MINIMALIST ENZYMOLOGY: NEW IN VIVO CATALYSTS OF PROTEIN DISULFIDE BOND ISOMERIZATION

by

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To my parents

MINIMALIST ENZYMOLOGY: NEW IN VIVO CATALYSTS OF PROTEIN DISULFIDE BOND ISOMERIZATION

Peter Tristram Chivers

Under the supervision of Dr. Ronald T. Raines

at the University of Wisconsin-Madison

This Thesis examines the minimal structural and functional requirements of the enzymic catalyst, protein disulfide isomerase (PDI). PDI is an essential protein in *Saccharomyces cerevisiae*, required for its ability to isomerize non-native disulfide bonds. Thus, proteins with PDI activity can be isolated by a powerful genetic selection. Eukaryotic protein disulfide isomerases are homologous to thioredoxin (Trx), a ubiquitous small (12 kDa) dithiol-disulfide oxidoreductase. Each PDI molecule contains two Trx domains per polypeptide chain and two other domains unrelated to Trx.

Wild-type Trx is unable to complement *pdi1Δ S. cerevisiae*. However, randomization of the CGPC active site to all twenty amino acids (*i.e.*, CXXC Trx) yields enzymes–CWGC, CVWC, and CGHC–that can substitute for PDI in *S. cerevisiae*. The redox inactive CGPS Trx mutant also complements *pdi1Δ S. cerevisiae*. Each of the mutant CXXC enzymes has an elevated reduction potential compared to wild-type Trx ($E^{\circ\prime} = -0.270$ V). pH titrations of the CWGC and CVWC mutants monitored by ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectroscopy revealed that the first cysteine of the CXXC motif has a lowered pK_a relative to

wild-type Trx. These results confirm the hypothesis that the essential function of PDI resides in its isomerase activity, which is catalyzed by the dithiol form of the CXXC motif.

Mutants of Asp26 of Trx–D26N and D26L–have altered catalytic properties from the wild-type enzyme. Kinetics experiments with these mutant enzymes show that Asp26 is a general acid/base catalyst in the disulfide reduction and dithiol oxidation activities of Trx. Further, the mutation of Asp26 simplifies the pH-titration behavior of reduced Trx, revealing the presence of microscopic pK_a's in the active-site of the reduced wild-type enzyme.

The experimental results presented in this Thesis illuminate the minimum functional group requirements for enzymic catalysis of disulfide bond isomerization. The interplay of the biophysical properties of the CXXC motif explains the wide presence in nature of CXXC-containing enzymes of different functions.

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

CXXC	Cys-Xaa-Xaa-Cys
DCl	deuterium chloride
DNA	deoxyribonucleic acid
DSS	2,2-dimethyl-2-silapentane-5-sulfonate
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	DL-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
5-FOA	5-fluoroorotic acid
GSH	reduced glutathione
GSSG	oxidized glutathione
HCl	hydrochloric acid
KOD	potassium deuteroxide
KOH	potassium hydroxide
NMR	nuclear magnetic resonance
$NADP^+$	β-nicotinamide adenine dinucleotide phosphate
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PMSF	phenylmethanesulfonyl fluoride
RNase A	ribonuclease A
SDS	sodium dodecyl sulfate
TCEP•HCl	tris(2-carboxyethyl)phosphine hydrochloride
TNB	5-thionitrobenzoate

TR	thioredoxin reductase
Tris-HCl	tris(hydroxymethyl)aminomethane hydrogen chloride
Trx	thioredoxin

Chapter 1

Introduction

This chapter was published in part as:

Chivers, P. T., Laboissière, M. C. A., and Raines, R. T. (1996). Protein disulfide isomerase: cellular enzymology of the CXXC motif. In Prolyl 4-hydroxylase, protein disulfide isomerase, and other structurally-related proteins. (N. A. Guzman, Ed.), In press.

Proteins are central to biology. Collectively, these molecules provide the myriad of activities necessary for life. Proteins (enzymes) that catalyze chemical reactions offer remarkable rate enhancements over uncatalyzed reactions (Radzicka & Wolfenden, 1995). Although enzymes are powerful catalysts, changes in pH, temperature, or solvent composition can seriously affect enzyme activity. Additionally, enzyme specificity is dictated by evolutionary pressures. These restrictions limit the in vitro applications of enzymic catalysts.

The modification of existing enzymes and the design of new enzymic catalysts are two approaches to expanding the catalytic repertoire of enzymes, in vitro and in vivo. Using the tools of protein chemistry and recombinant DNA technology, protein engineers have learned how to design new proteins and to enhance the stability of existing proteins (Cunningham & Wells, 1987; Regan & DeGrado, 1988; Chen & Arnold, 1991; Federov *et al.*, 1992; Davidson & Sauer, 1994). Still, the design of new catalysts is limited by knowledge of the relationship between amino acid sequence and protein structure-function.

This Thesis describes the first steps of the design of a minimalist enzymic catalyst. A minimal catalyst should contain only the functional groups necessary for catalysis arrayed on a small, stable protein scaffold. Because enzymes use several means to achieve efficient catalysis (Jencks, 1987), identification of the essential elements of catalysis is necessary for the successful design of a minimalist catalyst. The model for the studies described here is protein disulfide isomerase, an essential protein in yeast responsible for the correct formation of native disulfide bonds in secreted proteins (Scherens *et al.*, 1991; Laboissière *et al.*, 1995).

Disulfide Bonds in the Eukaryotic Cell

Disulfide bonds between the thiol groups of cysteine residues stabilize the native structures of many proteins (Creighton, 1988). The pathway for the formation of native disulfide bonds is complex (Creighton, 1977; Weissman & Kim, 1991). This complexity arises because the

thiol groups and disulfide bonds of proteins can undergo three distinct chemical reactions: dithiol oxidation, disulfide bond reduction, and disulfide bond isomerization (Gilbert, 1990). Of the three, only disulfide bond isomerization does not require the concomitant reduction or oxidation of another molecule (Figure 1-1).

The tendency of two thiols to form a disulfide bond or of an existing disulfide bond to be broken depends on the reduction potential of the environment (Gilbert, 1990). The cytosol of eukaryotic cells has a low reduction potential of $E^{\circ\prime} = -0.230$ V (Hwang *et al.*, 1992). In comparison, the endoplasmic reticulum (ER) has a high reduction potential of $E^{\circ\prime} = -0.172$ to -0.188 V (Hwang *et al.*, 1992). These values largely dictate the redox state of disulfide bonds in cellular proteins. In a typical unfolded protein two thiols have an effective molarity near 50 mM relative to two molecules of reduced glutathione (Lin & Kim, 1989; Lin & Kim, 1991). Using the reduction potential of oxidized glutathione [$E^{\circ\prime} = -0.252$ V (Lees & Whitesides, 1993)] and the Nernst equation, the reduction potential of a typical disulfide bond in an unfolded protein is calculated to be $E^{\circ\prime} = -0.22$ V. This value is between that of the cytosol and the ER, indicating that the formation of disulfide bonds in unfolded proteins is favored in the ER but not in the cytosol. In contrast, disulfide bonds in folded proteins have reduction potentials as low as $E^{\circ\prime} = -0.45$ V (Gilbert, 1995). Thus, reduction potentials tend to increase in the order: folded protein > cytosol > unfolded protein > ER. This order suggests that disulfide bonds form in unfolded proteins as they are being translocated into the ER. Some of these bonds will be those found in the properly folded protein, but others will be non-native (Wittrup, 1995).

Properties of Protein Disulfide Isomerase

Eukaryotic cells are now known to contain an ensemble of proteins that orchestrate the proper folding of other proteins (Freedman, 1992). The search for an enzymic catalyst of oxidative protein folding began more than 30 years ago. In 1963, an enzyme capable of

catalyzing the reactivation of reduced ribonuclease A was discovered in microsomal preparations from rat liver (Goldberger *et al.*, 1963; Venetianer & Straub, 1963). This enzyme was eventually named protein disulfide isomerase (PDI; EC 5.3.4.1). Subsequently, it was shown that the rate of thiol disappearance during the air oxidation of reduced ribonuclease A is independent of PDI, but that the rate of reactivation relies on the enzyme (Givol *et al.*, 1964). These results indicated that PDI does not catalyze the air oxidation of dithiols. Rather, air oxidation involves the uncatalyzed formation of non-native disulfide bonds followed by the PDI-catalyzed isomerization to the disulfide bonds in the native protein (Givol *et al.*, 1964). This discovery, along with the observation that PDI enhances the rate of reactivation of both reduced ribonuclease A and reduced lysozyme (Goldberger *et al.*, 1964), led Anfinsen and coworkers to favor "the hypothesis that the enzyme is a general and nonspecific catalyst for disulfide interchange in proteins containing disulfide bonds" (Givol *et al.*, 1964).

Mammalian PDI is a 57-kDa protein that constitutes approximately 2% of the protein in the ER (Zapun *et al.*, 1992). The enzyme can catalyze each of the reactions shown in Fig. 1-1—the oxidation of dithiols, and the reduction and isomerization of disulfide bonds—as shown by the in vitro assays listed in Table 1-1 . The products of redox catalysis by PDI depend on the dithiol/disulfide reduction potential of the substrate and the solution (Gilbert, 1990).

Early work on PDI implicated one or more cysteine residues as being necessary for its enzymatic activity. Carboxymethylation or carbamoylmethylation of PDI caused irreversible inactivation (Fuchs *et al.*, 1967; Hawkins & Freedman, 1991). In addition, PDI was shown to be inhibited by arsenite or Cd^{2+}, behavior diagnostic of enzymes with active-site dithiol groups (Ramakrishna Kurup *et al.*, 1966; Hillson & Freedman, 1980).

Rutter, Roth, and their coworkers verified the existence of the inferred cysteine residues by cloning and sequencing the cDNA that codes for rat PDI (Edman *et al.*, 1985). The cDNA sequence revealed that the rat enzyme is synthesized as a 528-residue precursor that contains

a 19-residue signal sequence. The mature enzyme contains 509 residues, resulting in a molecular mass of 56.8 kDa (Edman *et al.*, 1985; Gilbert *et al.*, 1991). Rat PDI consists of two pairs of homologous regions: amino acid residues 9 – 90 (region a) and 353 – 431 (region a′), and amino acid residues 153 – 244 (region b) and 256 – 343 (region b′). Regions a and a′ each contain an active site with the sequence WCGHCK (or, simply CXXC), which is homologous to the single active site in *Escherichia coli* thioredoxin (Holmgren, 1968). The C-terminus of PDI ends with the sequence KDEL, which has been implicated as the signal for retention of a protein in the ER of mammalian cells (Munro & Pelham, 1987). cDNA's encoding PDI have also been sequenced from human (Pihlajaniemi *et al.*, 1987), mouse (Gong *et al.*, 1988), and bovine (Yamauchi *et al.*, 1987) tissues.

The gene that codes for yeast PDI has also been isolated (Scherens *et al.*, 1991). Yeast PDI is a 522-residue protein that contains a C-terminal HDEL sequence—the yeast ER retention signal (Pelham *et al.*, 1988). Yeast PDI also contains five consensus N-glycosylation sites, each of which appears to be modified in the protein found in wild-type cells. The yeast PDI amino acid sequence shares 29% identity and 44% similarity with mammalian PDI. Most significantly, by cloning the yeast *pdi* gene, Scherens and coworkers were able to demonstrate that the *PDI1* gene is essential for the viability of *S. cerevisiae* cells (Scherens *et al.*, 1991).

Determining that yeast require PDI did not reveal what cellular process is impaired by the absence of PDI. The enzyme catalyzes dithiol oxidation, and disulfide bond reduction and isomerization (Freedman *et al.*, 1994). Yet, PDI can bind to peptides (Morjana & Gilbert, 1991; Noiva *et al.*, 1991). Moreover, PDI is the β subunit of prolyl 4-hydroxylase, an $\alpha_2\beta_2$ tetramer that catalyzes the formation of 4-hydroxyproline residues in collagen strands (Koivu *et al.*, 1987; Pihlajaniemi *et al.*, 1987). PDI is also a subunit of the heterodimeric microsomal triglyceride transfer protein (Wetterau *et al.*, 1990; Gordon *et al.*, 1995). But neither of these complexes is known to be present in yeast cells.

Two other functions have been ascribed erroneously to PDI. Although it has some affinity for thyroid hormones and has been deemed a thyroid-hormone binding protein (Boado *et al.*, 1988), PDI is not involved in thyroid hormone metabolism (Schoenmakers *et al.*, 1989). Similarly, PDI has an affinity for peptides, including glycosylated peptides (Geetha-Habib *et al.*, 1988), but it is not the glycosylation site binding protein responsible for core glycosylation of nascent chains in the ER (Noiva *et al.*, 1991).

Role of Protein Disulfide Isomerase in the Cell

PDI is the most efficient known catalyst of oxidative protein folding (Freedman, 1989; Noiva & Lennarz, 1992). Surprisingly, the catalysis by the PDI subunit is unnecessary for the function of either prolyl 4-hydroxylase (Myllylä *et al.*, 1989; Vuori *et al.*, 1992) or the microsomal triglyceride transfer protein [(Lamberg *et al.*, 1996), A.D. Attie, personal communication]. Still, the evolution of a protein catalyst is demanding and unlikely to be without purpose.

Complementation studies of *PDI1* null mutants of *Saccharomyces cerevisiae* have provided clues as to the essential role of PDI in eukaryotic cells. Tachibana and Stevens (1992) showed that overexpression of the *EUG1* gene, which codes for an ER protein with CLHS and CIHS sequences, allows *pdi1Δ* cells to grow. This important finding showed that the C-terminal cysteine residue of the CXXC motif is not essential to eukaryotic cells.

LaMantia and Lennarz (1993) used genetic and enzymatic data to claim that PDI is not needed for the catalysis of disulfide bond isomerization in *S. cerevisiae* . These workers found that a cDNA encoding CLHS/CIHS PDI (which mimics Eug1p) can complement *pdi1ΔS. cerevisiae* but does not catalyze the oxidation of dithiols. This observation spawned the widespread notion that PDI acts in vivo primarily as a chaperone or even as an "anti-chaperone" (Noiva *et al.*, 1993; Wang & Tsou, 1993; Puig & Gilbert, 1994; Puig *et al.*, 1994). These data only show, however, that PDI is unnecessary for the *formation* of disulfide bonds.

They do not address the role of PDI in the *isomerization* of existing disulfide bonds (Figure 1-1).

Is the isomerization activity of PDI essential for the viability of S. cerevisiae? The Raines laboratory used wild-type and mutant rat PDI to replace PDI in *S. cerevisiae* and to catalyze each of the activities in Figure 1-1 (Laboissière *et al.*, 1995). Double mutants of rat PDI were created in which either the N-terminal or C-terminal cysteine residue in each CGHC active site is replaced by a serine. The results of genetic analyses indicated that a cDNA for wild-type or CGHS PDI is able to complement *pdi1Δ S. cerevisiae*. In contrast, a cDNA for SGHC PDI is unable to compensate for this deficiency.

Using in vitro assays, the wild-type and mutant proteins were tested for each enzymatic activity demonstrated by PDI (Figure 1-1). In comparison to wild-type (CGHC) PDI, SGHC and CGHS PDI have negligible dithiol oxidation activity [measured by an increase in activity of reduced ribonuclease A] and negligible disulfide reduction activity (measured by the cleavage of porcine insulin). In contrast, CHGS PDI has isomerization activity (measured by an increase in activity of scrambled ribonuclease A) comparable to that of the wild-type enzyme. SGHC PDI has negligible isomerization activity. The essential function of PDI is therefore enzymic, but does not relate to the net formation of protein disulfide bonds. Rather, the role of PDI in vivo is to act as a "shufflease"—a catalyst of disulfide bond isomerization (Figure 1-1, bottom).

The properties of the Eug1p protein support this conclusion. Overexpresssion of the *EUG1* gene complements *pdi1Δ S. cerevisiae*. Wild-type Eug1p is analogous to the shufflease mutant of PDI in that each active site contains only the N-terminal cysteine residue (Tachibana & Stevens, 1992). Thus, the essential functional atom in the CXXC motif is the sulfur of the N-terminal cysteine residue.

Properties of Thioredoxin

PDI is homologous to thioredoxin (Trx), a 12-kDa cytosolic reducing agent for ribonucleotide reductase and other proteins (Holmgren, 1985). Trx does not possess the multiplicity of non-redox functions exhibited by PDI (Pihlajaniemi *et al.*, 1987; Wetterau *et al.*, 1990). For example, Trx does not bind to a peptide that was used to identify a peptide binding region in PDI (Noiva *et al.*, 1993). Still, the amino acid residues that surround the CXXC motifs of PDI and Trx are those that are most conserved between the two proteins (Edman *et al.*, 1985).

The presence of Trx in all types of organisms (Eklund *et al.*, 1991) argues for its early evolutionary appearance. The discovery of other proteins with Trx domains suggests that these domains were recruited for a specific purpose (Figure 1-2). As well as PDI, other proteins contain the Trx domain. ERp72, which encodes a protein of the eukaryotic ER, has three CXXC motifs and is able to complement *pdi1Δ* yeast (Günther *et al.*, 1993). Likewise, the yeast gene *MPD1* encodes an ER protein that contains a single CXXC sequence and can complement *pdi1Δ* yeast (Tachikawa *et al.*, 1995). DnaJ, an *E. coli* chaperone, has four CXXC sequences and catalyzes each reaction in Figure 1-1 (de Crouy-Chanel *et al.*, 1995). DsbA, a protein of the *E. coli* periplasm, has a CXXC motif and was discovered because of its ability to rescue *E. coli* mutants defective in disulfide bond formation (Bardwell *et al.*, 1991). DsbA shares no sequence similarity with *E. coli* Trx apart from the active site, but the two proteins do have similar three-dimensional structures (Martin *et al.*, 1993; Martin, 1995)

Overall, PDI and Trx share approximately 30% amino acid identity, and have similar active sites: CGHC in each Trx domain of PDI (Edman *et al.*, 1985) and CGPC in Trx (Höög *et al.*, 1984). Unlike any PDI to date, *E. coli* Trx is a small, well-characterized protein (Holmgren, 1985). A crystalline structure of the oxidized form is known to 1.68 Å resolution (Katti *et al.*, 1990), and solution structures are known of both the oxidized and the reduced forms (Jeng *et al.*, 1994) (Figure 3) A solution structure of the N-terminal domain of human PDI confirms that the Trx domains of PDI possess the thioredoxin fold (Kemmink *et al.*, 1996). Although

PDI1 is essential for the viability of *S. cerevisiae* (Scherens *et al.*, 1991), *trxA* is not essential for *E. coli* (Holmgren, 1976).

The active sites of PDI and Trx are similar. The results of chemical modification studies and pK_a determinations on PDI are parallel to those on Trx (Freedman *et al.*, 1988), suggesting that the reactivities of the active sites are similar. In addition, PDI is a substrate for thioredoxin reductase (Lundström & Holmgren, 1990), an indication that the three-dimensional structures of the active sites are alike. In native Trx, the most pronounced deviation from an almost spherical surface is a protrusion formed by residues 29 – 37, which includes the active site. The thiol of Cys32, which has a lowered pK_a, is exposed to the solvent but the thiol of Cys35 is recessed.

PDI is an attractive starting point for the design of new catalysts of protein disulfide bond formation *in vivo*. Because of its essential function in yeast, a powerful genetic selection exists for the isolation of new catalysts of protein disulfide bond formation. Additionally, the active-site region of the essential domain consists of a simple amino acid motif—CXXC—that protrudes into the solvent. This feature simplifies the design of new catalysts because features of the active-site microenvironment (*i.e.*, local dielectric constant, relative solvation) are less important. Further, the intervening residues of the active site motif may be randomized to permit selection of effective catalysts. Finally, dithiol-disulfide exchange reactions are a rare example of the reversible cleavage of a strong covalent bond (~65 kcal/mol) in aqueous solution (Burns & Whitesides, 1990).

This thesis describes the determination of the features required of a minimalist PDI. Chapter 2 decribes the isolation of mutants of *E. coli* thioredoxin that catalyze native protein disulfide bond formation in vivo. Chapter 3 reports the pH-titration behavior of mutants of *E. coli* Trx. Chapter 4 examines the role of a conserved non-cysteine residue in the mechanism of disulfide reduction by Trx. Chapter 5 discusses a link between thiol pK_a and reduction

potential in CXXC-containing proteins. Finally, the Appendix presents complete derivations of equations used in Chapters 2 and 5.

