SMALL-MOLECULE CATALYSIS OF NATIVE DISULFIDE BOND FORMATION IN PROTEINS

by

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Abstract

Native disulfide bond formation is essential for the folding of many proteins. The enzyme protein disulfide isomerase (PDI) catalyzes native disulfide bond formation using a Cys-Gly-His-Cys active site. The active-site properties of PDI (thiol pK_a = 6.7 and disulfide $E^{\circ\prime}$ = –180 mV) are critical for efficient catalysis. This Dissertation describes the design, synthesis, and characterization of small-molecule catalysts that mimic the active-site properties of PDI.

Chapter Two describes a small-molecule dithiol that has disulfide bond isomerization activity both *in vitro* and *in vivo*. The dithiol trans-1,2-bis(mercaptoacetamido)cyclohexane (BMC) has a thiol pK_a value of 8.3 and a disulfide $E^{\circ\prime}$ value of –240 mV. *In vitro*, BMC increases the folding efficiency of disulfide--scrambled ribonuclease A (sRNase A). Addition of BMC to the growth medium of yeast cells causes an increase in the heterologous secretion of *Schizosaccharomyces pombe* acid phosphatase, which contains eight disulfide bonds.

Chapter Three describes a tripeptide that is a functional mimic of PDI. The disulfide bond stability of the peptide Cys-Gly-Cys-NH_2 ($E^{\circ\prime}$ = –167 mV) matches closely that of the PDI active sites. *In vitro*, the reduced form of this peptide has higher disulfide bond isomerization activity than does the reduced form of BMC. The CGC sequence was incorporated into a protein scaffold via deletion of the proline residue from the CGPC active site of *Escherichia coli* thioredoxin (Trx). This deletion destabilizes the active-site disulfide bond of Trx and confers substantial disulfide bond isomerization activity.

Chapter Four describes the synthesis of an immobilized dithiol that is useful for protein folding *in vitro*. Tris(2-mercaptoacetamidoethyl)amine was synthesized and coupled to two

different solid supports: NovaSyn® TG Br resin (TentaGel resin, a co-polymer of styrene and bromine-functionalized polyethylene glycol) and styrene-glycidyl methacrylate microspheres. TentaGel presenting dithiol shows little disulfide bond isomerization activity with a sRNase A substrate, due to steric inaccessibility of the dithiols within the interior of the TentaGel resin. In contrast, microspheres present dithiol with a high surface density and show disulfide bond isomerization activity that is both substantial and greater than microspheres presenting a monothiol.

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List of Abbreviations

A	absorbance
BMC	(±)-trans-1,2-bis(mercaptoacetamido)cyclohexane
BPTI	bovine pancreatic trypsin inhibitor
CPY	carboxypeptidase Y
CXC	Cys-Xaa-Cys
CXXC	Cys-Xaa-Xaa-Cys
DTNB	5, 5′-dithiobis(2-nitrobenzoic acid)
DTT	D,L-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
Ero1p	endoplasmic reticulum oxidoreductin 1 protein
GSH	reduced glutathione
GSSG	oxidized glutathione
HPLC	high perfomance liquid chromatography
IGF-I	insulin-like growth factor-I
IR	infrared
βME	beta-mercaptoethanol
βME^{ox}	2-hydroxyethyl disulfide
MBA	4-mercaptobenzeneacetate
mp	melting point

$NADP^+$	β-nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
NMA	*N*-methylmercaptoacetamide
NMR	nuclear magnetic resonance
NTB	2-nitro, 5-thiobenzoate
PAGE	polyacrylamide gel electrophoresis
PDI	protein disulfide isomerase
poly(C)	poly(cytidylic acid)
Rnase A	ribonuclease A
SDS	sodium dodecyl sulfate
sRNase A	scrambled ribonuclease A
TCEP–HCl	tris(2-carboxyethyl)phosphine hydrochloride
T_m	midpoint of the thermal denaturation curve
tPA	tissue plasminogen activator
TR	thioredoxin reductase
Tris–HCl	tris(hydroxymethyl)aminomethane hydrochloride
Trx	thioredoxin
UV	ultraviolet
vtPA	truncated tissue plasminogen activator

Chapter One

Introduction

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1.1 Disulfide Bonds and Protein Folding

Covalent bonds between the sulfur atoms of cysteine residues stabilize the three-dimensional structures of many proteins. These disulfide bonds are common in eukaryotic secretory proteins and form post-translationally, during protein folding. The native (active) state of such a protein possesses a singular cysteine-pairing arrangement (Anfinsen, 1973). The attainment of native disulfide bonds can be a significant challenge, in both chemical and biological settings.

The importance of disulfide bonds in biochemistry has long been recognized (Jocelyn, 1972). Cystine (the disulfide-linked dimer of cysteine) was first identified in 1810. It was another 89 years, however, before cystine was found in proteins. The importance of disulfide bonds for protein function was first realized in 1931 during studies on insulin (Du Vigneud *et al.*, 1931). The amino acid sequence of insulin also provided the first assignment of the native disulfide bond arrangement in a protein (Ryle *et al.*, 1955). In 1939, Neurath and Saum discovered that the disulfide bonds of horse serum albumin contribute to structural stability (Neurath & Saum, 1939). The mechanism by which disulfide bonds stabilize proteins remains unclear (Betz, 1993).

While native disulfide bonds are beneficial to proteins, forming them can be problematic. Non-native disulfide bonds can arise from incorrect cysteine-pairing. As the number of cysteine residues in a given amino acid sequence increases, the chances for misoxidation increase dramatically (Anfinsen & Scheraga, 1975). For example, if a protein containing eight cysteines, and there are >300 such yeast open reading frames, has four intramolecular disulfide bonds in the native state, then it must find this unique state out of 105 possible fully oxidized species.

Misoxidized proteins are useless unless they can rapidly sort through these combinations and isomerize to their native states.

Misfolded proteins are inactive and waste cellular resources. Seminal experiments by Anfinsen and co-workers showed that purified, reduced ribonuclease A (RNase A) could spontaneously oxidize in air to the native state, but that this process occurs slowly and gives a low yield (Sela *et al.*, 1957). Additionally, small disulfides such as 2-hydroxyethyldisulfide can serve as the oxidant in place of atmospheric O_2, although folding is still slow on a biological time scale (Anfinsen *et al.*, 1961). The efficiency of protein folding *in vivo* was explained by the discovery of the enzyme protein disulfide isomerase (PDI) (Goldberger *et al.*, 1963; Venetianer & Straub, 1963). In fact, an ensemble of enzymes (such as PDI) and chaperones exist in the cell and interact with newly biosynthesized protein chains to assure their efficient folding (Zapun *et al.*, 1999).

1.2 Pathway of Disulfide Bond Formation in the Endoplasmic Reticulum

The formation of disulfide bonds in proteins requires a sufficiently oxidizing environment (Anfinsen, 1973; Gilbert, 1990; Raines, 1997). Eukaryotic cells contain compartments of widely varying reduction potential ($E^{\circ\prime}$) (Hwang *et al.*, 1992). Those proteins destined for secretion are co-translationally translocated into the oxidizing environment of the endoplasmic reticulum (ER), $E^{\circ\prime} = -0.18$ V, where they can fold and acquire their native disulfide bonds. Oxidative folding in the ER of the yeast *Saccharomyces cerevisiae* requires two proteins (Figure 1-1): protein disulfide isomerase (PDI) and endoplasmic reticulum oxidoreductin 1 protein (Ero1p) (Frand *et al.*, 2000).

Ero1p is a 65-kDa, membrane-associated resident of the ER lumen that is essential for *S. cerevisiae* viability. *In vivo,* functional Ero1p is required for protein disulfide bond formation (Figure 1-1). Ero1p was identified using genetic screens for proteins that either, when overexpressed, confer resistance to dithiothreitol (DTT, a small-molecule reductant) or, when mutated, cause sensitivity to DTT. Addition of diamide (a thiol oxidant) to the growth medium can complement an Ero1p deficiency, presumably by performing the oxidative function of the missing gene product. These results indicate that the essential function of Ero1p is to oxidize newly synthesized proteins (Frand & Kaiser, 1998; Pollard *et al.*, 1998).

PDI is a 57-kDa resident of the ER that is essential for *S. cerevisiae* viability (Farquhar *et al.*, 1991; LaMantia *et al.*, 1991; Scherens *et al.*, 1991; Tachikawa *et al.*, 1991). PDI possesses two active sites, and each one contains a CXXC motif with the sequence Cys-Gly-His-Cys (Edman *et al.*, 1985). *In vitro*, PDI catalyzes the oxidation of thiols and the reduction and isomerization of disulfide bonds (Figure 1-2) (Freedman *et al.*, 1994). Experiments using active-site variants of PDI indicate that its essential function is the isomerization of non-native disulfide bonds (Figure 1-1) (Laboissière *et al.*, 1995b; Chivers *et al.*, 1998).

Oxidative protein folding involves both the oxidation of thiols and the isomerization of non-native disulfide bonds. Kaiser and Frand (Frand & Kaiser, 1999) delineated how Ero1p and PDI accomplish the first step of this process (Figure 1-1). Ero1p and PDI form a disulfide-linked complex *in vivo*. PDI and carboxypeptidase Y (CPY), a protein that contains five disulfide bonds in its native state, also form mixed-disulfide bonds. Reduced forms of both PDI and CPY accumulate in cells lacking functional Ero1p. Reduced CPY also accumulates in the ER of PDI-

depleted cells. Thus, oxidizing equivalents flow from Ero1p to PDI to substrate proteins (Figure 1-1).

The formation of a mixed disulfide bond between Ero1p and PDI suggests that the mechanism of PDI oxidation by Ero1p involves a thiol–disulfide interchange reaction (Frand & Kaiser, 1999). *In vitro*, efficient catalysis of oxidative protein folding requires only Ero1p, PDI, and FAD, suggesting that flavin is the primary oxidant of proteins in the ER. Further, no protein folding activity was detected for Ero1p in the absence of PDI, suggesting that Ero1p has specificity for PDI (Tu *et al.*, 2000). Mutational analysis of Ero1p has identified two pairs of conserved cysteines (composed of CXXXXC and CXXC motifs, respectively) that are critical for protein disulfide bond formation in the ER (Frand & Kaiser, 2000). The likely mechanism for catalysis of PDI oxidation by Ero1p involves direct thiol–disulfide interchange between PDI and the CXXXXC motif of Ero1p. Ero1p can then shuttle the electrons from its CXXXXC motif to its CXXC motif and, ultimatately, to FAD.

GSSG, the oxidized dimer of the tripeptide glutathione, has long been thought the primary source of protein oxidation, with the accumulation of GSSG being a result of its selective import into the ER (Hwang *et al.*, 1992). Yet, GSSG can be formed in the ER from GSH by the action of Ero1p. Mutant strains of yeast unable to synthesize glutathione show no defect in protein disulfide bond formation. Indeed, knocking out glutathione synthesis restores viability to cells lacking functional Ero1p. These cells efficiently form protein disulfide bonds in the ER. Taken together, these results indicate that glutathione contributes net reducing equivalents to the ER and actually competes with protein thiols for oxidation. Thus, a role for GSSG in the oxidation of

proteins has been ruled out (Cuozzo & Kaiser, 1999). The role of glutathione in the ER may be to buffer against transient changes in oxidative stress.

If cells lacking Ero1p and glutathione can exhibit normal oxidation kinetics, then the ER must possess other oxidants. One candidate is sulfhydryl oxidase, an enzyme that catalyzes the oxidation of thiols by O_2 (Hoober *et al.*, 1999; Thorpe *et al.*, 2002). Indeed overexpression of the yeast sulhydryl oxidase Erv2p restores viability to *S. cerevisiae* cells lacking functional Ero1p. Further, Erv2p can oxidize PDI *in vitro*, suggesting a similar pathway for protein oxidation to that of Ero1p (Sevier *et al.*, 2001). The crystal structure of the yeast sulfhydryl oxidase Erv2p suggests a relay mechanism, whereby electrons are shuttled from substrate proteins to a CGC disulfide bond. The resulting CGC dithiol is then oxidized by a CGEC disulfide bond within Erv2p. From CGEC, the electrons are transferred to FAD and, ultimately, to O_2 (Gross *et al.*, 2002). The contribution of Erv2p to protein oxidation in the ER is likely minor compared with that of Ero1p because *S. cerevisiae* cells depleted of Erv2p show no detectable growth defects (Gerber *et al.*, 2001). Another putative electron acceptor is flavin-containing monooxygenase (FMO), an O_2- and NADPH-dependent catalyst of thiol oxidation that is localized to the cytoplasmic face of the ER (Suh *et al.*, 1999). The appeal of both sulfhydryl oxidase and FMO as putative oxidants is the direct link they provide to the ultimate electron acceptor: O_2.

1.3 PDI Structure

The three-dimensional structure of PDI remains unknown. PDI consists of five structural domains (named a, b, b′, a′, and c, Figure 1-3). The first four domains have structural homology to the CXXC-containing oxidoreductase thioredoxin (Ferrari & Söling, 1999). The a and a′

domains each contain a single active site with the sequence CGHC. Recent studies have identified an important role for the b′ domain in substrate binding (Klappa *et al.*, 1998; Cheung & Churchich, 1999). The c domain is not required for catalytic activity (Koivunen *et al.*, 1999).

The thioredoxin fold of the a and a′ domains consists of a β–α–β–α–β–α–β–β–α structure with the CGHC active sites at the *N*-terminus of the second α-helix (Figure 1-3) (Kemmink *et al.*, 1996). The more *N*-terminal active site cysteine residue has high reactivity at pH 7 that results in part from its location on the exterior surface of the protein and from its thiol pK_a value of 6.7 (Hawkins & Freedman, 1991). Alignment of this cysteine residue with the helix dipole stabilizes the thiolate form (Kortemme *et al.*, 1996). The two cysteine residues within the active site are able to form a cyclic disulfide bond with $E^{\circ\prime} = -180$ mV (Lundström & Holmgren, 1993), and this ability is crucial for redox catalysis (Figure 1-2) (Laboissière *et al.*, 1995b; Walker *et al.*, 1996).

Crystal structures of a PDI homolog composed solely of two catalytic domains from *Pyrococcus furiosus* (Ren *et al.*, 1998) and of a dimeric *E. coli* thioredoxin active-site variant with enhanced isomerase activity (Schultz *et al.*, 1999) provide clues as to how the thioredoxin-like domains of PDI may interact. The thioredoxin domains in both structures form a continuous β-sheet (although the interfaces occur at opposite edges of the β-sheet), suggesting a fairly linear arrangement of the domains within PDI. A crystal structure of the dimeric bacterial PDI homolog DsbC provides a slightly different model for the structure of PDI (McCarthy *et al.*, 2000). The DsbC dimer contains two catalytic domains and two dimerization domains. This arrangement is reminiscent of the four thioredoxin-like domains in PDI. The dimer structure is V-shaped, with the two CXXC active sites pointing towards each other across a large, hydrophobic cleft. This

cleft seems well-suited for the binding of unfolded proteins. The catalytic and dimerization domains in a DsbC monomer are connected by a hinged linker helix that allows for conformational changes associated with substrate recognition. Such flexibility has been implicated in PDI-catalyzed protein folding (Cheung & Churchich, 1999), though it is not known if PDI possesses interdomain linkers similar to those in DsbC (Darby *et al.*, 1999). Knowledge of the structure of intact PDI will allow for a more detailed model of the mechanism by which PDI catalyzes native disulfide bond formation.

1.4 Catalytic Mechanism of PDI

PDI is the most efficient known catalyst of protein folding (Weissman & Kim, 1993). The rate-determining step in oxidative protein folding often involves the isomerization of disulfide bonds (Narayan *et al.*, 2000). During protein folding, disulfide bonds tend to accumulate rapidly relative to the appearance of the native state. The presence of PDI may change the distribution, but not the identity of intermediates during protein folding (Shin & Scheraga, 1999; Vinci *et al.*, 2000). Although PDI catalyzes each of the individual steps in oxidative protein folding, the acceleration of the overall folding rate stems from an increase in the isomerization rate of specific, slowly rearranging intermediates (Creighton *et al.*, 1980; Weissman & Kim, 1993; Shin & Scheraga, 2000).

The simplest mechanism for catalysis of disulfide bond isomerization by PDI begins with the attack of a thiolate on a substrate disulfide bond (Figure 1-4) to form a covalent enzyme-substrate complex and a substrate-bound thiolate (Darby *et al.*, 1994; Chivers *et al.*, 1998). The *N*-terminal cysteine of the PDI active site has a thiol pK_a value of 6.7 (Hawkins & Freedman,

1991). The rate of thiol–disulfide interchange is maximal when pH = pK_a (Szajewski & Whitesides, 1980). The first thiol pK_a of the PDI active site matches the physiological pH to provide optimal reactivity *in vivo*. The next step in the catalytic mechanism of disulfide bond isomerization involves thiol–disulfide interchange reactions within the substrate. These reactions ultimately lead to the formation of native disulfide bonds and, eventually, release of the enzyme.

Support for this mechanism is provided by kinetic studies on active-site variants of PDI (Laboissière *et al.*, 1995b; Walker & Gilbert, 1997). Changing the *N*-terminal active-site cysteine residue to serine completely obliterates disulfide bond isomerization activity. In contrast, a PDI active-site variant with the *C*-terminal cysteine residue changed to serine retains substantial disulfide bond isomerization activity. In support of the covalent PDI-substrate complex shown in Figure 1-4, disulfide-linked complexes between PDI and protein substrates have been trapped, both *in vitro* (Walker & Gilbert, 1997) and *in vivo* (Molinari & Helenius, 1999), and visualized by SDS–PAGE. The disulfide bond isomerization activity of PDI is lost upon oxidation or alkylation by iodoacetate (Hawkins & Freedman, 1991). PDI is also inhibited by the arsenite or Cd^{2+}, behavior indicative of active-site dithiol groups (Ramakrishna Kurup *et al.*, 1966; Hillson & Freedman, 1980). Taken together, these results demonstrate that PDI requires an active-site thiolate for the formation of a covalent PDI-substrate complex during catalysis of disulfide bond isomerization, and that this thiolate is provided by the *N*-terminal cysteine residue in the sequence CGHC.

A striking feature of this mechanism is that it requires only the provision of a reactive thiolate on the part of the enzyme. While active-site variants of PDI lacking the *C*-terminal cysteine show significant catalysis of disulfide bond isomerization, these variants are somewhat

less efficient than wild-type PDI (Laboissière *et al.*, 1995b; Walker & Gilbert, 1997). This decrease in catalytic efficiency may arise from kinetic trapping of the covalent enzyme-substrate complex. Wild-type PDI can escape from such a trap through the formation of a disulfide bond within its CGHC active site (Figure 1-4). Such an escape mechanism produces oxidized enzyme and partially reduced substrate. Native disulfide bonds can then form via isomerization of the partially reduced substrate and subsequent reoxidation by PDI (Gilbert, 1997).

Non-covalent interactions also contribute to catalysis of disulfide bond isomerization by PDI. Often, rate-limiting disulfide rearrangements of protein folding intermediates involve structured species. The disulfide bonds in these species are typically buried within the protein structure and require transient conformational unfolding prior to rearrangement via thiol–disulfide interchange. PDI utilizes binding affinity, in addition to the reactivity of its active site, to efficiently capture these transiently unfolded species. A binding site for peptides and unfolded proteins has been identified within the b′ domain of PDI (Klappa *et al.*, 1998). Additionally, PDI, for catalysis of oxidative protein folding has K_m values on the order of 1 – 10 μM for peptides (Darby *et al.*, 1994) and proteins (Walker *et al.*, 1996). The isolated catalytic domains of PDI, which lack the principal peptide binding site, have 7- to 12-fold lower disulfide bond isomerization activity with RNase A containing randomized disulfide bonds as a substrate and have no detectable disulfide bond isomerization activity with an isolated, highly structured BPTI folding intermediate as a substrate (Darby & Creighton, 1995b). Non-covalent interactions are critical for the efficient catalysis of the disulfide rearrangements in structured folding intermediates by PDI (Weissman & Kim, 1993).

1.5 Role of Protein Disulfide Isomerase in the Cell

PDI is a multi-functional, ER-resident protein (Freedman, 1989) that is essential for viability in *S. cerevisiae* (Scherens *et al.*, 1991) and *C. elegans*(Winter & Page, 2000). In addition to catalysis of thiol–disulfide interchange reactions during protein folding (Figure 1-2), PDI also functions as the β-subunit of prolyl-4-hydroxylase (Koivu *et al.*, 1987; Pihlajaniemi *et al.*, 1987), as a subunit of the microsomal triglyceride transfer protein complex (Wetterau *et al.*, 1990), and as both an inhibitor (Wang & Tsou, 1993) and promoter (Puig & Gilbert, 1994; Puig *et al.*, 1994) of non-specific, hydrophobically-driven protein aggregation. Given the multiplicity of known functions for PDI, it is not immediately obvious which function is required for cell viability.

Complementation studies with *PDI1* null mutants of *S. cerevisiae* have shown that the essential role of PDI in eukaryotic cells is the isomerization of disulfide bonds during protein folding. Overproduction of Eug1p, which contains CLHS and CIHS sequences, restores viability to *pdi1Δ* cells (Tachibana & Stevens, 1992). Variants of PDI with CLHS and CIHS active sites (designed to mimic Eug1p) also allow yeast cells lacking wild-type PDI to grow (LaMantia & Lennarz, 1993). These proteins lack the ability to form a disulfide bond within the active site and, therefore, cannot catalyze reduction or oxidation reactions. The essential function of PDI does not reside in its redox activity.

Mammalian PDI can also complement a PDI deficiency in yeast (Günther *et al.*, 1993). The Raines laboratory constructed plasmids that encoded wild-type rat PDI and mutants of rat PDI in which either the *N*-terminal or *C*-terminal cysteine residues of each CGHC active site were replaced by a serine (Laboissière *et al.*, 1995b). Plasmids encoding either wild-type PDI or its

CGHS variant are able to complement *pdi1Δ S. cerevisiae*. In contrast, plasmid encoding SGHC PDI is unable to compensate for this deficiency (Table 1-1).

The wild-type rat PDI and its two variants were purified (Laboissière *et al.*, 1995a) and their abilities to catalyze each reaction shown in Figure 1-2 were assayed (Laboissière *et al.*, 1995b). In comparison to wild-type PDI, both CGHS and SGHC PDI have negligible dithiol oxidation activity and negligible disulfide bond reduction activity. CGHS PDI has disulfide bond isomerization activity comparable to the wild-type protein, but SGHC PDI has negligible disulfide bond isomerization activity. The essential function of PDI is therefore to act as a catalyst of disulfide bond isomerization.

Random mutation of the -XX- residues in the CXXC motif of PDI can create PDI variants with decreased thiol oxidation activity. *pdi1Δ S. cerevisiae* cells producing such PDI variants have heightened sensitivity to the reductant DTT. This sensitivity correlates with the rate of protein disulfide bond formation during CPY folding *in vivo* (Holst *et al.*, 1997). The oxidizing function of PDI, while not essential, is also important for optimal protein folding.

Neither of the two PDI active sites is, by itself, essential for cell viability (Holst *et al.*, 1997). The individual catalytic domains of PDI can also support growth of *pdi1Δ S. cerevisiae* (Xiao *et al.*, 2001). *In vitro*, these domains retain disulfide bond isomerization activity, although their activities are 7- to 12-fold lower than wild-type PDI (Darby & Creighton, 1995b). The ability of the individual catalytic domains to compensate for a deficiency in full-length PDI demonstrates that the multi-domain structure of PDI is not required for its essential cellular function.

The *S. cerevisiae* genome encodes non-essential homologs of PDI, such as Eug1p (Tachibana & Stevens, 1992), Mpd1p (Tachikawa *et al.*, 1995), and Mpd2p (Tachikawa *et al.*, 1997). Each

of these *S. cerevisiae* PDI homologs, when overproduced, can restore viability to *pdi1Δ* cells. In strains where *PDI1*, *EUG1*, *MPD1*, and *MPD2* were simultaneously deleted, only PDI and Mpd1p, when individually overproduced, restore viability (Nørgaard *et al.*, 2001). Eug1p and Mpd2p both require low levels of one or more other homologues to restore viability. The homologues of PDI are not functionally interchangeable, and, of these three proteins, only Mpd1p is capable of carrying out all the essential functions of PDI. A variant of Eug1p with CLHC and CIHC active sites is also capable of carrying out all the essential functions of PDI and increases the growth rate compared to cells complemented with wild-type Eug1p (Holst *et al.*, 1997). This variant highlights the importance of the second cysteine residue of the CXXC motif for the efficient function of PDI. Covalent complexes between Mpd2p and Ero1p have been trapped *in vivo*, suggesting that Mpd2p may catalyze protein oxidation in the cell (Frand & Kaiser, 1999). An oxidative function for Mpd2p *in vivo* is supported by the inability of a PDI variant with CGHS active sites to complement *pdi1Δ*, *mpd2Δ S. cerevisiae* (Nørgaard *et al.*, 2001).

