PDI, Reactive oxygen species stress and Polyphenolic Phytochemicals: Implications for Neurodegenerative Diseases

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PDI, reactive oxygen species stress and Polyphenolic Phytochemicals: Implications for neurodegenerative diseases

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Dedication

I dedicate this thesis and all the hard work that came with it to my dear family who always encouraged me to further my education and told me no matter what path I choose, they would always support me in reaching my ultimate goal. I am especially grateful to my mom who always reminded me to put the petal to the metal, I am thankful to my father to allow me to come all the way away from my country for better science. This is dedicated to my brother Dr. Angshuman Pal and my uncle Dr. Uttam Khamrai who I love with all my heart and hope I have served enough to reach their expectation in the field of science where both of them are role model for me. Lastly, I would thank to my sister and I believe I have served as a good enough role model for her. Thank you all for being there with me in every step of my way. I couldn’t have done it without you.

Thank you with all my love,

Rituraj
PDI, reactive oxygen species stress and Polyphenolic Phytochemicals: Implications for neurodegenerative diseases

By

RITURAJ PAL, B.S.

THESIS

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<tr>
<td>DsbA</td>
<td>Disulfide bond promoting enzyme encoded by dsbA</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
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<tr>
<td>GR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>Grx</td>
<td>Glutaredoxin</td>
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<tr>
<td>GSH</td>
<td>Glutathione (Reduced form)</td>
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<td>GSSH</td>
<td>Glutathione disulfide</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adeninedinucleotide phosphate</td>
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<tr>
<td>HCL</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectroscopy</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>RNaseA</td>
<td>RibonucleaseA</td>
</tr>
<tr>
<td>SH</td>
<td>Thiol group</td>
</tr>
<tr>
<td>sRNase</td>
<td>Ribonuclease with non-native disulfide bonds</td>
</tr>
<tr>
<td>SS</td>
<td>Disulfide bond</td>
</tr>
<tr>
<td>Trx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TNM</td>
<td>Tetra-nitromethane</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
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Abstract

PDI, protein disulfide isomerase, is one of the most versatile proteins and highly expressed in mammalian cells because there is a vast number of proteins that must undergo processing before secretion to their final destinations. PDI has several functions: oxidation of nascent proteins and isomerization of existing disulfide bonds. It also possesses chaperone activity and participates in protein degradation. Because of its structure, PDI can exist in a reduced or oxidized form. In mammalian cells it is mostly reduced due to the high demand of disulfide bond shuffling of secreted proteins within the ER, PDI a, a’, and b’ posses the ability to facilitate substrate folding. In our experiments we are using rat PDI and according to the sequence there are cysteine residues located in a and a’ domains. These cysteine residues may contribute to the catalytic activity of this domain and will be analyzed in future studies. Misfolded proteins, and the associated endoplasmic reticulum (ER) stress, are emerging as hallmarks of age- and neurodegeneration-related disorders such as Huntington’s disease (HD), Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis. Recent and compelling evidence has linked nitrosative stress and the ER-resident oxidoreductase, protein disulfide isomerase (PDI), to the pathogenesis of PD and AD. Overexpression of PDI has been found to reduce the formation of polyubiquitinated proteins, making the oxidoreductase an important target for therapeutic intervention in PD, AD and other age- and neurodegeneration-related disorders. We demonstrate the NO-scavenging ability of the biphenolic natural products curcumin and masoprocol and the concomitant prevention of S-nitrosylation of PDI by a model NO-donor. Furthermore, both ethnopharmaceuticals accelerate protein fold acquisition in their neat and nitrated forms, making them attractive candidates for prevention of age- and neurodegeneration-related diseases.
Chapter 1: Introduction

Protein Disulfide Isomerase (PDI)

What is PDI?

Protein disulfide isomerase (PDI), a very abundant protein in the endoplasmic reticulum of the cell, facilitates the formation and rearrangement of disulfide bonds using two nonequivalent redox active-sites, located in two different thioredoxin homology domains. Each dithiol/disulfide active-site contains the thioredoxin consensus sequence CXXC. Four domains of protein disulfide isomerase were constructed that contain only a single active-site cysteine. Kinetic analysis of these mutants show that the first (more N-terminal) cysteine in either active site is indispensable for catalysis of oxidation and rearrangement during the refolding of reduced bovine pancreatic ribonuclease A (RNase A). [1][2] PDI was first isolated from liver in 1963 by the pioneer of protein folding, Christian Anfinsen and characterized depending on its ability to catalyze the refolding of ribonuclease A, an enzyme with 4-disulfide bridges. The protein-thiol oxidoreductase activity of PDI appears to differ based upon the redox environment and cellular requirements. [3]

General functions of PDI

PDI catalyzes the formation of disulfide bonds and isomerizes incorrect disulfide in the nascent polypeptides undergoing folding in the oxidizing environment of the endoplasmic reticulum. The bulk of PDI is localized to the ER, consistent with a signal sequence and an ER retention signal. But the availability of PDI has been shown on the plasma membrane as well as in the cytosol,
endosome and at the plasma membrane, where PDI reduces disulfide bridges of proteins in as an upshot of the more reducing environments of those locales. Although PDI was initially characterized as an oxidoreductase, it is now known as a multifunctional contributor in the folding, assembly and post-translational modification of many proteins. It displays chaperone and anti-chaperone activities in vitro and in vivo (Cai et al., 1994; Primm et al., 1996; Puig and Gilbert, 1994b). In Saccharomyces cerevisiae, PDI is an indispensable protein (Scherens et al., 1991) and its crucial role in this organism is to catalyze disulfide bond formation (Solovyov et al., 2004; Xiao et al., 2004). PDI is also a subunit of two well characterized ER enzymes, proly 4 hydroxylase (P4H) (Pihlajaniemi et al., 1987) and microsomal triglyceride transfer protein (MTP) (Wetterau et al., 1990).

**PDI structure**

PDI is a member of the thioredoxin superfamily. The multidomain nature of PDI was initially recognized from an analysis of its amino acid sequences, in which four domains, denoted \( \text{a}, \text{b}, \text{b}' \) and \( \text{a}' \) were identified. These structural domains are followed by an extended acidic residue at the C terminus (designated \( \text{c} \)) (Edman et al., 1985). Proteolytic mapping and heterologous expression of individual domains established and refined the domain boundaries of PDI (Darby et al., 1996) (Fig.1). \( \text{a} \) and \( \text{a}' \) domains are homologous to each other (47% identity) and to thioredoxin (27% identity). Each of these domains contains an active site CGHC motif that can be reduced mammalian thioredoxin reductase, which further support a close structural relationship to thioredoxin (Lundstrom and Holmgren, 1990). In isolation, the PDI-a domain behaves as a folded globular protein in both its dithio and disulfide forms (Darby and Creighton, 1995b), and the structure of the disulfide form has been shown by NMR analysis to be very
similar to that of thioredoxin (Kemmink et al., 1996). On the other hand, the PDI-a’ domain is less stable than expected, as it unfolds at least partly upon formation of its disulfide bond (Darby and Creighton et al., 1995b). The b and b’ have similar sequences (28% identity), but show or very low sequence relatedness to thioredoxin and do not contain symbolic CGHC motif.

(A)
Fig. 1. The structure of mammalian PDI. (A) Domain boundaries have been determined by a combination of protein engineering and limited proteolysis studies (Darby et al., 1996). The first and last residues of each folded structure are indicated. The c domain at the C-terminus comprises a 24 acidic segment followed by KDEL sequence for retention in the endoplasmic reticulum. (B) Three dimensional structure of PDI.

Fig. 2. Electrostatic surface representation of the structures of the individual PDI domains. The structures of a and b are from NMR determinations. The arrow denotes the nucleophilic active site cysteine. The top and bottom rows represent 180 degree rotations of each domain around the
The b’ and a’ structures are homology models and should be considered as such. The anionic c domain is arbitrarily shown in an extended conformation.

Although a high resolution X-ray crystal structure of PDI has not been determined, the NMR structures of the isolated a and b domains provided the first detailed structural information about PDI. [7][8]. The a domain of PDI, as expected from its primary structure homology, has a thioredoxin fold with all the secondary structural elements of thioredoxin and two grooves that are sites of enzyme-substrate interaction (Kemmink et al., 1996). Surprisingly, NMR studies of the b domain of PDI indicates that like a domain, the b domain also has a thioredoxin fold despite the absence of any CXXC redox active sites or sequence similarity to thioredoxin (kemmink et al., 1997). Sequence homology between the b and b’ and a and a’ imply that PDI consists of four tandem domains with thioredoxin structure. [9] The c domain has been defined as the region 465 to the C terminus of the PDI polypeptide. This region of PDI may not be an actual domain as there is no evidence of defined secondary or tertiary structure. However, the c domain is of considerably interest because of its high content of acidic residues and the presence of the C terminal KDEL retention signal. The C-terminal KDEL motif of the PDI polypeptide is involved in the ER localization of the enzyme (Pelham, 1990). The acidic residues of the c domain participate in the extensive calcium binding activity of PDI (Lebeche et al., 1994). Although there is no structure data on the entire PDI molecule, sedimentation velocity and equilibrium measurements suggest that PDI is an elongated monomer with an axial ratio of ~5, consistent with nearly linear arrangement of the domains (Solovyov and Gilbert, 2004). [10][11]
Peptide binding

The ability to interact with polypeptides and unfolded proteins is the foundation for the activity of a chaperone and perhaps equally important for the activity of a disulfide isomerase. The most striking feature of the wild-ranging catalytic activities of PDI is its ability to catalyze steps in protein folding pathways which involve significant conformational change in protein substrate, associated with protein disulfide bond formation or isomerization. Full length PDI is much more effective than the individual a and a’ domains as a catalyst for such steps in the folding pathway of reduced RNase (Darby et al., 1998a), although the isolated domains are equally effective as catalysts of simple thiol-disulfide interchange reaction (Darby and Creighton, 1995b). This suggests that the complex natural substrate of PDI make extensive contacts with the PDI molecule, which may facilitate transactions between different conformational states of the protein substrate. [12] Peptide binding studies using either somatostatin or a 28-residue peptide from the N-terminus of staphylococcal nuclease indicate that the interaction of peptides with PDI competes with the refolding of reduced, denatured RNase and scrambled RNase, reduces the efficiency of catalysis of insulin reduction, inhibits the chaperone function of PDI in the refolding of denatured Glyceraldehyde 3-phosphate dehydrogenase (Klappa et al., 1997; Morjana and Gilbert, 1991; Quan et al., 1995). This indicates not only that both peptide and polypeptide binding sites are the same or spatially juxtaposed, but also that they are close enough to the active site to inhibit the redox activity when occupied by a polypeptide chain. PDI interact weekly (KD>100 µM) with polypeptide (Morjana and Gilbert, 1991). [36][37] The binding increases with increasing length of substrate backbone, but for peptides of similar length, those containing cysteine residues bind 4-8 folds more strongly to PDI (Klappa et al., 1997; Morjana
and Gilbert, 1991). The hydrophobic interaction may determine the efficiency of binding. Photo affinity labeling studies with a radioactive tripeptide probe have localized the first peptide-binding site that begins at the end of a’ and includes most of domain c (Noiva et al., 1993). Deletion of extreme C-terminal helix of domain a’ has an inhibitory effect on the assembly of recombinant proly-4 hydroxylase in insect cells or on the in vitro chaperone activity or disulfide isomerase activity of PDI (Koivunen et al., 1999). The mutations in the C-terminal part of a’ domain can also affect the peptide binding activity of PDI, a function associated with b’ domain (Klappa et al., 2000). Using a cross-linking method, Klappa and colleagues has demonstrated that b’ domain of PDI provides the principal peptide-binding site of PDI. The b’a’c construct is the smallest fragment that is able to bind a large misfolded protein as well as small peptides and a’ domain contributes more to the binding of full length PDI than does the a domain (Klappa et al., 1998a).