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

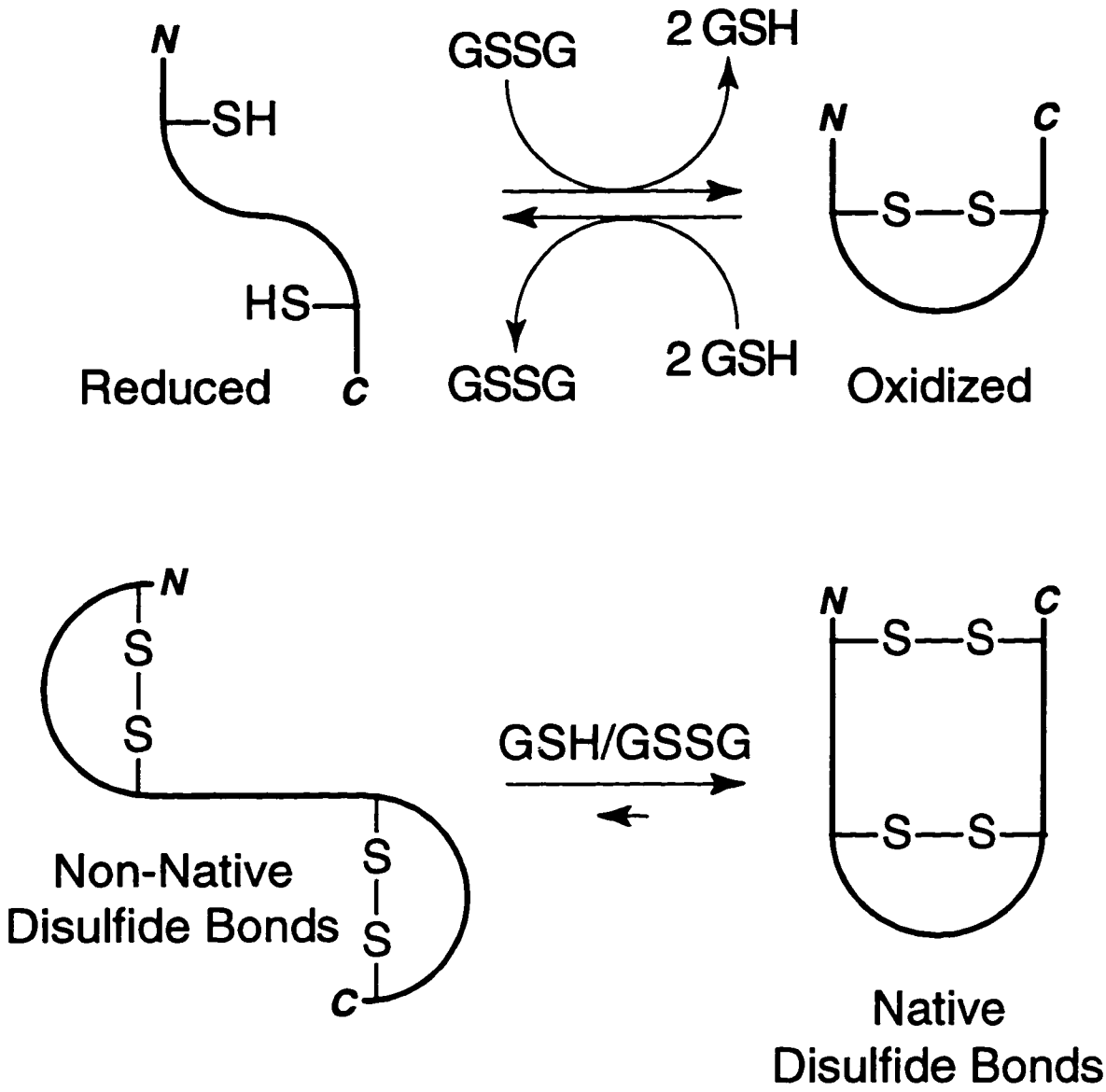
N
SH
HS
Reduced
C
GSSG
2 GSH
GSSG
2 GSH
N
C
S—S
Oxidized
N
S
S
Non-Native
Disulfide Bonds
S
S
C
GSH/GSSG
N
C
S—S
S—S
Native
Disulfide Bonds

Figure 1-2 Alignment of the active-site residues of CXXC–containing proteins. The references from which the sequences were obtained are listed in the table.

Protein		Residues	Sequence	Reference
E. coli	Trx	26-37	D F W A E W C G P C K M	Höög et al (1984)
human	Trx	26-37	D F S A T W C G P C K M	
human	PDI		E F F A P W C G H C K A	Pihlajaniemi et al (1987)
			E F V A P W C G H C K Q	
rat	PDI	29-40	E F Y A P W C G H C K A	Edman et al (1985)
		373-384	E F Y A P W C G H C K Q	
murine	PDI	49-60	E F Y A P W C G H C K A	Mazzarella et al (1990)
		393-404	E F Y A P W C G H C K Q	
murine	ERp72	78-89	E F Y A P W C G H C K Q	Mazzarella et al (1990)
		193-204	E F Y A P W C G H C K K	
		542-553	E F Y A P W C G H C K Q	
S. cerevisiae	PDI	33-44	E F F A P W C G H C K N	Scherens et al (1991)
		378-389	L Y Y A P W C G H C R R	
S. cerevisiae	Eug1p	33-44	E F F A P W C L H S Q I	Tachibana and Stevens (1992)
		377-388	K Y Y A T W C I H S K R	
S. cerevisiae	Mpd1p	32-43	E F Y A P W C G H C K K	Tachikawa et al (1995)
E. coli	DsbA	24-35	E F F S F F C P H C Y Q	Bardwell et al (1991)
E. coli	Grx		I F G R S G C P Y C V R	Björnberg and Holmgren (1991)
T4	Grx		D S N I H K C V Y C D N	LeMaster (1986)

Figure 1-3 Solution structure of the reduced form of *E. coli* Trx (Jeng *et al.*, 1994). Only the active site cysteine residues are shown.

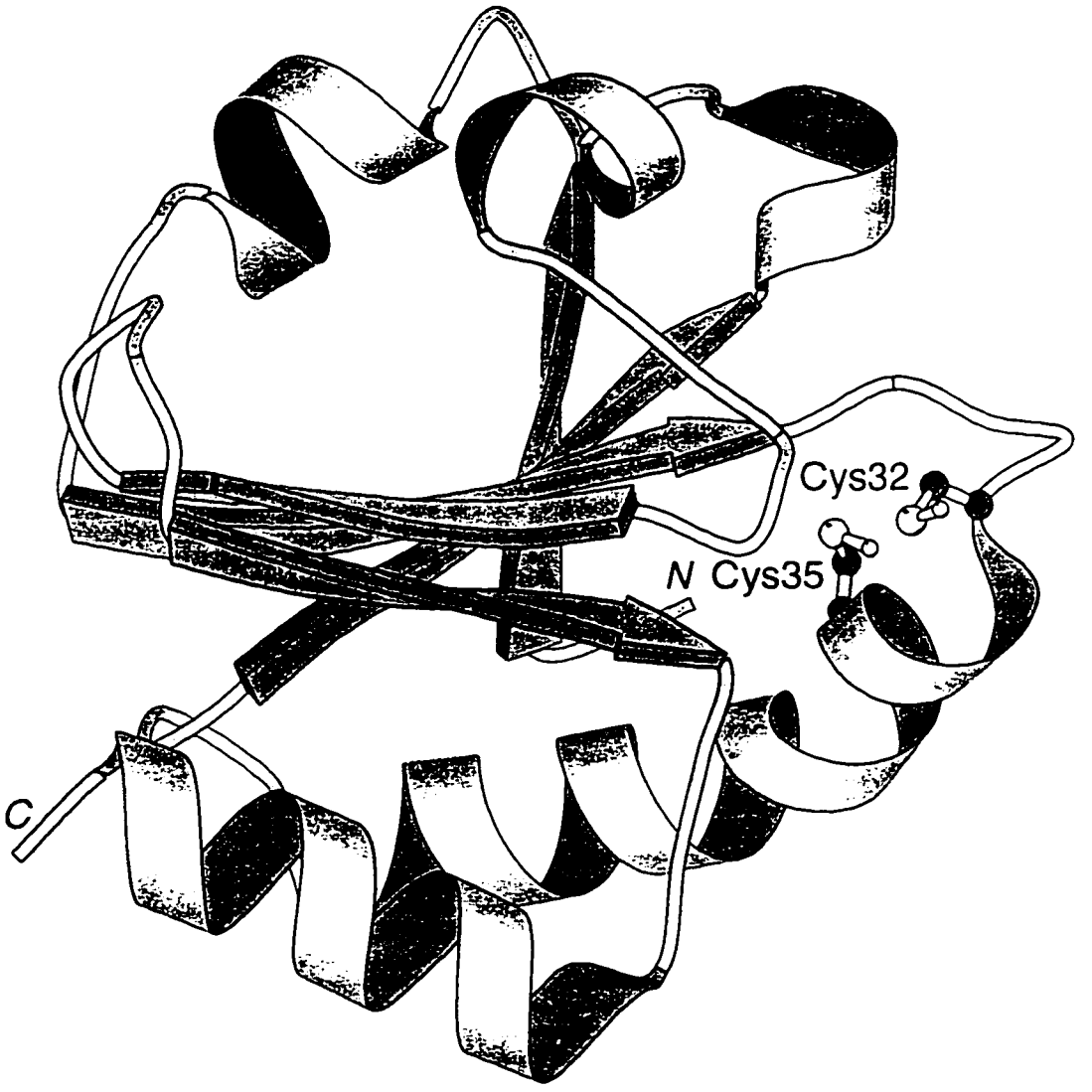
Cys32
N Cys35
C

Table 1-1

Assays for PDI activities

Activity	Substrate
Dithiol oxidation	Reduced ribonuclease A[a]
	Reduced lysozyme[b]
	Reduced bovine pancreatic trypsin inhibitor[c,d]
	Reduced human gonadotropic hormone[e]
	Reduced Fab fragment[f]
	Reduced synthetic peptide[g]
Disulfide bond reduction	Insulin[h]
	Fab fragment[f]
	Synthetic peptide[g]
Disulfide bond isomerization	Scrambled ribonuclease A[i]
	Partially reduced bovine pancreatic trypsin inhibitor[d]

a, (Lyles & Gilbert, 1991); b, (Goldberger *et al.*, 1964; Puig & Gilbert, 1994); c, (Creighton *et al.*, 1980); d, (Weissman & Kim, 1993); e, (Huth *et al.*, 1993); f, (Lilie *et al.*, 1994); g, (Darby *et al.*, 1994); h, (Lu *et al.*, 1992); i, (Lambert & Freedman, 1983).

Chapter 2

The CXXC Motif—Imperatives for Disulfide Bond Formation in the Cell

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ABSTRACT

The rapid formation of disulfide bonds in cellular proteins is necessary for the efficient use of cellular resources. This process is catalyzed in vitro by protein disulfide isomerase, with the *PDI1* gene being essential for the viability of *Saccharomyces cerevisiae*. PDI is a member of the thioredoxin family of proteins, which have the active-site motif CXXC. PDI contains two Trx domains as well as two domains unrelated to the Trx family. We find that the gene encoding *Escherichia coli* Trx is unable to complement *PDI1* null mutants of *S. cerevisiae*. Yet, Trx can replace PDI if it is mutated to have a CXXC motif with a disulfide bond of high reduction potential and a thiol group of low pK_a. Thus, an enzymic thiolate is both necessary and sufficient for the formation of native disulfide bonds in the cell.

INTRODUCTION

Misfolded proteins are costly to a cell. Nonfunctional, they have consumed valuable cellular resources in their synthesis. Moreover, their accumulation can limit cell growth by saturating secretory and other pathways (Robinson & Wittrup, 1995).

Eukaryotic cells contain an ensemble of proteins that orchestrate the proper folding of other proteins (Freedman, 1992). Of these proteins, only protein disulfide isomerase (PDI) is known to catalyze the formation of native disulfide bonds. PDI, a 57-kDa resident of the ER, was first isolated 30 years ago by Anfinsen and contemporaries (Goldberger *et al.*, 1963; Venetianer & Straub, 1963), who favored "the hypothesis that the enzyme is a general and nonspecific catalyst for disulfide interchange in proteins containing disulfide bonds" (Givol *et al.*, 1964). These early studies demonstrated that PDI can act as a catalyst for the isomerization of disulfide bonds in vitro.

In contrast, LaMantia and Lennarz (1993) claimed that PDI is not needed for the catalysis of disulfide bond isomerization in *Saccharomyces cerevisiae*. This claim has spawned the notion that PDI acts instead as a chaperone or "anti-chaperone" (Noiva *et al.*, 1993; Wang & Tsou, 1993; Puig & Gilbert, 1994; Puig *et al.*, 1994). The data of LaMantia and Lennarz (1993) only show, however, that PDI is not essential for the *formation* of disulfide bonds. Their results do not address the role of PDI in the catalysis of disulfide bond *isomerization* (Figure 1-1). Recent results from our laboratory indicate that catalysis of isomerization is indeed the essential function of PDI in the cell (Laboissière *et al.*, 1995).

We have used *E.coli* Trx to reveal which domains of PDI are essential in vivo and the properties of the Cys-Xaa-Xaa-Cys (hereafter, CXXC) motif that are most important to eukaryotic cells. Specifically, we have assessed the viability of *pdi1Δ S.cerevisiae* cells containing CGPC (that is, wild-type) or CGHC (which has a PDI-like active site) Trx in their ER. We did the same with cells containing Trx mutants in which the two residues between the

active-site cysteine residues had been made random. Finally, we tested the viability of cells containing CGPS or SGPC Trx, two enzymes that cannot form a disulfide bond within their active sites. Our results reveal that the Trx domains of PDI are essential to *S.cerevisiae*, and that an enzymic thiolate is both necessary and sufficient for the catalysis of native disulfide bond formation in cellular proteins.

MATERIALS AND METHODS

*Plasmids, strains, and reagents*Plasmid pTK10 was a generous gift of J.A. Fuchs (University of Minnesota). *E.coli* thioredoxin reductase (TR) was a generous gift of C.H. Williams, Jr. (University of Michigan). Wild-type *E.coli* Trx was from Promega (Madison, WI). All enzymes for the manipulation of DNA were from Promega except *Ava*II, which was from New England Biolabs (Beverly, MA).

S.cerevisiae BJ2168 **a** (*prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) was obtained from the Yeast Genetic Stock Center (Berkeley, CA). *S.cerevisiae* strain YPH 274 α/a *pdi1Δ::HIS3* (*ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) was a generous gift from T. H. Stevens (University of Oregon). *E.coli* strain DH11S *dut⁻ung⁻* was a generous gift of D. Polayes (BRL; Gaithersburg, MD).

5-FOA was a generous gift of M. R. Culbertson (University of Wisconsin – Madison). Reagents for DNA synthesis were from Applied Biosystems (Foster City, CA) except for acetonitrile, which was from Baxter Healthcare (McGaw Park, IL). Bacto yeast extract, Bacto peptone, and Bacto yeast nitrogen base without amino acids (YNB) were from Difco (Detroit, MI). Pepstatin A, phenylmethanesulfonyl fluoride (PMSF), NADPH, $NADP^+$, 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), dithiothreitol (DTT), and 400 – 600 μm glass beads for yeast transformation were from Sigma (St. Louis, MO). Ultrapure dioxynucleotide triphosphates for

mutagenesis were from Pharmacia (Piscataway, NJ). Concentrated phosphoric acid was from Mallinckrodt (Paris, KY). All other chemicals and reagents were of commercial or reagent grade or better, and were used without further purification.

General methods Manipulations of DNA, *E.coli*, and *S.cerevisiae* were performed as described (Ausubel *et al.*, 1989; Shermann, 1991). DNA oligonucleotides were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer using the β-cyanoethyl phophoramidite method (Sinha *et al.*, 1984), and were purified with Oligonucleotide Purification Cartridges from Applied Biosystems. DNA synthesis by the PCR was performed with a DNA thermal cycler from Perkin Elmer Cetus (Norwalk, CT). DNA was purified with the Wizard MaxiPreps DNA Purification System (Promega), PCR Magic Preps (Promega), or GeneClean (Bio101; Vista, CA). Plasmids were transformed into *E.coli* cells with a GenePulser electroporator (BioRad; Richmond, CA), and into *S.cerevisiae* cells as described (Elble, 1992). Dideoxynucleotide sequencing was performed with the Sequenase Version 2.0 kit from United States Biochemical (Cleveland, OH).

Yeast minimal medium (SD) contained (in 1 L) Bacto YNB (6.7 g), dextrose (2% w/v), and a supplemental nutrient mix (Ausubel *et al.*, 1989). Yeast rich medium (YEPD) contained (in 1 L) Bacto yeast extract (10 g), Bacto peptone (20 g), and dextrose (2% w/v). Variations of SD and YEPD media were also used. For example, YEP(1%)D medium contained 1% w/v dextrose. All media were prepared in distilled, deionized water and autoclaved before use.

*Spectroscopy*The absorbance of ultraviolet and visible light was measured with a Cary Model 3 spectrophotometer equipped with a Cary temperature controller (Varian; Sugar Land, TX). The absorbance of cell cultures at 600 nm was recorded after diluting the cells such that $A = 0.1 - 0.5$. Fluorescence was recorded on an SLM 8000 fluorescence spectrometer from SLM

Instruments (Urbana, IL) equipped with photon counting electronics and data collection software from ISS (Champaign, IL).

Expression and mutagenesis of trxA The *trxA* gene was excised from pTK10 by digestion with *Xba*I and *Pst*I. The resulting fragment was purified and inserted into the corresponding sites in pBluescript II S/K, to yield plasmid pBST1. To signal for secretion, the α-factor pre–pro segment (Brake *et al.*, 1984) was added to the 5′ end of *trxA* by oligonucleotide-mediated site-directed mutagenesis (Kunkel, 1985) using oligonucleotide PC01 (5′ ACGCCAGGTTATGGTAC CGCTGGATAAAAGAAGCGATAAAATT 3′) and single-stranded pBST1 prepared from *E.coli* strain DH11S. This mutation also created an *Acc65*I site for the in-frame insertion of *trxA* into yeast expression plasmid YEpWL.RNase A (delCardayré *et al.*, 1995).

To signal for ER retention (Pelham *et al.*, 1988), an HDEL sequence was added to the 3′ end of *trxA* by site-directed mutagenesis using oligonucleotide PC02 (5′ GCTAACCTG GCGCACGATGAGTTGTAACCGCGGTGCCCCGTCGCT 3′), to yield plasmid pBST7. This insertion displaced the stop codon by twelve nucleotides. The *Acc65I/SalI* cassette from pBST7 was inserted into YEpWL.RNase A, to yield plasmid YEpWL.TRX. Single amino acid mutants of *trxA* were created in pBST7: P34H with oligonucleotide PC06 (5′ GGTGCGGTCACTGCAAAATG 3′), C35S with PC08 (5′ GCGGTCCGTCTA AAATGATC 3′), and C32S with PC21 (5′ GCAGAGTGGTCTGGTCCGTGCA 3′). After dideoxynucleotide sequencing, the mutated DNA fragments were subcloned into YEpWL.RNase A as described above.

*Randomization of Gly33 and Pro34*The codons for Gly33 and Pro34 were mutated randomly by the PCR method of overlap extension (Ho *et al.*, 1989) using pBST7 as the template. The first round of mutagenesis used two separate reactions with oligonucleotides

PC23 [5′ TGGGCAGAGTGGTGCNN (GCT)NN(GCT)TGCAAAATGATC 3′] and PC05 (5′ AACGTGTTCAACCAAGTCGAC GGTATCGATAAGC 3′), or PC24 [5′ GGCGATCATTTTGCA(GCA)NN(GCA)NNGC ACCACTCTGC 3′] and PC01. The two fragments were purified, an aliquot of each was combined, and the resulting mixture was amplified by the PCR with oligonucleotides PC01 and PC05 to generate randomized *trxA*.

After fragment purification and restriction digestion, the *Acc65I/SalI* cassette was inserted into YEpWL.RNase A that had been digested with *Acc65I* and *SalI*. The ligation product was transformed into *E.coli* TG1 by electroporation. After overnight growth at 37 °C, each plate was washed with LB (2 mL) to suspend the colonies. The resulting suspension was diluted to 10 mL and grown for 6 h in LB containing extra ampicillin (300 μg/ml). An aliquot of this culture (1 mL) was used to inoculate LB (100 mL) containing ampicillin (100 μg/ml), and the resulting culture was grown overnight at 37 °C. The plasmid library purified from this culture encoded Gly33All/Pro34All Trx.

Transformation of S.cerevisiae *and plasmid shuffling*Plasmid shuffling refers to replacing a wild-type gene for a mutant gene in a haploid *S.cerevisiae* strain that has the wild-type gene on a plasmid but not a chromosome (Sikorski & Boeke, 1991). Cells that undergo plasmid shuffling initially contain two different plasmids—wild-type and mutant, each with a different selectable marker. By selecting against the plasmid containing the wild-type gene, mutant genes that confer viability (*i.e.*, complement) can be identified. Here, we have selected against the *URA3* gene on plasmid pCT37, which directs the expression of *S.cerevisiae* PDI. The enzyme encoded by *URA3* produces a toxic catabolite from 5-FOA.

Transformed *S.cerevisiae* cells were plated on solid medium appropriate for plasmid selection. Wild-type and single-site mutants were transformed into haploid *pdi1Δ* YPH 274 containing pCT37. Twenty fresh transformants for each construct were picked and plated on an SD – trp master plate and a 5-FOA selection plate, both of which were incubated at 30 °C. An

aliquot of the plasmid library that codes for Gly33All/Pro34All Trx was also transformed into haploid *pdi1Δ* YPH 274 containing pCT37. Approximately 300 *trp*$^+$ transformants were plated on both SD – trp and 5-FOA media, which were then incubated at 30 °C. Yeast *PDI1* on *TRP* plasmid pRS424 (Laboissière *et al.*, 1996) was used as a positive control, and YEpWL without an insert was used as a negative control. After growth on 5-FOA, colonies were replica plated on SD – trp and SD – ura media to confirm shuffling. Positive complementants were able to retain the *trp*$^+$ marker after several rounds of growth on solid YEPD medium, indicating the requirement of YEpWL.TRX in the absence of a selection for *trp*$^+$ (data not shown).

Yeast cell extracts After plasmid shuffling, *trp*$^+$ *ura*$^-$ YPH274 cells were grown at 30 °C in YEP(1%)D medium (100 mL) until $A = 0.7$ at 600 nm. Cell extracts were then prepared as described (Bostian *et al.*, 1983). Pepstatin, EDTA, and PMSF were added at standard concentrations to extraction buffers to inhibit protease activity (Deutscher, 1990).

Polyclonal antibodies to Trx A chicken was injected with wild-type *E.coli* Trx (200 μg) and 7 days later boosted with additional Trx (100 μg). Eggs were collected 21 days after the initial injection and IgY isolated as described (Polson *et al.*, 1980).

Immunoblotting Yeast cells were grown to late log phase in liquid medium (15 mL). Cell extracts were prepared as described above. Denaturing polyacrylamide gel electrophoresis was done as described (Ausubel *et al.*, 1989). Protein samples were electroblotted onto nitrocellulose (BioRad; Richmond, CA) with a Mini Trans-Blot apparatus from BioRad. Chicken polyclonal antibodies against *E.coli* Trx (1:250 dilution), peroxidase-conjugated affinity-purified rabbit antibody to chicken IgG (Cappel Research Products; Durham, NC), and a Renaissance chemiluminescence detection kit (Dupont NEN; Boston, MA) were used to visualize protein according to the kit manufacturer's instructions.