Plasmid encoding rat PDI fused to the *C*-terminus of the α-pre-pro sequence (which signals for translocation into the ER) is able to complement *pdi1Δ S. cerevisiae* during vegetative cell growth. In contrast, this plasmid is unable to complement *pdi1Δ S. cerevisiae* during spore germination. Mutation of the C-terminal KDEL sequence (which is the mammalian ER retention signal) to HDEL (which is the yeast ER retention signal) causes higher levels of rat PDI to accumulate in the yeast ER. Plasmid encoding a *C*-terminal HDEL variant of rat PDI are able to complement *pdi1Δ S. cerevisiae* during spore germination (Laboissière *et al.*, 1997). Apparently, the essential function of PDI – the catalysis of disulfide bond isomerization – is of greater

consequence to germinating spores than to dividing cells.

1.6 Thioredoxin as a Substitute for Protein Disulfide Isomerase

Thioredoxin (Trx) is a 12 kDa, single domain, cytosolic reducing agent for ribonucleotide reductase and other proteins (Holmgren, 1985). The catalytic domains of PDI have similar tertiary structure to Trx (Figure 1-3B) and similar active site sequences: CGHC in each catalytic domain of PDI and CGPC in Trx. Overall, PDI and Trx share approximately 30% amino acid identity (Edman *et al.*, 1985).

Despite their similar sequence and structure, PDI and Trx possess distinct physical properties. The active-site disulfide bond of Trx (disulfide $E^{\circ\prime} = -270$ mV (Moore *et al.*, 1964)) is 4.2 kcal/mol more stable than the active-site disulfide bond of PDI (disulfide $E^{\circ\prime} = -180$ mV (Lundström & Holmgren, 1993)). The more *N*-terminal cysteine residue in the Trx active site possesses microscopic thiol pK_a values of 7.5 and 9.2, which arise from interaction with a neighboring aspartate residue (Chivers *et al.*, 1997b). This aspartate residue functions as a general base to deprotonate the more *C*-terminal active-site cysteine during disulfide bond formation within the Trx active site (Chivers & Raines, 1997).

Escherichia coli Trx possesses none of the non-redox functions exhibited by PDI. To examine the minimal requirements of PDI for cell viability, *E. coli* Trx variants were tested for the ability to support the growth of PDI-deficient *S. cerevisiae* cells (Table 1-2). Wild-type Trx, which has measurable oxidative protein folding activity *in vitro* (Pigiet & Schuster, 1986), does not support the growth of these cells (Chivers *et al.*, 1996). In analogy to PDI (Laboissière *et al.*, 1995b), CGPS Trx, but not SGPC Trx, can replace PDI *in vivo* (Chivers *et al.*, 1996). A Trx

variant possessing a PDI-like CGHC active site has a destabilized active-site disulfide bond ($E^{\circ\prime}$ = –235 mV) and is a more efficient catalyst of disulfide bond isomerization *in vitro* (Lundström *et al.*, 1992). Production of this CGHC variant of Trx restores viability to PDI-deficient cells (Chivers *et al.*, 1996).

To further explore the requirements for functional replacement of PDI by Trx, the codons for the active-site glycine and proline residues were mutated in tandem to codons for all 20 amino acid residues. The resulting library encodes 400 double muants of Trx. Plasmid encoding either CWGC Trx or CVWC Trx is able to complement *pdi1Δ S. cerevisiae*. These Trx variants both possess higher disulfide $E^{\circ\prime}$ values and lower thiol pK_a values than does wild-type Trx (Table 1-2).

Complementation of *pdi1Δ S. cerevisiae* with plasmids encoding active-site variants of Trx demonstrates that the only function of PDI required for cell viability is the catalysis of native disulfide bond formation in proteins (Figure 1-1), and that only the catalytic domains of PDI are required for this function. These studies indicate that the minimal requirement for replacement of PDI in the cell is the provision of a reactive thiolate on the part of the catalyst (Figure 1-3).

1.7 Properties of the CXXC Motif

Both PDI and Trx contain CXXC active-site motifs. Two properties of the CXXC motif, thiol pK_a and disulfide $E^{\circ\prime}$, are apparently critical for the catalysis of disulfide bond formation in the ER. An increase in disulfide $E^{\circ\prime}$ increases the fraction of reduced molecules in an environment of a given reducing potential (E = –180 mV for the ER). Of those reduced molecules, those with a lower thiol pK_a will ionize to a greater extent in a solution of given pH (for the ER, pH = 7). A

decrease in thiol pK_a will also help to further increase disulfide $E^{\circ\prime}$. The ability of a CXXC-containing protein to confer viability to *pdi1Δ S. cerevisiae* requires that a high fraction of these molecules form a thiolate in the solution conditions of the ER (Chivers *et al.*, 1998). Mutation of non-cysteine residues in the CXXC motifs of PDI (Holst *et al.*, 1997) and Trx (Chivers *et al.*, 1996) can alter the redox properties of these proteins and thus alter their biological function. The CXXC motif is found in many thiol-disulfide oxidoreductases. These proteins display a wide variety of CXXC sequences, thiol pK_a values, and disulfide $E^{\circ\prime}$ values (Table 1-3). This variation tunes the active-site reactivity to fit the biological function of the individual enzyme.

In the bacterial periplasm, the Dsb family of CXXC-containing proteins comprise a pathway for native disulfide bond formation (Debarbieux & Beckwith, 1999). DsbA possesses a CPHC active site (thiol pK_a = 3.5, disulfide $E^{\circ\prime}$ = –122 mV) is the primary catalyst of protein oxidation in the periplasm (Bardwell *et al.*, 1991; Zapun *et al.*, 1993). DsbC possesses a CGYC active site (disulfide $E^{\circ\prime}$ = –143 mV) and catalyzes disulfide bond isomerization in the periplasm (Missiakas *et al.*, 1994; Shevchik *et al.*, 1994; Zapun *et al.*, 1995). Despite possessing a more stable disulfide bond, the fraction of DsbC molecules that are reduced *in vivo* is much higher than the fraction of reduced DsbA molecules (Joly & Swartz, 1997). This observation is consistent with the assigned functional roles of these proteins and implies that the redox states of these proteins are under kinetic control in the bacterial periplasm.

The CPHC disulfide bond in the active site of DsbA is 6.8 kcal/mol less stable than the CGPC active-site disulfide bond in Trx (Table 1-3). The structural basis for this stability

difference is unclear (Grauschopf *et al.*, 1995; Chivers *et al.*, 1997a). The lower thiol pK_a value of DsbA can explain some, but not all, of this stability difference (Chivers *et al.*, 1997a).

The disulfide bond in a DsbA variant with a Trx-like CGPC active site ($E°'$ = –214 mV) is stabilized by 4.3 kcal/mol relative to the disulfide bond in wild-type DsbA (Huber-Wunderlich & Glockshuber, 1998). The disulfide bond in a Trx variant with a DsbA-like CPHC active site ($E°'$ = –204 mV) is destabilized by 3.1 kcal/mol relative to the disulfide bond in wild-type Trx. The change in disulfide bond stability arising from mutations of the central residues in the CXXC motif of DsbA correlates quantitatively with the thiol pK_a of the N-terminal cysteine residue (Grauschopf *et al.*, 1995; Huber-Wunderlich & Glockshuber, 1998). Active-site variants of Trx with CXXC sequences that mimic those of other naturally-occurring oxidoreductases also show a correlation between disulfide $E°'$ and thiol pK_a (Mössner *et al.*, 1998). This correlation does not hold for the CWGC and CVWC variants of Trx (Chivers *et al.*, 1997a). The intervening residues are able to regulate disulfide bond stability in the CXXC motif by stabilizing the reduced, deprotonated form and by other, pH-independent factors (Grauschopf *et al.*, 1995; Chivers *et al.*, 1997a). Variations in the identity of the intervening –XX– residues in the CXXC motif underlie the diversity of biological function in this family of thiol–disulfide oxidoreductases.

1.8 Strategies for Improving Native Disulfide Bond Formation *in Vivo*

High levels of secreted protein can be obtained through heterologous overproduction in eukaryotes. Overexpression in yeast of genes on multi-copy plasmids can saturate the protein folding machinery and cause unfolded protein to accumulate in the ER and limit the yield of secreted protein (Parekh *et al.*, 1995). Indeed, the extractable levels of PDI in the ER decrease

upon prolonged constitutive overproduction of heterologous, disulfide-bonded proteins (Robinson & Wittrup, 1995). Co-overexpression of PDI increases the folding capacity of the ER and also increases the secretion of heterologously overproduced proteins in yeast (Robinson *et al.*, 1994; Shusta *et al.*, 1998). Manipulation of PDI levels in cells overproducing disulfide-bonded proteins is a promising means for increasing the yield of active protein (Wittrup, 1995).

Overproduction of eukaryotic proteins in bacteria can yield insoluble, misfolded aggregates because stable disulfide bonds rarely form in the cytosol (Marston, 1986; Gilbert, 1990). The bacterial cytoplasm ($E^{\circ\prime} = -0.27$ V) is normally even more reducing than its eukaryotic counterpart ($E^{\circ\prime} = -0.23$ V) (Hwang *et al.*, 1992) and is not a good environment for the production of properly folded, multiply disulfide-bonded proteins. Of course, bacterial expression systems can produce large quantities of heterologous proteins. Unfortunately, insoluble aggregates of these proteins tend to form when they misfold, and the *in vitro* folding of these inclusion bodies can be difficult and time-consuming (Rudolph & Lilie, 1996).

One approach to this problem has been to secrete disulfide-bonded proteins along with PDI to the more oxidizing periplasm in *E. coli* (Zhan *et al.*, 1999). This approach is hit-or-miss, as rat PDI does not improve yields of human tissue plasminogen activator (tPA), a 17-disulfide protein, but yeast PDI increases the yield by 50%. In addition to problems of specificity, PDI activity may be hindered by the redox environment of the periplasm. The yield of active tPA obtained by coproduction of yeast PDI was lower than that obtained from cells overproducing DsbC (Qiu *et al.*, 1998), whose CXXC active site is maintained in the reduced state (Debarbieux & Beckwith, 1999). Perhaps the periplasmic environment is too oxidizing for efficient catalysis of disulfide bond isomerization by PDI. The ability of PDI to function in place of the periplasmic

oxidant DsbA during the oxidative folding of *E. coli* alkaline phosphatase supports this idea (Zhan *et al.*, 1999).

In the *E. coli* periplasm, the prior overproduction of DsbC or DsbA (the bacterial analogue of Ero1p) doubles production of insulin-like growth factor-I (IGF-I) relative to the simultaneous overproduction of the proteins (Joly *et al.*, 1998). The abundance of Dsb proteins in the periplasm during production of IGF-I leads, however, to increased aggregation rather than more efficient *in vivo* folding. Fortunately, IGF-I can be folded easily from inclusion bodies, and 8.5 g of IGF-I can be isolated from 1 L of cell culture. This high yield may be due to protection of translocated IGF-I from proteolysis or assistance in its translocation. Surprisingly, most of the DsbA in cells after its transient overproduction is found in the reduced form rather than the oxidized form that normally accumulates and is responsible for catalysis of protein oxidation in the periplasm (Debarbieux & Beckwith, 1999). The decrease in folding efficiency when IGF-I is overproduced subsequent to DsbA or DsbC overproduction argues against improved thiol–disulfide interchange as a cause of the boost in yield (Joly *et al.*, 1998).

Manipulation of the redox environment in the bacterial cytosol may allow for more efficient oxidative protein folding *in vivo* (Bessette *et al.*, 1999). Such a strategy has been made possible by the development of *E. coli* strains that grow normally despite having an oxidizing cytosol. These strains were isolated as suppressors of the slow-growing phenotype displayed by cells lacking the genes for thioredoxin reductase and glutathione synthesis. The cytosolic folding of four normally secreted, multiply disulfide-bonded proteins was more efficient in these strains than in wild-type cells. Thioredoxin, which normally acts as a cytosolic reductant, acts as a protein oxidant in these strains (Stewart *et al.*, 1998), consistent with its ability to act as an

oxidant when exported to the periplasm (Debarbieux & Beckwith, 1998; Jonda *et al.*, 1999).

The cytosolic folding of truncated tPA (vtPA), which has 9 disulfide bonds, can be improved by coproduction of active-site thioredoxin variants with higher $E^{\circ\prime}$. Optimum yield of vtPA in the cytosol is obtained with coproduction of DsbC in these mutant strains, which gives a 200-fold increase in folding efficiency relative to wild-type cells (Table 1-4). This result underscores the importance of maintaining a dithiol catalyst for the shuffling of non-native disulfide bonds (Figure 1-4). Surprisingly, the coproduction of PDI in the cytosol has little effect on the cytosolic folding of vtPA. The folding of vtPA in the cytosol of the mutant strains is more efficient than is its folding in the periplasm of wild-type cells (Table 1-4) (Bessette *et al.*, 1999). Oxidation may be slower in the cytosol than in the periplasm, decreasing the need for disulfide bond isomerization, and thus explaining the greater efficiency of cytosolic folding.

Modulation of the redox environment in the cytosol may be an effective strategy for the production of eukaryotic secretory proteins in bacteria. Folding efficiency in this system could be improved further by coproduction of a disulfide bond isomerase with active-site properties appropriate to the redox environment. The mechanism of disulfide bond isomerization (Figure 1-4) can guide the design and choice of this catalyst.

1.9 Strategies for Improving Native Disulfide Bond Formation *in Vitro*

Oxidative protein folding involves the formation and isomerization of disulfide bonds (Narayan *et al.*, 2000). The addition of β-hydroxyethyl disulfide and β-mercaptoethanol can increase the *in vitro* folding efficiency of reduced and scrambled RNase A, respectively (Anfinsen *et al.*, 1961; Haber & Anfinsen, 1962). A redox buffer consisting of oxidized and

reduced glutathione (a physiologically abundant tripeptide) was utilized to improve the efficiency of *in vitro* oxidative folding for lysozyme (Saxena & Wetlaufer, 1970) and for RNase A (Hantgan *et al.*, 1974; Ahmed *et al.*, 1975). The fundamental requirements of redox buffers for oxidative protein folding are the provision of a thiol oxidant (such as GSSG) and a catalyst of disulfide bond isomerization (such as GSH). Several other small-molecule thiol–disulfide reagents have also been used for protein folding (Table 1-5).

The rate and yield of protein folding in a glutathione redox buffer does not correlate with increasing concentration of either GSH or GSSG (Wetlaufer *et al.*, 1987). Instead, the glutathione redox buffer system shows a distinct optimum for protein folding around 1 mM GSH and 0.2 mM GSSG (Hantgan *et al.*, 1974; Wetlaufer *et al.*, 1987; Lyles & Gilbert, 1991a). The catalytic activity of PDI is also optimal in this redox buffer (Lyles & Gilbert, 1991a) and these concentrations of glutathione are similar to those in the ER (Hwang *et al.*, 1992). Further increases in glutathione concentration decrease folding efficiency, even when the reduction potential of the solution is kept constant (Wetlaufer *et al.*, 1987), presumably because mixed disulfide bonds between the protein and glutathione accumulate and hinder protein folding.

Lowering the pK_a of the thiol reagent can increase the oxidative protein folding activity of the redox buffer. A nucleophilic thiolate is required for catalysis of disulfide bond isomerization (Figure 1-4). Further, lowering the thiol pK_a increases the leaving group ability, and increases the likelihood of escape from a mixed disulfide bond with a protein. The disulfide form of a thiol with lowered pK_a will also be a stronger oxidant. The aromatic monothiol 4-mercaptobenzeneacetate (MBA) has a thiol pK_a value of 6.6 and has a 32-fold higher rate of disulfide bond reduction than does glutathione (thiol pK_a = 9.0) (De Collo & Lees, 2001). A

redox buffer composed of MBA and GSSG can enhance the rate of reduced RNase A folding by up to 6-fold (Gough *et al.*, 2002).

Dithiol reagents can form cyclic disulfide bonds and are less prone to mixed disulfide bond formation than are monothiols. A redox buffer composed of the dithiol and disulfide forms of DTT can fold reduced RNase A (Creighton *et al.*, 1980; Rothwarf & Scheraga, 1991). A large excess of oxidized DTT is required for protein oxidation because the disulfide bond of DTT is extremely stable ($E^{\circ\prime} = -327$ mV) (Lees & Whitesides, 1993). DTT (thiol pK_a = 9.2 (Lamoureux & Whitesides, 1993)) is able to catalyze the isomerization of disulfide-containing intermediates more rapidly than is glutathione (Lambert & Freedman, 1983; Rothwarf & Scheraga, 1993), indicating that dithiols are better catalysts of disulfide bond isomerization than are monothiols. CXXC-containing peptides have also been shown to fold reduced RNase A (Moroder *et al.*, 1996). The folding rates correlate qualitatively with the disulfide $E^{\circ\prime}$ of the peptides. The folding activities of these peptides were further improved by cyclizing the peptide backbone, which increased their disulfide $E^{\circ\prime}$ values and decreased their thiol pK_a values (Cabrele *et al.*, 2002; Cattani-Scholz *et al.*, 2002). Knowledge of the properties and mechanism of PDI can be applied to the creation of new and better reagents for native disulfide bond formation in proteins.

An understanding of the mechanism by which PDI catalyzes protein folding can lead to more efficient strategies for protein production and also to new treatments for diseases caused by protein misfolding. Harnessing the power of PDI for these applications will require knowledge of thiol–disulfide interchange chemistry, the physical properties of the PDI active site, and how the interplay of the PDI active site and its environment promotes catalysis. Studies on PDI and

active-site variants have provided insight into its catalytic mechanism. In turn, knowledge of the PDI mechanism has inspired new biological and chemical catalysts of protein folding.

Table 1-1. Properties of wild-type and variant PDIs[a].

PDI active-site	Dithiol oxidation activity?	Disulfide bond reduction activity?	Disulfide bond isomerization activity?	Complementation of *pdi1Δ S. cerevisiae*?
CGHC	Yes	Yes	Yes	Yes
SGHC	No	No	No	No
CGHS	No	No	Yes	Yes

[a]Data from Laboissière *et al.*, 1995b.

Table 1-2. Properties of wild-type and variant Thioredoxins[a].

Trx active-site sequence	First thiol pK_a	Disulfide $E°'$ (mV)	% Trx thiolate in the ER	Complementation of *pdi1Δ S. cerevisiae*?
CGPC	7.5	–270	0.025	No
CGHC	7.5	–235	0.35	Yes
CWGC	5.86	–230	1.8	Yes
CVWC	5.94	–200	16	Yes

[a]Data from Chivers *et al.*, 1996

Table 1-3. Properties of CXXC-containing proteins.

Protein	Active-site sequence	First thiol pK_a	Disulfide $E^{\circ\prime}$ (mV)	Biological function
Trx	CGPC	7.5[a]	–270[b]	Disulfide bond reduction
PDI	CGHC	6.7[c]	–180[d]	Disulfide bond isomerization
DsbC	CGYC	unknown	–143[e]	Disulfide bond isomerization
DsbA	CPHC	3.5[f]	–122[g]	Dithiol oxidation

[a]Chivers *et al.*, 1997b.

[b]Moore *et al.*, 1964.

[c]Hawkins and Freedman, 1991.

[d]Lunström and Holmgren, 1993.

[e]Zapun *et al.*, 1995.

[f]Zapun *et al.*, 1993.

[g]Huber-Wunderlich and Glockshuber, 1998.

Table 1-4. Production of vtPA in bacteria[a].

Condition	Relative yield
Wild-type cytosol	0.1
Oxidizing cytosol	1
Wild-type periplasm plus DsbC	10
Oxidizing cytosol plus DsbC	21

[a]Data from Bessette *et al.*, 1999.

Table 1-5. Thiols used for oxidative protein folding.

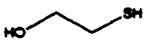
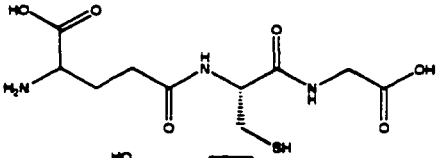
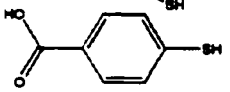
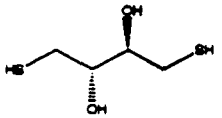
Name	Structure	Disulfide $E^{\circ\prime}$ (mV)	Thiol pK_a	Reference
βME		–260	9.2	Haber and Anfinsen, 1962
GSH		–252	9.0	Saxena and Wetlaufer, 1970
MBA		unknown	6.6	Gough *et al.*, 2001
DTT		–327	9.2	Rothwarf and Scheraga, 1991
CPYC	linear peptide backbone	–227	unknown	Moroder *et al.*, 1996
CPYC	cyclic peptide backbone	–191	7.9	Cabrele *et al.*, 2002

Figure 1-1. Primary pathway of native disulfide bond formation in the ER. PDI transfers oxidizing equivalents from Ero1p to reduced, unfolded proteins (Frand and Kaiser, 1999). Cells lacking Ero1p cannot oxidize newly synthesized proteins in the ER and are inviable. Those proteins that become incorrectly oxidized must rearrange to the native state. Cells lacking a catalyst of disulfide shuffling (such as PDI) are inviable (Laboissière *et al.*, 1995b).

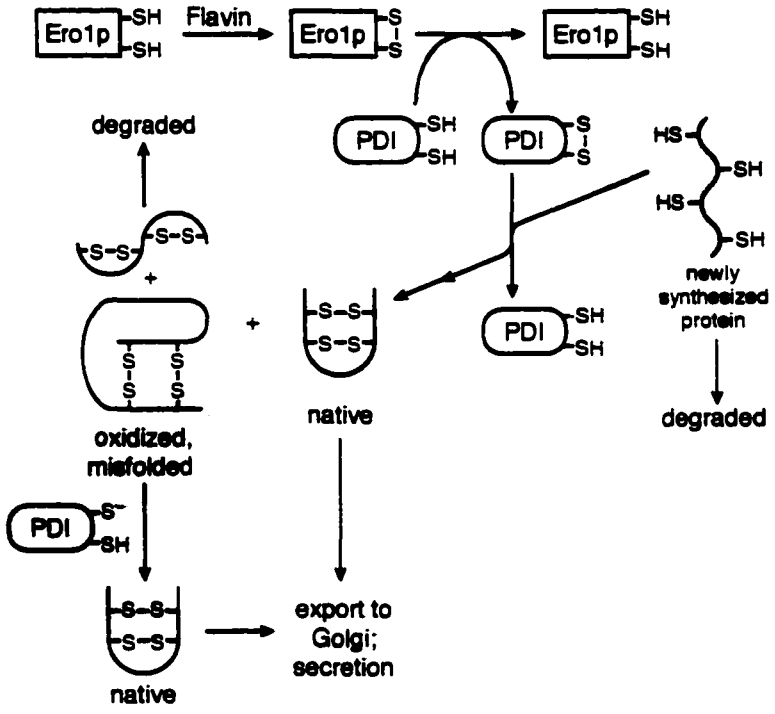

Ero1p
SH
SH
Flavin
Ero1p
S
S
Ero1p
SH
SH
PDI
SH
SH
PDI
S
S
HS
SH
HS
SH
newly synthesized protein
degraded
S-S
S-S
+
S
S
S
S
oxidized, misfolded
+
S-S
S-S
native
PDI
SH
SH
degraded
PDI
S
SH
S-S
S-S
native
export to Golgi; secretion

Figure 1-2. **Reactions catalyzed by PDI *in vitro*. The enzyme catalyzes the oxidation of thiols and the reduction of disulfide bonds (top), and the isomerization of disulfide bonds (bottom).**

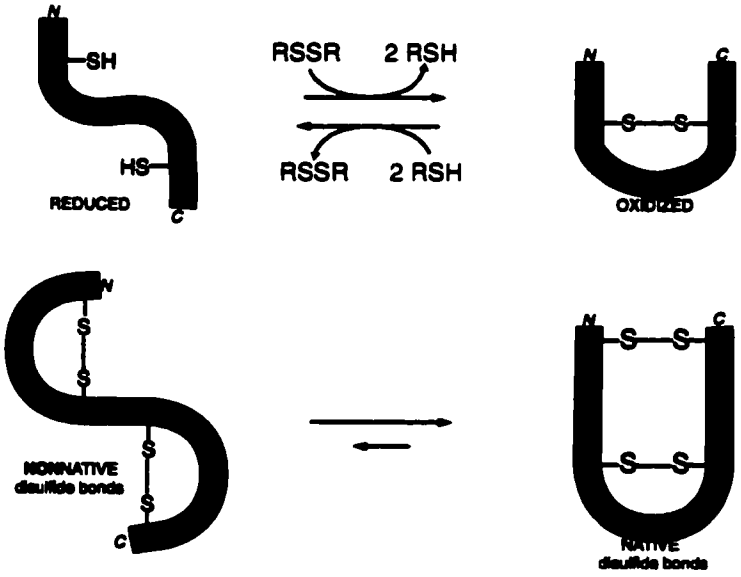
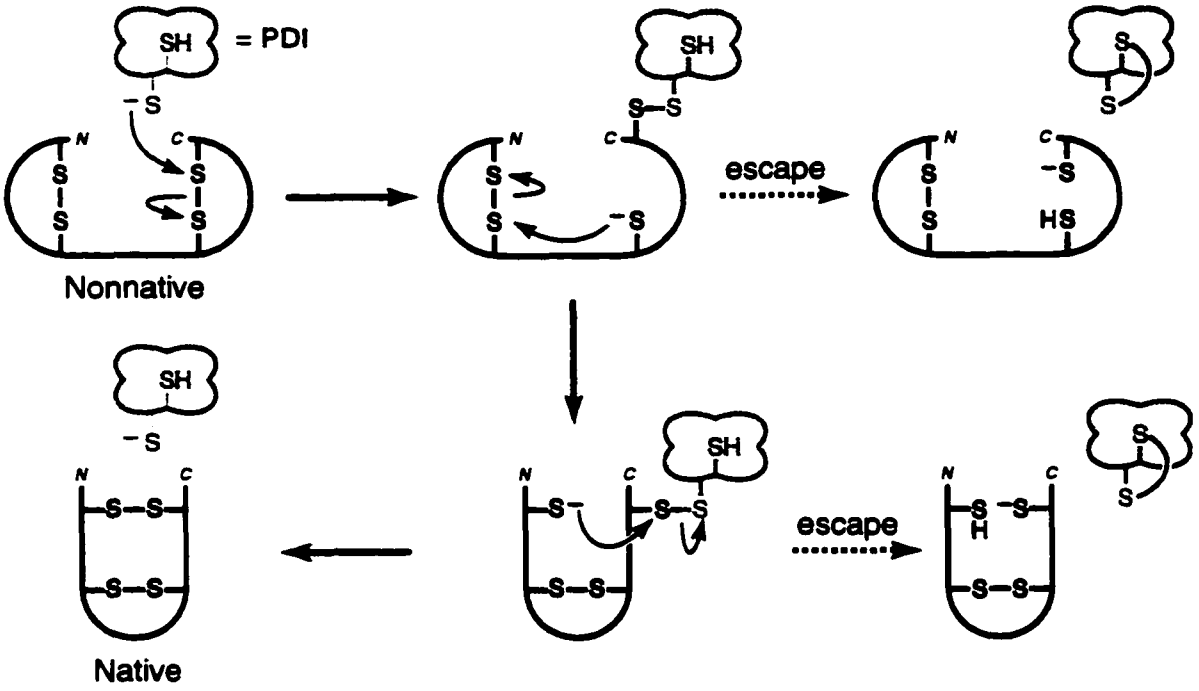

N
SH
HS
REDUCED
C
RSSR 2 RSH
RSSR 2 RSH
N
C
S S
OXIDIZED
N
S
S
S
S
C
NONNATIVE
disulfide bonds
N
C
S S
S S
NATIVE
disulfide bonds

Figure 1-3. The domain structure of PDI. (A) PDI consists of five structural domains, named a, b, b′, a′, and c. The a and a′ domains each contain a CGHC active site. (B) A ribbon diagram of *E. coli* thioredoxin, a protein of similar structure to the individual a, a′, b, and b′ domains of PDI. The cysteine residues in the thioredoxin active site are shown in a ball and stick rendering.

