Nε-acetylation of Residues K57 and K401 is a Potential Posttranslational Modulator of the Multiple Catalytic Activities of Protein Disulfide Isomerase

Cody T. Caba
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N^ε-acetylation of Residues K57 and K401 is a Potential Posttranslational Modulator of the Multiple Catalytic Activities of Protein Disulfide Isomerase

By

Cody Taylor Caba

A Thesis
Submitted to the Faculty of Graduate Studies through the Department of Chemistry and Biochemistry in Partial Fulfillment of the Requirements for the Degree of Master of Science at the University of Windsor

Windsor, Ontario, Canada

2017

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N\textsuperscript{\textalpha }-acetylation of Residues K57 and K401 is a Potential Posttranslational Modulator of the Multiple Catalytic Activities of Protein Disulfide Isomerase

by

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15/ 09/ 2017
DECLARATION OF ORIGINALITY

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ABSTRACT

Despite its study since the 1960's, very little is known regarding the regulation of the multiple catalytic activities performed by protein disulfide isomerase (PDI). A variety of conserved residues have been implicated as either important or vital for activity. This work ventures to identify a functional role for the highly conserved CGHC-flanking residues Lys$^{57}$ and Lys$^{401}$ of human PDI in vitro. Site-directed mutagenesis studies have revealed a role for the active site lysine residues in modulating the oxidoreductase activity of PDI in a pH-dependent manner. The effects of mutagenesis indicated that, along with the oxidoreductase activity, the kinetics of thiol-reductase and thiol-oxidase catalysis were also attenuated. Substitution of the aforementioned residues to glutamine, alanine or glutamic acid resulted in an enzyme variant 20 to 35% as efficient as wild type. This was found to translate to the decreased rate of electron shuttling between PDI and ERO1α to as little as 54% that of wild type. Consistent with this, molecular dynamic simulations suggest a role for Lys$^{57}$ and Lys$^{401}$ in differentially mediating the accessibility of the nucleophilic cysteine of each active site.

The possibility of lysine acetylation at residues Lys$^{57}$ and Lys$^{401}$ was assessed by in vitro treatment using Aspirin, coupled with mass spectrometry. A total of 18 acetyllysine (acK) residues were identified reproducibly, including acK$^{57}$ and acK$^{401}$. Altered enzyme kinetics as a result of acetylation by Aspirin corroborate with site-directed mutagenesis data. This provides a strong indication that acetylation of residues Lys$^{57}$ and Lys$^{401}$ is a potential modulator of the catalytic activities of PDI.
DEDICATION

To my parents and Olivia
ACKNOWLEDGEMENTS

I would first like to acknowledge and thank Dr. Bulent Mutus for his enthusiastic and unwavering support throughout my studies. As my supervisor, he was a profound role model for me and largely responsible for instilling my love for research through creativity and independence.

I graciously thank the members of my supervisory committee, Dr.’s Panayiotis Vacratsis and John Hudson. Their meaningful insight was a great help to me and the success of my research project.

For the smooth operation of the Department of Chemistry and Biochemistry, I give thanks to Beth Kickham, Marlene Bezaire, Cateherine Wilson, Jerry Vriesacker, and Joe Lichaa. Thank you all for your administrative services and going above and beyond to help students and faculty in need.

Research is always a team effort, and the work herein is no exception to that. To the past and present Mutus lab members, all your hard work has lead me to where I am now. Special thank you goes to Hyder Ali Khan for mentoring me during my undergraduate studies and providing me with a solid understanding of research. As well, previous alums Terence Yep, and Dr.’s Artur Jarosz and Bei Sun for their contributions and lending a helping hand in times of need. To Dr. Lana Lee for her construction of the PDI construct utilized throughout the studies, as well as her advice and wisdom. I would also like to thank Wanlei Wei, Paul Meister, Dr. James Gauld, and the entire Gauld research group for their help and expertise in performing molecular dynamics simulations. I extend gratitude to Dr. Janeen Auld for her help and persistence in performing mass spectrometry analysis.
of the many samples I brought forth. Last but not least I give thanks to the current graduates of the Vacratsis lab. Thank you to Ashley, Justin, and Rob for making themselves available and able to provide a helping hand during my years of research.

Other notable contributions came from the very gracious individuals whom provided me with some of the vital materials to carrying out my experiments. Firstly, I thank Dr. Luigi Puglielli (University of Wisconsin–Madison) for providing us with the N-acetyltransferase constructs utilized in these studies. Thank you to Dr. Kazuhiro Nagata (Kyoto Sanyo University) for generously providing recombinant Ero1α enzymes.
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<tr>
<td>2×YT</td>
<td>Yeast Extract Tryptone</td>
</tr>
<tr>
<td>Ala</td>
<td>Alanine</td>
</tr>
<tr>
<td>ATase</td>
<td>N-acetyltransferase</td>
</tr>
<tr>
<td>BL21</td>
<td><em>E. coli</em> strain deficient in Lon proteases</td>
</tr>
<tr>
<td>Cys</td>
<td>Cysteine</td>
</tr>
<tr>
<td>CoA</td>
<td>Coenzyme A</td>
</tr>
<tr>
<td>di-E-GSSG</td>
<td>Dieosin glutathione disulfide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EGSH</td>
<td>Eosin glutathione</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FSQ</td>
<td>Fluorescence self-quenching</td>
</tr>
<tr>
<td>Glu</td>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Glu-C</td>
<td>Endopeptidase Glu-C</td>
</tr>
<tr>
<td>Gln</td>
<td>Glutamine</td>
</tr>
<tr>
<td>GSSG</td>
<td>Glutathione disulfide</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>His</td>
<td>Histidine</td>
</tr>
<tr>
<td>kcat</td>
<td>Turnover number; Catalytic constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;M&lt;/sub&gt;</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass to charge ratio</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt;</td>
<td>Acid dissociation constant</td>
</tr>
<tr>
<td>RFU</td>
<td>Relative fluorescence units</td>
</tr>
<tr>
<td>UPLC-ESI MS</td>
<td>Ultra-high-performance liquid chromatography electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum rate of reaction</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Cys-S-S-Cys</td>
<td>Cysteine disulfide</td>
</tr>
<tr>
<td>Cys-SH</td>
<td>Cysteine thiol</td>
</tr>
<tr>
<td>Cys-S⁻</td>
<td>Cysteine thiolate anion</td>
</tr>
<tr>
<td>CysN</td>
<td>N-terminal cysteine of the CxxC motif</td>
</tr>
<tr>
<td>CysC</td>
<td>C-terminal cysteine of the CxxC motif</td>
</tr>
<tr>
<td>K57X</td>
<td>Mutation to residue K⁵⁷, where X is Q, A or E</td>
</tr>
<tr>
<td>K401X</td>
<td>Mutation to residue K⁴⁰¹, where X is Q, A or E</td>
</tr>
<tr>
<td>K57/401X</td>
<td>Simultaneous mutation to residues K⁵⁷ and K⁴⁰¹, where X is Q, A or E</td>
</tr>
<tr>
<td>redPDI</td>
<td>Reduced PDI</td>
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<tr>
<td>oxPDI</td>
<td>Oxidized PDI</td>
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CHAPTER 1

INTRODUCTION
1.1 Thiol-Disulfide Chemistry in Biological Systems

1.1.1 The Biochemistry of Thiols and Disulfides: Implications in Cellular Homeostasis and Protein Folding

There are 21 naturally occurring amino acids that can be incorporated into polypeptides during the process of translation in mammals. Cysteine (Cys, C) is one of the least abundant of these building blocks at only about 2.26% [1], yet it is the only residue containing the thiol functional group (R-SH); exhibiting the vital physicochemical properties associated with such. Despite its low abundance in relation to other amino acids, its prevalence and incorporation in the mammalian proteome is vast and believed to be an evolutionarily driven phenomenon of miscoding [1-4]. Cysteine residues are essential for a variety of biochemical processes such as protein folding, enzymatic redox reactions, multiprotein complex formation, as well as acting as key centers for modulation of enzymatic activity via posttranslational modifications that extend far past disulfide bond formation.

Biological thiols and disulfides do not only present themselves in the form of protein cysteine residues. Low molecular weight (LMW) thiols make up a large family of compounds that bear one or more thiol functional groups. These compounds define the redox potential of the cellular environment, protect the cell from oxidative stress, and facilitate the execution of many physiological pathways. In particular, some such molecules are able to carry out reversible S-thionylation of active cysteine residues in proteins to effectively protect against irreversible thiol-overoxidation to sulfinic and/ or sulfonic acid moieties [5]. Some of the most common LMW thiols- cysteine, glutathione and Coenzyme A are presented in Figure 1.1.
Figure 1.1: The most common low molecular weight (LMW) thiols found in eukaryotic organisms.
Cysteine predominantly exists in the free reduced state, with the ability to form a symmetric redox couple through metal-catalyzed autoxidation to cystine using O$_2$ as the oxidant [6, 7]. Total cysteine concentrations ($C_{\text{tot}} = \text{cysteine} + \text{cystine}$) have been measured at about 150 µM in human HT29 cells and up to 60 µM in human plasma [8, 9]. Another ubiquitous LMW thiol is glutathione. Glutathione is by and large the most prevalent LMW thiol in eukaryotes. It is an atypical tripeptide of glutamine, cysteine and glycine. Like cysteine, it exists in either a free reduced state (GSH) or in a symmetric oxidized form (GSSG). The ratio of GSH to GSSG is what defines the redox state of a particular cellular environment as this couple is the primary redox buffer of the cell (refer to section 1.1.2 for more information). The GSH:GSSG ratio is maintained enzymatically primarily through the work of glutathione reductase which functions to reduce GSSG to GSH [10]. Reports have shown the presence of glutathione in plasma at about 3 µM [11] and up to 10 mM in the secretory compartments, such as the lumen of the endoplasmic reticulum (ER) [12]. A last vital LMW thiol of note is Coenzyme A (CoA). In mammalian metabolism CoA is a widely used enzyme cofactor and substrate. The free thiol termini is often charged with functional moieties in the form of a thioester- \textit{i.e.} acetyl-CoA, propionyl-CoA, succinyl-CoA, malonyl-CoA, among many others (refer to section 1.3 for more information) [13]. The thiol functional group of CoA is primarily recognized for its role in making the molecule an acyl carrier, with little relevant implications to redox homeostasis within the cell. Although, in various prokaryotic organisms lacking GSH, CoA has been implicated in performing intracellular redox functions in conjunction with a widely distributed bacterial and archaeal enzyme, NAD(P)H-dependent CoA disulfide reductase (CoADR) [6].
In terms of protein folding, disulfide bond formation (R-S-S-R') is a reversible crosslink between two non-contiguous cysteine residues of a polypeptide chain or constituent polypeptides [14]. In conjunction with the non-covalent forces- van der Waals interactions, salt bridges, hydrogen bonds and the hydrophobic effect, this covalent interaction drives the formation of a three-dimensionally (3D) structured polypeptide; wherein the 3D conformation is directly related to its intrinsic function [15]. It is important to note that not all proteins contain cysteine residues, and even those that do contain two or more, will not always harbour a structural disulfide bond. It is a feature most prevalent in proteins of the secretory pathway and recycled membrane proteins in order to confer added stability in the presence of the extracellular milieu and acidic endocytic vesicles, respectively [16, 17].

Structurally, disulfide bonds are thought to be advantageous in two ways. First, by promoting structural constraints such that the most thermodynamically stable conformation is attained- thereby destabilizing non-native or unfolded states. Although, it is possible that disulfide bonds of a native protein be those that restrict the dynamics of even entropically favourable fluctuations [18, 19]. Nonetheless, a second advantage is their ability to enhance stability by protecting against oxidative damage or proteolytic degradation, effectively increasing a protein's half-life [14]. This is an aspect that is very pertinent to the biotechnology and pharmaceutical industries as many batch processes and therapeutics work through the use of oligopeptides and proteins- for example, insulin, oxytocin, antibodies, papain, to name a few [20]. Even still, the way in which disulfide bonds bring about structural stability is not well understood, and introducing engineered disulfides into a recombinant protein does not always result in enhanced stability. In a
natively folded protein the arrangement of disulfide bonds often represents intramolecular linkages (or intermolecular in the case of multi-subunit proteins) that have achieved their most thermodynamically favourable state in relation to a myriad of less favourable configurations. Figure 1.2 illustrates a general free energy profile or funnel diagram for the landscape of protein folding leading to a stable native conformation [21]. It is assumed that under set conditions the native state of a protein is at the energy minimum of the landmark; this is regarded as the folding funnel hypothesis [22]. It should be understood that many non-native conformations may exist in local energy minima. This is because the native state is a dynamic- not static- ensemble of many sub-states [23-25].

Despite folding being largely driven by the hydrophobic effect, for those proteins requiring the formation of disulfide bonds, it is the work of intracellular chaperones, foldases and dithiol-disulfide oxidoreductase enzymes that facilitate the native fold. One such enzyme being protein disulfide isomerase (PDI), found within the lumen of the ER. The activity of PDI during oxidative protein folding (refer to section 1.2) is to optimize the kinetics of both the introduction of disulfide bonds and the rearrangement of mispaired disulfides in a misfolded protein substrate [26]. It is understood that the rate limiting process of protein folding is the direct thiol-disulfide interchange reactions that take place, where appropriate [27]. PDI along with other PDI family members provide a catalytic architecture that is optimized to provide a minimization of the folding timescale.
**Figure 1.2:** Landscape of protein folding as depicted by an energy funnel diagram of conformational state vs. free energy. The native conformation is regarded as the lowest energy state at the bottom of the funnel. The bottom of the folding funnel is populated by multiple energy minima representing distinct native and stable non-native conformations. (adapted from [21])
1.1.2 Biochemical Considerations for Thiol-Disulfide Exchange Reactions

Disulfide bonds exist as either structural or functional elements of a protein. Functional disulfides are either catalytic centers or allosteric switches [14]. The ability of functional and structural disulfides (and their free thiol counterparts) to participate in chemical reactions is not surprisingly dependent on factors that are dictated by the microenvironment at any given time—such as pH, ionic strength, electrostatic interactions with neighbouring groups, accessibility, etc. [26-28]. These factors have a direct influence on thiol pKₐ and ionization state, as well as the disulfide redox potential (E); intrinsically linked properties for any thiol-disulfide couple. Protein microenvironments work exceptionally well at controlling these factors and as a result, modulating thiol and disulfide reactivity. It is common to observe biological thiols with pKₐ values in the range of 3 to 11, markedly different from the pKₐ of ca. 8.6 for the thiol of free cysteine [29, 30].

Thiol-disulfide exchange is a widely used enzymatic process that, as the name implies, is the exchange of thiol and disulfide groups between two reacting molecules. The process is a simple 1-step S₈2 reaction whereby a nucleophilic thiolate (R-S⁻) attacks an oxidized sulfur of a target disulfide [26, 27, 31]. The nucleophile is recognized as the deprotonated thiol functional group, a thiolate anion, as it has been demonstrated that R-SH is essentially unreactive as a nucleophile under standard conditions with nearly 10¹⁰-fold slower rates [31, 32]. For exchange to occur, the attacking thiolate must approach in-plane with the disulfide axis. The collinearity of thiol-disulfide exchange results in a trisulfide transition state with a delocalized electron (Figure 1.3). This is an oft-forgotten consideration to the chemistry of thiol-disulfide exchange that defines an extremely
important steric requirement for the kinetics of the reaction [26]. Furthermore, the concerted mechanism showcases negatively charged species in both the initial ground state and the transition state, making it apparent that the rate be greatly affected by electrostatic and through-bond inductive effects [28].

Thiol pK$_a$ and nucleophilicity are not mutually exclusive traits in terms of thiol-disulfide exchange rates. Rather, they are intrinsically linked as it has already been discussed that the thiolate anion is a 10$^{10}$-fold more reactive nucleophile. Considering this, it is logical to state that under set (standard) conditions, increasing the pK$_a$ of a nucleophile (thiol) will result in a decrease of reaction rate; a direct result of the existence of only a small proportion of thiolate anion (when pK$_a$ > pH). Meaning, the rate of thiol-disulfide exchange is expected to be inversely proportional to the pK$_a$ of the attacking thiol. Contrary to this, it is understood that the ability of a sulfur atom to retain its proton is somewhat representative of its inherent nucleophilicity [28, 31]. The linear-free energy relationship shows that thiolate nucleophilicity actually increases with pK$_a$, leading to enhanced reaction rates [27, 33]. This has been demonstrated in research using model systems with both small-molecule and peptide-based thiols [28]. Furthermore, one must consider the charge transfer that occurs within the transition state itself. The attacking nucleophile must pass its charge to the leaving-group sulfur about the trisulfide intermediate. Thus, the pK$_a$ of the leaving-group thiol is another factor influencing thiol-disulfide exchange rates. Research has shown that a more acidic (lower pK$_a$) leaving-group thiol mediates a greater rate of exchange [28, 33, 34]. This is due to a drop in the transition state energy as a lower pK$_a$ of the leaving-group thiol stabilizes the thiolate state in relation to the oxidized state [27].
Figure 1.3: Representative reaction coordinates for a simple bimolecular thiol-disulfide exchange reaction, highlighting the $\text{S}_2\text{N}_2$ type nucleophilic substitution along with the dependence on a thiolate nucleophile and a collinear orientation about the disulfide axis. (adapted from [27])
To further appreciate the mechanism of thiol-disulfide exchange it is useful to examine the correlation between thiol pK$_a$, nucleophilicity and reaction rate. This can be achieved using the pseudo-Brønsted correlation. Given by **Equation 1.1**, this is a linear relationship between the common logarithm of reaction rate, $k$, and the pK$_a$ of the participating sulfur atoms: $\text{nuc}$, nucleophile; $c$, central; $\text{lg}$, leaving group. The Brønsted coefficient of basicity is given by $\beta$. This defines the slope of the line, where a larger value (between 0 and 1) defines the extent of charge-transfer about the transition state. In this equation, the pK$_a$ of the attacking nucleophilic thiol ($\text{nuc}$) and the leaving-group thiol ($\text{lg}$) are most influential on rate, consolidating the idea that thiol-disulfide exchange is a finely tuned process of optimized nucleophilicity of the attacking thiolate and a depressed pK$_a$ of the $\text{lg}$ thiol [27].

$$\log k = \beta_{\text{nuc}}pK_{a,\text{nuc}} + \beta_c pK_{a,c} + \beta_{\text{lg}}pK_{a,\text{lg}} + C$$

**Equation 1.1**

Because $\beta$ is directly related to the transfer of charge about the transition state, any apparent differences in the value of this coefficient when comparing various reacting thiol-disulfide couples is believed to be useful in indicating differences in the transition states themselves [28]. A caveat to this arises when studying ever more complex systems, such as those brought about by enzyme active sites. Authors have expressed that apparent Brønsted correlations do not reflect purely the electronic structure of the transition state. They can be skewed by nearby charges and the interactions between the reacting molecules of the bimolecular reaction itself [28]. For instance, if two reacting molecules undergoing thiol-disulfide exchange are more likely to form an encounter-complex because the charge of one is net positive while the other is net negative.
(opposites attract; like-charges tend to repel), this will lead to artificially high $\beta$ values that are irrespective of any actual changes to transition state dynamics [28].

Nagy elegantly described a situation where lowering the $pK_a$ of an attacking thiol does not always correspond with enhanced nucleophilicity (reaction rate, Figure 1.4) [27]. Using $\beta = 0.5$ for the reaction of various thiols with GSSG at pH 7.0, it is apparent that a nucleophilic thiol with a $pK_a$ ca. 6-7 holds ideal nucleophilic power for optimized thiol-disulfide exchange rates. Nagy stated that under these conditions, decreasing the $pK_a$ of the attacking thiol will only increase the overall rate until the thiolate becomes the dominant species. Past this point the nucleophilicity of the sulfur center will decrease such that the rate slows [27]. Many enzymes that partake in the catalysis of thiol-disulfide exchange reactions, such as PDI, have abnormally low thiol $pK_a$ values. These would seemingly go against the characteristics of optimizing exchange rate (the proportion of thiolate vs. nucleophilicity). In such cases it is apparent that these enzymes rely on additional means to optimize both the kinetics and thermodynamics of their catalytic mechanisms. A hallmark of many thiol-disulfide oxidoreductases that perform thiol-disulfide exchange mechanisms is the presence of one or more CxxC motifs. It is the strategic spatial arrangement of these motifs that provide the catalytic power and tuning for these enzymes.

Thus far the kinetics of thiol-disulfide exchange have been addressed with little regard for the thermodynamics of the reaction. For a bimolecular thiol-disulfide exchange reaction an equilibrium may be established, directly related to free energy (Equation 1.2):
Figure 1.4: Representation of the dependence of the thiol-GSSG exchange rate on the p$K_a$ of the attacking thiol at pH 7.0.
(adapted from [27])
\[ aA_{\text{red}} + bB_{\text{ox}} \rightleftharpoons ^{k} cA_{\text{ox}} + dB_{\text{red}} \]

\[ K = \frac{[A_{\text{ox}}]^c[B_{\text{red}}]^d}{[A_{\text{red}}]^a[B_{\text{ox}}]^b} \]

\[ \Delta G = \Delta G^0 + RT \ln K \]

Equation 1.2

Now, one may describe the equilibrium (or non-equilibrium steady state) of a redox environment as defined by the redox couple. Schafer and Buettner stated that the redox environment is a sum of the reduction potential and the reducing capacity of a couple [35]. They went on to express that the redox potential can be estimated using the Nernst equation, and the reducing capacity be estimated by determining the concentration of the reduced species of the couple [35]. The Nernst equation can be used to find the voltage potential (Equation 1.3) and relate it to the Gibb's free energy change (Equation 1.4):

\[ \Delta E = \Delta E^o' - \frac{RT}{nF} \ln Q \]

Equation 1.3

\[ \Delta G = -nF \Delta E \]

Equation 1.4

Where \( n \) is the number of exchanged electrons, \( F \) is the Faraday constant, \( R \) is the gas constant, \( T \) is temperature, \( \Delta E^o' \) is the change in the reduction potential under standard conditions (\( pH = 7.0, T = 298 \text{ K}, 1 \text{ atmosphere} \)), and \( Q \) is the reaction quotient (reminiscent of the equilibrium constant, \( K \)). It is apparent that the more positive \( \Delta E \) becomes the more exergonic the reaction, where a positive value represents a spontaneous reaction. For thioredoxin-like enzymes, the reduction potential of the active site vicinal thiol couple varies markedly due to the architecture of the protein and the intervening residues of the CxxC motif having a direct impact on the \( pK_a \) of the thiols (see section 1.2.4). Lower \( pK_a \)'s result in more positive values of \( E \); a tendency towards
being reduced [35]. Because the redox poise of the cell is convoluted with its many intertwined pathways, it is difficult to observe true equilibria. Furthermore, in the complex system of the cell, the kinetic pathways (previously discussed) dominate reaction dynamics more so than the thermodynamic driving forces [26, 35, 36].

1.2 Protein Disulfide Isomerase (PDI)

The pioneering work by independent researchers Straub [37-39] and Anfinsen [40] in the 1960's allowed for the discovery and characterization of an enzyme with the ability to correctly fold and reactivate reduced ribonuclease A (RNase A). This enzyme was later named protein disulfide isomerase (PDI) [41]. PDI (EC 5.3.4.1) has since been studied extensively in literature, affording it its own gene family of 21 orthologues and paralogues dubbed the PDI family of proteins (Figure 1.5) [42, 43]. The PDI family as a whole is part of the thioredoxin (TRX) superfamily of enzymes. This classification is based on the presence of at least one TRX-like domain with the secondary element sequence: \( \beta\alpha\beta\alpha\beta\alpha\beta\alpha \), and often the subsequent presence of the CxxC thioredoxin motif [44]. The definition of a PDI family member has been altered to specify the need for not only a TRX-like domain, but also the exhibition of redox-reactivity and/ or chaperone-like activity [45]. There is little sequence homology amongst the PDI family members, with comparisons between any two members resulting in reported sequence identities ranging from 15 to 65% [42], along with variations in domain organization. It is believed that the multi-domain arrangement of PDI proteins is a result of evolutionary gene duplication [42, 46]. Some speculate that the existence of so many enzymes with PDI activity is indicative of a specialization each has with specific substrates [26]. Although,
PDI has been described as a promiscuous enzyme with few known bona fide substrates [47].

Herein, focus will be placed on the PDI family member PDIA1, the gene product of P4HB; said to comprise up to 1% of the cell's total protein content [43]. For simplicity, PDIA1 will be referred to as PDI or hPDI (human PDI) synonymously. Residue numbering is based on the accepted sequence for PDI from Homo sapiens (Uniprot: PDIA1, P07237).

### 1.2.1 The Structure and Localization of Human PDI

PDI is a 57 kDa horseshoe-shaped monomer that is comprised of 4 discreetly folded TRX domains: a, b, b', and a'. Separating domains b' and a' is a 19-residue long interdomain loop denoted as the x-linker. A 29-residue region at the C-terminus of PDI makes up the c-extension (a highly disordered and unstructured segment—referring to this as a domain is a misnomer). Thus, the fully mature enzyme takes on an a-b-b'-x-a'-c configuration and is 491 residues in length (Figure 1.6) [43]. Full-length PDI has yet to be crystalized in its entirety. The currently available structures of hPDI are of individually isolated domains (a [48], b [49], and a') or of the partial-length polypeptide (b'-x [50], b-b' [51], and b-b'-x-a' [52]). Only recently have Wang and coworkers been successful in crystalizing nearly full-length PDI, with the exception of the highly flexible c-extension, in both the reduced and oxidized states (PDB entries: 4EKZ and 4EL1, respectively. See later) [53]. This has since made it possible to study PDI to much greater depths than ever before.
Figure 1.5: Schematic representation of the PDI family of proteins. Domain organization is given along with the CxxC motifs where applicable. Domains $a$ and $a'$ represent catalytic domains based on the presence of the CxxC oxidoreductase active site motif. Domains $b$ and $b'$ are the non-catalytic domains.

(reference [43])
Figure 1.6: Structure and features of PDI. (A) Crystal structure of PDI in the reduced state. The four thioredoxin domains are indicated, along with the conserved CGHCK active motifs of the $a$-type domains. Ball and stick representation of the active site vicinal thiols (yellow) and the highly conserved neighbouring lysine (red). PDB: 4EKZ [53]. Image generated using Visual Molecular Dynamics software (VMD) [54]. (B) Simplified schematic of PDI.
The existence and configuration of the \( a, b, b', \) and \( a' \)-domains of PDI are central to its many enzymatic and non-enzymatic functions. The \( a \)-type domains are designated as such due to the presence of the catalytically active CxxC motif. There is only 33.6% sequence identity between these domains, and each harbours the conserved active site motif: FYAPWCGHCK \([43, 55]\). The arrangement of the vicinal active site thiols is quite different, with the N-terminal cysteiny1 thiol (Cys\(^{53}\) and Cys\(^{397}\)) being more solvent exposed than the corresponding C-terminal cysteiny1 thiol (Cys\(^{56}\) and Cys\(^{400}\)) \([43]\). The \( a' \)-active site also differs from that of \( a \) as it is shielded by a hydrophobic tunnel when PDI is in the reduced state \([53]\). Both active site regions are similar in displaying a hydrophobic surface \([53]\).

The non-catalytic \( b \)-type domains are placed between the \( a \)-type, rendering PDI in the aforementioned U-shape, and show only 16.5% sequence identity \([43]\). It is this overall conformation that allows PDI to bind large protein substrates using a continuous hydrophobic surface along the inside of its structure \([53]\). In particular, the \( b' \)-domain exhibits a hydrophobic pocket that is believed to help PDI distinguish between unfolded and folded substrates- perhaps owing to the fact that in unfolded protein substrates there is a larger propensity of surface exposed hydrophobic regions \([56]\).

The structural dynamics of PDI are extensive, and redox-regulated \([53]\). When PDI is in the reduced state \( (i.e., \) both CxxC active sites bearing free thiols) the enzyme takes on a non-planar U-shape with an inter-domain distance between the CxxC active sites of about 28 Å. Once oxidized \( (i.e., \) intramolecular disulfide between the CxxC vicinal thiols\) the \( a' \)-domain rotates about 45° in plane with domains \( a, b \) and \( b' \), rendering an inter-domain distance of about 41 Å \([53]\). This redox-state dependent conformational
change occurs about the flexible x-linker, and indicates that PDI has a much more open conformation when oxidized.

The highly acidic C-terminal extension of PDI has the classical tetrapeptide ER-retention signal KDEL. Because of this, PDI is primarily localized to the ER lumen and regularly recycled from the cis Golgi network back to the ER via a retrograde pathway [44, 57]. Despite this, it is found almost ubiquitously on the subcellular level, including the cytosol, cell surface and extracellular space. It was once speculated that specialized secretory cells underwent saturation of the KDEL receptors of the Golgi membranes, leading to an apparent leakage of PDI [44]. This has since been deemed slightly inaccurate with the realization that the secretion of PDI is likely mediated by the KDEL retention motif itself, and that such an event is specific and regulated [58]. Although, the mechanism of secretion is still under investigation.

1.2.2 The Enzymatic and Non-Enzymatic Activity of PDI and Methods of Detection

PDI is a multifunctional enzyme capable of performing both enzymatic and non-enzymatic activities. Non-enzymatically, PDI functions as a chaperone and anti-chaperone. Enzymatically, PDI is able to reduce, oxidize and isomerize disulfide bonds; making it a thiol-reductase, thiol-oxidase and thiol-disulfide isomerase (Figure 1.7).

The catalytic activities of PDI are centered on its two CxxC active site motifs. For hPDI, this sequence is CGHC. Each active site performs independent of the other in vitro [43, 59, 60] (refer to results- Chapter 3), although research has demonstrated that upon reduction of the a-domain, the a'-domain will subsequently re-oxidize it in vivo [56, 61] (see later). When in the reduced state PDI is capable of reducing the disulfide bond of
some substrate, rendering itself oxidized. The opposite can be said for when PDI oxidizes a substrate's dithiols. As for isomerization, this does not result in a change of the redox-state of PDI.

For thiol-disulfide exchange to occur the N-terminal cysteine (Cys$^{53}$ of the $a$-domain and Cys$^{397}$ of the $a'$-domain: CxxC) is required for the initial nucleophilic attack of a substrate disulfide. It has been described to have a $pK_a$ in the range of 3.8 [62] to 5.6 [63, 64]. The highly acidic nature of these thiols as compared to the typical cysteinyl-thiol $pK_a$ (ca. 8.6) is a result of the architecture of PDI. Each is found at the N-cap of the $\alpha$2 helix of the discretely folded TRX domain. As well, there are contributions from the intervening histidine residue of the CGHC motif (His$^{55}$ of the $a$-domain and His$^{399}$ of the $a'$-domain) [65].