Doubling time of S.cerevisiae Haploid *pdi1Δ* cells complemented with *E.coli trx* or yeast *PDI1* were grown in YEP(1%)D medium. These cultures were diluted with the same medium to a cell density of 3 x 10^6 cells/mL (A = 0.1 at 600 nm), and the resulting cultures (25 mL in a 125-mL culture flask) were grown at 30 °C with shaking at 250 rpm. At 2-h intervals, an aliquot was removed and its A was measured at 600 nm. Log$A$ was plotted vs time, and the slope of the linear portion of the curve was determined by linear least-squares analysis. At least 5 different clones from each construct were analyzed. For each clone, doubling time was calculated by dividing log2 by the slope of the curve. For each construct, the mean and standard deviation of the doubling times was calculated. Growth rates were normalized to the growth rate of *pdi1Δ* cells complemented with yeast *PDI1*.

*Protein purification*Mutant and wild-type Trx's were produced in vacuolar protease-deficient *S.cerevisiae* BJ2168 using a protocol similar to that of Laboissière *et al.* (1995a). Briefly, to select for plasmid YEpWL.TRX, starter cultures (10 mL) were grown at 30 °C in SD – trp media. To produce protein, cultures (1.0 L) were grown at 30 °C in YEP(1%)D medium with an initial A = 0.025 at 600 nm. Medium was collected when A = 6 (~24 h). Cells were removed by centrifugation at 4,000 x g for 10 min. The liquid media was chilled by stirring in an ice-water bath for 30 min. The pH of the chilled media was lowered to 3.5 by the addition of concentrated phosphoric acid. The precipitated protein was separated by centrifugation at 16,000 x g for 45 min. The supernatant was discarded, and the pellet resuspended in a minimal volume (<10 mL) of 100 mM potassium phosphate buffer, pH 8.0, containing EDTA (1 mM). The resulting solution was filtered through a 0.2 μm cellulose acetate filter (Nalgene; Rochester, NY).

The filtrate was applied (flow rate: 1.5 mL/min) to a Pharmacia Hi Load 26/60 G-75 gel filtration column that had been equilibrated with sample resuspension buffer. Fractions

containing Trx activity were pooled and loaded (flow rate: 1 mL/min) on a Pharmacia MonoQ column that had been equilibrated with 20 mM imidazolium chloride buffer, pH 7.6. Trx eluted in the flowthrough, and was reloaded on the MonoQ column and eluted with a linear gradient (12 + 12 mL) of NaCl (0.1 – 0.3 M) in 20 mM imidazolium chloride buffer, pH 7.6. Fractions containing Trx activity were pooled and concentrated with a 10 kDa-cutoff Centriprep concentrator (Amicon; Beverly, MA).

Enzymatic activity To aid protein purification, Trx activity was detected by using the coupled assay of Luthman and Holmgren (1982). In this assay, active Trx is detected by an increase in *A* at 412 nm due to the reduction of DTNB to produce 3-carboxylato-4-nitrothiophenolate (ε = 14,140 $M^{-1}cm^{-1}$). The oxidized Trx formed during the reaction is reduced by TR and NADPH. Thioredoxin activity was assayed in a solution (0.80 mL) of 0.10 M Tris-HCl buffer, pH 8.0, containing EDTA (2 mM), NADPH (250 μM from a 20 mM stock solution in assay buffer), and DTNB (1.0 mM from a 100 mM stock solution in ethanol). The reaction was initiated by the addition of TR (2 μL of a 0.78 μg/μL solution).

Reduction potential The reduction potential of Trx was determined by using the assay of Moore (1964). In this assay, the reduction of Trx is accompanied by a decrease in *A* at 340 nm due to the TR-catalyzed reduction of NADPH (ε = 6200 $M^{-1}cm^{-1}$). Here, TR (2 μL of a 0.78 μg/μL solution) was added to a solution (0.80 mL) of 0.10 M Tris-HCl buffer, pH 7.0, containing Trx (7 – 96 μg), NADPH (40 – 60 μM), and EDTA (2 mM). After equilibrium had been reached, $NADP^+$ (1.1 mM, final concentration) was added to generate a new equilibrium. Using the equilibrium concentrations of all species, $E^{o\prime}_{Trx}$ was determined using the equation

$$E^{o\prime}_{Trx} = E^{o\prime}_{NADP^+} + \frac{RT}{nF}\ln\frac{[Trx(SH)_2][NADP^+]}{[TrxS_2][NADPH]} \qquad (2\text{-}1)$$

where $E^{\circ\prime}_{NADP+} = -0.315$ V (Clark, 1960), $R = 8.314$ J/(K·mol), $T = 298$ K, $n = 2$, and $F = 96{,}500$ C mol^{-1}. At least 3 different starting concentrations of Trx were used to obtain a mean value of $E^{\circ\prime}$.

Thiol pK_a. The pK_a of Cys32 of Trx was determined by using fluorescence spectroscopy, as described by Holmgren (1972). Here, the fluorescence intensity (I) of reduced Trx (10 μg in 800 μL) was determined at pH 4.0 – 9.0 with buffers: sodium acetate, pH 4.0 – 5.5; potassium phosphate, pH 5.8 – 7.4; and Tris-HCl, pH 8.0 – 9.0. Potassium chloride was added to each buffer to maintain an ionic strength of 0.10 M at each pH. The midpoint (pK_a) for the transition near neutral pH was calculated from a plot of $\log[(I_{max} - I)/(I - I_{min})]$ vs pH (Lee, 1970).

RESULTS

Production of Trx in S.cerevisiae. To illuminate the function of the CXXC motif in eukaryotic cells, we designed a way to target and retain *E.coli* Trx in the ER of *S.cerevisiae*. Previously, we had used plasmid YEpWL to direct the expression of rat PDI (Laboissière *et al.*, 1995; Laboissière *et al.*, 1995; Laboissière *et al.*, 1996) and bovine pancreatic ribonuclease A (delCardayré *et al.*, 1995) in *S.cerevisiae*. Here, we constructed a plasmid, YEpWL.TRX, that directs the expression of Trx fused to the C-terminus of the α-factor pre–pro segment (Brake *et al.*, 1984) and the N-terminus of HDEL (Pelham *et al.*, 1988). This plasmid can replicate in both *E.coli* and *S.cerevisiae*, and contains the selectable *TRP1* and *LEU2*-d genes. The expression of the *trxA* gene in YEpWL.TRX is controlled by the glucose-regulated ADH2–GAPDH promoter. We used oligonucleotide-mediated site-directed mutagenesis to change the codons for the CGPC active site of wild-type Trx to those for CGHC, CGPS, and

SGPC (Table 2-1). Molecular modeling and structural studies suggest that these mutants are likely to adopt a structure similar to that of wild-type Trx (Eklund *et al.*, 1991; Krause *et al.*, 1991; Dyson *et al.*, 1994).

Our goal in replacing Pro34 with a histidine residue was to make the reduction potential of the CXXC motif of Trx more like that of PDI (Krause *et al.*, 1991; Lundström *et al.*, 1992). To generate enzymes with a still wider range of reduction potentials, we mutated the codons for Gly33 and Pro34 in tandem to codons for all 20 amino acid residues. The resulting pool codes for 400 double-mutants of Trx. Codons 33 and 34 were considered to be random in this pool because G, A, T, and C were found in positions one and two and G, C, and T were found in position three of each codon (data not shown). A plasmid library was obtained from approximately 200 bacterial transformants.

Our goal in replacing Cys32 or Cys35 with a serine residue was to alter a fundamental property of Trx—its ability to form a disulfide bond within its active site. Redox catalysis by the CXXC motif occurs in two steps. First, a disulfide bond is lost in a substrate (such as an oxidized protein) as one is gained in the CXXC motif. To complete the catalytic cycle, a disulfide bond is formed in a second substrate (such as two molecules of glutathione) as one is lost in the CXXC motif. Enzymes with CXXC motifs thus interact with two substrates in two distinct steps. In contrast, since CGPS and SGPC Trx cannot form a disulfide bond in their CXXC motifs, these enzymes cannot catalyze a redox reaction except in a single step that involves two substrates. Apparently, this constraint makes these enzymes inefficient catalysts of redox reactions (LaMantia & Lennarz, 1993; Laboissière *et al.*, 1995; Wunderlich *et al.*, 1995).

Complementation of pdi1Δ S.cerevisiae. Plasmids that code for CGHC and CGPS Trx, but not wild-type or SGPC Trx, can complement *pdi1Δ* yeast (Table 2-1). The ability of a plasmid to complement was determined by plasmid shuffling. Here, *trp*$^+$ *ura*$^+$ cells, which contain

plasmids pCT37 (*S.cerevisiae PDI1* and *URA3*) and YEpWL.TRX (*TRX* and *TRP1*), were grown on solid medium containing 5-fluoroorotic acid (5-FOA) to select against pCT37. Colonies of *pdi1Δ S.cerevisiae* complemented with pRS424 (*S.cerevisiae PDI1*) were visible after 2 – 3 days. In contrast, colonies of cells complemented with a mutated YEpWL.TRX appeared in 6 – 8 days. After this time, the appearance of colonies of cells transformed with YEpWL (no insert) precluded differentiating between true and false positives by their growth alone. True complementing cells were trp^+ ura^-. Immunoblot analysis confirmed the presence of Trx in *pdi1Δ* yeast cells after plasmid shuffling with YEpWL.TRX but not pRS424 (data not shown).

Complementation by a plasmid that codes for CGHC Trx but not by one that codes for wild-type Trx points to reduction potential as a key determinant in the efficient catalysis of disulfide bond formation in vivo. Complementation by a plasmid that codes for CGPS but not by one that codes for SGPC Trx indicates that Cys32 but not Cys35 is an essential residue in catalysis of native disulfide bond formation. Two plasmids from the random pool are also able to complement *pdi1Δ* yeast (Table 2-1). These plasmids coded for a protein that has a tryptophan residue in either position 33 or 34. (Recently, we found that CWDC Trx can also replace PDI in yeast.) Because only a single codon codes for Trp, its appearance in the genetic selection attests to the extensive scope of mutants in the random pool. The inability of our selection to uncover a plasmid encoding CGHC Trx is not unexpected because we did not survey the entire CXXC pool.

Function of Trx in vivo. The ability of Trx to function in vivo was assessed by the growth rate of complemented *pdi1Δ S.cerevisiae* cells. The doubling time of *pdi1Δ S.cerevisiae* cells complemented with yeast *PDI1* is 3.0 h. Complemented cells containing CWGC Trx double 2.2-fold more slowly than do those containing yeast PDI (Table 2-1). Cells containing CVWC, CGPS, or CGHC Trx double 3.8- to 4.4-fold more slowly than do those containing yeast PDI.

Purification of Trx from S.cerevisiae. The purification of Trx from *S.cerevisiae* was made facile in two ways. First, experiments were designed so that the same plasmid could be used for both complementation analysis (in *pdi1Δ* strain YPH274) and protein production (in vacuolar protease-deficient strain BJ2168). Second, much of the Trx produced is secreted into the growth medium—away from intracellular proteins. Trx produced in this system has the same mobility during SDS-PAGE as does Trx isolated from *E.coli*, indicating that the α-factor pre–pro segment is removed. [For a detailed discussion of this expression system, see: Laboissière *et al.* (1995a)].

Pure protein was obtained from the medium by precipitation with phosphoric acid, gel filtration chromatography, and anion-exchange chromatography. After gel filtration chromatography, the fractions containing Trx also contain a high concentration of inorganic phosphate. Accordingly, Trx elutes in the flowthrough from the anion-exchange column. Reloading of this flowthrough and application of an NaCl gradient yields 2 mg of pure Trx per L of *S.cerevisiae* culture. This yield is adequate for the biophysical characterization of mutant Trx's.

Reduction potential of CXXC motif. The reduction potential of the CXXC motif provides a measure of the relative stability of its dithiol and disulfide forms. The interconversion between these two forms is catalyzed by thioredoxin reductase (TR), with the concomitant interconversion of $NADP^+$ and NADPH. Thus in the presence of thioredoxin reductase, the characteristic ultraviolet absorbance of NADPH reports on the relative stability of the dithiol and disulfide forms of Trx.

We used the TR-coupled assay to determine the reduction potential of the active-site disulfide bonds of the CXXC motifs. CGHC, CWGC, and CVWC Trx are substrates for TR, and each has a reduction potential that is higher than that of wild-type Trx (Table 2-1). The

measured reduction potential of CWGC Trx is actually a lower limit. Even at low initial concentrations of NADPH and CWGC Trx, the addition of a high concentration (5 mM) of $NADP^+$ oxidizes only a small fraction of CWGC Trx. The increase in reduction potential indicates that the mutations have stabilized the reduced form of Trx.

pK_a of thiol in CXXC motif. The pK_a of the Cys32 (underlined) thiol in the CXXC motif provides a measure of the relative stability of its protonated and unprotonated forms. The fluorescence intensity of the indole sidechain of Trp28 in reduced Trx changes with pH. This change had been proposed to arise from the titration of the thiol in the sidechain of Cys32 (Holmgren, 1972; Reutimann *et al.*, 1981), and the $pK_a = 6.35$ first assigned to Cys32 by fluorescence titration was later confirmed by NMR spectroscopy (Forman-Kay *et al.*, 1992).

We used the change in fluorescence intensity to determine the pK_a of Cys32 in Trx. For wild-type Trx, we obtained $pK_a = 6.28$ (Table 2-1), which does not differ significantly from values reported previously. The pK_a's of the two mutants of Trx were depressed by 0.4 – 0.5 pH units relative to that of wild-type Trx. This decrease indicates that the mutations at positions 33 and 34 have stabilized the thiolate form of Cys32.

Recently, Jeng *et al.* (1994) asserted that the pK_a monitored by fluorescence titration of Trx is not that of Cys32. Rather, they suggested that titration of His6 gives rise to the observed pK_a. Accordingly, we also used two-dimensional ^1H NMR spectroscopy as an independent method to determine the pK_a of Cys32 in our mutant enzymes (Chapter 3). Preliminary NMR data indicate that the pK_a's of Cys32 in the mutant enzymes are approximately 0.5 units lower than that of Cys32 in wild-type Trx (Dyson *et al.*, 1991; Jeng *et al.*, 1995). In contrast, the pK_a's of His6 in the mutant enzymes remain unchanged. Thus, fluorescence and NMR titrations yield consistent results—Cys32 in a mutant Trx that can replace PDI has a lower pK_a than does Cys32 in wild-type Trx. Finally, it is worth noting that discrepancies between thiol pK_a's determined by NMR and fluorescence spectroscopy may result from the low deuterium

fractionation factor of sulfhydryl groups (Schowen, 1977). NMR titrations performed in deuterated water must be corrected for this anomaly.

DISCUSSION

In all species, the dominant role of Trx is as a cytosolic reducing agent (Holmgren, 1985; Buchanan *et al.*, 1994). Because of its small size, Trx does not possess the multiplicity of non-redox functions exhibited by PDI (Pihlajaniemi *et al.*, 1987; Wetterau *et al.*, 1990). For example, Trx does not bind a tripeptide that was used to identify a peptide binding region in PDI (Noiva *et al.*, 1993). Still, the amino acid residues that surround the CXXC motifs of PDI and Trx are those that are most conserved between the two proteins (Edman *et al.*, 1985).

The presence of Trx in all types of organisms (Eklund *et al.*, 1991) argues for its early evolutionary appearance. The discovery of other proteins with Trx domains suggests that these domains were recruited for a specific purpose. For example, PDI has two Trx domains. ERp72, a protein of the eukaryotic ER, has three CXXC motifs and is able to complement *pdi1Δ* yeast (Günther *et al.*, 1993). DsbA, a protein of the *E.coli* periplasm, has a CXXC motif and was discovered because of its ability to rescue *E.coli* mutants defective in disulfide bond formation (Bardwell *et al.*, 1991). DsbA shares no sequence similarity with *E.coli* Trx apart from the active site, but the two proteins do have similar three-dimensional structures (Martin *et al.*, 1993). The continued and widespread presence of the CXXC motif points to a critical role in cellular function (Chivers *et al.*, 1996).

By complementing *pdi1Δ* yeast with Trx, we have demonstrated that any roles ascribed to PDI, other than its catalysis of the formation of native disulfide bonds, are not essential. In other words, PDI is first-and-foremost a catalyst of the activity for which it was named and needs only its Trx domains for this activity. In addition, the ability of a cytosolic protein from a

prokaryotic cell to function in the ER of an eukaryotic cell heralds the versatility of the CXXC motif. Further, our genetic and biophysical analyses of the CXXC motif reveals the requirements for catalysis of protein disulfide bond isomerization in the eukaryotic cell.

Role of PDI in the cell. Catalysis of disulfide bond reduction or dithiol oxidation depends upon the redox environment (Gilbert, 1990). In contrast, during catalysis of disulfide bond isomerization, the substrate does not undergo a net change in oxidation state (Figure 1-1). Thus neither a redox-active catalyst nor a redox buffer (*e.g.*, a mixture of reduced and oxidized glutathione) is necessary for catalysis. The simplest mechanism for catalysis of an isomerization reaction begins with the attack of a thiolate ion on a protein disulfide, forming a mixed disulfide (Darby & Creighton, 1995). Then, the protein thiolate produced can attack another protein disulfide bond. Finally, the resulting thiolate can attack the mixed disulfide to release the catalyst, unaltered. The energetics of such an isomerization reaction would be driven by the search for the most stable conformation of the substrate protein.

A redox-inactive catalyst, CGPS Trx, restores the viability of *pdi1Δ* yeast. Thus, reduction or oxidation of the catalyst itself is not necessary for the cell to live. This result indicates either that catalysis of disulfide bond isomerization is the essential function of the CXXC motif, or that this motif is unnecessary altogether. The lack of complementation with a plasmid encoding the redox-inactive SGPC Trx mutant indicates that Cys32 (Figure 1-3) is a critical residue, and that the function of the CXXC motif is to catalyze the isomerization of existing protein disulfide bonds (Figure 1-1). In vitro evidence supports this view. SGHC PDI is an ineffective catalyst of disulfide bond reduction and isomerization, and dithiol oxidation (Laboissière *et al.*, 1995). CGHS PDI can catalyze only disulfide bond isomerization. Also, wild-type PDI acts after oxidizing equivalents have been introduced into a reduced substrate (Haggren & Kolodrubetz, 1988; Lyles & Gilbert, 1991).

Interestingly, a protein with a CXXS sequence already exists in the ER of *S.cerevisiae*, and this protein is able to complement *pdi1Δ S.cerevisiae* (Tachibana & Stevens, 1992). Its gene, *EUG1*, is not normally expressed well and complements only weakly when expressed at high levels. We propose that a CXXS sequence is less efficacious because of its inability to rescue itself from potentially harmful side reactions. A CXXC motif would be less susceptible to such inactivation because it could escape by forming an intramolecular disulfide bond. For example, a CXXS sequence is prone to irreversible oxidation—a sulfenic acid [RS–OH $\rightleftarrows$ RS(O)H] formed in a CXXS sequence could be oxidized further to a sulfinic acid [RS(O)–OH $\rightleftarrows$ $RS(O)_2H$] and then to a sulfonic acid [$S(O)_2$–OH]. Such a cascade, which would be initiated by molecular oxygen in the ER, could account for the wide variation observed in the doubling times of cells containing CGPS Trx (Figure 2-1). In contrast, a sulfenic acid formed in a CXXC motif could be converted to a half-cystine. Preliminary evidence suggests that a CXXS sequence is indeed more useful to cells grown under less oxidizing conditions (P.T. Chivers, D.J. Bauhs, Jr., and R. T. Raines, unpublished results).

Trx mutants that can replace PDI. The substitutions for Gly33 and Pro34 found in the complementing Trx mutants are quite drastic. The success of these particular substitutions does, however, have a physical basis. An increase in reduction potential means that the stability of the reduced form has increased relative to the oxidized form. Mutation of a proline to another amino acid residue increases the conformational entropy of a polypeptide chain. The additional entropy would favor the reduced form because the oxidized form would be more strained if the protein were more flexible (Gilbert, 1990). Direct support for this argument comes from the reduction potentials of CGHC Trx (Krause *et al.*, 1991) and CGSC Trx (Lin & Kim, 1991), which are higher than that of wild-type Trx.

The appearance of a tryptophan, the most rare residue, in the complementing CXXC motifs may also be explicable. Tryptophan has the largest sidechain of all natural amino acids. Its

presence in the CXXC motif may serve to increase the reduction potential simply by providing a steric hindrance to disulfide bond formation. In addition, a hydrophobic residue in the CXXC motif could enhance the interaction of Trx with a misfolded protein. Analogously, a hydrophobic residue in T_4 glutaredoxin has been postulated to aid in the interaction of T_4 glutaredoxin with glutathione (Nikkola *et al.*, 1991).

The decreased pK_a's of the complementing CXXC motifs may be another manifestation of increased mainchain flexibility. The likely physical basis for the anomalously low pK_a of Cys32 in wild-type Trx is its position at the N-terminus of an α-helix (Figure 1-3) (Hol, 1985). There, the positive dipole of the helix can stabilize the negative thiolate form of Cys32 (Forman-Kay *et al.*, 1991). Molecular modeling of our Trx mutants suggests that the replacing Pro34 allows the thiol of Cys32 to interact even more strongly with the N-terminus of the helix.

Requisite properties of a protein disulfide isomerase. Two biophysical properties of the CXXC motif are apparently critical to cell viability (Table 2-1; Figure 2-1). An increase in the reduction potential of a CXXC motif increases the fraction of the motif that is present in the reduced form. The higher reduction potential that we observed for Trx complementants is consistent with the necessity of having an enzymic thiol or thiolate in the ER. The ability of CGPS Trx but not wild-type Trx to confer viability to *pdi1Δ S.cerevisiae* also supports this conclusion. Reduction potential is linked to another important equilibrium—a thiol must be deprotonated to act as a nucleophile. We observed that the mutation of the non-cysteine residues in the CXXC motif can lower the pK_a of the nucleophilic thiol (Figure 2-1), thereby increasing at any pH the fraction of the enzyme that exists in the thiolate form. Still, the pK_a for wild-type Trx is already below the ambient pH of the yeast ER. Thus in our system, cell viability is less sensitive to thiol pK_a than to CXXC reduction potential.