**Ribonuclease A**

Before describing the products of the artificial oligomerisation of bovine pancreatic ribonuclease A, a short description of some of the main features of RNase A is appropriate. The enzyme which is one of the most studied proteins in the world is composed of 124 amino acids (molecular mass, 13,686 Da), among which basic residues (10 Lys, 4 Arg, besides 4 His) prevail over acidic residues (5 Glu, 5 Asp), making therefore the protein definitely basic, with a pI of 9.3. The protein’s secondary structure consists of three α-helices and seven β-strands, and the molecule contains four disulphide bonds (26-84, 40-95, 58-110, 65-72) (Fig.2) that definitely contribute, in particular the two terminal ones (26-84 and 58-110), to the remarkable stability of the protein. It might be worth pointing out that RNase A, in contrast with many other proteins
with disulphide bridges, has rather long termini outside the disulphide-bonded core, in which the
two active-site histidines (His-12 and His-119) are indeed located. The N-terminus (residues 1-
15), comprising one of the α-helices (residues 3-13), is connected with the body of the protein by
a flexible loop (residues 16-23) that, under controlled conditions, may be cleaved by subtilisin
forming RNase S, without any loss of activity. The C-terminal β-hairpin of RNase A is
composed by two β-strands (residues 105-124) relatively richer in hydrophobic residues than the
N-terminus. Both termini can become mobile under defined experimental conditions that weaken
the noncovalent bonds between them and the protein core. The protein can be viewed as a kidney
bean whose two main lobes are separated by a positively charged region which binds the RNA
substrate. [42][43][44] We just mention here some details concerning its enzymatic activity that
can be useful in the further discussion, referring the interested reader to the many excellent
reviews available for more detailed information concerning the structural and functional
properties of RNaseA. The active site of the enzyme is formed by His-12, His-119 and Lys-41.
The well-known “in line” mechanism of the endonucleolytic cleavage of single-stranded RNA
by RNase A consists of a first transesterification step (leading to 2’, 3’-cyclic phosphate
intermediates), and a following hydrolytic action on these intermediates. To take place the
reaction requires that the 2’ oxygen of the ribose hydroxyl group, which acts as an internal
nucleophile, and the 5’ oxygen leaving group simultaneously occupy the two apical positions
within the trigonal bipyramidal phosphorane intermediate. This steric requirement makes a
fruitful interaction of the enzyme with double stranded RNA impossible, and therefore it is
chiefly responsible for the high resistance of this RNA species to attack by native, monomeric
RNase A. It must be remembered, however, that other variables, such as the ionic strength and
the presence of carbohydrate chains on the enzyme protein, influence the resistance of double-helical RNA to ribonuclease attack. [16][17][89]

**Fig.3.** A ribbon diagram of RNase A with the disulfide bonds highlighted by spheres. The disulfide bonds 26–84 and 58–110 are completely buried, whereas disulfide bonds 40–95 and 65–72 are partially exposed (Koradi et al., 1996)

**Oxidative folding of RNaseA**

Bovine pancreatic ribonuclease A (RNase A) is ideally suited for elucidating the oxidative protein folding events because RNase A contains disulfide bonds, and its folding has been studied in depth. The protein consists of 124 amino acid residues and contains four intramolecular disulfide bonds (26-84, 40-95, 58-110 and 65-72). Recent studies in our laboratory involve DTTox/DTTred-coupled refolding experiments using 2 aminoethyl methanethiosulfonate (AEMTS) as a rapid thiol-blocking agent. The use of DTTox/DTTred instead of the commonly used GSSG/GSH as the redox reagent significantly simplifies protein-folding studies because the number of possible disulfide-bonded intermediates is drastically reduced, from 7191 to 762, by eliminating the formation of mixed disulfide bonds (Rothwarf & Scheraga, 1993a). [91][92][93]

The oxidative folding of the four-disulfide protein bovine pancreatic ribonuclease A (RNase A) by DTTox is done at 25°C and pH 8, the native protein is regenerated from the fully reduced form through two major (Rothwarf et al., 1998a, b) and two minor (Iwaoka et al., 1998; Xu and
Scheraga, 1998) pathways, involving the formation of two intermediate three-disulfide species, des-[65–72] and des-[40–95]4, which have native-like structures (Shimotakahara et al., 1997; Laity et al., 1997). [18][45]

![Diagram of protein folding pathways](image)

**Fig. 4. A model for the regeneration of native protein from reduced RNase A with DTTox at 25°C and pH 8.0.** R represents the fully reduced protein, nS represents an ensemble of species with n disulfide bonds.

These two intermediates, which appear in the rate-determining step from the unstructured 3S ensemble, are then oxidized to the native protein by DTTox. Two additional intermediates with three native disulfide bonds, des-[26–84] and des-[58–110], become observable when oxidative folding is carried out at lower temperatures (15°C) (Welker et al., 1999). However, these two intermediates do not oxidize to the native protein at a significant rate; instead, they reshuffle back to the unstructured 3S ensemble before proceeding along the major pathways, ultimately to the native protein (Welker et al., 2001). Formation of the native-like structure in des-[65–72] and des-[40–95] protects their disulfides from reshuffling back to the 3S ensemble, thereby enabling them to proceed to the native protein in the presence of DTTox (Narayan et al., 2000). It is, therefore, of interest to examine the conditions that stabilize native-like structure and protect the already-formed disulfides in folding intermediates, thus enabling them to proceed to the native protein. [46][47][48] One stabilizing additive is Dipotassium phosphate (K2HPO4).
Salts, such as K2HPO4, can alter the stability and folding of proteins by three different processes: by specific binding to the active site, by nonspecific electrostatic interactions, and by influencing hydrophobic interactions. Anions, such as phosphate, are known to stabilize proteins (Hermans and Scheraga, 1961; Ginsburg and Carroll, 1965; von Hippel and Wong, 1965; Collins and Washabaugh, 1985; Ahmad and Bigelow, 1986; Baldwin, 1996) and peptides (Jelesarov et al., 1998) and to increase the folding rate of denatured proteins (Schaffer et al., 1975; Prajapati et al., 1998; Low et al., 2000). An earlier report from our laboratory suggests that phosphate may catalyze the rearrangement of unstructured three-disulfide intermediates to form des-[65–72] and des-[40–95] (Low et al., 2000). It was also shown (Low et al., 2000) that des-[26–84] and des-[58–110], which accumulate at low temperature (Welker et al., 1999), can be observed at 25°C if phosphate ions are present. Among the various observed pathways for the oxidative folding of RNase A, the dominant one (to the extent of about 80%) involves the early formation of the [65-72] disulfide bond to a considerable extent in the 1S ensemble, and the subsequent formation of des [40-95] (a three-disulfide native-like intermediate) by thiol-disulfide reshuffling reactions of the 3S ensemble in the rate-determining step. A minor, albeit parallel pathway (to the extent of about 20%) involves the formation of des [65-72] (another three-disulfide native-like intermediate) in the rate-determining step by similar thiol-disulfide reshuffling reactions of the 3S ensemble. Both des 40-95] and des [65-72] are then oxidized to the native protein. [19][20][21]
Catalysis of the Oxidative Folding of Bovine Pancreatic Ribonuclease A by Protein Disulfide Isomerase (PDI)

PDI accelerates the folding rate of RNaseA by inducing two novel ways, by showing its chaperon activity and by disulfide isomerization.

Chaperon activity of PDI

The mechanism by which a protein molecule folds into the native conformation still remains an unsolved problem in structural biology. Understanding the folding process will not only elucidate the fundamental relationship between an amino acid sequence and the three-dimensional structure of a protein, but will also provide invaluable information to a variety of related fields, such as the rational design of novel protein molecules and the understanding of the pathological states associated with protein misfolding (Dobson & Ellis, 1998). Protein folding in vitro is generally a spontaneous process leading to a native three-dimensional structure depending on the amino acid sequence of the protein (Anfinsen, 1973). [22][23] In many cases, however, folding does not proceed efficiently because of intermolecular aggregation and slow folding reactions such as disulfide formation or proline isomerization. The folding process can be particularly slow in the case of cysteine-containing proteins that require the formation of disulfide bonds (Huppa & Ploegh, 1998). [50][51] Disulfide formation in vivo is catalyzed by specialized enzymes belonging to a thioredoxin super family, such as PDI (protein disulfide isomerase) in eukaryotes and Dsb (disulfide bond) proteins in prokaryotes. PDI is a 55 kDa protein found in the endoplasmic reticulum (ER) where it catalyzes disulfide bond formation and rearrangement during the maturation of most secretary and membrane bound proteins (Freedman
et al., 1994; Gilbert, 1998). PDI is not a particularly effective enzyme, consistent with its abundance in the ER, and high concentrations are often required to observe catalysis of protein folding both in vivo and in vitro (Lyles & Gilbert, 1991). Despite the information available for the general effects on the oxidation and rearrangement of the folding of disulfide-containing proteins, detailed knowledge about the effect of PDI on the mechanism of protein folding still remains to be elucidated. Depending on the concentrations of foldase and denatured substrate and the order in which they are added to initiate folding, PDI can exhibit chaperon activity. When it is present at high concentrations relative to unfolded protein substrate, PDI functions as a chaperon making folding more productive by increasing the folding rate and by inhibiting aggregation both for denatured proteins with no sulfide bonds and the proteins containing disulfide bonds.[24][52][53]

**PDI catalyzed disulfide isomerization**

Non-covalent interactions within the protein that is undergoing folding specify which disulfides will form most effectively. When disulfide formation occurs early in folding, before the correct non-covalent structure is developed, cysteine pairing is most likely to be incorrect. Thus protein folding is error-prone. For some proteins like BPTI, the specification of cysteine connectivity occurs early in folding and is directed by the formation of native like structure that are interconverted by intramolecular disulfide rearrangements (Goldenberg,1992). [54] In other cases such as RNaseA (Creighton, 1979; Konishi et al., 1982) or hirudin (Chatrenet and Chang, 1992), disulfides are formed randomly in early folding intermediates. [25] Thus, protein folding is a process of trial and error. As a catalyst, PDI must deal with different mechanisms for
directing disulfide formation. The cysteine near the N-terminal of each active site (\textbf{CGHC}) housed in two, internally homologous thioredoxin-like domain, is essentially for all catalytic activity, both oxidation and isomerization (Laboissiere et al., 1995; LaMantia and Lennarz, 1993; Walker et al., 1996). Mutations of the active site cysteine closer to the carboxyl terminus (\textbf{CGHC}) selectively destroy the ability of PDI to catalyze disulfide formation. However, these mutants still remain a low but measurable amount of isomerase activity in the presence of glutathione redox buffer (Laboissiere et al., 1995; Walker et al., 1996). [26][27][28]

Formally, the rearrangements of substrate disulfide do not require the net oxidation or reduction of PDI or the substrate, but a substrate disulfide must be broken to initiate rearrangement. The first step of isomerization involves a reduced PDI active site attacking a substrate disulfide. After the initial reaction, two different mechanisms could result in substrate isomerization depending on the covalent PDI-substrate intermediate partitions. In the first mechanism, an intramolecular rearrangement in the substrate may occur when the sulfhydryl of the substrate cysteine generated by PDI reacts intramolecularly with another substrate disulfide. In this mechanism, PDI facilitates reactions between the thiols and disulfides of the substrates while the substrate is covalently bound to PDI. The intramolecular rearrangement concludes when a substrate disulfide cysteine displaces PDI from the covalent complex, forming another in the substrate and regenerating reduced PDI for another round of catalysis. [56][57] In the second mechanism, PDI can resolve the covalent intermediate by reducing cycles of substrate reduction and reoxidation in a different configuration lead to isomerization, eventually leading to the native structure. Several lines of evidences suggest that the reduction oxidation pathway contributes to the isomerization of scrambled sRNaseA by PDI; 1) Isomerization is inhibited at high concentration...
of RNaseA; such inhibition can be relieved by addition of oxidized PDI (Schwaller et al., 2003). For an intramolecular isomerization, there is no role for oxidized PDI. 2) In the absence of redox buffer, PDI mutants with only a nucleophilic cysteine (CGHS) have no isomerase activity. The second active site cysteine is essential for isomerization of sRNaseA (Walker et al., 1996), but it plays no role in an intramolecular mechanism. 3) Reduced PDI rapidly oxidized when mixed with sRNaseA before any isomerization occurs suggesting that substrate is released before native RNaseA forms (Schwaller et al., 2003). 4) Low levels of covalent PDI-substrate intermediates are detected with wild type PDI, but such intermediates build up with CGHS mutants (Walker and Gilbert, 1997) indicating that second cysteine limits their accumulation. While isomerization of sRNaseA by PDI may proceed through the reduction-oxidation pathway, other substrate may utilize the intramolecular pathway and even sRNaseA may utilize it with low frequency. [93][94][95]
We already know that wild type PDI has tremendous potentiality to accelerate the oxidative folding of RNaseA or formation of similar protein disulfide bond formation by its catalytic and chaperone activity. In our lab, we are trying to see the potency of each domain of wild type PDI if they are showing same functional activity or more or less activity. Our primary aim is to use small molecule to catalyze disulfide bond formation. The small is the molecule the better is in the body. If we find any of the PDI domains are showing same potency as wild type PDI or showing better activity than the wild type PDI we can use that very small domain instead of a whole big molecule. We tried with two different domains, PDI $ab$ and PDI $a'b'$ to see their potency in disulfide bond formation comparing to wild type PDI. PDI $ab$ and PDI $a'b'$ both are

**Fig. 5. PDI dependant thiol oxidation and isomerization activity.**
thioredoxin like domains of wild type PDI, so, we are expecting acceleration in disulfide bond formation. But, to know the actual potency of these two domains we have to perform the oxidative protein folding experiment with the substrate protein RNaseA which has four disulfide bonds (8 free thiol groups). [29][30][59][60]

Materials and Methods

Materials: RNase A was purchased from Sigma and was purified properly (Rothwarf D.M., and Scheraga H.A., 1998 et al.,)

Wild type PDI, PDI ab and PDI a’b’ plasmids were brought from Sweden and were expressed in E.Coli (BL21 strain).

Methods: PDI ab and PDI a’b’ expression. 100 µl aliquot BL21(DE3) competent cell was taken out from -80ºC, and was Thawed on ice bucket for 10 min. PDI plasmid was taken out from -20ºC and was thawed too. 1 µl of the plasmid was added into 100 µl BL21 cell tube, and was kept on Ice for 30 min.