The C-terminal active site cysteine residue (Cys$^{56}$ of the $a$-domain and Cys$^{400}$ of the $a'$-domain: CxxC) is recognized as the resolving cysteine as it functions to release PDI from a mixed-disulfide intermediate state following the initial nucleophilic attack of a substrate disulfide via the N-terminal vicinal thiol. The $pK_a$ of the C-terminal cysteine is largely transient and dependent on the state of the PDI ensemble. When PDI is in a free reduced state (i.e., not in an intermediate complex with substrate) the C-terminal active site thiol is reported to have a $pK_a$ between 8.6 [63] and >10 [62]. When PDI is occupying a mixed-disulfide state, the $pK_a$ has been observed to be as low as 6.1 [62, 63]. The transient nature of this thiol $pK_a$ has been shown to be mediated by the strategic movement of an arginine residue (Arg$^{120}$ of the $a$-domain and Arg$^{461}$ of the $a'$-domain) to the active site; facilitating direct electrostatic interactions with the C-terminal- but not the N-terminal, cysteinyi thiol [63]. Unfortunately, the ability of the resolving cysteine to be
regioselective and form an intramolecular disulfide with the N-terminal vicinal active site thiol rather than an intermolecular disulfide with the substrate is not understood. Many researchers attempt to describe the observed regioselectivity using the hard-soft acids and bases (HSAB) principle [66].

Computational analyses have helped to further characterize the potential specificity of PDI based on the dihedral angles about a substrate's disulfide bond [58, 67]. Chiu and coworkers noted that of the implicated disulfide targets of PDI (substrates of thrombosis, refer to section 1.2.4), all harbour a disulfide conformation that is indicative of an allosteric linkage [58]. There are 20 characterized disulfide conformations found in protein crystal structures (Figure 1.8 A), three have been correlated as being allosteric disulfides- two of such showing enhanced bond strain about the S-S bond, resulting in it being "pre-stressed" [67]. This leads to accelerated rates of reduction. An example of a pre-stressed disulfide is given in Figure 1.8 B.

The ability of PDI to distinctly assign a nucleophilic cysteine (CxxC) and a resolving cysteine (CxxC) in its catalytic mechanism provides assurance that that the enzyme will not be trapped in a mixed-disulfide intermediate state for extended periods of time, particularly during protein folding. This characteristic has been deemed the "scan and escape" mechanism [68]. Liberation of the mixed-disulfide intermediate is the rate-limiting step [68]. Gilbert and coworkers indicated that because disulfide bond rearrangement within a protein is slow, it is advantageous for PDI to liberate itself from the covalent complex and initiate isomerization successively anew [68]. The process continues until the most stable disulfide remains, one by which PDI cannot act to reduce; a disulfide with sufficient kinetic and thermodynamic stability [68, 69].
Figure 1.7: The enzymatic dithiol-oxidoreductase activities of PDI. Illustrating the ability of PDI to reduce substrate disulfides, oxidize substrate thiols and isomerize different configurations of disulfides on a substrate. PDI is initially depicted as being in a mixed-disulfide intermediate state with the substrate. For simplicity only the $a$-domain active site is depicted.
Figure 1.8: Disulfide bond configurations identified in proteins. (A) The 20 possible disulfide bond configurations of proteins, highlighting the three configurations that represent allosteric disulfides in yellow, orange and blue. (B) An example of the geometry of a pre-stressed disulfide configuration with inter-sulfur distance ($d$) and the disulfide bending angles ($\alpha$).
(adapted from [58])
Should the resolving cysteine be sufficiently slow in releasing the intermediate complex, there is a possibility that a thiolate from the substrate attack the mixed disulfide instead. This can potentially lead to an unproductive catalytic cycle should the now free substrate be back to its original disulfide state.

When these reactions occur, there is a need for the regeneration of the PDI active sites back to their original states- the oxidized state when considering oxidative protein folding. *In vitro*, many small molecule electron donors (dithiothreitol, DTT; GSH) and electron acceptors (GSSG) are used to reduce or oxidize PDI active sites, respectively. Within the ER it is the resident oxidase, a flavo-enzyme, that preferentially mediates the oxidation of PDI. This enzyme is endoplasmic reticulum oxidoreductin-1 (ERO1). Depending on the mode of protein folding, PDI may act in a PDI-1st or a PDI-2nd manner. PDI-1st pathways see PDI perform the initial oxidation of substrate thiols, then the subsequent reduction and isomerization. In PDI-2nd pathways, PDI is purely an isomerase, as other enzymes such as quiescin-sulphydryl oxidase (QSOX), catalyze the initial oxidation of substrate thiols [26]. Therefore, the reliance on ERO1 comes from PDI-1st pathways. When the a-domain active site of PDI is reduced during PDI-1st oxidative protein folding, the a'-domain is required to subsequently re-oxidize it, rendering itself reduced [70]. In order for the a'-domain active site to be re-oxidized, ERO1 facilitates the oxidation using molecular oxygen as the oxidant, forming hydrogen peroxide as a by-product (H₂O₂, refer to section 1.2.3 for more details) [43, 56].

Irrespective of the catalytic CxxC motifs is the chaperone activity PDI performs. The presence of a large hydrophobic groove on the b'-domain facilitates the interaction with unfolded nascent polypeptides or misfolded proteins. PDI has been shown to
prevent the aggregation of unfolded proteins that do not bear disulfide bonds [71, 72]. Using reduced, denatured lysozyme, Puig and Gilbert demonstrated that PDI functions as both a chaperone at high concentrations and an anti-chaperone when at low concentrations [73]. They speculated that this concentration-dependence be linked directly to the chaperone capacity and folding capacity of the ER, where PDI is highly expressed to near millimolar quantities [74, 75].

Although it may appear that domain-related activity be relatively independent, it has been demonstrated that there is cooperativity that occurs amongst them. The $a$ and $a'$-domains are required for the binding of larger substrates, while the $b$ and $b'$-domains actually enhance reductase activity [76-79].

Seeing as PDI is such a multifunctional protein it is unsurprising to realize that there exists a plethora of techniques to monitor each of its respective functions (Figure 1.9). Each assay developed comes with its own inherent advantages and limitations. Although, when used in conjunction with one another, these techniques provide powerful tools for elucidating the mechanism of PDI and providing a means for potential drug discovery. Depending on the initial state of the substrate and PDI itself (i.e., reduced or oxidized), one type of PDI activity will be biased in relation to the others [80].

To measure PDI reductase activity, reduced PDI (redPDI) and an oxidized substrate are required. Advances in the measurement of reductase activity have brought about the development of small molecule and protein substrates with an emphasis on UV-Vis and fluorescence detection methods. The most cost-effective reductase assay is the insulin turbidity assay. Here, PDI catalyzes the reduction of insulin using GSH or DTT as the electron donor source, resulting in the liberation of free A and B chains- thereby
increasing the solution's turbidity (OD$_{600}$, Figure 1.9 A) [43]. This method may be continuously monitored by coupling it to the formation of GSSG- making it an indirect assay. A less cost-effective, but direct and continuous method to measure PDI reductase activity is through the use of fluorescently-quenched probes. Mutus and coworkers developed a fluorimetric assay using GSSG as a means to link two identical fluorescent molecules in close proximity in order to take advantage of fluorescence self-quenching (FSQ); diaminobenzoyl-GSSG (Figure 1.9 C) [81] and diosin-GSSG [82]. When PDI catalyzes the reduction of diabz/diosin-GSSG, two free abz/eosin-GSH molecules result, leading to a loss of fluorescence quenching and an increase in signal proportional to PDI activity. This fluorescence turn-on assay is capable of reaching detection limits of 1 nM PDI. DTT is often used as the source of electrons to regenerate redPDI as it has no impact on the stability of the probe itself.

To measure thiol-oxidase activity, PDI must initially be in the oxidized state (oxPDI) and the substrate be reduced. Using RNase A, it is possible to measure the thiol-oxidase activity of PDI by monitoring the rate of hydrolysis of cyclic cytidine monophosphate (cCMP) to CMP (Figure 1.9 D). An increase in the absorbance at 296 nm is proportional to the oxidative renaturation of RNase A [43]. To regenerate oxPDI, GSSG is used as the oxidant. Another method, based on fluorescence quenching, uses a synthetic decapeptide substrate (Figure 1.9 F). Designed by Freedman and coworkers [83], it contains two cysteine residues separated by a flexible linker, a fluorescent tryptophan residue adjacent one of the cysteines, and a protonatable arginine adjacent the other. The tryptophan and arginine residues allow for the rate of PDI-catalyzed peptide oxidation to be measured by monitoring the quenching of tryptophan fluorescence ($\lambda_{ex}$:
280 nm, $\lambda_{\text{em}}$: 360 nm) due to the close proximity with arginine, *i.e.* fluorescence turn-off [83]. Again, GSSG is used to regenerate oxidized enzyme active sites.

Measuring the isomerase activity of PDI requires the use of substrates containing mispaired disulfides. Some such examples include the scrambled RNase (scRNase) assay and the refolding of bovine pancreatic trypsin inhibitor (BPTI). Here, PDI catalyzes the rearrangement of disulfide bonds until the native conformation of the substrate is attained. In each case, that would be one displaying native activity as for the scRNase assay (*Figure 1.9 E*), or the correct disulfide arrangement as determined by mass spectrometry as for the BPTI refolding assay (*Figure 1.9 G*). The scRNase assay is a time-consuming reaction that indirectly measures PDI isomerase activity. The BPTI refolding assay is also very time consuming in-part due to the requirement of sample preparation for mass spectrometry. Thus, these methods are inadequate as high throughput techniques [43].

Monitoring the chaperone-like activity of PDI requires the use of completely denatured substrates by which refolding follows a pathway that does not require disulfide bond formation [80]. Of note is the acid-denatured green fluorescent protein (GFP) refolding assay (*Figure 1.9 I*). The refolding of GFP by PDI results in a native conformation exhibiting an increase of fluorescence over time. This allows for the determination of rates directly [43].
**Figure 1.9:** The various methods for detecting the different PDI activities *in vitro*. Boxed in red are those methods used for monitoring reductase activity (A, insulin turbidity assay; B, insulin degradation assay; C, fluorimetric di-abz-GSSG assay). In blue are methods of detecting PDI thiol-oxidase activity (D, RNase oxidation assay; F, peptide dithiol oxidation assay). In green are techniques used to detect PDI isomerase activity (E, scrambled RNase assay; G, bovine pancreatic trypsin inhibitor (BPTI) refolding assay). Boxed in purple are the common methods of measuring PDI chaperone activity (H, rhodanese aggregation assay; I, acid-denatured green fluorescent protein (GFP) refolding assay).

(adapted from [43])
The reader should note that for the assays described capable of monitoring the enzymatic activities of PDI (reductase, thiol-oxidase and isomerase), there is often the presence of an electron donor or acceptor molecule to mediate the regeneration of the PDI active sites. This is especially meaningful when considering reductase and thiol-oxidase assays. It means that no matter the method employed, the rates obtained represent the oxidoreductase activity of PDI, and often not purely the respective reductase or thiol-oxidase rates. The process of active site regeneration can at times be rate-limiting, and unless this caveat be addressed, reported rates of catalysis may be inherently skewed (refer to sections 3.3 and 3.4).

1.2.3 Interactions Between PDI and ERO1

Thiol-disulfide redox reactions performed by PDI directly affect the redox state of the active sites. When PDI is oxidized (bearing an intramolecular disulfide between the CxxC vicinal thiols) it functions to oxidize substrate thiols, thereby rendering the CxxC reduced (dithiol CxxC motif). The opposite can be said for PDI-mediated substrate reduction. This change of redox state indicates a need for mechanisms able to regenerate PDI active sites to either their reduced or oxidized forms. During oxidative protein folding within the lumen of the ER, the oxidized a-domain active site of PDI has been found to introduce disulfides into reduced nascent substrates. The now reduced a-domain active site is re-oxidized via a thiol-disulfide exchange reaction with the a'-domain disulfide. With the regeneration of the oxidized a-domain achieved, there is now a need for the re-oxidation of the a'-domain CxxC; catalyzed by ERO1 [61, 84].
ERO1 is an ER-resident oxidase with two isoforms: ERO1α and ERO1β. Research has demonstrated that the *in vitro* sulfhydryl oxidase activity of the β isoform be approximately 2-fold greater, likely owing to the variation in sequence of the first CxxC active site (CFKC and CDKC for α and β isoforms, respectively) [85]. ERO1α is expressed in many different cell types in a near ubiquitous fashion, whilst ERO1β has been described as pancreas-specific with expression being inducible via ER-stress, such as the unfolded protein response (UPR) [86-88]. A β-hairpin of ERO1 binds the hydrophobic pocket of the *b*-domain of redPDI allowing it to selectively oxidize it over other PDI family members [89, 90].

ERO1α contains regulatory disulfides that occur between various catalytic and non-catalytic cysteines. The catalytic cysteines constitute two distinct regions required for activity: C\(^{94}\)xxxxC\(^{99}\) and C\(^{394}\)xxC\(^{397}\). The two primary redox states ERO1α takes on in living cells is Ox1 and Ox2 [91, 92]. Ox1 is an active form that exhibits intramolecular disulfides about the CxxxxC and CxxC motifs. Ox2 is the inactive form where C\(^{94}\)-C\(^{131}\) and C\(^{99}\)-C\(^{104}\) disulfides exist. Ox2 is the predominant form of ERO1α, with Ox1 levels being enhanced upon PDI over-expression and PDI binding [92-94]. The C\(^{94}\)-C\(^{99}\) disulfide is located on a flexible loop (the regulatory loop) and is referred to as the shuttle disulfide. During sulfhydryl oxidation, it passes electrons to the active site C\(^{394}\)-C\(^{397}\) disulfide which is proximal the Flavin adenine dinucleotide (FAD) cofactor. Reduction of FAD to FADH\(_2\) leads to the reduction of molecular oxygen (O\(_2\)), forming hydrogen peroxide (H\(_2\)O\(_2\)).

The detailed molecular mechanisms are as follows. When active ERO1α (Ox1) binds the *b*-domain of redPDI, it selectively oxidizes the *a*-active site via a dithiol-
disulfide exchange reaction - that being a transfer of two electrons from PDI to ERO1. The electrons from PDI are accepted by the regulatory loop cysteines, C^{94} and C^{99} (C^{94}xxxxC^{99}) of ERO1α. Residue C^{99} effectively acts as the shuttle cysteine by performing internal dithiol-disulfide exchange with the active site disulfide C^{394}-C^{397}, rendering the regulatory loop re-oxidized [61, 95, 96]. Electron transfer to the active site leads to a reduced dithiol state that is in close proximity with bound FAD. The electron flow is furthered by transfer from C^{397} to FAD, resulting in the reduced form, FADH\textsubscript{2}. This occurs via a charge-transfer complex between the C^{397} thiolate anion and the carbon C(4a) of FAD. The covalent adduct is liberated by the subsequent nucleophilic attack by the C^{394} thiolate. The result is a regenerated C^{394}-C^{397} disulfide and the aforementioned FADH\textsubscript{2} [92]. The terminal electron acceptor is O\textsubscript{2}, where a single molecule accepts one of the two electrons of the exchange pathway, therefore reduction of two O\textsubscript{2} molecules occurs. Reduction of two equivalents of O\textsubscript{2} by FADH\textsubscript{2} yields two superoxide molecules and FADH\textsubscript{2} being re-oxidized to FAD. Superoxide dismutates to H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2} (Figure 1.10) [27].

1.2.4 Influence of CxxC Intervening Residues on PDI Activity

The specific sequence of the CxxC motif is widely understood to directly influence the redox characteristics of the active site. For instance, PDI bears two CGHC motifs and thioredoxin (TRX) contains a single CGPC motif sequence. This results in PDI being a better thiol-oxidase, while TRX is a better thiol-reductase. The reason being the effect of the intervening residues on the thermodynamic stability (redox potential: E) of the vicinal thiol couple.
Figure 1.10: Electron flow during oxidative protein folding mediated by the PDI-ERO1α interaction pathway of thiol-disulfide exchange. The a-domain of PDI oxidizes a reduced substrate, thereby becoming reduced (accepting two electrons denoted in red as e'). The a'-domain of PDI re-oxidizes the a-domain resulting in electron flow between PDI active sites. ERO1α binds the b'-domain of redPDI and selectively oxidizes the a'-domain by facilitating electron transfer to its regulatory loop disulfide. The electrons are subsequently shuttled to the active site CxxC of ERO1α, which ultimately results in the reduction of FAD to FADH₂. FADH₂ reduces molecular oxygen to form hydrogen peroxide- regenerating oxidized ERO1α.
The presence of histidine electrostatically stabilizes the reduced state of the active site in relation to the oxidized (intramolecular disulfide) state- thereby increasing $E$ ($-160$ to $-180$ mV: a better oxidizer/ tendency to be reduced). On the other hand, a proline in the same position with respect to TRX results in a decrease of $E$ ($-270$ mV: a better reducer/ tendency to be oxidized) [97-100]. The impact on $E$ is correlated directly with a change of thiol $pK_a$, dependent on the xx-intervening dipeptide sequence. The CGHC motif of PDI primes it as a better thiol-oxidase, in-part due to the His-Cys$_N$ (N-terminal Cys thiol) interactions. The electrostatic stabilization of the Cys$_N$-$S^\cdot$ consequently results in a low thiol $pK_a$ of about 4.5 [64]. Substitution to proline (reminiscent of TRX) increases the $pK_a$ to 6.6, rendering the enzyme a better reductase as the oxidized form is now more stable [64, 99]. The requirement of positively charged, ionizable groups at intervening positions for thiolate stabilization is not absolute. Aromatic residues also mediate thiolate stabilization by ring-edge ($\delta^-$) interactions [101-105].

The redox activity of enzymes functioning through a CxxC motif do not rely solely on the motif sequence itself (as discussed). Using the *E. coli* thioredoxin DsbA (a potent oxidizer with motif sequence: CPHC, $E = -121$ mV [105]) and TRX, Bardwell and coworkers demonstrated that mutations to TRX that resulted in either complementation or non-complementation to the intervening sequence of DsbA consistently resulted in increased $E$ values, with no discernable correlation in DsbA-like activity [101]. They went on to conclude that although the CxxC motif sequence is extremely important for biasing redox activity by affecting $E$, this is only a "prerequisite." The activity of thiol-disulfide oxidoreductases is largely impacted by other specific interactions that occur *in vitro* and *in vivo*, likely a result of protein architecture [101]. Even so, the intermediate
redox potential of PDI as compared to TRX and DsbA allows for its participation in a wider variety of thiol-disulfide exchange reactions [100]. The glutathione redox pool of the ER lumen has been most accurately estimated to average $-225$ mV, allowing for the prediction of a GSH:GSSG ratio of 35:1 [26]. This is in contrast to earlier reports estimating the redox poise of the ER lumen as being much more oxidizing with GSH:GSSG ratios of 1:1 to $\sim 5:1$ [26]. This would suggest that upon equilibration, redPDI would predominate over oxPDI within the ER lumen, purely from a thermodynamics standpoint [26]. Indeed, this is observed [12, 90, 106-109].

1.2.5 Physiological and Pathophysiological Roles of PDI

The physiological and pathophysiological events by which PDI has been found to be associated include NAD(P)H oxidase regulation [110], thrombus formation [111], and nitric oxide (NO) transport from red blood cells to the endothelium tissue in an oxygen-dependent manner [56, 112], among many others. Moreover, PDI has garnered much attention in view of its postulated roles in the promotion of pathogen internalization [113, 114], cancer [115], coagulation disorders [116, 117], and SNO-PDI (S-nitrosylated PDI) mediated protein aggregation in neuronal diseases including Alzheimer's, Parkinson's and Amyotrophic Lateral Sclerosis (ALS) [118]. The involvement of PDI in such an array of cellular processes renders it infeasible to appreciably review all current literature on the matter. Thus, a few primary examples will be briefly addressed herein.

Only recently has PDI become realized for its role in cancer progression. Neamati and coworkers assessed the upregulation of PDI in various cancer types using published microarray data in their most recent review [43]. Their findings indicated that in
comparison to normal, healthy cells, PDI is upregulated with a >2-fold change in brain, lymphoma, kidney, prostate, and lung cancers [43]. Furthermore, cell surface PDI has been linked to cancer invasion and metastasis, an event that can be inhibited through the use of PDI inhibitors and antibodies [43]. It is very possible that the onset of disease is not so much associated with PDI itself, rather it could be that the metabolic load of proliferating cancer cells is so high that it requires more PDI to facilitate the need for more protein folding. Neamati stated that because of this fact, cancer cells are much more sensitive to PDI inhibition [43].

Cell surface PDI is particularly important when it comes to thrombus formation and coagulation. Upon vascular injury, platelets and endothelial cells secrete PDI. One such role for PDI in thrombosis stems from its ability to influence the binding of von Willebrand factor of the injured vessel wall with the α-subunit of glycoprotein 1b (GP1bα) of the GP1b-IX-V receptor complex of activated platelets [58, 119]. On the cell surface of platelets or nucleated cells PDI catalyzes thiol-disulfide exchange to mediate the cleavage of disulfide bonds of GP1bα. Furthermore, PDI has been implicated in tissue factor decryption [120], factor XI activation [121] and the activation of integrin [58, 122, 123]. Recent findings using substrate-trapping mutants of PDI identified thrombus-specific substrates, such as plasma vitronectin [124], and others [99].

Neurodegenerative diseases such as ALS, Alzheimer's and Parkinson's are common in that they exhibit an abnormal accumulation of misfolded proteins. These misfolded proteins aggregate and form large inclusion bodies within and outside of cells, leading to plaques (aggregates of β-amyloid in Alzheimer's, α-synuclein in Parkinson's, and mutant superoxide dismutase (mtSOD1), in ALS disease patients). PDI, along with many other
chaperones, is used to prevent the aggregation of terminally misfolded proteins, primarily via a response called the unfolded protein response (UPR); an up-regulation of PDI [118, 125]. It has been shown that overexpression of PDI in neurodegenerative cell models of Parkinson's and ALS results in a decrease of respective aggregates, but overexpression in cancerous hepatocytes did not alleviate the levels of α1-antitrypsin inclusions [125]. This lead Grek and Townsend to suggest that cellular protection by PDI is disease and protein-specific [125]. Furthermore, the protective effects of PDI have been shown to be largely influenced by its localization [126] and oxidative stress levels within the cell [56, 125, 127]. PDI is primarily cell-protective for these reasons, but it has been speculated that its over-accumulation in response to stress initiates cell-death pathways [125, 128].

Events that lead to ER-stress, such as the accumulation of misfolded proteins, can initiate the UPR in two distinct ways. If the stress is transient and reversible, the response will be pro-protective. If the stress is chronic and irreversible the response will be pro-apoptotic. Neurodegeneration tends to follow the latter case [129]. Even though PDI has been classically described as cell-protective, S-nitrosylation is prevalent in neurodegenerative disease, a modification which acts to limit and even prevent its protective functions [127]. The pro-apoptotic role of PDI is furthered by its discovered ability to induce oxidative stress [130], as well as results that have indicated reduced cytotoxicity in Huntington's disease models via the selective inhibition of PDI [128, 131].

1.2.6 Recent Advances in PDI-Targeted Therapeutics and Activity Regulation

The role of PDI in disease is indicative of a need for commercially available potent and selective inhibitors. To date, no such therapeutics exist in the clinical setting [43].
Because PDI functions through its CxxC motifs and/or the hydrophobic surface about its U-shaped structure (especially via the \( b' \) hydrophobic cleft), inhibitors will often aim to target these sites. It is well understood that the catalytic activity of PDI may be attenuated or inhibited by reversible or irreversible thiol-modifying compounds. *In vivo*, increased levels of nitric oxide (NO) leads to the S-nitrosylation (Cys-SNO, see previous) of the thiols of PDI. This has been linked to neurodegenerative disorders [125]. As well, there are implications that the thiols of cellular PDI may be nitro-alkylated by nitroarachadonic acid [132]- but further investigations are required. Detailed in this section are some common inhibitors of PDI activity. The reader is directed to a recent publication by Neamati and coworkers for a more extensive review [43].

There exists a plethora of thiol-modifying small-molecules for the inhibition of PDI. Due to the chemistry of such compounds, they exhibit little to no specificity, but their ability to modify the active site thiols renders PDI catalytically inactive. Commonly used *in vitro* are iodoacetamide (IAM), \( N \)-ethylnaleimide (NEM) and 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB), which function to irreversibly alkylate free thiols. IAM and NEM are cell permeable [43]. Slightly more specific is phenylarsine oxide (PAO). This compound is cell permeable and targets CxxC motifs exclusively by forming an irreversible complex with the vicinal thiols. The family of propynoic acid carbamoyl methyl amides (PACMA) are able to react with the thiols of PDI and alter its secondary structure. PACMA 31 was found to have potency towards ovarian cancer *in vitro* and *in vivo*, with little cytotoxicity to normal, healthy cells [43, 133].

Antibodies, antibiotics and plant metabolites have also been shown to exude PDI inhibition. The very first reported inhibitor of PDI activity was bacitracin, a cyclic
dodecapeptide isolated from *Bacillus subtilis* [43, 134]. Isolation results in a complex mixture of bacitracin analogs, where bacitracin F has the lowest reported IC$_{50}$ at 20 µM. Bacitracin does not significantly inhibit PDI reductase, thiol-oxidase or isomerase activities [135]. This compound binds to the $b'$-domain of PDI through disulfide bond formation with the domain thiols of C$^{312}$ and C$^{343}$, resulting in attenuated chaperone activity [43, 136, 137]. Furthermore, the monoclonal anti-PDI antibody (RL90) has been shown to inhibit PDI activity with great specificity [80].

Even though PDI is implicated in many disease states, with its inhibition being cell-protective in certain cases, its role in physiological processes such as thrombus formation indicates that therapeutics need not only focus on its inhibition. Recent work by Flaumenhaft and coworkers has demonstrated a class of reversible therapeutics that paradoxically enhance PDI activity *via* binding to the $b'$-domain [76]. These compounds, bepristats, target the $b'$ binding cleft, acting as allostERIC switches to enhance the rate of reductive cleavage of di-E-GSSG, yet inhibit the reduction of insulin [76]. Researchers indicate that the mode of action be the movement of the $x$-linker from the "capped position" (blocking the hydrophobic cleft) to the "uncapped position." This result correlated strongly with other $b'$-directing agonists such as somatostatin, indicating that the conformational control of PDI is not only influenced by the redox environment (see section 1.2.1), but also the binding events at the $b'$-domain [76]. It is speculated that the augmented rate of oxidoreductase activity be a result of the conformational change promoting the reduced state of the PDI active sites. This is a recent example of how there remains a need to fully understand the means by which PDI functions both *in vitro* and *in vivo* with its various substrates.
1.3 $\text{N}^\varepsilon$-lysine Acetylation

Posttranslational modifications (PTMs) further the central dogma by effectively expanding structural and functional characteristics of any single gene product (protein). A hallmark of protein modifications is $O$-phosphorylation. Of the 461 reported PTMs, phosphorylation is the most well-studied and abundant [138]. The addition and removal of a charged phosphate to and from the hydroxyl-$O$ of serine, threonine and tyrosine residues is mediated by the vast family of protein kinases and protein phosphatases, respectively. An emerging PTM newly found to rival that of phosphorylation in terms of abundance is lysine acylation [139-141]. Acetylation, one such form of acylation (Figure 1.1), was first discovered in 1964 on histone lysine residues [142]. Since then the existence of lysine acetylation on thousands of proteins beyond histone and nuclear proteins has been described, leading to the realization of the acetylome [143].

$\text{N}^\varepsilon$-lysine acetylation is the transfer of an acetyl moiety from acetyl-CoA to the side chain amine ($\epsilon$-amine) of a protein's lysine residue. This results in a neutralization of the positive charge and added steric bulk. Acetylation is a very pertinent signalling event that works through the activity of "writers," "readers" and "erasers" [138]. The process occurs either enzymatically through the activity of lysine acetyltransferases (KATs, the "writers"), or non-enzymatically [144]. There are five distinct families of KATs: HAT1, Rtt109, Gcn5-related $N$-acetyltransferases (GNATs), E1A-associated protein of 300 kDa (p300)/CREB-binding protein (CBP), and MYST proteins. The MYST and GNAT families showcase an acetyl-CoA binding motif, while the p300/CBP family proteins do not [145]. "Readers" are any proteins influenced by acetylation, leading to an intracellular signaling event/ response.
Figure 1.11: $N^\varepsilon$-lysine acylation is a reversible modification that takes on many different forms. Acyl-CoA acts as the donor substrate for acylation to occur either non-enzymatically or via lysine acyltransferases to modify acceptor substrate lysine residues. Acyl-modification of lysine residues is reversed by the Sirtuin family of deacylase enzymes.
Proteins containing a bromodomain may be able to recognize acetylated residues, as has been implicated in histone and chromatin regulation [138]. "Erasers" mediate the removal of the acetyl moiety, such as the sirtuin family of deacetylases, akin to phosphatases with respect to dephosphorylation. The mechanism of deacetylation varies depending on the deacetylase enzyme: NAD$^+$-dependent or zinc-containing [146].

Each KAT has a common structural core region for acetyl-CoA and substrate binding (β-sheet-helix), but there is little sequence homology amongst them. Furthermore, KAT substrate specificity is defined by the flanking regions about the common core [147]. It has been found that the various KATs also employ very different enzymatic mechanisms for the acetylation of their respective acceptor substrate proteins [147].

Only recently has acetylation been identified specifically to occur within the ER. Research has demonstrated the presence of two ER-resident KATs of the GNAT family [148]. These isoforms, N-acetyltransferase 8B (ATase-1) and N-acetyltransferase 8 (ATase-2) are ER luminal transmembrane KATs containing a single transmembrane domain. They may exist as monomers, homodimers or heterodimers [148]. The mechanism of catalysis is not understood for these KATs, but an estimation can be made based on their relation to other GNAT family proteins such as Gcn5/ PCAF.

The reaction likely initiates once both acetyl-CoA and the substrate protein are bound to the enzyme (a bi-bi ternary complex, and not through a ping-pong mechanism). The acceptor lysine is deprotonated by a general base catalytic mechanism, rendering it a suitable nucleophile for accepting the electrophilic carbonyl carbon of the acetyl moiety [144, 147]. Substrate recognition cannot be speculated due to structural variance among
the family members. Proteomic efforts have explored the possibility of there being a recognition motif for acetylation, though more research is required [149, 150]. Acetylation as a PTM does not solely rely on the work of "writers." The process has been described as occurring non-enzymatically due to the inherent reactivity of the thioester linkage present in acetyl-CoA (or any acyl-CoA for that matter) [144].