Catalysis is a cyclic process (Raines & Knowles, 1987). A redox-active catalyst must cycle between reduced and oxidized forms. A balance must be achieved between the stability of the dithiol and disulfide forms for both substrate turnover and catalyst regeneration to occur efficiently. For example, a CXXC motif with a high reduction potential (such as that in DsbA; Figure 2-2) would not necessarily be a good catalyst of disulfide bond formation in the ER because it exists almost exclusively in the dithiol form, rather than in the disulfide form that initiates disulfide bond formation in a substrate. The reduction potential of PDI matches that of the ER (Figure 2-2). Thus, PDI (and our Trx mutants) exist in the ER as a near equimolar mixture of reduced and oxidized forms, allowing substrate turnover and catalyst regeneration to proceed with comparable facility. In contrast, wild-type Trx and DsbA have reduction potentials that are too extreme for significant recycling to occur in the ER (Figure 2-2). In vivo, the recycling of the CXXC motifs in Trx and DsbA is coupled to other enzyme-catalyzed redox reactions (Holmgren, 1985; Bardwell *et al.*, 1993).

The effects of pK_a on catalysis are more complex (Gilbert, 1990). An isomerization reaction (Figure 1-1) begins with the nucleophilic attack of a thiolate on a disulfide bond. Thiols of low pK_a are ionized more often (giving higher reaction rates) but are intrinsically less nucleophilic. These effects counteract such that rate constants for thiol – disulfide exchange reactions are maximal when the pK_a of the thiol equals the pH of the solution (Gilbert, 1990), which is approximately 7.0 in the ER (Hwang *et al.*, 1992). The pK_a of a thiol also affects its electrophilicity, which is necessary for regenerating the catalyst. Thiols of low pK_a leave more rapidly from a disulfide. A compromise must therefore be achieved to maximize catalytic efficiency. A typical cysteine residue has sidechain $pK_a \approx 8.7$. The CXXC motifs that complement *pdi1Δ* yeast have thiol pK_a's of 6 – 7 (Figure 2-1). Apparently, the need to catalyze native disulfide bond formation has resulted in a lower pK_a so as to maximize its ionization and electrophilicity without minimizing its nucleophilicity.

Nature often resorts to compromise. For example, enzymes have evolved to function under a variety of constraints (Benner, 1989; Burbaum *et al.*, 1989). Since cells containing different CXXC motifs grow at different rates (Figure 2-1), an attribute of the CXXC motif must be of great consequence. The correlation shown in Figure 2-1 indicates that this attribute is the fraction of the CXXC motif that is in the thiolate form.

CONCLUSIONS

We have used the complementation of *PDI1* null mutants of *S.cerevisiae* to reveal the imperatives for the formation of native disulfide bonds in cellular proteins. Our genetic and biophysical data demonstrate that the primary role of PDI is catalysis by its Trx domains—other functions ascribed to PDI (such as chaperone and anti-chaperone activity) are less relevant. Our analyses indicate that the critical attribute of a CXXC motif like that in PDI is its ability to provide a thiolate in the cell (Figure 2-1). Although a CXXS sequence can substitute for CXXC, we propose that the C-terminal cysteine residue is important because it provides an intramolecular anti-oxidant. Finally, our results and those of Laboissière *et al.* (1995b) suggest that nonnative disulfide bonds do exist in vivo, and that the role of the CXXC (or CXXS) motif is to unscramble these bonds (Figure 1-1; Chivers et al., 1996).

Figure 2-1 In vivo (ordinate) and in vitro (abscissa) properties of CXXC motifs. Data are from Table 2-1. A linear least-squares fit of the data gives: doubling time (rel.) = – 0.79 x log(fraction thiolate) + 3.8. This correlation and the doubling time (3.0 h) of *pdi1*Δ cells complemented with *PDI1* suggests that if wild-type Trx could replace PDI, then the doubling time of the resulting cells would be 20 h.

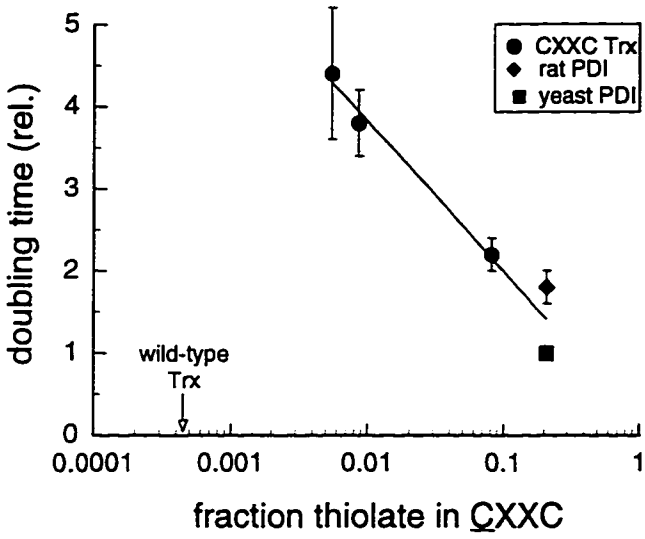

5
4
3
2
1
0
doubling time (rel.)
CXXC Trx
rat PDI
yeast PDI
wild-type
Trx
0.0001
0.001
0.01
0.1
1
fraction thiolate in CXXC

Figure 2-2 Spectrum of known reduction potentials for CXXC motifs. Trx mutants that can replace PDI have reduction potentials closer to that of the ER than does wild-type Trx. The reduction potential of DsbA is from Wunderlich and Glockshuber (1993).

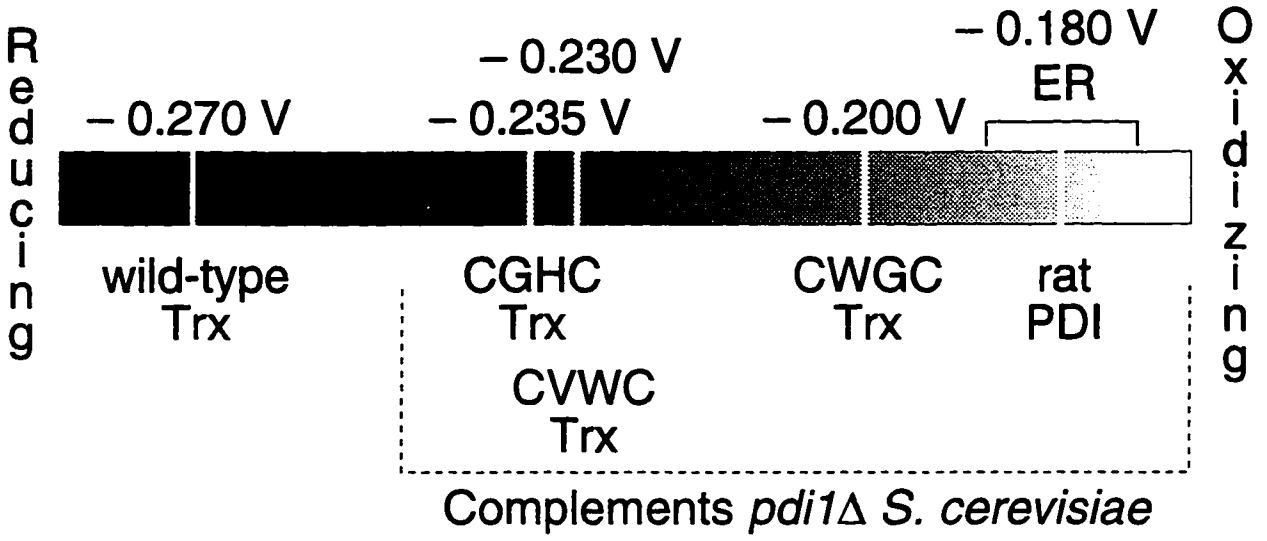

Reducing
– 0.270 V
– 0.230 V
– 0.235 V
– 0.200 V
– 0.180 V
ER
Oxidizing
wild-type
Trx
CGHC
Trx
CVWC
Trx
CWGC
Trx
rat
PDI
Complements pdi1Δ S. cerevisiae

Table 2-1

In vivo and in vitro properties of PDI and Trx

Protein	Nucleotide Sequence of CXXC[a]	Doubling Time of Complemented *pdi1Δ* Yeast (rel.)	$E^{o\prime}$ of CXXC	pK_a of CXXC	Thiolate Form of CXXC in ER (%)[b]
yeast PDI	TGTGG(CT)CACTGT	1.0	nd	nd	nd
rat PDI	TGTGG(CT)CACTGC	1.8 ± 0.2[c]	– 0.180[d]	6.7[e]	21
wild-type Trx	TGCGGTCCGTGC	nc	– 0.270[f]	6.28	0.034
CGHC Trx	TGCGGTCACTGC	4.4 ± 0.8	– 0.235[g]	nd	0.52[h]
CVWC Trx	TGCGTGTGGTGC	3.8 ± 0.4	– 0.230	5.86	0.86
CWGC Trx	TGCTGGGGTTGC	2.2 ± 0.2	– 0.200	5.94	8.2
CGPS Trx	TGCGGTCCGTCT	4.3 ± 0.5	—	nd	83[h]
SGPC Trx	TCTGGTCCGTGC	nc	—	—	—

a, PDI has two CXXC motifs; underlined sequences were created in this work; b, Calculated for the thiol of the N-terminal cysteine residue of the CXXC motif by using the Nernst and Henderson–Hasslebalch equations at pH 7.0 with $E^{o\prime}$ = – 0.180 V (Hwang *et al.*, 1992); c, Laboissière *et al.* (1995); d, Lundström and Holmgren (1993); e, Hawkins and Freedman (1991); f, Moore *et al.* (1964); g, Krause *et al.* (1991); h, Assuming that pK_a of CXXC is unchanged by the mutation; nd, not determined; nc, no complementation.

Chapter 3

The Microscopic pK_a's of *E. coli* Thioredoxin

ABSTRACT

The pK_a of the first cysteine (CXXC) of dithiol-disulfide oxidoreductases is a determinant of their catalytic activity. *E. coli* thioredoxin is an efficient protein disulfide reductant. The nucleophilic cysteine (Cys32) should have a pK_a close to physiological pH to optimize the reductive activity of the protein. Previous determinations of the pK_a of Cys32 by NMR methods have been complicated by ambigious titration curves that suggest the presence of more than one acidic group. Here, using mutants of Trx we have resolved the ambiguities of the titration of the wild-type enzyme (CGPC). CVWC and CWGC Trx have a lowered Cys32 pK_a (6.1 - 6.2) relative to the wild-type enzyme (7.5 and 9.5) . D26N and D26L Trx have a Cys32 pK_a of 7.5, and exhibit well defined, single pK_a titration curves. These mutants reveal that the *microscopic* pK_a's of Asp26 and Cys32 are responsible for the complex titration behavior of wild-type Trx. In reduced wild-type Trx, Cys32 has pK_a 7.45 when Asp26 is protonated and pK_a 9.2 when Asp26 is deprotonated. Similarly, Asp26 has pK_a 7.5 when Cys32 is protonated and pK_a 9.25 when Cys32 is deprotonated. Indeed, Cys32 has a pK_a close to physiological pH. The presence of microscopic pK_a's in the active-site of Trx is unlikely to have a significant effect on the kinetic activity of the wild-type enzyme.

INTRODUCTION

Disulfide oxidoreductases containing the Cys-Xaa-Xaa-Cys (CXXC) motif are found in diverse intracellular environments (Goldberger *et al.*, 1963; Venetianer & Straub, 1963; Moore *et al.*, 1964; Bardwell *et al.*, 1991). The reduction potential ($E^{o\prime}$) of the active-site disulfides of CXXC-containing proteins is determined by its location in the cell. For example, *E. coli* Trx (CGPC), $E^{o\prime} = -0.270$ V (Moore *et al.*, 1964), exists in the cytosol, $E = -0.270$V (Gilbert, 1990). Protein disulfide isomerase [(PDI), CGHC], $E^{o\prime} = -0.180$ V (Lundström & Holmgren, 1993), is found in the endoplasmic reticulum, $E = -0.180$ V (Hwang *et al.*, 1992). The pK_a of the first cysteine (Cys_N) is one of several determinants of $E^{o\prime}$ of the CXXC motif (Chapter 5). In general, a lower Cys_N pK_a correlates with a higher reduction potential of the active-site disulfide.

This cysteine residue also has kinetic importance. In aqueous conditions, thiols are most reactive when pH = pK_a (Szajewski & Whitesides, 1980). At physiological pH, when pK_a >> pH, the thiol group of Cys_N is its non-reactive protonated form. Yet, when the pK_a is low, the thiol group is a much better electrophile than a nucleophile. For example, Cys_N of *E. coli* DsbA has pK_a = 3.5 (Nelson & Creighton, 1994) and in most *in vivo* dithiol-disulfide exchange reactions, Cys_N of DsbA will have the lower pK_a and therefore be a better leaving group when it is in mixed disulfide.

E. coli Trx is a strong reductant *in vivo* (Holmgren, 1985). Thus, Cys_N (Cys32) is likely to have a pK_a close to physiological pH. Indeed, previous studies have determined this pK_a to be slightly above 7 (Dyson *et al.*, 1991; Li *et al.*, 1993; Jeng *et al.*, 1995; Wilson *et al.*, 1995). Cys_N in human Trx has a slightly lower pK_a of 6.35 (Forman-Kay *et al.*, 1992). Recently, however, Takahashi & Creighton (1996)proposed that the pK_a of Cys_N is actually 9, or even higher.

We have isolated two mutants of *E. coli* Trx that complement *pdi1Δ S. cerevisiae* (Chivers *et al.*, 1996). These mutants have altered CXXC sequences (CWGC and CVWC) compared to wild-type Trx (CGPC). These mutants have elevated $E^{o'}$ values relative to wild-type Trx, characteristic of an optimization of their catalytic activity (Burbaum *et al.*, 1989) for the environment of the endoplasmic reticulum (Hwang *et al.*, 1992). An elevated reduction potential should correlate with a lowered Cys32 pK_a [Chapter 5; (Szajewski & Whitesides, 1980; Grauschopf *et al.*, 1995)] Our initial pK_a measurements employed a fluorescence technique that may not directly measure cysteine pK_a (Jeng *et al.*, 1994). Here, we have used ^{1}H- and ^{13}C-NMR to observe the pH-titration behavior of the CXXC mutants. We find that the pK_a of Cys32 in the mutant enzymes is indeed depressed. We have also carried out titrations of two other mutant Trx's. In these enzymes, Asp26 (which is proximal to Cys_C) has been changed to an asparagine or leucine residue. Our results show that the pK_a of Cys32 in these mutant enzymes is close to the physiological pH. The titrations of these mutant enzymes provide new insight into the controversial titration behavior of wild-type Trx (Dyson *et al.*, 1991; Li *et al.*, 1993; Jeng *et al.*, 1995; Wilson *et al.*, 1995; Jeng & Dyson, 1996; Takahashi & Creighton, 1996). The confusion present in the literature over the assignment of pK_a values to the active-site residues (Cys32, Cys35, Asp26) of reduced wild-type Trx is a consequence of the electrostatic interaction of functional groups, which produces microscopic pK_a values for the sidechains of Asp26 and Cys32.

MATERIALS AND METHODS

Strains and Reagents. E. coli strain BL21(DE3) was from Novagen (Madison, WI). *Taq* polymerase and restriction enzymes were from Promega (Madison, WI). Deuterium oxide, deuterium chloride, and potassium deuteroxide were from Isotec (Miamisburg, OH). [3-

^{13}C]cysteine was from Cambridge Isotopes (Andover, MA). Potassium phosphate was from Fisher.

Construction of pTRX. The genes for the CXXC mutants of Trx were amplified from YepWL.TRX (Chivers *et al.*, 1996) by the PCR with oligonucleotides PC26 (5′ AAGAAGGAGTTATACATACATATGAGCGATAAAATTATT 3′) and PC05 (5′ AACGTGTTCAACCAAGTCGACGGTATCGATAAGC 3′). The PCR products were digested at the underlined sites with *Nde*I and *Sal*I, and the resulting fragments were inserted into pET22b(+) that had been digested with the same enzymes. The gene for wild-type Trx without its HDEL tail were amplified using oligonucleotides PC26 and PC27 (5′ GGGGCACCCAACGTCGACATTCCCTTACGCCAGGTTAGCGTC 3′). Genes for CWGC and CVWC Trx without their HDEL tails were constructed by subcloning of the *Cla*I/*Sal*I fragment of the wild-type Trx gene into vectors coding for the mutant enzymes. The creation of genes for D26L and D26N Trx is described in Chapter 4.

Production and Purification of Trx in E. coli. A plasmid coding for wild-type or mutant Trx was transformed into *E. coli* BL21(DE3). A single transformant was picked and replated at least two times on LB plates containing ampicillin (400 μg/mL) to amplify plasmid copy number (Hoffman *et al.*, 1995). A single colony was picked and grown at 37 °C in 2.0 mL of LB medium containing ampicillin (400 μg/ml). While $A < 0.1$ at 600 nm, an aliquot (10 μL) of this culture was transferred to fresh LB medium (2.0 mL) containing ampicillin (400 μg/ml). This culture was used to inoculate fresh LB medium (20 mL) containing ampicillin (400 μg/ml). Before incubation, this culture was used for sequential 1:1000 dilutions into two additional fresh LB medium (20 mL) containing ampicillin (400 μg/ml). All three cultures were grown overnight at 37 °C. An aliquot (10 mL) of the least dense culture ($A < 1$ at 600 nm) was used to inoculate 0.5 or 1.0 L cultures of TB media. Expression of *trx* was induced when A =

1.9 at 600 nm by the addition of IPTG (to 1 mM). After a 3 h induction, cells were harvested by centrifugation at 7000 *g* for 10 min. The cell pellet was resuspended in lysis buffer (125 mL per 0.50 L of cell culture), which was 20 mM Tris-HCl buffer, pH 7.8, containing urea (6.0 M) and EDTA (1.0 mM). The suspension was shaken for 20 min at 37 °C to ensure complete lysis. The lysate was centrifuged at 30 000 *g* for 15 min, and the urea-soluble fraction (125 mL) was dialyzed against 4 L of 20 mM Tris-HCl buffer, pH 7.8, containing EDTA (1.0 mM). The dialysate was then centrifuged at 30 000 *g* for 30 min to remove any precipitate. The resulting supernatant was concentrated using a microconcentrator from Amicon (Beverly, MA) with a YM10 ultrafiltration membrane. The concentrate (<10 mL) was loaded with a flow rate of 1.5 mL/min onto a Pharmacia FPLC Hi-Load Sephadex G-75 column, which had been equilibrated with 0.10 M potassium phosphate buffer, pH 7.6 containing EDTA (1.0 mM). SDS-PAGE showed that the protein was >98% pure at this stage (data not shown).

Protein solutions were concentrated for NMR experiments using a Amicon Centriprep 10 concentrator. For experiments in D_2O, the concentrated protein solution was lyophilized, and the lyophilisate was resuspended in D_2O and incubated at 37 °C for at least 4 h to allow for proton exchange. Then, the protein solution was lyophilized, and the lyophilisate was resuspended in D_2O (0.5 mL).

When necessary (*e.g.*, to remove excess salts or DTT) samples were desalted using a Pharmacia FPLC Fast Desalt 10/10 column, which had been equilibrated with 0.10 M potassium phosphate buffer, pH 6.2, in H_2O.

Preparation of Site-Specifically ^{13}C-Labeled Trx. To enhance the resolution of NMR experiments, Trx was prepared with an enrichment of ^{13}C at Cβ of the two cysteine residues. This ^{13}C-labeled enzyme was prepared as described above from growth medium containing [3-^{13}C]cysteine (40 μg/mL). The resulting protein had levels of ^{13}C incorporation that were low, but adequate for the analysis of wild-type Trx. For production of ^{13}C-labeled mutant Trx

mutants, pTRX was transformed into *E. coli* strain JM15, a cysteine auxotroph. Transformed cells were grown in LB medium containing [3-^{13}C]cysteine (40 μg/mL), and enzyme was produced as described above. Because of the auxotrophic host, the incorporation of label into the mutant enzymes was much higher than for the wild-type enzyme. Labelled mutant enzymes were purified as described above.

Protein Concentrations. Protein concentrations were determined by measuring *A* at 280 nm. The extinction coefficients of the CWGC and CVWC Trx were estimated to be ε_{280} = 18470 $M^{-1}cm^{-1}$ by using the method of Gill and von Hippel (1989). The extinction coefficients of D26N and D26L Trx were assumed to be the same as wild-type Trx (ε_{280}=13 700 M^{-1} cm^{-1}).

NMR Experiments. NMR experiments were performed on solutions with protein concentrations of 1 – 3 mM. Proteins were reduced prior to each experiment by the addition of DTT (to 5 mM). DSS was added as a chemical shift reference. The pH of solutions was adjusted by the addition of aliquots (<4 μL) of either 1.0 M KOH or 1.0 M HCl for samples in H_2O, or 1.0 M KOD or 1.0 M DCl for samples in D_2O. The pH of the sample was measured before and after each experiment and the latter reading was used for data analysis. pH measurements of samples containing D_2O were corrected for the deuterium isotope effect (Glasoe & Long, 1960). ^{1}H NMR in H_2O used WATERGATE water suppression (Piotto *et al.*, 1992). ^{13}C-NMR spectra were collected using WALTZ-16 proton-decoupling (Shaka *et al.*, 1983). All spectra were collected at 308 K.

Fluorescence Experiments. Fluorescence experiments were carried out as described previously (Holmgren, 1972; Reutimann *et al.*, 1981; Chivers *et al.*, 1996). The final protein concentration was 0.050 mg/mL. Potassium phosphate buffers were made from a 0.10 M

stock solution containing EDTA (1.0 mM). The pH of the stock solution was adjusted with either HCl or NaOH, and an aliquot was removed to serve as the buffer for that pH. The same procedure was used for potassium phosphate in D_2O, except DCl and NaOD were used to adjust the pH* of the solution. The initial pH or pH* of the stock solution was 7.0. The pH* measurements of the D_2O buffer were corrected for the deuterium isotope effect (Glasoe & Long, 1960).

Preparation of Carboxymethylated Thioredoxin. A solution (200 μL) containing wild-type Trx (5 mg/mL) in 0.10 M potassium phosphate buffer, pH 8.0, containing 0.001 M EDTA was reduced by adding DTT (to 5 mM) and incubating at 20 °C for 15 min. The reduced enzyme was carboxymethylated by adding a 10-fold molar excess of iodoacetic acid (10 mM) (Sigma Chemical; St. Louis, MO) in 0.25 M Tris-HCl buffer, pH 8.0.. The resulting solution was incubated at 20 °C for 15 min. The reaction was quenched by lowering the temperature to 0 °C, and removing excess reagents with a Pharmacia FPLC Fast Desalt 10/10 column, which had been equilibrated with 0.10 M potassium phosphate buffer, pH 6.2.