# the amount of plasmid may vary depending on the concentration of plasmid and the competitiveness of BL21.

Heat shock was given for 60 sec. at 42ºC. (Isotemp should be pre-equilibrated).Then the cell was Put it back on ice for 2 min. (meanwhile, prepare 1 ml LB in a 15 ml Falcon tube). 100 µl transformed BL21 was transferred to the 1 ml LB, and kept in 37ºC incubator with shaking at 150 rpm for 45 min. (meanwhile, LB-Amp plate was taken out from 4ºC and equilibrated to room temp.) 100 µl of the LB (45 min incubated 1 ml LB) was spread onto the LB-Amp plate. The LB/AMP plate was Incubated at 37ºC for 13 hrs. (It may vary ± 2hrs).After overnight incubation,
next day, a single colony was picked up into 50ml LB and add 0.1 ml of Ampicillin (Stock concentration is 50 mg/ml and final concentration in 50 ml LB is 0.1 mg/ml) in 250 ml Erlenmeyer flask, and incubate at 37°C with shaking at 150 rpm until OD<sub>600</sub> reaches 0.5. Starter culture O.D should not be allowed to exceed 0.5.

- It will take ≈ 4hr. Check OD<sub>600</sub> after 3 hr.
- OD doubles, generally in 20 min.

25 ml of the starter culture was incubated into 1 L LB and add 2 ml of ampicillin stock was added, and Incubated until OD<sub>600</sub> reaches 0.55-0.6.

- It will take ≈ 3-4 hrs.

When the OD<sub>600</sub> reaches 0.55-0.6, 1 ml of IPTG (0.5 M) was added, and it was shaken 4 more hours. Then, ells were harvested by centrifuging at 5,000 rpm for 10 min at 4°C. Supernatant was discarded and resuspended with lysis buffer (No more than 20 ml).

Lysis buffer: 300 mM NaCl, 50 mM PO<sub>4</sub>, and 10 mM Imidazole, pH 7.5

Resuspended cells were placed in 50 ml Falcon Tube and were sonicated. The condition must be set up with each machine.

# Four 30-sec-sonications and 1 min cooling between each sonication.

# Sonication must be done in Ice-Bucket.

Then the cells were centrifuged at 11,000 rpm for 40 min at 4°C. Supernatant was taken and the pellet was discarded. The supernatant was poured onto Ni-NTA resin in column and purified. Then the protein was run on SDS gel and Experian gel to for confirmation by molecular weight with proper protein ladder.
Fig. 6. Wild type PDI in SDS gel. L- ladder, 1-First wash (300 mM NaCl, 50 mM PO₄, and 50 mM Imidazole, pH 7.5), 2- Second wash (300 mM NaCl, 50 mM PO₄, and 100 mM Imidazole, pH 7.5), and 3- Third wash (300 mM NaCl, 50 mM PO₄, and 300 mM Imidazole, pH 7.5). PDI protein came out as 55 KD molecular weight.

Fig. 7. Experion gel picture for wild type PDI shows molecular weight of wild type PDI is 55KD.
Preparation of Fully Reduced RNase A. Purified native RNase A was reduced by incubating the protein in a buffer containing 100 mM DTTred and 6 M GnHCl (100 mM Tris-HCl, 1 mM EDTA, pH 8.3, 25 °C). After incubation for 3h, the pH of the solution was lowered to 3 by addition of glacial acetic acid. The reduced protein was desalted (and DTTred was
removed) on a reversed-phase HPLC column (C-18) and lyophilized to remove the organic solvent and water. Lyophilized reduced protein was dissolved in a dilute acetic acid solution (1µl of glacial acetic acid with 100 ml of mili Q, pH 3.7) to obtain a stock solution (5.0 mg/mL) and was stored at -20 °C. [31]

**Oxidative Folding of RNase A.** Oxidative folding of RNase A was carried out at 25 °C in four different test tubes with different reagents by incubating 0.5 mg/mL (33 µM) reduced protein in a oxidizing buffer of 100 mM DTTox (pH 8.3, 100 mM Tris-HCl, 1 mM EDTA). No DTTrEd was present in the original oxidation buffer, but DTTrEd accumulated during the oxidation reaction. Aliquots of 200 µL were withdrawn at different times after the initiation of oxidative folding, and any free thiols were blocked by an excess amount of AEMTS (final concentration of 50 mM, pH 8.3, 100 mM Tris-HCl and 1 mM EDTA). Five minutes after the addition of the AEMTS, the pH of the solution was lowered to 3 by addition of 20 µL of glacial acetic acid. All samples were desalted on a Hi-Trap G25 column by exchange against a 0.2% acetic acid solution before analysis using cation-exchange HPLC. Prior to the addition of AEMTS which blocks any free thiols, some samples were subjected to a reduction pulse (5 mM DTTrEd at pH 8.3 for 2 min) which results in the reduction of all unstructured species to the fully reduced protein (R), a procedure that has been used successfully to identify and isolate structured species in the oxidative folding of RNase A. [31][32]
Results and discussions

Results

First test tube contains RNaseA (Final conc. 30µM) and oxidizing buffer. In second test tube RNaseA (Final conc 30µM) added with oxidizing buffer and wild type PDI (Final conc. 6µM). In the next one we added RNaseA (Final conc. 30µM), oxidizing buffer, PDI ab (Final conc. 6µM), and in last test tube we added RNaseA (Final conc. 30µM), oxidizing buffer, PDI a’b’ (Final conc. 6µM). No DTTred was present in the original oxidation buffer, but DTTred accumulated during the oxidation reaction. Aliquots of 200 µL were withdrawn at different times after the initiation of oxidative folding, and any free thiols were blocked by an excess amount of AEMTS (final concentration of 50 mM, pH 8.3, 100 mM Tris-HCl and 1 mM EDTA). Five minutes after the addition of the AEMTS, the pH of the solution was lowered to 3 by addition of 20 µL of glacial acetic acid.

The table (1) below is the typical calculation formula for calculation of fraction of reduced and native protein. Results from these experiments showed that the rate of formation of the native protein (N) which is indicated by Ln (1-n) where (1 – n) is the fractional concentration of non-native RNaseA. Ti, T2 and so on are the different time points where 200µl of aliquots were withdrawn. Times are calculated in minutes and each aliquot was withdrawn at different time intervals. All four experiments were started with fully reduced (R) RNaseA, as the time increases RNaseA gradually become folded and as a result native (N) fraction of RNaseA increases with time. In HPLC chromatogram two peaks are visible, one for reduced protein and another for native protein. As the protein enrich by native fraction the hydrophobic part goes in the core of the protein, unlikely, in the reduced protein hydrophobic portion will be exposed outside of the
amino acid chain, which leads the native peak to come before the reduced one because our HPLC column has long hydrocarbon (C18) chain which is very strong hydrophobic. As the reduced protein has exposed hydrophobic part it binds strongly with the column comparing to the native protein and stick to the column for a longer time than the native protein and as a consequence the reduced protein comes after the native one. With the help of area calculator, we can calculate the area of reduced (R) RNaseA and the area of native (N) RNaseA. Once we get all the Reduced and Native area at different time interval we can calculate the formation of native fraction of RNaseA by the following calculation.

**Table 1**

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Time(mins)</th>
<th>Native(N)</th>
<th>reduced®</th>
<th>N+R</th>
<th>N/N+R</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>M0</td>
<td>N1</td>
<td>R1</td>
<td>X1</td>
<td>Y1</td>
<td>Z1</td>
<td>F1</td>
</tr>
<tr>
<td>T1</td>
<td>M1</td>
<td>N2</td>
<td>R2</td>
<td>X2</td>
<td>Y2</td>
<td>Z2</td>
<td>F2</td>
</tr>
<tr>
<td>T2</td>
<td>M2</td>
<td>N3</td>
<td>R3</td>
<td>X3</td>
<td>Y3</td>
<td>Z3</td>
<td>F3</td>
</tr>
<tr>
<td>T3</td>
<td>M3</td>
<td>N4</td>
<td>R4</td>
<td>X4</td>
<td>Y4</td>
<td>Z4</td>
<td>F4</td>
</tr>
<tr>
<td>T4</td>
<td>M4</td>
<td>N5</td>
<td>R5</td>
<td>X5</td>
<td>Y5</td>
<td>Z5</td>
<td>F5</td>
</tr>
<tr>
<td>T5</td>
<td>M5</td>
<td>N6</td>
<td>R6</td>
<td>X6</td>
<td>Y6</td>
<td>Z6</td>
<td>F6</td>
</tr>
<tr>
<td>T6</td>
<td>M6</td>
<td>N7</td>
<td>R7</td>
<td>X7</td>
<td>Y7</td>
<td>Z7</td>
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<tr>
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<td>N8</td>
<td>R8</td>
<td>X8</td>
<td>Y8</td>
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</tr>
<tr>
<td>T8</td>
<td>M8</td>
<td>N9</td>
<td>R9</td>
<td>X9</td>
<td>Y9</td>
<td>Z9</td>
<td>F9</td>
</tr>
<tr>
<td>T9</td>
<td>M9</td>
<td>N10</td>
<td>R10</td>
<td>X10</td>
<td>Y10</td>
<td>Z10</td>
<td>F10</td>
</tr>
</tbody>
</table>

**Experiment 1:** first test tube contains RNaseA (Final conc. 30µM) and oxidizing buffer.
Table (2) below comprises all the data we have from HPLC.

**Table 2.**

<table>
<thead>
<tr>
<th>Time point</th>
<th>Time (Mins)</th>
<th>Native (N)</th>
<th>Reduced (R)</th>
<th>N+R</th>
<th>N/N+R (n)</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>40</td>
<td>1.7</td>
<td>105.3</td>
<td>107</td>
<td>0.0159</td>
<td>0.9841</td>
<td>-0.016</td>
</tr>
<tr>
<td>T2</td>
<td>60</td>
<td>29.23</td>
<td>382.44</td>
<td>411.67</td>
<td>0.071</td>
<td>0.929</td>
<td>-0.0736</td>
</tr>
<tr>
<td>T3</td>
<td>80</td>
<td>62.24</td>
<td>722.4</td>
<td>784.64</td>
<td>0.0793</td>
<td>0.9207</td>
<td>-0.0826</td>
</tr>
<tr>
<td>T4</td>
<td>100</td>
<td>81</td>
<td>908.47</td>
<td>989.47</td>
<td>0.082</td>
<td>0.918</td>
<td>-0.0856</td>
</tr>
<tr>
<td>T5</td>
<td>120</td>
<td>81.9</td>
<td>858</td>
<td>939.9</td>
<td>0.0871</td>
<td>0.913</td>
<td>-0.091</td>
</tr>
<tr>
<td>T6</td>
<td>140</td>
<td>104.8</td>
<td>881</td>
<td>985.8</td>
<td>0.1063</td>
<td>0.8937</td>
<td>-0.1124</td>
</tr>
<tr>
<td>T7</td>
<td>160</td>
<td>104.1</td>
<td>781</td>
<td>885.1</td>
<td>0.1176</td>
<td>0.8824</td>
<td>-0.1251</td>
</tr>
<tr>
<td>T8</td>
<td>180</td>
<td>110.2</td>
<td>781.34</td>
<td>891.6</td>
<td>0.1236</td>
<td>0.8764</td>
<td>-0.1319</td>
</tr>
<tr>
<td>T9</td>
<td>200</td>
<td>76.6</td>
<td>458.9</td>
<td>535.5</td>
<td>0.143</td>
<td>0.857</td>
<td>-0.1543</td>
</tr>
</tbody>
</table>

After we calculated native fraction (ln (1-n)) at different time interval we plotted the data in excel and we get a figure like below.

From **table 2** we get the following **figure**

![Figure 10](image)

**Fig. 10.** gives us different native fractions at different time points when oxidative folding of RNaseA is done with only oxidizing buffer. We added a trend line through the curve we get from different data point. It gives us a straight line equation (Y = mX + C), where m is the slope of the straight line.
Experiment 2: second test tube contains RNaseA (Final conc. 30µM) added with oxidizing buffer and PDI (Final conc. 6µM). Table (3) below comprises all the data we have from HPLC.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Time (Mins)</th>
<th>Native (N)</th>
<th>Reduced (R)</th>
<th>N+R</th>
<th>N/N+R (n)</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>40</td>
<td>29.7</td>
<td>339</td>
<td>368.7</td>
<td>0.0855</td>
<td>0.9145</td>
<td>-0.0894</td>
</tr>
<tr>
<td>T3</td>
<td>60</td>
<td>40.75</td>
<td>340</td>
<td>380.75</td>
<td>0.107</td>
<td>0.893</td>
<td>-0.1132</td>
</tr>
<tr>
<td>T4</td>
<td>80</td>
<td>65.3</td>
<td>292.5</td>
<td>357.8</td>
<td>0.1825</td>
<td>0.8175</td>
<td>-0.2015</td>
</tr>
<tr>
<td>T5</td>
<td>100</td>
<td>73.6</td>
<td>287.84</td>
<td>361.44</td>
<td>0.2036</td>
<td>0.7964</td>
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</tr>
<tr>
<td>T6</td>
<td>120</td>
<td>87.1</td>
<td>286.44</td>
<td>373.5</td>
<td>0.2331</td>
<td>0.7669</td>
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</tr>
<tr>
<td>T7</td>
<td>140</td>
<td>111.5</td>
<td>256.3</td>
<td>367.8</td>
<td>0.3031</td>
<td>0.6969</td>
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</tr>
<tr>
<td>T8</td>
<td>160</td>
<td>127.73</td>
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<td>415.29</td>
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<tr>
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<td>180</td>
<td>160.4</td>
<td>309.4</td>
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<tr>
<td>T10</td>
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<td>163.3</td>
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<td>0.6509</td>
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<tr>
<td>T11</td>
<td>220</td>
<td>149.5</td>
<td>286.1</td>
<td>335.6</td>
<td>0.4204</td>
<td>0.5796</td>
<td>-0.5454</td>
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</tbody>
</table>

From table 3 we get the following figure
Fig. 11. gives us different native fractions at different time points when oxidative folding of RNaseA is done with oxidizing buffer and wild type PDI (Final conc. 6µM). We added a trend line through the curve we get from different data point. It gives us a straight line equation ($Y = mx + C$), where $m$ is the slope of the straight line.