A

B

Figure 1-4. Mechanism of disulfide bond isomerization (Woycechowsky *et al.*, 1999). Rearrangement begins with nucleophilic attack of a thiolate provided by the catalyst (such as PDI) on a non-native disulfide bond. The resulting, covalent substrate–catalyst complex contains a substrate thiolate and can perform intramolecular thiol–disulfide exchange reactions to form native disulfide bonds and, eventually, release of the catalyst (redox-inactive mechanism). The second thiol in the active site of the catalyst can act as a clock to ensure timely rearrangement of the substrate. Those substrates that are slow to rearrange can be partially reduced (and subsequently reoxidized) by a dithiol catalyst (reduction-reoxidation mechanism). Those catalysts lacking a second thiol can become trapped in mixed disulfide complexes with the substrate.


SH
= PDI
N
C
escape
HS
Nonnative
Native

Chapter Two

A Small-Molecule Catalyst of Protein Folding *in Vitro* and *in Vivo*

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2.1 Abstract

The formation of native disulfide bonds between cysteine residues often limits the rate and yield of protein folding. The enzyme protein disulfide isomerase (PDI) catalyzes the interchange of disulfide bonds in substrate proteins. The two –Cys–Gly–His–Cys– active sites of PDI provide a thiol of low pK_a and a disulfide bond of high reduction potential ($E^{\circ\prime}$). A synthetic small-molecule dithiol, (±)-*trans*-1,2-bis(2-mercaptoacetamido)cyclohexane (BMC), has pK_a = 8.3 and $E^{\circ\prime} = -0.24$ V. These values are similar to those of the PDI active sites. BMC catalyzes the activation of scrambled ribonuclease A, an inactive enzyme with non-native disulfide bonds, and doubles the yield of active enzyme. A monothiol analog of BMC, *N*-methylmercaptoacetamide, is a less efficient catalyst than BMC. BMC in the growth medium of *Saccharomyces cerevisiae* cells increases by >3-fold the heterologous secretion of *Schizosaccharomyces pombe* acid phosphatase, which has eight disulfide bonds. This effect is similar to that from the overproduction of PDI in the *S. cerevisiae* cells, indicating that BMC (like PDI) can catalyze protein folding *in vivo*. A small-molecule dithiol with a low thiol pK_a and high disulfide $E^{\circ\prime}$ can mimic PDI by catalyzing the formation of native disulfide bonds in proteins, both *in vitro* and *in vivo*.

2.2 Introduction

The formation of native disulfide bonds is the rate-determining step in the folding of many proteins. Reduced, unfolded proteins containing multiple cysteine residues tend to oxidize rapidly and non-specifically. The non-native disulfide bonds formed during oxidative folding must then be isomerized to achieve the correct cysteine pairings. The greater the number of cysteine residues in an amino acid sequence, the more possibilities arise for incorrect disulfide arrangements. Given that the native structure is required for protein function and that disulfide bonds are often crucial for structural stability, it is not surprising that, *in vivo*, native disulfide bond formation is an enzyme-catalyzed process (Guzman, 1998).

Protein disulfide isomerase (PDI; EC 5.3.4.1) is essential for the viability of *Saccharomyces cerevisiae* cells (Scherens *et al.*, 1991). Although PDI has many known functions (Freedman, 1989), its essential role is to catalyze the unscrambling of non-native disulfide bonds in other proteins (Laboissière *et al.*, 1995b). PDI is a 57-kDa resident of the endoplasmic reticulum (ER). Each PDI molecule has two active sites with the sequence: –Cys–Gly–His–Cys– (Edman *et al.*, 1985). Only the *N*-terminal cysteine residue in each active site is necessary for catalysis of disulfide bond isomerization (Laboissière *et al.*, 1995b; Walker *et al.*, 1996).

A simple chemical mechanism for catalysis by PDI begins with the nucleophilic attack of its active-site thiolate on a non-native disulfide bond (Figure 2-1 (Darby & Creighton, 1995a; Chivers *et al.*, 1998)). This attack results in an intermolecular (*i.e.*, mixed) disulfide and a substrate thiolate. Further disulfide rearrangements are then induced by this substrate thiolate. The attainment of the native three-dimensional structure guides the formation of native disulfide

bonds and, ultimately, the release of PDI. A striking feature of this mechanism is that it requires the enzyme to provide only a reactive thiolate (Darby & Creighton, 1995a; Chivers *et al.*, 1998).

In a given pH and redox environment, the fraction of PDI active sites that have a thiolate is governed by two factors (Gilbert, 1990; Chivers *et al.*, 1997a). One is the value of the formal reduction potential ($E^{\circ\prime}$) of the disulfide bond that forms between the two cysteine residues in each active site. The other factor is the value of the acid dissociation constant (K_a) of the nucleophilic thiol. In PDI, the active-site disulfide bond has an $E^{\circ\prime}$ of – 0.18 V, and the thiol of the *N*-terminal active-site cysteine residue has a pK_a of 6.7 (Hawkins & Freedman, 1991; Lundström & Holmgren, 1993). These two physical parameters can be combined with the properties of the ER ($E_{solution}$ = – 0.18 V and pH 7 (Hwang *et al.*, 1992)) to reveal that 33% of the PDI active sites in the ER contain a thiolate (Chivers *et al.*, 1996).

The –Cys–Xaa–Xaa–Cys– (CXXC) active-site motif of PDI is also found in homologous thiol:disulfide oxidoreductases. The best characterized thiol:disulfide oxidoreductase is *Escherichia coli* thioredoxin (Trx), a 12-kDa cytosolic reducing agent for ribonucleotide reductase and other proteins (Holmgren, 1985; Buchanan *et al.*, 1994). Trx has a –Cys–Gly–Pro–Cys– active site with an $E^{\circ\prime}$ of – 0.27 V (Moore *et al.*, 1964) and pK_a of 7.5 (Chivers *et al.*, 1997b). Wild-type Trx cannot replace PDI in *S. cerevisiae* cells because only 0.021% of Trx active sites would contain a thiolate in the ER. Trx variants with higher fractions of thiolate can, however, substitute for PDI *in vivo* (Chivers *et al.*, 1996). This finding demonstrates that a small homolog can mimic PDI. Here, we take the next step.

We reasoned that the two active-site thiols of PDI are its most important functional groups, and that a small-molecule dithiol with appropriate $E^{\circ\prime}$ and pK_a values could likewise be an

effective catalyst of disulfide bond isomerization. The dithiol (±)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane (BMC) had been studied previously as a technetium ligand (Kasina *et al.*, 1986) and as a reducing agent (Lamoureux & Whitesides, 1993). Here, we synthesize BMC and show that it has $E^{\circ\prime}$ and pK_a values similar to those of PDI. We find that BMC catalyzes disulfide bond isomerization, both *in vitro* and *in vivo*, and is more efficient than its monothiol analog, *N*-methylmercaptoacetamide (NMA).

2.3 Materials and Methods

General. (±)-*trans*-1,2-Diaminocyclohexane, chloroacetyl chloride, βME, and 2-hydroxyethyldisulfide (βME^{ox}) were from Aldrich Chemical (Milwaukee, WI). Thioacetic acid was from Acros Organics (Pittsburgh, PA). NMA was from Fluka (Buchs, Switzerland). sRNase A, GSH, and GSSG were from Sigma Chemical (St. Louis, MO). Poly(C) was from Midland Certified Reagents (Midland, TX) and was precipitated from aqueous ethanol (70% v/v) before use. Yeast nitrogen base was from Difco (Detroit, MI). All other chemicals were of reagent grade or better, and were used without further purification.

Thiol concentrations were determined using the method of Ellman (Ellman, 1959). Ultraviolet absorbance measurements were made on a Cary Model 3 spectrophotometer equipped with a Cary temperature controller, from Varian (Sugar Land, TX).

Synthesis of BMC. (±)-*trans*-1,2-Bis(mercaptoacetamido)cyclohexane (BMC) was synthesized by a route similar to those described previously (Kasina *et al.*, 1986; Lamoureux & Whitesides, 1993). This route is outlined in Figure 2-2.

(±)-*trans*-1,2-Diaminocyclohexane (22 g, 0.20 mol) and K_2CO_3 (56 g, 0.40 mol) were dissolved in 1 l of ice-cold distilled water. Chloroacetyl chloride (34 ml, 0.42 mol) was added

dropwise and with stirring to this solution. The resulting cloudy white mixture was stirred at 0 °C for 1 h. The precipitate was collected by filtration, washed with cold distilled water, and dried under reduced pressure. The bis(choroacetyl) product (17.5 g, 33%) was obtained as a white powder, which was judged to be pure by ^{1}H NMR analysis (Lamoureux & Whitesides, 1993).

The bis(chloroacetyl) product (5.6 g, 21 mmol) was dissolved in 1 l of methylene chloride, and this solution cooled to 0 °C. Thioacetic acid (6 ml, 83 mmol) was added dropwise and with stirring to the cooled solution. Then, triethylamine (12 ml, 83 mmol) was added dropwise and with stirring. The resulting solution was allowed to warm slowly to room temperature and stirred under N_2(g) for 40 h. The reaction was quenched by the addition of 0.5 l of 0.35 M sodium acetate buffer (pH 4). The layers were separated, and the organic layer washed with an ice-cold solution of saturated aqueous sodium bicarbonate. The organic layer was dried, filtered, and concentrated to yield a white powder. This powder was purified by recrystallization from ethyl acetate. The bis(thioacetate) product (6.2 g, 86%) was obtained as a white powder, which was judged to be pure by ^{1}H NMR analysis (Lamoureux & Whitesides, 1993).

The bis(thioacetate) product (3.0 g, 8.75 mmol) was dissolved in 125 ml of 1.2 M HCl in methanol. The resulting clear solution was incubated at room temperature under N_2(g) for 20 h. The excess acid was then removed by bubbling nitrogen gas through the solution, and the solvent was removed under reduced pressure to yield a yellow oil. The oil was dissolved in a minimal amount of methanol, and the resulting solution was cooled in a dry ice/acetone bath. The supernatant was decanted, and the white precipitate was dried under reduced pressure to yield BMC (1.8 g, 80%) as a white solid, which was judged to be pure by spectroscopic analysis: mp 193 – 194 °C; IR (thin film) 3272, 3083, 2917, 2852, 2547, 1650, 1554, 1419, 1328, 1241, 1162,

974, 742, 700 cm^{-1}; ^1H NMR (CD_3OD, 300 MHz) δ 3.66 (br, s, 2 H, NC**H**), 3.15 and 3.14 (2d, J = 14.5 Hz, 4 H, COC**H**$_2$S), 1.94 (m, 2 H, NCHC**H**$_2$ eq.), 1.80 (m, 2 H, NCHC**H**$_2$ ax.), 1.37 (m, 4 H, NCHCH$_2$C**H**$_2$) ppm; MS (EI) calcd for $C_{10}H_{18}N_2O_2S_2$ m/z 262, found 262.

Synthesis of BMC-disulfide (BMC^{ox}). The disulfide form of BMC was synthesized from BMC by oxidation with I_2. Briefly, BMC (0.05 g, 0.19 mmol) was dissolved in 0.19 l of ethyl acetate at 0 °C. $KHCO_3$ (25 ml of a 10% w/v solution in water) was added. A concentrated solution of I_2 (0.088 g in 6 ml ethyl acetate) was added dropwise and with stirring. I_2 addition was stopped when the BMC solution turned brown, and the solution was then stirred on ice for 1 h. The reaction was quenched by the dropwise addition of aqueous sodium thiosulfate until the solution became colorless. The organic layer was separated, dried with Na_2SO_4, filtered, and concentrated to yield a white powder. This powder was purified by recrystallization from ethyl acetate. The BMC^{ox} product (56%) was judged to be pure by spectroscopic analysis: mp 247 °C; IR (thin film) 3246, 3066, 2937, 2845, 1633, 1558, 1400, 1326, 1151, 974, 746 cm^{-1}; ^1H NMR (d^6-DMSO, 300 MHz) δ 8.0 (N**H**), 3.8 (NC**H**), 3.1 (COC**H**$_2$S), 1.7 (NCHC**H**$_2$), 1.3 (NCHCH$_2$C**H**$_2$) ppm; MS (EI) calcd for $C_{10}H_{16}N_2O_2S_2$ m/z 260, found 260.

Determination of thiol pK_a. Thiol titration curves were obtained by measuring A_{238} as a function of pH. Buffer stock solutions of KH_2PO_4, K_2HPO_4, and K_3PO_4 (0.10 M each) were degassed and flushed with Ar(g). A stock solution of thiol (1.5 mM BMC or 2.1 mM NMA) was prepared using KH_2PO_4. Different ratios of the buffer stock solutions were combined in duplicate to give two identical sets of 1 ml solutions. These sets of solutions varied in pH according to the ratio of each buffer stock solution added. To each duplicate pair of solutions, 70 μl of KH_2PO_4 was added to one and used to set the A_{238} at zero. Next, 70 μl of thiol solution was added to the

corresponding sample, the A_{238} measured immediately, and the solution recovered. After measuring the A_{238}, the pH of each solution was measured using a Beckman (Fullerton, CA) pH meter freshly calibrated with pH 4.00 and pH 10.00 standard solutions from Fisher (Pittsburgh, PA). This process was repeated for each pair of solutions to generate a plot of A_{238} vs pH.

To determine pK_a values, the A_{238} vs pH data were fitted to either a single (for NMA) or a double (for BMC) titration model (equations 1 and 2, respectively) derived from Beer's Law and the

$$A_{238} = C_T\left(\frac{\varepsilon_{SH} + \varepsilon_{S^-}10^{(pH-pK_a)}}{1+10^{(pH-pK_a)}}\right) \quad (1)$$

$$A_{238} = C_T\left(\frac{\varepsilon_{S^-}^{S^-}10^{(pH-pK_{a2})} + \varepsilon_{SH}^{S^-} + \varepsilon_{SH}^{SH}10^{(pK_{a1}-pH)}}{10^{(pH-pK_{a2})}+1+10^{(pK_{a1}-pH)}}\right) \quad (2)$$

definition of the acid dissociation constant. In equations 1 and 2, C_T is the total thiol concentration, ε_{SH} and ε_{SH}^{SH} are the extinction coefficients of the fully protonated forms, ε_{S^-} and $\varepsilon_{S^-}^{SH}$ are the extinction coefficients of the singly unprotonated forms, and $\varepsilon_{S^-}^{S^-}$ is the extinction coefficient of the doubly unprotonated form. Both the extinction coefficients and pK_a's were obtained as parameters of the curve fit.

Determination of disulfide reduction potential. Thiol – disulfide interchange equilibria were established between BMC and βME. In a typical reaction, 25-ml flask containing solid BMC (1 mg) was sealed with a septum and flushed with Ar(g) for 10 min. Potassium phosphate buffer (pH 7.0; 10 ml of a 10 mM solution), that had been saturated with Ar(g) was added, and the resulting mixture was stirred for 10 min to dissolve the solid. Then, βME^{ox} (0.1 ml of a 40 mM stock solution in 10 mM sodium phosphate buffer, pH 7.0) was added, and the ensuing thiol

– disulfide interchange reaction was allowed to reach equilibrium at 25 °C for 24 h. Similar equilibria were established with different starting concentrations of BMC and βME^{ox}.

The equilibrated mixture was quenched with HCl (1:100 dilution of a 3 M solution) to prevent further reaction. An aliquot (0.1 ml) of the quenched mixture was analyzed immediately by HPLC using Waters system equipped with a Spectra-Physics integrator and a Vydac analytical C18 reverse phase column. The loaded column was eluted at 1.0 ml/min with water (5.0 ml) followed by a linear gradient (20 ml + 20 ml) of acetonitrile (0 – 40% v/v). Compounds were detected by their absorption at 205 nm. Four peaks were evident in the chromatograms, and were anticipated to arise from BMC, BMC^{ox}, βME, and βME^{ox}. HPLC analysis of standard solutions revealed that the four peaks did indeed correspond to βME (retention time: 7 min), βME^{ox} (19 min), BMC^{ox} (26 min), and BMC (28 min). No evidence for mixed disulfides was apparent. To correlate peak area with concentration, calibration curves were developed for these compounds and were linear over the concentration range used (data not shown). From these calibration curves, the equilibrium concentration of each component was determined, allowing calculation of K_{eq} and, from the Nernst equation, here: $E^{\circ\prime}_{BMC} = E^{\circ\prime}_{\beta ME} - \frac{RT}{nF} \ln \frac{[BMC^{ox}][\beta ME]^2}{[BMC][\beta ME^{ox}]}$.

Assay for isomerase activity in vitro.The activation of sRNase A was monitored essentially as described previously (Laboissière *et al.*, 1995a). Refolding reactions were performed at 30 °C in 50 mM Tris-HCl buffer (pH 7.6) containing GSH (1.0 mM), GSSG (0.2 mM), and catalyst (either 1.0 mM BMC or 2.0 mM NMA). BMC was delivered from a 100-fold concentrated stock solution in methanol. Reactions (in triplicate) were initiated by the addition of sRNase A (1.0 μl of a 0.5 mg/ml stock) to a final volume of 0.10 ml. At timed intervals, aliquots

were assayed for poly(C) cleavage activity as described until no further increase in activity was observed. Data were fitted to the equation:

$$[\text{active RNase A}] = [\text{sRNase A}]_{t=0}(1 - e^{-[\text{catalyst}]kt}) \qquad (3)$$

to obtain the value of the second-order rate constant, k. The difference in k values for catalyzed and uncatalyzed reactions was used to calculate the specific activity of the catalyst.

Assay for heterologous secretion. S. cerevisiae cells were engineered to overproduce *S. pombe* acid phosphatase. *S. cerevisiae* strain BJ5464 (α *ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) was obtained from the Yeast Genetic Stock Center (Berkeley, CA). BJ5464 was transformed with a multicopy plasmid directing overproduction of *S. pombe* acid phosphatase, as described previously (Robinson *et al.*, 1994). Yeast cultures (10 replicates) were grown in liquid medium of 50 mM HEPES buffer (pH 6.5) containing BMC (0, 0.1, 0.2, or 0.4 mg/ml from a stock solution in dimethyl sulfoxide), glucose (2% w/v), yeast nitrogen base (0.67% w/v), and synthetic amino acid supplement [arginine (190 mg/L), methionine (108 mg/L), tyrosine (52 mg/L), isoleucine (290 mg/L), lysine (440 mg/L), phenylalanine (200 mg/L), glutamic acid (1260 mg/L), aspartic acid (400 mg/L), valine (380 mg/L), threonine (220 mg/L), glycine (130 mg/L), and the nucleotide adenine (40 mg/L)]. After 48 h of growth at 30 °C, the cells were harvested and the concentration of secreted active *S. pombe* acid phosphatase in the supernatant was determined by a colorimetric assay as described previously (Robinson *et al.*, 1994).

2.4 Results

Synthesis and properties of BMC. We synthesized BMC from (±)-*trans*-1,2-diaminocyclohexane by the 3-step route outlined in (Figure 2-2). The overall yield was 23%, or 9 g of BMC from 24 mL of starting material.

The acid dissociation constant of a titratable group can be determined by measuring the equilibrium concentrations of its protonated and unprotonated forms over a pH range broad enough to encompass the transition from largely protonated to largely unprotonated. The absorbance at 238 nm (A_{238}) of a thiolate exceeds that of its corresponding protonated form. We measured the thiol pK_a values of BMC and NMA using this UV signature. Our analysis of A_{238} as a function of pH indicates that the mercaptoacetamido groups of BMC have thiol pK_{a1} = 8.3 and pK_{a2} = 9.9 (Figure 2-3A). The pK_{a1} for BMC is in good agreement with that obtained for NMA, which has pK_a = 8.3 (Figure 2-3B). As a control, a pH-titration of glutathione was also performed, giving a thiol pK_a of 9.0 (data not shown).

The reduction potential of a redox-active group can be determined by measuring the equilibrium concentrations of its oxidized and reduced forms in the presence of the oxidized and reduced forms of a reference molecule of known reduction potential. To determine the reduction potential of BMC, we developed an HPLC method using β-mercaptoethanol (βME; $E^{\circ\prime}_{\beta ME}$ = –0.260 V (Lees & Whitesides, 1993)) as a reference. As measured by HPLC analysis of the thiol – disulfide exchange equilibrium between BMC and βME, the $E^{\circ\prime}$ of BMC is –0.241 ± 0.002 V. This value is the mean (± SD) of five independent assays starting with different concentrations of BMC and βME.

Having characterized its relevant chemical properties (Table 2-1), we subjected BMC to two distinct assays for biological catalysis, one *in vitro* and one *in vivo*.

Activity in vitro. Bovine pancreatic ribonuclease A (RNase A; EC 3.1.27.5) has eight cysteine residues, which in the native enzyme form 4 disulfide bonds. The ability of BMC to catalyze *in vitro* disulfide bond isomerization was assessed by monitoring the activation of scrambled RNase A (sRNase A), which is fully oxidized RNase A with a random distribution of disulfide bonds. sRNase A is a poor catalyst of RNA cleavage, as few of the enzyme molecules are folded properly. An increase in the rate of RNA cleavage is indicative of an increase in the concentration of RNase A molecules with native disulfide bonds.

The presence of BMC increased the rate of activation of sRNase A (Figure 2-4). Perhaps more significantly, the presence of BMC enhanced by twofold the final yield of native RNase A. NMA also showed unscrambling activity in the *in vitro* assay (Figure 2-4). Unlike BMC, NMA was not able to activate sRNase A completely. Still, the final concentration of native RNase A produced by NMA was greater than that produced by the glutathione redox buffer alone. Apparently, a small-molecule dithiol is more efficient at unscrambling than is a small-molecule monothiol. Assays with other concentrations of BMC and NMA (or with the glutathione redox buffer alone) gave results similar to those in Figure 2-4 (data not shown).