1.4 Research Objectives and Rationale

There is increasing evidence regarding the importance of PDI in physiology and disease. Despite its scrutiny since the 1960's little is known regarding the regulation of this enzyme. Recently unpublished work performed by our group showed that the lysine-directing fluorophore, eosin-5-isothiocyanate (EITC), abolished PDI oxidoreductase activity when carrying out in vitro treatment at as low as a 2:1 molar ratio (EITC: PDI). In conjunction with these findings, Kimura et al. highlighted the presence of a highly conserved lysine residue found neighbouring immediately downstream the CxxC motif of many PDI family members [55] (refer to Figures 1.5 and 1.6). In the same work, they showed that a CxxCK active site motif resulted in enhanced rates of isomerase activity. The human PDI family member PDIA6, regarded as hP5, has two CxxC motifs- the first is CGHCQ and the second is CGHCK. Interestingly, they demonstrated that substitution of Gln in the first active site to Lys resulted in a 45% increase of isomerase activity [55]. Involvement in such an array of cellular processes tends to be indicative of a need for posttranslational regulation and subsequently, the modulation of activity. This is especially true for PDI when considering its ubiquitous nature, lengthy half-life and high expression levels at up to 1% the total cellular protein content of certain cell types [43].
The goal of this research project is to characterize the oxidoreductase, thiol-reductase and thiol-oxidase activities of hPDI in relation to the conserved active site lysine residues Lys$^{57}$ and Lys$^{401}$. Site directed mutagenesis of these residues will be performed individually and simultaneously to the non-conservative amino acids glutamine (Gln), alanine (Ala) and glutamic acid (Glu). A focus will be placed on the Gln mutants of PDI as it is believed that this mutation most closely mimics the highly abundant and physiologically relevant posttranslational modification of $N^\epsilon$-lysine acetylation (a neutral, yet still polar acetyllysine residue: acK). That being the case whilst limited to canonical amino acid substitution. Research in the lab of Dr. Luigi Puglielli has linked lysine acetylation to the ER and Golgi intermediate complex [151], where it has been shown that an acetyl-CoA transporter and two distinct ER-luminal transmembrane acetyltransferases are expressed [148, 152]. Proteomic assessment has identified PDI and many other ER-resident proteins as being targets of lysine acetylation [153, 154].

Physiological significance will be addressed by assessing the interaction of ERO1$\alpha$ with wild type and mutant PDI enzymes. Furthermore, the acetylation of PDI will be performed using acetylsalicylic acid- the active ingredient in Aspirin. Mass spectrometry will elucidate the target residues of in vitro acetylation, complemented by steady state activity assays to discern the general impact of this modification. Structural analyses will be performed in order to propose a mechanism by which lysine acetylation of residues Lys$^{57}$ and Lys$^{401}$ affects the catalytic activity of PDI, with reference to other PDI family member proteins.
CHAPTER 2

MATERIALS AND METHODS
2.1 Chemicals and Materials Tabulated

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<tr>
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<td>N0636</td>
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<tr>
<td>Protease Inhibitor Cocktail</td>
<td>P8340</td>
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<td>G2580</td>
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<td>T1952</td>
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| Maxi-Prep Plasmid Kit                            | 12162 |

*Ms: Mouse
2.2 Methods

2.2.1 Site Directed Mutagenesis

Mutagenesis of residues Lys$^{57}$ and Lys$^{401}$ was carried using the Q5® site-directed mutagenesis kit (NEB). Primers were designed end-to-end wherein the forward contained the mutagenic sequence that resulted in a single amino acid substitution to either Ala, Gln, or Glu. Similarly, mutagenesis of the active site vicinal thiols (Cys$^{53}$ and Cys$^{56}$; Cys$^{397}$ and Cys$^{400}$) followed similar principles. The polymerase chain reaction (PCR) conditions were optimized for each primer set (appendix A Table A.1). Thermal cycling was performed using a BioRad® T-100 Thermal Cycler carrying out 25 cycles of denaturation at 98 °C for 10 seconds, primer annealing (variable temperatures; appendix A Table A.1) for 30 seconds, and plasmid elongation and extension at 72 °C for 4.5 min. The resulting PCR products were treated with a kinase-ligase-DpnI (KLD) enzyme mix as per the manufacturer's instructions. Plasmids were transformed into NEB® 5-alpha competent E. coli cells using the heat-shock method. Transformed cells were then plated onto LB agar containing kanamycin antibiotic (LB-Kan 50 µg/mL) and grown overnight at 37 °C. Individual colonies were selected, inoculated in liquid LB-Kan for overnight growth, then used for plasmid isolation via a standard phenol-chloroform bacterial miniprep procedure (Qiagen, see below). Isolated plasmids were sequenced by Robart's Research Institute (London Regional Genomics Center, London, Ontario, Canada) to identify the successful mutagenesis of the doubly His$_6$-tagged (N- and C-termini) hPDI gene present in a pET-28b(+) vector. The resulting mutants were as follows; K57A, K57Q, K57E, K401A, K401Q, K401E, K57/401A, K57/401Q, and K57/401E for the kinetic analysis of hPDI activity in relation to a CxxC-neighbouring lysine residue. As
well, the vicinal active site thiols of each active site (α and α'-domain) were substituted to alanine residues for the determination of the reductive potential on a per-active site basis. These consisted of CxxC(K/Q)\(^{57}\)-AxxA(K/Q)\(^{57}\) and CxxC(K/Q)\(^{401}\)-AxxA(K/Q)\(^{401}\) mutant enzymes, as well as a control CxxCK-AxxAK mutation to both active sites simultaneously resulting in a catalytically inactive enzyme.

### 2.2.2 Bacterial Plasmid Miniprep

Isolation of plasmid DNA from *E. coli* was adapted from a standard protocol (Qiagen). 1.5 mL of previously transformed cells were harvested by centrifugation at 12,000 \(\times\) g for 1 min. The cell pellet was resuspended in 100 µL of buffer P1 (25 mM Tris-HCl pH 8, 50 mM glucose, 10 mM EDTA). To this, 200 µL of buffer P2 (0.2 M NaOH, 1% SDS) was added, followed by neutralisation with 150 µL buffer P3 (3 M NaOAc pH 5.2). The solution was kept on ice for 20 min prior to centrifugation at 12,000 \(\times\) g for 10 min at 4 °C. The supernatant was collected and 400 µL of phenol:chloroform isoamylalcohol was added. The solution was vortexed and centrifuged at 12,000 \(\times\) g for 1 min. The aqueous phase layer was collected and 800 µL ice cold anhydrous EtOH was added to it. The sample was vortexed and centrifuged at 12,000 \(\times\) g for 10 min at 4 °C. The pelleted plasmid DNA was rinsed with 70% ice cold EtOH, dried, then reconstituted in TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA). Purity and concentration was assessed using a NanoDrop 2000 spectrophotometer (ThermoFisher).
2.2.3 Protein Purification and Standardization

2.2.3.1 Human Protein Disulfide Isomerase (hPDI)- WT and Mutants

WT and mutated hPDI plasmid constructs, as described previously, were used to transform competent BL21 (DE3) E. coli cells (NEB) via the heat-shock method. Successfully transformed cells were grown overnight at 37 °C in 100 mL 2×YT media containing kanamycin (2×YT-Kan: 50 µg/mL). Fresh 1.5 L of 2×YT-Kan was inoculated with overnight culture and grown at 37 °C until a cell density (O.D_{600}) of 0.6 was reached. Expression of PDI was then induced via the addition of 1 mM IPTG with incubation for 4 hours at 37 °C. Cells were harvested and resuspended in lysis buffer (50 mM Tris pH 8.0, 1mM NaCl, 2 mM EDTA, 1% Triton X-100, 2 mM PMSF, 125 µg/mL lysozyme, and 75 µg/mL DNase) followed by 10 rounds of sonication on ice at 20 second pulses (Sonic Dismemberator, Fisher Scientific). Cellular lysates were clarified by centrifugation at 12,000 ×g for 30 min. Clarified lysates were passed over a gravity-fed HIS-Select™ Ni-affinity resin (Sigma) with a 5 mL bed volume and subsequently washed with 3 column volumes of wash buffer (50 mM phosphate pH 8, 150 mM NaCl) containing 10 mM imidazole, followed by another wash using 2 column volumes of buffer containing 40 mM imidazole. His_{6}-PDI was eluted using a 500 mM imidazole buffer. The eluted sample was incubated with 100 mM DTT for 20 min on ice, then desalted and buffer exchanged with PDI assay buffer (0.1 M potassium phosphate pH 7.4, 0.1 mM DTPA) using Amicon® ultracentrifuge filter units (MWCO: 30 kDa) as per the manufacturer's instructions (EMD). Protein concentration was determined using the BCA assay [155], and purity was assessed via SDS-PAGE (refer to appendix A Figure
A.2). Enzyme preparations were aliquoted, snap-frozen in liquid nitrogen and stored at −80 °C.

For kinetic studies of PDI, the functional concentration of all enzymes was determined through the use of the di-E-GSSG assay (see below) by analyzing the burst phase kinetics. Under standard conditions, 10 µL of affinity purified PDI was incubated with 800 nM di-E-GSSG in PDI assay buffer and allowed to react for 20 min, or until maximum fluorescence was reached (indicating that all catalytically active enzyme had reduced di-E-GSSG to EGSH. Refer to appendix A Figure A.3). The resulting fluorescence was plotted against a standard curve to determine the concentration of active (functional) PDI on a per-active site basis.

For the determination of successful mutagenesis of the active site vicinal thiols of the CxxC-AxxA mutant enzymes, the di-E-GSSG assay was employed. In relation to WT PDI, the activity of the CxxCK\textsuperscript{57}-AxxAK\textsuperscript{57}, CxxCK\textsuperscript{401}-AxxAK\textsuperscript{401} and CxxCK\textsuperscript{57/401}-AxxAK\textsuperscript{57/401} mutants was determined. This involved measuring the activity of 10 nM of PDI with 800 nM of the di-E-GSSG substrate. In a similar fashion, the activity of the CxxCQ\textsuperscript{57}-AxxAQ\textsuperscript{57} and CxxCQ\textsuperscript{401}-AxxAQ\textsuperscript{401} mutants was assessed in relation to the K57/401Q variant bearing all intact active site thiols, the template to which the aforementioned mutants were engineered (appendix A Figure A.4).

2.2.3.2 Endoplasmic Reticulum Oxidoreductin-1α (ERO1α)

Recombinant His-tagged ERO1α was graciously provided by Dr. Nagata. In this study, a constitutively active human ERO1α mutant (C104/131A) was used [84]. Purification followed an adapted protocol from both Araki and Nagata [84] and that of
the hPDI purification procedure described. BL21 (DE3) pLysS E. coli cells (NEB) containing the his-tagged ERO1α plasmid construct were grown overnight in the dark in 100 mL 2×YT media containing ampicillin and chloramphenicol antibiotics (2×YT-AmpCam; 100 and 25 µg/mL, respectively) and 10 µM FAD. 1.5 L of fresh 2×YT-AmpCam media (ampicillin: 50 µg/mL, chloramphenicol: 12.5 µg/mL, and 10 µM FAD) was inoculated at 37 °C with the overnight culture. At a cell density (OD$_{600}$) of 0.8, ERO1α expression was induced using 0.5 mM IPTG for 16 hours at room temperature in the absence of light. Cells were harvested, lysed in the presence of 5 µM FAD, and Ero1α was isolated and purified as per the methods described previously for the purification of hPDI. Once eluted from the Ni-affinity chromatography column, Ero1α was completely oxidized using 20 mM potassium ferricyanide (KFe(CN)$_6$) for 20 min on ice. After desalting and buffer exchange the isolated enzyme was stored at -80 °C in storage buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol). Protein concentration was determined using the BCA assay [155], and purity was assessed via SDS-PAGE (appendix A Figure A.5).

To determine the relative purity of the holoenzyme (FAD-bound ERO1α) an absorbance spectrum of the purified protein was taken in 6 M guanidine-HCl. Using the characteristic absorbance peak at 440 nm and (ε: 12.5 mM$^{-1}$ cm$^{-1}$) [92], purity of the FAD-bound enzyme was determined to be > 92%.

2.2.3.3 Green Fluorescent Protein (GFP)

Recombinant His-tagged GFP was prepared as per the protocol described for hPDI with some modifications. The entirety of the procedure was performed in the dark to
maintain the integrity of the intrinsic GFP fluorescence. Briefly, BL21 (DE3) *E. coli* cells were grown in fresh 2×YT media containing 100 µg/mL ampicillin to a density (OD$_{600}$) of 0.6. His-GFP expression was induced for 4 hours at 37 °C using 1 mM IPTG. After isolation and purification of his-GFP using Ni-affinity chromatography, the protein preparation was desalted and buffer exchanged to GFP storage buffer (50 mM Tris-HCl pH 7.4, 0.3 mM EDTA) using Amicon® ultracentrifuge filter units (MWCO: 10 kDa). Protein concentration was determined using the BCA assay [155], and purity was assessed via SDS-PAGE. UV-Vis and fluorescence spectra of the purified protein were recorded (appendix A Figure A.6).

2.2.3.4 *N*-acetyltransferase-1 and -2 (ATase-1 and ATase-2)

Maltose Binding Protein (MBP) fusion proteins of ATase-1 and ATase-2 were graciously provided by Dr. Luigi Puglielli. Purification of MBP-ATase followed the procedure previously described with some modifications [156]. Rosetta (DE3) pLysS *E. coli* cells were grown at 37 °C overnight in 100 mL of fresh 2×YT media containing ampicillin and chloramphenicol antibiotics (2×YT-AmpCam; 100 and 25 µg/mL, respectively) and 0.2% glucose. 1.5 L of fresh 2×YT AmpCam media (with 0.2% glucose) was inoculated with the overnight culture and grown at 37 °C until a density (OD$_{600}$) of 1.0. MBP-ATase expression was induced by adding 0.5 mM IPTG followed by incubation for 18 hours at room temperature. Cells were harvested by centrifugation at 4 °C and lysed as described previously for the purification of hPDI. Whole cell lysates were incubated with 3 mL of amylose resin for 2 hours at 4 °C. The lysate-amylose mixture was transferred to a glass-walled Econo-Column® (BioRad) for the purposes of
gravity-fed washing and elution of MBP-ATase. Amylose resin was washed with 5 column volumes of AT buffer (20 mM HEPES pH 8, 150 mM NaCl, 1 mM EDTA). MBP-ATases were eluted from the resin using AT buffer containing 30 mM maltose. The enzyme preparation was then desalted and buffer exchanged to AT buffer using Amicon® ultracentrifuge filter units (MWCO: 30 kDa). Protein concentration was determined using the BCA assay [155], and purity was assessed via SDS-PAGE (appendix A Figure A.7).

Acetyltransferase activity was assayed using a commercially available histone acetyltransferase (HAT) inhibitor screening kit (Cayman Chemicals). The adapted method involved using affinity purified MBP-ATase enzyme as donor of the acetyl group from acetyl-CoA to an acceptor substrate, that being a human histone H3 peptide. The assay consisted of incubating various concentrations (10 nM to 2 µM) of ATase-1 or ATase-2 with 50 µM acetyl-CoA and the H3 peptide (appendix A Figure A.7). Reactions were carried out for 1 hour at 30 °C, quenched with ice cold isopropanol, and activity was quantified using a thiol-reactive CPM molecule that becomes fluorescently active upon reaction with free CoASH (see manufacturer's notes).

2.2.4 Dieosin Glutathione Disulfide (di-E-GSSG) Synthesis and Standardization

Synthesis of the dieosin glutathione disulfide (di-E-GSSG) self-quenching pseudo substrate of PDI was performed using an adapted protocol from Raturi et al. [82]. All steps were performed in the dark to limit photo bleaching of the eosin fluorophore. 30 mg of oxidized glutathione (GSSG) was dissolved in 1.5 mL of 100 mM ammonium bicarbonate buffer (pH 9) containing 2 mM EDTA. A 5-fold excess of eosin-5-isothiocyanate (EITC; 173 mg) was dissolved in 5 mL of DMSO and added to the GSSG
solution dropwise with stirring. The reaction was then brought to a final volume of 15 mL through the addition of 100 mM ammonium bicarbonate (pH 9) containing 2 mM EDTA. Protected from light, the reaction proceeded overnight at 4 °C. The labelling reaction was quenched by snap freezing in liquid nitrogen, then lyophilized to a dry, red, flakey powder. This was then dissolved in a minimal amount of milli-Q grade water (1-2 mL). The concentrate was then applied to a long-body sephadex G-25 column (~123 mL bed volume; height: 25 cm, diameter: 2.5 cm) with a flow rate of 0.5 mL per min (controlled by a Model EP-1 EconoPump, BioRad) using milli-Q grade water as the eluent. The progress of gel-filtration was monitored by observing the elution profile using the BioLogic LP system (BioRad) by which fractions of the first elution peak were collected as product di-E-GSSG. It should be noted that free, unreacted EITC binds to the sephadex resin resulting in convenient isolation of the desired product. Furthermore, G-25 gel filtration is an appropriate means of sample workup as separation based on hydrodynamic volume means di-E-GSSG may be reliably obtained as the first eluate. A total of 192 fractions of 1 mL volume were collected. The presence of di-E-GSSG was confirmed by assessing both the self-quenching properties and the fold-increase of fluorescence for each fraction. Using black, opaque fluorescence-optimized 96-well plates (Corning™), the fluorescence of 50 µL of each fraction was analyzed before and after the addition of 1 M DTT (λ_{ex}: 525 nm; λ_{em}: 545 nm) using a Wallac 1420 Victor3 Microplate Reader (Perkin Elmer). Incubation with DTT was for 30 min to ensure complete reduction of the probe. Fractions that exhibited a >30-fold increase of fluorescence were pooled, snap-frozen in liquid nitrogen, and lyophilized. The resulting dry powder was then reconstituted in milli-Q water to a stock concentration of 375 µM.
using \( \varepsilon: 176,000 \text{ M}^{-1} \text{ cm}^{-1} \) at 525 nm. Aliquots of the stock solution were then snap-frozen in liquid nitrogen and stored at \(-80^\circ\text{C}\).

### 2.2.5 Synthesis of Gold Nanoparticles (AuNPs)

Synthesis of AuNPs followed the procedure described by Freeman et al. [157]. Briefly, 200 mg of gold (III) chloride trihydrate was dissolved in 500 mL of high purity milli-Q water in a 1 L double-neck round bottom flask. The solution was left to reflux with vigorous stirring for 1 hour in a hot oil bath \((100^\circ\text{C})\). Next, 600 mg of sodium citrate dihydrate was dissolved in 50 mL of milli-Q water then added quickly to the gold chloride solution. The reaction was allowed to proceed for 10 min before being removed from the heat and cooled to room temperature. The formation of a colloidal solution was realized by the change in colour from clear to deep purple, exhibiting a characteristic absorbance maxima at 529 nm (appendix A Figure A.8).

### 2.2.6 Preparation of CNBr-activated and Epichlorohydrin-activated Mercury Sepharose

Generation of activated sepharose herein was performed by Milosz Brzozowski of the Mutus research group (University of Windsor, Windsor, Ontario, Canada).

The preparation of cyanogen bromide (CNBr) activated organomercurial sepharose (Hg-sepharose) followed an adapted procedure from Wilchek et al. and Sluyterman and Wijdenes [158, 159]. First, 10 mL of packed Sepharose 6B was washed with 100 mL ddH\(_2\)O and then suspended in a beaker on ice with 20 mL ddH\(_2\)O to maintain the reaction temperature at 15 \(^\circ\text{C}\). To the suspension was added 1 g of CNBr and a pH of 11 was
maintained throughout the reaction with the drop wise addition of 2 M NaOH. Once the pH was stabilized the CNBr-activated Sepharose 6B beads were washed with 200 mL of 0.1 M NaHCO₃ by vacuum filtration. The beads were then suspended in 20 mL of a 10% DMSO solution and 0.15 g of 4-aminophenylmercuric acetate (APMA) dissolved in DMSO was added to the activated bead solution. The CNBr-activated Sepharose 6B and APMA were tumbled gently overnight at 4 °C to ensure completion of the reaction. The resulting CNBr-activated Hg-sepharose was then brought to room temperature and washed with 20% DMSO to remove any remaining unreacted APMA, and then washed with 200 mL of 100mM EDTA (pH 8.0) to chelate any free mercury. Lastly, the beads were washed with 200 mL ddH₂O. This procedure was repeated four times to produce approximately 50 mL of packed CNBr-activated Hg-sepharose.

An alternative approach was taken, utilizing the activating compound epichlorohydrin (Epi) in place of the more expensive and toxic CNBr reagent. Preparation of Epi-activated Hg-sepharose followed the synthesis as performed by Matsumoto et al. with some alterations [160]. First, 12 mL of packed Sepharose 6B was washed with 100 mL ddH₂O and suspended in 35 mL of a 0.4 M NaOH solution. The Sepharose 6B solution was then reacted with 1.5 mL Epi and tumbled gently at 40 °C for 2 hours in order to encourage homogeneous mixing of the Epi reagent. The Epi-activated Sepharose 6B beads were brought to room temperature before washing with 200 mL ddH₂O. The resin was then suspended in 15 mL of 0.1 M NaOH (pH 10.5) and reacted with 0.15 g APMA (dissolved in DMSO) by tumbling gently for 24 hours at 40 °C. The resulting Epi-activated Hg-sepharose was washed with 200 mL ddH₂O. This procedure
was repeated three times to produce approximately 40 mL of packed Epi-activated Hg-sepharose.

2.2.7 In Vitro Kinetic Assays

Herein, all respective kinetic assays described were performed at 22 ± 2 °C using fresh enzyme having gone through no more than 1 freeze-thaw cycle. Furthermore, each respective assay was performed in parallel with the WT or untreated control to add to the validity of the results. Data are reported as the mean and standard deviation (± S.D.) of n replicates. All fluorescence data was collected using a Cary Eclipse fluorescence spectrophotometer (5 nm slit width and medium gain settings; Agilent Technologies), unless otherwise stated.

2.2.7.1 Steady State Oxidoreductase di-E-GSSG Assay

The kinetic assay used to measure the oxidoreductase activity of PDI was performed in PDI assay buffer of varying pH at a final volume of 500 µL. PDI (10 nM) was added to a cuvette containing 10 µM dithiothreitol (DTT) and varying concentrations of di-E-GSSG (25 nM to 5 µM). The reduction of di-E-GSSG was monitored for 60 seconds and the initial rate of EGSH production was determined from the linear portion of the curve (RFU vs. time; λex: 525 nm, λem: 545 nm). Kinetic data was fitted to a Michaelis-Menten hyperbolic curve (Equation 2.1) using non-linear regression analysis [161] in Excel with the Solver data analysis tool.
pH-dependence experiments were performed under steady state conditions using a single di-E-GSSG concentration of 800 nM. The data was fitted to a two-pKa bell shaped curve (Equation 2.2).

\[
v = \frac{10^{pH-pK_1}}{(1 + 10^{pH-pK_1})} - \frac{10^{pH-pK_2}}{(1 + 10^{pH-pK_2})}
\]

Equation 2.2

Where \( v \) represents the rate of fluorescence increase at any given pH.

Mathematical simulations of the pH-dependence of PDI activity were performed using Equation 2.3. Here, \( pK_1 \) represents the average value of the experimentally determined macroscopic \( pK_a \) of the ascending limb of all PDI variants tested (Figure 3.1), \( x \) is the factor (between 0 and 1) of relative contribution to di-E-GSSG reduction kinetics with respect to the active site lysine residues (where 0 would represent no alteration and 1 would represent complete removal, i.e. mutation), \( pK_x \) is the average value of the experimentally determined \( pK_a \) of the descending limb of the K57/401(Q/A/E) mutants studied, and \( pK_K \) is the value of \( pK_2 \) of WT PDI as determined experimentally using Equation 2.2.

\[
v = \frac{10^{pH-pK_1}}{(1 + 10^{pH-pK_1})} - \left( x \cdot \frac{10^{pH-pK_x}}{(1 + 10^{pH-pK_x})} \right) + \left( (1-x) \cdot \frac{10^{pH-pK_K}}{(1 + 10^{pH-pK_K})} \right)
\]

Equation 2.3
2.2.7.2  Single Turnover Thiol-reductase di-E-GSSG Assay

The single-turnover rate of PDI was measured under pseudo-first order conditions; 80 nM of PDI was incubated with 1 µM di-E-GSSG in the absence of DTT. The reaction was monitored until completion at varying pH. A time-course fluorescence increase was related to the consumption of di-E-GSSG over time, resulting in exponential decay plots which were fit to Equation 2.4.

\[
[\text{di} - \text{E} - \text{GSSG}]_f = [\text{di} - \text{E} - \text{GSSG}]_o e^{-k_{obs}t}
\]

*Equation 2.4*

Where \([\text{di}-\text{E}-\text{GSSG}]_f\) and \([\text{di}-\text{E}-\text{GSSG}]_o\) represent the final and initial concentrations of the substrate over the course of the reaction, respectively. The observable rate, \(k_{obs}\), was taken as the slope of the natural log of the integrated rate law above (*Equation 2.4*).

2.2.7.3  Thiol-oxidase Assay

PDI-catalyzed dithiol-oxidation was monitored using the assay described by Ruddock *et al.* [83]. The synthetic decapeptide substrate; NRCSQGSCWN, was synthesized by CPC Scientific (Sunnyvale, California, USA) as a trifluoroacetic acid (TFA) salt (MW: 1154.3, purity: 97.4%). A stock solution was made at 1.71 mM (confirmed by absorbance at 290 nm; \(\varepsilon = 5600 \text{ M}^{-1} \text{ cm}^{-1}\)) by reconstituting the peptide in 30% acetonitrile with 0.1% TFA and stored at -80 O°C. PDI enzymes were oxidized with 10 mM GSSG for 1 hour at room temperature prior to performing the experiment. Removal of excess GSSG was performed using Zeba™ Spin Desalting columns as per the manufacturer's instructions. A 250 µL reaction volume was utilized. In a cuvette, 2 µM PDI and 1 mM GSSG were mixed in PDI assay buffer of varying pH. The reaction was initiated by the addition of 50 µM of the peptide substrate and the rate of oxidation...
was monitored by fluorescence ($\lambda_{ex}: 280$ nm, $\lambda_{em}: 360$ nm). Data analysis was performed as previously described [83]. The rate of oxidation was determined as the inverse half-time of reaction; that is, the inverse of the time at which mid-point fluorescence was reached. The mid-point fluorescence of the reaction was taken as the mean of the initial and final fluorescence outputs.

2.2.7.4 ERO1α Oxidase Assay

ERO1α activity was measured using an oxygen consumption assay monitored by an Oxygraph Plus oxygen electrode unit (Hansatech) with a stirrer speed of 70%. The electrode was calibrated as per the manufacturer's instructions. Experiments were performed in air-saturated ERO1α assay buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA) at pH 7.4 in a final reaction volume of 800 µL. Air-saturation of the buffer was completed by bubbling with air for 30 min prior to each experiment. Catalytic oxygen consumption was initiated by the addition of 2 µM ERO1α to a reaction mixture containing 5 µM PDI and 20 mM GSH. The time-course of the reactions were reported as a percentage of oxygen in solution to provide a semi-quantitative analysis of the interaction between ERO1α and the PDI enzymes. Reactions were performed in the dark to prevent the photo-degradation of the FAD cofactor. The initial linear portion of the curve was used to report observable rates as determined by the absolute slope of the best-fit line ($R^2 \geq 0.99$).

2.2.8 pH-Induced Substrate Trapping

To trap PDI in a mixed disulfide state with the fluorescent eosin-glutathione substrate (EGSH), iodoacetamide (IAM)-mediated thiol blocking was utilized. Under
similar steady state conditions as employed previously, 150 nM PDI was incubated with 5 µM di-E-GSSG in PDI assay buffer of varying pH containing 10 µM DTT. The reaction was quenched at defined time points by the addition of 30 mM IAM for a 20 min incubation at room temperature. Samples were desalted and buffer exchanged to PDI assay buffer pH 7.4 using Zeba™ Spin Desalting columns (MWCO: 7 kDa) as per the manufacturer's instructions. Samples were then loaded to a black, opaque fluorescence-optimized 96-well plate (Corning™) for fluorescent measurements using a SpectraMax® M5E microplate reader. The fluorescence readings were related to an EGSH standard curve to quantify the amount of PDI trapped in a mixed-disulfide intermediate state.

2.2.9 Chemical Acetylation of PDI

\( N^\varepsilon \)-lysine acetylation of PDI was carried out using acetic anhydride (AA) and acetylsalicylic acid (ASA). For acetylation by AA, 2 µM PDI was diluted in 100 mM sodium bicarbonate buffer pH 8.5 and incubated with 450 µM AA (7 mM stock solution in acetonitrile) for 1 hour at room temperature in the presence of 0.5 mM DTT. This reaction represents ~1:1 molar ratio between PDI lysine residues and AA. The use of ASA for acetylating PDI was performed at 37 °C for 4 hours in a 0.1 M Tris-HCl buffer pH 8.5 using 0.1 to 15 mM ASA in the presence of 0.5 mM DTT.

To quench the acetylation of primary amines and to revert undesirable O-acetylation, hydroxylamine (HA) was added [162-167]. HA-mediated acyl removal was accomplished by adding 100 mM HA to the acetylation mixture and allowing it to react for 30 min at room temperature. Acetylated PDI was desalted and buffer exchanged to 50 mM ammonium bicarbonate (ABC) buffer using Zeba™ Spin Desalting columns.
(MWCO: 7 kDa). Lysine acetylation was confirmed by Western blot analysis and ultra-high-performance liquid chromatography mass spectrometry.

2.2.10 Western Blotting

All primary and secondary antibodies were utilized as dilutions in 1X TBST (tris-buffered saline 0.1% Tween-20) containing 1% skim milk. Primary antibodies (mouse IgG, see section 2.1) used to probe for acetylated lysine residues (acK) and PDI were used at 1:1000 and 1:3000 dilutions, respectively. Secondary antibody conjugated to horseradish peroxidase (HRP; goat α-mouse, see section 2.1) was used at a dilution of 1:5000.

Western blotting was performed on 10% or 12% SDS-PAGE with transfer to a PVDF membrane. Membranes were blocked with 5% skim milk in 1X TBST for 1 hour at room temperature. Primary antibodies were then added and incubated overnight at 4°C with gentle rocking. Before the addition of the secondary antibody, membranes were washed for 5 min with 1X TBST at room temperature, this was repeated 3 times. Secondary antibody incubation was for 1 hour at room temperature with gentle rocking. Prior to imaging, the membranes were washed with 1X TBST as described previously. Detection of chemiluminescence was performed using a FluoroChem® Q quantitative imaging system (Alpha Innotech).
2.2.11 High Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry (HPLC-ESI MS)

2.2.11.1 Proteolysis and Sample Preparation

Digestion of affinity purified hPDI followed an in-solution protocol using Glu-C endopeptidase. 100 µg of PDI was diluted 1:1 with 50 mM ammonium bicarbonate buffer pH 8.0 (ABC, unadjusted) containing 4 M urea. To this, 2 mM DTT was added and allowed to fully reduce PDI disulfides for 30 min and 60°C. The fully reduced PDI preparation was desalted and buffer exchanged to 25 mM ABC pH 8.0 using Zeba™ Spin Desalting columns (MWCO: 7 kDa). Lyophilized Glu-C (10 µg; Promega) was reconstituted in 25 mM ABC pH 8.0 to 0.1 µg/µL. 30 µg of PDI was then digested with Glu-C at a protein:protease ratio of 10:1 for 16 hours at room temperature with shaking. Proteolysis was quenched by the addition of 1% formic acid (FA) and the peptides were desalted using C₁₈ ZipTips® (EMD Millipore) according to the manufacturer. Peptides were dried and resuspended in 0.1% FA for ESI-MS analysis. An unquenched fraction of the digest was saved for the purpose of enrichment of thiol-containing peptides.