Experiment 3: Third test tube contains RNaseA (Final conc. 30µM) added with oxidizing buffer and PDIab (Final conc. 6µM). Table (4) below comprises all the data we have from HPLC.
From **table 4** we get following figure

![Graph](image)

**Fig.12.** gives us different native fractions at different time points when oxidative folding of RNaseA is done with oxidizing buffer and PDI ab (Final conc. 6µM). We added a trend line through the curve we get from different data point. It gives us a straight line equation \( Y = mX + C \), where \( m \) is the slope of the straight line.
Experiment 4: Last test tube contains RNaseA (Final conc. 30µM) added with oxidizing buffer and PDI a’b’ (Final conc. 6µM). Table (5) below comprises all the data we have from HPLC.

**Table 5**

<table>
<thead>
<tr>
<th>Time point</th>
<th>Time (Mins)</th>
<th>Native (N)</th>
<th>Reduced (R)</th>
<th>N+R</th>
<th>N/N+R</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T0</td>
<td>5</td>
<td>55.3</td>
<td>565.5</td>
<td>620.8</td>
<td>0.089</td>
<td>0.911</td>
<td>-0.093</td>
</tr>
<tr>
<td>T1</td>
<td>30</td>
<td>59.6</td>
<td>600.3</td>
<td>659.9</td>
<td>0.09</td>
<td>0.91</td>
<td>-0.094</td>
</tr>
<tr>
<td>T2</td>
<td>60</td>
<td>79</td>
<td>558.3</td>
<td>637.3</td>
<td>0.124</td>
<td>0.876</td>
<td>-0.132</td>
</tr>
<tr>
<td>T3</td>
<td>150</td>
<td>107.7</td>
<td>665.2</td>
<td>772.9</td>
<td>0.139</td>
<td>0.861</td>
<td>-0.15</td>
</tr>
<tr>
<td>T4</td>
<td>180</td>
<td>155.8</td>
<td>448.6</td>
<td>604.4</td>
<td>0.258</td>
<td>0.742</td>
<td>-0.298</td>
</tr>
<tr>
<td>T5</td>
<td>240</td>
<td>208.2</td>
<td>584.6</td>
<td>792.8</td>
<td>0.263</td>
<td>0.737</td>
<td>-0.305</td>
</tr>
<tr>
<td>T6</td>
<td>300</td>
<td>180</td>
<td>386.4</td>
<td>566.4</td>
<td>0.318</td>
<td>0.682</td>
<td>-0.383</td>
</tr>
<tr>
<td>T7</td>
<td>330</td>
<td>229.2</td>
<td>473.2</td>
<td>702.4</td>
<td>0.326</td>
<td>0.674</td>
<td>-0.395</td>
</tr>
<tr>
<td>T8</td>
<td>360</td>
<td>214.2</td>
<td>408.3</td>
<td>622.5</td>
<td>0.344</td>
<td>0.656</td>
<td>-0.422</td>
</tr>
<tr>
<td>T9</td>
<td>420</td>
<td>220.4</td>
<td>358.6</td>
<td>579</td>
<td>0.381</td>
<td>0.619</td>
<td>-0.48</td>
</tr>
</tbody>
</table>

From table 5 we get the following figure.
Fig. 13. gives us different native fractions at different time points when oxidative folding of RNaseA is done with oxidizing buffer and PDI a’b’ (Final conc. 6µM). We added a trend line through the curve we get from different data point. It gives us a straight line equation ($Y = mX + C$), where $m$ is the slope of the straight line.
**Fig. 14.** Folding rate from experiment 1, 2, 3, 4 together.

**Table 6.** Rate constant from experiment 1, 2, 3, and 4 together.

<table>
<thead>
<tr>
<th>Reaction For RNaseA</th>
<th>Rate constant (Mmin$^{-1}$)</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncatalyzed</td>
<td>$6.0 \times 10^{-4}$</td>
<td>-</td>
</tr>
<tr>
<td>Catalyzed with WT PDI</td>
<td>$23.0 \times 10^{-4}$</td>
<td>3.83</td>
</tr>
<tr>
<td>Catalyzed with PDI ab</td>
<td>$8.0 \times 10^{-4}$</td>
<td>1.33</td>
</tr>
<tr>
<td>Catalyzed with PDI a’b’</td>
<td>$12.0 \times 10^{-4}$</td>
<td>2.00</td>
</tr>
</tbody>
</table>


**Discussions:**

From fig 10, 11, 12, 13 we get protein folding curves which are been formed by different native fractions in respect to different time. Every curve has a straight line equation with a specific slope (m). This “m” indicates rate of protein folding. If we consider experiment 1 (RNaseA and Oxidizing buffer only) and experiment 2 (Wild type PDI is added to RNaseA and Oxidizing buffer mixture) we can see wild type PDI is accelerating oxidative folding of RNaseA to a significant amount. When we performed oxidative folding of RNaseA with oxidizing buffer only we get slope “m” of 0.0006, after adding wild type PDI in experiment 2 slope “m” is 0.0023. First experiment was carried out for 200 minutes where second one was for 220 minutes. When we added wild type PDI to RNaseA folding rate is much higher and gave high native fraction, that means, wild type PDI is typically active with its catalytic and isomerization. Wild type PDI is increasing the folding rate around 4 folds.

In our third experiment, we added PDI ab instead of wild type PDI and experiment was carried out for 420 minutes, here, RNaseA folding rate is 0.0008 and which is little faster than first experiment which was done without any chaperon protein. Again, this rate is very less than second experiment which is done with wild type PDI (slope was 0.0023). So, PDI ab domain makes the folding rate faster to some extent but it has very less activity than wild type PDI. PDI ab domain is increasing the folding rate around 1.33 folds.

In our fourth experiment, we added PDI a’b’ to RNaseA and oxidizing buffer mixture and the experiment was carried out for 420 minutes as well. Here, we get a slope of 0.001 which is little
higher than PDI ab, fraction of native RNaseA is little higher than fraction of native produced in experiment with PDI ab. But, the rate of folding is much less than wild type PDI, so as native protein fraction is also very less than it produced in case of wild type PDI. PDi a’b’ is increasing the folding rate around 2 folds.

From above results, we see that PDI is losing its typical activity when it is used as different single domain, like PDIab and PDIa’b’ and subsequently protein folding rate is also decreasing when PDI is used as different domain instead of multidomain PDI. But, still single domain of PDI (PDIab and PDIa’b’) has some activity which can accelerate the folding rate to a certain amount and production of native protein is relatively higher than it’s being done without single or multidomain PDI.

PDI consists of four tandem domains with thioredoxin structures, two with redox active sites (a and a’), and two with no redox activity (b and b’). To understand the relationship between individual domains and their contributions to the high isomerase activity displayed by full length of PDI, this experiment was done with various domains combinations constructed using a systemic genetic engineering approach. We isolated each protein in soluble and sufficiently pure form to characterize their activity. The activity of ab and a’b’ in RNaseA folding is very low. As, b and b’ has no redox activity the isomerase activity of ab and a’b’ is significantly lower than of full length PDI. Our data suggest that although, PDI ab and PD Ia’b’ getting lowered in it’s isomerase activity than full length PDI these two constructs accelerate RNaseA folding rate greater than RNaseA been folded without any PDI and PDI a’b’ accelerate RNaseA folding little higher than PDI ab.
Conclusion

From all above information we can conclude with following collusions:

Ability of different PDI domains to contribute to a significant isomerase activity is a function of context rather than the property of single domain.

PDIab and PDIa’b’ have much lower isomerase activity than multidomain PDI, so as full length PDI is required to accelerate thiols containing protein folding for its high isomerase activity.

PDIa’b’ is little more active than PDIab construct as it increases RNaseA folding rate in a little higher account.

pKa factor might be the reason for this type of result. Normally pKa value of wild type PDI is around 5.4 and our experiment is done in pH8. While pH is much greater than pKa thiols become active thiolate. But normal pKa of cysteine is around 8 which is very high. When we do the experiment with PDI domains the all thiol groups don’t turn in to active thiolate. This might results less activity of PDI domains in compare to wild type PDI.
Chapter 3

Targeting Nitrosative Stress Effects on an Er-Resident Oxidoreductase through Small-Molecule Ethnopharmaceuticals Intervention: Implications for Age- And Neurodegeneration-Related Disorders

Introduction

Protein quality control is a critical feature of intracellular homeostasis. In particular, unfolded or misfolded proteins resulting from environmental stresses or free radicals are rapidly degraded via the ubiquitin-proteasome pathway. Nitric oxide (NO), a free radical gas, has been reported to be involved in such processes as vasorelaxation and neurotransmission. Conversely, NO also is implicated in neuronal cell death or neurodegeneration. Recent reports suggest that S-nitrosylation of proteins is a significant cause of neural dysfunction leading to neurodegenerative disorders. Specifically, S-nitrosylation of Parkin eventually leads to the accumulation of unfolded proteins and subsequent neuronal death. The focus of this review is the identity of the target of NO. [61][62] Nitrosative stress prevents normal functioning of the endoplasmic reticulum (ER) via S-nitrosylation of protein-disulfide isomerase (PDI), which is located in the ER lumen. This may contribute to the accumulation of misfolded proteins, as well as sustained activation of the unfolded protein response (UPR) pathway. These phenomena may be linked to the development of sporadic neurodegenerative diseases. Over activation of N-methyl-D-aspartate (NMDA)-type glutamate receptors accounts, at least in part, for excitotoxicity neuronal damage, potentially contributing to a wide range of acute and chronic neurologic disorders. Recent studies have suggested that generation of excessive nitric oxide (NO) and reactive oxygen species (ROS) can mediate excitotoxicity, in part by triggering protein misfolding. S-Nitrosylation, which is a covalent reaction of a NO group with a cysteine thiol, represents one such mechanism that can contribute to NO-induced neurotoxicity. The ubiquitin-proteasome
system (UPS), in conjunction with molecular chaperones, can prevent accumulation of aberrantly-folded proteins. Chaperon PDI can provide neuroprotection from misfolded proteins or endoplasmic reticulum stress through its molecular chaperone and thiol-disulfide oxidoreductase activities. [63][64] Here, the authors present recent evidence suggesting that NO contributes to degenerative conditions by S-nitrosylating PDI (forming SNO-PDI) and the ubiquitin protein ligase, parkin (forming SNO-parkin). Moreover, it is demonstrated for the first time that inhibition of excessive NMDA receptor activity by memantine, via a mechanism of uncompetitive open-channel block with a relatively rapid off-rate, can ameliorate excessive production of NO, protein misfolding, and neurodegeneration. Misfolded proteins, and the associated endoplasmic reticulum (ER) stress, are emerging as hallmarks of age- and neurodegeneration-related disorders such as Huntington’s disease (HD), Alzheimer’s disease (AD), Parkinson’s disease (PD) and amyotrophic lateral sclerosis. Recent and compelling evidence has linked nitrosative stress and the ER-resident oxidoreductase, protein disulfide isomerase (PDI), to the pathogenesis of PD and AD. Overexpression of PDI has been found to reduce the formation of polyubiquitinated proteins, making the oxidoreductase an important target for therapeutic intervention in PD, AD and other age- and neurodegeneration-related disorders. We demonstrate the NO-scavenging ability of the biphenolic natural products curcumin and masoprocol and the concomitant prevention of S-nitrosylation of PDI by a model NO-donor. Furthermore, both ethnopharmaceuticals accelerate protein fold acquisition in their neat and nitrated forms, making them attractive candidates for prevention of age- and neurodegeneration-related diseases. [65][66][67][69]
Protein misfolding in neurodegenerative diseases