Activity in vivo. Many of the proteins secreted from eukaryotic cells have multiple disulfide bonds. Those proteins that do not fold quickly and properly are degraded rather than secreted (Bonifacino & Lippincott-Schwartz, 1991; Biederer *et al.*, 1997). Increasing the endogenous levels of PDI can increase the efficiency of secretion for some of these proteins (Robinson *et al.*, 1994; Shusta *et al.*, 1998). Presumably, a molecule of small mass and little charge, such as BMC, could gain access to the ER of a yeast cell, where these secretory proteins fold. An increase in heterologous secretion would be consistent with BMC having disulfide

isomerization activity inside the cell, because only properly folded proteins are secreted by eukaryotes (Hammond & Helenius, 1995).

Native *Schizosaccharomyces pombe* acid phosphatase is a 30-kDa homodimer with 8 disulfide bonds (6 intrachain and 2 interchain) and many possibilities for incorrect pairings. Not surprisingly, increased expression of endogenous PDI increases the yield of secreted enzyme (Robinson *et al.*, 1994; Wittrup, 1995). The addition of BMC to the growth medium of *S. cerevisiae* cells producing basal levels of PDI increased the yield of secreted *S. pombe* acid phosphatase (Figure 2-5). The presence of 0.1 mg/ml BMC in the growth medium increases phosphatase secretion by over 3-fold, equivalent to the increase achieved with 15-fold overproduction of PDI (Robinson *et al.*, 1994). Higher concentrations of BMC do not lead to higher levels of phosphatase. This accordance of achievable yield suggests that a step other than catalysis of disulfide bond isomerization limits heterologous secretion from *S. cerevisiae* cells treated with BMC or excess PDI. Addition of BMC to cells overproducing PDI produces no incremental increase in phosphatase secretion (data not shown), consistent with a shared mechanism of action between BMC addition and PDI overproduction.

2.5 Discussion

Disulfide bond interchange is a facile chemical reaction (Raines, 1997). The high nucleophilicity of thiolates and high electrophilicity of disulfide bonds allow for rapid thiol – disulfide interchange. Nevertheless, enzymes have evolved to catalyze this reaction (Guzman, 1998). The enzyme PDI catalyzes the isomerization of non-native disulfide bonds in substrate proteins. The chemical basis for catalysis by PDI results from thiol pK_a and disulfide $E^{\circ\prime}$ values

that produce a high fraction of thiolate in its native environment—the ER of eukaryotic cells (Chivers *et al.*, 1996; Chivers *et al.*, 1997a). If a high fraction thiolate is the sole requirement for catalysis of disulfide bond isomerization, then it should be possible to design and synthesize an effective small-molecule catalyst of protein folding.

In 1962, Haber and Anfinsen demonstrated that low concentrations of βME could activate sRNase A (Haber & Anfinsen, 1962). Glutathione redox buffers have been used extensively to study *in vitro* oxidative protein folding (Saxena & Wetlaufer, 1970; Hantgan *et al.*, 1974; Konishi *et al.*, 1982; Wetlaufer *et al.*, 1987; Lyles & Gilbert, 1991b). The use of monothiols to refold proteins is complicated by the observation that the rates and yields of folding reactions do not parallel the redox potential of the solution (Wetlaufer *et al.*, 1987). The glutathione redox buffer composition for optimum folding of reduced RNase A has been determined empirically by systematic variation of both the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) and the total concentration of the two components. At higher total glutathione concentrations, mixed disulfide intermediates accumulate and become trapped. Dithiols have an advantage over monothiols (such as glutathione) in that mixed disulfide intermediates can be cleaved by intramolecular disulfide bond formation.

Previous attempts at using small dithiols to refold proteins have met with only moderate success. Short peptides containing a PDI-like –Cys–Gly–His–Cys– sequence have modest PDI-like activity (Ookura *et al.*, 1995). The low activity of these peptides relative to PDI likely stems from the loss of the protein context. Specifically, the nucleophilic thiol in the active sites of known thiol:disulfide oxidoreductases is positioned at the *N*-terminus of an α-helix, which provides a depressed thiol pK_a due to interaction with the helix dipole (Kortemme & Creighton,

1995). Moreover, the CXXC sequence is constrained structurally to provide an active-site disulfide bond of appropriate $E^{\circ\prime}$.

Dithiothreitol (DTT) is a poor oxidant of dithiols ($E^{\circ\prime} = -0.327$ V (Lees & Whitesides, 1993)). Yet, native RNase A has been produced from the reduced enzyme by using only oxidized DTT (Rothwarf & Scheraga, 1991). The concentration of DTT used in this experiment was high (0.1 M), and still the refolding rate was slower than that with much lower concentrations of oxidized glutathione. The purpose of using DTT as an oxidant was, however, to demonstrate that a dithiol can oxidize reduced RNase A without the accumulation of mixed disulfide intermediates. Further experiments showed that although DTT oxidizes RNase A >100-fold more slowly than does glutathione, DTT is able to rearrange disulfide-containing folding intermediates slightly more quickly than is glutathione (Rothwarf & Scheraga, 1993). It is difficult to create conditions that would favor the isomerization, rather than the reduction, of disulfide bonds by DTT. The stability of its disulfide bond and its high thiol pK_a of 9.2 (Lamoureux & Whitesides, 1993) combine to combine to give an exceedingly low fraction of DTT molecules in the thiolate form. At pH = 7 and $E_{solution} = -0.18$ V, fewer than one-in-a-million DTT molecules would contain a thiolate, severely limiting its efficacy as a catalyst of disulfide bond isomerization.

Thiol pK_a of reduced BMC. Reduced BMC has a low (first) pK_a. Inductive effects from the electronegative oxygen and nitrogen lower the thiol pK_a from 10.6 (which is the pK_a of propyl mercaptan (Kreevoy *et al.*, 1960)) to 8.3 (Figure 2-3A). This pK_a value differs from that reported previously for mercaptoacetamido groups. Using a similar method, Lamoureux and Whitesides (Lamoureux & Whitesides, 1993) reported pK_a values of 7.6 – 7.8 for the thiols of this group. Their values were determined by fitting the pH-titration data to an equation different from ours

and derived after making the assumptions: (1) the extinction coefficient of the fully protonated form is zero and (2) the extinction coefficient of the doubly unprotonated form is twice that of the singly unprotonated form. Indeed, when using the equation of Lamoureux and Whitesides to fit our pH-titration data, the pK_a value is 7.7. This fit is, however, much worse than that using our equation (Figure 2-3A). For NMA, a thiol pK_a of 8.05 was determined previously by electrometric titration (Danehy & Parameswaran, 1968). The (macroscopic) thiol pK_a measured here for glutathione is 9.0, which is in the range of those previously reported (8.6 – 9.2) (Danehy & Parameswaran, 1968; Jung *et al.*, 1972; Rabenstein, 1973; Reuben & Bruice, 1976; Kanchuger & Byers, 1979), and precisely between the two microscopic thiol pK_a values of 8.93 and 9.08 determined in a careful study using ^{1}H NMR spectroscopy (Rabenstein, 1973).

Disulfide reduction potential of oxidized BMC. The disulfide bond of oxidized BMC has a reduction potential of –0.24 V. This value differs from that reported previously. From ^{1}H NMR spectra of solutions containing DTT and BMC, Lamoureux and Whitesides (Lamoureux & Whitesides, 1993) reported that the $E°'$ of BMC was ≥ –0.21 V. This method is imprecise because the reduction potential of BMC is much greater than that of DTT. In a redox equilibrium between equal amounts of BMC ($E°'$ = –0.24 V) and DTT ($E°'$ = –0.327 V (Lees & Whitesides, 1993)), only 0.1% of the BMC molecules will be in the oxidized form.

Two factors are responsible for this value being higher than that of, say, DTT. The major contributor to the increase is that a ten-membered ring has conformational strain, which is relieved in the reduced molecule. A minor factor arises from the inductive effect—disulfide bonds are more easily reduced if they are formed from thiols with lower pK_a values (Chivers *et*

al., 1997a). As calculated from eq 5 of ref (Chivers *et al.*, 1997a), having mercaptoacetamido groups rather than two propyl mercaptan groups increases the $E^{\circ\prime}$ value of BMC by 0.0007 V.

Catalysis in vitro. The relevant chemical and catalytic parameters of BMC are listed in Table 2-1. These parameters highlight the value of the mercaptoacetamido group, which has much merit for small-molecule catalysts of protein folding. In particular, the mercaptoacetamido group will be 10^2-fold more ionized at pH 7 than a simple alkyl thiol. Moreover, virtually any primary or secondary amine can be elaborated into a mercaptoacetamido group by a synthetic route analogous to that in Figure 2-2.

Although BMC is an effective catalyst of disulfide bond isomerization, its specific activity is approximately 500-fold less than that of PDI (data not shown). PDI has three attributes that could be responsible for its greater efficacy as a catalyst. First, PDI has a lower thiol pK_a, a higher disulfide $E^{\circ\prime}$, and hence a higher fraction thiolate. Under any solution conditions, the fraction of PDI molecules in the thiolate form would always exceed that for BMC. Second, PDI has a peptide binding site that enables the formation of a noncovalent complex with a substrate (Gilbert, 1997; Noiva *et al.*, 1998). The use of binding energy likely lowers the free energy of the chemical transition state (Jencks, 1987; Hansen & Raines, 1990). In contrast, BMC collides indiscriminately with other molecules. Third, PDI could interact preferentially with proteins and so could discriminate against glutathione. For example, the glutamate and cysteine residues of glutathione are linked by an irregular (and thus easily distinguishable) amide bond to the γ-carboxyl group of the glutamate residue. BMC cannot discriminate against glutathione and may be compromised by the formation of mixed disulfides with the redox buffer.

NMA, a small molecule monothiol, is a less efficient catalyst of disulfide bond

isomerization than is BMC. A comparison of these two catalysts provides insight on the role of the second thiol group. In the presence of 2.0 mM NMA (which provides the same concentration of thiol as 1.0 mM BMC), full activation of substrate is not achieved (Figure 2-4). This result is interpretable in terms of the mechanism shown in Figure 2-1. The product of the first step of this mechanism is a covalent catalyst–substrate complex. Further productive steps in this mechanism rely on the ability of the substrate thiolate to induce further rearrangements. If a disulfide bond isomerization is hindered, the substrate will be slow to rearrange. A monothiol catalyst would then be trapped. In contrast, a dithiol catalyst could escape from the trapped complex via the formation of an intramolecular disulfide bond within the catalyst. This disulfide can then be converted back into a dithiol by reaction with the redox buffer or reduced substrate, enabling another attempt at catalysis. In effect, this second thiol group acts like a clock, providing a set amount of time for the substrate to rearrange and free the catalyst before an intramolecular attack within the catalyst disrupts the complex (Walker *et al.*, 1996; Walker & Gilbert, 1997). The difference between the concentration of substrate ultimately activated by a dithiol (BMC) and that activated by a monothiol (NMA or the glutathione redox buffer alone) likely reflects the production of such trapped complexes.

The greater activation rate and increased yield of active RNase A observed for NMA over glutathione alone (Figure 2-4) may result from the decreased pK_a of the thiol in NMA (pK_a = 8.3) relative to that in glutathione (pK_a = 9.0). This pK_a difference results in an increased fraction of thiolate in NMA, which increases the population of catalytically active species and thus the activation rate. In addition, the lower pK_a of NMA makes it a better leaving group from mixed disulfides, making it easier for NMA to escape from trapped complexes and thereby increasing

the yield of folded protein.

Catalysis in vivo. DTT can enter the ER of mammalian cells and prevent the formation of disulfide bonds there (Braakman *et al.*, 1992). For BMC to exhibit PDI-like activity in vivo, it too must enter the ER. The heterologous secretion data suggest that BMC does do so (Figure 2-5). We suspect that the cell permeability of BMC is enhanced by its lipophilic cyclohexyl ring.

Misfolded proteins with non-native disulfide bonds do occur *in vivo* and are costly to a cell. Co-overproduction of PDI has been a successful strategy for increasing the output efficiency of some overproduced secretory proteins (Robinson *et al.*, 1994). The *in vivo* activity of BMC suggests that a similar increase can be obtained simply by adding BMC to the growth medium. Indeed, BMC is the first small-molecule thiol shown to increase the secretion of a heterologous protein.

The pinnacle of biochemical understanding is de novo design. To design a minimized mimic of a protein requires a detailed understanding of its function, as a minimal molecule would contain only those chemical groups that are required for function (Cunningham & Wells, 1987). Some notable success has been attained in creating small mimics of proteins and peptides (Cunningham & Wells, 1997). For example, a dimer of 20-residue peptides can substitute for erythropoiten, which is a 34-kDa glycoprotein (Livnah *et al.*, 1996; Wrighton *et al.*, 1996). The 28-residue atrial natriuretic peptide has been shrunk to 15 residues with only a moderate loss in activity (Li *et al.*, 1995). A few enzymes have been minimized effectively (Kirby, 1996). A 14-residue peptide can catalyze the decarboxylation of oxaloacetate à la acetoacetate decarboxylase (Johnsson *et al.*, 1993). Organoselenium compounds can imitate the active-site selenocysteine residue of glutathione peroxidase (Wilson *et al.*, 1989). To the best of our

knowledge, however, BMC is the first small-molecule mimic of an enzyme that has been shown to function both *in vitro* and *in vivo*.

2.6 Prospectus

An effective, small-molecule mimic of PDI could have numerous practical applications. BMC is useful for *in vitro* protein folding on a preparative scale. Often, overproduction of proteins in *E. coli* leads to the formation of inclusion bodies (Marston, 1986). These insoluble aggregates must be solubilized by reduction and denaturation, and then refolded by dilution into an appropriate redox buffer. The most commonly used redox buffer contains reduced and oxidized glutathione, as used herein. As a monothiol, glutathione lacks the ability to escape from nonproductive complexes with protein, and can lead to low yields of properly folded protein (Figure 2-4). Also, the high thiol pK_a of glutathione (9.0) leads to slow disulfide bond isomerization at pH 7. The use of PDI itself for preparative-scale protein folding is hindered by the high cost and instability of enzymic catalysts and by the problem of separating PDI from the target protein. In contrast, a small-molecule catalyst is inexpensive, inert, and readily separable from a target protein by filtration, dialysis, or size exclusion chromatography.

Some proteins cannot be refolded efficiently *in vitro* and are best produced in their native state. Creating a new strain of a microorganism that overproduces PDI (or other enzymic catalyst) can improve the isolated yield of a secreted protein (Robinson *et al.*, 1994), but is tedious to effect. Small molecules such as BMC may obviate the need for such manipulations by achieving a similar effect via addition to the growth medium (Figure 2-5).

In addition to improving the production of heterologous proteins, small-molecule catalyst

mimicked in a small-molecule catalyst of significant practical utility.

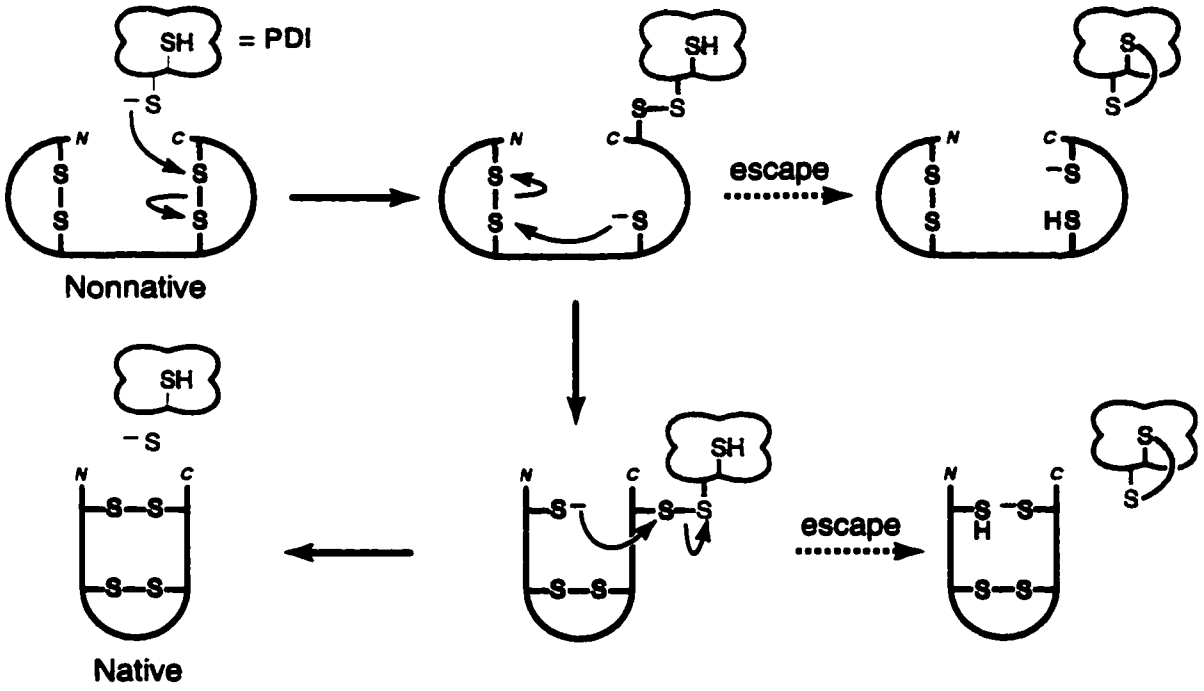
Table 2-1. Properties of (±)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane

Property	Value
Molecular mass (Da)	262
Disulfide E°′ (mV)	–240
Thiol pK_a	8.3; 9.9
Fraction thiolate[a]	0.16
Specific activity (U/mg)[b]	0.4

[a] Fraction thiolate was calculated using the procedure outlined in (Chivers *et al.*, 1998) for a pH 7.6 solution containing 1.0 mM GSH and 0.2 mM GSSG, like that used in the *in vitro* assays.

[b] One unit (U) catalyzes the activation of 1 pmol of sRNase A per minute in Tris-HCl buffer (pH 7.6) containing GSH (1.0 mM) and GSSG (0.2 mM).

Figure 2-1 Putative chemical mechanism for catalysis of disulfide bond isomerization by protein disulfide isomerase (Darby & Creighton, 1995a; Chivers *et al.*, 1998). The enzymic active site has two thiols, which enables the enzyme to escape from nonproductive mixed disulfides. After escape, additional redox reactions can lead ultimately to native protein and reduced catalyst.


SH
= PDI
−S
N
C
S
S
S
S
Nonnative
SH
S
S
N
C
S
S
−S
escape
S
S
N
C
S
S
−S
HS
SH
−S
N
C
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Native
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escape
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N
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H
−S
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S
S

Figure 2-2 Synthetic route to (±)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane (BMC).

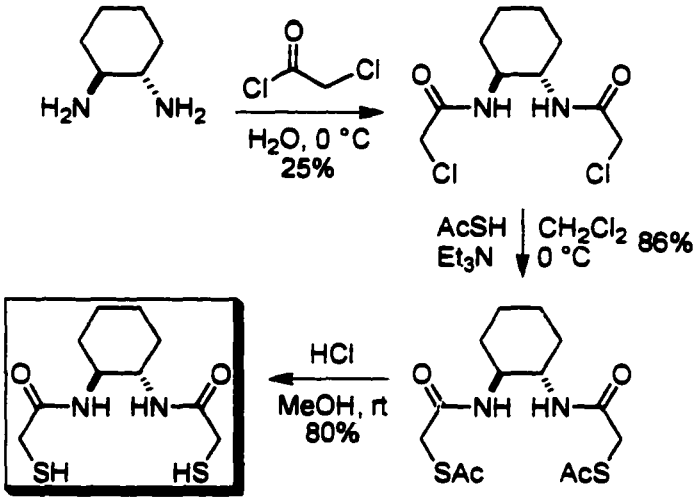

H2N
NH2
Cl
Cl
O
H2O, 0 °C
25%
O
NH
HN
O
Cl
Cl
AcSH
Et3N
CH2Cl2
0 °C
86%
SAc
AcS
HCl
MeOH, rt
80%
SH
HS

Figure 2-3 Effect of pH on the absorbance at 238 nm (A_{238}) of **(A)** BMC (0.10 mM) and **(B)** NMA (0.14 mM) in 0.10 M potassium phosphate buffer. Fitting the BMC data to equation 2 ($r^2 > 0.99$) and the NMA data to equation 1 ($r^2 > 0.99$) gives $pK_{a1} = 8.3$ and $pK_{a2} = 9.9$ for BMC and $pK_a = 8.3$ for NMA.

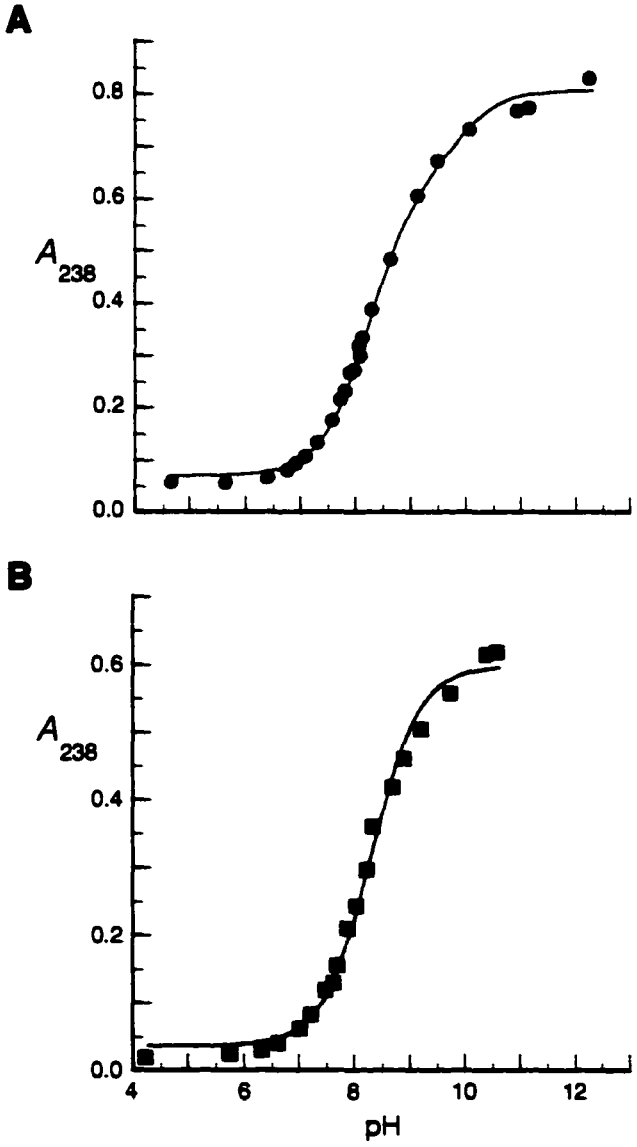

A
0.8
0.6
A_{238}
0.4
0.2
0.0
B
0.6
A_{238}
0.4
0.2
0.0
4
6
8
10
12
pH

Figure 2-4 Time course for the activation of scrambled ribonuclease A by various thiols. All unscrambling reactions were performed at 30 °C in 50 mM Tris-HCl buffer (pH 7.6) containing glutathione alone (1.0 mM GSH; 0.2 mM GSSG; black triangles) or glutathione (1.0 mM GSH; 0.2 mM GSSG) plus BMC (1.0 mM = 0.26 mg/ml; red circles) or NMA (2.0 mM; blue squares). Data points are the average of triplicate measurements, and are fitted to equation 3.