Thioredoxin/Protein Disulfide Isomerase Activity. Catalysis of protein disulfide bond isomerization was assayed by monitoring the regain in activity of scrambled RNase A (Sigma Chemical), which has non-native disulfide bonds, as described (Laboissière *et al.*, 1995).

Data Fitting. Titration data were fit to either one or two pK_a titration curves using the program MACCURVEFIT. Single proton titrations were fit using eq 3-1:

$$\delta = \delta_{HA} - \left(\frac{\left(\delta_{HA} - \delta_{A^-}\right)}{\left(1 + 10^{(pK_a - pH)}\right)} \right) \qquad (3\text{-}1)$$

Complex titration curves were fit with equations that take into account multiple pK_a's (Shrager *et al.*, 1972). Two proton titrations with non-interacting pK_a's were fit using eq 3-2:

$$\delta = \left(\frac{\left(\delta_{H_2A}[H^+]^2 + \delta_{HA^-} K_1[H^+] + \delta_{A^{2-}} K_2[H^+] + \delta_{A^{2-}} K_1 K_2 \right)}{\left([H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1 K_2 \right)} \right) \tag{3-2}$$

Two proton titrations with interacting pK_a's were fit using eq 3-3:

$$\delta = \left(\frac{\left(\delta_{H_2A}[H^+]^2 + \delta_{HA^-} K_1[H^+] + \delta_{-AH} K_2[H^+] + \delta_{A^{2-}} K_1 K_{12} \right)}{\left([H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1 K_2 \right)} \right) \tag{3-3}$$

Eq 2-3 and eq 3-3 differ in the last two terms of the numerator. Eq 3-3 accounts for a change in the second proton dissociation constant (site 2) due to the presence (K_2) or absence (K_{12}) of the first proton (site 1). Eq 3-3 also accounts for the effect of K_2 on the chemical shift (δ_{-AH}) while the first proton (site 1) is still present.

RESULTS

Production and Purification of Mutant Trx's. Our protocol for protein production followed that used for the expression of RNase A in *E. coli* (delCardayré *et al.*, 1995). Pure wild-type Trx was produced at 150 mg/L of culture. Pure D26L, D26N, and C35S Trx were produced at levels slightly less than that of wild-type Trx. Pure CVWC and CWGC Trx were produced at somewhat lower levels (~50 mg/L). Chromatography on a single gel filtration column produced protein that was sufficiently pure (>98%) for NMR experiments.

Titration of Thioredoxins Monitored by Fluorescence Spectroscopy. The first use of fluorescence spectroscopy to monitor the pH-titration of reduced thioredoxin was carried out by Holmgren (1972). We used this method to characterize the titration of CVWC and CWGC Trx. The apparent pK_a values we obtained for these enzymes were lower than that observed for wild-type (Chivers *et al.*, 1996). Since the XX mutations should only affect local pK_a's, we reasoned that the XX mutations had lowered the pK_a of Cys_N. Dyson and coworkers, however, have claimed that the pH-dependence of the fluorescence of reduced Trx is due to a hydrogen bond between Nε of His6 and NεH of Trp28 (Jeng *et al.*, 1994). They base this claim on detailed solution structures of oxidized and reduced *E. coli* Trx. ^{1}H-NMR experiments indicate a pK_a of 6 for His6 in reduced Trx (Hiraoki *et al.*, 1988; Dyson *et al.*, 1991). This value is similar to the apparent pK_a determined by fluorescence spectroscopy. Because of the complexity of the apparent pK_a value determined by fluorescence spectroscopy, we performed fluorescence experiments using solution conditions that mimicked those used for NMR spectroscopy.

First, we used 0.10 M potassium phosphate buffer to determine if the higher ionic strength of this buffer was responsible for the change in apparent pK_a. The pK_a values determined in this experiment (Table 3-1; Figure 3-1) are within error of those determined using a constant ionic strength of 0.10 M (Chivers *et al.*, 1996). The quenching of the fluorescence of Trp28 in both CVWC and CWGC Trx are less than that in the wild-type enzyme. Interestingly, the titration of wild-type Trx using the potassium phosphate buffer shows a slight inflection at pH > 8. Using eq 3-2 to fit this titration curve yields pK_a values of 6.1 and 7.4, with the second pK_a being poorly determined. The first pK_a matches closely that determined for reduced Trx using ^{1}H-NMR spectroscopy (Hiraoki *et al.*, 1988; Dyson *et al.*, 1991).

Next, we carried out the fluorescence titrations using D_2O as the solvent. These data show a dramatic effect of solvent on the value of the apparent pK_a (Table 3-1; Figure 3-1). These

results are anomalous, either with or without the necessary correction for the deuterium isotope effect. From the pK_a's in H_2O and D_2O, the apparent fractionation factor (ϕ) of the titrating group can be determined by using eq 3-4:

$$\Delta pK_a = \log \frac{\phi_{BH}\phi_{H_2O}}{\phi_{H_3O^+}} \tag{3-4}$$

and $\phi_{H2O} = 1$ and $\phi_{H3O+} = 0.33$ (Klinman, 1977). The high values of ϕ_{BH} (>2; Table 3-1) indicate that a much more stable protonated species of reduced Trx exists in D_2O than in H_2O. A limitation of eq 3-4 is the assumption that the unprotonated species (B) is identical in H_2O and D_2O. Differences in solvation of the unprotonated species in H_2O vs D_2O are possible because these solvents have different physical properties (Némethy & Scheraga, 1964). An additional constraint is that eq 3-4 requires that the observed titration reflects a single functional group. Because of the large number of titratable groups on Trx, more than one fractionation factor could be responsible for the effect that we observe (Kresge, 1973). Still, these results demonstrate that properties of wild-type Trx differ in H_2O and D_2O.

Yet, even after consideration of the isotope effect, the pH-titration of wild-type Trx monitored by fluorescence spectroscopy may not directly monitor the titration of Cys32 (or Cys35). How then do perturbations to the CXXC motif affect the apparent titration of residues other than the active-site cysteines?

pH titrations of Thioredoxins by NMR spectroscopy. One-dimensional ^{1}H- or ^{13}C-NMR experiments were used to determine directly the pK_a's of the histidine and cysteine residues in H_2O and D_2O.

His6 and His109. His109, which is in the HDEL tail of the CXXC mutants, provides a useful control for the titration of a histidine residue in H_2O vs D_2O. It exists in a disordered

region of the enzyme away from the active site, and its titration should be insensitive to the redox state of the active site. The titration of His109 should also exhibit a normal isotope effect. The pK_a of His109 in CVWC Trx is 6.9 in both oxidized and reduced Trx, in both H_2O and D_2O (Table 3-2; Figure 3-3). The chemical shift of C2H decreases by 0.95 ppm upon deprotonation of His 109 in each case. The close proximity of two acidic sidechains (Asp110 and Glu111) as well as the C-terminal carboxyl group likely elevate the pK_a of His109. The difference in pK_a between the H_2O and D_2O measurements is ~0.4 units. The value of ϕ_{BH} determined for His109 (Table 3-5) using eq 3-4 is slightly lower than that normally seen for histidine sidechains (Schowen, 1972). This decrease may result from the anionic environment, which can decrease fractionation factors (Schowen & Schowen, 1982).

The titration of His6 in H_2O vs D_2O is more complex than expected (Figure 3-3). Clearly there is a difference in the titration behavior of His6 in the reduced and oxidized forms of the enzyme. As a result, data for the reduced and oxidized enzymes were fit independently to eq 3-2. In H_2O, the first pK_a of His6 changes little between the oxidized and reduced molecules (Table 3-3; Figure 3-3). In contrast, the second pK_a differs by at least 1 unit. The presence of a second pK_a is supported by the difference in the C2H chemical shifts of cationic and neutral imidazolyl sidechains. This difference is about 0.9 – 1.0 ppm for most histidine residues (Bundi & Wüthrich, 1979) and His109 titrates normally in this respect in all cases ($\Delta\delta = 0.95$ ppm). For the titrations of His6 in wild-type and CVWC Trx in H_2O, $\Delta\delta$ for C2H is 1.1 ppm. In D_2O, the pK_a of His6 in the reduced protein is slightly higher than that in the oxidized protein, with the size of the observed difference varying in each Trx. The value of $\phi_{BH} \approx 1.2$ determined by NMR spectroscopy for the His6 titrations in reduced Trx are considerably lower than those observed by fluorescence spectroscopy.

Titration of His6 in D26N and D26L Trx differs from that in wild-type, CVWC, and CWGC Trx (Figure 3-3). The second titration that can be seen in D26N and D26L Trx occurs at pH > 9, which is higher than in enzymes containing Asp26. The isotope effect on the

titration of His6 in the D26N and D26L Trx are, however, similar to those observed for wild-type, CVWC, and CWGC Trx, as are the values of $\Delta\delta = 1.04$ ppm. Finally, D26N Trx has a different pK_a for His6 in its oxidized and reduced forms.

Trp28 and Trp31. The indole protons of the Trp residues in Trx should provide clues to their environment in H_2O. If His6 is involved in a hydrogen bond to NεH of Trp28, then the indole proton should exhibit a pH-titration confirming the hypothesis that His6 quenches the fluorescence of this tryptophan (Jeng *et al.*, 1994). Indeed, a significant change in the chemical shift of NεH of Trp28 is observed in each enzyme as the pH increases to 7 (Figure 3-4). Unfortunately, the aggregation of Trx at pH < 5.5 prevents the accurate determination of δ in this pH range.

Interestingly, although there is a difference at high pH in the chemical shift of NεH of Trp28 between reduced (10.9 ppm) and oxidized proteins (10.75 ppm), the pH-dependence of this proton is the same in the two redox forms at pH < 7. This result suggests either that Nε of His6 forms a hydrogen bond with NεH of Trp28 in both oxidized and reduced Trx [which is contrary to the solution structures of the oxidized and reduced proteins (Jeng *et al.*, 1994)] or that the perturbation of the chemical shift of $N_\varepsilon H$ of Trp28 is effected in another manner.

The chemical shift of NεH of Trp28 in D26N and D26L Trx does not vary with the redox state at pH > 7. In D26N Trx, the maximal chemical shift is ~10.95 ppm in the reduced and oxidized enzymes. In D26L Trx, the maximum is 10.8 ppm in the oxidized form. In the reduced form, this proton exhibits three different pH-dependent perturbations of its chemical shift. The oxidized form of the D26L enzyme shows the approach to an endpoint at low pH. A one pK_a fit to the titration data of Trp28 in this case yielded a value within error of that determined for His6 in D26L Trx (Table 3-3).

These results suggest that the environment around Trp28 is very sensitive to the oxidation state of the protein as a result of its proximity to Asp26. The D26L mutants indicate that residue 26 is also senstive to the protonation state of the active-site cysteines.

The chemical shift of NεH of Trp31 also exhibits a pH-dependence (Figure 3-4). In the oxidized protein, it shows a two pK_a titration. The lower value cannot be accurately determined without a baseline at low pH but this chemical shift change is likely due to Asp61 which is involved in a H-bond with this proton (Katti *et al.*, 1990; Jeng *et al.*, 1994). The second pK_a is approximately 7.5, which agrees with previous values assigned to Asp26 in the oxidized protein (Dyson *et al.*, 1991; Langsetmo *et al.*, 1991). The absence of this titration in the Asp26 mutants supports this assignment.

In reduced Trx the chemical shift behavior of Trp31NεH is more complex, a likely reflection of the titration of both proximal and distal functional groups, such as those of Cys32, Cys35, Asp26, and Asp61.

The influence of Asp26 is most apparent in D26L Trx. In that protein, two titrations above pH 6.5 are visible in the chemical shift of NεH of Trp31. It is compelling that the pH-dependence of the Trp28NεH chemical shift in this mutant has a similar appearance to that of Trp31NεH, suggesting that both residues are sensitive to pH-dependent changes in Trx structure. In some cases, these structural perturbations can be effected over a relatively long distance (>10 Å).

pH-Dependence of $N_\varepsilon H$ of Trp33 and Trp34. In contrast to the behavior of Trp28 and Trp31, the pH-dependence of the chemical shift $N_\varepsilon H$ of the new tryptophan residues in CVWC and CWGC Trx mutants appear to be sensitive only to the titration of Cys32 (*vide infra*), with pK_a values of 6.26 for CWGC and 6.15 for CVWC, respectively (Figure 3-4). There was no significant change in the chemical shift ($\Delta\delta < 0.04$ ppm) of $N_\varepsilon H$ upon oxidation of these enzymes.

^{13}C-NMR Spectrocopy. The ^{1}H-NMR experiments described thus far do not report directly on the pK_a's of the active-site cysteine residues. To determine these values, we used enzymes that were labeled with ^{13}C only at Cβ of their two cysteine residues. This technique enabled us to monitor directly the titration of the thiol protons by following the change in ^{13}C chemical shift. The titration curves of [^{13}C]Trx in D_2O appeared the same as those observed by Jeng et al. (1995) and Woodward et al. (1995). We also performed pH-titrations of reduced [^{13}C]Trx in H_2O and oxidized [^{13}C]Trx in D_2O. The titration behavior of the reduced protein in H_2O was very similar to that observed in D_2O (data not shown). Interestingly, the fractionation factors for the thiols of Cys32 and Cys35 calculated with eq 3-4 were close to 1, which is an unusual value for a thiol (Belasco *et al.*, 1986; Wong *et al.*, 1988). The titration of oxidized [^{13}C]Trx showed a single pK_a of approximately 7.5 (Figure 3-6; Table 3-5). We conclude that this value reflects the titration of Asp26. This conclusion was supported by the titration of oxidized D26L Trx (Figure 3-6; Table 3-5), which lacks a pK_a near 7.5. Most importantly, these results demonstrate that the environment of Cys32 is sensitive to the ionization state of Asp26. The converse must necessarily be true. The ionization state of Cys32 in reduced Trx could therefore influence the titration behavior of Asp26.

The ^{13}C chemical shifts of Cβ of the cysteine residues in CWGC and CVWC Trx changed dramatically with pH at pH< 7 (Figure 3-5). An additional chemical shift change was observed above 8.5. This second transition clearly results from the titration of a functional group. This functional group could be either Asp26 or Cys35. The chemical shift change observed for Cys35 does not support the latter conclusion.

The titration of the ^{13}C chemical shifts of Cβ of the cysteine residues in D26L Trx reveals a pK_a of 7.5. The titration of D26N Trx reveals a pK_a of 7.4. The similarity of these values in Cys32 and Cys35 suggests that they report on the titration of the same functional group with a pK_a near 7.5. These values are similar to those previously reported for D26A Trx (Wilson *et*

al., 1995; Jeng & Dyson, 1996). At pH >10, a second titration was observed for D26L Trx (Figure 3-5). Without a well-defined endpoint at high pH, however, any pK_a determination is just an estimate. Still, two pK_a fits of both the Cys32 and Cys35 titration curves yielded values for this pK_a of ~11. However, no titration at high pH was observed for D26N Trx. The $\Delta\delta$ values for these titrations were 3 – 4 ppm. The higher value was observed for the D26N and D26L enzymes.

Disulfide Isomerase Activity of Trx. Wild-type and CXXC enzymes were assayed for the ability to isomerize non-native disulfide bonds in scrambled RNase A. The data obtained are shown in Table 3-8. All three enzymes catalyze protein disulfide bond isomerization at a level above the rate of glutathione buffer (1.0 mM GSH and 0.2 mM GSSG). The heterogeneous substrate used for this assay may explain the high activity exhibited by the wild-type enzyme. Scrambled RNase A is prepared by air oxidation and it is possible a significant number of native disulfide bonds could be present in the substrate. RNase A molecules with some native disulfide bonds may be more stable to the reducing ability of wild-type Trx than a nascent peptide chain in the ER.

DISCUSSION

The titration behavior of reduced thioredoxin has defied straightforward interpretation (Holmgren, 1972; Reutimann *et al.*, 1981; Dyson *et al.*, 1991; Li *et al.*, 1993; Jeng *et al.*, 1995; Wilson *et al.*, 1995; Jeng & Dyson, 1996; Takahashi & Creighton, 1996). The difficulty in determining pK_a values in reduced Trx results from the proximity of three titrating groups (Asp26, Cys32, and Cys35) in the active site. Here, we have used mutagenesis and site-specific labeling to overcome this complexity.

Nearby titrating groups with similar pK_a's exhibit *microscopic* pK_a's (Edsall & Wyman, 1958). [For a recent example of an analysis of microcopic pK_a's, see: McIntosh et al. (1996).] Microscopic pK_a's result from Coulombic interactions that create either positive or negative cooperativity. Wyman and Gill (1990) have presented a comprehensive analysis of such cooperativity in the titration of protein functional groups. In addition, a series of equations for fitting both independent (eq 3-4) or dependent titration (eq 3-4) curves is known (Shrager *et al.*, 1972). Previous interpretations of the pH-titration of Trx have not considered the microsopic pK_a's (Dyson *et al.*, 1991; Jeng *et al.*, 1995; Wilson *et al.*, 1995; Jeng & Dyson, 1996).

Determining Microscopic pK_a's. The removal of Asp26 from Trx by mutagensis produces a well-defined, single pK_a titration curve for Cys32 [Figure 3-5; Wilson et al. (1995)(Wilson *et al.*, 1995)]. The pK_a value determined from this curve, about 7.5, is likely to be similar to the pK_a of Cys32 in wild-type Trx when Asp26 is protonated because the net charge on both enzymes is the same. In an analogous manner, the pK_a of Asp26 when Cys32 and Cys35 are protonated can be determined from the titration of oxidized Trx. This pK_a is also about 7.5 (Figure 3-6). In addition, ^{1}H- and ^{13}C-NMR titrations of Trx in which Cys32 has been modified with a carbamoylmethyl group reveals a pK_a of 7.58 (LeMaster, 1996). The structure of this modified Trx is virtually identical to that of the reduced enzyme. These similar pK_a values for Asp26 and Cys32 as well as the effects of Asp26 on the ^{13}C chemical shift of Cβ of Cys32 necessitate the consideration of microscopic pK_a's for Asp26 and Cys32. Here, these Coulombic interactions are likely to yield *negative* cooperativity because both Asp26 and Cys32 become anionic upon deprotonation.

The equilibria depicted in Figure 3-7 can be used to determine the extent of the interaction between Asp26 and Cys32. Because pK_a is a state function, complete titration by path $K_{SH}K_{COOH*}$ is identical to that by the path $K_{COOH}K_{SH*}$. The amount of Cys32 present in the thiolate form will be the sum of species **II** and **IV** over the concentration of all species (**I-IV**).

A model titration curve can be constructed when microscopic pK_a values for the two titrating groups and chemical shift values for species **I-IV** are known (eq 3-4) (Shrager *et al.*, 1972).

Model fits using eq 3-4 are shown in Figure 3-8 and Table 3-7. Clearly, microscopic pK_a's are present in the active-site of Trx. When *Asp26 is protonated* the *microscopic* pK_a of Cys32 is 7.46, which is 0.3 units higher than the value determined by a simple two-pK_a fit of the titration data. When *Cys32 is protonated* the *microscopic* pK_a of Asp26 is 7.5, similar to the oxidized protein. In contrast, when *Cys32 is deprotonated* the *microscopic* pK_a of Asp26 is about 9.3. From Figure 3-8, when *Asp26 is deprotonated* the *microscopic* pK_a of Cys32 is about 9.2. The large perturbation of the pK_a's of Asp26 and Cys32 signal significant negative cooperativity between the functional groups of these two residues in reduced Trx. The extent of this interaction can be described by the ratio pK_{aCO2H^*}/pK_{aCO2H}, which is 0.017 in the wild-type enzyme (the ratio of pK_{a32SH^*}/pK_{a32SH} yields the same value). This value is two orders of magnitude lower than the value for non-interacting functional groups ($pK_a/pK_{a^*} = 1$).

The degree of this cooperativity is illuminated by comparison with CWGC or CVWC Trx. In these mutant enzymes, when Asp26 is protonated the pK_a of Cys32 is 6.15. As a consequence, just a small fraction of reduced Trx (>4%) will be found as species III (Figure 3-9). When pH equals the pK_a of Cys32, the fraction of deprotonated Cys32 will be approximately 0.5 for CVWC Trx but only 0.35, for wild-type Trx (Figure 3-11). In the wild-type enzyme, the upper pathway in Figure 3-7 is only slightly favored, so that at pH 7, Trx is 35% thiolate (**II**) and 32% carboxylate (**III**) (Figure 3-10).

None of the published NMR titration data is in discord with this analysis. In the direct monitoring of the Asp26 titration in reduced Trx, a second titration was observed (Jeng & Dyson, 1996). This second pK_a is likely to be the remaining Asp26-COOH dissociating to Asp26-COO^-. None of the previous NMR studies supports the titration of Cys35 with a pK_a value < 10.

The pK_a values we have determined for reduced wild-type Trx do not conflict with any of the experimental data presented by Takahashi and Creighton (1996). The interpretation of equilibrium measurements, such as the pH-dependence of Trx in equilibrium with glutathione, without full consideration of the titration behavior of all the equilibirum species is prone to (sometimes large) errors. Certainly, the measurements of Takahashi and Creighton revealed that a simple interpretation (Dyson *et al.*, 1991; Jeng *et al.*, 1995; Wilson *et al.*, 1995; Jeng & Dyson, 1996) of the titration of reduced Trx was not accurate.

We can conclude that Cys32 has a pK_a of 7.5 in the folded protein. This value is remarkably similar to that obtained for an α-helical peptide containing a cysteine at the N-terminus (Kortemme & Creighton, 1995). Thus, the net stabilization of the pK_a of Cys32 in wild-type Trx relative to a a normal cysteine (pK_a 8.5) could derive solely from its presence at the N-terminus of an α-helix.

Many of the ^{1}H chemical shift changes presented earlier support an elevated pK_a for Asp26 in reduced Trx. The titration of His6 in D26L and D26N is identical in the oxidized and reduced forms of Trx unlike His6 in the wild-type and CXXC mutants. The indole NεH chemical shifts in the reduced and oxidized forms of Trx change markedly in the absence of Asp26. Thus, the downfield shift of Trp31NεH of CWGC (Figure 3-4) at pH > 8 is likely due to the titration of Asp26. This conclusion is corroborated by the lack of any cysteine titration for this mutant above pH 8 (Figure 3-4).