For the enrichment of thiol-containing peptides, a procedure was first used to test the efficiency thiol-protein binding of the prepared AuNPs and Hg-sepharose [168]. Briefly, 500 µL of AuNPs were collected by centrifugation at 15 000 ×g for 15 min. Similarly, 100 µL of a ~50% slurry of Hg-sepharose (Epi- or CNBr-activated) and 100 µL of unmodified sepharose 6B (~60% slurry; control) was collected by centrifugation at 5000 ×g for 1 min. The supernatant was removed from the collected AuNPs and sepharose supports and 30 µg of native redPDI (50 µL of a 0.6 µg/µL stock) was added and incubated for 30 min at 37°C. The solutions were then appropriately centrifuged to
isolate the supernatants. The pelleted matrices (AuNPs and sepharose) were washed 3 times with 200 µL of 25 mM ABC buffer pH 8.0 (unadjusted). To elute PDI from the AuNPs and sepharose beads, 25 µL of 100 mM DTT (in ddH2O) was added followed by sonication in a water bath for 5 min. Following sonication, the suspensions were incubated with DTT for 30 min at 37 °C with occasional mixing. The suspensions were centrifuged and the elution fraction saved for analysis. This was repeated 2 times, with all eluted fractions being pooled. Various fractions were assessed using SDS-PAGE, indicating the Epi-activated Hg-sepharose resin as being the most efficient method for isolation of PDI via thiol-enrichment (appendix A Figure A.9). For the enrichment of thiol-containing peptides, a similar procedure was followed, except 30-50 µg of an unquenched Glu-C digest of redPDI was applied.

2.2.11.2 Data Acquisition

Mass spectrometry was performed by Dr. Janeen Auld (University of Windsor, Windsor, Ontario, Canada).

Samples were analyzed using a Waters SYNAPT G2-Si time-of-flight mass spectrometer configured for nano-ESI operated in positive-ion mode coupled to a Waters nanoACQUITY UPLC system (UPLC-ESI MS). The UPLC was configured for 1D single pump trapping with a Waters ACQUITY UPLC Symmetry C18 2G V/M trap column coupled with a Waters ACQUITY Peptide BEH C18 with 0.5 to 1 µL sample injections. Mobile phase buffer A was 0.1% formic acid (FA). Mobile phase buffer B was acetonitrile with 0.1% FA. Samples were loaded on the trap column for 3 min at a flow rate of 5 µL/ min in 97% buffer A, 3% buffer B. A 7-step, 60 min gradient was run at a flow rate of 0.3 µL/ min where buffer A concentration was 97% at 0 min to 90% at 5
min, 75% at 33 min, 50% at 41 min, 15% at 42 min to 44 min, and 97% at 45 min to 60 min. Samples were first analyzed in MS\textsuperscript{e} mode prior to MS/MS sequencing. MS/MS followed a data-dependent acquisition (DDA) method by targeting the precursor ions of interest identified by MS\textsuperscript{e}.

\subsection{Molecular Dynamics Simulations}

Computational analyses were performed by Wanlei Wei and Paul Meister of the Gauld research group (University of Windsor, Windsor, Ontario, Canada). The X-ray crystal structures of reduced (redPDI) and fully oxidized (oxPDI) PDI were obtained from the Protein Data Bank (PDB: 4EKZ and 4EL1, respectively) [53]. The enzyme structures were hydrogenated in accordance with the protonation states of the various charged side chains using PROPKA [169]. Using the AMBER14 program package with the cuda-enabled version of the pme md module [170, 171], the system was solvated with TIP3P [171] water molecules generating a cubic solvation space with edge length of \(\sim105.9\ \text{Å}\). Solvation achieved a density of 1.06 g/cm\(^3\) after equilibrium, containing 37 071 water molecules.

Simulations were performed on WT and the K57/401Q double mutant PDI enzyme. All simulations were performed through the ff14SB [172] and TIP3P [171] force fields for protein and water, respectively. Periodic boundary conditions were applied, and an NVT ensemble was used with Anderson temperature coupling scheme [173]. The Particle-Mesh Ewald (PME) procedure was used to calculate long-range electrostatic interactions and a cut-off of 8 Å was applied in real space. The SHAKE algorithm was used to restrain the bond stretches involving hydrogen atoms, which allowed a 2 fs time step to be used for both equilibration and production. The equilibrations were conducted
in five stages after energy minimization. (1) To ensure the proper geometry of the hydrogen atoms, all heavy atoms, including water oxygen, were restrained with a harmonic potential using a force constant of 50 kcal mol\(^{-1}\) Å\(^{-2}\) for 100 ps, at 10 K. (2) The same potential and conditions were applied for an additional 100 ps, but with the removal of the restraint on the water oxygens to ensure optimized positions of water with respect to the protein environment. (3) The harmonic potential constant was decreased to 5 kcal mol\(^{-1}\) Å\(^{-2}\) for 100 ps. (4) Subsequently, the harmonic potential force constant was removed for 100 ps. (5) The system was gradually heated to 300 K over 2000 ps. The velocities were randomly updated every 10 steps for the equilibration stages 1-4 and every 100 steps for stages 5. The production run was conducted for 50 ns following equilibration.

To determine the pKₐ of the active site cysteiny1 thiols (of redPDI) and lysine residues Lys\(^{57}\) and Lys\(^{401}\), the PROPKA feature within the MOE2016 program package was employed [174]. Predictions were done on a representative structure obtained from the MD simulation based on the most populated RMSD values of the protein over the course of the simulation.

### 2.2.13 Statistical Analysis

One-way ANOVA followed by Dunnett's multiple comparisons test was performed on all kinetic data using GraphPad Prism version 6.0e (GraphPad Software, La Jolla California, USA). Differences were deemed significant if \(P < 0.05\). Significance is denoted with respect to WT control unless otherwise stated; * \(P < 0.05\), ** \(P < 0.01\), *** \(P < 0.005\), **** \(P < 0.001\). No significance is denoted by ns where appropriate.
CHAPTER 3

RESULTS
A total of nine hPDI mutants were designed and purified. Successful mutagenesis was confirmed via cDNA sequencing. Rational design involved targeting the highly conserved active site lysine residues Lys$^{57}$ and Lys$^{401}$ of the $a$ and $a'$-domains, respectively [55, 62]. Amino acid substitution of the aforementioned residues was performed (appendix A Table A.1). Residue numbering is based on the accepted sequence for PDI from *Homo sapiens* (Uniprot: PDIA1, P07237). Sequential mutagenesis of Lys$^{57}$ and Lys$^{401}$ to the non-conservative amino acids glutamine (Gln), alanine (Ala), or glutamic acid (Glu) resulted in single mutant progenitor enzymes by which double mutant hPDI constructs were also generated (K57/401A, K57/401Q and K57/401E). The purity of the isolated PDI proteins was assessed using SDS-PAGE (appendix A Figure A.2), and standardization of active enzyme was determined on a per-active site basis by single turnover, burst-phase kinetic analysis using the dicosin-glutathione disulfide (di-E-GSSG) assay, followed by interpolation of a standard curve (appendix A Figure A.3).

### 3.1 Substitution of the CGHC-neighbouring Residues K$^{57}$ and K$^{401}$ Alters the pH-Dependence of PDI Oxidoreductase Activity

In order to assess the potential catalytic contributions of Lys$^{57}$ and Lys$^{401}$, these amino acids were substituted to neutral-polar glutamine (Gln), nonpolar alanine (Ala), or to negative-polar glutamic acid (Glu) residues via site-directed mutagenesis. The pH profiles of the wild type (*WT*) and doubly-mutated PDI enzymes (10 nM) were determined at a constant concentration of di-E-GSSG (800 nM) in buffers of varying pH.
First, the structural integrity of PDI and the fluorescence properties of the di-E-GSSG pseudo-substrate were assessed within the pH range studied. For PDI structural integrity, the intrinsic tryptophan fluorescence spectrum was monitored as a simple reporter of pH-induced conformational changes. The PDI-fluorescence spectrum was stable and consistent over the pH range of 5.5 to 9.5. However, the emission peak began to increase at pH 4.5, an indication that the enzyme was undergoing substantial global conformational changes under acidic conditions (Figure 3.1A). The pH stability of the di-E-GSSG substrate (800 nM) was also tested by completely reducing the GSSG disulfide with excess dithiothreitol (DTT, 1 M) in the pH range employed. At low pH, the apparent rate of reduction was significantly slower owing to the low amounts of ionized DTT, however, the substrate gave a consistent 56-fold fluorescence-enhancement upon disulfide reduction irrespective of pH (Figure 3.1B).

The WT enzyme and the mutants all exhibited bell-shaped activity vs. pH profiles. The activity vs. pH data was fit to a double-ionization model (Equation 2.2) which allowed for the estimation of 2 macroscopic pKₐ's (\(\text{app}pK_1\) and \(\text{app}pK_2\)). The pH\(_{\text{max}}\) (pH of maximum activity) as well as the maximum rate of EGSH formation were affected by the simultaneous substitutions of Lys\(^{57}\) and Lys\(^{401}\) to Gln, Ala or Glu. The most obvious macroscopic effect of the double mutations was on the descending limb midpoints which were shifted to the left by \(~0.5\) pH units (\(\text{app}pK_2\), Table 3.1). The net result of this was the shift of pH\(_{\text{max}}\) by \(~0.3\) pH units in comparison to WT (pH\(_{\text{max}}\) = 6.77, Table 3.1 and Figure 3.1C). The double mutant PDI variants also displayed activities at pH\(_{\text{max}}\) that were \(~68\%\), \(~50\%\) and \(~38\%\) of the WT enzyme. The mutation-dependent activity decrease was more dramatic at physiological pH (7.4) where Q, A and E double mutants had
oxidoreductase activities that were ~36%, 33% and 21% of the WT enzyme (Figure 3.1 C).

It is proposed that the ascending limb of all respective profiles represents enzyme activation as a result of active site thiol deprotonation. Therefore, \(^{\text{app}}pK_1\) is likely attributed to a combination of the deprotonation events of the N-terminal nucleophilic cysteine (Cys\(_N\)) and the C-terminal resolving cysteine (Cys\(_C\)) from both active sites of PDI, yielding the more reactive thiolate anion state. The \(^{\text{app}}pK_1\) from this was found to be independent of the residue at positions 57 and 401, with only nominal shifts occurring as a result of mutation (Table 3.1).

Inactivation of the enzyme, as seen by the descending limb of the pH profiles, indicated that \(^{\text{app}}pK_2\) was directly dependent on the residues at positions 57 and 401. Upon substitution of Lys\(^{57}\) and Lys\(^{401}\), PDI variants exhibited a narrower range of oxidoreductase activity (breadth of the pH profile). The apparent enzyme inactivation may be attributed to the deprotonation of the intervening histidine residues His\(^{55}\) and His\(^{397}\) (\(a\) and \(a'\)-domain CGHC, respectively) in conjunction with other ionizable residues important to the catalytic function of PDI. Furthermore, the fact that the \(\varepsilon\)-amine of Lys\(^{57}\) and Lys\(^{401}\) (for WT) supports a wider range of enzyme activity; whereby substitution to Gln, Ala or Glu shifts \(^{\text{app}}pK_2\) to lower pH, clearly indicates that an active site lysine is a player in determining the pH-dependence of enzyme inactivation. Equation 2.3 provides a mathematical model by which this proposed scheme of ionization may be tested in relation to the experimentally generated results (Figure 3.1 C inset). Considering the apparent contribution to \(^{\text{app}}pK_2\) by residues Lys\(^{57}\) and Lys\(^{401}\), simulated curves (light purple, solid line) provided a fit that was unsurprisingly close to that of the experimental
data set for WT PDI (black dotted line). However, decreasing the relative contribution provided by the active site Lys residues lead to simulated curves (darker purple, solid lines) that tended to mimic the mutants. By eliminating the contribution of Lys entirely, i.e. mimicking mutation, the curve (darkest purple, solid line) most closely fit to that observed for the K57/401A mutant (green dotted line). Both the Gln and Glu variants under study did not corroborate as closely with the theoretical model. This may be an indication of other residue-specific effects occurring that cannot be concluded upon with the present data, potentially impacting the structural characteristics of PDI.

3.2 Steady State Oxidoreductase Kinetics are Attenuated in the Absence of K57 and K401

The pH-dependent activity profiles at a single substrate concentration, as in Figure 3.1 A, did not give a complete picture as to the pH effects on the kinetic parameters. To circumvent this, the turnover number ($k_{cat}$), $K_M$ and catalytic efficiency ($k_{cat}/K_M$) were determined as a titration of pH from 6.0 to 8.0. Due to very low initial rates, steady state kinetic data could only be obtained at pH ≥ 6.0 (see later). Data was collected for all nine mutant PDI variants in relation to WT (Figure 3.2 and appendix B Tables B.1 to B.5).

The $k_{cat}$ for hPDI- WT and all mutant variants, was at a minimum at pH 6.0, with a gradual increase towards more physiological conditions at pH 7.0 and 7.4, before again declining under basic conditions of pH 8.0 (Figure 3.2 A and appendix B Tables B.1 to B.5). The trend for the apparent $K_M$ was different, showing a gradual increase across the tested pH range; this held true of all PDI enzymes (Figure 3.2 B and appendix B Tables B.1 to B.5).
Figure 3.1: pH-dependent oxidoreductase activity of hPDI. (A) Tryptophan fluorescence of redPDI (6.175 µM) was monitored over the indicated pH range as a reporter of pH-induced conformational changes. (B) The di-E-GSSG probe maintained its fluorescence properties over the tested pH range. The fluorescence fold increase of 800 nM di-E-GSSG was monitored after the addition of 1 M DTT (black arrow). The retarded rate of reduction at pH 5.5 (black circles) is attributed to DTT being only ~0.02% deprotonated (more reactive thiolate form) as a result of its thiol $pK_a$'s (ca. 9.2). (C) Activity profiles of WT (black), K57/401Q (blue), K57/401A (green) and K57/401E (red). 10 nM PDI was incubated with 800 nM di-E-GSSG in PDI assay buffer of varying pH. pH-dependent activity profiles represent the mean ± S.D. of $n = 3$ experiments fit to Equation 2.2. (C inset) Simulated pH profiles generated using Equation 2.3 (purple curves: light to dark) as compared to experimentally observed curves for WT (black dotted lines) and the Q, A and E mutants (blue, green and red dotted lines, respectively).
Table 3.1: Apparent pK\textsubscript{a} values and the pH of maximum activity of WT and double-mutant enzymes. Data extracted from the fits seen in Figure 3.1.

<table>
<thead>
<tr>
<th>hPDI</th>
<th>\textsuperscript{app}pK\textsubscript{1}</th>
<th>\textsuperscript{app}pK\textsubscript{2}</th>
<th>pH\textsubscript{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.16 ± 0.01</td>
<td>7.37 ± 0.02</td>
<td>6.77 ± 0.16</td>
</tr>
<tr>
<td>K57/401Q</td>
<td>6.05 ± 0.01 \textsuperscript{**}</td>
<td>6.90 ± 0.00 \textsuperscript{****}</td>
<td>6.44 ± 0.00 \textsuperscript{**}</td>
</tr>
<tr>
<td>K57/401A</td>
<td>6.11 ± 0.03 \textsuperscript{*}</td>
<td>6.87 ± 0.04 \textsuperscript{****}</td>
<td>6.49 ± 0.04 \textsuperscript{**}</td>
</tr>
<tr>
<td>K57/401E</td>
<td>6.20 ± 0.01</td>
<td>6.80 ± 0.01 \textsuperscript{****}</td>
<td>6.55 ± 0.01 \textsuperscript{*}</td>
</tr>
</tbody>
</table>
Following this, the catalytic efficiency ($k_{\text{cat}}/K_M$) of the enzymes were found to be variable when comparing WT to any of the $a$ or $a'$-domain single mutants, as well as the double mutant enzymes. PDI WT as well as the K57A and K401A single substitution mutants were most efficient at pH 6.5 whilst all other enzymes were apparently most efficient oxidoreductases at pH 6.0 (Figure 3.2 C, appendix B Tables B.1 and B.2). The pH dependence of $k_{\text{cat}}/K_M$ was most notably influenced by the trend described for $K_M$ rather than $k_{\text{cat}}$. The increase of $K_M$ from pH 6.0 to 8.0 was greater than 165-fold for all enzymes. This is in contrast to the ~5-fold variation in $k_{\text{cat}}$. This resulted in an overall trend of catalytic efficiency decreasing with increasing pH, with the exception of WT exhibiting a stark increase at pH 6.5 (Figure 3.2 C).

To our knowledge, few studies to date that have assessed the pH-dependence of PDI activity. Moreover, these studies fell short of describing PDI activity over such a wide range of pH. A more efficient enzyme at lower pH seems counter intuitive in relation to the ionization states of the catalytic thiols, as well as those of DTT (present in excess, required for the regeneration of redPDI. See later). It would make sense for efficiency to increase with pH as this would promote a higher proportion of the more reactive thiolate state, but this was not seen. This provides strong evidence for the interplay between nucleophilicity and $pK_a$- indicating that PDI is optimized to perform oxidoreductase catalysis at a neutral to slightly acidic pH, such as that observed in the ER and advanced secretory compartments of the Golgi apparatus [175].
Figure 3.2: pH-dependent kinetic parameters for the oxidoreductase kinetics of WT (black) and double mutant PDI enzymes (K57/401Q, blue; K57/401A, green; K57/401E, red) towards the di-E-GSSG pseudo-substrate. Data for (A) $k_{cat}$, (B) $K_M$ and (C) $k_{cat}/K_M$ are reported as the mean ± S.D. of $n = 3$ experiments.
When examining the Michaelis-Menten profiles at each pH it becomes apparent that $K_M$ (the substantiating factor in the pH-dependence of $k_{cat}/K_M$) may be biased as a result of enzyme saturation ($V_{max}$) being approached at much lower substrate concentrations under acidic conditions (Figure 3.3 A). This means that it is possible PDI appears most efficient at pH 6.0 or 6.5 under the *in vitro* conditions employed due to the extensively small values of $K_M$, yet the neutral pH of the ER lumen may still be preferred with respect to the complex cellular environment *in vivo*.

Another interesting phenomenon was seen as a result of acidity. As mentioned, steady state kinetics could not be performed below pH 6.0 due to low initial rates. At pH 5.5 a rate which was independent of substrate concentration was observed (Figure 3.3 A and B). Upon addition of PDI, fluorescence increased rapidly before plateauing to a negligible rate (Figure 3.3 B). In comparison to the raw kinetic data at pH 7.4, this was surprising (Figure 3.3 B inset). Based on this, an experiment was performed in order to identify if PDI was becoming trapped in a mixed-disulfide state for extended periods of time. To test this, we mimicked the *in vitro* di-E-GSSG assay under similar steady state conditions as described previously, following it with a rapid thiol-quenching step using iodoacetamide (IAM). After separating PDI from the unreacted di-E-GSSG, free EGSH, and excess IAM, any detectable fluorescence would be attributed to PDI being trapped in a mixed-disulfide state with eosin glutathione (see later, Figure 3.12). Should fluorescence be absent, this would be indicative of a high turnover rate of the enzyme, thereby minimizing the time in which the enzyme-substrate complex exists- or, be evidence of a predominantly oxidized PDI active site.
By comparing the effects of substrate trapping at pH 5.5 and 7.4 it was observed that acidity did promote an enhanced level of the mixed-disulfide state of PDI (Figure 3.3 C). Between the time points of 0 and 20 seconds, ~92% of PDI active sites were occupying a mixed-disulfide state at pH 5.5, while only ~30% were determined to be at pH 7.4. These findings were corrected in relation to a negative control which consisted of PDI alkylated by IAM prior to the addition di-E-GSSG. This is suggestive of two things; (1) there is a greatly attenuated rate of substrate release under acidic conditions, while the initial nucleophilic attack is likely only marginally affected. (2) PDI is adopting a stabilized intermolecular disulfide with substrate, rather than an intramolecular disulfide between the vicinal active site cysteines.

The effectiveness of DTT as a reductant at such low pH is poor, but this is not a factor based on the observed results. The detection of trapped PDI indicates that the resolving cysteine of the active site is unable to facilitate substrate release as efficiently. This may be from altered structural dynamics of the active site and/ or a small proportion of the reactive thiolate state at pH 5.5 in comparison to pH 7.4 (considering a Cys\textsubscript{C} thiol pK\textsubscript{a} of about 6.1, see section 1.2.2). This result was proven true for the WT PDI enzyme, but a similar trend is expected of all PDI variants herein.

It has been demonstrated that pH is a major factor in defining PDI activity. Acidic conditions seemed to promote a stabilized mixed-disulfide intermediate between PDI and eosin glutathione. More importantly, optimal steady state oxidoreductase activity appears to be dependent on a WT CGHCK active site motif and mutant CGHC(Q/A/E) motifs support a less efficient enzyme.
Figure 3.3: PDI undergoes acid-induced substrate trapping via markedly attenuated substrate turnover. (A) Representative plots of the Michaelis-Menten kinetic relationships over the range of pH tested for the reduction of di-E-GSSG by 10 nM PDI in the presence of 10 μM DTT. (B) Raw data of the fluorescence (RFU) increase over time depicting a comparison of the initial rates of catalysis at pH 5.5 and 7.4 (inset). The addition of WT PDI (10 nM) to the reaction mixture is indicated by an arrow. (C) The impact of pH on PDI-substrate intermediate complex trapping was assessed under steady state conditions similar to those of the kinetic assays of A, with the addition of iodoacetamide (IAM) as the "trapping" reagent. Results are reported as the fraction of trapped PDI active sites as determined by the fluorescence carry-over after protein desalting and reference to a standard curve of fluorescent signal vs. [EGSH]. Data from A and C represent the mean ± S.D. of n = 3 experiments.
3.3 Residues K\textsuperscript{57} and K\textsuperscript{401} Potentially Mediate Active Site Flexibility - A Case Study with Related PDI Family Members

In an attempt to explain the shifted pH-profiles as a result of mutation, we sought to examine the structural aspects of related PDI family members naturally expressing a CxxC(K/Q/A/E) active site motif. The focus here was on NMR solution structures because these provide a dynamic view of protein conformations as compared with X-ray crystallographic structures. Interestingly, by analyzing the solution structures of homologous mammalian PDI family members PDIA3 (PDB: 2DMM [176]) and PDIA6 (PDB: 2DML [177]) (refer to Figure 3.4) strong hints as to the potential role of Lys\textsuperscript{57} and Lys\textsuperscript{401} have been elucidated. The PDB entry 2DMM contains 20 solution structures of a 142-amino acid peptide from of the \textit{a}'-domain of human PDIA3; naturally expressing a CGHCK active site motif (similar to PDIA1) [176]. Whereas the PDB entry 2DML represents 20 solution structures of a 130-amino acid peptide from the \textit{a}-domain of mouse PDIA6; naturally expressing a CGHCQ active site sequence (similar to the K57/401Q PDI variants engineered here) [177].

A comparison of the overlaid structures of 2DMM (CGHCK) (Figure 3.4 A) to that of 2DML (CGHCQ) (Figure 3.4 B) shows that when Lys is the C-terminal amino acid to the CGHC motif, the residue's side chain is very mobile with little interaction between the \textit{ε}-amine and the peptide backbone. This notion is confirmed by previous reports demonstrating the short-lived, transient nature of backbone hydrogen bonds with lysine side chains [178]. This results in a seemingly flexible active site. It is believed that the motion of the Lys side chain is translated through the conserved active site region to the intervening His, causing it to also be mobile and able to move away from the active site.
cysteines. The minimum apparent distance was determined to be about 5.6 Å and 7.0 Å from the N- and C-terminal cysteiny1 sulfurs, respectively (measurements taken from the imidazolium N\textsubscript{ε} of His). This provides an indication that the intervening His is in closer proximity with Cys\textsubscript{N} in the NMR solution structures. Full or partial positive charges <6.5 Å away are able to stabilize thiolate anions (decrease thiol pK\textsubscript{a}) [65, 102], thus the distance of 5.6 Å as measured here supports the role for His as a stabilizing factor for Cys\textsubscript{N}-S\textsuperscript{−} and not the Cys\textsubscript{C}-S\textsuperscript{−}.

The presence of two nitrogen atoms on the imidazole ring of His (N\textsubscript{δ} and N\textsubscript{ε}) means there are three relevant ionization states: HN\textsubscript{δ}, HN\textsubscript{ε} or H\textsubscript{2}N\textsubscript{δ}N\textsubscript{ε} (cationic state), respectively representing the protonation of the N\textsubscript{δ}, N\textsubscript{ε}, and both N\textsubscript{δ} and N\textsubscript{ε} nitrogen atoms. Although, the energetically favourable tautomeric state of the neutral imidazole moiety is the protonation of N\textsubscript{ε} and subsequently a lone-pair of electrons on N\textsubscript{δ} [179, 180]. To this end, the rotameric state of the imidazole ring largely dictates the influences on neighbouring side chains.

In contrast to the structure observed for 2DMM (bearing a CGHCK motif, Figure 3.4 A), when Lys is replaced with a Gln as in 2DML, the δ-amide group is seemingly immobilized by hydrogen bonding interactions with the backbone carbonyl oxygen of the upstream active site cysteine (n−4) and proline (n−6) residues (FYAP\textsubscript{W}GHCQ), leading to an apparently more rigid active site (Figure 3.4 B). Backbone hydrogen bonding by the Gln side chain is a well characterized feature of many protein motifs [181-183]. As a result, the motion of His is apparently restricted, potentially allowing it to interact \textit{via} a salt bridge with the active site thiolates.
Figure 3.4: NMR solution structures of the active site CGHCK and CGHCQ motifs naturally expressed by the PDI family members PDIA3 and PDIA6, respectively. (A) Superimposition of 20 solution structures of the $\alpha'$-domain active site of PDIA3 (APWCGHCK), PDB: 2DMM [176]. (B) Superimposition of 20 solution structures of the $\alpha$-domain active site of PDIA6 (APWCGHCQ), PDB: 2DML [177]. Hydrogen bonding represented by dotted lines. Visualized using UCSF Chimera software [184].
The effect here is likely two-fold, where His causes a decrease of thiol pKₐ as well as a steric impedance to the active site. A more acidic active site thiol would likely result in a concomitant decrease of activity due to the balance between thiol pKₐ, nucleophilicity and environment pH (refer to section 1.1.2).

Measurements taken from the solution structure overlays indicate that the imidazolium Nₑ is about 2.6 Å and 3.7 Å from the N- and C-terminal cysteiny1 sulfurs, respectively (Figure 3.4 B). This again indicates that the intervening His is in closer proximity to Cysₙ. In contrast to 2DMM, His is within hydrogen bonding distance to Cysₙ. The activity vs. pH data may support this notion. When substituting Lys to Gln, Ala or Glu in the double mutants, the activity at pHₘₐₓ (where both His and Lys are likely protonated) decreased by ~32%, 50% and 62% in comparison to the WT enzyme, respectively. For WT, as His deprotonates with increasing pH (likely represented by the descending limb, _app pK₂ Figure 3.1 C and Table 3.1_) it no longer imparts as strong of a stabilization effect to the Cysₙ thiolate, contributing to the apparent enzyme inactivation. The shifted maximum and _app pKₐ ultimately indicates a potential role for residues Lys₅⁷ and Lys₄₀¹ in providing active site flexibility such that the intervening His is incapable of forming unfavourably strong ion-pairs with the Cysₙ thiolate, those that would lead to suboptimal nucleophilicity. The active site flexibility is proposed to arise as a result of the apparent motions of the Lys side chain. It has been shown that Gln is unable to impart such flexibility as it is strongly hydrogen bonded with the backbone. As for a Glu mutation, this would be reminiscent of Gln, also able to hydrogen bond with the peptide backbone. An Ala mutation results in the introduction of a small, immobile side chain
that also would not display the apparent flexibility required for optimal activity due to a more mobile His.

3.4 Identification of Distinct Roles for K$^{57}$ and K$^{401}$ in Mediating Active Site Accessibility- An Alternative Explanation Uncovered by Molecular Dynamics

In order to further characterize the proposed scheme above (see previous, section 3.2) molecular dynamics (MD) simulations were performed using full-length hPDI in both the reduced (redPDI, PDB: 4EKZ [53]) and oxidized (oxPDI, PDB: 4EL1 [53]) forms. Four simulations were performed in total: redPDI WT, oxPDI WT, redPDI K57/401Q, and oxPDI K57/401Q. Despite the previous data strongly indicating a role for residues Lys$^{57}$ and Lys$^{401}$ in enhancing activity by mediating a flexible active site, resulting in an apparently highly mobile intervening His, this was not found in our simulations. Instead, it was interesting to observe Lys$^{57}$ and Lys$^{401}$ performing roles in apparently modulating active site accessibility in very distinct manners. These dynamic changes of accessibility are viewed as open and closed active site conformations herein.

The orientation of the $a$ and $a'$-domain active sites are quite different. As shown for redPDI, the $a$-domain active site is very surface-exposed on the posterior side of the PDI architecture (Figure 3.5). In contrast, the $a'$-domain active site is less exposed, yet still visible from the anterior side, oriented at the $b'$-$a'$ domain interface (see later, Figure 3.8). It was determined that for each active site region, the apparent accessibility/exposure of Cys$_{SN}$ was differentially hindered by the dynamics of key hydrophobic residues. A focus was placed on Cys$_{SN}$ as it is the residue responsible for initiating
catalysis, therefore its orientation is much more important with respect to PDI activity than is Cys$_C$, in comparison [60].

For the $a$-domain, this Cys$_N$ accessibility was modulated via the movement of a conserved tryptophan residue, Trp$^{52}$ (FYAPWCGHK). With regards to the $a'$-domain, this was a result of a phenylalanine, Phe$^{440}$, found to reside $n-1$ in relation to a conserved $cis$-Proline, Pro$^{441}$. These residues effectively result in active site conformations that are either more or less accessible with respect to Cys$_N$; acting as lids to each respective active site.