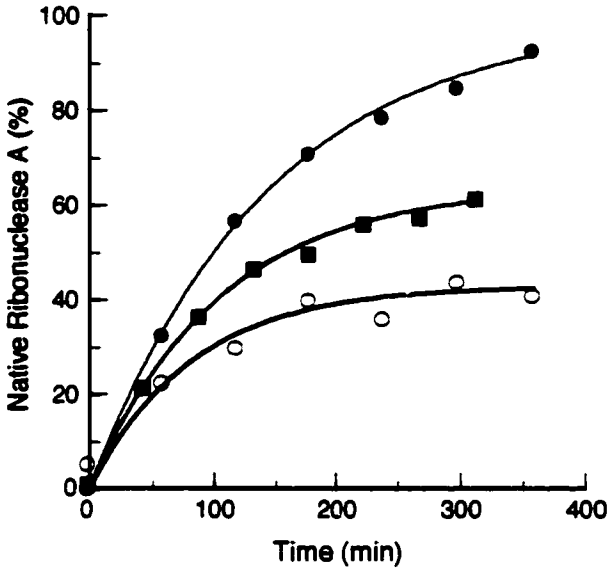
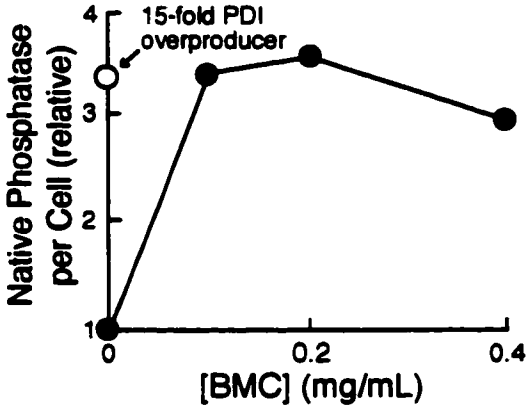

Native Ribonuclease A (%)
100
80
60
40
20
0
0
100
200
300
400
Time (min)

Figure 2-5 Dependence of *Schizosaccharomyces pombe* acid phosphatase secreted from *Saccharomyces cerevisiae* on BMC concentration. Cells were grown for 48 h before assay. In the absence of BMC, $A_{435}/OD_{600} = 0.014$ (see: ref (Robinson *et al.*, 1994)). Data points are the average of 10 replicate measurements. The dotted line represents phosphatase secretion from an isogenic strain in which yeast PDI is overproduced by 15-fold from a single integrated gene (Robinson *et al.*, 1994).


15-fold PDI
overproducer
Native Phosphatase
per Cell (relative)
4
3
2
1
0
0.2
0.4
[BMC] (mg/mL)

Chapter Three

The CXC Motif – A Functional Mimic of the Protein Disulfide Isomerase Active Site

3.1 Abstract

In vivo, native disulfide bond formation in proteins is an enzyme-catalyzed reaction. The essential enzyme protein disulfide isomerase (PDI) utilizes a Cys-Gly-His-Cys (CGHC) active site with disulfide $E^{\circ\prime} = -180$ mV to effect thiol-disulfide exchange reactions with proteins undergoing oxidative protein folding. A CGHC peptide ($E^{\circ\prime} = -220$ mV) poorly mimics the PDI active site. In contrast, CGC peptide ($E^{\circ\prime} = -167$ mV) more closely mimics the PDI active site. This CGC peptide has 2.2-fold higher activity than does *trans*-1,2-bis(mercaptoacetamido)cyclohexane, a small organic dithiol with high disulfide bond unscrambling activity. *Escherichia coli* thioredoxin (Trx) utilizes a CGPC active site ($E^{\circ\prime} = -270$ mV) to effect disulfide bond reduction in the cytosol. Removal of the proline residue from the Trx active site yields a CGC active site with a highly destabilized disulfide bond ($E^{\circ\prime} \geq -200$ mV). This deletion causes minimal disruption of Trx structure and retains high conformational stability in both the reduced and oxidized forms. In contrast to the reduced form of wild-type Trx, the reduced form of this variant has disulfide bond isomerization activity *in vitro*. Creation of a CGC active site in Trx improves disulfide bond isomerization activity 25-fold relative to the CGC peptide. Thus, CGC, in both a peptide and a protein, provides functional mimicry of PDI.

3.2 Introduction

Proper disulfide bond formation is a necessary prerequisite for the folding of many proteins. The enzyme protein disulfide isomerase (PDI) is the most efficient known catalyst of native protein disulfide bond formation (Weissman & Kim, 1993). A member of the family of thiol-disulfide oxidoreductases that possess the Cys-Xaa-Xaa-Cys (CXXC, where X is any amino acid) active-site motif, PDI can catalyze the formation, reduction, or isomerization of disulfide bonds within a substrate protein (Freedman *et al.*, 1994; Laboissière *et al.*, 1995b). The product contains the most stable arrangement of disulfide bonds in equilibrium with the redox environment (Anfinsen, 1973). The design of new redox reagents with properties similar to CXXC-containing oxidoreductases, particularly PDI, is a promising strategy for the development of more efficient protein folding strategies (Woycechowsky *et al.*, 1999).

CXXC-containing oxidoreductases are localized to subcellular compartments with widely varying redox environments (Rietsch & Beckwith, 1998). The two variable amino acids (-XX-) allow for the tuning of disulfide bond stability within the active site (Table 3-1) to suit the redox function and environment within the cell (Grauschopf *et al.*, 1995; Chivers *et al.*, 1997a). For example, in *Escherichia coli*, thioredoxin (Trx) has a CGPC active site, with $E^{\circ\prime} = -270$ mV (Moore *et al.*, 1964), and functions as a cytosolic reductant (Holmgren, 1985). DsbA has a CPHC active site, with $E^{\circ\prime} = -122$ mV (Huber-Wunderlich & Glockshuber, 1998), and catalyzes the oxidation of proteins that have been secreted to the *E. coli* periplasm (Bardwell *et al.*, 1991). PDI, a resident of the ER, has a CGHC active site, with $E^{\circ\prime} = -180$ mV (Lundström & Holmgren, 1993), and, in *Saccharomyces cerevisiae*, is essential for catalysis of the isomerization of non-native disulfide bonds (Laboissière *et al.*, 1995b). These three proteins are

believed to possess a high degree of tertiary structural similarity (Martin *et al.*, 1993; Kemmink *et al.*, 1996; Ferrari & Söling, 1999). Yet, their active-site disulfide bond stabilities differ by up to 143 mV (or, 6.8 kcal/mol at 30 °C) (Table 3-1).

The sequence of -XX- residues within the same active site can likewise make a large contribution to disulfide bond stability (Huber-Wunderlich & Glockshuber, 1998; Mössner *et al.*, 1998). For example, thioredoxin variants with CGHC and CPHC active sites (resembling those of PDI and DsbA, respectively) have active-site disulfide bonds that are destabilized by 35 and 75 mV (1.6 and 3.5 kcal/mol), respectively (Krause *et al.*, 1991; Mössner *et al.*, 1998). CGHC Trx also possesses greater functional resemblance to PDI than does wild-type Trx. *In vitro*, CGHC Trx is 10-fold more active than the wild-type enzyme at catalyzing native disulfide bond formation in ribonuclease A (RNase A) (Lundström *et al.*, 1992). *In vivo*, CGHC Trx, but not the wild type enzyme, can complement a PDI deficiency (Chivers *et al.*, 1996). The more PDI-like properties of the CGHC Trx variant demonstrate the importance of the active-site -XX- residues in determining the function of CXXC-containing proteins.

Removing the protein structural context can also change the stability of a CXXC disulfide (Table 3-1). For example, the stability of the active-site disulfide in CGPC (wild-type) Trx decreases by 75 mV (3.5 kcal/mol) upon denaturation in urea (Lin & Kim, 1989). The disulfide bond in the octapeptide WCGPCKHI is destabilized by 70 mV (3.3 kcal/mol) relative to that in native CGPC Trx (Siedler *et al.*, 1993). In contrast to the proteins mentioned above, CXXC-containing peptides lack tertiary structure and have a narrow range of disulfide bond stabilities, with $E^{\circ\prime}$ values typically in the range of –200 to –230 mV (Zhang & Snyder, 1989;

Siedler *et al.*, 1993). Thus, small CXXC-containing peptides are generally poor mimics of CXXC-containing proteins.

To mimic the $E^{\circ\prime}$ of PDI, a CXXC-containing peptide would be a poor choice because the disulfide bonds that form within these peptides are too stable. However, changing the number of intervening residues in $C(X)_nC$ peptides, where $0 \leq n \leq 5$, increases the range of accessible $E^{\circ\prime}$ values increases. Of these $C(X)_nC$ peptides, CXC peptides – those with only one intervening amino acid – tend to have the least stable disulfides (Zhang & Snyder, 1989). We synthesized the tripeptide CGC and found that its physical properties (disulfide $E^{\circ\prime}$ and thiol pK_a) are similar to those of PDI and, moreover, that CGC is a better catalyst of disulfide bond isomerization than is the dithiol BMC. Further, we created a variant of Trx with a CGC active site and find that this variant has much improved disulfide isomerization activity relative to the wild-type enzyme.

3.3 Materials and Methods

General. Fmoc-amino acids were purchased from Novabiochem (San Diego, CA). β-Hydroxyethyldisulfide was from Aldrich (Milwaukee, WI). RNase A (Type XII-A from bovine pancreas), reduced glutathione, DTNB, and *E. coli* thioredoxin reductase were from Sigma Chemical (St. Louis, MO). DTT and NADPH were from Fisher (Pittsburgh, PA). TCEP was from Fluka (Buchs, Switzerland). Enzymes used for DNA manipulations were from either Promega (Madison, WI) or New England BioLabs (Beverly, MA). Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). *E. coli* strain BL21(DE3) was from Promega (Madison, WI). All other reagents were of commercial grade or better and were used without further purification.

Manipulations of DNA and *E. coli*were carried out as described previously (Ausubel *et al.*, 1989). DNA was purified using either QIAprep spin minipreps or QIAquick gel extraction kits from QIAGEN (Valencia, CA). Plasmids were transformed into *E. coli* cells with a GenePulser electroporator (Bio-Rad, Richmond, CA). DNA sequencing was performed using a BigDye cycle sequencing kit from Perkin Elmer Life Sciences (Foster City, CA) and a DNA thermal cycler from Perkin Elmer Cetus (Norwalk, CT). The DNA sequencing reactions were analyzed at the University of Wisconsin–Madison Biotechnology Center with an ABI 377XL automated DNA sequencer from PE Biosystems (Foster City, CA).

The absorbance of UV and visible light was measured on a Cary Model 3 spectrophotometer equipped with a Cary temperature controller (Varian, Sugar Land, TX). HPLC was performed using a Waters system equipped with either a Vydac analytical C18 column or a Dynamax preparative C18 column.

Thioredoxin concentrations were determined using UV spectroscopy, with $\varepsilon = 13{,}700\ M^{-1}\cdot cm^{-1}$ at 280 nm (Krause *et al.*, 1991). Thiol concentrations were determined by using the method of Ellman (Ellman, 1959).

Synthesis of CGC. The tripeptide Cys-Gly-Cys-NH_2 was synthesized at the University of Wisconsin–Madison Biotechnology Center on an amide resin (Applied Biosystems) using an Applied Biosystems 432A peptide synthesizer (25 μmol scale) with Fmoc amino acids. The thiol groups of cysteine were protected with trityl groups. The peptide was deprotected and cleaved from the resin with 95:2.5:2.5 TFA:EDT:H_2O. The resin was removed by filtration and the filtrate was concentrated under reduced pressure.

CGC was purified by precipitation in ice cold ether (10 mL), dissolution of the resulting white solid in 1% (w/v) aqueous acetic acid, and then reversed-phase HPLC. This method produced 2.9 mg CGC (41% yield). The peptide was found to be pure by electrospray ionization mass spectrometry (*m/z* calculated 281, found 281).

Reduction Potential of CGC. To assess the stability of the cyclic disulfide bond formed by CGC, thiol-disulfide equilibria were established between the dithiol CGC (370 μM) and β-hydroxyethyldisulfide (810 μM) and analyzed by HPLC as described previously (Woycechowsky *et al.*, 1999). The equilibrium constant was determined from the peak areas of reduced and oxidized β-hydroxyethyldisulfide. The reduction potential for the CGC disulfide ($E^{\circ\prime}_{CGC}$) was calculated from the equilibrium constant using the Nernst equation and $E^{\circ\prime}_{\beta\text{-hydroxyethyldisulfde}} = -260$ mV (Lees & Whitesides, 1993). The reported values are the average (± SD) of three measurements.

Thiol pK_a values of CGC. Thiol titration curves were obtained by measuring A_{238} as a function of pH for a series of solutions containing reduced CGC (63 μM) in 0.10 M potassium phosphate buffers and analyzed using a double titration model as described previously (Woycechowsky *et al.*, 1999).

Disulfide Bond Isomerization Activity. The activation of scrambled RNase A (sRNase A), a fully oxidized protein containing predominantly non-native disulfide bonds, is indicative of disulfide bond isomerization. Only those RNase A molecules possessing a full set of four native disulfide bonds show ribonucleolytic activity. sRNase A was obtained by air oxidation of fully reduced RNase A (70 μM) in 20 mM Tris–HCl buffer, pH 8.0, containing guanidine–HCl (6.0 M) and EDTA(1.0 mM) over a period of several days followed by exhaustive dialysis into

0.10 M Tris–HCl buffer, pH 7.6, containing EDTA (1.0 mM). This method produced RNase A with <0.1 mol thiol/mol protein and approximately 1% of the ribonucleolytic activity of native RNase A. The activation of sRNase A was monitored and analyzed as described previously (Woycechowsky *et al.*, 1999).

Construction of plasmid coding for ΔP34 Trx. pTRX is a plasmid that directs the expression of wild-type Trx in *E. coli* (Chivers *et al.*, 1997b). The codon for Pro34 was deleted from the *trxA* gene by oligonucleotide-mediated site-directed mutagenesis using the oligonucleotide KW08 (5′ GGGCAGAGTGGTGCGGTTGCAAAATGATCGCCCC 3′) to yield plasmid pΔP34TRX.

Production and Purification of Wild-Type and ΔP34 Trx. Production of wild-type and ΔP34 Trx was carried out by the expression of wild-type or mutant *trxA* in *E. coli* BL21(DE3) cells that had been transformed with either pTRX or pΔP34TRX, as described previously (Chivers *et al.*, 1997b). The Trx proteins isolated by this procedure are oxidized completely.

Oxidized wild-type Trx was purified as described previously (Chivers *et al.*, 1997b). Reduced wild-type Trx was generated by the addition of DTT (15 mg, 0.10 mmol) to the purified protein (5 mg, 0.43 μmol) in 1.0 mL of 0.10 M Tris–HCl buffer, pH 7.6, containing EDTA (1.0 mM). Following incubation overnight at 4 °C, reduced wild-type Trx was purified with a Pharmacia Hi-Trap desalting column and found to contain 2.0 mol thiol/mol protein.

Purification of ΔP34 Trx on a Pharmacia FPLC Hi-Load Sephadex G-75 column followed by SDS–PAGE in the presence and absence of DTT revealed that most of the protein formed disulfide-linked oligomers, as was seen for a Trx containing a CAC active site (Gleason *et al.*, 1990). To disrupt these oligomers, 0.10 M DTT was added to the cell lysis buffer, which

was 20 mM Tris–HCl buffer, pH 7.8, containing urea (6.0 M) and EDTA (1.0 mM). The crude, reduced protein was purified in the same manner as was oxidized wild-type Trx. To produce oxidized monomers of ΔP34 Trx, 0.05 mL of a solution of DTNB (25 mM, 1.3-fold molar excess) was added to 55 mL of a solution of reduced, purified protein (18 μM) in 0.10 M potassium phosphate buffer, pH 7.6. The reaction mixture was stirred in the dark at room temperature for 1 h, and then dialyzed against 4 L of 20 mM imidazole–HCl buffer, pH 7.6, at 4 °C. The protein was concentrated to <10 mL with a Vivaspin concentrator (Vivascience, MWCO 5000) and purified by Mono-Q column chromatography (Pharmacia) as described previously (Chivers *et al.*, 1996). The protein eluted as two peaks and neither contained detectable thiols. Both proteins were monomeric, as judged by SDS–PAGE. The protewin in the first peak, constituting 82% of the total protein, co-eluted with wild-type Trx, and this protein was used in all subsequent experiments. The protein in the second peak eluted later on the NaCl gradient and could arise from deamidation of an asparagine residue.

Thermal Stability of ΔP34 Trx. The value of T_m, which is the temperature at the midpoint of the thermal unfolding transition, provides a measure of the conformational stability of a protein. To assess the consequences of Pro34 deletion on conformational stability, T_m values were determined for both reduced and oxidized ΔP34 Trx. The decrease in A_{280} upon unfolding of Trx was used to report on the equilibrium between native and unfolded Trx. To obtain thermal unfolding curves, a solution of oxidized ΔP34 Trx was exchanged into 20 mM potassium phosphate buffer, pH 7.0, using a Vivaspin concentrator. A buffer versus buffer blank was performed in matched cuvettes at 280 nm. The absorbance of protein (1.6 mL of a 0.16 mg/mL solution) was recorded at the initial temperature (25 °C). The temperature was increased slowly

(0.15 °C/min) to 80 °C, and the A_{280} was recorded in 1-°C increments. Upon reaching 80 °C, a similar temperature scan was performed in the reverse direction, and the temperature-dependent change in A_{280} was found to be >90% reversible. The thermal unfolding curves for reduced ΔP34 Trx were obtained in the same manner, except for the presence of 1 mM TCEP in the buffer to protect the protein from oxidation. A two-state model was used to fit the data in a plot of the fraction of unfolded protein molecules versus temperature. Values of T_m were determined as described previously (Pace *et al.*, 1989; Klink *et al.*, 2000).

Thioredoxin reductase assay of wild-type Trx and its ΔP34 variant. To test for perturbation of Trx structure caused by the deletion of Pro34, TR activity was measured with wild-type Trx and its ΔP34 variant as substrates. In this assay, TR (1 μL of a 0.1 mg/mL solution) was added to 0.6 mL of 10 mM potassium phosphate buffer, pH 7.0, containing NADPH (25 μM), and wild-type Trx or its ΔP34 variant (10 μM). The decrease in A_{340} over time upon reduction of NADPH ($\varepsilon = 6200\ M^{-1}\cdot cm^{-1}$) was measured and used to assess the ability of ΔP34 Trx to act as a substrate for TR, relative to wild-type Trx.

Reduction potential of ΔP34 Trx. The reduction potential of the active-site disulfide bond in oxidized ΔP34 Trx was estimated by measuring the equilibrium constant for the TR-catalyzed oxidation of NADPH ($E^{\circ\prime} = -315$ mV (Clark, 1960)) as described previously (Chivers *et al.*, 1996).

3.4 Results

Design and Properties of CGC. The simplest mechanism for catalysis of disulfide bond isomerization begins with the nucleophilic attack of a thiolate by the catalyst on a substrate

disulfide bond to form a covalently-linked catalyst-substrate complex (Darby *et al.*, 1994; Chivers *et al.*, 1998). Subsequent thiol–disulfide exchange reactions within the substrate lead to native disulfide bonds and, eventually, release of the catalyst. For a given dithiol, the fraction of molecules in the thiolate form under specified solution conditions (pH and $E_{solution}$) is governed by both the disulfide $E^{\circ\prime}$ and thiol pK_a. CGC was chosen as a promising reagent for the isomerization of disulfide bonds for two reasons. First, the disulfide bond in CXC peptides is relatively unstable (Zhang & Snyder, 1989). Second, a favorable Coulombic interaction with the *N*-terminal amino group stabilizes a thiolate in the sidechain of the *N*-terminal cysteine residue. The *C*-terminus was amidated to prevent the carboxylate from destabilizing a thiolate in the side chain of the *C*-terminal cysteine residue. The tripeptide CGC (Table 3-2) was synthesized by standard solid-phase peptide synthesis methods. The HPLC-purified peptide was subjected to tests of its physical properties (disulfide $E^{\circ\prime}$ and thiol pK_a) and disulfide isomerization activity (Table 3-2).

Thiol groups undergo a large increase in absorbance at 238 nm (A_{238}) upon deprotonation (Benesch & Benesch, 1955). The A_{238} was used to measure the equilibrium between dithiol, thiolate-thiol, and dithiolate over a pH range spanning mostly protonated to mostly unprotonated species (Figure 3-1). A fit of the data to a double titration model gives thiol pK_a values of 8.7 and 9.8 (Table 3-2).

The reduction potential of the CGC disulfide bond was determined by measuring the equilibrium constant for reduction of a reference molecule with a known reduction potential, β-hydroxyethyldisulfide ($E^{\circ\prime} = -260$ mV) (Lees & Whitesides, 1993). Using HPLC analysis of the thiol–disulfide exchange equilibrium between CGC and β-hydroxyethyldisulfide, an $E^{\circ\prime}$ value of

-167 ± 1 mV was determined for CGC (Table 3-2). This value is similar to the $E^{\circ\prime}$ value of -169 mV reported for the peptide YSRCGC (Zhang & Snyder, 1989).

Catalysis of disulfide bond isomerization requires the provision of a reactive thiolate. The disulfide bond isomerization activity of the dithiol CGC was assayed by monitoring the increase in ribonucleolytic activity upon addition of randomly oxidized, predominantly misfolded RNase A (scrambled RNase A). Figure 3-2 shows that reduced CGC folds scrambled RNase A more efficiently than does either reduced glutathione, a tripeptide monothiol commonly used for protein folding *in vitro*, or BMC, a dithiol previously shown to possess high RNase A unscrambling activity (Woycechowsky *et al.*, 1999). The specific activity of CGC (Table 3-2) is 2.2-fold higher than that of BMC (56 μmol RNase A activated per min per mol BMC). This rate increase is particularly striking given that the first thiol pK_a is 0.4 units higher for CGC than for BMC (thiol pK_a = 8.3).

Having characterized the physical properties and disulfide bond isomerization activity of CGC (Table 3-2), we next explored the consequences of inserting this sequence of three amino acids into the context of a folded protein.

Design and Properties of ΔP34 Trx. E. coli Trx is a small (108 residues, 11.7 kDa), stable, single-domain protein that is thought to act as a cellular reductant for ribonucleotide reductase and other proteins. Trx effects redox catalysis through its CGPC active site (residues 32–35) (Chivers & Raines, 1997). The reducing activity of Trx requires a stable oxidized form (disulfide $E^{\circ\prime} = -270$ mV) and a reactive reduced form (thiol pK_a = 7.5) (Chivers *et al.*, 1997b). Althogh Trx can catalyze native disulfide bond formation (Pigiet & Schuster, 1986; Boniface & Reichert, 1990; Hawkins *et al.*, 1991), the stability of its active-site disulfide bond limits its

effectiveness. Trx variants with destabilized disulfide bonds do, however, show enhanced ability to catalyze native disulfide bond formation (Lundström *et al.*, 1992; Chivers *et al.*, 1996). Given the instability of CXC disulfide bonds in peptides relative to CXXC disulfide bonds (Zhang & Snyder, 1989), we hypothesized that deletion of the active-site proline in wild-type (CGPC) Trx would increase catalytic activity.

ΔP34 Trx was produced by heterologous overexpression in *E. coli* of the mutant *trxA* gene (Chivers *et al.*, 1997b). Gel filtration chromatography of cell lysates gave multiple peaks containing ΔP34 Trx. SDS–PAGE indicated that the majority of the ΔP34 Trx formed disulfide-linked oligomers. After addition of DTT to the cell lysis buffer, ΔP34 Trx eluted from the gel filtration column as a monomer in a single peak and was fully reduced (2 mol thiol/mol Trx). The yield of purified protein was typically 100 mg per L of culture.

The tendency of ΔP34 Trx to form disulfide-linked oligomers indicates that the deletion of Pro34 destabilizes the active-site disulfide bond. This deletion may also disrupt the Trx structural scaffold. We used both thermodynamic and functional assays to search for deleterious effects on the structure and stability of ΔP34 Trx.

To discern any destabilization to the Trx tertiary structure caused by deletion of Pro34, we examined the thermal denaturation behavior of ΔP34 Trx. The value of T_m is used to report on conformational stability. Both reduced and oxidized ΔP34 Trx show reversible thermal unfolding behavior (Figure 3-3), and both redox states of ΔP34 Trx are less stable than are the corresponding redox form of wild-type Trx (Table 3-3). Interestingly, the oxidized form of ΔP34 Trx is slightly less stable than is the reduced form. The increase in thermal stability upon disulfide bond reduction indicates that the active-site disulfide bond destablizes the Trx tertiary

structure, in marked contrast to the increased stability imparted by the wild-type disulfide bond (Ladbury *et al.*, 1994). The thermal denaturation data also indicate that, while deletion of Pro34 causes significant destabilization of both redox states, ΔP34 Trx still maintains high conformational stability (Figure 3-3).

Wild-type Trx is a substrate for thioredoxin reductase, an enzyme that catalyzes the specific reduction of the Trx active-site disulfide bond by NADPH to form $NADP^+$ (Moore *et al.*, 1964). The rate of decrease in A_{340} upon oxidation of NADPH can be used to report on the ability of a Trx variant to act as a substrate for TR and thus, indirectly, on the structural similarity between the variant and wild-type Trx. The rate of TR-catalyzed NADPH oxidation was 3.9-fold lower with ΔP34 Trx than with wild-type Trx. This only slight decrease provides a further indication that any structural perturbation induced by deletion of Pro34 is small and is in gratifying agreement with the 2.1-fold lower k_{cat} reported for CAC Trx as a TR substrate (Gleason *et al.*, 1990).

The TR assay could in theory be used to estimate the equilibrium constant for reduction of ΔP34 Trx by NADPH ($E^{\circ\prime} = -310$ mV (Clark, 1960)). Yet, NADPH reduces ΔP34 Trx beyond the detection limit of this assay. Attempts to shift the equilibrium by the addition of excess $NADP^+$ resulted in no detectable NADPH formation (Figure 3-4). Although this assay failed to establish an $E^{\circ\prime}$ value for ΔP34 Trx, it did provide a lower limit of $E^{\circ\prime} \geq -200$ mV. The active-site disulfide of ΔP34 Trx is markedly destabilized relative to that of wild-type Trx, which has $E^{\circ\prime} = -270$ mV.