What is the pK_a of Cys35? The titration data of the Trx mutants show no sign of a titration for Cys35. This result indicates that Cys35 does not titrate in the pH range spanned in these experiments. The data for D26L Trx suggests that this pK_a is at least 11. The titration of Cys35 in a carboxyamido-C32 form of Trx also shows a titration for Cys35 above pH 10.5 (LeMaster, 1996). Such a high pK_a value for this residue is not surprising. Cys35 is a buried residue in an environment of low dielectric constant (LeMaster, 1996). Additionally, as the pH

of the solution is increased, both Cys32 and Asp26 will be negatively charged. It is unlikely then that Cys35 will titrate with a pK_a close to either Cys32 or Asp26.

Relevance of Microscopic pK_a's to Thioredoxin Kinetics. The microscopic pK_a's observed in the active-site of *E. coli* Trx will not appreciably affect its kinetic activity. Proton-transfer rates in aqueous solutions are very rapid, usually approaching the diffusion limit. Only in very rapid reactions which require Cys32 as a nucleophile should biphasic kinetics be observable (Chapter 5).

Figure 3-1. Titration of CXXC Trx by fluorescence spectroscopy in H_2O (filled symbols) and D_2O (open symbols). Panel A, wild-type Trx. Panel B, CVWC Trx (HDEL). Panel C, CWGC (HDEL). The apparent pK_a values determined from the curve fits are presented in Table 3-1. The lowest pH point for the CVWC titration in D_2O was not included in the fit. Titrations were carried out in 0.1 M potassium phosphate buffer at 293 K, containing 1.0 mM DTT.

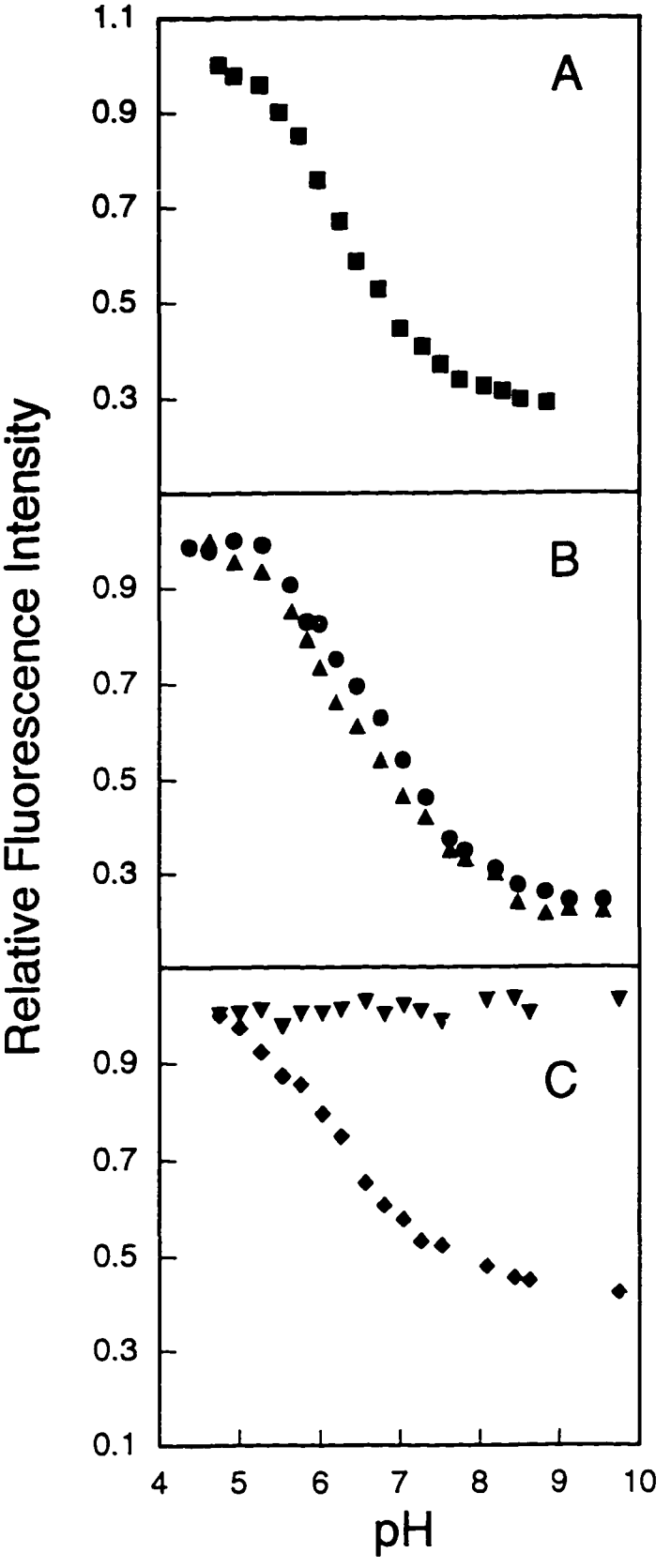

Relative Fluorescence Intensity
pH
A
B
C
1.1
0.9
0.7
0.5
0.3
0.1
4
5
6
7
8
9
10

Figure 3-2. Titration of thioredoxins by fluorescence spectroscopy in H_2O. Panel A, wild-type, ■. Panel B, D26L ●; D26N ▲. Panel C, carboxymethylated wild-type, ◆; C35S, ▼. The apparent pK_a values determined from the curve fits are presented in Table 3-1. Titrations were carried out in 0.1 M potassium phosphate buffer at 293 K, containing 1.0 mM DTT

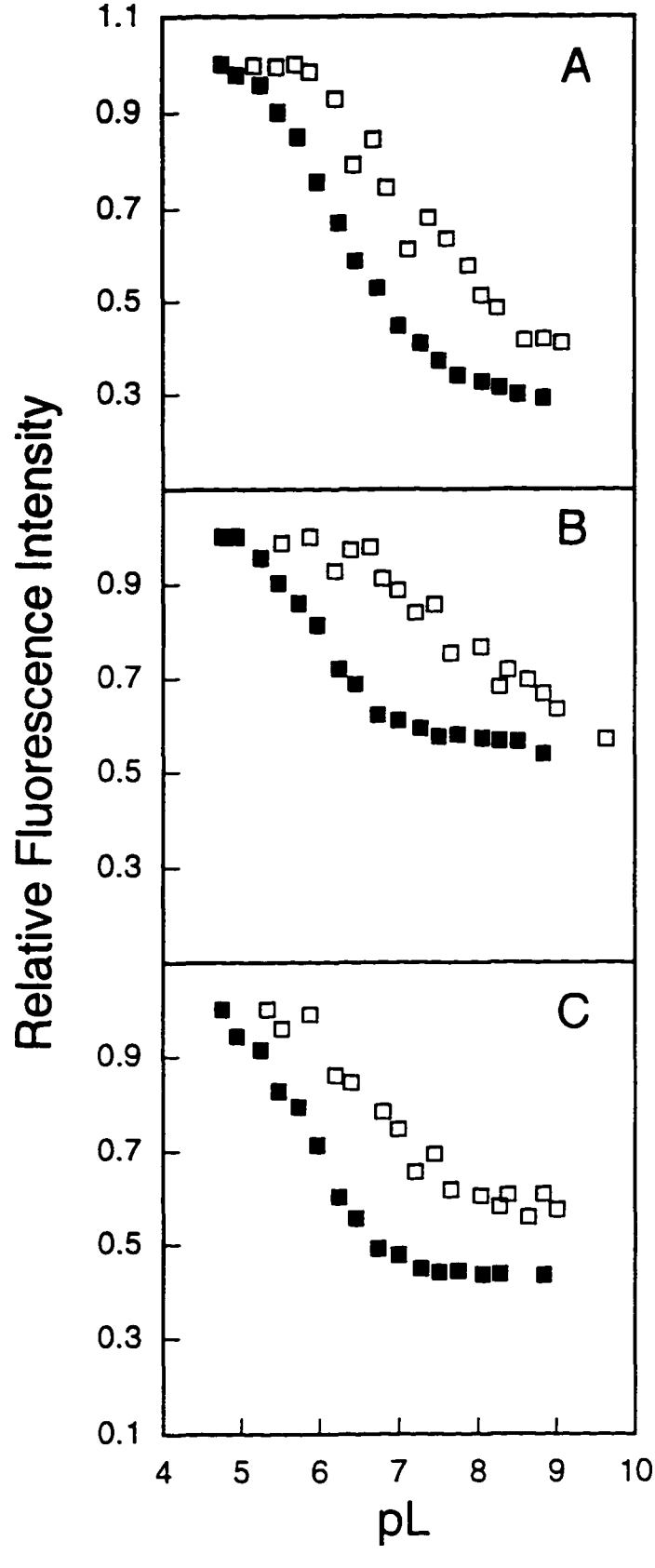

Relative Fluorescence Intensity
1.1
0.9
0.7
0.5
0.3
0.1
A
B
C
4
5
6
7
8
9
10
pL

Figure 3-3. Titration of histidine residues in thioredoxins by ^{1}H-NMR spectroscopy. The titration was monitored by following the chemical shift change of C2H of each histidine. Filled symbols, H_2O; open symbols, D_2O. ■ and □, Trx-S_2; ● and O, Trx-$(SH)_2$. Panel A, His6 from CVWC Trx; Panel B, His6 from D26L Trx; Panel C, His109 from CVWC Trx. pK_a values determined from curve fits are presented in Tables 3-2 and 3-3. Titrations were carried out in 0.1 M potassium phosphate buffer at 308 K. Reduced protein solutions contained 5.0 mM DTT.

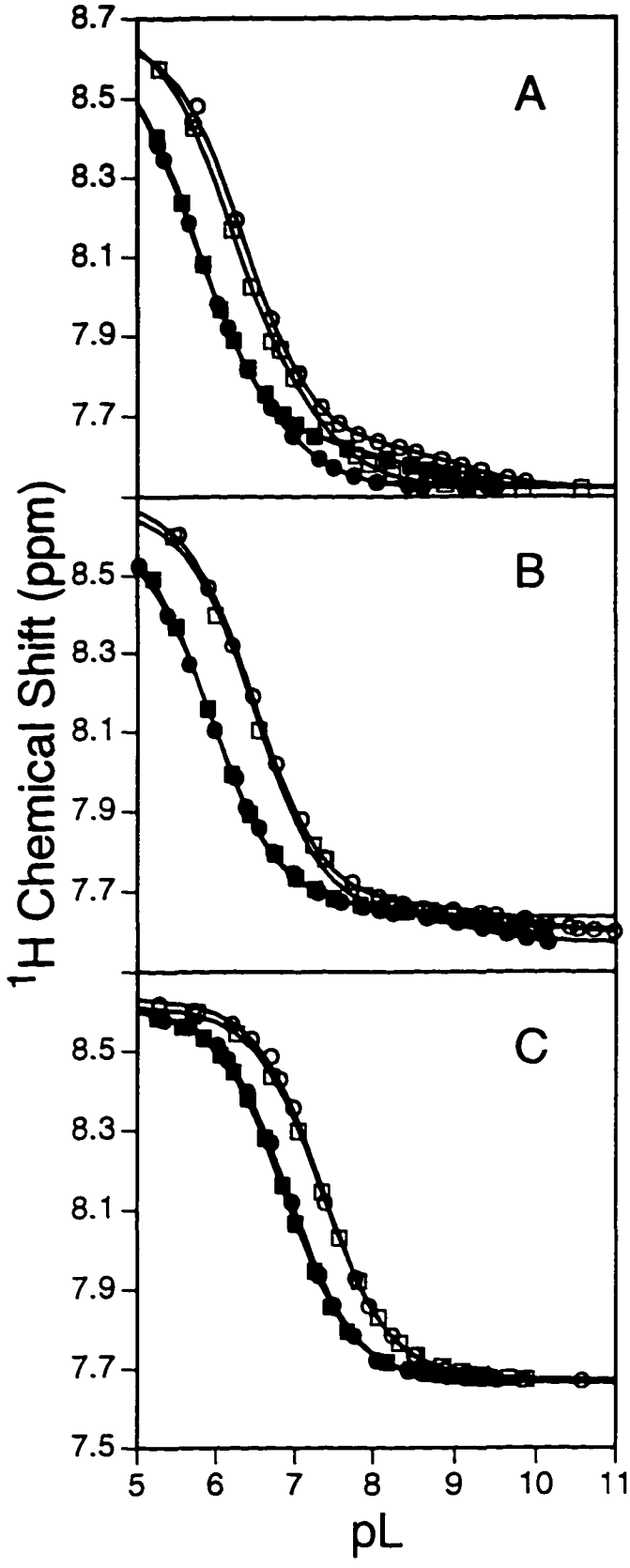

A
B
C
8.7
8.5
8.3
8.1
7.9
7.7
7.5
5
6
7
8
9
10
11
pL
^{1}H Chemical Shift (ppm)

Figure 3-4. pH-dependence of the ^{1}H-chemical shift of NεH of tryptophan residues. Open symbols, Trx-S_2; closed symbols, Trx-$(SH)_2$; ● and O, Trp31; ■ and □, Trp28; ▲ Trp33 or 34. Panel A, CWGC; Panel B, CVWC; Panel C, D26L and D26N (Trp28, ▼ and ∇; Trp31, ◆ and ◇).Titrations were carried out in 0.1 M potassium phosphate buffer at 308 K. Reduced protein solutions contained 5.0 mM DTT.

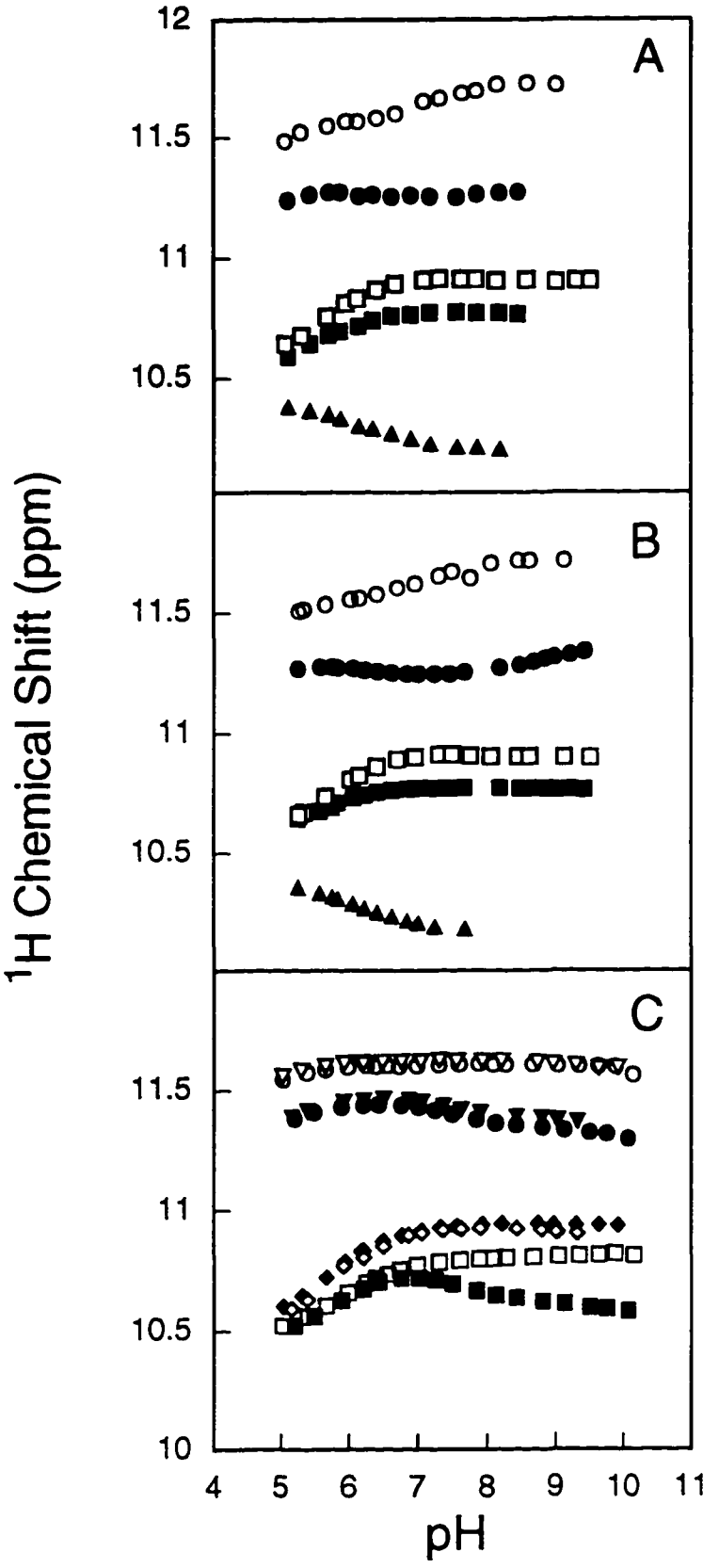

A
B
C
12
11.5
11
10.5
10
4
5
6
7
8
9
10
11
pH
^{1}H Chemical Shift (ppm)

Figure 3-5. Titration of reduced Trx in D_2O by ^{13}C-NMR spectroscopy. Closed symbols, Cys32; open symbols, Cys35. Panel A, wild-type, ▼. Panel B, D26L, ■; D26N; ▲. Panel C, CVWC, ●; CWGC, ◆. pK_a values determined from curve fits using eq 3-2 are presented in Table 3-4.Titrations were carried out in 0.1 M potassium phosphate buffer at 308 K. Reduced protein solutions contained 5.0 mM DTT.

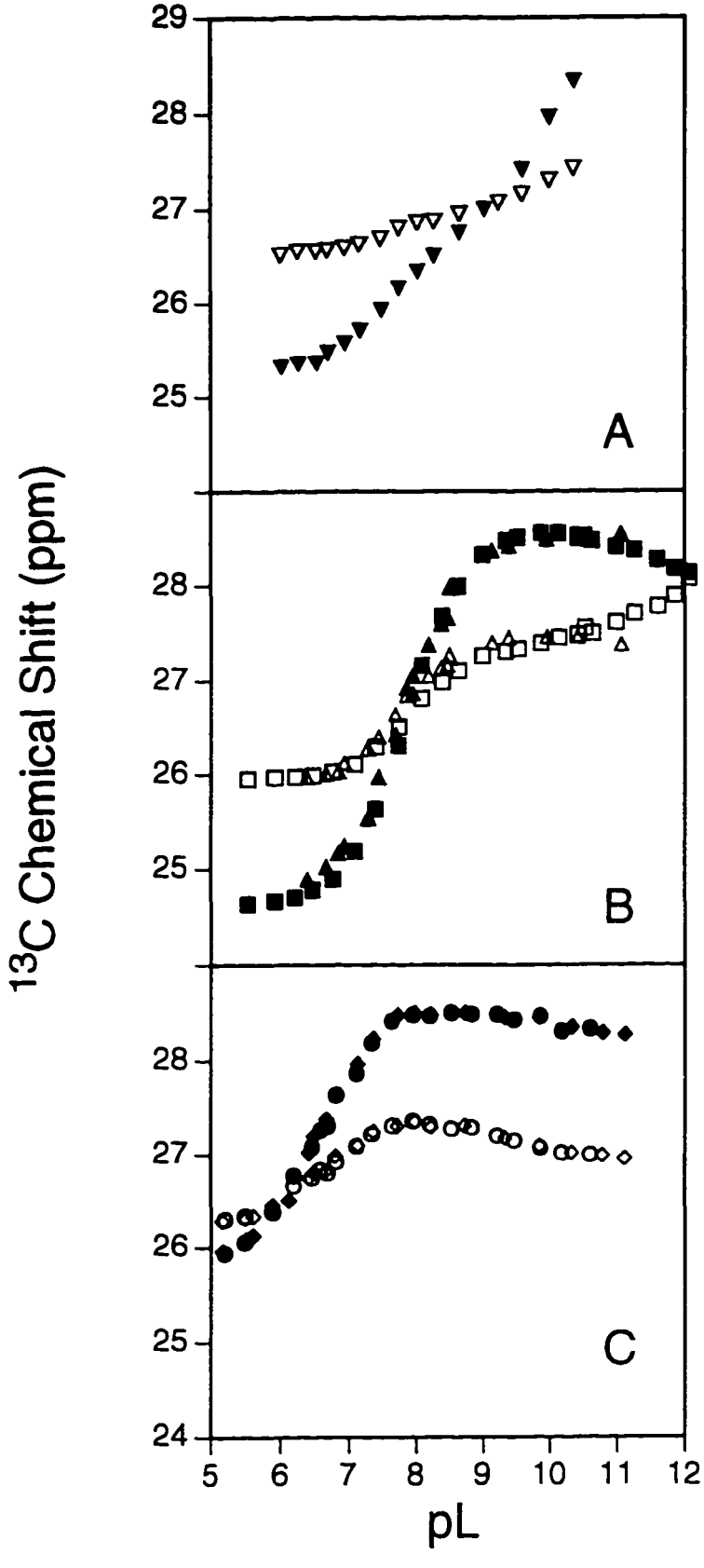

29
28
27
26
25
A
28
27
26
25
B
28
27
26
25
24
C
5
6
7
8
9
10
11
12
pL
^{13}C Chemical Shift (ppm)

Figure 3-6. Titration of oxidized thioredoxin in D_2O by ^{13}C-NMR spectroscopy. Panel A, Cys32, wild-type, ■; D26L, ●. Panel B, Cys35, wild-type, □; D26L, O.Titrations were carried out in 0.1 M potassium phosphate buffer at 308 K. Reduced protein solutions contained 5.0 mM DTT.