With respect to the $a$-domain, the dynamics of Trp$^{52}$ were determined to be dictated by Lys$^{57}$. The MD simulations indicated the presence of a cation-$\pi$ interaction between the $\varepsilon$-amine of Lys$^{57}$ and the aromatic indole ring of Trp$^{52}$. This interaction apparently resulted in an open conformation of the active site as the side chain of Trp$^{52}$ was directed away from Cys$_N$ (Cys$^{53}$, Figure 3.6). By substituting Lys$^{57}$ for Gln, this interaction was unsurprisingly lost, leading to an apparently closed active site as the Trp$^{52}$ side chain was able to rotate and project towards the active site and hinder the apparent exposure of Cys$^{53}$ (Figure 3.7). Furthermore, the Gln side chain was determined to have a high propensity to hydrogen bond with the backbone carbonyl of an upstream proline, Pro$^{51}$ (FYAPWCGHK, refer to appendix B Figure B.4 C). This was not seen for the Lys side chain, indicating that the primary interaction likely dictating the accessibility of Cys$^{53}$ is the specific cation-$\pi$ interactions fulfilled by Lys$^{57}$. Previous research showed that a K-R mutation had only a nominal impact on PDI activity, this is likely because Arg may also fulfill the cation-$\pi$ interaction determined here [185, 186].
**Figure 3.5:** Surface structure of the a-domain active site of redPDI generated by molecular dynamics (MD). Posterior view of the enzyme showing the accessibility of the active site thiol of the N-terminal nucleophilic cysteine, Cys$^{53}$. Arrows indicate plausible access points for small-to-intermediate sized substrates. Residue Trp$^{52}$ mediates exposure of Cys$^{53}$ by acting as a lid, providing an open (upper panel, redPDI WT) or closed (lower panel, redPDI K57/401Q) configuration. Visualized using Visual Molecular Dynamics software (VMD) [54].
Figure 3.6: Residue Lys$^{57}$ of the $\alpha$-domain participates in cation-$\pi$ interactions with the upstream Trp$^{52}$. The electrostatic cation-$\pi$ interaction between Lys$^{57}$ ($\alpha_2$-helix) and Trp$^{52}$ ($\alpha_2$-$\beta_3$ loop) maintains an open active site conformation, rendering Cys$^{53}$ more solvent exposed (arrows). The $N^\epsilon$-amine of lysine is positioned 2.2 Å away from the aromatic center (lower panel). A wire-frame space-filling model further highlights the interaction (upper panel). In green, the conserved cis-Pro loop motif (Tyr$^{99}$ and Pro$^{100}$ shown). Hydrogen atoms have been omitted for simplicity. Visualized using Visual Molecular Dynamics software (VMD) [54].
Figure 3.7: Mutation of residue Lys$^{57}$ to Gln eliminates cation-π interactions with the upstream Trp$^{52}$, resulting in a less accessible active site. The mutant residue, Gln$^{57}$, forms hydrogen bond interactions with the peptide carbonyl of Pro$^{51}$ (upper left panel). In this conformation Trp$^{52}$ acts as a lid, effectively limiting the accessibility of Cys$_{53}$: Cys$_{53}$ (arrows). In green, the conserved cis-Pro loop motif (Tyr$^{99}$ and Pro$^{100}$ shown). Hydrogen atoms have been omitted for simplicity. Visualized using Visual Molecular Dynamics software (VMD) [54].
Stark differences between the orientation of the active sites lead to the realization that residues Lys\textsuperscript{57} and Lys\textsuperscript{401}, although conserved with respect to the primary sequence, modulate Cys\textsubscript{N} exposure distinctly. In the \alpha'-domain, Lys\textsuperscript{401} did not participate in cation-\pi interactions with the equivalent tryptophan, Trp\textsuperscript{396} (contrary to that seen in the \alpha-domain). Instead, Trp\textsuperscript{396} appeared to be fulfilling this with an arginine residue of the \beta'-domain, Arg\textsuperscript{300} \[53, 187\]. As a result, the side chain of Lys\textsuperscript{401} projected freely into the solvent, with no indication of any bona fide interactions (Figure 3.9). In this conformation, the active site appeared \emph{open}, with Cys\textsubscript{N} (Cys\textsuperscript{397}) relatively solvent-exposed. Although, it is possible for Lys\textsuperscript{401} to form transient hydrogen bonds with the carbonyl oxygen of the peptide backbone of an upstream proline, Pro\textsuperscript{395} (refer to appendix B Figure B.4 B). In doing so, there was an indication that the apparent access to Cys\textsuperscript{397} be hindered by Phe\textsuperscript{440} (Figure 3.10). Furthermore, it was noted that the R\textsuperscript{300}-Trp\textsuperscript{396} cation-\pi interaction was abolished as a result. With this, we examined the MD simulation for a mutated active site by introducing Gln in place of Lys\textsuperscript{401}. The propensity for Gln to hydrogen bond with the backbone of an upstream Pro was again determined to be high (akin to those results for the \alpha-domain, refer to appendix B Figure B.4 D). As a result, the \alpha'-domain active site was apparently \emph{closed}, as Phe\textsuperscript{440} projected toward Cys\textsuperscript{397} rather than away as in the \emph{open} configuration (Figure 3.11).

These results indicate that, for redPDI, residues Lys\textsuperscript{57} and Lys\textsuperscript{401} differentially maintain the accessibility of the active site Cys\textsubscript{N}. Residue Lys\textsuperscript{57} of the \alpha-domain does so \emph{via} cation-\pi interactions with the conserved, upstream residue Trp\textsuperscript{52}. Mutation to Gln abolished this interaction, resulting in a rotation of the indole ring over Cys\textsuperscript{53}, making an apparently \emph{closed} \alpha-domain active site.
Figure 3.8: Surface structure of the $a'$-domain active site of redPDI generated by molecular dynamics (MD). Anterior view of the enzyme showing the accessibility of the active site thiol of the N-terminal nucleophilic cysteine, Cys$^{397}$, situated near the $b'$-$a'$ domain interface. Arrows indicate plausible access points for small-to-intermediate sized substrates. Residue Phe$^{440}$ mediates access to Cys$^{397}$ by acting as a lid, providing an open (upper panel, redPDI WT) or closed (lower panel, redPDI K57/401Q) configuration. Visualized using Visual Molecular Dynamics software (VMD) [54].
Figure 3.9: Residue Lys\textsuperscript{401} of the \textit{a}'-domain freely projects into the solvent. The upstream Trp (Trp\textsuperscript{396}, \(\alpha_2-\beta_3\) loop) is incapable of participating in electrostatic cation-\(\pi\) interactions with Lys\textsuperscript{401} (\(\alpha_2\)-helix) contrary to that observed in the \(a\)-domain active site. Residue Arg\textsuperscript{300} of the \(b\)'-domain forms strong cation-\(\pi\) interactions with Trp\textsuperscript{396} (right panel), likely sequestering it from Lys\textsuperscript{401}. In this conformation, the N-terminal active site thiol, Cys\textsuperscript{397}, is apparently solvent accessible in two general directions (arrows). In green, the conserved \textit{cis}-Pro loop motif (Phe\textsuperscript{440} and Pro\textsuperscript{441} shown). Note, Phe\textsuperscript{440} of the \textit{cis}-Pro loop projects away from the active toward the interior of the \(b\)'-\(a\)' domain interface. Hydrogen atoms have been omitted for simplicity. Visualized using Visual Molecular Dynamics software (VMD) [54].
Residue Lys$^{401}$ of the $\alpha'$-domain may form hydrogen bonds with upstream residues. The carbonyl oxygen of the upstream Pro (Pro$^{395}$, $\alpha_2$-$\beta_3$ loop) provides a hydrogen bond acceptor site by which the $\varepsilon$-amine of Lys$^{401}$ may interact (dotted line; 2.8 Å, left panel). Hydrogen bonding results in a loss of the cation-$\pi$ interaction between Arg$^{300}$ and Trp$^{396}$. This, in turn, results in a 3.2 Å translation of Phe$^{440}$ to a position that partially impedes access to Cys$^{397}$. Hydrogen atoms have been omitted for simplicity. Visualized using Visual Molecular Dynamics software (VMD) [54].
Figure 3.11: Mutation of residue Lys\textsuperscript{401} to Gln introduces hydrogen bonding to the α'-domain active site, leading to a less accessible N-terminal thiol. Hydrogen bonding by the mutant residue Gln\textsuperscript{401} and the peptide carbonyl of Pro\textsuperscript{395} (dotted line and right panel) causes Phe\textsuperscript{440} of the cis-Pro loop to sterically hinder access to Cys\textsuperscript{397} (arrows). In green, the conserved cis-Pro loop motif (Phe\textsuperscript{440} and Pro\textsuperscript{441} shown). Hydrogen atoms have been omitted for simplicity. Visualized using Visual Molecular Dynamics software (VMD) [54].
As for the $a'$-domain, it has been suggested that the ability of Lys$^{401}$ to form no bona fide interactions is what provides the open conformation of the active site, specifically, the exposure of Cys$^{397}$. Substitution of Gln in the place of Lys$^{401}$ introduced hydrogen bonding, leading to structural changes that propagated through the active site region leading to the projection of Phe$^{440}$ towards Cys$^{397}$, limiting its accessibility.

Active site dissimilarities accounts for the reasons why Lys$^{57}$ does not seem to perform in a manner similar to Lys$^{401}$. The conserved Trp$^{52}$ of the $a$-domain is free to participate in cation-π interactions with Lys$^{57}$, whilst in the $a'$-domain, the equivalent residue, Trp$^{395}$, is essentially sequestered by preferential cation-π interactions with Arg$^{300}$. Furthermore, the reason that residue Phe$^{440}$ ($n-1$ to conserved cis-Pro) is the lid to the $a'$-domain, yet the equivalent residue of the $a$-domain (Tyr$^{99}$, $n-1$ to conserved cis-Pro) does not function as such is due to hydrogen bonding with the phenolic alcohol group, rendering Tyr$^{99}$ incapable of projecting toward the $a$-domain Cys$_N$ (refer to appendix B Figure B.1).

The global conformation of PDI is redox-state dependent (refer to appendix B Figure B.3) [53], therefore it was important to assess the contributions of Lys$^{57}$ and Lys$^{401}$ (as well as mutations to Gln) with respect to oxPDI. In either domain, it was apparent that an intramolecular disulfide between the active site vicinal cysteines elicited a conformational constraint. For the $a$-domain, this resulted in the loss of the K$^{57}$-W$^{52}$ cation-π interaction seen previously in redPDI. Furthermore, substituting Lys$^{57}$ for Gln indicated that the propensity for Gln to hydrogen bond with Pro$^{51}$ was disrupted as a result of this constraint brought about by the disulfide (refer to appendix B Figures B.2 A and C, and B.5 A and C). This seemed to result in a more closed conformation. When
looking at the $a'$-domain, it was interesting to note that the large dynamic changes in domain organization showed a marked separation of the $b'$-$a'$ domain interface, so much so that the $R^{300}$-$W^{305}$ cation-$\pi$ interaction observed for redPDI was no longer present (refer to appendix B Figure B.2 B). Furthermore, apparent constraints brought about by the disulfide-linked vicinal cysteines was supported by disrupted hydrogen bonding when assessing the MD simulation of the Q mutant (refer to appendix B Figure B.5 D). As a result of this, only very limited backbone hydrogen bonding was able to occur, in comparison to that seen for redPDI $WT$, leading to an apparently open active site, irrespective of mutation. Therefore, it is possible that for $WT$ PDI, the $a$-domain active site transitions between open and closed conformations when reduced and oxidized, respectively, whilst the $a'$-domain active site remains apparently open, independent of redox state.

We next went on to investigate the impact such structural dynamics had on the $pK_a$'s of the vicinal active site thiols. Analyses indicated no meaningful perturbations to thiol $pK_a$ when looking at $WT$ or K57/401Q PDI (Table 3.2). This may suggest that the proposed scheme of open and closed active site dynamics is likely a factor influencing only substrate binding/ PDI-substrate encounter complex formation, rather than thiol-disulfide exchange rates in relation to nucleophilic attack. Although, it remains likely that the rate of exchange be ultimately attenuated simply due to the limited accessibility of the nucleophile ($\text{Cys}_N$) [26-28]. The unfounded, previously proposed scheme (see section 3.3) is further refuted by the indication that the $\text{Cys}_N$ $pK_a$'s apparently increased slightly upon Lys mutation, rather than decreasing via electrostatic stabilization from an intervening His.
Table 3.2 Computational analyses of the reduced PDI (redPDI) active site vicinal thiols and lysine residues; presenting pK\textsubscript{a} estimations for WT and K57/401Q.

<table>
<thead>
<tr>
<th>redPDI</th>
<th>a-domain</th>
<th>a'-domain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(pK_a)\textsubscript{Cys53}</td>
<td>(pK_a)\textsubscript{Cys56}</td>
</tr>
<tr>
<td>WT</td>
<td>4.4</td>
<td>8.7</td>
</tr>
<tr>
<td>K57/401Q</td>
<td>5.0</td>
<td>8.5</td>
</tr>
</tbody>
</table>
3.5 A CxxCK Motif Supports Enhanced Thiol-reductase Activity as Evidence by Single-turnover Kinetics

There is an important distinction to be made when describing the catalytic activity of PDI. The di-E-GSSG assay as employed thus far has included an excess of the reducing agent DTT. This is a vital consideration to the experiment as it is required for the regeneration of redPDI, thus allowing for successive catalytic turnovers and facilitating a steady state (Figure 3.12).

In the absence of DTT, or other excess reducing agent, the rate of di-E-GSSG reduction would be solely attributed to the reductase activity of hPDI and not the entirety of its oxidoreductase catalytic cycle. The inherent rate constants are thus somewhat attributable to not only the rate of reduction of the di-E-GSSG substrate, but also the rate of regeneration of redPDI by DTT. Although, it has been described that the rate of oxidation of PDI by GSSG is rate-limiting (akin to the reduction of di-E-GSSG) [83]. This means that the steady state data (section 3.4) should be telling of the thiol-reductase activity of PDI as the process of enzyme regeneration via DTT likely has negligible contribution. This notion was supported by varying the amount of DTT and assessing the impact on the rate of di-E-GSSG reduction. Should the steady state oxidoreductase activity be rate-limiting with respect to DTT-mediated redPDI regeneration, one would expect a doubling of the concentration of DTT to result in a doubling of the rate of reaction. As demonstrated in appendix B Figure B.7, this was not the case. Instead, the rate was not dependent on the concentration of DTT when at or above 1 µM.
Figure 3.12: Schematic of PDI-catalyzed reduction of di-E-GSSG. Cys$_N$ of redPDI initiates the reaction by nucleophilic attack of the GSSG disulfide, resulting in the formation of one free EGSH molecule and a mixed disulfide intermediate with a second EGSH molecule. The Cys$_C$ resolving cysteine liberates PDI from the mixed-disulfide state, releasing EGSH. redPDI is regenerated by the reducing agent DTT, allowing for successive catalytic turnovers. Di-E-GSSG reduction results in a detectable increase of fluorescence (RFU). For simplicity only the $a$-domain active site is depicted.
In light of these findings, it still remained purposeful to examine the single turnover event of di-E-GSSG reduction to discern the observable rates with respect to a WT and mutated active site motif. To study only the reductase portion of the oxidoreductase cycle, the di-E-GSSG assay was performed such that pseudo-first order kinetics (Equation 2.4) would be favoured as to provide an accurate estimation of reaction rate ($k_{obs}$) with comparisons between WT and the K-Q mutants. We chose to focus on the K-Q hPDI mutants as it is believed that mutation to glutamine most closely mimics the physiologically relevant posttranslational modification of $N^e$-lysine acetylation (a neutral, yet still polar acetylysine residue: acK). This being the case while limited to canonical amino acid substitution.

At a pH of 5.5, $k_{obs}$ for each enzyme was unchanged, while at pH 7.4 $k_{obs}$ of K57/401Q was only 43% that of WT, and the single domain Q mutants showed about 68% the activity of WT (Figure 3.13 B). With reference to the K57/401Q mutant, a significant decrease in $k_{obs}$ was also seen at pH 6.5, but not as much so at the more basic conditions tested. It is interesting to note that WT PDI outperformed the mutants to the greatest extent at physiological pH (7.4), with an observable rate of 0.75 s$^{-1}$, in comparison to 0.32 s$^{-1}$, 0.50 s$^{-1}$ and 0.52 s$^{-1}$ for K57/401Q, K57Q and K401Q, respectively. Furthermore, it follows that no statistically significant differences were observed between the respective single Q mutants. This indicates that each active site ($a$ and $a'$-domain) performed nearly identically under the particular in vitro conditions.
Figure 3.13: Single turnover reductase activity of PDI. (A) Representative data for the progress of the reduction of 1000 nM di-E-GSSG by 80 nM PDI WT (black) and K57/401Q (blue). Fluorescence data was related to the depletion of substrate (di-E-GSSG) over time. (B) The relative \( k_{\text{obs}} \) of all K-Q mutants (K57/401Q, blue; K57Q, green; K401Q, orange) was plotted as a percentage of WT (black). Kinetic data represents the mean ± S.D. of \( n = 3 \) experiments.
It is apparent that a CGHC-flanking Lys is a key residue for the function of PDI, both as a thiol-reductase and oxidoreductase. More importantly, the attenuation of thiol-reductase activity upon mutation of either or both Lys\textsuperscript{57} and Lys\textsuperscript{401} is greatest at the physiologically relevant pH range of 7-7.4, conditions by which PDI primarily functions \textit{in vivo}. By removing DTT from the assay and effectively negating the kinetic contributions of enzyme regeneration, the role of residues Lys\textsuperscript{57} and Lys\textsuperscript{401} has been shown to be substantial with regards to the initial nucleophilic attack of the di-E-GSSG substrate by Cys\textsubscript{N}.

### 3.6 Dithiol Oxidation by PDI is Attenuated Upon Substitution of K\textsuperscript{57} and K\textsuperscript{401}

Findings thus far have demonstrated the importance of residues Lys\textsuperscript{57} and Lys\textsuperscript{401} for PDI when bearing reduced active site motifs and catalyzing the reduction of substrate under both steady state and pseudo-first order conditions. It remains to be seen whether these residues are influential to PDI activity when functioning as a thiol-oxidase. Results here should shed light on the potential of these residues as participating in redox state-dependent enzyme regulation. The global protein conformation is largely impacted by the redox state of the active sites, whereby such conformational fluxionality may translate to stark differences for the role of the active site lysines when comparing redPDI and oxPDI activity; strongly suggested by MD analyses (refer to section 3.3).

As a complement to the previous findings, the activity of \textit{WT} and K-Q PDI enzymes were studied in relation to their thiol-oxidase activity using a self-quenching peptide substrate (\textbf{Figure 3.14}) [83]. Oxidation of a reduced, dithiol-containing peptide (sequence: NRCSQGSCWN) is catalyzed by oxPDI. The decapeptide substrate was
designed by Freedman and coworkers [83] such that it contains two cysteine residues separated by a flexible linker. A fluorescent tryptophan residue is adjacent one of the cysteines and a protonatable arginine is adjacent the other cysteine. The linker region of the peptide satisfies the need for no propensity to form secondary structure and so, the allowance for thiol-disulfide redox. The tryptophan and arginine residues allow for the rate of peptide oxidation to be measured by monitoring the quenching of tryptophan fluorescence upon peptide oxidation due to the close proximity with arginine [83].

Again, the distinction must be made in this instance as the measured rates are a result of an oxidoreductase catalytic cycle, as described previously. This is due to the presence of excess GSSG required for the regeneration of oxPDI. Despite this, contribution to the determined rates by peptide oxidation is substantial enough when compared to that of enzyme regeneration by GSSG. This was found to be true by Alanen and coworkers, whom demonstrated that the overall reaction rate be governed by both peptide oxidation and enzyme regeneration by GSSG [188]. With this, results may loosely represent the dithiol-oxidase activity of PDI.

The activity of the PDI enzymes, WT and K-Q mutants, were studied over the pH range of 5.5 to 8.0. This was the determined range as conditions more acidic than pH 5.5 were found to induce marked conformational changes of PDI (Figure 3.1 A), as well as markedly slow rates of peptide oxidation (Figure 3.15 A)- likely due to unproductive cycles with PDI or exceedingly stabile active site disulfides. Above pH 8.0 resulted in substantial non-enzymatic rates of peptide oxidation (results not shown). The peptide thiol-oxidase activity of all enzymes tended to increase with increasing pH, where maximal activity was determined to be at either pH 6.9 or 7.4 (Figure 3.15 B).
Figure 3.14: Schematic of PDI-catalyzed oxidation of a dithiol-containing peptide. A nucleophilic thiol of the substrate peptide attacks the CxxC disulfide of oxidized PDI (oxPDI), resulting in the formation of a mixed disulfide intermediate state. The peptide substrate autoxidizes, liberating PDI from the mixed-disulfide. Regeneration of oxPDI is accomplished using the oxidizing agent GSSG, allowing for successive catalytic turnovers. Peptide oxidation results in a detectable decrease of fluorescence (RFU) via self-quenching between the Arg and Trp side-chains. For simplicity only the $a$-domain active site is depicted.
The only statistically significant difference between WT and the single Q mutants was found with the K401Q enzyme at pH 6.3, 6.5 and 6.9. Here, the mutant exhibited 82%, 77% and 79% the activity of WT, respectively. As well, K57Q at pH 6.3 displayed 82% the activity of WT. The most notable change in activity was demonstrated by the double mutant K57/401Q enzyme, with as little as 57% the activity of WT at pH 6.9. This is suggestive of a need for active site residues Lys^57 and Lys^401 for the catalytic function of PDI even when catalyzing dithiol oxidation reactions, where in this case, mutation of Lys^401 is more detrimental to PDI activity that Lys^57.

This assay is not without its inherent flaws. The effectiveness of accurate rate determination is questionable in comparison to the di-E-GSSG reductase assay seen previously. Optimization of the assay conditions indicated a need for a relatively high concentration of PDI (2 µM) and substrate (50 µM) to generate a detectable signal. This is likely due to marked differences in sensitivity. Being a method that monitors the turn-off of fluorescence, signal acquisition is dramatically less sensitive than a comparable fluorescence turn-on assay, due to high background. Furthermore, tryptophan is a moderate fluorophore compared to others with a reported quantum yield (Φ) of 0.2, molar absorptivity (ε) of 5600 M^-1 cm^-1, and lifetime of 3.1 ns in neutral pH water (λ_ex = 280 nm; λ_em = 350 nm) [189-191]. In comparison to the parameters reported for eosin in water (eosin Y: Φ = 0.2, ε = 88, 000 M^-1 cm^-1, lifetime = 0.95 ns; λ_ex = 525 nm, λ_em = 545nm) [192]. It can be seen that a fluorophore such as eosin is better than tryptophan primarily as a result of ε. Lastly, the activity of the enzyme towards the peptide substrate may be inherently low in comparison to the di-E-GSSG substrate, adding to the abatement of sensitivity.
**Figure 3.15**: Thiol-oxidase activity of hPDI studied over the pH range 5.5 to 8.0. (A) Representative data for the time-course oxidation of 50 µM peptide substrate by 2 µM WT PDI in the presence of 1 mM GSSG, where red dotted lines depict the half-time of reaction. (B) The inverse of the half-time was reported as the quantitative kinetic parameter (after correction for the uncatalyzed rate) by which WT PDI (black) was compared to K57/401Q (blue), K57Q (green) and K401Q (orange). (C) As a control, the fidelity of the peptide substrate (NRCSQGSCWN) was proven by demonstrating its complete oxidation by copper (II) sulfate to yield low fluorescence (red spectrum). Addition of DTT resulted in the regaining of fluorescence via peptide reduction. Data in B represents the mean ± S.D. of n = 3 experiments.
It is possible that the advantages of an active site lysine to PDI activity be primarily through thiol-reductase and not thiol-oxidase activity. The altered kinetics of peptide oxidation may be a result of PDI-mediated GSSG reduction (i.e., enzyme regeneration). What remains unknown is the true relative rates of dithiol-oxidation alone, in the absence of enzyme regeneration. Unfortunately, it was not possible to omit GSSG from the assay mix to provide single-turnover rate determinations (reminiscent of those performed in section 3.5), as sensitivity was an issue.

3.7 **Mutation of K\textsuperscript{57} and K\textsuperscript{401} Impedes the Transfer of Electrons Between PDI and the ER-Resident Oxidase, ERO1α**

The physiological significance of a potential modification of Lys\textsuperscript{57} and Lys\textsuperscript{401} was examined by assessing the activity of PDI towards the well characterized ER-resident oxidase, endoplasmic reticulum oxidoreductin-1α (ERO1α). It has been shown that a CGHCK motif facilitates the greatest rates of both oxidoreductase, thiol-reductase and thiol-oxidase activities of PDI using novel pseudo-substrates. It remains to be seen whether this conserved motif performs in such a manner when PDI participates in redox interactions with the known ER-luminal interacting partner, ERO1α.

The oxidase activity of ERO1α was assessed by monitoring the catalytic consumption of oxygen in the presence of PDI \textit{WT} and the K-Q variants at pH 7.4 using a Clark type electrode [193]. The ERO1α enzyme used was a constitutively active mutant lacking the regulatory cysteines Cys\textsuperscript{104} and Cys\textsuperscript{131} (mutation: C104/131A) [84, 92, 97].

Under standard assay conditions, control rates of oxygen consumption were established for GSH+ERO1α in the absence of PDI (Figure 3.16 dark grey line) and for GSH + PDI in the absence of ERO1α (Figure 3.16 light grey line). As for that
determined for ERO1 in the presence of PDI, the largest rate of oxygen consumption
\(~\sim 0.12 \pm 0.0057 \text{ s}^{-1}\) (Figure 3.16 black line, Table 3.3) was obtained for the interaction
with WT. In the presence of the K57/401Q double mutant ERO1α was only capable of a
rate of oxygen consumption that was \(~\sim 54\%\) of that of WT PDI (0.064 \pm 0.011 s\(^{-1}\), Figure
3.16 blue line, Table 3.3). The single K-Q mutants mediated rates that were very similar,
at 76\% and 83\% for K57Q and K401Q, respectively in relation to WT.

Results indicate that an active site Lys residue is a requisite for efficient electron
shuttling between the \(\alpha\)-type domains of PDI and ultimately to ERO1α. Thus, the
CGHCK motif is favoured for the oxidation of PDI by ERO1α, a characteristic event
during oxidative protein folding within the ER. The oxidase activity of ERO1α was
greatest when in the presence of WT PDI. It is unlikely that the affected rate of oxygen
consumption be a result of altered ERO1α binding to PDI as it has been reported that the
primary site of binding be through the \(b'\)-domain of PDI with no indications of Lys\(^{57}\) or
Lys\(^{401}\) as being significant in this case [89, 92, 194]. Instead, these data suggest that
mutation may cause a hindrance to the efficiency of \(\alpha\)-domain reduction by GSH, thiol-
disulfide exchange between PDI's \(\alpha\) and \(\alpha'\)-domains, and/ or the shuttling of electrons
from the reduced \(\alpha'\)-domain of PDI to the regulatory loop disulfide of ERO1α.

3.8 \(N^e\)-lysine Acetylation of PDI May be A Potent Posttranslational Modulator
of its Multiple Catalytic Activities

To date, no naturally occurring mutations or disease-related single-nucleotide
polymorphisms (SNPs) have been identified for PDI at residues Lys\(^{57}\) and Lys\(^{401}\).
**Figure 3.16:** Mutation of an active site lysine residue affects the interaction between PDI and ERO1α. Oxygen consumption by 2 µM ERO1α in the presence of 5 µM WT PDI (black), K57/401Q (blue), K57Q (green), and K401Q (orange) with 20 mM GSH. Shaded regions represent the standard deviation of the mean of $n = 3$ experiments. As controls, the rate of oxygen consumption was determined for 5 µM PDI in the presence of 20 mM GSH and for 2 µM ERO1α in the presence of 20 mM GSH. All experiments were performed in air-saturated ERO1α assay buffer at pH 7.4.
Table 3.3: Observed rates of ERO1α oxidase activity as determined from the slopes of the initial linear phase of the oxygen consumption profiles in Figure 3.16.

<table>
<thead>
<tr>
<th>hPDI</th>
<th>$k_{obs}$ (s$^{-1}$)</th>
<th>%$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.12 ± 0.0057</td>
<td>100</td>
</tr>
<tr>
<td>K57/401Q</td>
<td>0.064 ± 0.011***</td>
<td>54</td>
</tr>
<tr>
<td>K57Q</td>
<td>0.091 ± 0.011*</td>
<td>76</td>
</tr>
<tr>
<td>K401Q</td>
<td>0.10 ± 0.0035*</td>
<td>83</td>
</tr>
</tbody>
</table>

$^a$ Relative to WT
Thus, a possible means of naturally modulating activity may be via lysine posttranslational modifications, such as acetylation.

To identify if such a modification is possible in targeting the active site lysine residues specifically, an *in vitro* acetylation procedure was performed using acetylsalicylic acid (ASA), the active ingredient of the popular therapeutic Aspirin. By incubating 2 µM PDI with various concentrations of ASA at 37 °C for 4 hours, Western blot analysis revealed the presence of acetyllysine residues in a dose-dependent manner (Figure 3.17). As little as 2 mM ASA was capable of acetylating PDI and providing detectable chemiluminescence at short exposure times (~ <7 minutes).

Next, PDI was acetylated using 15 mM ASA by the same *in vitro* technique described, followed by mass spectrometry (MS) analysis to discern the specific sites of ASA-mediated acetylation. Of the 48 lysine residues present in the PDI construct used (refer to appendix A Figure A.1), 18 were identified as being modified reproducibly (Table 3.4). This included the active site residues Lys57 and Lys401. To unambiguously confirm the presence of acK57 and acK401, the thiol-containing peptides were first enriched using gold nanoparticles (AuNPs) prior to performing tandem mass spectrometry (MSMS; refer section 2.2.13.1). Alternative methods using mercury-activated sepharose were also employed for thiol-enrichment. Despite preliminary results indicating successful pull-down of native redPDI (appendix A Figure A.9), digested peptides applied to the sepharose supports exhibited high carry-over as evidence from the total ion chromatogram (TIC) generated from the UPLC-ESI MS analyses performed. This was determined by comparison to an unenriched PDI digest (results not shown). For this reason, AuNPs were used exclusively for thiol-containing peptide enrichment. The
MSMS data obtained was successful in unambiguously identifying the presence of acetylation on Lys$^{57}$ (Figure 3.18) and Lys$^{401}$ (Figure 3.19). This indicated the active site lysine residues as being solvent accessible and capable of being modified chemically.

All 18 acetyllysine residues identified by MS (Table 3.4) were mapped to a MD simulated structure of redPDI (Figure 3.20). Each appears to be solvent accessible—perhaps unsurprising as lysine residues are inherently preferentially located on the protein surface. Furthermore, there is a propensity for acetylation to occur on the active site helix ($\alpha_2$-helix of the $a$ and $a'$-domains), with 5 total acetyllysine residues identified. The $a$-type domains together accounted for 14 of the 18 sites of acetylation. Alongside the active site residues Lys$^{57}$ and Lys$^{401}$, residues Lys$^{81}$ and Lys$^{424}$ were also acetylated by ASA. These have been implicated as forming charged-ion pairs with conserved glutamic acid residues ($E^{47}$ and $E^{391}$, respectively) situated under the CxxC active sites of each respective domain, facilitating proton-transfer during catalysis [195]. Despite the presence of 8 candidate lysine residues in the $b$-domain (residues 138 to 243) no reproducible acetylation patterns were exhibited. As for the $b'$-domain, only a single site of acetylation was determined on residue K$^{247}$. Lastly, 3 lysine residues of the $x$-linker were determined to be acetylated as well.