The sRNase A assay was performed to assess the disulfide bond isomerization activity of reduced ΔP34 Trx *in vitro*. As shown in Figure 3-5, reduced ΔP34 Trx reactivated sRNase A. In

contrast, reduced wild-type Trx did not significantly reactivate sRNase A. Deletion of Pro34 endows reduced Trx with a greatly enhanced ability to catalyze disulfide bond isomerization. Incorporation of CGC into this protein scaffold enhances the specific activity of the CGC sequence by 25-fold, relative to the tripeptide (Table 3-2).

3.5 Discussion

Comparison of the CXC and CXXC Motifs. The rational design of catalysts of protein folding is an important and ongoing challenge. Enzymes, such as PDI, that catalyze native protein disulfide bond formation utilize CXXC active sites (Chivers *et al.*, 1998; Rietsch & Beckwith, 1998). This active-site motif imparts a great deal of functional flexibility to thiol-disulfide oxidoreductases. Variations in the sequence of -XX- residues give rise to significant changes in both disulfide $E^{\circ\prime}$ and thiol pK_a and allow for tuning of the active site to suit a biological function (Chivers *et al.*, 1997a).

For example, Trx and DsbA are two *E. coli* CXXC-containing proteins with high structural similarity. Yet, the CPHC active site of DsbA has a disulfide $E^{\circ\prime}$ that is 145 mV more positive (that is, the disulfide bond is 6.8 kcal/mol less stable) and a thiol pK_a that is 4 units lower (that is, the thiolate is 5.6 kcal/mol more stable) than that of the CGPC active site in Trx. These physical differences are important for the proper function of each CXXC protein in its cellular environment. Moreover, for both Trx and DsbA the $E^{\circ\prime}$ and pK_a can be changed by changing the sequence of -XX- residues. Most attempts at tuning the physical properties of CXXC proteins have relied on simple swapping of one naturally occurring -XX- sequence for another (Huber-Wunderlich & Glockshuber, 1998; Mössner *et al.*, 1998) or making libraries that

randomly replace each X residue with any of the twenty amino acids (Grauschopf *et al.*, 1995; Chivers *et al.*, 1996; Holst *et al.*, 1997). Only one attempt has been made to examine the functional consequences of altering the number of intervening amino acids in a CXXC motif (Gleason *et al.*, 1990).

Small peptides are good candidates for the design of new protein folding reagents. Their desirable traits include the ease of peptide synthesis, the chemical variety of the constituent amino acid building blocks, and the ability to incorporate interesting sequences into proteins by using the genetic code. Short CXXC-containing peptides (7–10 residues) with sequences corresponding to naturally occurring oxidoreductases have been shown to catalyze native disulfide bond formation in RNase A (Moroder *et al.*, 1996). The activities of these peptides correlate qualitatively with their disulfide $E^{\circ\prime}$ values. Peptides with less negative (that is, more PDI-like) $E^{\circ\prime}$ values tend to have higher activities. Still, there is much room for improvement, as a CXXC peptide will likely form a more stable disulfide bond than that of the CGHC active site in PDI. Recently, the properties of these CXXC peptides have been improved upon through backbone cyclization, which can both destabilize the disulfide bond and lower the thiol pK_a (Cabrele *et al.*, 2002; Cattani-Scholz *et al.*, 2002).

Rather than mimic structure, that is, the amino acid sequence and disulfide loop size of PDI, we suspected that it could be better to base the design of a peptide mimic on function, that is the values of disulfide $E^{\circ\prime}$ and thiol pK_a. CXC peptides can form 11-membered disulfide-bonded rings within a γ-turn (Kishore & Balaram, 1985) and have $E^{\circ\prime}$ values that are typically 30–40 mV (1.4–1.9 kcal/mol) less stable than CXXC peptides, whose 14-membered rings can be stabilized by a β-turn (Moroder *et al.*, 1996). The structural basis for the relative instability of

CXC disulfide-bonded rings relative to CXXC disulfide-bonded rings is not entirely clear. For cycloalkanes, 11-membered rings have higher strain energy than do 14-membered rings (Dunitz, 1968). This higher strain probably results from unfavorable transannular steric interactions in 11-membered rings that are relieved by enlarging the ring to 14 atoms (Smith & March, 2001). Further, a 14-membered ring in a CXXC peptide may have more favorable transannular hydrogen bonding (Moroder *et al.*, 1996) than an 11-membered ring in a CXC peptide. The destabilization caused by shortening the disulfide ring from 14 to 11 amino acids provides a good starting point in the design of short peptides with improved disulfide bond isomerization activity.

Cyclic CXC disulfide bonds are rarely found in Nature. The few known examples include CSC in Mengo encephalomyelitis virus coat protein (Krishnaswarmy & Rossman, 1990), CDC in Bacillus Ak.1 protease (Smith *et al.*, 1999), and CTC in the *E. coli* chaperone Hsp33 (Jakob *et al.*, 1999). The last case is particularly interesting because the CXC motif is thought to act as a redox switch for chaperone function. CXC sequences are commonly found in cystine-rich peptides such as endothelins, sarafotoxins, and bee and scorpion venom toxins, but the bioactive forms of these peptides never contain a cyclic CXC disulfide bond. Indeed, these peptides may utilize the CXC sequence as a means of decreasing the number of accessible disulfide-bonded isomers. During oxidative folding, non-native CXC disulfide bonds form less easily than the native disulfide bonds, which form larger rings. The high energetic cost of forming a non-native CXC disulfide bond may preclude its formation and by thus restricting the set of oxidized intermediates, help to guide proper folding of these peptides (Tamaoki *et al.*, 1998).

Recently, a CXC motif was found in the thiol oxidase Erv2p, a yeast flavo-enzyme. The mechanism of this enzyme is thought to involve a disulfide relay, with a CGC sequence near the C-terminus shuttling electrons from substrate proteins to a CGEC sequence, adjacent to the flavin (Gross *et al.*, 2002). Apparently, the enzyme takes advantage of a relatively strained CXC disulfide bond to effect efficient oxidation.

The instability of cyclic CXC disulfide bonds relative to CXXC disulfides may make the CXC motif more desirable than the CXXC motif in the design of new catalysts of disulfide bond isomerization. The smaller number of accessible conformations in CXC than CXXC may also restrict the perturbation of disulfide bond stability induced by tertiary structural context. If so, the CXC motif may prove to be a useful module that can be inserted into novel protein folding enzymes.

Design and properties of CGC. The tripeptide CGC was synthesized and found to have a disulfide $E^{\circ\prime}$ more similar to that of PDI than is the disulfide $E^{\circ\prime}$ of a CGHC-containing peptide (which matches the sequence of the PDI active site (Tables 3-1 and 3-2)). The CGC disulfide bond is 50 mV (or, 2.3 kcal/mol) less stable than that of the CGHC-containing peptide. The narrow range of disulfide $E^{\circ\prime}$ in CXC peptides (-167 to -190 mV) (Zhang & Snyder, 1989) clusters near that of the PDI active site ($E^{\circ\prime} = -180$ mV (Lundström & Holmgren, 1993)) and suggests that a CXC module may be insensitive to substitution at the X position.

An additional strategy in the design of our CGC peptide was to stabilize the formation of a thiolate through Coulombic interactions. The sulfur of the *N*-terminal cysteine is 3 atoms away from a positively charged nitrogen atom and this positive charge should lower the thiol pK_a. The carboxylate of the *C*-terminal cysteine was amidated to prevent any additional negative charge

from elevating the thiol pK_a. Indeed this strategy was successful in lowering the first thiol pK_a of CGC by 0.3 units relative to glutathione (thiol pK_a = 9.0 (Woycechowsky *et al.*, 1999)). Nonetheless, the thiol pK_a is still 2 units higher than that of PDI (pK_a = 6.7) (Hawkins & Freedman, 1991). The addition of cationic residues (such as arginine or lysine) to a CXC-containing peptide could further depress the thiol pK_a.

The CGC peptide is able to use its favorable disulfide $E^{\circ\prime}$ and thiol pK_a to fold sRNase A. At the same total thiol concentration, the protein folding activity of reduced CGC is superior to that of both the monothiol glutathione and the dithiol BMC. Glutathione is a constituent of the redox buffers most commonly used for *in vitro* protein folding (Saxena & Wetlaufer, 1970; Lyles & Gilbert, 1991a). The higher yield of active RNase A relative to glutathione is likely attributable to the favorable properties of CGC. Glutathione likely becomes trapped in mixed disulfide bonds with RNase A. The $E^{\circ\prime}$ for CGC is approximately 70 mV higher than that for BMC ($E^{\circ\prime}$ = –240 mV) (Woycechowsky *et al.*, 1999). The relative instability of its disulfide bond may underlie the superior activity of CGC. BMC is more likely to form a cyclic disulfide bond, releasing a partially reduced substrate. Protein folding then requires the reoxidation of this reduced substrate by oxidized BMC. CGC could rely less on the reduction-reoxidation pathway because of its more favorable $E^{\circ\prime}$. Also, the unstable CGC disulfide bond is likely to be a more efficient oxidant of reduced substrates. The higher activity of CGC relative to BMC, despite its higher thiol pK_a, underscores the importance of disulfide $E^{\circ\prime}$ for catalysis of disulfide bond isomerization.

Design and Properties of ΔP34 Trx. It is unlikely that non-covalent interactions contribute to the protein folding activity of the peptide CGC. The peptide was designed as a

minimal unit for the induction of covalent rearrangements within misfolded proteins and likely uses only covalent catalysis. CXXC-containing oxidoreductases can, however, take advantage of both covalent and non-covalent interactions in the catalysis of disulfide bond rearrangements. For example, PDI has a substrate binding site with affinity for unfolded proteins (Noiva *et al.*, 1993; Klappa *et al.*, 1998). Such binding affinity increases the effective concentration of the CXXC active site relative to substrate sulfur atoms. The isolated catalytic domains of PDI, lacking the peptide binding site, suffer a 7- to 12-fold decrease in their ability to fold scrambled RNase A (Darby & Creighton, 1995b). Further, isomerization of partially oxidized proteins with a high degree of tertiary structure is often the rate limiting step in protein folding. Such intermediates have cysteine residues that are buried within a stable structure, making them inaccessible to thiol-disulfide exchange. Isomerization of these intermediates requires a transient global unfolding step followed by thiol–disulfide interchange, leading to native disulfide bond formation (Narayan *et al.*, 2000; Chang & Li, 2002). Capture of these transiently unfolded species is critical for efficient catalysis of protein folding (Weissman & Kim, 1993; Darby *et al.*, 1998; Freedman *et al.*, 2002).

Insertion of a CGC module into a stable protein scaffold with affinity for unfolded proteins could result in a molecule with enhanced catalytic activity. Trx is a single-domain, heat-stable protein with affinity for proteins such as thioredoxin reductase and ribonucleotide reductase (Holmgren, 1985). Although Trx acts as a cellular reductant, it can also catalyze native disulfide bond formation using its CGPC active site (Pigiet & Schuster, 1986; Boniface & Reichert, 1990; Hawkins *et al.*, 1991). Yet, its catalytic efficiency is diminished by the stabilization imparted to its active-site disulfide bond by the protein scaffold, and efficient

folding requires a large excess of Trx (Pigiet & Schuster, 1986). Studies on a Trx variant with a CAC active site have indicated that the protein scaffold is robust enough to withstand deletion of Pro34 (Gleason *et al.*, 1990). The diminished disulfide reductase activity observed for the CAC Trx variant suggest that, in the Trx active site, a CXC disulfide bond is less stable than is the naturally occurring CGPC disulfide bond. Deletion of the active-site proline residue gives an active-site sequence of CGC with a destabilized active-site disulfide and improved disulfide bond isomerization activity.

The destabilization of the Trx active-site disulfide bond upon Pro34 deletion is at least as great as the stabilization imparted to the CGPC disulfide bond by the Trx scaffold (Table 3-1). The CGC peptide disulfide bond is 30 mV (1.4 kcal/mol) less stable than is the CGPC peptide disulfide bond. In the context of the Trx active site, the CGC disulfide bond is ≥70 mV (≥3.3 kcal/mol) less stable than is the CGPC disulfide bond. Within the Trx active site, some of the increased destabilization of the CGC disulfide relative to the CGPC disulfide bond (≥1.9 kcal/mol) could stem from stabilization of the Cys32 thiolate, as has been observed for CXXC Trx variants (Chivers *et al.*, 1996; Mössner *et al.*, 1998). Such thiolate stabilization could stem from better alignment of Cys32 with an α-helix dipole (Kortemme & Creighton, 1995) or from enhanced electrostatic interactions with neighboring residues (Kortemme *et al.*, 1996), such as Lys36 (in the wild-type Trx numbering system). Alternatively, CXXC could possess stabilizing packing interactions with the Trx scaffold that are absent in ΔP34 Trx. In this case, differences in $E^{\circ\prime}$ result from changes in standard state reduction potential (E°) rather than changes in the stability of titratable groups (pK_a) (Chivers *et al.*, 1997a).

The active site (Cys32–Cys35) of Trx spans the first turn of an α-helix (Katti *et al.*, 1990). Therefore, it was important to examine the consequences of deleting Pro34 on Trx structure and stability. ΔP34 Trx suffers only a modest (3.9-fold) drop in activity as a substrate for TR. The activity of ΔP34 Trx in the TR assay indicates that deletion of Pro34 does not drasticaly alter the structure of Trx.

Deletion of Pro34 is deleterious to the conformational stability of the Trx fold. The T_m values of reduced and oxidized ΔP34 Trx are 61 and 65 °C, respectively (Table 3-3). In wild-type Trx, the linkage relationship between disulfide bond formation and protein stability is well-established (Lin & Kim, 1989). The T_m of wild-type Trx decreases by 12 °C upon disulfide bond reduction (Ladbury *et al.*, 1994). In contrast, the stability of the variant Trx increases slightly upon reduction (Figure 3-3), providing an additional indication of the relative instability of the CGC disulfide bond in the Trx active site.

Reduced ΔP34 Trx has greater disulfide bond isomerization activity than does wild-type Trx. Indeed, fully reduced wild-type Trx showed no detectable activation of sRNase A (Figure 3-5). Protein folding with wild-type Trx requires a large excess of the oxidized form (Pigiet & Schuster, 1986), as wild-type Trx is more likely to cause substrate reduction rather than isomerization. The much greater activity of the variant provides an indication of the importance of $E^{\circ\prime}$ for catalysis of disulfide bond isomerization. Deletion of Pro34 creates a Trx active site with a more favorable $E^{\circ\prime}$ value, and thus endows reduced Trx with a new and useful property.

The higher specific activity of ΔP34Trx relative to the CGC peptide (Table 3-2) could result from a higher substrate binding affinity for ΔP34Trx relative to the CGC peptide. The CGC peptide likely utilizes only covalent catalysis. In addition to optimizing the covalent

interactions of the catalyst with disulfide-bonded proteins, through manipulation of thiol pK_a and disulfide $E^{\circ\prime}$, the design of non-covalent interactions of the catalyst with substrate proteins could lead to further enhancements in catalytic efficiency.

3.6 Prospectus

Small-molecule redox reagents can be useful for protein folding *in vitro* because they tend to be less expensive and more easily separable from the folded protein product than PDI. The CXC motif is a promising lead for the design of improved peptide redox buffers for protein folding. The placement of appropriately charged amino acids within or around a CXC motif could improve further the efficiency of thiol–disulfide interchange reactions either through decreased thiol pK_a (and, consequently, increased $E^{\circ\prime}$) or increased affinity for charged substrates. Additional hydrophobic residues could also provide non-covalent interactions with unfolded proteins. Recently, a 19-residue peptide corresponding to the functional element of α-A-crystallin was reported to have chaperone activity (Bhattacharyya & Sharma, 2001), suggesting that it is possible to endow peptides with affinity for unfolded proteins. The fusion of CXC to such a peptide could enhance further its protein folding activity.

Engineered thiol–disulfide oxidoreductases could be useful for protein folding *in vivo*. The co-production of such an enzyme, with disulfide $E^{\circ\prime}$ and thiol pK_a values suited to the particular redox function and environment, could help to increase the yield of soluble, active protein in heterologous expression systems (Ostermeier *et al.*, 1996; Joly *et al.*, 1998; Bessette *et al.*, 1999; Mössner *et al.*, 1999). Changing the number as well as the nature of the intervening

residues in the CXXC motif provides a further means of engineering thiol-disulfide oxidoreductases.

The covalent attachment of a CXC-containing peptide sequence to the terminus of a protein folding substrate could provide a novel means for the acceleration of oxidative protein folding. *In vivo*, many proteins are synthesized and fold as pro-proteins (Eder & Fersht, 1995). A pro sequence is usually attached to the *N*-terminus of a newly synthesized protein and is cleaved by a protease after folding. *In vitro*, pro-BPTI , which is a protein containing three disulfide bonds in its native state, folds about three times faster and with an approximately two-fold higher yield of native protein than does mature BPTI (Weissman & Kim, 1992). The pro-sequence of BPTI contains a single cysteine residue that is required for this rate enhancement. Further, a single cysteine residue tethered to the *C*-terminal end of mature BPTI through an artificial $(Ser\text{-}Gly\text{-}Gly)_3$ linker provides rate and yield enhancements similar to those seen with pro-BPTI. Although the pro-sequence may not be important for the physiological folding of BPTI (Creighton *et al.*, 1993), these results suggest a general strategy for more efficient oxidative protein folding *in vitro*. Specifically, a CXC sequence could be attached to the terminus of a protein through an engineered linker containing a protease recognition site. This CXC-containing pro-protein should fold more efficiently and, after cleavage of the artificial pro sequence, yield the fully folded, native protein. Such "catalysis-in-cis" could provide a new route to more efficient protein production.

Table 3-1. Reduction potentials of CXXC sequences

Protein	–XX–	Native Protein E°′ (mV)[a]	Denatured Protein E°′ (mV)	Peptide Fragment E°′ (mV)
Trx	–GP–	–270[b]	–195[c]	–200[d]
PDI	–GH–	–180[e]	not determined	–220[d]
DsbA	–PH–	–122[e]	–200[f]	not determined

[a]Relative to $E^{\circ\prime}$ = –252 mV for glutathione (Lees *et al.*, 1993).

[b]Moore, 1964.

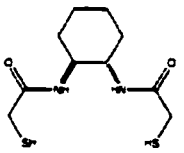
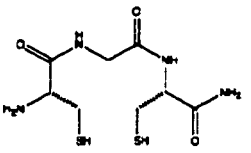
[c]Lin and Kim, 1989.

[d]Siedler *et al.*, 1993.

[e]Lundström and Holmgren, 1993.

[f]Huber-Wunderlich *et al.*, 1998.

Table 3-2. Properties of BMC, CGC, and ΔP34Trx.

	 BMC	 CGC	ΔP34Trx
Disulfide $E^{\circ\prime}$ (mV)	–240	–167	≥ –200
Thiol pK_a	8.3, 9.9	8.7, 9.8	not determined
Specific activity (U/mol)	56	132	3300

[a]Woycechowsky *et al.*, 1999.

[b]One unit will activate 1 μmol RNase A per min.

Table 3-3. Conformational stabilities of wild-type Trx and ΔP34Trx.

Trx	Oxidized T_m (°C)[a]	Reduced T_m (°C)[a]
wild-type[b]	85	73
ΔP34	61	65

[a]In 20 mM phosphate buffer, pH 7.0.

[b]Ladbury *et al.*, 1994.

Figure 3-1 Titration of CGC by UV spectroscopy. Titrations were carried out in 0.1 M potassium phosphate buffers at 25 °C. The pK_a values, obtained from a fit of the data to a double titration model (Woycechowsky *et al.*, 1999), are presented in Table 3-2.

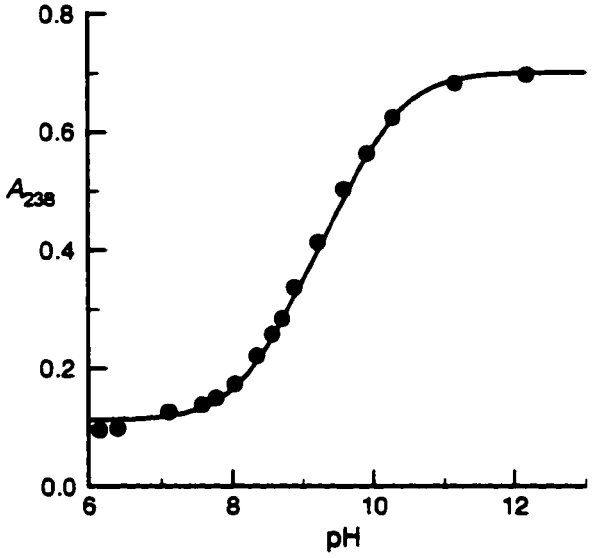

0.8
0.6
A_{238}
0.4
0.2
0.0
6
8
10
12
pH

Figure 3-2. Reactivation of sRNase A by CGC (0.5 mM), ●; BMC (0.5 mM), ■; and GSH (1.0 mM), O. The specific activities for CGC and BMC were determined from a fit of the data to a single exponential, as described previously (Woycechowsky *et al.*, 1999).

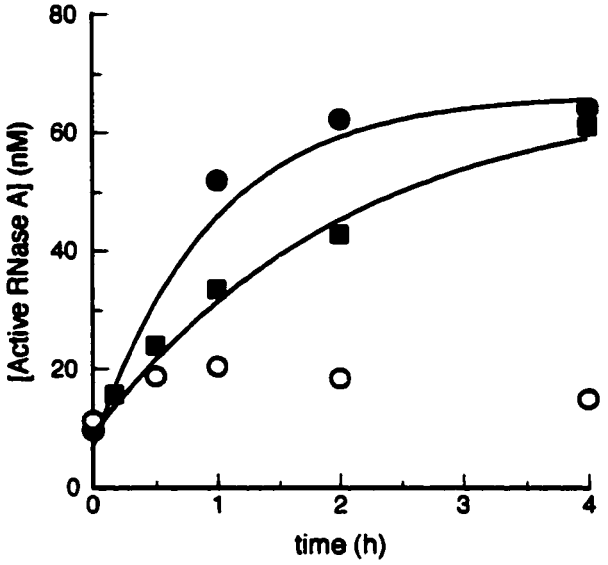

80
60
40
20
0
[Active RNase A] (nM)
0
1
2
3
4
time (h)

Figure 3-3. Thermal unfolding of reduced (◆) and oxidized (■) ΔP34 Trx. Values of T_m were determined from a plot of the fraction of unfolded ΔP34 Trx molecules (fraction unfolded) versus temperature (T). Fraction unfolded values were calculated by applying a two-state unfolding model to a plot of A_{280} versus temperature, as described previously (Klink *et al.*, 2000). Experiments were performed in 20 mM potassium phosphate buffer, pH 7.0, containing either 1 mM (reduced protein) or 0 mM (oxidized protein) TCEP. Values of T_m are listed in Table 3-3.

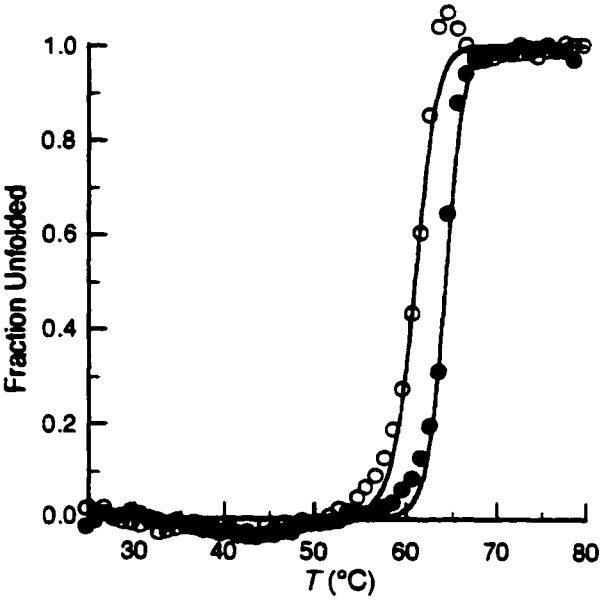

Fraction Unfolded
1.0
0.8
0.6
0.4
0.2
0.0
30
40
50
60
70
80
T (°C)

Figure 3-4 Redox equilibria between ΔP34 Trx and NADPH. TR (2.3 μg) was added to a 0.6 mL solution containing oxidized ΔP34 Trx (41 μM) and NADPH (33 μM) in 0.01 M potassium phosphate buffer, pH 7.0, at 25 °C. The concentration of NADPH at equilibrium was determined by using $\varepsilon = 6200\ M^{-1}\cdot cm^{-1}$ at 340 nm. To shift the equilibrium in the reverse direction, the concentration of $NADP^+$ was increased in 1.3 mM- increments. No increase in A_{340} was observed beyond that due to the intrinsic absorbance of $NADP^+$ ($\varepsilon = 60\ M^{-1}\cdot cm^{-1}$). Such an increase would be indicative of the formation of NADPH.