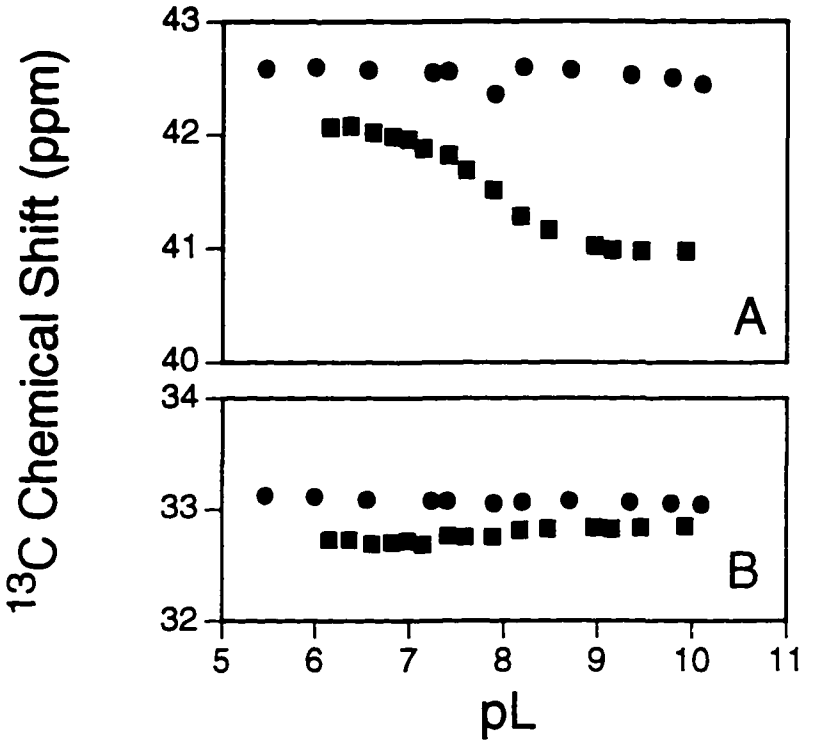

43
42
41
40
34
33
32
A
B
5
6
7
8
9
10
11
pL
^{13}C Chemical Shift (ppm)

Figure 3-7. Fit of experimental data to model titration curves with dependent pK_a's (eq 3-3). Panel A, CWGC Trx. Panel B, wild-type Trx.

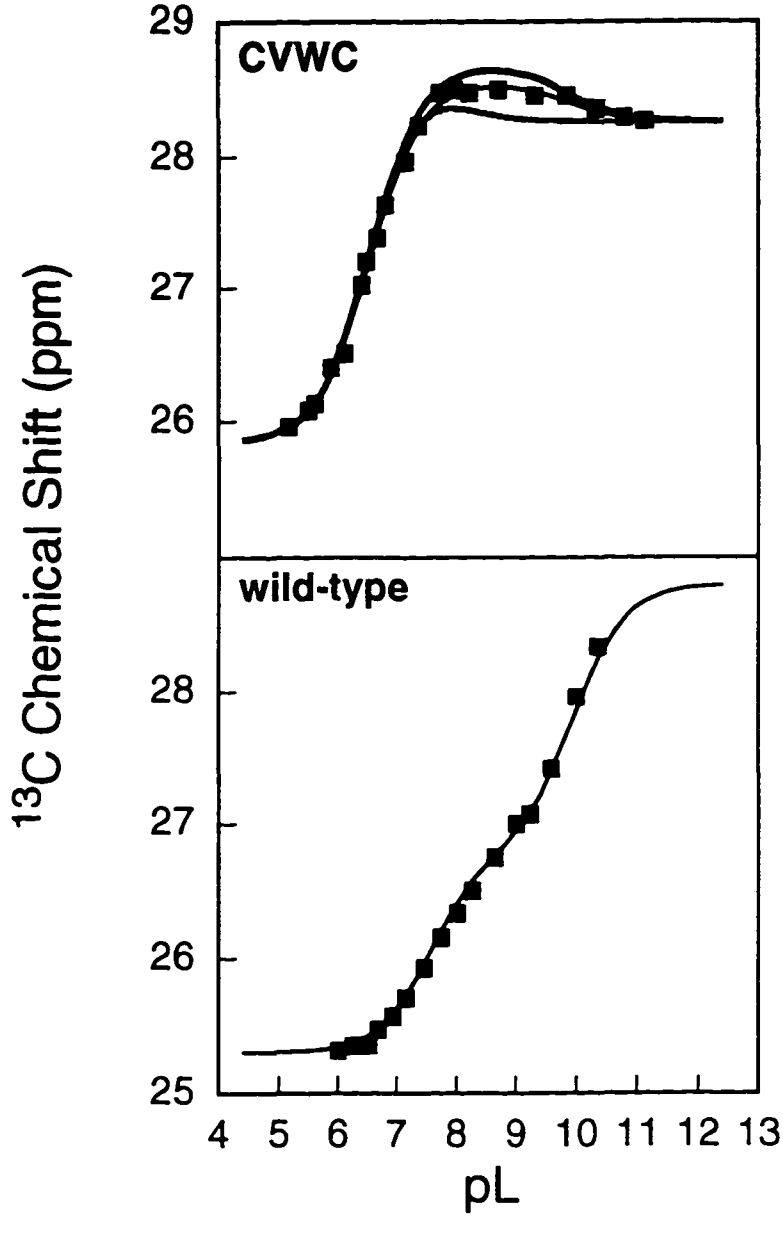

CVWC
wild-type
^{13}C Chemical Shift (ppm)
29
28
27
26
28
27
26
25
4 5 6 7 8 9 10 11 12 13
pL

Figure 3-8. Microscopic acid-dissociation equilibria of reduced thioredoxin.

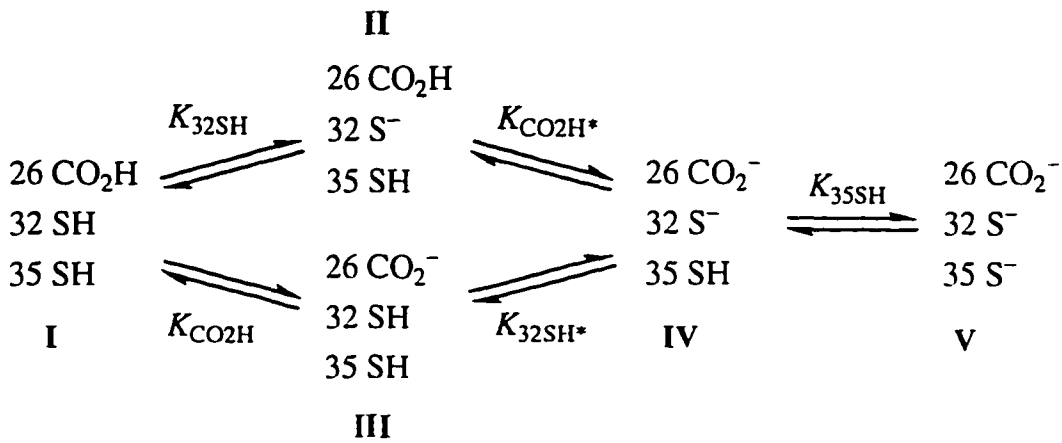

II
26 CO_2H
32 S^-
35 SH
K_{32SH}
K_{CO2H*}
26 CO_2H
32 SH
35 SH
I
K_{CO2H}
26 CO_2^-
32 SH
35 SH
III
K_{32SH*}
26 CO_2^-
32 S^-
35 SH
IV
K_{35SH}
26 CO_2^-
32 S^-
35 S^-
V

Figure 3-9. Fraction of species **I-IV** of wild-type Trx as a function of pH. Curves were calculated using eq A-19 for each species and the data from Figure 3-8, Panel B.

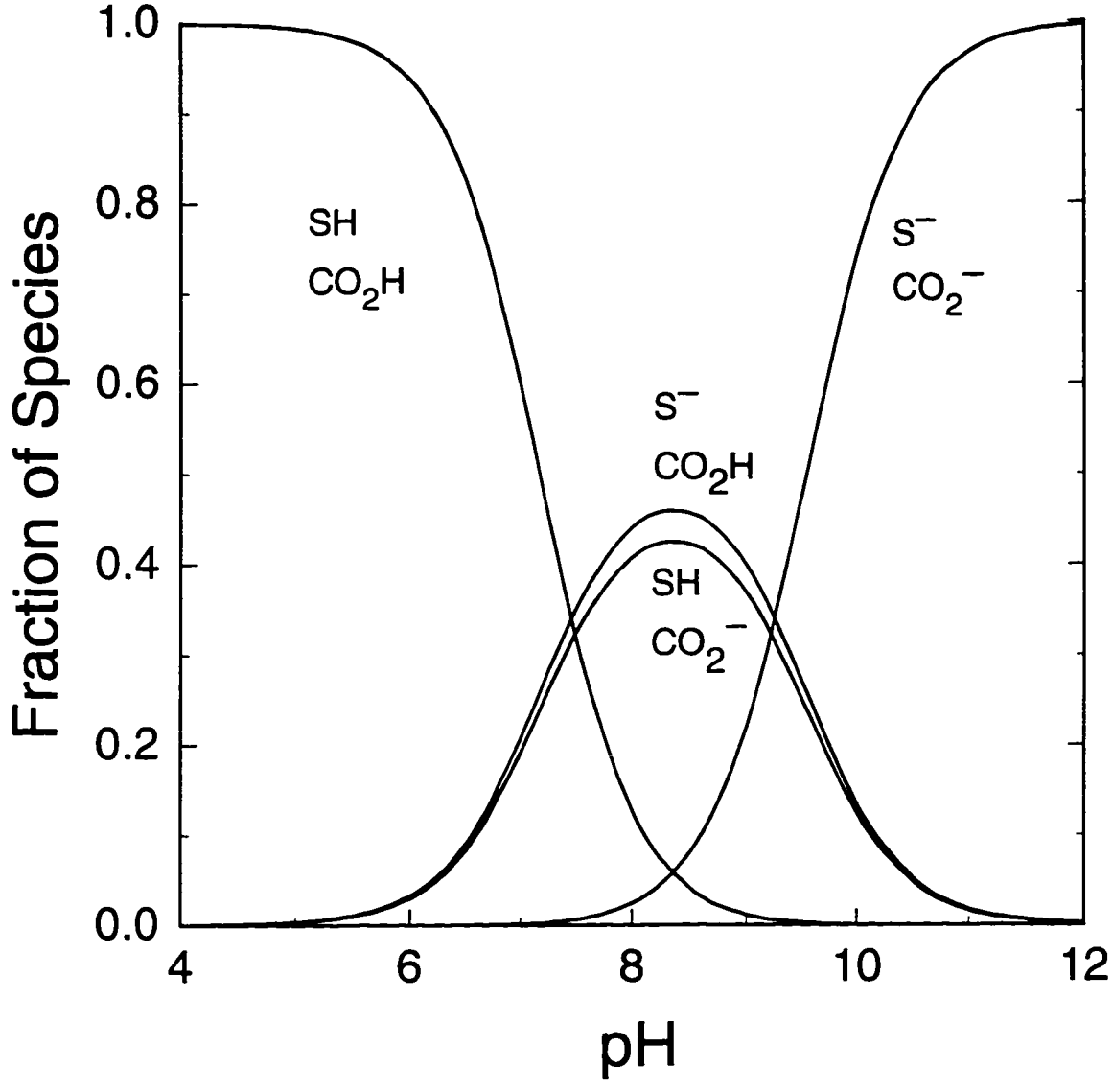

1.0
0.8
0.6
0.4
0.2
0.0
Fraction of Species
SH
CO2H
S−
CO2−
S−
CO2H
SH
CO2−
4
6
8
10
12
pH

Figure 3-10. Fraction of species **I-IV** of CVWC Trx as a function of pH. Curves were calculated using eq A-19 for each species and the data from Figure 3-8, Panel A.

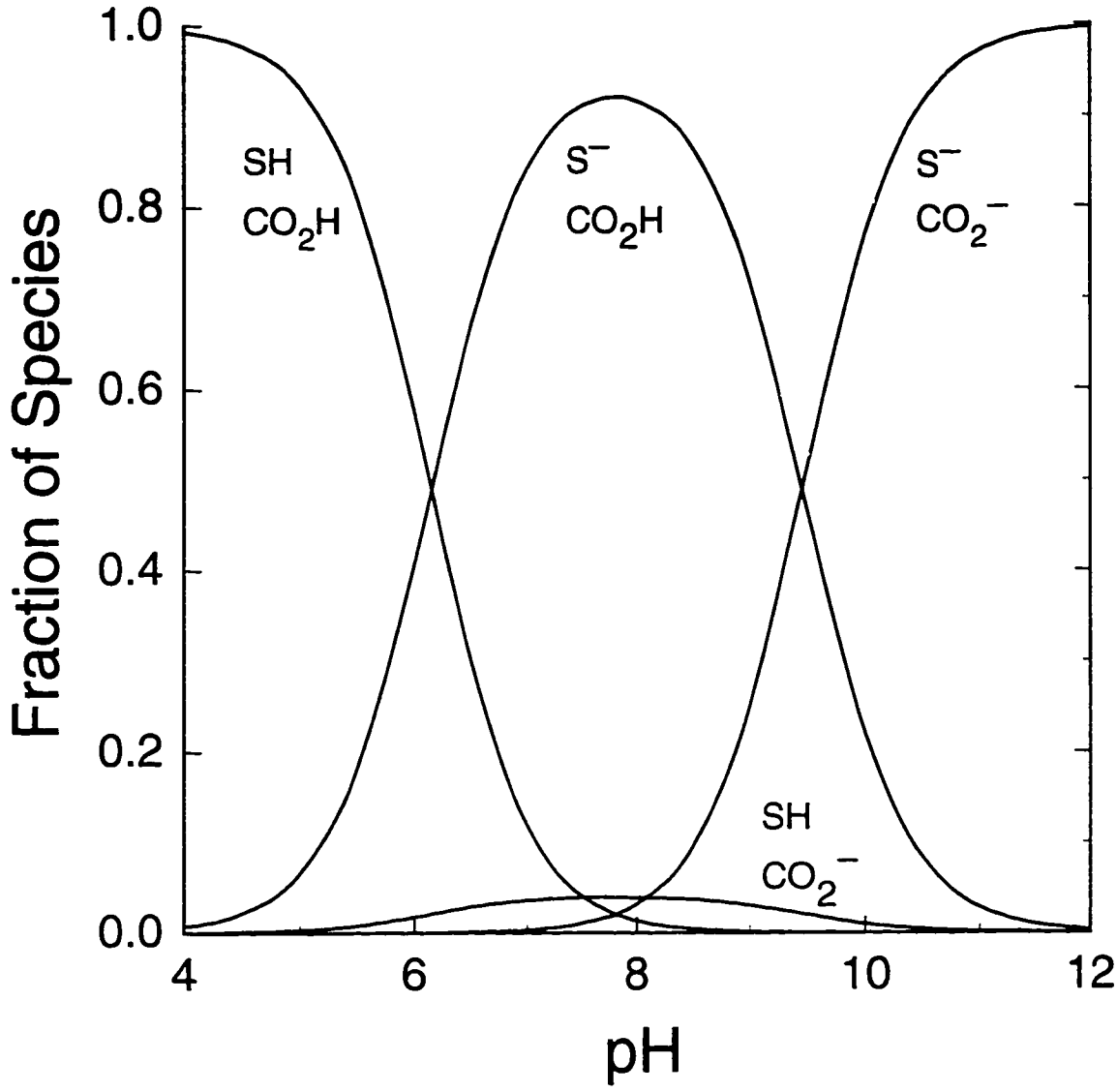

1.0
0.8
0.6
0.4
0.2
0.0
Fraction of Species
SH
CO2H
S−
CO2H
S−
CO2−
SH
CO2−
4
6
8
10
12
pH

Figure 3-11. Fraction of Trx present as thiolate as a function of pH. Calculated using the sum of species **II** and **IV** (eq A-19) over the sum of species **I-IV** and data from Figure 3-8.

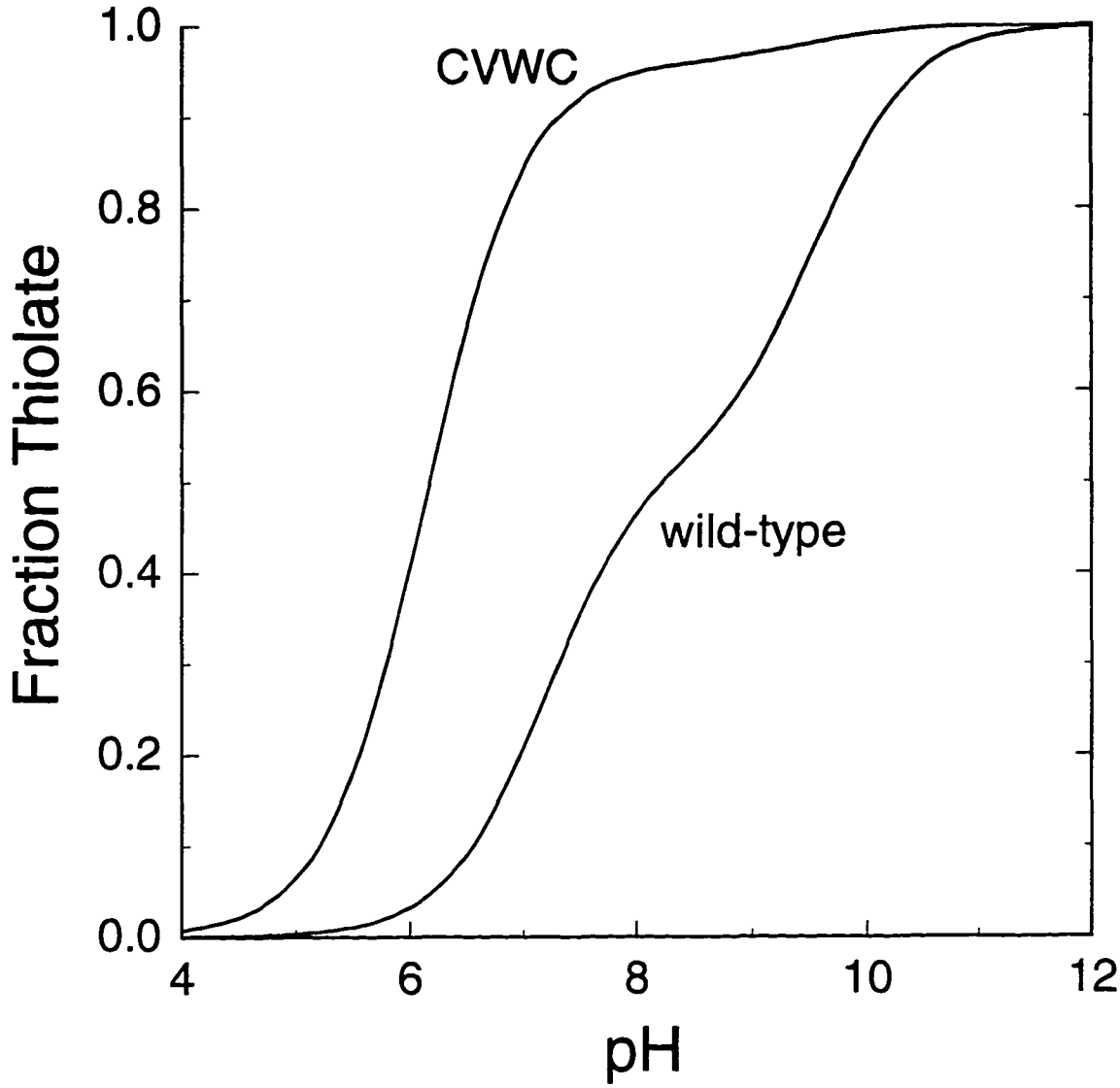

1.0
0.8
0.6
0.4
0.2
0.0
Fraction Thiolate
CVWC
wild-type
4
6
8
10
12
pH

Table 3-1

Apparent pK_a values of thioredoxins from fluorescence spectrocopy

Protein	I = 0.1 M [a]	0.1 M KPO_4 H_2O	0.1 M KPO_4 D_2O [b]	Fractionation Factor [c]
wild-type	6.28	6.30 (0.04)	7.0 (0.11)	1.65 (0.02)
		6.08 (0.05), 7.4 (0.2)		2.74 (0.02)
CWGC	5.94	5.88 (0.03)	6.71 (0.09)	2.23 (0.04)
CVWC	5.86	5.99 (0.03)	7.56 (0.14)	12.3 (0.01)
D26L	—	—	—	—
		6.04 (0.14), 7.36 (0.13)		
D26N	—	—	—	—
		5.96 (0.06), 7.63 (0.14)		
CM-Trx	—	6.35	—	—
C35S	—	nt	—	—

a, Data from Chapter 2 [also Chivers *et al* (1996)]. b, Corrected for deuterium isotope effect. c, Calculated using eq 3-4; nt, no titration observed.

Table 3-2

pK_a values and ^{1}H-chemical shift changes (C2H) for titration of His109 in CVWC and CWGC Trx

Protein		$TrxS_2$	$Trx(SH)_2$	$TrxS_2$*	$Trx(SH)_2$*
CVWC	pK_a	6.93 (0.01)	6.88 (0.01)	—	6.91 (0.02)
	δ_{HA}	8.61 (0.004)	8.61 (0.003)		8.61 (0.002)
	δ_{A^-}	7.66 (0.003)	7.67 (0.002)		7.67 (0.002)
CWGC	pK_a	6.91 (0.01)	6.89 (0.01)	—	—
	δ_{HA}	8.60 (0.01)	8.60 (0.001)		
	δ_{A^-}	7.67 (0.001)	7.68 (0.002)		

*, titration carried out in D_2O.