It should be noted that the acetylation reaction employed likely results in a low stoichiometry of acetylated lysine residues for two primary reasons. First, in aqueous solution ASA is susceptible to hydrolysis (base-catalyzed deacetylation) resulting in salicylic acid and acetate. This is particularly important to consider because the in vitro conditions for lysine acetylation used here were alkaline (pH ~8.5).
Figure 3.17: PDI is acetylated by Aspirin (acetylsalicylic acid, ASA) *in vitro*. Purified hPDI *WT* (2 μM) was treated with varying concentrations of ASA (0.25, 0.5, 0.75, 1, 2, 5, 10, and 15 mM) for 4 hours at 37°C in a 0.1 M Tris-HCl buffer pH 8.5. Immunoblotting was performed using anti-acetyllysine pan-specific antibody for the detection of acetylated lysine residues and anti-PDI RL90 antibody to determine consistent PDI loading.
Table 3.4: Acetylated peptides detected using UPLC-ESI MS for ASA-acetylated PDI. Peptides were identified from an un-enriched PDI digest (Glu-C protease) confirmed by MS² and MSMS sequencing. Reported acetylation sites (K_{ac}) represent those found to be common among \( n = 2 \) samples.

<table>
<thead>
<tr>
<th>Precursor Sequence</th>
<th>Acetylation Site on PDI (^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FYAPWCGHK{\textsuperscript{ac}}ALAPE(^{62})</td>
<td>K(^{57}); ( \alpha )-domain active site; ( \alpha_{2} )-helix</td>
</tr>
<tr>
<td>YAK{\textsuperscript{ac}}AAGK{\textsuperscript{ac}}LK{\textsuperscript{ac}}AE(^{73})</td>
<td>K(^{65}), K(^{69}) and K(^{71}); ( \alpha )-domain; ( \alpha_{2} )-helix</td>
</tr>
<tr>
<td>IRLAK{\textsuperscript{ac}}VDAT(^{86})</td>
<td>K(^{81}); ( \alpha )-domain; ( \beta_{3} )-sheet</td>
</tr>
<tr>
<td>SDLAQGYGVRGYTI{\textsuperscript{ac}}FRNGD{\textsuperscript{ac}}TASPK{\textsuperscript{ac}}E(^{115})</td>
<td>K(^{114}); ( \alpha )-domain; ( \beta_{3} )-sheet</td>
</tr>
<tr>
<td>FTEQTapK{\textsuperscript{ac}}IFGGE(^{252})</td>
<td>K(^{247}); ( b' )-domain; ( \alpha_{1} )-helix</td>
</tr>
<tr>
<td>G{\textsuperscript{ac}}K{\textsuperscript{ac}}I{\textsuperscript{ac}}PHLMSQELPE(^{362})</td>
<td>K(^{350}) and K(^{352}); ( x )-linker; loop</td>
</tr>
<tr>
<td>WDK{\textsuperscript{ac}}QPVKVVLGVKNFE(^{378})</td>
<td>K(^{366}); ( x )-linker; loop</td>
</tr>
<tr>
<td>K{\textsuperscript{ac}}QPVK{\textsuperscript{ac}}VVLGV{\textsuperscript{ac}}NFEDVAFD(^{383})</td>
<td>K(^{366}) (see above). K(^{370}) and K(^{375}); ( a' )-domain; ( \beta_{1} )-sheet and ( \alpha_{1} )-helix, respectively</td>
</tr>
<tr>
<td>K{\textsuperscript{ac}}NVFVEFYAPWCGHK{\textsuperscript{ac}}QLAPIWDK{\textsuperscript{ac}}LGE(^{412})</td>
<td>K(^{385}) and K(^{386}); ( a' )-domain; ( \alpha_{1}-\beta_{2} ) loop</td>
</tr>
<tr>
<td>FYAPWCGHK{\textsuperscript{ac}}QLAPIWDKLGE(^{412})</td>
<td>K(^{409}); ( a' )-domain; ( \alpha_{2} )-helix</td>
</tr>
<tr>
<td>NIVIAK{\textsuperscript{ac}}MDSTANE(^{431})</td>
<td>K(^{401}); ( a' )-domain active site; ( \alpha_{2} )-helix</td>
</tr>
<tr>
<td>GFK{\textsuperscript{ac}}KFLE(^{471})</td>
<td>K(^{424}); ( a' )-domain; ( \beta_{3} )-sheet</td>
</tr>
</tbody>
</table>

\(^a\) (residue number); (domain location); (secondary structural element location)
Figure 3.18: Residue Lys$^{57}$ of the $\alpha$-domain active site is acetylated by Aspirin in vitro. Peptides of GluC-digested PDI underwent gold nanoparticle (AuNP) thiol enrichment prior to performing UPLC-ESI MS/MS (see section 2.2.11).
Figure 3.19: Residue K^{401} of the α'-domain active site is acetylated by Aspirin in vitro. Peptides of GluC-digested PDI underwent gold nanoparticle (AuNP) thiol enrichment prior to performing UPLC-ESI MS/MS (see section 2.2.11).
Figure 3.20: Location of the 18 identified acetyllysine residues mapped to the lowest energy structure of a molecular dynamics simulation of redPDI (PDB: 4EKZ [53]). MS was used to identify the acetylated lysine residues of 2 µM PDI treated with 15 mM ASA. Those residues found to be acetylated are shown in red and labelled respectively. Hydrogens of the side chain have been omitted. Yellow spheres represent the active site cysteines. Visualized using Visual Molecular Dynamics software (VMD) [54].
These conditions are to promote a larger proportion of the reactive deprotonated state of the ε-amine of lysine residues; conditions that contrarily enhance aqueous ASA hydrolysis. Secondly, ASA is not purely amine-directing. It is possible to observe O-acetylation of tyrosine (Tyr), serine (Ser) and threonine (Thr) residues. The most notable example of this is the irreversible acetylation of the catalytic serine of cyclooxygenase enzymes COX-1 and COX-2, resulting in the hallmark antithrombotic (low dose) and anti-inflammatory (high dose) responses elicited by Aspirin [196]. For the purposes of this study, O-acetylation of PDI was reverted by incubation with hydroxylamine (HA) prior to treatment with ASA (see section 2.2.9). Nevertheless, the possibility of O-acetylation occurring would further limit the stoichiometry of lysine acetylation via direct competition for ASA.

The effect of ASA-mediated acetylation on the steady state kinetics of PDI activity was assessed using the di-E-GSSG assay (Figure 3.21). To discern the impact of acetylation on only the active site residues Lys\textsuperscript{57} and Lys\textsuperscript{401} in relation to the other acK residues generated from the \textit{in vitro} treatment with ASA, the kinetics of ASA-acetylated \textit{WT} PDI (\textit{WT}_{ASA}, Figure 3.21 A, black dotted line) was compared to ASA-acetylated K57/401Q (K57/401Q\textsubscript{ASA}, Figure 3.21 A, blue dotted line). Negative controls consisted of unacetylated \textit{WT} (\textit{WT}_{control}) and K57/401Q PDI (K57/401Q\textsubscript{control}, Figure 3.21 A, black and blue solid lines, respectively). By comparing the kinetics between \textit{WT}_{ASA} and K57/401Q\textsubscript{ASA} it was possible to realize the kinetic contributions of only Lys\textsuperscript{57} and Lys\textsuperscript{401} acetylation. As discussed, the stoichiometry of acetylation is by no means perfect, resulting in heterogeneous mixtures of modified PDI molecules. Thus, the results should
be taken as a qualitative trend because the presence of non-acetylated active site lysine residues will likely persist to some extent for \( WT_{ASA} \).

As illustrated by Figure 3.21, \( WT_{control} \) performed optimally compared to the other enzymes, with an apparent \( k_{cat} \), \( K_M \) and \( k_{cat}/K_M \) of \( (0.688 \pm 0.0277) \times 10^{-2} \) s\(^{-1}\), \( 1.27 \pm 0.114 \) µM and \( 5400 \pm 356 \) M\(^{-1}\) s\(^{-1}\), respectively. \( WT_{ASA} \) resulted in an enzyme that was functionally very similar to \( K57/401Q_{control} \). The only statistically significant difference was observed for \( K_M \) (Figure 3.21 C), where \( WT_{ASA} \) had an apparent \( K_M \) of \( 2.13 \pm 0.0679 \) µM, and \( K57/401Q_{control} \) exhibited a \( K_M \) of \( 2.51 \pm 0.0542 \) µM. Both \( k_{cat} \) and \( k_{cat}/K_M \) were relatively the same, with no significant difference determined: \( (0.307 \pm 0.0249) \times 10^{-2} \) and \( (0.287 \pm 0.0486) \times 10^{-2} \) s\(^{-1}\), and \( 1440 \pm 396 \) and \( 1140 \pm 290 \) M\(^{-1}\) s\(^{-1}\) for \( WT_{ASA} \) and \( K57/401Q_{control} \), respectively. When examining \( k_{cat} \) further (Figure 3.21 B), it was observed that a significant variation occurred when comparing \( WT_{ASA} \) and \( K57/401Q_{control} \) with \( K57/401Q_{ASA} \). This can be concluded as being a result of acetylation at residues other than Lys\(^{57}\) and Lys\(^{401}\), in particular, Lys\(^{81}\) and Lys\(^{424}\). The difference of the apparent \( K_M \) determined for \( WT_{ASA} \) and \( K57/401Q_{ASA} \) was also significant, with the ASA-treated mutant exhibiting a larger value of \( 2550 \pm 55.7 \) µM. A similar comparison between \( K57/401Q_{control} \) and \( K57/401Q_{ASA} \) drew no significance, indicating that Lys\(^{57}\) and Lys\(^{401}\) are likely the substantiating factors in determining \( K_M \). Furthermore, enzyme efficiency was only nominally impacted when comparing \( WT_{ASA} \) to \( K57/401Q_{control} \) and \( K57/401Q_{ASA} \), consolidating the importance of Lys\(^{57}\) and Lys\(^{401}\) (Figure 3.21 D).
Figure 3.21: ASA-mediated acetylation of PDI attenuates oxidoreductase kinetics for the reduction of di-E-GSSG. PDI at a concentration of 2 µM was treated with 15 mM ASA for 4 hours at 37 °C in the presence of 0.5 mM DTT. Untreated, parallel controls were also performed in the absence of ASA. (A) Using the di-E-GSSG assay, the steady state oxidoreductase kinetics of 20 nM untreated control WT (black solid line, WTcontrol), ASA-treated WT (black dotted line, WTA SA), untreated control K57/401Q (blue solid line, K57/401Qcontrol), and ASA-treated K57/401Q (blue dotted line, K57/401QASA) was assayed at pH 7.4. The apparent kinetic parameters (B) $k_{\text{cat}}$, (C) $K_M$ and (D) $k_{\text{cat}}/K_M$ were calculated from the Michaelis-Menten fits in A. Statistical significance with respect to WTcontrol is denoted by asterisks (*). Hashtags (#) denote statistically significant differences between any indicated pair of data. Data represents the mean ± S.D. of $n = 3$ experiments.
CHAPTER 4

DISCUSSION
The prevalence of PDI (PDIA1, EC: 5.4.3.1) in human physiology and pathophysiology has provided evidence of a need for a better understanding of its multiple enzymatic and non-enzymatic functions- both *in vitro* and *in vivo*. Despite its discovery and extensive study since the 1960's [37-40], disparate results have been observed quite recently with respect to the possible mechanisms by which PDI follows. For instance, it has long been understood that the multi-domain organization of PDI and other PDI family members is intrinsically linked to specific activities [53, 76-79]. Even though the catalytic centers are the CxxC motifs of the *a*-type domains, the non-enzymatic *b*-type domains are equally as important with regards to substrate binding and the overall conformation.

Conventionally, it has been shown that many potent, specific inhibitors of PDI function *via* binding (reversibly or irreversibly) to the *b*' domain (substrate binding domain), leading to abrogated or attenuated activity. Compounds that lead to direct modification of the active site thiols are unsurprisingly the most potent inhibitors; nonspecific in function [43]. Even still, a class of compounds has recently been described as inhibitory with respect to substrate binding (inhibiting platelet aggregation), yet hold the ability to *enhance* enzymatic activity. Regarded as bepristats, these reversibly bind the *b*' hydrophobic cleft. Using the di-E-GSSG oxidoreductase assay, research has demonstrated this to be positive allostery enhancing catalysis [76]. Interestingly, bepristats were actually found to *inhibit* PDI activity when using the insulin turbidity assay, contrary to those findings with the di-E-GSSG assay. These indications of inhibitor-induced positive allostery of PDI catalytic activity are in stark contrast to the effects imposed by the bacitracin family of inhibitory compounds, those which bind the *b'*
hydrophobic cleft and inhibit chaperone activity with no discernable impact on catalytic activity [43, 134]. This is a key example of how PDI activity is largely substrate-dependent, and why the dynamics of its catalytic mechanisms are not solely dependent on the CxxC motif, but the entirety of the protein architecture. For this reason, it is vitally important to conduct complementary PDI assays when assessing activity.

Many highly conserved residues have been identified as important to the redox reactivity of PDI. The active site sequence of hPDI is CGHC, where the N-terminal cysteine (CysN) is the nucleophile responsible for the initial attack of a substrate disulfide, and the C-terminal cysteine (CysC) is the resolving cysteine required to liberate PDI from a mixed-disulfide intermediate state [62-64]. The intervening residues His and Gly act to mediate stabilization of the CysN thiolate and provide a degree of flexibility to the active site, respectively [65]. An arginine residue situated away from the active site has been shown to effectively move in close proximity to CysC whilst PDI is occupying a mixed-disulfide intermediate state. This functions to stabilize the CysC thiolate anion by depressing its pKa and making it a better nucleophile, thereby mediating substrate release [63]. Lastly, a buried K-E charged-ion pair resides below the active site vicinal thiols of each active site. This has been implicated in facilitating proton-transfer during thiol-disulfide exchange reactions [62, 195, 197].

The functions of the aforementioned residues have been scrutinized extensively and their roles well characterized. In this thesis, we characterize and provide evidence for the functional role provided by the highly conserved active site lysine residues, Lys57 and Lys401 (of the a and a'-domains, respectively). The primary sequence of the active site region of PDI is invariant and highly conserved from yeast to human:
FYAPWCGHCK [55, 185]. Despite such a high degree of conservation, PDI is able to accommodate many alterations to the amino acid sequence of this motif with only minor changes to redox activity. This demonstrates that only the catalytic cysteine residues are required for catalysis, while the other conserved residues are non-essential, but still important to the specific functions of PDI. Lu et al. studied the active site-flanking Lys in the first domain of rat PDI [185]. They concluded that such does not perform an essential, "specific" function when substituting it for Arg and measuring the relative kinetics for the reduction of insulin and refolding of RNase A, \textit{in vitro}. They went on to state that the \~15\% loss of activity likely indicates that arginine may fulfill functions similar to that of lysine [185]; that which has yet to be elucidated (see later).

We demonstrate here that substitution to non-conservative amino acids does indeed result in marked attenuation of PDI kinetics using a variety of \textit{in vitro} assays. This includes the indication that residues Lys$^{57}$ and Lys$^{401}$ are important for ERO1$\alpha$-mediated oxidation of redPDI. Furthermore, analysis of genetic variations of PDI did not indicate the presence of single nucleotide polymorphisms (SNP's) at either active site residue. Coupled with the kinetic data, it is apparent that a logical means of modulating PDI activity may potentially be \textit{via} posttranslational acylation of Lys$^{57}$ and Lys$^{401}$. In an attempt to determine acetylation as the potential acylation event, PDI was treated with Aspirin, and the modified lysine residues were identified unambiguously using mass spectrometry (MS), with reference to the impact on oxidoreductase kinetics.
4.1 The CxxCK Motif Enhances the Full Complement of PDI's Catalytic Activities

The rate by which PDI catalyzes thiol-disulfide exchange reactions is of vital importance to cellular homeostasis. A primary example being the ability of PDI to minimize the folding timescale of nascent polypeptides within the lumen of the endoplasmic reticulum (ER). This effectively prevents the build-up of unfolded or misfolded proteins that would otherwise lead to aggregates. Proliferating or stressed cells will exhibit a higher rate of protein synthesis that can become pro-apoptotic should PDI as well as other foldases and chaperones be unable to handle the "work load." The primary result of such is chronic stress and the initiation of the unfolded protein response (UPR) [118, 125, 128]. Therefore, the optimal catalytic performance of PDI is required to prevent such detrimental processes from occurring. It is well documented that the reversible oxidation of the active site thiols of PDI by S-nitrosylation abrogates activity-inducing the UPR, thereby resulting in neuronal cell death. This is a hallmark of the neurodegenerative diseases, ALS, Parkinson's and Alzheimer's [125, 127, 130].

As mentioned, the mechanistic details by which PDI performs its functions optimally is not completely understood. For instance, the pH-dependence of PDI activity has seldom been studied to date. An investigation of the pH-dependence of PDI-catalyzed RNase A refolding focused only on a narrow range of pH, from 7 to 9 (at 1 pH unit increments) [198]. Based on the data, it was concluded that the isomerase activity of PDI was most optimal at pH 7 [198]. Without expanding the work to more acidic conditions, it cannot be said for certain whether this is true of PDI or not. Other such research focusing on the pH-dependent dithiol-oxidase activity of PDI indicated that the maximum activity be centered on about pH 7, when studying over the pH range of 4.5 to
7.5 [83, 188]. In this case, data were undeterminable above pH 7.5 due to exceedingly high uncatalyzed rates of substrate peptide oxidation.

To our knowledge, we thus present for the first time the full pH-dependent oxidoreductase activity profile for hPDI over a comprehensive range of pH. This was achieved utilizing the di-E-GSSG assay [82] as the pseudo-substrate was deemed stable over the employed range (Figure 3.1 B). The determined pH range was 5.5 to 8.5, with the indication that PDI undergoes substantial pH-induced global conformational changes below pH 5.5 (Figure 3.1 A). Intrinsic tryptophan fluorescence was used to monitor the conformational stability of redPDI, where an increase of the emission peak at pH 4.5 was the indication of denaturation. This emission increase was likely a result of the altered solvent accessibility of residue Trp\(^{364}\), with the active site neighbouring residues Trp\(^{52}\) and Trp\(^{396}\) being a non-factor due to their close proximity to the respective catalytic sites, leading to quenching [199, 200].

Full bell-shaped activity vs. pH profiles were generated and fitted to a double-ionization model (Equation 2.2). Estimation of the apparent macroscopic ionization events leading to PDI activation (\(\text{app}pK_1\), ascending limb) and inactivation (\(\text{app}pK_2\), descending limb) provided interesting findings suggesting that the enzyme functions over a relatively narrow range of pH as an oxidoreductase (Figure 3.1 C and Table 3.1). WT PDI displayed optimal activity at pH 6.77. This is in close agreement with previous studies using differing assay methods [83, 188, 198]. This further demonstrates that catalysis is optimal at near-neutral to slightly acidic pH in vitro, conditions similar to the in vivo environment of the secretory compartments (ER lumen and Golgi) where PDI is found to primarily localize and function [44, 175, 201].
The breadth of oxidoreductase activity was determined as the difference between \( \text{app} pK_1 \) and \( \text{app} pK_2 \). For \( WT \) PDI this was just 1.21 pH units. Mutation of the conserved residues Lys\(^{57} \) and Lys\(^{401} \) simultaneously to either Gln, Ala or Glu, resulted in mutant PDI enzymes with narrower ranges of activity, at only \( \sim 0.6 \) to 0.85 pH units. The factor at play here was the shift of \( \text{app} pK_2 \), whilst \( \text{app} pK_1 \) remained relatively unchanged at \( \sim 6.16 \), with only nominal deviations brought about the varying mutations. This was suggestive of there being no discernable alterations to the pK\(_a\) of the active site Cys\(_N\) and Cys\(_C\) thiols as \( \text{app} pK_1 \) likely represents a combination of the ionization of both. This was further supported by molecular dynamics (MD) simulations that estimated the active site thiol pK\(_a\)'s as being \( \sim 4.5 \) and \( \sim 8.5 \) for Cys\(_N\) and Cys\(_C\) of \( WT \) PDI, respectively. When substituting both Lys\(^{57} \) and Lys\(^{401} \) to Gln, the Cys\(_N\) thiol pK\(_a\) of each active site increased slightly by \( \sim 0.6 \) units, while the Cys\(_C\) pK\(_a\) exhibited no significant perturbation (Table 3.2).

The shift of the descending limb midpoint (\( \text{app} pK_2 \)) by \( \sim 0.5 \) units as a result of mutation indicated an important role for residues Lys\(^{57} \) and Lys\(^{401} \) in modulating the pH-dependence of PDI activity. It was speculated that the apparent inactivation of PDI be a result of the ionization of key residues, such as the intervening His of the CGHC motifs. Should an active site Lys be functioning to modulate the pK\(_a\) of this residue in either a direct or indirect fashion, a decrease of \( \text{app} pK_2 \) brought about by mutation would indicate that its orientation be important for favouring the cationic state of the imidazolium side chain. The intervening His residue is known to stabilize the thiolate state of Cys\(_N\), thereby increasing \( E \) (redox potential); rendering PDI a more effective thiol-oxidase in comparison to other TRX enzymes [64, 65, 97, 99]. Additionally, \( \text{app} pK_2 \) may represent a
macroscopic ionization event that is a combination of the intervening His, the Lys side chain, as well as other residues that cannot be inferred upon at this time. Although, the pKₐ of residues Lys⁵⁷ and Lys⁴⁰¹ were estimated to be ~10 using MD analyses (Table 3.1). It can thus be speculated that under the in vitro conditions employed, ionization of either active site lysine of WT PDI would have been negligible, therefore likely not being a factor in directly contributing to apppK₂.

The pH-dependent activity profiles at a single substrate concentration did not provide a complete picture of the pH effects on the kinetic parameters. To circumvent this, the kinetic parameters: turnover number (kₐₜ), Kₘ and catalytic efficiency (kₐₜ/Kₘ) were determined as a titration of pH from 6.0 to 8.0. The substitution of residues Lys⁵⁷ and Lys⁴⁰¹ indicated that mutation to either domain of PDI was nearly equivalent on the basis of kinetics. In general, both kₐₜ and Kₘ were only marginally variable when comparing K₅⁷X and K₄₀¹X variants (where X is Q, A or E) at any given pH (appendix B Tables B.1 to B.5). Despite the understanding that each active site is actually inequivalent in vivo [56, 61], as well as under saturating conditions in vitro [60], our results show that they may perform very similarly. Such has been observed elsewhere as well [59]. This is further supported when assessing the activity of active site thiol mutants (CxxC-AxxA, appendix A Figure A.4).

Over the pH range of 6.5 to 8.0, WT PDI was determined to be the most efficient enzyme. The double mutant PDI variants were unsurprisingly less efficient than the respective single mutants. Despite being apparently most efficient at pH 6.5, WT PDI outperformed all mutant variants to the greatest extent under physiologically relevant conditions, pH 7.4. Here, the single mutant enzymes were ~40 to 55% as efficient, while
the double mutants were ~20 to 34% as efficient in relation to \( WT \) (Figure 3.1 and appendix B Table B.4). The kinetic parameters \( k_{\text{cat}} \), \( K_M \) and \( k_{\text{cat}}/K_M \) for \( WT \) PDI at pH 7.4 were determined to be 0.0347 s\(^{-1}\), 777 nM and \( 4.46 \times 10^3 \) M\(^{-1}\) s\(^{-1}\), respectively (Figure 3.1 and appendix B Table B.4). This is in fairly close agreement with previous reports [81].

It was realized that the large determinant for enzyme efficiency was the trend observed for \( K_M \) in relation to pH. \( K_M \) exhibited a consistent increase, with a net change of ~165-fold. This is in contrast to the ~5-fold variation of \( k_{\text{cat}} \), which did not increase consistently across the pH range- rather, it approached a maximum at pH 7.0 before declining at pH 7.4 and 8.0 (this was true of all PDI enzymes tested; Figure 3.1 A and B, and appendix B Tables B.1 to B.5). This likely indicates the optimal pH for the balance with thiol nucleophilicity of both Cys\(_N\) and Cys\(_C\) [27]. The result was \( WT \) PDI being an apparently more efficient enzyme across the entirety of the pH range, except for at pH 6.0 where no statistically significant differences were observed as compared to the mutants (appendix B Table B.1). These data indicate that at pH 6.0, the activity of PDI was not significantly reliant on either Lys\(^{57}\) or Lys\(^{401}\), as mutation to Gln, Ala or Glu did not affect \( K_M \) or \( k_{\text{cat}} \) (thereby not altering enzyme efficiency). It is possible to explain this result when examining Figure 3.3. Under acidic conditions the rate of substrate release was exceedingly slow, rendering PDI trapped in a mixed-disulfide state. This was likely a result of slowed kinetics of the resolving cysteine, Cys\(_C\). It is therefore possible that the kinetics of PDI activity are most influenced by this acid-induced substrate trapping event, thereby negating any possible contributions brought about by the neighbouring lysine residues, Lys\(^{57}\) and Lys\(^{401}\) (or any residue in these positons, for that matter).
No statistically significant differences in $K_M$ were observed at pH 8.0. Furthermore, this was also true at pH 6.0, with the exception of the K57/401Q variant, which exhibited a near 2-fold increase. Between pH 6.5 and 7.4 the single mutant enzymes displayed $K_M$ values about 1.5 to 2-fold greater than $WT$, whilst the double mutant enzymes were 2 to >3-fold larger. This is consistent with the notion that mutation to residues Lys$^{57}$ and Lys$^{401}$ results in diminished substrate (di-E-GSSG) binding to the active site.

It was interesting to note that, in relation to the differences of $K_M$ between $WT$ and the mutant PDI enzymes, $k_{cat}$ was also impacted. The significance, though, was much less so. Across the range of pH, only some mutants displayed altered $k_{cat}$ values that were deemed statistically significant, but none at pH 6.0 (with the exception of K57/401E, see appendix B Table B.1). In general, the single mutant variants displayed nominal changes to $k_{cat}$ in relation to $WT$. On the other hand, the double mutants K57/401(Q/A/E) exhibited markedly attenuated turnover rates. This provides an indication that mutation to Lys$^{57}$ and Lys$^{401}$, simultaneously, retards PDI substrate turnover significantly, likely a result of changes to the environment of the Cys$_N$ nucleophile (see later). Surprisingly, at pH 8.0 $k_{cat}$ was the only parameter affected by mutation, while $K_M$ was relatively unchanged (appendix B Table B.5). It is possible that under these conditions the ability of PDI to accommodate and bind the di-E-GSSG substrate is limited to such an extent that mutation to the active site region likely does not invoke structural variations that dictate substrate binding to the same extent in comparison. At basic conditions such as this, the surface charge of PDI (and the substrate for that matter) may be altered, leading to the observed result.
Taken together, $k_{\text{cat}}$ may be telling of the possible mutation-induced changes to cysteiny1 $pK_a$ and/or nucleophilicity that affect the kinetics of substrate attack and release, whilst the $K_M$ data may be a valid reporter of active site steric hindrance mediated by the substitution of an active site lysine residue to non-conservative amino acids.

Interpretation of the thermodynamic and kinetic effects of the respective single and double mutations can provide insight to the relatedness of residues to substrate binding and catalysis. Results indicate that the effects to $K_M$ by double mutation are apparently additive or partially additive with respect to the single mutations. This means that the sum of the contribution of the K57X and K401X single mutants is about equal to that observed for the respective K57/401X double mutants [202]. The observation for $k_{\text{cat}}$, is only partial additivity (where significant). This makes sense as these residues are non-interacting yet likely help mediate similar steps of catalysis [202]; localized to either of the two distinct active sites of PDI that act in a near-equivalent, yet individual manner 	extit{in vitro} [59, 60]. Furthermore, the impact on PDI activity is such that Lys$^{57}$ and Lys$^{401}$ are non-catalytic residues that likely do not largely hinder the rate-limiting steps of catalysis—that is, they facilitate non-rate-limiting steps [83, 202]. Interestingly, additivity with respect to $K_M$ was not consistently seen over the entire pH range, or with each substituted residue (Gln, Ala and Glu). At pH 6.5 and 7.0 (appendix B Tables B.2 and B.3) both Gln and Ala mutants were additive, with partial additivity for Gln and nearly complete additivity for Ala at pH 7.4 (appendix B Table B.4). In contrast, mutation to Glu displayed only partial additivity from pH 6.5 to 7.4. This, taken together with the varied
enzyme efficiencies, suggests possible residue-specific effects at these flanking regions within the PDI active sites.

Mutation to Gln is the least detrimental to activity (enzyme efficiency). This makes sense as it is most similar both structurally and chemically to lysine (compared to Ala and Glu). An Ala mutation removes both the steric bulk and charge of lysine in place of a small, hydrophobic residue. The impact on activity brought about by this substitution is less still when compared to Glu. Mutation to Glu results in an anionic side chain which presents the most drastic alteration in microenvironment.

The di-E-GSSG pseudo-substrate takes on a net negative charge (appendix B Figure B.6 for structure and estimated pKₐ's [203, 204]). It is possible that mutation to Glu may effectively result in electrostatic repulsion of the substrate from either the active site or the general encounter-complex association with PDI. All the while, Lys may take on a stabilizing role in this regard. As for Ala, it would present somewhat of an intermediate effect, supported by the observed kinetics of di-E-GSSG reduction. It is interesting to note that an Ala mutation may also out-perform Glu in certain other cases, such as thiol-disulfide exchange reactions and chaperone interactions between PDI and larger substrates, perhaps nascent polypeptides. This can be speculated as Ala could potentially complement the hydrophobic surface of the substrate binding surface of PDI [53].

In order to characterize the functional role of the active site-flanking residues Lys⁵⁷ and Lys⁴⁰¹, we first sought to examine the NMR solution structures of homologous mammalian PDI family members PDIA3 (PDB: 2DMM [176]) and PDIA6 (PDB: 2DML [177]) (Figure 3.2). For the PDB entry 2DMM, this was 20 structural overlays of the α'-
domain active site of human PDIA3, bearing a CGHCK motif that is reminiscent of WT PDI (Figure 3.2 A). For the PDB entry 2DML, this was 20 structural overlays of the α-domain active site of mouse PDIA6, bearing a CGHCQ motif that is reminiscent of our designed K-Q mutants (Figure 3.2 B). It was interesting to note the apparent flexibility of the WT-like CGHCK active site motif (2DMM/ PDIA3) in relation to the apparently ordered and rigid active site region of the CGHCQ motif (2DML/ PDIA6).