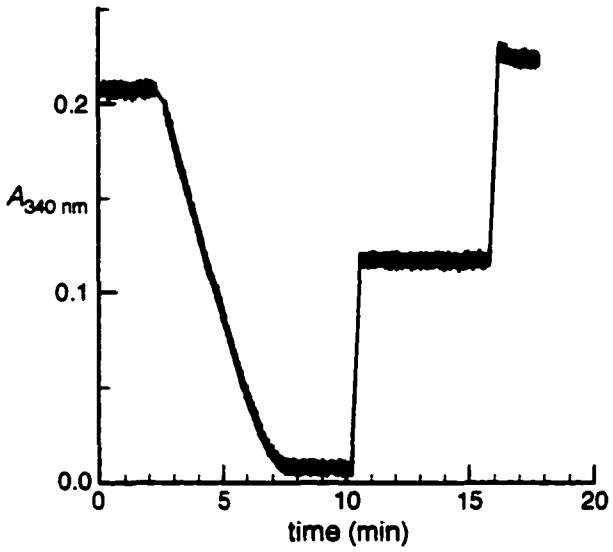
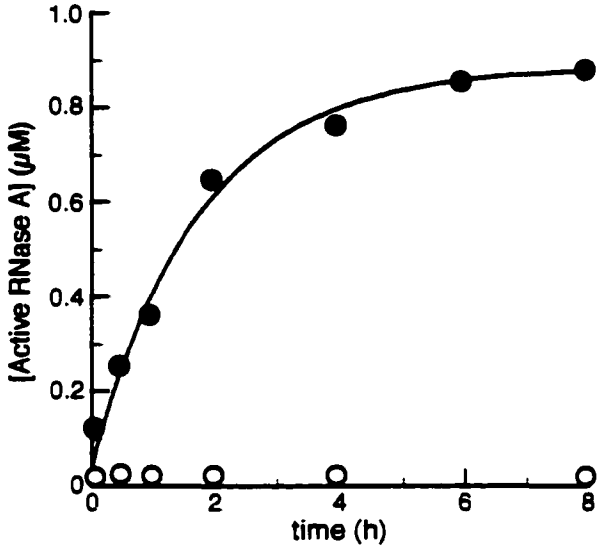

0.2
$A_{340\ nm}$
0.1
0.0
0
5
10
15
20
time (min)

Figure 3-5 Reactivation of sRNase A by reduced ΔP34 Trx (0.14 mM), ●; and reduced wild-type (0.10 mM), O. The specific activities for ΔP34 Trx was determined from a fit of the data to a single exponential, as described previously (Woycechowsky *et al.*, 1999), and is listed in Table 3-2.


1.0
0.8
0.6
0.4
0.2
0
[Active RNase A] (μM)
0
2
4
6
8
time (h)

Chapter Four

Protein Folding Using an Immobilized Dithiol

4.1 Abstract

The isomerization of disulfide bonds is often the rate-limiting step during protein folding. Dithiols can efficiently catalyze this process. Here, a symmetric trithiol is immobilized to two different solid supports. Coupling of an excess of trithiol to TentaGel resin produced immobilized dithiol in good yield. Incubation of a ribonuclease A substrate containing scrambled disulfide bonds (sRNase A) with dithiol immobilized on a TentaGel resin produces a 2-fold increase in ribonucleolytic activity relative to incubation of sRNase A with unmodified TentaGel resin. Coupling of excess trithiol to styrene-glycidyl methacryate microspheres produced a poor yield of immobilized dithiol. This poor yield is attributed to the high surface density of functional groups on the microspheres, which may make the coupled trithiol susceptible to cross-linking. Capping some of the reactive sites with *N*-methyl mercaptoacetamide prior to trithiol coupling decreases the density of reactive sites and increases the yield of coupled thiol by 14-fold relative to trithiol coupling with uncapped microspheres. Incubation of sRNase A with dithiol immobilized on microspheres produces produces a 17-fold increase in ribonucleolytic activity relative to incubation of sRNase A with unmodified microspheres, and this activity is 1.5-fold higher than that of a monothiol immobilized on the microspheres. These results show that both polymer structure and thiol structure require careful consideration in the design of solid-supported reagents for use in protein folding. The dithiol-decorated microsphere reported here provides a new strategy for increasing the efficiency of protein folding *in vitro*.

4.2 Introduction

Many useful proteins contain disulfide bonds. In such proteins, the attainment of the proper disulfide bonds during folding is critical for function. The formation of disulfide bonds in proteins requires a sufficiently oxidizing environment (Raines, 1997). For any given protein, the native disulfide bond arrangement is that which is most thermodynamically stable in its native environment (Anfinsen, 1973). *In vivo*, enzymes, such as protein disulfide isomerase (PDI), exist to increase protein folding efficiency. Much effort has been expended to improve the efficiency of *in vitro* folding for disulfide-bonded proteins (De Bernadez Clark, 2001).

The mechanism of oxidative folding includes both the oxidation of thiols and the isomerization of disulfide bonds. Often, protein disulfide bonds accumulate rapidly and the isomerization of oxidized intermediates is the rate-limiting step in protein folding (Givol *et al.*, 1964; Narayan *et al.*, 2000). The active site of PDI, the most efficient known catalyst of oxidative protein folding (Weissman & Kim, 1993), utilizes a -Cys-Gly-His-Cys- active site with a high disulfide $E^{\circ\prime}$ (–180 mV) and low thiol pK_a (6.7) to catalyze this step (Chivers *et al.*, 1998).

In vitro, oxidative protein folding is usually initiated by the addition of reduced and unfolded protein to a redox buffer. The most commonly used redox buffer consists of reduced and oxidized glutathione (GSH and GSSG, respectively). The glutathione system uses the dimeric disulfide GSSG to provide oxidizing equivalents and the monothiol GSH to catalyze disulfide bond isomerization (Saxena & Wetlaufer, 1970). The folding efficiency of this system shows a narrow optimum at concentrations around 1 mM GSH and 0.2 mM GSSG (Lyles & Gilbert, 1991a). These concentrations produce a solution potential similar to that of the endoplasmic reticulum ($E_{solution} = -180$ mV) (Hwang *et al.*, 1992). Increasing the total

concentration of glutathione decreases the yield of correctly folded protein because the stability of mixed disulfides between glutathione and protein increases at higher glutathione concentrations (Wetlaufer *et al.*, 1987; Rothwarf & Scheraga, 1993). Monothiols with lower thiol pK_a, such as *N*-mercaptoacetamide or 4-mercaptobenzoic acid, form less stable mixed disulfide bonds and can be used at higher concentrations to give faster folding rates than can glutathione (Woycechowsky *et al.*, 1999; Gough *et al.*, 2002).

Dithiols can form cyclic disulfides, and are thus less apt to form mixed disulfide bonds. At a constant solution potential, the folding efficiencies of dithiol-based redox buffers should show optima at much higher concentrations of thiol and disulfide than is seen with monothiol-based systems. Indeed, reduced ribonuclease A (RNase A) can fold in a redox buffer consisting of 0.06 mM reduced dithiothreitol (DTT) and 100 mM oxidized DTT ($E_{solution} = -230$ mV) with minimal mixed disulfide bond accumulation (Rothwarf & Scheraga, 1993). The addition of reduced *trans*-1,2-bis(mercaptoacetamido)cyclohexane (BMC) to a glutathione redox buffer increases the rate and yield of folding for an RNase A substrate containing non-native disulfide bonds (Woycechowsky *et al.*, 1999). Dithiol-based redox buffers with properties (disulfide $E^{\circ\prime}$ and thiol pK_a) similar to the PDI active site can provide a means for more rapid protein folding.

Immobilization of the redox reagent to a solid support could lead to improved folding efficiency by providing a facile means for both recycling of the redox reagent and separation of the folded protein from the redox reagent. Herein, a microsphere presenting a symmetric dithiol is shown to fold RNase A efficiently, and more efficiently than one presenting a monothiol. The immbilized dithiol is a new and useful tool for the production of proteins for pharmaceutical and biotechnical applications.

4.3 Materials and Methods

General. NovaSyn® TG bromo resin (bromo-Tentagel resin) was from NovaBiochem (San Diego, CA). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride, glycidyl methacrylate, lithium hydride, lithium *tert*-butoxide, styrene, triethylamine, and tris-(2-aminoethyl)amine were from Aldrich (Milwaukee, WI). Chloroacetyl chloride and thioacetic acid were from Acros Organics (Pittsburgh, PA). GSH and GSSG were from Sigma Chemical (St. Louis, MO). DTT was from Fisher (Pittsburgh, PA). NMA was from Fluka (Buchs, Switzerland). DMF was from J.T. Baker (Phillipsburg, NJ). All other reagents were of commercial grade or better and were used without further purification.

Synthesis of trithiol ***1***. The coupling of a trithiol with three-fold symmetry to a solid support through any one of its nucleophilic thiols results in the presentation of equivalent dithiols. Trithiol **1** (Figure 4-1) was synthesized by a route similar to that used in the previous syntheses of dithiols (Lamoureux & Whitesides, 1993; Woycechowsky *et al.*, 1999).

tris(2-chloroacetamidoethyl)amine. Chloroacetyl chloride (37.2 g, 0.33 mol) was added dropwise to water (0.50 L) containing tris(2-aminoethyl)amine (14.6 g, 0.10 mol) and K_2CO_3 (45.6 g, 0.33 mol). The solution was stirred on ice for 90 min. A white solid was collected by filtration and washed with 2 M K_2CO_3(aq). Solid K_2CO_3 was added to the filtrate until the pH reached 11. A second crop of white solid was collected by filtration. The two solid products were combined and the tris(chloroacetyl) product (10.6 g, 37%) was judged to be pure by spectroscopic analysis. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.09 (br, 3 H), 4.08 (s, 6 H), 3.37 (q, J = 5.7 Hz, 6 H), 2.65 (t, J = 5.6 Hz, 6 H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.4, 53.5, 42.6, 37.6 ppm; MS (ESI) calculated for $C_{12}H_{22}Cl_3N_4O_3$ m/z 375.1, found 375.2.

tris(2-acetylmercaptoacetamidoethyl)amine. The tris(2-chloroacetamidoethyl)amine product (5.0 g, 13 mmol) was dissolved in dichloromethane (0.15 L), and the resulting solution was cooled to 0 °C. Thioacetic acid (5.7 mL, 80 mmol) was added dropwise and with stirring to the cooled solution. Then, triethylamine (11 mL, 0.080 mol) was added dropwise and with stirring. The resulting yellow solution was allowed to warm slowly to room temperature and stirred under Ar(g) for 40 h. The reaction was quenched by the addition of 2 M K_2CO_3(aq). The layers were separated and the organic layer was washed a second time with 2 M K_2CO_3(aq). The organic layer was dried, filtered, and concentrated to yield a white solid. The powder was twice recrystallized from ethyl acetate. The tris-(thioacetate) product (4.5 g, 69%) was judged to be pure by spectroscopic analysis. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.05 (br, 3 H), 3.64 (s, 6 H), 3.28 (q, J = 5.5 Hz, 6 H), 2.56 (t, J = 5.6 Hz, 6 H), 2.40 (s, 9 H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 195.9, 168.2, 53.8, 37.9, 33.1, 30.1 ppm; MS (ESI) calculated for $C_{18}H_{31}N_4O_6S_3$ m/z 495.1, found 495.4.

tris(2-mercaptoacetamidoethyl)amine. The tris(2-acetylmercaptoacetamidoethyl)amine product (2.0 g, 40 mmol) was dissolved in 0.10 L of 1.2 M HCl in methanol. The resulting clear solution was incubated at room temperature under Ar(g) for 20 h. The acetic acid by-product was removed by bubbling Ar(g) through the solution and the solvent was removed under reduced pressure to yield a yellow oil. The resulting oil was dissolved in a minimal volume of methanol, and a white precipiate formed upon cooling to –78 °C. The slurry was filtered at room temperature and, during filtration, the white precipitation redissolved. A small amount of brown oil remained on the filter. The filtrate was dried under reduced pressure to yield trithiol **1** (1.5 g, 90%) as a white solid. The product was judged pure by spectroscopic analysis. ^{1}H NMR (D_2O,

300 MHz) δ 3.25 ppm (t, J = 7.0 Hz, 6 H), 3.08 (s, 6 H), 2.67 (t, J = 6.5 Hz, 6 H) ppm; ^{13}C NMR (D_2O, 75 MHz) 84.3, 65.4, 57.8 ppm; MS (ESI) calculated for $C_{12}H_{24}N_4O_3S_3$ m/z 368.1, found 366.8.

Coupling of trithiol 1 to Bromo-TentaGel. Bromo-TentaGel resin (5 mg) was allowed to swell in water (1.0 mL) at room temperature for 1 h. The resin was then washed with 1.0 mL of 1.0 M Tris–HCl buffer, pH 7.6. Trithiol **1** (0.9 mL of a 40 mM aqueous solution) and Tris–HCl buffer, pH 7.6 (0.10 mL of a 1.0 M solution), were added to the resin. The suspension was agitated gently on a platform shaker at room temperature for 2 h. The resin was separated by centrifugation. To block unreacted sites, the resin was incubated overnight at room temperature with NMA (1.0 mL of a 0.3 M aqueous solution in 0.3 M Tris–HCl buffer, pH 7.6). The resin was then washed extensively with water and the coupling yield was determined by the reaction of resin-bound thiols with DTNB (*vide infra*).

Synthesis of epoxy-microspheres. A co-polymer of styrene and glycidyl methacrylate forms microspheres in water that present surface epoxy groups (Kawaguchi *et al.*, 1989). Styrene-glycidyl methacrylate microspheres were synthesized via a seeded emulsion polymerization. Both the styrene and the glycidyl methacrylate were distilled under reduced pressure in the dark before use. Styrene (11.4 mmol, 1.32 mL) and glycidyl methacrylate (12.6 mmol, 1.73 mL) were added to a flask containing degassed water (110 mL). Polymerization was initiated by the addition of aqueous 2,2′-azobis(2-methylpropionamidine) dihydrochloride (10 mL of a 6 mg/mL solution). The reaction was stirred under Ar(g) at 70 °C for 2 h and the initally clear, colorless solution turned cloudy and white. Glycidyl methacrylate (21.1 mmol, 2.88 mL) was added to coat the styrene-glycidyl methacrylate seeds with surface epoxy groups. The

polymerization was stirred under Ar(g) at 70 °C for an additonal 13 h. The epoxy-microspheres were isolated by centrifugation at 20,000 x *g* for 10 min at 4 °C. The pellet was washed by three cycles of resuspension in water (120 mL) followed by centrifugation. The epoxy-microspheres were dried under reduced pressure to give a fine, white solid (5.85 g, 98%).

Coupling of DTT to epoxy-microspheres. DTT was immobilized to microspheres via reaction with the exposed epoxidemoieties of styrene-glycidyl methacrylate microspheres. DTT (1.0 g, 6.5 mmol) was added to LiH (0.11 g, 13 mmol) in dry DMF (3.5 mL). The resulting slurry was stirred under Ar(g) on ice for 10 min and then transferred to a flask containing microspheres (0.10 g, 0.56 mmol total epoxide groups) dispersed in DMF (3.5 mL). The reaction was stirred on ice and under Ar(g) for 4 h and then quenched by the addition of saturated NH_4Cl(aq). The microspheres were collected by centrifugation (20,000 x *g*, 20 min) and the pellet was washed three times with water (30 mL per wash). The pellet was then transferred to a 2-mL eppendorf tube and washed by three more cycles of resuspension in water (1 mL per wash) followed by centrifugation for 2 min in a Sorvall benchtop centrifuge (maximum speed). A final wash with 1.0 mL 0.10 M potassium phosphate buffer, pH 7.6, was performed in a similar fashion.

*Coupling of trithiol **1** to epoxy-microspheres*. To minimize cross-linking of the trithiol to the epoxy-microsphere, a fraction of the epoxide groups were blocked. To accomplish this blocking, the monothiol NMA (10 μL, 0.11 mmol) was dissolved in 1.0 mL of dry DMF containing lithium *tert*-butoxide (10 mg, 0.12 mmol). The mixture was stirred on ice for 10 min and then microspheres (50 mg, 0.28 mmol total epoxide groups) were added and the blocking reaction was allowed to proceed on ice under Ar(g) for 2 h. Trithiol 1 (46 mg, 0.13 mmol) in dry

DMF (5 mL) and lithium *tert*-butoxide (20 mg, 0.24 mmol) were added sequentially to the NMA-capped microspheres. The coupling reaction was stirred on ice under Ar(g) for 3 h and then quenched with saturated aqueous NH_4Cl. The microspheres were collected by centrifugation (20,000 x *g* for 20 min) and then washed extensively with water and 0.10 M potassium phosphate buffer, pH 7.6.

Determination of coupling yields. To ensure complete washing, the solid support (TentaGel resin or microsphere) was washed with 0.10 M potassium phosphate buffer, pH 7.6, and pelleted by centrifugation. The supernatant was collected, and its thiol content was assayed by the addition of DTNB (10 μL of a 10 mg/mL solution). The solid support was judged clean (free of significant solution-phase thiol) if the supernatant had an $A_{412} < 0.1$. The yield of immobilized thiol was determined by the addition of DTNB (1 mL of a 10 mg/mL solution in 0.10 M potassium phosphate buffer, pH 7.6) to the solid support. The particles were dispersed in the DTNB solution by repeated inversion and then agitated on a platform shaker for 30 min at room temperature. The solid support was pelleted by centrifugation and the supernatant decanted. The moles of immobilized thiol were determined from the A_{412} of the supernatant (Ellman, 1959).

Mixed disulfide bond formation between resin-bound thiols and DTNB. The tendency of resin-bound thiols to form mixed disulfide bonds was assessed by monitoring the amount of NTB released upon reduction of DTNB-oxidized resin by DTT. Briefly, the fully reduced, thoroughly washed resin was incubated with a stoichiometric amount of DTNB relative to thiol. The DTNB solution was collected, and its NTB concentration ($[NTB]_1$) was measured by UV spectroscopy. The DTNB-oxidized resin was washed thoroughly and then incubated with an

excess of DTT. The DTT solution was collected, and its NTB concentration ($[NTB]_2$) was measured likewise. The ratio of NTB released upon reduction to NTB released during oxidation gives the fraction of thiols that formed mixed disulfides with NTB. This ratio, $[NTB]_2/[NTB]_1$, is unity if all of the resin-bound thiols form mixed disulfide bonds upon oxidation by DTNB. This ratio is zero if all of the resin-bound thiols form cyclic disulfide bonds upon oxidation by DTNB.

Disulfide bond isomerization activity of thiols bound to a solid support. Scrambled RNase A (sRNase A) results from the oxidation of the fully reduced protein under denaturing solution conditions (*i.e.*, 6 M guanidine–HCl). To determine the ability of resin-bound thiols to catalyze disulfide bond isomerization in proteins, sRNase A (1.0 mL of a 0.5 mg/mL solution in 0.10 M Tris–HCl buffer, pH 7.6) was added to fully reduced resin presenting 1.0 μmol thiol. The resin was agitated gently at room temperature for 20 h, and the RNase A solution was separated from the solid support by centrifugation. The concentration of native RNase A was determined from the spectrophotometric measurement of ribonucleolytic activity using poly(cytidylic acid) as the substrate, as described previously (Woycechowsky *et al.*, 1999). The fold reactivation was calculated relative to the incubation of sRNase A with uncoupled resin (*i.e.*, resin presenting 0 μmol thiol) and is the mean (± S.D.) of three experiments.

Determination of apparent E_{resin}. The apparent disulfide reduction potential of a dithiol immobilized on a solid support (E_{resin}) can be estimated by measuring the fraction of thiol groups remaining after incubation of the fully reduced resin in a redox buffer of known solution potential ($E_{solution}$). Trithiol-coupled TentaGel resin was incubated in 0.10 M Tris–HCl buffer, pH 7.6 (0.90 mL), containing 6.5 mM GSH and 16 mM GSSG ($E_{solution} = -180$ mV) at room temperature for 1h. The buffer was removed by centrifugation and the resin was washed

thoroughly with 50 mM Tris–HCl buffer, pH 7.6. The amount of thiol remaining on the resin was measured using DTNB, as described above. E_{resin} was calculated from the fraction of dithiols remaining after incubation in the redox buffer, using Equation 4-1, where $(\text{moles dithiol})_{final}$ is the number of moles of dithiol remaining on the resin after incubation with the redox buffer and $(\text{moles dithiol})_{initial}$ is the number of moles of dithiol present on the fully reduced resin. The value of E_{resin} is reported as the mean ± S.D. of duplicate experiments.

$$E_{resin} = E_{solution} - \frac{RT}{nF} \ln \frac{(\text{moles dithiol})_{final}}{(\text{moles dithiol})_{inital} - (\text{moles dithiol})_{final}} \qquad (4-1)$$

4.4 Results

Design and synthesis of trithiol 1. The symmetric trithiol **1** (Figure 4-1) was designed as a means for presenting a dithiol of defined structure on a solid support. Covalent attachment of **1** can occur via attack of a thiol on electrophilic groups presented by the solid support. The three-fold symmetry means that any of the three sulfhydryl groups can serve as the point of attachment, leaving dithiols of identical structure. The tertiary amine will possess a positive charge and help to stablize a thiolate within the molecule. A positive charge will also increase the accessibility of the coupled trithiol molecule by increasing its hydrophilicity. The synthesis of **1** was performed in three steps with a 13% overall yield (Figure 4-1).

Trithiol-coupled TentaGel resin. Bromo-TentaGel resin is a composite of polystyrene and polyethylene glycol (PEG) in which each PEG chain contains a terminal bromo group and was initially chosen as the solid support because of its high capacity (0.28 mmol Br/g resin) and water compatability. Immobilization of trithiol **1** can occur through displacement of bromide,

creating a stable thioether bond (Figure 4-2A). Coupling of a 30-fold excess of trithiol **1** to Bromo-TG resin gave a yield of 0.28 mmol thiol/g resin (Table 4-1), as measured by the reactivity of the coupled resin with DTNB.

One molecule of trithiol can displace up to three bromide ions from the resin (Figure 4-3). A singly attached molecule presents a dithiol, a doubly attached molecule presents a monothiol, and a triply attached molecule has no free thiols. To gauge the valency of the thiol groups presented by the coupled resin (monothiol vs dithiol), the propensity of the coupled resin to form mixed disulfide bonds was measured. Reduction of DTNB-oxidized resin by DTT indicated that only 14% of the thiols formed mixed disulfide bonds when oxidized with NTB (Table 4-1). Thus, the trithiol-coupled TG resin can readily form cyclic disulfide bonds, which is indicative of dithiol presentation (Figure 4-3A).

The trithiol-coupled TG resin readily engages in thiol–disulfide interchange with small molecules such as glutathione, DTT, and DTNB. Incubation of the resin with sRNase A produced only a small increase in enzyme activity (Figure 4-4 and Table 4-1). Incubation of sRNase A and uncoupled Br-TG resin in a solution of 1 mM GSH and 0.2 mM GSSG gave a 30-fold increase in RNase A activity, indicating that the TG resin itself does not prevent RNase A folding. The trithiol-coupled TG resin cannot effciently perform the thiol–disulfide interchange reactions necessary for the isomerization of non-native disulfide bonds in RNase A.

Determination of apparent E_{resin}. For efficient catalysis of disulfide bond isomerization by a dithiol, it is important that the dithiol have an appropriate disulfide $E^{\circ\prime}$. If the dithiol is too reducing (*i.e.*, if the disulfide $E^{\circ\prime}$ is too low), then the dithiol will prevent protein folding because reducton of protein disulfde bonds will be favored over isomerization of protein disulfide bonds.

After incubation in a glutathione redox buffer with $E_{solution} = -180$ mV, the trithiol-coupled TG resin contained about 10% of its original thiol content. Assuming that trithiol-coupled resin exclusively forms cyclic disulfides upon oxidation in this redox buffer, the apparent $E_{resin} = -208 \pm 8$ mV.

Thiol-Presenting Micrsopheres. Microspheres composed of styrene-glycidyl methacrylate co-polymers are water-compatible and have a high capacity of electrophilic epoxide groups (5.6 mmol epoxide/g microsphere). The structural integrity of these microspheres stems from the burial of hydrophobic styrene units and the surface presentation of hydrophilic glycidyl methacrylate units. The surface enrichment of glycidyl methacrylate units provides a high density of exposed epoxide groups. Immobilization of a thiol can occur via reaction with the epoxide groups on the surfaces of these microspheres.