A shared histological feature of many neurodegenerative diseases is the accumulation of misfolded proteins that adversely affect neuronal connectivity and plasticity, and trigger cell death signaling pathways. For example, degenerating brain contains aberrant accumulations of misfolded, aggregated proteins, such as a-synuclein and synphilin-1 in PD, and amyloid-b (Ab) and tau in AD. The inclusions observed in PD are called Lewy bodies and are mostly found in the cytoplasm. AD brains show intracellular neurofibrillary tangles, which contain tau, and extracellular plaques, which contain Ab. Other diseases with inclusions include Huntington’s (polyQ), ALS, and prion disease. The above-mentioned aggregates consist of oligomeric complexes of non-native secondary structures, and demonstrate poor solubility in aqueous or detergent solvent. [70] It has been suggested that either genetic mutations or an increase in nitrosative/oxidative stress can facilitate protein aggregation. In general, protein aggregates do not accumulate in unstressed, healthy neurons due in part to the existence of cellular quality control machineries. For example, molecular chaperones are believed to provide a defense mechanism against the toxicity of misfolded proteins because chaperones can prevent inappropriate interactions within and between polypeptides, and can promote refolding of proteins that have been misfolded because of cell stress. In addition to the quality control of proteins provided by molecular chaperones, the ubiquitin-proteasome system (UPS) is involved in the clearance of abnormal or aberrant proteins. When chaperones cannot repair misfolded proteins, they may be tagged via addition of polyubiquitin chains for degradation by the proteasome. [71] In neurodegenerative conditions, intra- or extracellular protein aggregates are thought to accumulate in the brain as a result of a decrease in molecular chaperone or proteasome activities. In fact, several mutations that disturb the activity of molecular chaperones or UPS-
associated enzymes can cause neurodegeneration. Historically, lesions that contain aggregated proteins were considered to be pathogenic. Recently, several lines of evidence have suggested that aggregates are formed through a complex multi-step process by which misfolded proteins assemble into inclusion bodies; currently, soluble oligomers of these aberrant proteins are thought to be the most toxic forms via interference with normal cell activities, while frank aggregates may be an attempt by the cell to wall off potentially toxic material. [72][73]

**Protein S-nitrosylation and neuronal cell death**

Early investigations indicated that the NO group mediates cellular signaling pathways, which regulate broad aspects of brain function, including synaptic plasticity, normal development, and neuronal cell death. In general, NO exerts physiological and some pathophysiological effects via stimulation of guanylate cyclase to form cyclic guanosine-3', 5'-monophosphate (cGMP) or through S-nitros(y)lation of regulatory protein thiol groups. SNitrosylation is the covalent addition of anNOgroup to a critical cysteine thiol/sulfhydryl (RSH or, more properly, thiolate anion, RS−) to form an S-nitrosothiol derivative (R-SNO). Such modification modulates the function of a broad spectrum of mammalian, plant, and microbial proteins. In general, a consensus motif of amino acids comprised of nucleophilic residues (generally an acid and a base) surrounds a critical cysteine, which increases the cysteine sulfhydryl's susceptibility to S-nitrosylation. Our group first identified the physiological relevance of S-nitrosylation by showing that NO and related RNS exert paradoxical effects via redox-based mechanisms – NO is neuroprotective via S-nitrosylation of NMDA receptors (as well as other subsequently discovered targets, including caspases), and yet can also be neurodestructive by formation of
peroxynitrite (or, as later discovered, reaction with additional molecules such as MMP-9 and GAPDH). Over the past decade, accumulating evidence has suggested that S-nitrosylation can regulate the biological activity of a great variety of proteins, in some ways akin to phosphorylation. Chemically, NO is often a good “leaving group,” facilitating further oxidation of critical thiol to disulfide bonds among neighboring (vicinal) cysteine residues or, via reaction with ROS, to sulfenic (-SOH), sulfinic (-SO2H) or sulfonic (-SO3H) acid derivatization of the protein. Alternatively, S-nitrosylation may possibly produce a nitroxyl disulfide, in which the NO group is shared by close cysteine thiols. Analyses of mice deficient in either nNOS or iNOS confirmed that NO is an important mediator of cell injury and death after excitotoxic stimulation; NO generated from nNOS or iNOS is detrimental to neuronal survival. In addition, inhibition of NOS activity ameliorates the progression of disease pathology in animal models of PD, AD, and ALS, suggesting that excess generation of NO plays a pivotal role in the pathogenesis of several neurodegenerative diseases. Although the involvement of NO in neurodegeneration has been widely accepted, the chemical relationship between nitrosative stress and accumulation of misfolded proteins has remained obscure. Recent findings, however, have shed light on molecular events underlying this relationship. Specifically, we recently mounted physiological and chemical evidence that S-nitrosylation modulates the (i) ubiquitin E3 ligase activity of parkin, and (ii) chaperone and isomerase activities of PDI, contributing to protein misfolding and neurotoxicity in models of neurodegenerative disorders. Additionally, Cohen et al. Recently demonstrated that insulin/insulin-like growth factor-I (IGFI) signaling, which influences longevity and lifespan in many species in part via down-regulation of ROS/ RNS generation, can affect aggregation of toxic proteins such as Ab. This finding potentially provides an additional
link between ROS/RNS production during the normal aging process and protein aggregation in neurodegenerative conditions. [74][75][76]

**Unfolded protein response (UPR) and PDI**

The ER normally participates in protein processing and folding but undergoes a stress response when immature or misfolded proteins accumulate. ER stress stimulates two critical intracellular responses. The first represents expression of chaperones that prevent protein aggregation via the unfolded protein response (UPR), and is implicated in protein refolding, post-translational assembly of protein complexes, and protein degradation. This response is believed to contribute to adaptation during altered environmental conditions, promoting maintenance of cellular homeostasis. At least three ER transmembrane sensor proteins are involved in the UPR: pancreatic ER kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1). The activation of all three proximal sensors results in the attenuation of protein synthesis via eukaryotic initiation factor-2 (eIF2) kinase and increased protein folding capacity of the ER. The second ER stress response, termed ER-associated degradation (ERAD), specifically recognizes terminally misfolded proteins for retro-translocation across the ER membrane to the cytosol, where they can be degraded by the UPS. Additionally, although severe ER stress can induce apoptosis, the ER withstands relatively mild insults via expression of Cell. These proteins behave as molecular chaperones that assist in the maturation, transport, and folding of secretory proteins. [77][78] During protein folding in the ER, PDI catalyzes thiol/ disulfide exchange, thus facilitating disulfide bond formation, rearrangement reactions, and structural stability. PDI has four domains that are homologous to
thioredoxin (TRX) (termed a, b, b_, and a_). Two of the four TRX-like domains (a and a_) contain a characteristic redox-active CXXC motif, and these two-thiol/disulfide centers function as independent active sites. The recently determined structure of yeast PDI revealed that the four TRX-like domains form a twisted “U” shape with the two active sites facing each other on opposite sides of the “U”. Hydrophobic residues line the inside surface of the “U,” facilitating interactions between PDI and misfolded proteins. In many neurodegenerative disorders and cerebral ischemia, the accumulation of immature and denatured proteins results in ER dysfunction, but up-regulation of PDI represent an adaptive response promoting protein refolding and may offer neuronal cell protection. In a recent study, we reported that the S-nitrosylation of PDI (to form SNO-PDI) disrupts its neuroprotective role. [79][80]

S-Nitrosylation of PDI mediates protein misfolding and neurotoxicity in cell models of PD or AD

In contrast to the highly reducing environment of the cytosol and mitochondria, the ER manifests a relatively positive redox potential (e.g., the ratio of reduced: oxidized glutathione is approximately 3:1). [81]

This redox environment can contribute to the stability of protein S-nitrosylation or oxidation. Recently, we reported that excessive NO can lead to SNitrosylation of the active site thiol groups of PDI, and this reaction inhibits both its isomerase and chaperone activities. Mitochondrial complex I insult by rotenone can also result in S-nitrosylation of PDI in cell culture models. Moreover, we found that PDI is Snitrosylated in the brains of virtually all cases examined of sporadic AD and PD. Additionally, it is possible that vicinal (nearby) cysteine thiols reacting
with NO can form nitroxyldisulfide [82][83], and such reaction may potentially occur in the catalytic side of PDI to inhibit enzymatic activity. To determine the consequences of S-nitrosylated PDI (SNO-PDI) formation in neurons, we exposed cultured cerebrocortical neurons to neurotoxic concentrations of NMDA, thus inducing excessive Ca2+ influx and consequent NO production from nNOS. Under these conditions, we found that PDI was S-nitrosylated in a NOS dependent manner. SNO-PDI formation led to the accumulation of polyubiquitinated/misfolded proteins and activation of the UPR. Moreover, S-nitrosylation abrogated the inhibitory effect of PDI on aggregation of proteins observed in Lewy body inclusions. [84] S-Nitrosylation of PDI also prevented its attenuation of neuronal cell death triggered by ER stress, misfolded proteins, or proteasome inhibition. Further evidence suggested that SNO-PDI may in effect transport NO to the extracellular space, where it could conceivably exert additional adverse effects. In addition to PDI, S-nitrosylation is likely to affect critical thiol groups on other chaperones, such as HSP90 in the cytoplasm and possibly GRP in the ER. Normally, HSP90 stabilizes misfolded proteins and modulates the activity of cell signaling proteins including NOS and calreticulin. [85][86] In AD brains, levels of HSP90 are increased in both the cytosolic and membranous fractions, where HSP90 is thought to maintain tau and Ab in a soluble conformation, thereby averting their aggregation. (Martinez-Ruiz et al). Recently demonstrated that SNitrosylation of HSP90 can occur in endothelial cells, and this modification abolishes its ATPase activity, which is required for its function as a molecular chaperone. These studies imply that S-nitrosylation of HSP90 in neurons of AD brains may contribute to the accumulation of tau and Ab aggregates. The UPS is apparently impaired in the aging brain. Additionally, inclusion bodies similar to those found in neurodegenerative disorders can appear in brains of normal aged individuals or those with
subclinical manifestations of disease. These findings suggest that the activity of the UPS and molecular chaperones may decline in an age-dependent manner. Given that we have not found detectable quantities of SNO parkin and SNO-PDI in normal aged brain, we speculate that S-nitrosylation of these and similar proteins may represent a key event that contributes to susceptibility of the aging brain to neurodegenerative conditions. With bacitracin can increase aggregation of mtSOD1 in neuronal cells. Moreover, PDI co-localized and bound to intracellular aggregates of mtSOD1. [87][88] Upregulation of the UPR was also observed in mtSOD1 mice. These findings suggest that ER stress may contribute to the pathophysiology of familial ALS, and PDI could potentially reduce mtSOD1 aggregation and affect neuronal survival. Interestingly, S-nitrosothiol levels have also been found to be abnormal in the spinal cords of mtSOD1 transgenic mice. Whether SNO-PDI is involved in SOD1 aggregation and motor neuron injury in ALS remains to be studied. Finally, transmissible spongiform encephalopathy (TSE), also known as prion diseases, is transmissible neurodegenerative disorders and includes Creutzfeldt-Jacob disease, bovine spongiform encephalopathy, and scrapie. Cerebral accumulation of misfolded prion protein (PrP) and extensive neuronal apoptosis represent pathological hallmarks of these prion diseases. Recent reports have suggested that a prolonged UPR due to PrP misfolding in the ER may contribute to neuronal dysfunction. This ER stress response is mainly associated with up-regulation of Grp58, an ER chaperone with PDI-like activity, suggesting that this chaperone may represent a cellular response to prion infection. In fact, in vitro studies on Grp58, either over expressing (via transfection) or down-regulating (via RNAi), demonstrated that this ER chaperone protects cells against PrP misfolding and toxicity. Collectively, these studies raise the possibility that SNO-PDI and S-nitrosylation of other
Chaperone molecules may represent potential therapeutic targets to prevent protein aggregation in several neurodegenerative diseases. [89]

**Fig. 15.** Diagram shows how PDI is getting nitrosylated and producing ER stress which leads to cell death.

**Small molecule like masoprocol and Curcumin can scavenge the nitro group and protect PDI to be nitrated**

**Masoprocol**

Plants used to treat non-insulin-dependent diabetes mellitus (NIDDM) offer valuable leads for the development of pharmaceuticals (Luo et al., 1998; Oubre’ et al., 1997). Oral decoctions and extracts of the creosote bush *Larrea tridentata* have been used by the Pima Indians in the
Masoprocol, a phenolic (fig.16) pure compound from the creosote bush, was evaluated in mouse models of NIDDM for its ability to lower blood glucose concentration.