It was initially theorized that the flexibility of the Lys side chain propagated through the active site, leading to the mobilization of the intervening His imidazolium moiety. This was supported by the solution structures showing that when Gln was in place of K, the His side chain was within hydrogen bonding distance with the active site Cys$_N$ (2.6 Å away) [205]. The WT CGHCK motif seemed to maintain the His side chain at >5.6 Å from Cys$_N$. This may indicate that a CGHC-flanking Lys residue mediates an active site conformation that allows for Cys$_N$ thiolate stabilization via long-range electrostatic interactions with the intervening His, at the same time preventing the imidazolium from being too near to form unfavourably strong ion pairs and/ or steric hindrance [65, 102]. The attenuated activity observed by the K-Q mutants was thus initially believed to be a result of the intervening His over-stabilizing the Cys$_N$ thiolate (decreasing its pK$_a$) due to its close proximity. This would effectively result in a concomitant decrease of nucleophilicity [27, 28, 31]; a less reactive enzyme. Though, this was unfounded when looking at the MD-estimated thiol pK$_a$'s that were nearly unchanged as a result of mutation, and if anything, the Cys$_N$ thiol pK$_a$'s actually increased slightly as a result of substituting Lys$_{57}$ and Lys$_{401}$ to Gln (Table 3.2). When looking at the entirety of the domain overlays, rather than just the active site motifs as shown in
Figure 3.2, it was apparent that 2DMM (PDIA3 $a'$-domain) as a whole is slightly more disordered than 2DML (PDIA6 $a$-domain). Therefore, the proposed active site "floppiness" of 2DMM may simply be an artifact of data acquisition during the NMR experiment itself. The reader should note that depositions 2DMM or 2DML originate from unpublished research.

Due to the controversial results observed when assessing the NMR solution structures of CGHCK and CGHCQ PDI motifs, we next performed full MD simulations of red- and oxPDI: $WT$ and K57/401Q. Here, the role of either active site flanking Lys was not identified as being related to any dynamic changes of the active site intervening His. Should the previous hypothesis have held fidelity, the mutation-induced movement of the intervening His in closer proximity to the Cys$_N$ thiol would have resulted in a better thiol-oxidase. This is because the thiolate state of PDI would have been stabilized and favoured. Meaning, mutation of Lys$^{57}$ and Lys$^{401}$ should have resulted in an enzyme with attenuated thiol-reductase activity (as has been observed, see later and Figure 3.13), yet augmented thiol-oxidase activity, that which was not observed (see later and Figure 3.15). Furthermore, if $a^{\text{app}}pK_2$ (Table 3.1) were an estimation of the ionization of the intervening H, then it's closer proximity to the active site thiolate as a result of mutation would have actually increased this parameter instead of decreasing it. Instead, residues Lys$^{57}$ and Lys$^{401}$ were found to apparently modulate the accessibility of Cys$_N$ in very distinct ways. Following this, it was realized that such processes may be redox-state dependent for the $a$-domain, but not the $a'$-domain.

For the $a$-domain of redPDI, Lys$^{57}$ was observed to participate in cation-π interactions with the indole side chain of an upstream tryptophan, Trp$^{52}$ (Figures 3.3 and
When mutating Lys\textsuperscript{57} to Gln this interaction was lost, resulting in the translation of the indole moiety over the active site Cys\textsubscript{N} (Cys\textsuperscript{53}), acting as a sort of "lid" (Figure 3.5). Based on this, it was suggested that the interactions between Lys\textsuperscript{57} and Trp\textsuperscript{52} be important for mediating an *open* active site as determined from the apparent accessibility of Cys\textsuperscript{53}. The accessibility/ exposure of Cys\textsubscript{N} is particularly important as this residue is required for the initiation of catalysis [62-64]. When assessing the structure of oxPDI, this same interaction was not observed, rendering the a-domain active site apparently *closed* (appendix B Figure B.2 A). It is likely that the Cys\textsuperscript{53}-Cys\textsuperscript{56} disulfide introduced some degree of constraint on the active site, leading to this result. By comparing the ability of the mutant Gln\textsuperscript{57} residue to hydrogen bond with the active site backbone in the simulations of redPDI (appendix B Figure B.4 C) and oxPDI (appendix B Figure B.5 C), an apparent disruption was observed in the oxPDI simulation. This may be telling of the speculated conformational constraints brought about by the active site disulfide. This strongly suggests that the a-domain active site of PDI undergoes redox state-dependent *open* and *closed* conformations as mediated by the cation-π interactions between Lys\textsuperscript{57} and Trp\textsuperscript{52}.

The work of Lu *et al.* [185] may suggest the presence of cation-π interactions as being a factor in determining PDI activity. In their work, substitution of Lys\textsuperscript{57} for Arg indicated that Lys performed no "specific" function as alterations to activity were only minor. Although this may be true, a similarity between Lys and Arg is the shared ability to form strong cation-π interactions within protein ensembles [186]. Therefore, the semi-conservative mutation of K\textsuperscript{57}R is likely not a large detriment to PDI activity as the proposed cation-π interaction with Trp\textsuperscript{52} remains, thereby rendering the active site of the
a-domain open [185]. Furthermore, studies of the equivalent (conserved) active site-flanking Lys in *E. coli* TRX showed that mutation to Glu did not greatly attenuate the efficiency of ribonucleotide reductase activity at pH 7.0 [206]. Our kinetic data oppose this finding, yet it is likely a result of the obvious amino acid sequence and structural differences between TRX and hPDI, as well as the assay conditions and substrate used. It is widely understood that the various structural differences between TRX and PDI family members is what accounts for the variety of specific functional roles and mechanisms of substrate recognition [26, 43, 207]. Thus, an active site flanking residue in TRX may not be as important as it is for PDI, as evidence from the hPDI kinetic data herein.

The active site Trp is a highly conserved residue of a vast majority of prokaryotic and eukaryotic TRX enzymes and PDI family members [208-210]. Its role has been debated in research, with the general conclusion that in TRX (from *E. coli* and *S. aureus*), it is important to the protein's thermodynamic stability [209, 211, 212]. Computational modelling and theoretical calculations coupled with experimental results proved that the active site Trp of TRX was unimportant for catalysis in the sense that mutation to A did not result in the perturbation of the active site thiol pKₐ's, nor did it affect the regioselectivity of the Cys₅₇ thiolate [212]. Furthermore, the structures of both redTRX and oxTRX display the indole ring as projecting toward, and partially covering the dithiol/ disulfide active site [208]. This is similar to that observed in the crystal structures of red- and oxPDI (PDB: 4EKZ and 4EL1, respectively [53]), as well as for the solution structures of PDIA3 [176] and PDIA6 [177] (Figure 3.4). The proposed cation-π interaction between Lys⁵⁷ and Trp⁵² was not observed in the available crystal structures
of PDI. In all cases, it appeared as though the postulated closed active site conformation of the a-domain persisted in relation to the open.

Disparity between the MD simulated structures here, and that of experimentally determined crystal structure data may lie in the dynamic low-energy minima populated by a given protein ensemble. At times, there can be considerable structural variation when comparing theoretical models (based upon experimentally determined structures), crystal structures and NMR solution structures [213]. Although the core-regions of proteins are well ordered and relaxed, rendering a high degree of agreement between the various methods of structural analyses, the more solvent-exposed loop regions often show greater disorder and variability in backbone and side chain conformations [213]. The a-domain active site of PDI (as well as the a'-domain active site, for that matter) is located on a loop region where such disorder may occur. Therefore, it remains probable that the observed open-closed phenomena determined here using computational modelling may still be realistic despite little indication of such when assessing experimentally determined structures. This notion may be further supported by the observation that the PDI molecule in solution is structurally quite different from that obtained in solid crystal form. This was confirmed by MD analyses and small angle X-ray scattering (SAXS; an in-solution procedure) experiments where PDI was found to adopt a more compact conformation [70, 214].

The MD simulated structures of the a'-domain provided interesting insight as to its inequivalent nature in comparison to that of the a-domain. The accessibility of CysN was modulated by Lys401, not via cation–π interactions with the upstream Trp396 (equivalent to Trp52), but through its inability to form any bona fide interactions. The side chain was
found to predominantly project into the solvent, thereby mediating an open conformation (Figure 3.9). This was deemed so as mutation to Gln resulted in the introduction of backbone hydrogen bonding, similar to that observed for the $a$-domain (Figure 3.11 and appendix B Figure B.4 D). As a result, dynamic changes in the active site conformation occurred, allowing residue Phe$^{440}$ (of the conserved cis-Pro loop) to project toward the active site and impede the apparent solvent accessibility of CysN. Residue Lys$^{401}$ of WT PDI may still form transient hydrogen bonds [178], similar to those of Gln, and it was interesting to note that when such occurred, the closed conformation was favoured (Figure 3.10 and appendix B Figure B.4 B). The oxidized conformation of the $a'$-domain was different from that of the $a$-domain. Here, the active site appeared open, irrespective of mutation, with a more solvent exposed CysN (Cys$^{396}$, appendix B Figure B.2 B and D). This is in contrast to the closed conformation observed for the oxidized $a$-domain. Unlike the $a$-domain, the $a'$-domain active site did not appear to exhibit redox state-dependent open and closed conformations.

As discussed, the K57Q mutation resulted in the introduction of hydrogen bonding within the active site region. This was due to the amide-N$_8$ of Gln being positioned perfectly in relation to the peptide carbonyl of the upstream active site Pro$^{55}$ (Figure 3.5 and appendix B Figure B.4 C). It is unlikely that this interaction be a player in defining the kinetic contributions of a residue at this position (residue 57) simply because mutation to Ala and Glu also resulted in attenuated kinetics, and to greater extents. It should be noted, though, that the structural similarities between Gln and Glu suggest that it may be possible for Glu to participate in similar hydrogen bonding interactions with the backbone of the active site (with the peptide N-H rather than the peptide carbonyl).
Unfortunately, the structural data observed for the $a'$-domain, and the speculated mechanism of open-closed dynamics, may only satisfy a K401Q and potentially a K401E substitution. This hypothesis falls short of providing reasoning for the attenuation of kinetics observed for the K401A variant of PDI (appendix B Tables B.1 to B.5). This is because, unlike Gln or Glu, Ala cannot participate in the backbone hydrogen bonding that lead to a closed active site (Figure 3.11). Mutagenesis of Lys$^{401}$ may present other structural effects that cannot be concluded upon at this time.

Even though the degree of burial of cysteine residues is high in proteins, it is not hydrophobic; displaying similar polarity to that of serine [36]. Cys$_N$ is in a more hydrophobic microenvironment when the active site is in the closed conformation (i.e., mutated). This likely accounts for both the variations of $K_M$ as well as $k_{cat}$ observed for the PDI variants. Steric hindrance to the active site results in an increase of $K_M$. Furthermore, a more hydrophobic microenvironment could effectively raise thiol p$K_a$, and destabilize the thiolate state [36]. Despite this not being discernable with the experimentally determined p$K_a$ (app$K_i$, Table 3.1), computational estimations did support this (Table 3.2). The p$K_a$ increase estimated for Cys$_N$ was modest, but it may support the steady state oxidoreductase kinetic data that indicated $K_M$ to be much more affected by mutation in relation to $k_{cat}$. Therefore, changes in active site accessibility as mediated by mutation of Lys$^{57}$ and Lys$^{401}$ modulate substrate binding to the active sites of PDI, with slight attenuation of substrate turnover due to the altered accessibility and microenvironment of Cys$_N$.

The single-turnover reduction of di-E-GSSG was also impeded as a result of mutating the active site flanking Lys residues (Figures 3.12 and 3.13), this was
determined under pseudo-first order conditions. An interesting feature of this assay was the ability to negate the kinetic contributions of enzyme regeneration. This provided further insight into the effects imposed by the K-Q mutation. Because single turnover reductase activity was being monitored, the rate of substrate release as mediated by CysC was able to be ruled out. This is because signal acquisition is obtained upon the initial nucleophilic attack of the di-E-GSSG self-quenching substrate by CysN (Figure 3.12). An increase of fluorescence in this case is not reliant on CysC. At pH 7.4 the WT enzyme outperformed the mutant variants to the greatest extent, a similar result to that observed for the steady state oxidoreductase kinetics (Figure 3.2 C and appendix B Tables B.1 to B.5). These data suggest that the mutation of active site flanking residues Lys57 and Lys401 affects the rate of thiol-reductase activity as a result of impeded accessibility and changes to the microenvironment about CysN.

Going further, the peptide thiol-oxidase activity of PDI was assessed (Figure 3.14). Here, oxPDI oxidizes a dithiol-containing peptide substrate. Results indicated that across the range of pH, substantial attenuation of kinetics was observed for the double mutant K57/401Q variant, while the respective single mutants displayed only nominal alterations (Figure 3.15 B). MD simulations pointed to a redox-regulated open and closed conformation of the a-domain active site; open when reduced and closed when oxidized, irrespective of mutation. Based on this, it would be anticipated that the K57Q variant function very similarly to WT. This was indeed observed, with the exception at pH 6.3 (82% of WT). In contrast, the K401Q single mutant deviated from WT more, with significant changes to thiol-oxidase rate at pH 6.3, 6.5 and 6.9 (82%, 77% and 79% with respect to WT, respectively). A caveat to this assay is that the altered kinetics of peptide
oxidation may be a result of oxPDI regeneration mediated by GSSG. Unfortunately, single turnover thiol-oxidase activity was not attainable. It therefore cannot be concluded if the observed results are due to the oxidation of the peptide substrate or if this assay simply provides an alternative approach to measuring the oxidoreductase activity of PDI, similar to the steady state experiments using di-E-GSSG.

During oxidative protein folding of nascent polypeptides within the ER, PDI may participate in PDI-1\textsuperscript{st}, PDI-2\textsuperscript{nd} or a mixture of PDI-1\textsuperscript{st}/2\textsuperscript{nd} pathways [26, 75]. The transfer of oxidizing equivalents by oxPDI to a reduced substrate is what defines PDI-1\textsuperscript{st} pathways. The opposite is true of PDI-2\textsuperscript{nd} pathways, where PDI does not introduce disulfides to some reduced substrate, rather it functions as a thiol-disulfide isomerase to correct mispaired disulfides. During PDI-1\textsuperscript{st} oxidative folding \textit{in vivo}, endoplasmic reticulum oxidoreductin-1\textalpha{} (ERO1\textalpha{}) is the interacting partner required to regenerate oxPDI from the growing pool of redPDI (\textbf{Figure 1.10}). We speculated that our K-Q mutant enzymes- having displayed markedly attenuated oxidoreductase kinetics towards smaller pseudo-substrates, would also exhibit decreased rates of thiol-disulfide exchange with ERO1\textalpha{}. It was observed that the rate of oxygen consumption by the oxidase, ERO1\textalpha{}, was greatest when in the presence of \textit{WT} PDI, indicating the most rapid rate of thiol-disulfide exchange. The PDI variants K57Q, K401Q and K57/401Q resulted in relative rates of oxygen consumption that were 76\%, 83\% and 54\% of \textit{WT}, respectively (\textbf{Figure 3.16} and \textbf{Table 3.3}).

The transfer of disulfides begins with active ERO1\textalpha{} (ox1) [96]. PDI accepts these disulfides and in turn transfers oxidizing equivalents to some substrate. Resultantly, electron flow occurs in the opposite direction, from said (reduced) substrate to PDI (first
the a-domain then a'-domain), then terminating at ERO1α where reduction of the FAD cofactor to FADH$_2$ results in a transfer of electrons to molecular oxygen (O$_2$), thereby generating hydrogen peroxide (H$_2$O$_2$) [91, 92]. The in vitro reaction here utilized GSH as the initial electron donor source. The apparently attenuated rates of oxygen consumption by ERO1α in the presence of mutant PDI is likely telling of the impedance of electron flow between the reduced a-domain active site of PDI and the oxidized a'-domain active site. As well, kinetic slowing may be occurring between the subsequently reduced a'-domain active site and the C$_{94}$-C$_{99}$ electron shuttling disulfide of ERO1α. This would make sense as previous in vitro oxidoreductase and thiol-reductase kinetics indicated that, when in the reduced state, the kinetics of PDI-catalyzed reduction were greatly attenuated when bearing a mutant CGHCQ motif (Figures 3.2 and 3.13). This being a result of the hindered accessibility to Cys$_N$ of the mutant redPDI active sites (Figures 3.7 and 3.11). It is therefore clear that such a mechanism is also relevant when assessing the redox-regulated interactions between PDI and ERO1α.

It is unlikely that mutation of either Lys$_{57}$ or Lys$_{401}$ impacted the binding of ERO1α to PDI, thereby leading to the attenuated rates of oxygen consumption observed. Binding has been found to occur between a β-hairpin loop of ERO1α and the hydrophobic pocket of the b'-domain of PDI [89, 92]. There is no indication of the active site flanking Lys residues as mediating this interaction [89]. Furthermore, mutation of all active site Cys residues of PDI to Ala was shown to display similar binding kinetics as that of WT PDI; further highlighting the non-covalent association as being relatively independent of the active site motif [92].
With this understanding, it is most plausible to say that the 24% decrease to the rate of oxygen consumption observed for ERO1α in the presence of the K57Q PDI variant be a result of the inaccessibility of Cys\textsuperscript{53} (Cys\textsubscript{N} of the a-domain). Thus, when the interdomain thiol-disulfide exchange reaction occurs between the reduced C\textsuperscript{53}GHCQ motif of the a-domain and the oxidized C\textsuperscript{597}GHCK motif of the a'-domain, it is slowed due to the closed active site and limited accessibility of the nucleophilic thiolate of Cys\textsuperscript{53} [26-28].

The observed 17% decrease of rate for ERO1α in the presence of the K401Q PDI variant was likely not a result of any impedance to the interdomain thiol-disulfide exchange reaction as noted above. Recall that the MD simulations of WT and mutant PDI showed the oxidized a'-domain active site open, irrespective of the residue at position 401 (appendix B Figure B.2 B and D). Therefore, the only differentiating factor between the WT and mutant K401Q a'-domain active sites are seen when in the reduced state; that which would follow the interdomain thiol-disulfide exchange. The reduced mutant active site was determined to be closed, with a less solvent exposed Cys\textsubscript{N} (C\textsuperscript{397}, Figure 3.11). As a result, the thiol-disulfide exchange reaction between PDI and the C\textsuperscript{94}-C\textsuperscript{99} electron shuttling disulfide of ERO1α was likely the hindered step.

As for the double mutant K57/401Q PDI variant, a combination of the aforementioned mechanisms of the single mutants occurs. This lead to drastically reduced rates of oxygen consumption by 46%. Simply put, substitution of active site flanking residues Lys\textsuperscript{57} and/ or Lys\textsuperscript{401} of PDI resulted in markedly slowed rates of electron transfer between redPDI and ERO1α. These active site residues can be viewed as highly important for the ERO1α-mediated regeneration of oxPDI, whereby absence of
the native CxxCK motif may effectively form a kinetic bottleneck, limiting the efficiency of electron transfer. Interestingly, the effects imposed by the single mutant enzymes are nearly additive with respect to the K57/401Q double mutant. In support of this, it is understood that each active site flanking residue (Lys\textsuperscript{57} and Lys\textsuperscript{401}) helps to mediate the same step of catalysis, that being the net transfer of electrons to ERO1\textalpha, yet are non-interacting [202].

This implies a potential for the build-up of redPDI within the ER lumen, \textit{in vivo}, should a modification of the active site neighbouring residues Lys\textsuperscript{57} and Lys\textsuperscript{401} occur. Such a modification is likely to be \textit{N}\textsuperscript{ε}-lysine acetylation. The resulting kinetic bottleneck of oxPDI regeneration can most certainly extend to other pathways outside of the PDI-ERO1\textalpha interaction, such as the H\textsubscript{2}O\textsubscript{2}-driven oxidation of PDI by peroxiredoxin 4 (PRDX4) [215, 216] and PDI peroxidases GPRx7/8 [217]. Although accurate estimations of the glutathione redox poise (GSH:GSSG = \textasciitilde35:1) of the ER lumen indicates that redPDI largely predominates, there is an inkling that oxidative "hot spots" exist [26]. These may present themselves in the form of intraluminal concentration gradients whereby PDI does not equilibrate with GSH, thus exhibiting temporal increases of oxPDI [26]. It is therefore interesting to theorize possible upstream and downstream perturbations to the redox equilibria that may occur between a kinetically abated PDI (modified Lys\textsuperscript{57} and Lys\textsuperscript{401}) and equilibrium partners within a given cellular redox environment [26]. These perturbations may reflect dynamic signalling events required by the cell, all stemming from the modulation of PDI activity as centered on the active site Lys residues.
A recent review by Hudson *et al.* provides curious speculation as to the existence and importance of redox-equilibria directly within protein networks (*i.e.*, PDI-ERO1α) and/or with small molecules such as glutathione [26]. Not only may the pool of redPDI increase as a result attenuated thiol-disulfide exchange kinetics, but reductive stress to the ER lumen may also persist due to compromised GSSG production. This likely implies detrimental effects to protein folding, including a build-up of improperly folded proteins containing mispaired disulfides. This would be particularly damaging within professional secretory cells such as pancreatic β cells [218]. Further, an increase of redPDI may ultimately lead to enhanced levels of active ERO1α (Ox1). The implication here being the hyperactivity of ERO1α as activated by redPDI. In short, this may lead to the hyperoxidaiton of other PDI family members, such as ERp57. The end result of such being UPR signaling, as demonstrated by Hansen *et. al.* [219].

### 4.2 Acetylation of K^{57} and K^{401} is a Means of Modulating PDI Activity

Site directed mutagenesis of Lys^{57} and Lys^{401} provided a framework for resolving the mechanisms by which an active site-flanking Lys facilitated enhanced rates of catalysis by mediating the solvent accessibility of Cys_N. These results do not directly relate to what may be expected to occur naturally *in vivo*. This is because, to our knowledge, there has been no research to date that has identified any naturally occurring mutations of these residues. It is therefore more relevant to assess the possibility of lysine posttranslational modifications with respect to Lys^{57} and Lys^{401}. Here, we investigate PDI as a target for N^-lysine acetylation.
The optimal catalytic activity of PDI is not an absolute requirement for a healthy physiology. The indication that many cancers are associated with its upregulation demonstrates that there exists a need for modulating PDI activity [43]. Using acetylsalicylic acid (ASA), the active ingredient in Aspirin, an in vitro shotgun approach lead to the identification of many lysine residues as being candidate targets of acetylation. In total, 18 acetyllysine residues (acK) were identified by mass spectrometry (MS) in a reproducible manner (Table 3.4). This included active site-flanking residues Lys\textsuperscript{57} and Lys\textsuperscript{401} (Figures 3.18 and 3.19, respectively). As well, the key catalytic residues Lys\textsuperscript{81} and Lys\textsuperscript{464} were found to be acetylated. The implication is that those residues determined to be acetylated by ASA may present themselves as viable targets of in cellulo acetylation either in Aspirin-treated cultures, via chemical interactions with cellular acetyl-CoA [144], or enzymatically through the work of N-acetyltransferase enzymes [138].

Quite recently it has been realized that reversible $N$-lysine acetylation is a posttranslational regulatory mechanism that goes beyond that of histone modification. Research has linked this PTM to various cellular compartments, including the ER and Golgi intermediate complex [141, 151]. It has, for the first time, been shown that an acetyl-CoA transporter and two distinct ER-luminal transmembrane acetyltransferases exist [148, 152]. Proteomic assessment has identified PDI and many other ER-resident proteins as being acetylated [141, 153, 154]. Specifically, the efforts of Pehar et al. identified residue Lys\textsuperscript{401} of hPDI as being acetylated when examining purified ER preparations from human neuroglioma (H4) cells [153]. Other proteomic studies probing for various lysine acylation events (such as acetylation, succination, etc.) have found PDI to be extensively modified in vivo [141, 220]. These studies were unable to characterize
any modifications to residues Lys\textsuperscript{57} or Lys\textsuperscript{401}, despite the use of enrichment strategies. The absence of such may be attributed to either an exceedingly low stoichiometry, or a lack of resolution in comparison to other, more readily modified peptides. In either case, the use of whole-cell, global proteomics may explain this, as the prepared samples are complex in nature. Furthermore, the use of deacylase inhibitors was omitted during cell lysis and sample preparations. This may also have a negative impact on the detectable levels of PDI acylation. It should be noted that, to our knowledge, specific examination of the modifications to hPDI lysine residues has yet to be performed. Therefore, our \textit{in vitro} experimentation using ASA provide important insights to the kinetic effects of acetylation.

The kinetic activity of PDI was determined under steady state conditions using the di-E-GSSG assay as described previously. By comparing ASA-treated and untreated \textit{WT} PDI (\textit{WT\textsubscript{ASA}} and \textit{WT\textsubscript{control}}, respectively) it was evident that global lysine acetylation was detrimental to activity (\textbf{Figure 3.21}). \textit{WT\textsubscript{ASA}} exhibited a near 75\% decrease of enzyme efficiency (\textbf{Figure 3.21 D}). This was attributable to a \textasciitilde68\% increase of \textit{K_m} and a 66\% decrease of \textit{k_{cat}} (\textbf{Figure 3.21 B and C}). As mentioned above, MS analysis identified 18 acK sites. Importantly, this included acK\textsuperscript{57} and acK\textsuperscript{401}, as well as acK\textsuperscript{81} and acK\textsuperscript{424} (\textbf{Table 3.4}). The importance of Lys\textsuperscript{81} and Lys\textsuperscript{424} to PDI activity has been identified previously, with the indication that each facilitates proton transfer during thiol-disulfide exchange reactions [195]. For this reason, a comparison of the kinetics between \textit{WT\textsubscript{ASA}} and \textit{WT\textsubscript{control}} cannot be conclusive in elucidating the impact of acetylation to Lys\textsuperscript{57} and Lys\textsuperscript{401} specifically. The concomitant acetylation of Lys\textsuperscript{81} and Lys\textsuperscript{424}, and others, likely infers contribution to the observed differences in \textit{k_{cat}}.
To circumvent this, the double mutant K57/401Q was either treated with ASA (K57/401QASA) or left untreated (K57/401Qcontrol). By comparing the kinetics of $WT_{ASA}$ to that of K57/401QASA and K57/401Qcontrol, the kinetic contribution of acK$^{81}$ and acK$^{424}$ in relation to acK$^{57}$ and acK$^{401}$ was able to be determined. Focusing on $K_M$, that determined for $WT_{ASA}$ was lower- albeit marginally, yet statistically so- than both K57/401Qcontrol and K57/401QASA (~16 to 18%, Figure 3.21 C). This is because the closed active site configurations brought on by mutation (Figures 3.7 and 3.11) can be anticipated to occur similarly for acK$^{57}$ and acK$^{401}$. Introduction of an $N^\varepsilon$-acetyl group to Lys$^{57}$ would result in the loss of cation-π interactions with Trp$^{52}$, resulting in a closed a-domain active site. Furthermore, acK$^{401}$ would lead to a closed $a'$-domain active site due to the introduction of backbone hydrogen bonding between the acetyl oxygen and the peptide N-H group. Therefore, the mutant K57/401Q enzyme may be viewed as PDI with a perfect stoichiometry (homogenous acetylation) of acK$^{57}$ and acK$^{401}$.

The modest variation of $K_M$ observed is likely due to the stoichiometry of the ASA-mediated acetylation reaction. Seeing as the in vitro reaction was performed at alkaline pH (8.5), ASA was susceptible to hydrolysis (base-catalyzed deacetylation) resulting in salicylic acid and acetate [221]. Additionally, ASA is capable of producing $O$-acetylated Ser, Thr and Tyr residues [196, 222]. Although $O$-acetylation was later reverted by incubation with excess hydroxylamine (HA) [162-167], this form of acetylation coupled with aqueous hydrolysis means an overall depletion of the ASA reagent available for modifying the lysine residues of PDI. Therefore, the ASA-treated and untreated K57/401Q enzymes each displayed similar $K_M$ values that were increased ~16 to 18% as compared to $WT_{ASA}$. This is because all PDI molecules of these mutants exhibit closed a-
and $\alpha'$-domain active sites as induced by mutation. In contrast, $WT_{ASA}$ represents a heterogeneous acetylation of PDI, with some molecules displaying no acetylation ($K_{57}$ and $K_{401}$), partial acetylation (ac$K_{57}$ and $K_{401}$, or $K_{57}$ and ac$K_{401}$) and full acetylation (ac$K_{57}$ and ac$K_{401}$) of the active site-flanking Lys residues. This means a mixture of natively open active sites as well as acetylation-induced closed active sites. This was confirmed by the presence of unacetylated active site peptides ($m/z$ 564.93 ($z = 3$) for the $\alpha$-domain and $m/z$ 616.30 ($z = 4$) for the $\alpha'$-domain, results not shown). Thus, acetylation of $Lys^{57}$ and $Lys^{401}$ can be concluded as the substantiating factor in the 68% increase of $K_M$ between $WT_{control}$ and $WT_{ASA}$.

Although a statistically significant difference between $K57/401Q_{control}$ and $K57/401Q_{ASA}$ was not seen in relation to $K_M$, it was observed for $k_{cat}$ (Figure 3.21 B). Treatment of the mutant resulted in a 55% decrease. This decrease was relatively the same when compared to $WT_{ASA}$. Comparing $WT_{ASA}$ and $K57/401Q_{control}$ (enzymes that are kinetically similar) to $K57/401Q_{ASA}$ indicated that acetylation of $Lys^{57}$ and $Lys^{401}$ is by-and-large the factor that influences PDI activity in comparison to the other 16 identified acK residues. Again, the heterogeneity of the in vitro acetylation reaction is most likely the reason $WT_{ASA}$ and $K57/401Q_{ASA}$ do not corroborate more closely, kinetically (see enzyme efficiency, Figure 3.21 D). Yet, it remains that other residues, including $Lys^{81}$ and $Lys^{424}$, are important for enhancing enzyme turnover, and acetylation of such decreases $k_{cat}$.

Enzymatic acetylation of PDI has yet to be observed directly. Lundby et al. described in vivo tissue and organelle-specific acetylation patterns in rats [141]. They identified compartment-specific acetylation motifs which may point to the existence of
compartment-specific lysine \(N\)-acetyltransferase enzymes (KATs) [141, 223]. As discussed previously, earlier studies by Puglielli and coworkers identified two ER-resident KATs, \(N\)-acetyltransferase 8B (ATase-1) and \(N\)-acetyltransferase 8 (ATase-2) [148]. Acetylation was found to generally be favoured in lysine-rich regions [141]. Granted, this may simply be an artifact of there being a greater potential of acetylation to occur in these regions. Within the ER specifically, a tendency for negative and hydrophobic amino acids at the \(-1\) and \(-2\) positions in relation to acK was observed [141]. The flanking sequence to residues Lys\(^{57}\) and Lys\(^{401}\) of PDI do not satisfy these findings (refer to appendix A Figure A.1). The apparent acetylation motifs described may be biased by compartment-specific protein expression, or mediated by lysine deacetylase enzymes, seeing as acetylation is a dynamic, reversible modification [141, 146].