A 12-fold excess of DTT relative to total epoxide groups was coupled to these microspheres and gave a yield of 0.30 mmol thiol/g microsphere. Using identical coupling conditions, reaction of trithiol **1** with epoxide-containing microspheres yielded 0.004 mmol thiol/g microsphere. Further, reduction of DTNB-oxidized resin with DTT showed that 65% of the thiol groups had formed mixed disulfide bonds, indicating a low propensity for cyclic disulfide bond formation by the immobilized thiol groups.

The low yield of immobilized thiol and high propensity for the formation of stable mixed disulfide bonds suggests that, during coupling, trithiol **1** often reacted with more than one microsphere epoxide group (Figures 4-3B and 4-2C). To decrease the likelihood of such cross-linking the microspheres were first treated with the monothiol NMA (0.4 equivalents relative to total epoxide groups) to block some of the reactive epoxides. Then, 0.5 equivalents of trithiol **1**

(relative to total epoxide) was coupled as before (Figure 4-2B). This procedure gave a yield of 0.056 mmol thiol/g microsphere, with 17% mixed disulfide bond formation upon oxidation with DTNB (Table 4-1).

The ability of thiol-presenting microspheres to fold sRNase A was assessed in the same fashion as for the trithiol-coupled TG resin. Incubation of sRNase A with either the DTT-coupled microspheres or the trithiol-microspheres resulted in an increase in enzyme activity (Figure 4-4 and Table 4-1). The trithiol-coupled microspheres, however, gave a 1.5-fold higher increase in activity than did the DTT-coupled microspheres.

4.5 Discussion

Heterologous gene expression often provides ready access to large quantities of protein (Swartz, 2001). These systems, however, can be so efficient at protein synthesis that the folding capacity of the cell becomes saturated and misfolded proteins accumulate, limiting the yield of active protein (Marston, 1986; Wittrup, 1995). For proteins containing disulfide bonds, folding in the reducing environment of the bacterial cytosol is especially challenging. In *E. coli*, misfolded proteins tend to form insoluble aggregates called inclusion bodies. To improve the yield of soluble, active protein from heterologus expression systems, Beckwith, Georgiou, and co-workers engineered *E. coli* strains with improved oxidative folding capabilities (Bessette *et al.*, 1999). Although such strains hold much promise for efficient production of soluble proteins containing disulfide bonds, inclusion body formation does have advantages (Guise *et al.*, 1996; Rudolph & Lilie, 1996). Those advantages include the high yield and relative purity of the

constituent protein, and the ability to produce large quantities of proteins that are otherwise toxic to the cell.

The *in vitro* folding of proteins from inclusion bodies commonly involves the dilution of denaturant-solubilized protein into solution conditions that favor protein folding (Rudolph & Lilie, 1996). Oxidative folding is plagued by two deleterious side reactions: hydrophobically-driven aggregation and non-native disulfide bond formation. To overcome these side reactions, cells utilize a full complement of chaperones (Bukau *et al.*, 2000; Walter & Buchner, 2002) and foldases (Debarbieux & Beckwith, 1999; Zapun *et al.*, 1999; Schiene & Fischer, 2000; Woycechowsky & R.T., 2000). The use of such proteins to effect folding *in vitro* is problematic because chaperones and foldases are expensive and because their subsequent removal requires an extra purification step.

Several methods have been developed to circumvent these limitations. For example, the GroEL/GroES chaperone system was successfully incorporated into an automated, solution-phase protein folding protocol (Kohler *et al.*, 2000). Although the chaperone could be recycled, its low stability prevented multiple reuses. PDI has been immobilized on an agarose support which demonstrated protein folding activity both in column-mode (Morjana & Gilbert, 1994) and batch-mode (Moutiez *et al.*, 1997). The covalent attachment of PDI was achieved through the non-specific reaction of lysine residues with activated agarose, a process that can render the active site inaccessible to substrate and thereby decrease the efficiency of catalysis. Finally, agarose gel beads decorated with a GroEL minichaperone and the foldases DsbA and peptidyl-prolyl isomerase were shown to facilitate the folding of proteins that are particularly intractable

towards *in vitro* folding (Altamiriano *et al.*, 1999; Karadimitris *et al.*, 2001). These proteins were also immobilized through non-specific attachment of lysine residues to activated agarose.

Low molecular weight additives have also proven to be useful for increasing the efficiency of protein folding *in vitro*. Aggregation can be minimized by the addition of a variety of low molecular weight folding enhancers, such as L-arginine (De Bernadez Clark *et al.*, 1999). An artificial molecular chaperone system has been developed and is based on the solubilization of unfolded proteins by detergent followed by the stripping of detergent from the protein by β-cyclodextrin (Rozema & Gellman, 1995). This method has also been adapted for use with insoluble, polymeric β-cyclodextrin (Mannen *et al.*, 2001).

Low molecular weight thiol–disulfide couples are useful for *in vitro* oxidative protein folding (Saxena & Wetlaufer, 1970; Rothwarf & Scheraga, 1991). The disulfide can oxidize the reduced protein; the thiol can isomerize non-native intermediates. The isomerization of these intermediates is often the rate-limiting step in oxidative protein folding (Creighton, 1978; Weissman & Kim, 1991; Narayan *et al.*, 2000). Dithiols are more efficient at disulfide bond isomerization than are monothiols (Rothwarf & Scheraga, 1993; Woycechowsky *et al.*, 1999). Presentation of a small-molecule cyclic dithiol/disulfide redox reagent on a solid support could provide an even more convenient means to improve *in vitro* folding efficiency.

To make an immobilized dithiol of defined structure for use in protein folding, the design, both of the dithiol and of the solid support, is of critical importance. Further, this redox reagent should have a reactive handle that allows for attachment to the solid support to yield the immobilized dithiol. The symmetric trithiol **1** can use any one of its three nucleophilic thiols to

react with an electrophile on a solid support and produce an immobilized dithiol of identical structure.

Three criteria were considered in the choice of solid support. First, the resin should have a high density of electrophilic groups. Second, the resin should be compatible with trithiol coupling conditions. Third, the resin should be compatible with the aqueous buffer conditions required for protein folding. The commercially available bromo-TentaGel resin meets these criteria, and the trithiol **1** was successfully coupled to it.

During coupling, if the trithiol displaces every bromine group on the TentaGel resin and every immobilized trithiol molecule is attached to the resin through only one of its thiols, then the coupling yield should be 2 mol thiol/mol bromine (or, 0.56 mmol thiol/g resin). Likewise, if the trithiol displaces every bromine group on the resin and every immobilized trithiol is attached to the resin through two of its thiol groups, then the coupling yield should be 0.5 mol thiol/mol bromine (0.14 mmol thiol/g resin). Coupling of the symmetric trithiol **1** to bromo-TentaGel resin gave a yield of 1 thiol group per bromine site on the TentaGel resin (0.28 mmol thiol/g resin). *In extrema*, this yield of immobilized thiol implies either that one-half of the coupled trithiol was singly attached while the other half doubly coupled to the TG resin or that the trithiol was predominantly singly coupled to the TG resin, but only coupled to half of the total bromine sites. The observation that only 14% of the TG-bound thiols formed mixed disulfide bonds upon oxidation with DTNB demonstrates that trithiol-coupled TG resin effectively forms cyclic disulfides.

To optimize its disulfide bond isomerization activity, the dithiol should have a low thiol pK_a and a high disulfide $E°'$. The mercaptoacetamido group has a thiol pK_a of 8.3

(Woycechowsky *et al.*, 1999), and a positive charge on the tertiary amine will extra stabilization of a thiolate on the mercaptoacetamido group of trithiol **1.** This positive chargeshould also increase the hydrophilicty, and thus accessibility, of immobilized trithiol **1**, an important factor in the reactivity of immobilized reagents with solution-phase molecules (Fields & Fields, 1991). Incubation of the fully reduced trithiol-coupled TentaGel resin in a glutathione redox buffer with $E_{solution} = -180$ mV causes approximately 90% oxidation of the resin-bound thiols. This observation indicates that the trithiol-coupled TentaGel resin has an apparent disulfide bond reduction potential of $E_{resin} = -208$ mV, which is not far removed from that of the PDI active site (Lundström & Holmgren, 1993).

Surprisingly, the trithiol-coupled TentaGel resin has little protein disulfide bond isomerization activity. This resin can readily undergo thiol-disulfide exchange reactions with a variety of small molecules (DTT, DTNB, glutathione) and the TentaGel resin itself does not significantly hinder RNase A folding. These results suggest that RNase A does not efficiently undergo productive thiol–disulfide interchange reactions with thiols attached to TentaGel resin. Trithiol-coupled TentaGel resin could fail to fold RNase A because thiols attached to TentaGel are inaccessible to RNase A (Figure 4-5A). The vast majority of thiol groups on the TentaGel resin are inside of pores within the polymer. Although small molecules ($M_r < 1000$) can diffuse readily through these pores, RNase A ($M_r = 13{,}700$) could be too large to access the thiol groups on the interior of TG beads. This size exclusion limit could be a general problem encountered when attempting to find interactions between proteins and small molecules immobilized to the high-capacity resins used commonly in solid-phase organic synthesis (Smith & Bradley, 1999).

A co-polymer of styrene and glycidyl methacrylate will form a microsphere in water. The styrene forms a hydrophobic core and the hydrophilic glycidyl methacrylate coats the solvent-exposed surface. Such microspheres provide a high surface density of electrophilic epoxide groups. The presentation of functional groups on the exterior of a microsphere circumvents the size exclusion limit presented by the porous TentaGel resin. Indeed, microspheres coupled to DTT present monothiols on their surfaces and have been shown to have RNase A folding activity (Shimizu *et al.*, 1999; Shimizu *et al.*, 2000). Further, these DTT-coupled microspheres have a higher affinity for unfolded RNase A relative to native RNase A (Shimizu *et al.*, 2000), which helps localize the substrate to the thiol groups while still allowing for easy separation of the folded protein from the solid support. These microspheres can be recycled readily and used for multiple folding reactions with only a slight loss of activity (Shimizu *et al.*, 1999).

The reported coupling of DTT to epoxy-microspheres was carried out in aqueous solution at pH 10 and 60 °C to produce a low yield of immobilized thiol (0.0074 mmol/g microsphere) (Shimizu *et al.*, 1999). These conditions are harsher than those used to couple trithiol **1** to bromo-TentaGel resin and are necessitated by the difficulty of opening an epoxide relative to displacing a bromide. Using a strong base and activation of the epoxide oxygen with a Lewis acid (Li^+) results in a 41-fold increase in DTT-coupling (0.30 mmol/ g microsphere) relative to the yield reported previously.

Yet, using these same conditions to couple the trithiol to the microspheres produced a low yield (0.004 mmol/g microsphere). Further, these trithiol-coupled microspheres had a high propensity for stable mixed disulfide bond formation upon oxidation with DTNB. These results indicate that the trithiol readily cross-links to the microsphere (Figures 4-3B and 4-3C). The

localization of thiol groups to the microsphere surface after the first coupling could increase the rate of attack by a second (and third) thiol group on neighboring epoxides by increasing the local concentration of thiol relative to epoxide. The larger number of atoms between thiols in **1** relative to DTT (11 versus 4) may allow the immobilized thiols easier access to neighboring epoxides and thus increase the susceptibility of the trithiol to cross-linking. This increased propensity for cross-linking may also account for the low yield of immobilized thiol from trithiol **1** relative to DTT, as trithiol **1** may cross-link more completely, leaving fewer free thiols among the coupled molecules (Figure 4-3). Indeed, a similar increase in local concentration has been harnessed for the synthesis of homodimeric molecules from reactive monomers bound to the same polymer bead (Blackwell *et al.*, 2001).

One advantage of microspheres relative to TentaGel resin is the high surface density of functional groups. This high surface density, however, also proved detrimental to efficient coupling and dithiol presentation. To improve the coupling efficiency, some of the surface density was sacrificed by blocking a fraction of the reactive epoxides with the monothiol NMA and then coupling the trithiol **1** (Figure 4-2B). This strategy provided a 14-fold increase in the yield of immobilized thiol. Further, the propensity of this trithiol-coupled microsphere to form stable mixed disulfide bonds was decreased greatly (Table 4-1).

Whether coupled to DTT or trithiol **1**, the microspheres (in contrast to the trithiol-coupled TG resin) showed substantial disulfide bond isomerization activity (Figure 4-4). The higher activity of the trithiol-coupled microsphere relative to trithiol-coupled TentaGel resin could stem from the greater surface display of dithiol by the microspheres (Figure 4-5B). The trithiol-coupled microsphere produced a 1.5-fold higher yield of active RNase A than did the DTT-

coupled microspheres. Such a difference in yield between a dithiol and a monothiol has also been observed in solution phase folding of RNase A (Woycechowsky *et al.*, 1999).

The increased yield of active protein is an obvious advantage of using trithiol-coupled microspheres relative to DTT-coupled microspheres (Figure 4-4). It could be argued, however, that if optimal coupling conditions were found thatsaturate the accessible epoxide groups with singly-attached molecules of DTT, then these immobilized thiol groups could form cyclic disulfide rings of 30 atoms. It is, however, difficult to ascertain the exact structure of the coupled species and thus know the spacing between the immobilized thiols. Single attachment of trithiol **1** to the microspheres always provides a dithiol of the same structure and the same, desirable disulfide $E^{\circ\prime}$. Oxidation of such an immobilized trithiol to the cyclic disulfide gives a 13-membered ring. These structural and energetic properties provide additional advantages for the use of the immobilized trithiol **1** realtive to the immobilized dithiol DTT.

Microspheres coupled to trithiol **1** are useful for catalyzing native disulfide bonds in proteins. In contrast to the immobilized protein catalysts in current use, this immobilized dithiol possesses a unique structure. The activity of this immobilized dithiol does not depend on tertiary structure. Consequently, this immobilized dithiol is not prone to denaturation and subsequent inactivation, as are protein catalysts. Further, styrene-glycidyl methacrylate micropheres are able to withstand exposure to many organic solvents or to high temperatures (Lai, 1976; Ley & Baxendale, 2002). The robust nature of microspheres displaying dithiol could be useful for industrial protein folding applications.

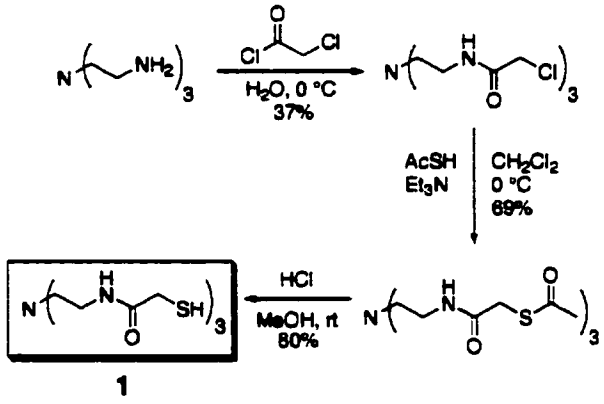
The use of solid-supported reagents is an emerging and useful strategy for solution-phase organic synthesis (Bhattacharyya, 2001; Ley *et al.*, 2002). Polymer-bound reagents, scavengers,

and catalysts can be used to effect transformations on solution-phase starting material in high yield, without the need for purification. The application of the principles of polymer-assisted solution-phase chemistry to protein folding provides a new approach to improving the efficiency of protein folding *in vitro*.

Table 4-1. Properties of thiol-bearing resins.

Resin	Coupling yield (mmol thiol/g resin)	% mixed disulfide bond formation with NTB	Fold reactivation of sRNase A
Trithiol-coupled TentaGel	0.28	14	2.0
DTT-coupled microspheres	0.30	Not determined	11
Trithiol-coupled microspheres	0.056	17	17

Figure 4-1. Synthetic route to tris(2-meraptoacetamidoethyl)amine.



1

Figure 4-2 Scheme for coupling trithiol **1** to resins. (A) Bromo-TentaGel resin (T). (B) Epoxy-microspheres (M).

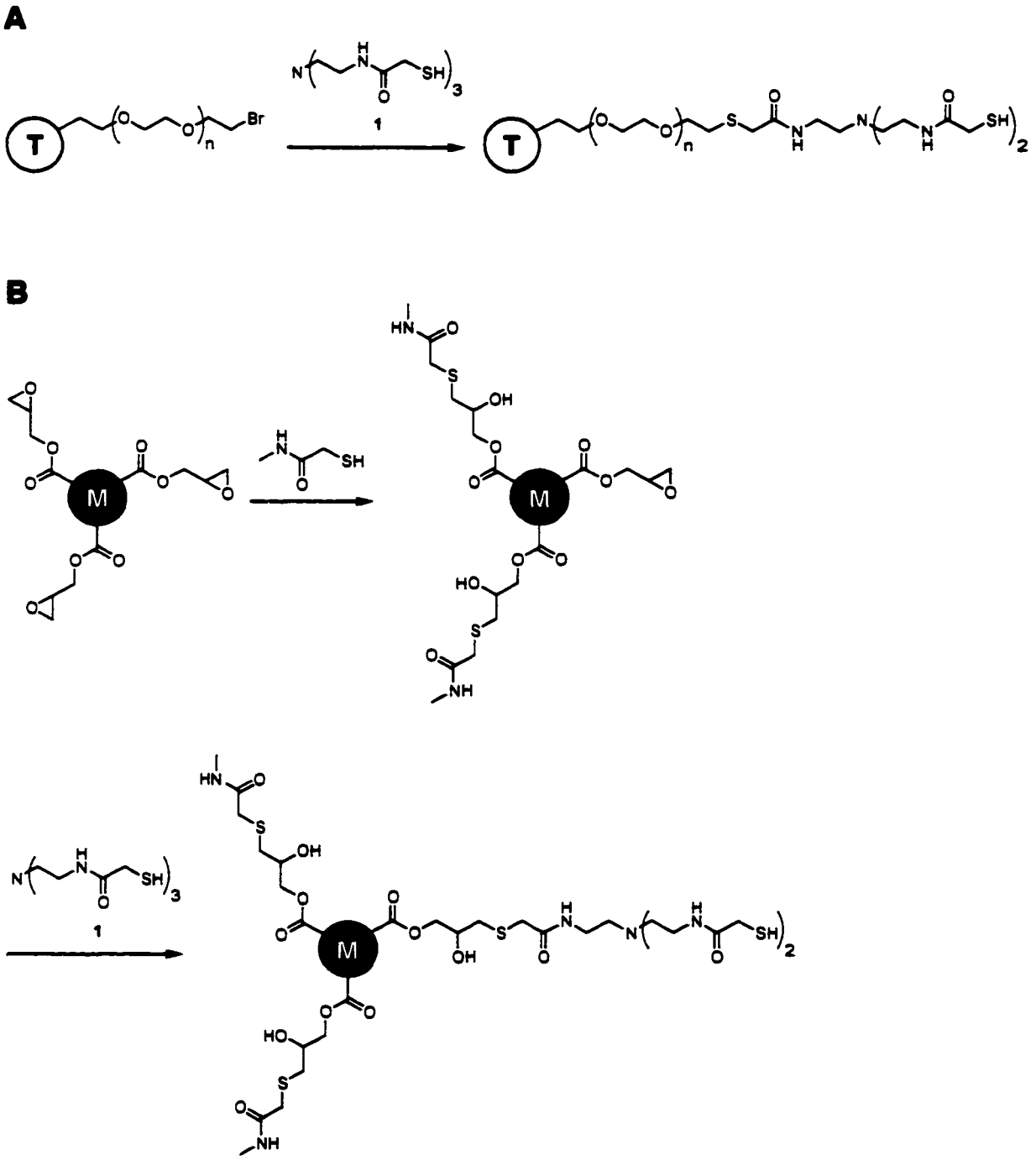

A
T
Br
n
1
T
S
N
H
SH
2
B
M
OH
HO
NH
1
M
SH
3
2

Figure 4-3 Different modes of trithiol immobilization. (A) Trithiol is singly attached to a resin and presents a dithiol. (B) Trithiol is doubly attached to a resin and presents a monothiol. (C) Trithiol is triply attached to a resin and this fully cross-linked species presents no thiols.

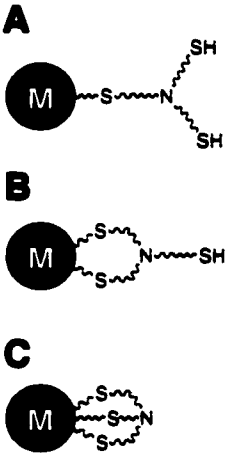

A
M
S
N
SH
SH
B
M
S
S
N
SH
C
M
S
S
N
S

Figure 4-4 Reactivation of scrambled RNase A by reduced resins. Resin displaying 1.0 μmol total thiol and unmodified resin were dispersed in parallel solutions of 0.10 M Tris–HCl, pH 7.6, containing sRNase A (0.5 mg/mL). The suspensions were gently agitated at room temperature for 20 h. The resin was removed by centrifugation and the ribonucleolytic activity of each solution was measured using a poly(cytidylic) acid substrate. The fold-reactivation of RNase A is calculated from the difference in RNase A activity between resin displaying 1.0 μmol total thiol and unmodified resin.

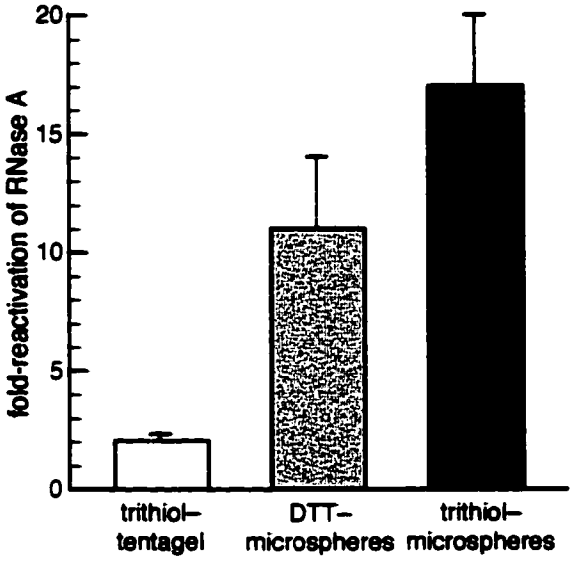
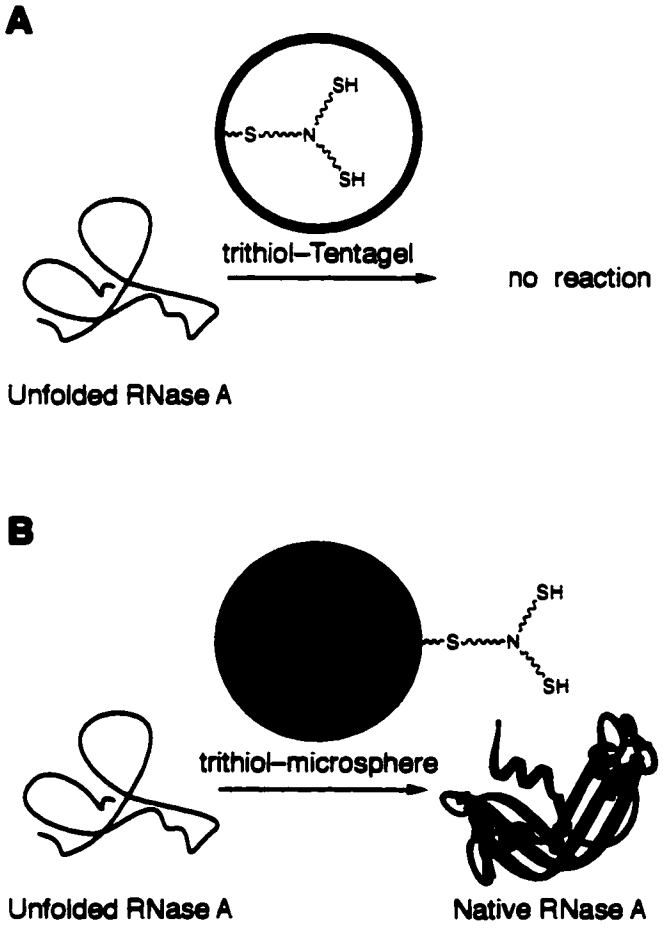

20
15
10
5
0
fold-reactivation of RNase A
trithiol–
tentagel
DTT–
microspheres
trithiol–
microspheres

Figure 4-5 Model for the higher activity of trithiol **1** immobilized to microspheres relative to trithiol **1** immobilized to TentaGel resin. (A) Thiols immobilized to TentaGel are located predominantly within the pores of the resin particles. Proteins, such as RNase A, cannot access the immobilized thiols, and thus do not fold efficiently. (B) Dithiols immobilized on microspheres have a high surface density. RNase A can readily interact with these sulfhydryl groups and thus folds efficiently.


A
SH
S
N
SH
trithiol–Tentagel
no reaction
Unfolded RNase A
B
SH
S
N
SH
trithiol–microsphere
Unfolded RNase A
Native RNase A

Chapter 5

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