Masoprocol, also known as Nordihydroguaiaretic (NDGA), is an antineoplastic drug used to treat skin growths caused by sun exposure. A form of nordihydroguaiaretic acid that is taken by mouth is being studied in the treatment of prostate cancer. Nordihydroguaiaretic acid is an antioxidant, and it may block certain enzymes needed for tumor growth. Masoprocol also called nordihydroguaiaretic acid, NDGA, and actinex. Systematic (IUPAC) name 4-[4-(3,4-dihydroxyphenyl)-2,3-dimethyl-butyl]benzene-1,2-diol. [90]

Masoprocol is a naturally occurring lignan from the creosote bush (Larrea tridentata). Masoprocol has been utilized in traditional healing practices for a wide range of ailments and was licensed for use as a topical treatment for actinic keratosis (Actinex, Chemex Pharmaceuticals, Denver, CO). Use of masoprocol for therapeutic purposes is currently limited due to reports of contact hypersensitivity, nephrotoxicity, hepatotoxicity, and cytotoxicity. It has been suggested that the toxicity is the result of oxidation to a reactive ortho-quinone species, the presence of which we recently confirmed via trapping of NDGA ortho-quinones as glutathione (GSH) adducts. [91]

Fig.16. Structure of masoprocol.
Curcumin

Turmeric (fig.17, 18) is a spice derived from the rhizomes of Curcuma longa, which is a member of the ginger family (Zingiberaceae). Rhizomes are horizontal underground stems that send out shoots as well as roots. The bright yellow color of turmeric comes mainly from polyphenolic pigments, known as curcuminoids. Curcumin (fig19) is the principal curcuminoid found in turmeric, and is generally considered its most active constituent. Other curcuminoids found in turmeric include demethoxycurcumin and bisdemethoxycurcumin. In addition to its use as a spice and a pigment, turmeric has been used in India for medicinal purposes for centuries. More recently, evidence that curcumin may have anti-inflammatory and anticancer activities has renewed scientific interest in its potential to prevent and treat disease. [92]

Fig.17, and Fig.18. Turmeric powder. Fig.19. Structure of Curcumin.
**Metabolism and Bioavailability**

Clinical trials in humans indicate that the systemic bioavailability of orally administered curcumin is relatively low. Curcumin is readily conjugated in the intestine and liver to form curcumin glucuronides and curcumin sulfates or reduced to hexahydrocurcumin. Curcumin metabolites may not have the same biological activity as the parent compound. [108] In one study, conjugated or reduced metabolites of curcumin were less effective inhibitors of inflammatory enzyme expression in cultured human colon cells than curcumin itself. In a clinical trial conducted in Taiwan, serum curcumin concentrations peaked 1-2 hours after an oral dose, and peak serum concentrations were 0.5, 0.6 and 1.8 micromoles/liter at doses of 4, 6 and 8 g/day, respectively. Curcumin could not be detected in serum at lower doses than 4 g/day. More recently, a clinical trial conducted in the UK, found that plasma curcumin, curcumin sulfate and curcumin glucuronide concentrations were in the range of 10 nanomoles/liter (0.01 micromole/liter) one hour after a 3.6 g dose of oral curcumin. Curcumin and its metabolites could not be detected in plasma at lower doses than 3.6 g/day. Curcumin and its glucuronidated and sulfated metabolites were also measured in urine at a dose of 3.6 g/day. There is some evidence that orally administered curcumin accumulates in gastrointestinal tissues. When colorectal cancer patients took 3.6 g/d of curcumin orally for 7 days prior to surgery, curcumin was detected in malignant and normal colorectal tissue. In contrast, curcumin was not detected in the liver tissue of patients with liver metastases of colorectal cancer after the same dose of oral curcumin, suggesting that oral curcumin administration may not effectively deliver curcumin to tissues outside the gastrointestinal tract. [93]
**Biological Activities**

Antioxidant activity

Anti-inflammatory activity

Glutathione synthesis

Effects on biotransformation enzymes involved in carcinogen metabolism

Induction of cell cycle arrest and apoptosis

Inhibition of tumor invasion and angiogenesis. [94]

Our first hypothesis was that masoprocol and curcumin are the two substances which can scavenge nitro group by getting ring nitration or aliphatic chain nitration. Our final aim is to block the nitro group by small molecule to prevent the thiol groups of PDI to be nitrated. When thiols are nitrated by nitro group PDI loses its catalytic activity and subsequently substrate proteins are misfolded in the Endoplasmic reticulum and bring ER stress which ultimately leads inclusion body formation. To retain the catalytic activity of PDI we have to protect the thiol groups of PDI from getting nitrated by nitro group. In vitro we have used two small molecules, like masoprocol and curcumin to protect PDI from nitro group. We added Tetra nitro methane (TNM), a nitro group donor, with these small molecules and by observing the absorbance in spectrometry it’s been confirmed that these two small molecules are getting nitrated.

We use UV spectrometry to prove that masoprocol is getting nitrated in the presence of nitro group (NO)
**Fig. 20.** shows absorbance of masoprocol is 0.9 at 280 nm wavelength.

**Fig. 21.** shows absorbance of TNM (nitro group donor) is 0.04 at 353 nm wavelength.
Fig. 22. shows absorbance of masoprococol after adding nitro group donor is at 280 and 353 nm wavelength and at 353 nm absorbance is 1.2

Fig. 23

NB: “Series 2” indicates the absorbance of masoprococol
    “Series 3” indicates the absorbance of TNM
    “All together” curve indicates the absorbance when masoprococol is added to TNM

In Fig. 23 three curves are plotted together, and we can see that TNM has some absorbance at 353 nm wavelength where as masoprococol only has no absorbance at 353 nm wavelength, but
masoprocol itself has absorbance at 280 nm wave length. Again when TNM is added with masoprocol we get two absorbance peak at 280 and 353 nm wavelengths and absorbance at 353 nm wavelength increases almost 30 times which clearly shows that masoprocol is getting nitrated which resulting a huge increase in the absorbance at 353 nm wavelength.

The results from above four figures prove that masoprocol is getting nitrated in the presence of nitro group (NO).

We used mass spec to prove that curcumin is getting nitrated in the presence of nitro group (NO).

**Figure** below is the mass spec result of curcumin with nitro group.

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**Fig. 24.** Mass spec. data shows phytochemicals are getting nitrated
Above mass spec data interprets that mass of curcumin is certainly increasing when TNM is added with curcumin which proves that curcumin is getting nitrated in the presence of nitro group (NO).

So, now we came to know that curcumin and masoprocol both phenolic compounds are getting nitrated in the presence of nitro group (NO). For our future research we will use masoprocol only as a nitro group scavenger.

Now, we added the small molecules to the chaperon protein PDI and have done oxidative protein folding of RNaseA to see if the small molecule can get nitrat ed faster than PDI and scavenges the nitro group to protect thiols group of PDI from getting nitrat ed. Our substrate protein is RnaseA which has four di- sulfide bonds (8 free thiols). So, this is one of the most convenient proteins for oxidative protein folding experiment.

**MATERIALS AND METHODS**

*Materials:* RNase A was purchased from Sigma and was purified properly (Rothwarf D.M., and Scheraga H.A., 1998 et al.,)

Tetranitromethane (TNM) was purchased from sigma.

Masoprocol was brought from a medicine company at El Paso.

Protein Di-sulfide Isomerase (PDI) plasmid was brought from Sweden and was expressed in E.Coli (BL21 strain).
**Methods:**

**PDI expression.** 100 µl aliquot BL21(DE3) competent cell was taken out from -80°C, and was Thawed on ice bucket for 10 min. WT PDI plasmid was taken out from -20°C and was thawed too. 1 µl of the plasmid was added into 100 µl BL21 cell tube, and was kept on ice for 30 min.

# the amount of plasmid may vary depending on the concentration of plasmid and the competitiveness of BL21.

Heat shock was given for 60 sec. at 42°C. (Isotemp should be pre-equilibrated). Then the cell was Put it back on ice for 2 min. (meanwhile, prepare 1 ml LB in a 15 ml Falcon tube). 100 µl transformed BL21 was transferred to the 1 ml LB, and kept in 37°C incubator with shaking at 150 rpm for 45 min. (meanwhile, LB-Amp plate was taken out from 4°C and equilibrated to room temp.) 100 µl of the LB (45 min incubated 1 ml LB) was spread onto the LB-Amp plate. The LB/AMP plate was Incubated at 37°C for 13 hrs. (It may vary ± 2hrs). After overnight incubation, next day, a single colony was picked up into 50ml LB and add 0.1 ml of Ampicillin (Stock concentration is 50 mg/ml and final con in 50 ml LB is 0.1 mg/ml) in 250 ml Erlenmeyer flask, and incubate at 37°C with shaking at 150 rpm until OD$_{600}$ reaches 0.5. Starter culture O.D should not be allowed to exceed 0.5.

- It will take ≈ 4hr. Check OD$_{600}$ after 3 hr.

- OD doubles, generally in 20 min.

25 ml of the starter culture was incubated into 1 L LB and add 2 ml of ampicillin stock was added, and Incubated until OD$_{600}$ reaches 0.55-0.6.

- It will take ≈ 3-4 hrs.
When the OD$_{600}$ reaches 0.55-0.6, 1 ml of IPTG (0.5 M) was added, and it was shaken 4 more hours. Then, cells were harvested by centrifuging at 5,000 rpm for 10 min at 4°C. Supernatant was discarded and resuspended with lysis buffer (No more than 20 ml).

Lysis buffer: 300 mM NaCl, 50 mM PO$_4$, and 10 mM Imidazole, pH 7.5

Resuspended cells were placed in 50 ml Falcon Tube and were sonicated. The condition must be set up with each machine.

# Four times 30-sec-sonications and 1 min cooling between each sonication.

# Sonication must be done in Ice-Bucket.

Then the cells were centrifuged at 11,000 rpm for 40 min at 4°C. Supernatant was taken and the pellet was discarded. The supernatant was poured onto Ni-NTA resin in column and purified.

![Fig.25. Wild type PDI in SDS gel. L- ladder, 1-First wash (300 mM NaCl, 50 mM PO$_4$, and 50 mM Imidazole, pH 7.5), 2- Second wash (300 mM NaCl, 50 mM PO$_4$, and 100 mM Imidazole, pH 7.5), and 3- Third wash (300 mM NaCl, 50 mM PO$_4$, and 300 mM Imidazole, pH 7.5). PDI protein came out as 55 KD molecular weight](image)
**Fig. 26.** Experion gel picture for wild type PDI shows molecular weight of wild type PDI is 55KD

**Fig. 27.** SDS gel pic which shows RNaseA with its 13.4KD molecular weight
**Preparation of Fully Reduced RNase A.** Purified native RNase A was reduced by incubating the protein in a buffer containing 100 mM DTTred and 6 M GnHCl (100 mM Tris-HCl, 1 mM EDTA, pH 8.3, 25 ºC). After incubation for 3h, the pH of the solution was lowered to 3 by addition of glacial acetic acid. The reduced protein was desalted (and DTTred was removed) on a reversed-phase HPLC column (C-18) and lyophilized to remove the organic solvent and water. Lyophilized reduced protein was dissolved in a dilute acetic acid solution (1µl of glacial acetic acid with 100 ml of mili Q, pH 3.7) to obtain a stock solution (5.0 mg/mL) and was stored at -20 ºC.

**Oxidative Folding of RNase A.** Oxidative folding of RNaseA was carried out at 25 ºC in four different test tubes with different reagents by incubating 0.5 mg/mL (33 μM) reduced protein in a oxidizing buffer of 100 mM DTTox (pH 8.3, 100 mM Tris-HCl, 1 mM EDTA).

Number one test tube contains RNaseA (Final conc. 30μM) and oxidizing buffer. In second test tube RNaseA (Final conc 30μM) added with oxidizing buffer and PDI (Final conc. 6μM).In the next one we added RNaseA (Final conc.30μM), oxidizing buffer, PDI (Final conc. 6μM) and Tetranitromethane (TNM, Final conc.360μM). In the last test tube we added RNaseA (Final conc. 30μM), oxidizing buffer, PDI (Final conc. 6μM), masoprocol (Final conc. 720μM) and Tetranitromethane (Final conc. 720μM). No DTTred was present in the original oxidation buffer, but DTTred accumulated during the oxidation reaction. Aliquots of 200 μL were withdrawn at different times after the initiation of oxidative folding, and any free thiols were blocked by an excess amount of AEMTS (final concentration of 50 mM, pH 8.3, 100 mM Tris-HCl and 1 mM EDTA). Five minutes after the addition of the AEMTS, the pH of the solution was lowered to 3.
by addition of 20 µL of glacial acetic acid. All samples were desalted on a Hi-Trap G25 column by exchange against a 0.2% acetic acid solution before analysis using cation-exchange HPLC. Prior to the addition of AEMTS which blocks any free thiols, some samples were subjected to a reduction pulse (5 mM DTTred at pH 8.3 for 2 min) which results in the reduction of all unstructured species to the fully reduced protein (R), a procedure that has been used successfully to identify and isolate structured species in the oxidative folding of RNaseA. [95]

Results and Discussion

Results

Regeneration of RNase A. Fig.28 shows a typical HPLC chromatogram 60 min after initiation of oxidative folding (100 mM DTTox, pH 8.3, 25 °C), and blocked with AEMTS. The native protein (N), the 3S, 2S, and 1S ensembles, and the fully reduced protein (R) are clearly visible.