ER-luminal acetylation is a very new phenomenon only just being evaluated now. Pehar and Puglielli offer interesting insight as to the likelihood of acetylation signaling playing a role in quality control mechanism of the ER [224]. A key finding being that knockout of the ER-based acetyl-CoA transporter (AT-1) resulted in cell death, an indication that ER-luminal acetyl-CoA (and thus protein \(N^e\)-lysine acetylation) is required for cell viability [153, 224, 225]. Additionally, it could be that acetylation of nascent polypeptides is required to help facilitate the native fold by alleviating the charge of lysine, making it more favourable for it to be buried within the protein conformation. Under conditions that promote the UPR, acetyl-CoA influx is increased as a result of AT-1 upregulation. This event occurs as a result of both transient and chronic stress-associated UPR response [224]. Therefore, under such conditions the likelihood of PDI
acetylation increases. Events that result in the hyperacetylation of PDI may be associated with pathophysiological states.

Research has demonstrated that PDI is extensively acylated in vivo [141, 153, 220]. Despite not exhibiting a favoured motif sequence, the active site-flanking residue Lys$^{401}$ of the $a'$-domain was identified as being acetylated in vivo [153]. Taken together with the kinetic data herein, evidence infers that an active site-flanking Lys may act as a regulator of PDI activity via its reversible, posttranslational acetylation. Results point to a role for these residues in maintaining the appropriate microenvironment for the nucleophilic active site cysteine, Cys$_N$. 
CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS
The discovery of PDI in the 1960's [37, 40] was ground-breaking for the understanding of protein folding and cellular redox biology. Since that time PDI has been identified as a multifunctional chaperone of the ER, linked to a variety of physiological and pathophysiological processes, including cancer [43, 111-113, 115]. Despite this, many characteristics regarding substrate recognition and details of the enzymatic mechanisms remain enigmatic. A variety of features have been identified as important for the specific activity of hPDI, such as domain organization [76-79], active site redox potential [97-100], and many conserved catalytic and non-catalytic residues. It remains that the conserved active site-flanking residues Lys$^{57}$ and Lys$^{401}$ have been seldom studied [55, 185].

The present study explored the roles of residues Lys$^{57}$ and Lys$^{401}$ in relation to a full complement of PDI activities. We demonstrated that mutation lead to attenuated rates of oxidoreductase, thiol-reductase and thiol-oxidase catalysis. Furthermore, a mutant CxxCQ motif at either or both active sites abated electron transfer during ERO1α-mediated oxidation of redPDI. The implication here is a kinetic bottleneck that likely translates to the impedance of oxidative protein folding in vivo. MD simulation studies established the importance of these active site-flanking Lys residues. It was determined that each function to mediate the solvent accessibility of Cys$_N$ in distinct manners. Conclusively, a WT CxxCK motif resulted in an open active site, with a more solvent exposed Cys$_N$ residue. A mutant CxxCQ motif, on the other hand, promoted a closed active site, with a less accessible Cys$_N$. As a result, enzyme efficiency was markedly reduced, with the indication that substrate binding ($K_M$) be largely compromised.
\(N^e\)-lysine acetylation of Lys\(^{57}\) and Lys\(^{401}\) was investigated as a potential posttranslational regulatory mechanism of PDI. Aspirin-mediated acetylation was capable of reproducibly generating 18 unique acK sites, including acK\(^{57}\) and acK\(^{401}\).

Interestingly, acetylated \(WT\) PDI performed catalytically very similarly to the K57/401Q control variant, with a near 75% loss of efficiency in relation to unacetylated \(WT\). Data here further pointed to residues Lys\(^{57}\) and Lys\(^{401}\) as being important for maintaining an optimal active site conformation with respect to Cys\(_N\). Overall, these data, coupled with previous reports indicating the acylation of PDI \textit{in vivo}, implicate the active site-flanking Lys residues as important to PDI activity. Acetylation of such is likely a viable regulatory mechanism of PDI, never before described.

Further studies are required to provide added insight to roles of Lys\(^{57}\) and Lys\(^{401}\). For instance, the kinetic data obtained is not telling of the thermodynamic implications these residues may have on the chemistry of the PDI active sites. Does mutation or acetylation result in altered redox potentials? This may be most simply investigated using a semi-quantitative \textit{in vitro} gel-shift assay [31, 91, 106]. It is recommended that each active site be investigated individually, therefore active site cysteine mutants (CxxC-AxxA, appendix A \textbf{Figure A.4}) have been designed. Additionally, it may be useful to perform kinetic assays as a titration of ionic strength to further scrutinize the proposed cation-\(\pi\) interaction between Lys\(^{57}\) and Trp\(^{52}\). In this case, shielding of the cationic amine of Lys\(^{57}\) by increasing salt concentrations may eliminate the interaction between with Trp\(^{52}\), thereby rendering the \(a\)-domain active site \textit{closed}; exhibiting similar kinetics to that of the K57(Q/A/E) PDI variants. Lastly, the non-catalytic chaperone activity of PDI should be assessed in relation to Lys\(^{57}\) and Lys\(^{401}\). A promising technique
that can be utilized is the refolding of acid-denatured green fluorescent protein (GFP, Figure 1.9 I and appendix A Figure A.6) [80].

In terms of acetylation, results provided by this thesis are quite rudimentary. The enzymatic acetylation of PDI has yet to be investigated. A first effort should be centered on identifying whether or not PDI is acetylated in vitro by the ER-resident KATs, ATase-1 and ATase-2. Preliminary trials have been performed using affinity purified ATase enzymes conjugated to maltose binding protein (MBP) at the N-terminus. Despite the KATs exhibiting in vitro lysine acetyltransferase activity towards human histone H3 peptide (appendix A Figure A.7), PDI has not yet been identified as a target. Cleavage of the cumbersome MBP affinity tag prior to performing reactions with PDI could provide a solution to this problem. Moreover, in cellulo acetylation of PDI should be investigated. First, one should determine the acetylation state of endogenous and/or overexpressed PDI in a variety of cell types. It may be useful to employ deacetylase inhibitor cocktails as a cell-treatment strategy to enhance the levels of acetylation. By Western blot analysis, it has been determined that a combination of pan-specific inhibitors, tricostatin-A (TSA, 5 µM), nicotinamide (NAM, 10 mM) and sodium butyrate (NaBut, 1 mM) effectively elevate levels of cellular acetylation in HeLa, human embryonic kidney (HEK-293) and human retinal pigmented endothelium (ARPE-19) cell cultures when treated for 6 hours (results not shown). It may prove useful to also incubate cells with varying amounts of Aspirin to provide an indication of the chemical acetylation of PDI upon drug treatment. A last avenue to explore is genetically encoding site-specific acetyllysine residues. This avoids the possibility of heterogeneous and undesirable acetylation patterns observed with in vitro chemical procedures [226, 227].
APPENDICES

Appendix A (Chapters 1, 2 and 5 supplementary material)

Figure A.1: Amino acid sequence of the recombinant N- and C-terminally His\textsubscript{6}-tagged human PDI used in this study. Active sites (CGHCK) of the $\alpha$- and $\alpha'$-domains are underlined.
**Table A.1**: PCR primers for site-directed mutagenesis of hPDI. Substituted nucleotides are in bold and underlined. $T_a$ represents primer annealing temperature.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5' – 3')</th>
<th>$T_a$ (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$a$-domain rev.</td>
<td>AGG GGC CAG AGC CGC GCA GTG GCC ACA CCA AGG</td>
<td>61-68</td>
</tr>
<tr>
<td>K57A $a$-domain fwd.</td>
<td>AGG GGC CAG AGC <strong>G</strong> GCA GTG GCC ACA CCA AGG</td>
<td>65</td>
</tr>
<tr>
<td>K57Q $a$-domain fwd.</td>
<td>AGG GGC CAG AGC <strong>C</strong> GCA GTG GCC ACA CCA AGG</td>
<td>68</td>
</tr>
<tr>
<td>K57E $a$-domain fwd.</td>
<td>AGG GGC CAG AGC <strong>C</strong> GCA GTG GC</td>
<td>61</td>
</tr>
<tr>
<td>$a'$-domain rev.</td>
<td>CCA TGG GGC ATA GAA TTC CAC AAA GAC</td>
<td>57-60</td>
</tr>
<tr>
<td>K401A $a'$-domain fwd.</td>
<td>TGT GGT CAC TGC <strong>G</strong> CAG TTG GCT CCC</td>
<td>57</td>
</tr>
<tr>
<td>K401Q $a'$-domain fwd.</td>
<td>TGT GGT CAC TGC <strong>C</strong> AAA CAG TTG GCT CCC</td>
<td>60</td>
</tr>
<tr>
<td>K401E $a'$-domain fwd.</td>
<td>TGT GGT CAC TGC <strong>C</strong> AAG CAG TTG GCT C</td>
<td>58</td>
</tr>
<tr>
<td>CxxCK$^{57}$–AxxAK$^{57}$ fwd.</td>
<td>AGG GGC CAG AGC CTT <strong>G</strong> GTG GCC <strong>A</strong> GCC CCA AGG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>GGC ATA G</td>
<td></td>
</tr>
<tr>
<td>CxxCQ$^{57}$–AxxAQ$^{57}$ fwd.</td>
<td>AGG GGC CAG AGC CTG <strong>G</strong> GTG GCC <strong>A</strong> GCC CCA AGG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>GGC ATA G</td>
<td></td>
</tr>
<tr>
<td>CxxCK$^{401}$–AxxAK$^{401}$ fwd.</td>
<td>CTA TGC CCC ATG <strong>G</strong> TGG TCA <strong>G</strong> CAA ACA GTT GCC TCC</td>
<td>54</td>
</tr>
<tr>
<td>CxxCQ$^{401}$–AxxAQ$^{401}$ fwd.</td>
<td>CTA TGC CCC ATG <strong>G</strong> TGG TCA <strong>G</strong> CCA ACA GTT GCC TCC C</td>
<td>54</td>
</tr>
<tr>
<td>CxxC–AxxA $a'$-domain rev.</td>
<td>AAC TCC ACA AAG ACG TTT TTT TTC TCA TC</td>
<td>54</td>
</tr>
</tbody>
</table>
Figure A.2: Purity of isolated His$_6$-PDI; wild-type (WT) and mutant variants was assessed via SDS-PAGE using a 10% polyacrylamide gel and visualized by Coomassie staining. N- and C-terminally His$_6$-tagged PDI (~59 kDa) is indicated with reference to a standard molecular weight ladder.
Figure A.3: The concentration of catalytically active PDI as determined by burst-phase analysis. In a cuvette, 10 uL of purified enzyme (16.9 µM stock) was incubated with 800 nM di-E-GSSG in PDI assay buffer (final volume 500 uL). (B-K) An increase in fluorescence (RFU) was monitored over 20 min. (A) The y-intercept of each respective plot represents the concentration of functional enzyme determined by relating the fluorescence of enzyme-mediated EGSH production to that of a standard curve. The standard curve was generated by fully reducing di-E-GSSG with 1 M DTT in PDI assay buffer. Burst-phase data are representative of an experiment performed in triplicate. The standard curve represents the mean ± S.D. of n = 3 experiments.
Figure A.4: Purity of affinity purified hPDI active site thiol mutants (CxxC-AxxA) was assessed via SDS-PAGE using a 10% polyacrylamide gel and visualized by Coomassie staining. N- and C-terminally His<sub>6</sub>-tagged PDI (~59 kDa) is indicated with reference to a standard molecular weight ladder. Using the di-E-GSSG (500 nM) assay the activity of 10 nM of each mutant was assayed in relation to an un-mutated control PDI; WT (A) or K57/401Q (B) as a simple reporter of active site inactivation due to cysteine–alanine mutation of the vicinal thiols of the respective motif(s). Mutation of a single active site resulted in a near-loss of 50% activity (A and B), while mutation of both active sites rendered PDI inactive (CxxCK<sup>57/401</sup>-AxxAK<sup>57/401</sup>, A).
Figure A.5: Purity of affinity purified endoplasmic oxidoreductin 1α (ERO1α) was assessed via SDS-PAGE using a 10% polyacrylamide gel and visualized by Coomassie staining. N-terminally His₆-tagged ERO1α (~55 kDa) is indicated with reference to a standard molecular weight ladder.
Figure A.6: Purity of affinity purified green fluorescent protein (GFP) was assessed via SDS-PAGE using a 10% polyacrylamide gel and visualized by Coomassie staining. N-terminally His6-tagged GFP (~29 kDa) is indicated with reference to a standard molecular weight ladder. The fluorescence spectroscopic properties of GFP (400 nM) was found to exhibit the characteristic excitation spectrum; primary peak at 395 nm, with secondary excitation at 480 nm (constant emission wavelength: 509 nm). The emission spectrum was also as expected for intact GFP, with a peak at 509 nm and a shoulder at 540 nm (constant excitation wavelength: 395 nm).
Figure A.7: Purity of affinity purified (A) N-acetyltransferase 1 (ATase-1) and (B) N-acetyltransferase 2 (ATase-2) was assessed via SDS-PAGE using a 10% polyacrylamide gel and visualized by Coomassie staining. ATase enzymes were fused to a maltose binding protein (MBP) epitope at the N-terminus. The fusion protein (~60 kDa) and free MBP (a product of degradation; ~40 kDa) are indicated with reference to a standard molecular weight ladder. The activity of the isolated enzymes was assayed using a commercially available fluorescent kit (ATase-1, A; ATase-2, B).
Figure A.8: UV-visible absorption spectra of gold nano particles (AuNPs). The concentrated reaction mixture was diluted 1:3 prior to spectral acquisition.
Figure A.9: SDS-PAGE analysis of the efficiency of thiol-mediated pull down of reduced PDI by gold nanoparticles (AuNPs), sepharose 6B, CNBr-activated Hg-sepharose, and Epi-activated Hg-sepharose. A consistent loading volume of 25 µL was used to compare the various fractions from the affinity enrichment procedure (see section 2.2.11.1). **Control** lane represents reduced PDI prior to affinity pull down (13 µg loading); **FT**, flow through following incubation with AuNPs or sepharose matrices, respectively; **W1** and **W3**, washes 1 and 3; **Elution**, elution fraction resulting from treatment with 100 mM DTT.
Appendix B (Chapters 3 and 4 supplementary material)

Table B.1: Apparent steady-state kinetic parameters at pH 6.0. Results are representative of the mean ± S.D. of \( n = 3 \) experiments. Data is also presented as the percentage of \( WT \) at each respective pH.

<table>
<thead>
<tr>
<th>hPDI</th>
<th>( k_{\text{cat}} \left( \times 10^{-2} \text{ s}^{-1}, % \right) )</th>
<th>( K_M ) (nM, %(^a))</th>
<th>( k_{\text{cat}}/K_M \left( \times 10^{3} \text{ M}^{-1} \text{ s}^{-1}, % \right) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.627 ± 0.043, 100</td>
<td>10.7 ± 1.50, 100</td>
<td>588 ± 31.5, 100</td>
</tr>
<tr>
<td>K57/401Q</td>
<td>0.720 ± 0.028, 115</td>
<td>20.3 ± 2.65, 190</td>
<td>355 ± 63.4, 60</td>
</tr>
<tr>
<td>K57Q</td>
<td>0.719 ± 0.056, 115</td>
<td>13.6 ± 3.34, 128</td>
<td>529 ± 118, 90</td>
</tr>
<tr>
<td>K401Q</td>
<td>0.695 ± 0.010, 110</td>
<td>10.3 ± 2.25, 97</td>
<td>675 ± 220, 115</td>
</tr>
<tr>
<td>K57/401A</td>
<td>0.610 ± 0.099, 92</td>
<td>11.3 ± 3.31, 106</td>
<td>539 ± 57.0, 92</td>
</tr>
<tr>
<td>K57A</td>
<td>0.640 ± 0.050, 84</td>
<td>13.0 ± 6.63, 122</td>
<td>492 ± 110, 84</td>
</tr>
<tr>
<td>K401A</td>
<td>0.695 ± 0.021, 117</td>
<td>10.1 ± 5.40, 95</td>
<td>687 ± 220, 117</td>
</tr>
<tr>
<td>K57/401E</td>
<td>0.434 ± 0.012, 76 ***</td>
<td>9.65 ± 0.95, 94</td>
<td>449 ± 65.4, 76</td>
</tr>
<tr>
<td>K57E</td>
<td>0.598 ± 0.041, 89</td>
<td>11.4 ± 2.23, 107</td>
<td>525 ± 86.4, 89</td>
</tr>
<tr>
<td>K401E</td>
<td>0.715 ± 0.048, 92</td>
<td>13.2 ± 2.94, 124</td>
<td>540 ± 97.2, 92</td>
</tr>
</tbody>
</table>

\(^a\) Relative to \( WT \)
Table B.2: Apparent steady-state kinetic parameters at pH 6.5. Results are representative of the mean ± S.D. of \( n = 3 \) experiments. Data is also presented as the percentage of \( WT \) at each respective pH.

<table>
<thead>
<tr>
<th>hPDI</th>
<th>( k_{\text{cat}} \times 10^{-2} \text{ s}^{-1}, %^a )</th>
<th>( K_M \text{ (nM, } %^a) )</th>
<th>( k_{\text{cat}}/K_M \times 10^3 \text{ M}^{-1} \text{ s}^{-1}, %^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( WT )</td>
<td>( 1.79 \pm 0.0133, 100 )</td>
<td>( 20.3 \pm 2.79, 100 )</td>
<td>( 882 \pm 90.9, 100 )</td>
</tr>
<tr>
<td>K57/401Q</td>
<td>( 2.30 \pm 0.0807, 128 **** )</td>
<td>( 67.8 \pm 10.47, 334 **** )</td>
<td>( 340 \pm 57.0, 39 **** )</td>
</tr>
<tr>
<td>K57Q</td>
<td>( 2.56 \pm 0.0848, 143 **** )</td>
<td>( 47.7 \pm 6.95, 235 *** )</td>
<td>( 537 \pm 78.3, 61 *** )</td>
</tr>
<tr>
<td>K401Q</td>
<td>( 2.30 \pm 0.140, 128 **** )</td>
<td>( 42.8 \pm 7.01, 211 *** )</td>
<td>( 537 \pm 55.7, 61 *** )</td>
</tr>
<tr>
<td>K57/401A</td>
<td>( 1.77 \pm 0.0611, 99 )</td>
<td>( 42.7 \pm 8.63, 210 *** )</td>
<td>( 414 \pm 75.9, 47 **** )</td>
</tr>
<tr>
<td>K57A</td>
<td>( 1.68 \pm 0.0001, 94 )</td>
<td>( 29.0 \pm 0.25, 143 )</td>
<td>( 579 \pm 44.0, 66 ** )</td>
</tr>
<tr>
<td>K401A</td>
<td>( 1.89 \pm 0.0477, 105 )</td>
<td>( 31.8 \pm 3.96, 157 )</td>
<td>( 590 \pm 79.0, 67 *** )</td>
</tr>
<tr>
<td>K57/401E</td>
<td>( 1.42 \pm 0.0880, 79 ** )</td>
<td>( 31.1 \pm 4.14, 153 )</td>
<td>( 455 \pm 83.5, 52 **** )</td>
</tr>
<tr>
<td>K57E</td>
<td>( 1.89 \pm 0.233, 105 )</td>
<td>( 30.8 \pm 2.94, 152 )</td>
<td>( 612 \pm 70.2, 69 ** )</td>
</tr>
<tr>
<td>K401E</td>
<td>( 2.04 \pm 0.0672, 113 * )</td>
<td>( 35.7 \pm 2.39, 176 * )</td>
<td>( 571 \pm 83.2, 65 *** )</td>
</tr>
</tbody>
</table>

* Relative to \( WT \)
Table B.3: Apparent steady-state kinetic parameters at pH 7.0. Results are representative of the mean ± S.D. of n = 3 experiments. Data is also presented as the percentage of WT at each respective pH.

<table>
<thead>
<tr>
<th>hPDI</th>
<th>$k_{cat}$ (×10^{-2} s^{-1}, %)</th>
<th>$K_M$ (µM, %)</th>
<th>$k_{cat}/K_M$ (×10^{-3} M^{-1} s^{-1}, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3.64 ± 0.164, 100</td>
<td>0.191 ± 0.0178, 100</td>
<td>190 ± 8.05, 100</td>
</tr>
<tr>
<td>K57/401Q</td>
<td>3.08 ± 0.0479, 85 ****</td>
<td>0.522 ± 0.0134, 273 ****</td>
<td>59.0 ± 5.61, 31 ****</td>
</tr>
<tr>
<td>K57Q</td>
<td>3.97 ± 0.123, 109 *</td>
<td>0.347 ± 0.0235, 181 ****</td>
<td>114 ± 5.08, 60 ****</td>
</tr>
<tr>
<td>K401Q</td>
<td>4.21 ± 0.0338, 116 ****</td>
<td>0.374 ± 0.0227, 195 ****</td>
<td>113 ± 7.40, 59 ****</td>
</tr>
<tr>
<td>K57/401A</td>
<td>2.48 ± 0.0522, 68 ****</td>
<td>0.397 ± 0.0131, 207 ****</td>
<td>62.4 ± 2.48, 33 ****</td>
</tr>
<tr>
<td>K57A</td>
<td>3.31 ± 0.0313, 91 *</td>
<td>0.288 ± 0.0106, 150 ***</td>
<td>115 ± 3.50, 60 ****</td>
</tr>
<tr>
<td>K401A</td>
<td>3.46 ± 0.193, 95</td>
<td>0.306 ± 0.0468, 160 ****</td>
<td>113 ± 12.2, 59 ****</td>
</tr>
<tr>
<td>K57/401E</td>
<td>1.98 ± 0.0541, 54 ****</td>
<td>0.379 ± 0.0253, 198 ****</td>
<td>52.1 ± 2.01, 27 ****</td>
</tr>
<tr>
<td>K57E</td>
<td>3.08 ± 0.131, 85 ****</td>
<td>0.288 ± 0.0123, 166 ****</td>
<td>96.8 ± 5.79, 51 ****</td>
</tr>
<tr>
<td>K401E</td>
<td>3.08 ± 0.164, 85 ****</td>
<td>0.306 ± 0.0133, 174 ****</td>
<td>92.4 ± 6.28, 49 ****</td>
</tr>
</tbody>
</table>

* Relative to WT
Table B.4: Apparent steady-state kinetic parameters at the physiological pH 7.4. Results are representative of the mean ± S.D. of $n = 3$ experiments. Data is also presented as the percentage of $WT$ at each respective pH.

<table>
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<tr>
<th>hPDI</th>
<th>$k_{\text{cat}}$ (×10^{-2} s^{-1}, %$^a$)</th>
<th>$K_M$ (µM, %$^a$)</th>
<th>$k_{\text{cat}}/K_M$ (×10^3 M^{-1} s^{-1}, %$^a$)</th>
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</thead>
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<tr>
<td>$WT$</td>
<td>3.47 ± 0.0435, 100</td>
<td>0.777 ± 0.0593, 100</td>
<td>44.6 ± 4.00, 100</td>
</tr>
<tr>
<td>K57/401Q</td>
<td>2.74 ± 0.344, 79 ***</td>
<td>1.83 ± 0.285, 235 ***</td>
<td>15.0 ± 0.590, 34 ****</td>
</tr>
<tr>
<td>K57Q</td>
<td>3.63 ± 0.129, 105</td>
<td>1.69 ± 0.264, 218 ***</td>
<td>21.5 ± 1.91, 48 ****</td>
</tr>
<tr>
<td>K401Q</td>
<td>4.03 ± 0.0955, 116 **</td>
<td>1.65 ± 0.179, 212 **</td>
<td>24.5 ± 1.43, 55 ****</td>
</tr>
<tr>
<td>K57/401A</td>
<td>2.26 ± 0.103, 65 ****</td>
<td>1.92 ± 0.252, 247 ****</td>
<td>11.8 ± 1.97, 26 ****</td>
</tr>
<tr>
<td>K57A</td>
<td>3.24 ± 0.103, 94</td>
<td>1.41 ± 0.288, 181 *</td>
<td>23.0 ± 3.39, 52 ****</td>
</tr>
<tr>
<td>K401A</td>
<td>3.41 ± 0.104, 98</td>
<td>1.45 ± 0.0858, 186 *</td>
<td>23.6 ± 0.876, 53 ****</td>
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<tr>
<td>K57/401E</td>
<td>1.68 ± 0.141, 48 ****</td>
<td>1.84 ± 0.256, 237 **</td>
<td>9.12 ± 0.489, 20 ****</td>
</tr>
<tr>
<td>K57E</td>
<td>2.94 ± 0.284, 85 **</td>
<td>1.66 ± 0.296, 214 **</td>
<td>17.7 ± 1.56, 40 ****</td>
</tr>
<tr>
<td>K401E</td>
<td>3.23 ± 0.154, 93</td>
<td>1.33 ± 0.148, 171 *</td>
<td>24.3 ± 1.56, 54 ****</td>
</tr>
</tbody>
</table>

$^a$ Relative to $WT$
Table B.5: Apparent steady-state kinetic parameters at pH 8.0. Results are representative of the mean ± S.D. of n = 3 experiments. Data is also presented as the percentage of WT at each respective pH.

<table>
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<tr>
<th>hPDI</th>
<th>$k_{\text{cat}} \times 10^2$ s$^{-1}$, %$^a$</th>
<th>$K_{\text{M}}$ (µM, %$^a$)</th>
<th>$k_{\text{cat}}/K_{\text{M}} \times 10^3$ M$^{-1}$ s$^{-1}$, %$^a$</th>
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<tr>
<td>WT</td>
<td>3.07 ± 0.396, 100</td>
<td>2.22 ± 0.271, 100</td>
<td>12.94 ± 0.695, 100</td>
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<tr>
<td>K57/401Q</td>
<td>1.17 ± 0.109, 38 ****</td>
<td>2.46 ± 0.156, 111</td>
<td>5.74 ± 0.483, 34 ****</td>
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<tr>
<td>K57Q</td>
<td>1.70 ± 0.0974, 56 ****</td>
<td>2.13 ± 0.0440, 96</td>
<td>8.37 ± 0.277, 58 ****</td>
</tr>
<tr>
<td>K401Q</td>
<td>2.20 ± 0.258, 72 **</td>
<td>2.46 ± 0.245, 111</td>
<td>9.78 ± 0.737, 65 ****</td>
</tr>
<tr>
<td>K57/401A</td>
<td>1.17 ± 0.166, 38 ****</td>
<td>2.53 ± 0.188, 114</td>
<td>4.26 ± 1.07, 33 ****</td>
</tr>
<tr>
<td>K57A</td>
<td>1.35 ± 0.121, 44 ****</td>
<td>1.60 ± 0.0159, 72</td>
<td>8.44 ± 0.745, 61 ****</td>
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<tr>
<td>K401A</td>
<td>2.01 ± 0.256, 66 ***</td>
<td>2.11 ± 0.607, 95</td>
<td>9.45 ± 1.17, 69 ****</td>
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<td>K57/401E</td>
<td>0.473 ± 0.0541, 15 ****</td>
<td>2.32 ± 0.264, 105</td>
<td>2.01 ± 0.763, 15 ****</td>
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<tr>
<td>K57E</td>
<td>1.20 ± 0.485, 39 ****</td>
<td>1.99 ± 0.325, 90</td>
<td>4.26 ± 0.983, 44 ****</td>
</tr>
<tr>
<td>K401E</td>
<td>1.99 ± 0.353, 65 ***</td>
<td>2.33 ± 0.164, 105</td>
<td>8.54 ± 1.10, 62 ****</td>
</tr>
</tbody>
</table>

$^a$ Relative to WT
Figure B.1: Residue Tyr$^{99}$ of the $a$-domain $cis$-Pro loop is immobilized by hydrogen bonding (dotted line) to the peptide carbonyl of $A^{84}$. Interaction found to be present in all representations of the $a$-domain (red/oxPDI $WT$ and K57/401Q); depicted is that of redPDI $WT$. Visualized using Visual Molecular Dynamics software (VMD) [54].
Figure B.2: MD simulations of the active site structural dynamics of oxPDI. (A) WT PDI a-domain, (B) WT PDI a'-domain, (C) K57/401Q PDI a-domain, and (D) K57/401Q PDI a'-domain. Visualized using Visual Molecular Dynamics software (VMD) [54].
Figure B.3: Redox-state dependent structural dynamics of the PDI ensemble as determined from MD simulations of WT PDI. redPDI takes on a much more closed conformation in relation to oxPDI. Note the dramatic rotation about the $a'$-domain of oxPDI, that not observed with redPDI. Visualized using Visual Molecular Dynamics software (VMD) [54].
Figure B.4: Plots of distance vs. frame of the redPDI MD simulation for the estimation of hydrogen bonding. Distances were taken as the measurement between hydrogen bond donor and acceptor heavy atoms; (A) Lys\textsuperscript{57} ε-amine to the peptide carbonyl oxygen of Pro\textsuperscript{51}, (B) Lys\textsuperscript{401} ε-amine to the peptide carbonyl oxygen of Pro\textsuperscript{395}, (C) mutant Gln\textsuperscript{57} δ-amide nitrogen to the peptide carbonyl oxygen of Pro\textsuperscript{51}, (D) mutant Gln\textsuperscript{401} δ-amide nitrogen to the peptide carbonyl oxygen of Pro\textsuperscript{395}. Red dotted lines represent the threshold distance of 3.5 Å for moderately strong hydrogen bonds, distances smaller than this would indicate strong hydrogen bonds and those larger would be weak to non-existent hydrogen bonds [205]. Plots generated using Visual Molecular Dynamics software (VMD) [54].
Figure B.5: Plots of distance vs frame of the oxPDI MD simulation for the estimation of hydrogen bonding. Distances were taken as the measurement between hydrogen bond donor and acceptor heavy atoms; (A) Lys$^{57}$ ε-amine to the peptide carbonyl oxygen of Pro$^{51}$, (B) Lys$^{401}$ ε-amine to the peptide carbonyl oxygen of Pro$^{395}$, (C) mutant Gln$^{57}$ δ-amide nitrogen to the peptide carbonyl oxygen of Pro$^{51}$, (D) mutant Gln$^{401}$ δ-amide nitrogen to the peptide carbonyl oxygen of Pro$^{395}$. Red, dotted lines represent the threshold distance of 3.5 Å for moderately strong hydrogen bonds, distances smaller than this would indicate strong hydrogen bonds and those larger would be weak to non-existent hydrogen bonds [205]. Plots generated using Visual Molecular Dynamics software (VMD) [54].
Figure B.6: Structure of dieosin glutathione disulfide (di-E-GSSG). In boxes are the ionizable groups and their respective $pK_a$'s [203, 204].
Figure B.7: Dependence of the rate of PDI oxidoreductase activity on the concentration of DTT. Using the same steady state conditions from before (see section 3.1) the concentration of DTT was varied and the rate of reduction of di-E-GSSG was monitored and reported as the production of EGSH.
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177. *The solution structure of the first thioredoxin domain of mouse Protein disulfide-isomerase A6.* To be published.


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