Fig.28. Regeneration of RNaseA in HPLC

The table (7) below is the typical calculation formula for calculation of fraction of reduced and native protein. Results from these experiments showed that the rate of formation of the native
protein (N) which is indicated by \( \text{Ln} (1-n) \) where \((1 – n)\) is the fractional concentration of non-native RNaseA. Ti, T2 and so on are the different time points where 200\( \mu \)l of aliquots were withdrawn. Times are calculated in minutes and each aliquot was withdrawn at 20 minutes interval. All four experiments were started with fully reduced (R) RNaseA, as the time increases RNaseA gradually become folded and as a result native (N) fraction of RNaseA increases with time. In HPLC chromatogram two peaks are visible, one for reduced protein and another for native protein. As the protein enrich by native fraction the hydrophobic part goes in the core of the protein, unlikely, in the reduced protein hydrophobic portion will be exposed outside of the amino acid chain, which leads the native peak to come before the reduced one because our HPLC column has long hydrocarbon (C18) chain which is very strong hydrophobic. As the reduced protein has exposed hydrophobic part it binds strongly with the column comparing to the native protein and stick to the column for a longer time than the native protein and as a consequence the reduced protein comes after the native one. With the help of area calculator, we can calculate the area of reduced (R) RNaseA and the area of native (N) RNaseA. Once we get all the Reduced and Native area at different time interval we can calculate the formation of native fraction of RNaseA by the following calculation.
### Table 7.

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Time (mins)</th>
<th>Native (N)</th>
<th>Reduced® (R)</th>
<th>N+R</th>
<th>N/N+R</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
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<td>T1</td>
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<td>N2</td>
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<td>N3</td>
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<td>X3</td>
<td>Y3</td>
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<td>R4</td>
<td>X4</td>
<td>Y4</td>
<td>Z4</td>
<td>F4</td>
</tr>
<tr>
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<td>120</td>
<td>N5</td>
<td>R5</td>
<td>X5</td>
<td>Y5</td>
<td>Z5</td>
<td>F5</td>
</tr>
<tr>
<td>T6</td>
<td>140</td>
<td>N6</td>
<td>R6</td>
<td>X6</td>
<td>Y6</td>
<td>Z6</td>
<td>F6</td>
</tr>
<tr>
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<td>N7</td>
<td>R7</td>
<td>X7</td>
<td>Y7</td>
<td>Z7</td>
<td>F7</td>
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<td>N8</td>
<td>R8</td>
<td>X8</td>
<td>Y8</td>
<td>Z8</td>
<td>F8</td>
</tr>
<tr>
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<td>200</td>
<td>N9</td>
<td>R9</td>
<td>X9</td>
<td>Y9</td>
<td>Z9</td>
<td>F9</td>
</tr>
<tr>
<td>T10</td>
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<td>N10</td>
<td>R10</td>
<td>X10</td>
<td>Y10</td>
<td>Z10</td>
<td>F10</td>
</tr>
<tr>
<td>T11</td>
<td>240</td>
<td>N11</td>
<td>R11</td>
<td>X11</td>
<td>Y11</td>
<td>Z11</td>
<td>F11</td>
</tr>
</tbody>
</table>

### Experiment 1: first test tube contains RNaseA (Final conc. 30µM) and oxidizing buffer.

Table (8) below comprises all the data we have from HPLC.

### Table 8.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Time (Mins)</th>
<th>Native (N)</th>
<th>Reduced (R)</th>
<th>N+R</th>
<th>N/N+R (n)</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>40</td>
<td>1.7</td>
<td>105.3</td>
<td>107</td>
<td>0.0159</td>
<td>0.9841</td>
<td>-0.016</td>
</tr>
<tr>
<td>T2</td>
<td>60</td>
<td>29.23</td>
<td>382.44</td>
<td>411.67</td>
<td>0.071</td>
<td>0.929</td>
<td>-0.0736</td>
</tr>
<tr>
<td>T3</td>
<td>80</td>
<td>62.24</td>
<td>722.4</td>
<td>784.64</td>
<td>0.0793</td>
<td>0.9207</td>
<td>-0.0826</td>
</tr>
<tr>
<td>T4</td>
<td>100</td>
<td>81</td>
<td>908.47</td>
<td>989.47</td>
<td>0.082</td>
<td>0.918</td>
<td>-0.0856</td>
</tr>
<tr>
<td>T5</td>
<td>120</td>
<td>81.9</td>
<td>858</td>
<td>939.9</td>
<td>0.0871</td>
<td>0.913</td>
<td>-0.091</td>
</tr>
<tr>
<td>T6</td>
<td>140</td>
<td>104.8</td>
<td>881</td>
<td>985.8</td>
<td>0.1063</td>
<td>0.8937</td>
<td>-0.1124</td>
</tr>
<tr>
<td>T7</td>
<td>160</td>
<td>104.1</td>
<td>781</td>
<td>885.1</td>
<td>0.1176</td>
<td>0.8824</td>
<td>-0.1251</td>
</tr>
<tr>
<td>T8</td>
<td>180</td>
<td>110.2</td>
<td>781.34</td>
<td>891.6</td>
<td>0.1236</td>
<td>0.8764</td>
<td>-0.1319</td>
</tr>
<tr>
<td>T9</td>
<td>200</td>
<td>76.6</td>
<td>458.9</td>
<td>535.5</td>
<td>0.143</td>
<td>0.857</td>
<td>-0.1543</td>
</tr>
</tbody>
</table>
After we calculated native fraction (ln (1-n)) at different time interval we plotted the data in excel and we get a figure like below.

![Figure 29](image)

**Fig.29.** gives us different native fractions at different time points when oxidative folding of RNaseA is done with only oxidizing buffer. We added a trend line through the curve we get from different data point. It gives us a straight line equation (Y = mX + C), where m is the slope of the straight line.
Experiment 2: second test tube contains RNaseA (Final conc 30µM) added with oxidizing buffer and PDI (Final conc. 6µM). Table (9) below comprises all the data we have from HPLC.

**Table 9**

<table>
<thead>
<tr>
<th>Time point</th>
<th>Time (Mins)</th>
<th>Native (N)</th>
<th>Reduced (R)</th>
<th>N+R</th>
<th>N/N+R (n)</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>40</td>
<td>29.7</td>
<td>339</td>
<td>368.7</td>
<td>0.0855</td>
<td>0.9145</td>
<td>-0.0894</td>
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<tr>
<td>T3</td>
<td>60</td>
<td>40.75</td>
<td>340</td>
<td>380.75</td>
<td>0.107</td>
<td>0.893</td>
<td>-0.1132</td>
</tr>
<tr>
<td>T4</td>
<td>80</td>
<td>65.3</td>
<td>292.5</td>
<td>357.8</td>
<td>0.1825</td>
<td>0.8175</td>
<td>-0.2015</td>
</tr>
<tr>
<td>T5</td>
<td>100</td>
<td>73.6</td>
<td>287.84</td>
<td>361.44</td>
<td>0.2036</td>
<td>0.7964</td>
<td>-0.2277</td>
</tr>
<tr>
<td>T6</td>
<td>120</td>
<td>87.1</td>
<td>286.44</td>
<td>373.5</td>
<td>0.2331</td>
<td>0.7669</td>
<td>-0.2714</td>
</tr>
<tr>
<td>T7</td>
<td>140</td>
<td>111.5</td>
<td>256.3</td>
<td>367.8</td>
<td>0.3031</td>
<td>0.6969</td>
<td>-0.3611</td>
</tr>
<tr>
<td>T8</td>
<td>160</td>
<td>127.73</td>
<td>287.56</td>
<td>415.29</td>
<td>0.3075</td>
<td>0.6925</td>
<td>-0.3674</td>
</tr>
<tr>
<td>T9</td>
<td>180</td>
<td>160.4</td>
<td>309.4</td>
<td>469.8</td>
<td>0.3414</td>
<td>0.6586</td>
<td>-0.4176</td>
</tr>
<tr>
<td>T10</td>
<td>200</td>
<td>163.3</td>
<td>304.67</td>
<td>467.97</td>
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<td>0.6509</td>
<td>-0.4294</td>
</tr>
<tr>
<td>T11</td>
<td>220</td>
<td>149.5</td>
<td>286.1</td>
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<td>0.4204</td>
<td>0.5796</td>
<td>-0.5454</td>
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</tbody>
</table>

From table 9 we get the following figure

![Graph](image-url)
Fig.30. gives us different native fractions at different time points when oxidative folding of RNaseA is done with oxidizing buffer and PDI (Final conc. 6µM). We added a trend line through the curve we get from different data point. It gives us a straight line equation (Y= mX + C), where m is the slope of the straight line.

Experiment 3: Third test tube contains RNaseA (Final conc.30µM), oxidizing buffer, PDI (Final conc. 6µM) and Tetranitromethane (TNM, Final conc.360µM). Table (10) below comprises all the data we have from HPLC.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Time (Mins)</th>
<th>Native (N)</th>
<th>Reduced (R)</th>
<th>N+R</th>
<th>N/N+R (n)</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>40</td>
<td>13.7</td>
<td>728.3</td>
<td>742</td>
<td>0.0185</td>
<td>0.9815</td>
<td>-0.0187</td>
</tr>
<tr>
<td>T3</td>
<td>60</td>
<td>24.7</td>
<td>829.1</td>
<td>853.8</td>
<td>0.0289</td>
<td>0.9711</td>
<td>-0.0293</td>
</tr>
<tr>
<td>T4</td>
<td>80</td>
<td>21</td>
<td>550</td>
<td>571</td>
<td>0.0368</td>
<td>0.9632</td>
<td>-0.0375</td>
</tr>
<tr>
<td>T5</td>
<td>100</td>
<td>30</td>
<td>544</td>
<td>574</td>
<td>0.0523</td>
<td>0.9477</td>
<td>-0.0537</td>
</tr>
<tr>
<td>T6</td>
<td>120</td>
<td>32.9</td>
<td>589</td>
<td>621.9</td>
<td>0.053</td>
<td>0.947</td>
<td>-0.0544</td>
</tr>
<tr>
<td>T7</td>
<td>140</td>
<td>41.3</td>
<td>680</td>
<td>721.3</td>
<td>0.0573</td>
<td>0.9427</td>
<td>-0.059</td>
</tr>
<tr>
<td>T8</td>
<td>160</td>
<td>28.3</td>
<td>461.9</td>
<td>490.2</td>
<td>0.0577</td>
<td>0.9423</td>
<td>-0.0594</td>
</tr>
<tr>
<td>T9</td>
<td>180</td>
<td>38.3</td>
<td>540.1</td>
<td>578.4</td>
<td>0.0662</td>
<td>0.9338</td>
<td>-0.0685</td>
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<tr>
<td>T11</td>
<td>220</td>
<td>10.6</td>
<td>146.1</td>
<td>156.7</td>
<td>0.0676</td>
<td>0.9324</td>
<td>-0.0699</td>
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</table>

From table10 we get the following figure
*Fig. 31.* gives us different native fractions at different time points when oxidative folding of RNaseA is done with oxidizing buffer, PDI (Final conc. 6µM) and Tetranitromethane (TNM, Final conc. 360µM). We added a trend line through the curve we get from different data point. It gives us a straight line equation ($Y = mX + C$), where $m$ is the slope of the straight line.

**Experiment 4:** Final test tube contains RNaseA (Final conc. 30µM), oxidizing buffer, PDI (Final conc. 6µM), masoprocol (Final conc. 720µM) and Tetranitromethane (Final conc. 720µM). Table (11) below comprises all the data we have from HPLC.
Table 11. 

<table>
<thead>
<tr>
<th>Time point</th>
<th>Time (Mins)</th>
<th>Native (N)</th>
<th>Reduced (R)</th>
<th>N+R</th>
<th>N/N+R (n)</th>
<th>(1-n)</th>
<th>Ln(1-n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
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<td>6.74</td>
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<td>114.74</td>
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<td>0.9413</td>
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<tr>
<td>T3</td>
<td>60</td>
<td>16.7</td>
<td>126</td>
<td>142.7</td>
<td>0.117</td>
<td>0.883</td>
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<tr>
<td>T4</td>
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<td>20.6</td>
<td>127.9</td>
<td>148.5</td>
<td>0.1387</td>
<td>0.8613</td>
<td>-0.1493</td>
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<tr>
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<td>129.8</td>
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<td>91.6</td>
<td>115.57</td>
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<td>0.7926</td>
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<tr>
<td>T7</td>
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<td>27.97</td>
<td>90.7</td>
<td>119.67</td>
<td>0.2337</td>
<td>0.7663</td>
<td>-0.2662</td>
</tr>
<tr>
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<td>160</td>
<td>25.4</td>
<td>67.58</td>
<td>92.98</td>
<td>0.2732</td>
<td>0.7268</td>
<td>-0.319</td>
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<tr>
<td>T9</td>
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<td>25.9</td>
<td>65.5</td>
<td>91.5</td>
<td>0.2831</td>
<td>0.7169</td>
<td>-0.3328</td>
</tr>
<tr>
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<td>200</td>
<td>27.7</td>
<td>60.5</td>
<td>88.2</td>
<td>0.3141</td>
<td>0.6859</td>
<td>-0.377</td>
</tr>
<tr>
<td>T11</td>
<td>220</td>
<td>36.4</td>
<td>62.1</td>
<td>98.5</td>
<td>0.3695</td>
<td>0.6305</td>
<td>-0.4612</td>
</tr>
</tbody>
</table>

From table 5 we get the following figure

Fig. 32. gives us different native fractions at different time points when oxidative folding of RNaseA is done with oxidizing buffer, PDI (Final conc. 6µM), masoprocol (Final conc. 720µM) and Tetenitromethane (TNM, Final conc.360µM). We added a trend line through the curve we get from different data point. It gives us a straight line equation (\( Y = mX + C \)), where \( m \) is the slope of the straight line.
**Fig. 33.** Folding rate from all above 4 experiments together

**Table 12.** Rate constant from above 4 experiment together

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate constant (Mmin)^{-1}</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uncatalyzed</td>
<td>6.0 × 10^{-4}</td>
<td>1</td>
</tr>
<tr>
<td>Catalyzed with WT PDI</td>
<td>23.0 × 10^{-4}</td>
<td>3.83</td>
</tr>
<tr>
<td>Catalyzed with WT PDI in the presence of TNM</td>
<td>4.0 × 10^{-4}</td>
<td>0.67</td>
</tr>
<tr>
<td>Catalyzed with WT PDI in the presence of masoprocol and TNM</td>
<td>22.0 × 10^{-4}</td>
<td>3.67</td>
</tr>
</tbody>
</table>
Discussion

In all above graphs we get protein folding curves which are been formed by different native fractions in respect to different time. Every curve has a straight line equation with a specific slope (m). This “m” indicates rate of protein folding. Slope for all 4 figures are 0.0006, 0.0023, 0.0004 and 0.0022 respectively. Number 28 and number 30 figures show very low and mostly close “m” values where as, number 29 and number 31 figures show very high and very close “m” values to each other as well. Figure number 28 produced from those data which we get when oxidative folding of RNaseA performed with oxidizing buffer only. In absence of chaperone protein PDI, RNaseA folding rate is very low. Figure number 29 is generated from those data which we obtained from folding of RNaseA with oxidizing buffer and chaperone protein PDI. The slope “m” is almost 4 times greater than the slope of number 28 figure, which interprets effects of chaperone protein PDI on RNaseA folding; PDI increases the folding rate at significant ratio. Slope of figure 30 is again very low and very close to figure 28. Figure 30 is derived from data generated from RNaseA folding with oxidizing buffer, PDI and Tetranitromethane (TNM), the NO donor. That means acceleration of protein folding by chaperone protein PDI disappeared and folding likely depend on oxidizing buffer only rather PDI. Most of PDI activities are blocked by nitro group donated by TNM. Thiol groups (SH-) are blocked by NO- and become SNO-, this process is known as S-nitrosylation of PDI where PDI lose its catalytic and chaperon activity resulting increased amount of misfolded protein in cell. These misfolded proteins aggregated in ER and generates ER stress as a result neuronal cells are damaged which leads to neurodegenerative diseases like Parkinson’s, Alzheimer’s, etc.
Slope of figure number 31 is again very high and very close to figure number 29 which typically shows regeneration of protein folding which was lost when PDI was blocked by nitro group of TNM. After adding masoprolcol with PDI protein folding rate is recovered which means that masoprolcol can scavenge nitro group from TNM instead of PDI get nitrated by nitro group. This result also support the fact that masoprolcol has much greater affinity towards nitro group than PDI. By having better competency masoprolcol can scavenge the nitro group to prevent PDI from S-nitrosylation. Thus PDI still remains with its typical catalytic and chaperone activity and accelerate protein folding to a certain rate which increases the fraction of native protein in short period of time. By accelerating protein folding PDI prevents protein misfolding which means no aggregation of misfolded protein, no inclusion or Lewy’s Body formation. As there is no inclusion body, no ER stress which leads no neuronal cell death.

![Graph showing protein folding rate](image)

**Fig.34.** shows that when PDI is added to substrate protein the rate of folding is increasing a certain amount and when TNM (nitro group donor) is added to PDI, PDI is getting nitrated and there is SNO-PDI formation which blocks PDI’s catalytic activity and certainly the rate of folding is decreasing a certain amount. Again, when we are adding TNM with a mixture of small
molecule and PDI the folding rate is increasing a certain amount as the folding rate of substrate protein with PDI. That means, PDI activity is expressed here. The nitro groups are scavenged by small molecule and PDI is not getting blocked by nitro group.

**Conclusion**

These studies concerning the influence of nitro group on S-nitrosylation of PDI and influence of masoprocrol on protection of PDI from S-nitrosylation. From all the above mentioned theoretical information and lab experimental data we can conclude with two very important facts which are very important in neurodegenerative diseases studies and for their prophylactic measurement. As our first conclusion, UV spectrometer investigations suggest that masoprocrol gets nitrated in the presence of nitro group and mass spec investigations support the fact that curcumin gets nitrated in the presence of nitro group. Second conclusion is even much more imperative in neurodegenerative diseases studies. Oxidative folding experiments of RNaseA suggest the underlying cause of S-nitrosylation of PDI and protection of S-nitrosylation by masoprocrol as well. Preventing S-nitrosylation of PDI by getting nitrated small molecules like, masoprocrol plays a crucial role in the circuit of neurodegenerative diseases.
Chapter 4

Curcumin has a novel prophylactic action to protect protein from hydroxyl stress

Introduction

Curcumin, the principle curcuminoids found in turmeric, and is generally considered its most active constituent. We already discussed about the background information of curcumin and its property of getting nitrated by free nitro group. Here, we tried a different experiment to emphasize on curcumin’s hydroxyl group scavenging property. The protective effects of curcumin against oxidative protein damage to free thiol containing protein, such as, β-lactoglobulin, treated with fenton reaction (hydroperoxide (H2O2) reacts with FeSO4 to produce free hydroxyl group) is studied in vitro. [96] We also investigated the effects of curcumin on the level of total free thiol groups and low-molecular-weight thiols (glutathione and homocysteine) in β-lactoglobulin treated with free hydroxyl (OH) group. The defense mechanisms against oxidative stress (free hydroxyl radical action) are very important for biological activities of cell. Moreover, the role of exogenous antioxidants in the defense against oxidative stress in cell is still unknown. Therefore, the protective effects of curcumin against the oxidative damage of free thiol containing proteins and low molecular-weight thiols (glutathione and HCSH as important components of redox thiol status) induced by free OH radicals were studied. Low molecular weight thiols and disulfides, including GSH, Cys, and HCys, are critical cellular components that play numerous important roles in metabolism and homeostasis. GSH, the most abundant non-protein thiol, has many wide-ranging functions within the cell including detoxification of free radicals and peroxides, regulation of cell growth and protein function, and maintenance of immune function.[97] Cys also plays numerous important roles as a key extracellular reducing agent, critical substrate for protein synthesis, and rate-limiting precursor
for GSH and taurine synthesis. HCys is a critical regulatory intermediate of the Met cycle that serves as a precursor for Cys via the transsulfuration pathway as well as for Met through remethylation. Thiols group of GSH, Cys and HCys maintain the nerve cell active by formation of di-sulfide bond. Free thiols always take a considerable account in neurodegenerative diseases, like Alzheimer’s, Parkinson’s, etc. In these cases free thiols are blocked by free radical, like NO, OH and form misfolded protein aggregation which disturbs the homeostasis. This underlying cause leads us to study the mechanism of curcumin on free hydroxyl radical (OH). [98][99]

**MATERIALS AND METHODS**

*Materials:*

β-lacto globulin, a free thiol containing protein was purchased from sigma. Molecular weight of this protein is **13.8 KD**

Curcumin, H2O2, and FeSO4 were also brought from sigma.

Diluted acetic acid solution was prepared by diluting glacial acetic acid in milliQ water (500ml milliQ water is added with 500µl of glacial acetic acid). A buffer solution is prepared to perform fenton reaction (20mM tris HCL, 1mM EDTA, pH7)
**Methods:**

10 mM β-lactoglobulin solution was prepared with diluted acetic acid solution. Fenton reaction is done in five different test tubes with five different concentration of H₂O₂, like 20µl, 30µl, 40µl, 50µl and 60µl with same amount of FeSO₄ each time. 4mM curcumin solution was prepared in acetonitrile. We took 60µl of protein solution in five test tubes and to it 50µl solution was added from five different fenton reactions. In another five test tubes, we took 60µl of protein in each test tube; 80µl of curcumin solution was added to each test tube which follows 50µl solution of five different fenton reactions in five consecutive tubes. Two test tubes contain only 20ml 10 mM β-lactoglobulin solution itself.

All these samples were prepared to run in 15% SDS (Sodium dodecyl sulphate) Page. Samples were run in SDS at 110 V for 1hr 45 minutes. In one gel added protein itself and protein after adding fenton reaction, where as in another gel we added protein itself and protein after adding curcumin and fenton reaction.
Results and discussions:

Results

In our first gel, we loaded pure protein in the first lane and after that we loaded protein affected by three different concentrations of fenton reactions. Figure below shows the result.

**Fig.35.** Shows β-lactoglobulin at 13.8 KD size and protein is forming polymer after adding fenton reaction, as the concentration of H2O2 increasing the amount of polymer is increasing as well.

*NB: lane 1, 2, 3 are protein with different concentrations (20, 30, 40, µlof H2O2) of fenton reaction. Lane 4 is for protein Ladder.*

Above figure shows protein starts forming polymer and increasing as we increase the concentration of H2O2 in fenton reaction. Hydroxyl radical production is consequently increased as we increased the amount of H2O2.
In our second gel, we loaded protein affected by curcumin and four different concentrations of fenton reactions. Figure below shows the result.

![Figure showing protein bands](image)

**Fig.36.** Shows β-lactoglobulin at 13.8 KD size. No polymer is found here.

*NB: lane 1, 2, 3, 4, are protein with curcumin and different concentrations (20, 30, 40, 50 µl of H2O2)) of fenton reaction. Lane 1 and 5 is for protein Ladder.*

Above figure is showing that proteins are not forming polymer in the presence of sufficient amount of curcumin, even, free hydroxyl radicals are there.
Discussions

In recent years it has been an increasing interest in the fate of thiols in cell when free radicals are produced. There are several technologies have been developed to observe this. However, there is a critical and recognized need to see the polymer formation in SDS Page. In this study we identified that the oxidative reactions are responsible to decrease the disulfide bond and results increased amount of misfolded protein aggregation which leads to neurodegenerative diseases. Fenton reaction is a potential index of oxidative reactions which produced free radicals like hydroxyl (OH).

As we increased the concentration of H2O2 in fenton reaction OH concentration is also increased at a subsequent rate. Fig.1 shows that as concentration of OH is increasing amount of protein polymer is increasing as well. That means, free OH radicals are blocking free thiols of protein and prevent the formation of disulfide bond which is imperative for thiol containing protein folding. When thiols are getting blocked by hydroxyl radical protein amount of misfolded protein will be increased which will aggregate and form inclusion body that leads to cell death and ultimately numerous neurodegenerative diseases.

In our second experiment we used curcumin to study the prophylactic action of its on generated free hydroxyl radicals. In presence of curcumin, proteins are not forming polymer, even in the presence of hydroxyl free radicals. Fig.2 shows in the presence of curcumin and fenton reaction proteins are not forming polymer, which typically proves free thiols are not getting hydroxylated by free hydroxyl molecule; the OH radicals are generated and scavenged by curcumin as curcumin gets ring and aliphatic chain hydroxylation. By scavenging the free hydroxyl radicals curcumin assist to form disulfide bond by keeping free thiols intact.
Conclusion

Our intensive study typically proves that curcumin has a prophylactic action to protect protein from free hydroxyl stress. By preventing free thiols from free hydroxyl radical, eventually curcumin takes a considerable account in protein folding and prevention of formation of misfolded protein aggregation. Curcumin prevents the inclusion body formation which causes stress in ER of the cell, and leads cell to die which is the obligatory cause for neurodegenerative diseases.
References:


Curriculum Vitae

Rituraj Pal was born as younger son of Madan Pal and Krishna Pal on November 23, 1982 in India. Rituraj is graduated with high school degree from Midnapur Collegiate School in May 2001. In July 2006 he received Bachelor’s degree in Veterinary Science from West Bengal University of Animal and Fishery Sciences. After serving 6 months as a veterinary doctor Rituraj came to USA on Dec, 2006.

Rituraj worked on a NSF funded project at University of North Texas for 6 months. He was accepted in the Graduate as a Master’s student under the direction of Dr. Mahesh Narayan, Ph.D. During this time, he worked with Protein Disulfide Isomerase, a chaperone protein believed to play crucial role in protein folding. In May of 2009, Rituraj successfully defended thesis titled PDI, reactive oxygen species and Polyphenolic Phytochemicals: Implications for neurodegenerative diseases to gain his degree of Master’s of Science.

The manuscripts of these findings are currently in progress and will be submitted for publication. During his time as a graduate student, Rituraj was employed by University of Texas at El Paso, Department of Chemistry as a Teaching Assistant teaching chemistry labs. Rituraj participated in SACNAS conference held in 2008 demonstrating his poster titled “Targeting nitrosative stress effect on an ER-resident oxidoreductase through small molecule ethnopharmaceuticals intervention: intervention for neurodegenerative disease.” Rituraj is going to start his Ph.D in Biochemistry at University of Texas at Austin.

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