Table 3-3

pK_a values and ^{1}H-chemical shift changes (C2H) for titration of His6 in thioredoxins

Protein		$TrxS_2$	$Trx(SH)_2$	$TrxS_2$*	$Trx(SH)_2$*
wild-type	pK_1	—	5.90 (0.02)	—	6.03 (0.048)
	pK_2		8.24 (0.16)		8.36 (0.39)
	δ_{HAH}		8.61 (0.02)		8.51 (0.036)
	δ_{HA^-}		7.60 (0.01)		7.60 (0.016)
	δ_{A2^-}		7.52 (0.009)		7.53 (0.009)
CVWC	pK_1	5.69 (0.02)	5.77 (0.01)	6.19 (0.03)	6.40 (0.02)
	pK_2	6.87 (0.04)	8.45 (0.08)	7.42 (0.11)	8.69 (0.01)
	δ_{HAH}	8.64 (0.007)	8.63 (0.01)	8.68 (0.01)	7.63 (0.007)
	δ_{HA^-}	7.77 (0.01)	7.63 (0.003)	7.76 (0.04)	
	δ_{A2^-}	7.52 (0.001)	7.52 (0.004)	7.52 (0.01)	
CWGC	pK_1	5.67 (0.06)	—	—	6.48 (0.01)
	pK_2	7.08 (0.26)			8.55 (0.10)
	δ_{HAH}	8.65 (0.03)			8.71 (0.004)
	δ_{HA^-}	7.70 (0.06)			7.64 (0.003)
	δ_{A2^-}	7.52 (0.005)			7.53 (0.006)
D26L	pK_1	5.94 (0.02)	5.91 (0.01)	—	6.08 (0.01)
	pK_2	9.00 (0.21)	8.99 (0.18)		9.32 (0.16)
	δ_{HAH}	8.61 (0.01)	8.65 (0.008)		8.69 (0.01)
	δ_{HA^-}	7.66 (0.005)	7.66 (0.003)		7.66 (0.004)
	δ_{A2^-}	7.57 (0.007)	7.61 (0.005)		7.60 (0.004)
D26N	pK_1	5.73 (0.02)	5.79 (0.02)	—	—
	pK_2	8.34 (0.28)	7.05 (0.10)		
	δ_{HAH}	8.62 (0.01)	8.69 (0.007)		
	δ_{HA^-}	7.64 (0.008)	7.74 (0.02)		
	δ_{A2^-}	7.57 (0.007)	7.58 (0.001)		

*, titration carried out in D_2O.

Table 3-4

pK_a values and ^{13}C-chemical shift changes for titration of Cys32 and Cys35 in reduced thioredoxins

		Cys32		Cys35	
Protein		Trx(SH)2	Trx(SH)2*	Trx(SH)2	Trx(SH)2*
wild-type	pK_1	7.19 (0.11)	7.59 (0.04)	7.15 (0.16)	7.62 (0.07)
	pK_2	9.37 (0.14)	9.88 (0.06)	9.44 (0.16)	9.89 (0.10)
	δ_{HAH}	26.78 (0.03)	26.72 (0.03)	27.98 (0.01)	27.95 (0.01)
	δ_{HA^-}	27.89 (0.10)	28.16 (0.04)	28.28 (0.04)	28.40 (0.02)
	$\delta_{A^{2-}}$	29.89 (0.18)	30.32 (0.09)	29.01 (0.08)	29.01 (0.04)
CVWC	pK_1	—	6.12 (0.36)	—	6.22 (0.057)
	pK_2				8.88 (0.023)
	δ_{HAH}		25.84 (0.06)		
	δ_{HA^-}		28.48 (0.03)		26.26 (0.03)
	$\delta_{A^{2-}}$				27.38 (0.03)
					26.97 (0.06)
CWGC	pK_1	—	6.14 (0.024)	—	6.16 (0.04)
	pK_2		9.45 (0.30)		9.44 (0.14)
	δ_{HAH}		25.84 (0.03)		26.22 (0.02)
	δ_{HA^-}		28.56 (0.03)		27.38 (0.02)
	$\delta_{A^{2-}}$		28.26 (0.04)		26.96 (0.02)
D26L	pK_1	—	7.46 (0.01)	—	7.56 (0.03)
	pK_2		10.92 (0.09)		11.0 (0.1)
	δ_{HAH}		24.60 (0.01)		25.94 (0.02)
	δ_{HA^-}		28.59 (0.01)		27.39 (0.02)
	$\delta_{A^{2-}}$		28.06 (0.03)		28.13 (0.06)
D26N	pK_1	—	7.39 (0.03)	—	7.32 (0.03)
	pK_2				
	δ_{HAH}		24.75 (0.06)		25.87 (0.02)
	δ_{HA^-}		28.49 (0.04)		27.45 (0.02)
	$\delta_{A^{2-}}$				

*, titration carried out in D_2O.

Table 3-5

^{13}C-Chemical shift changes of for the titration of [^{13}C]thioredoxins

Protein		Cys32	Cys35
wild-type	pK_1	7.45 (0.02)	7.51 (0.18)
δ_{HAH}		42.08 (0.01)	32.70 (0.01)
δ_{HA^-}		40.94 (0.01)	32.84 (0.01)
D26L	pK_1	nt[a]	nt

a, No titration observed.

Table 3-6

Fractionation factors in reduced Trx

		Residue			
Protein		Cys32	Cys35	His6	His109
wild-type	pK_1	0.84 (0.04)	0.97 (0.05)	1.12 (0.04)	—
	pK_2	1.10 (0.04)	0.93 (0.06)		
CVWC		—	—	1.23	0.83
CWGC		—	—	2.13 (0.02)	—
D26L		—	—	1.23 (0.02)	—

Table 3-7

Microscopic pK_a and chemical shift values from the fits of eq 3-3 to ^{13}C titration data

Protein	pK_{SH}	pK_{COOH}	pK_{SH*}[a]	pK_{COOH*}	c[b]
wild-type	7.46	7.50	9.23	9.26	0.017
CVWC	6.15	7.52	8.07	9.45	0.012

a, calculated using c and pK_{SH}; b, K_{a*}/K_a

Table 3-8

Assay of wild-type and CXXC enzymes for protein disulfide isomerase activity

Enzyme	Disulfide isomerization activity (units/mg)[a]
wild-type Trx	0.37
CWGC Trx	0.38
CVWC Trx	0.21
rat PDI	0.55[b]
bovine PDI	0.65[b]

a, One unit will catalyze the reactivation of 1 nmol of scrambled RNase A per min at pH 7.6 in the presence of GSH (1.0 mM) and GSSG (0.2 mM).

b, Data from Laboissière et al (1995).

Chapter 4

General Acid/Base Catalysis in the Active-Site of *E. coli* Thioredoxin

ABSTRACT

The essential catalysts of dithiol-disulfide exchange reactions contain two cysteine residues at their active-site (CXXC). In *E. coli* thioredoxin (Trx), the effective concentration of these cysteine residues (Cys32 and Cys35) makes this enzyme the most efficient reductant of the thiol-disulfide oxidoreductases family of enzymes. Another residue that is conserved in thioredoxins is Asp26. We have constructed D26N and D26L Trx to examine the role of Asp26 in catalysis by Trx. The kinetics of Trx reduction by thioredoxin reductase and the reduction of 5,5'-ditihobis(2-nitrobenzoic acid) (DTNB) by reduced Trx indicate that Asp26 is a general acid/base catalyst in the active-site of Trx. Asp26 is responsible for the protonation and deprotonation of Cys35 to facilitate its attack of Cys32 in mixed disulfides.

INTRODUCTION

Dithiol-disulfide exchange reactions are prevalent in biology (Gilbert, 1990). Essential catalysts of this reaction are found in both prokaryotic and eukaryotic cells. These disulfide oxidoreductases are part of a protein family whose common characteristic is the active-site sequence Cys-Xaa-Xaa-Cys (CXXC). In *Escherichia coli*, DsbA (CPHC) is found in the periplasm and is required for oxidation of protein dithiols to disulfide bonds in the periplasm (Bardwell *et al.*, 1991). In *Saccharomyces cerevisiae*, protein disulfide isomerase [(PDI), CGHC] is found in the endoplasmic reticulum and is necessary for the isomerization of non-native protein disulfide bonds (Laboissière *et al.*, 1995).

Dithiol-disulfide exchange equilibria are dependent on the reduction potentials of the solution and the reacting molecules (Gilbert, 1990). For example, in eukaryotes protein disulfide bond formation is not favored in the cytosol ($E^{o\prime}$ = -0.230 V) (Hwang *et al.*, 1992). However, when proteins are translocated to the more oxidizing enviroment of the endoplasmic reticulum ($E^{o\prime}$ = -0.180 V) (Hwang *et al.*, 1992), protein disulfide bond formation becomes favored. Dithiol-disulfide exchange equilibria also exhibit a pH-dependence because functional groups (*i.e.*, the thiols) of the reacting species have different pK_a's in the reduced and oxidized forms (Chapter 5) For example, lowering the thiol pK_a of the first cysteine by mutagenesis of the XX residues of the CXXC motif in DsbA (Grauschopf *et al.*, 1995) and *E. coli* thioredoxin (Krause *et al.*, 1991; Chivers *et al.*, 1996) correlates with an elevated reduction potential in these proteins.

Wild-type *E. coli* thioredoxin (CGPC) is an efficient reductant ($E^{o\prime}$ = -0.270 V) (Moore *et al.*, 1964). It is 1000-fold more efficient than DTT at reducing the disulfides of insulin (Holmgren, 1979). This enzyme achieves catalytic efficiency through several means. The active-site nucleophile (Cys32) has a pK_a close to physiological pH. This pK_a provides the optimal balance between nucleophilicity and the fraction of the cysteine present in the

catalytically functional thiolate form (Szajewski & Whitesides, 1980). Additionally, the proximity of Cys35 results in efficient intramolecular attack on a mixed disulfide involving Cys32 and another (*i.e.*, substrate) sulfur. Because the pK_a of Cys35 is 4 units higher than physiological pH, this residue is likely to be unreactive. The effectiveness of Cys35 as a nucleophile would be enhanced, however, by general base catalysis.

A good candidate for this general base catalyst in Trx is Asp26 (Figure 4-1). This residue is conserved in thioredoxins (Eklund *et al.*, 1991). At the analogous position in other CXXC-containing proteins (PDI, *E. coli* DsbA) this residue is a Glu. The role of Asp26 as a proton sink in thioredoxin activity has been proposed before (Jeng & Dyson, 1996; LeMaster, 1996), however, no mechanistic evidence exists to corroborate this hypothesis.

Here, we have used D26N and D26L Trx to reveal the role of Asp26 in catalysis by the wild-type enzyme. Kinetics experiments indicate that Asp26 plays an important role as a general acid/base catalyst in the reduction and oxidation reactions catalyzed by Trx .

MATERIALS AND METHODS

Construction of D26 mutants. pTRX, a pET vector (Novagen; Madison, WI) containing *E. coli trxA*, was constructed as described earlier (Chapter 3). Site-directed mutagenesis was performed using the method of Kunkel (1985). Oligonucleotides for site-directed mutagenesis were from IDT Technologies (Coralville, IA). Oligonucleotide p-ASN (5′ CTCTGCCCAGAAGTTAACGAGGATCGC 3′) and oligonucleotide p-LEU (5′ CTCTGCCCAGAAGAGTACTAGGATCGC 3′) were used to create the D26N and D26L mutants of *E. coli* Trx, respectively. The underlined sequence corresponds to restriction sites (p-ASN, *Hpa*I; p-LEU, *Sca*I) used to screen for mutant DNA. Dideoxynucleotide sequencing

of plasmids identified by restriction screens was used to verify the DNA sequence of the mutant Trx's.

Production and Purification of D26 Mutants. Mutant proteins were expressed and purified as described previously (Chapter 3).

Thioredoxin Reductase Assays. In *E. coli*, Trx is converted to its reduced form by NADPH in a reaction catalyzed by thioredoxin reductase. The reduction potential of Trx was determined by using the assay of Moore (1964)(Moore *et al.*, 1964). *E.coli* thioredoxin reductase (TR) was a generous gift of C.H. Williams, Jr. (University of Michigan). In this assay, the reduction of Trx is accompanied by a decrease in *A* at 340 nm due to the TR-catalyzed reduction of NADPH ($\varepsilon = 6200\ M^{-1}cm^{-1}$). Here, TR (1 μL of a 0.94 μg/μL solution) was added to a solution (1.0 mL) of 0.10 M Tris-HCl buffer, pH 7.0, containing Trx (100 – 120 μg), NADPH (25 μM), and EDTA (1 mM). After equilibrium had been reached, $NADP^+$ (10 μL of a 0.138 M solution) was added to generate a new equilibrium. Using the equilibrium concentrations of all species, $E^{o\prime}_{Trx}$ was determined using the equation

$$E^{o\prime}_{Trx} = E^{o\prime}_{NADP^+} + \frac{RT}{nF}\ln\frac{[Trx(SH)_2][NADP^+]}{[TrxS_2][NADPH]} \qquad (4\text{-}1)$$

where $E^{o\prime}_{NADP+} = -0.315$ V (Clark, 1960) $R = 8.314$ J/(K·mol), $T = 298$ K, $n = 2$, and $F = 96{,}500$ C mol^{-1}. Three different starting concentrations of Trx were used to obtain a mean value of $E^{o\prime}$.

Stopped-flow Kinetics. Reduced thioredoxin rapidly transfers a pair of electrons to the disulfide bond of a substrate. To measure the rate of this process, we used a stopped-flow technique. Stopped-flow visible spectroscopy was performed on an OLIS RSM1000 rapid-

scanning spectrometer equipped with a stopped-flow injection device (On-Line Instrument Systems; Bogart, GA). Experiments were performed at 22 °C. Injection volumes were 75 μL per buffer chamber, yielding a reaction mixture of 150 μL. At least 6 injections were performed for each reaction of protein and substrate. Reactions were performed in 0.050 M potassium phosphate buffer, pH 7.0, containing EDTA (1 mM). To eliminate O_2(g), the buffer solution was degassed under vacuum for 30 min and then saturated with N_2(g), which was bubbled through the buffer for 30 min. If necessary, the pH of the buffer was adjusted after degassing and nitrogen bubbling. Substrate solutions of DTNB (5mg/mL, 12.6 mM) were prepared in the degassed potassium phosphate buffer with and without 20 mM Tris free-acid.

Wild-type and mutant thioredoxins were reduced by the addition of a 10-fold molar excess of TCEP•HCl (Rockford, IL). After 30 min, the reducing agent was removed by injecting the protein solution onto a Pharmacia Fast Desalt FPLC column equilibrated with the degassed potassium phosphate buffer. Protein concentrations after buffer exchange (1.5 to 2.0 mg/mL) were determined by measuring A at 280 nm (ε_{280} = 13 700 M^{-1} cm^{-1}).

RESULTS

Reduction Thioredoxin Reductase. Replacing Asp26 with an asparagine or leucine residue slows the rate of reduction of Trx by TR (Figure 4-2). The initial rate of reduction of wild-type Trx is 5-10 times faster than the rate of reduction of D26N and D26L Trx. Similarly, the rate of oxidation of these mutants by TR in the presence of excess $NADP^+$ is also 5-10 times slower than that of the wild-type enzyme. Intriguingly, the reduction potentials of D26N and D26L calculated using eq 4-1 are both elevated by 0.010 to 0.015 V from that of wild-type Trx. This effect is slightly larger for the D26L enzyme. The properties of Trx that determine its reduction potential are complex (Chapter 5) but the results of this assay suggest that the pKa of Asp26 is

elevated in reduced wild-type Trx, favoring the oxidized form of the protein. The absence of this pK_a difference in the D26 mutants negates this component of the reduction potential. However, D26N and D26L do not have identical reduction potentials indicating other pH-independent factors also affect the reduction potential of these mutants.

Solvent Isotope Effects. The reduction of Trx by TR was performed in a final concentration of 90% (v/v) D_2O. In this solvent, the rate of reduction of wild-type Trx is slow relative to its reduction in H_2O (data not shown). The reduction of D26N Trx also proceeds more slowly in D_2O. The rate of reduction of D26L in D_2O is very similar to the rate in H_2O. The rate of oxidation in D_2O upon the $NADP^+$ is considerably slower for all three proteins. Only for the D26N enzyme was the equilibrium perturbed in D_2O, favoring the reduced protein more in this solvent than in H_2O. Because Trx has a large number of proton binding sites, analysis of the solvent isotope effect on its redox equilibrium is complicated (Kresge, 1973). Still, the different solvent isotope effects on the rate of reduction of these three enzymes suggest that the reduction of D26L proceeds by a mechanism different from the wild-type and D26N enzymes.

Stopped-Flow Kinetics. We used stoppedflow visible spectroscopy to monitor the kinetics of DTNB cleavage. DTNB was present in a final concentration that was a 30-fold molar excess of reduced Trx. Figure 4-3 shows that the reduction of DTNB by Trx started during the mixing time of the reaction. The curves in Figures 4-3 and 4-4 are fits of a two-exponential equation to the appearance of a single species (TNB). The first step was of comparable rate for all three proteins. In contrast, D26N and D26L enzymes show slower kinetics for the second step, the release of the second TNB molecule from a mixed disulfide with Cys32.

Because both steps of a dithiol-disulfide exchange reaction require a thiolate as the nucleophilic species (*i.e.*, initial attack of the disulfide and cleavage of the mixed disulfide intermediate), proton transfer will be important when the thiol pK_a is above that of the solution

pH. We also carried out the DTNB reduction reaction with Tris in the reaction mixture (final concentration 10 mM). Figure 4-4 shows the effect of Tris on the release of the second molecule of DTNB in D26N and D26L Trx. Apparently, in the absence of Asp26, the deprotonation of Cys35 limits the rate of the reduction of DTNB and the addition of Tris eases this constraint.

The first step of DTNB reduction by Trx is too fast under these conditions (0.050 M potassium phosphate buffer, pH 7.0) to detect the presence of microscopic pK_a's in the active-site of wild-type Trx. Detection of microscopic pK_a's, if they are present, will require reaction conditions where the rate of proton transfer between Cys32SH and solution is minimized. Only if k_{cat} is faster than k_t, the proton transfer rate between Cys32SH and solution, might biphasic kinetics for the first step of DTNB reduction be observable.

DISCUSSION

Dithiol-disulfide exchange reactions proceed as outlined in Figure 4-5. First, a thiolate attacks a disulfide (Step **I**). Ultimately, cleavage of the mixed disulfide intermediate (**III**) produces products (**IV**). For Trx, the cleavage of the mixed disulfide intermediate is determined by the rate at which Cys35 attacks Cys32. Two factors assist Cys35 in the attack of Cys32: the proximity of Cys35 to Cys32 and the deprotonation of Cys35 to generate thiolate nucleophile.

The proximity of Cys35 to Cys32 correlates with the effective concentration (EC) of the two thiol groups. EC refers to the preference of a dithiol-containing molecule to form an intramolecular disulfide bond relative to a reference monothiol-containing molecule that forms an intermolecular disulfide bond (see Chapter 5). EC correlates with reduction potential, so that

a higher value of EC is equivalent to a lower reduction potential. The CWGC and CVWC mutants described in Chapter 2 have values of EC lower than that of wild-type Trx.

The deprotonation of Cys35 in wild-type Trx is accomplished by Asp26. The results of the stopped-flow kinetics experiments (Figures 4-3 and 4-4) show direct evidence that the presence of Asp26 speeds cleavage of the mixed disulfide, Trx-TNB. D26N and D26L Trx require the assistance of an exogenous proton donor/acceptor to compensate for the absence of Asp26 (Figure 4-4). These results are not consistent with the proposed proton-sharing mechanism between Cys32 and Cys35 (Jeng *et al.*, 1995). Additionally, our results do not provide evidence for the role of Cys35 as proton donor in disulfide reduction by Trx.

The rate enhancement provided by the addition of Tris suggests that the mixed disulfide between Cys32 and TNB does not completely exclude water or buffer molecules from the active-site of Trx. Such small molecules can apparently deprotonate Cys35. This result is consistent with ability of small molecules to form covalent bonds with Cys35 (Kallis & Holmgren, 1980). Curiously, Asp26 (or Glu) is not always conserved in glutaredoxins, which catalyze the reduction of oxidized glutathione. Perhaps because glutaredoxin acts on a small substrate, deprotonation of the second cysteine in this protein does not require the assistance of a proximal general base.

A mixed disulfide intermediate between Trx and a larger substrate such as TR may slow the access of small molecules to the active site. The TR assay does not directly monitor the breakdown of the mixed disulfide intermediate. However, we can infer that this step is rate-limiting with D26N and D26L Trx. The presence of 10 mM Tris accelerated the cleavage of the mixed disulfide in the stopped-flow experiment, making the rate for the mutant enzymes close to wild-type. The TR assay is performed in a solution containing 0.1 M Tris and in this reaction this buffer does not provide a comparable rate enhancement.

The pK_a's of the sidechain of Asp26 is 7.5 in oxidized Trx, and 7.5 or 9.5 (depending on the protonation state of Cys32) in reduced Trx (Chapter 3). Thus, at physiological pH, Asp26

will not always be in the correct protonation state for catalysis. When Trx is acting as a reductant, Asp26 must be deprotonated to act as a general base once the mixed disulfide species forms. However, at pH 7, Asp26 will be protonated in most Trx molecules because it has a pK_a of 7.5 or higher. In these molecules, Asp26 must somehow lose its proton. A reasonable mechanism for this process involves a chain of water molecules that can approach Asp26 from the side of the enzyme distal to the active-site. Water molecules (3 to 4) are indeed observed in proximity to Asp26 in X-ray structures of different oxidized thioredoxins crystallized under different conditions (Katti *et al.*, 1990; Saarinen *et al.*, 1995).

The TR assay results support this mechanism. The presence of a leucine residue in place of Asp26 may access of proton transfer agents (water or buffer) to Cys35. In D26N Trx, however, water (or buffer molecules) may still be able to approach Cys35 because this mutant retains some of the non-covalent interactions that are present in the Asp-containing protein. Thus, the reduction of D26L Trx is slowed more than that of the D26N enzyme. The effect of D_2O on the rate of Trx reduction is not significant in D26L Trx but is apparent in the D26N and wild-type enzymes. This result suggests the mechanism of Cys35 deprotonation is differs in D26L Trx. An elaboration of this difference is complicated by the presence of multiple proton binding sites on Trx. If water shuttles protons between Asp26 and solvent, substitution of D_2O for H_2O would compromise this proton transfer network if the ordered water molecules necessary for this mechanism had fractionation factors that deviate from unity. An additional complication is the absence of any hydrogen bond between Cys35 and residue 26 in the D26L mutant. This interaction is possible in both wild-type and D26N Trx, and may account for the difference in observed rates.

The removal of Asp26 has some analogy to the removal of Cys35 from the CXXC motif. Without deprotonation of Cys35, the mixed disulfide of Trx and substrate is stable to intramolecular cleavage by the enzyme. In CXXS-containing proteins, such as Eug1p (Tachibana & Stevens, 1992) , CLHS/CIHS (LaMantia & Lennarz, 1993), and CGHS PDI

(Laboissière *et al.*, 1995; Walker *et al.*, 1996).the mixed disulfide cannot be cleaved by the enzyme but instead relies on either a substrate thiol or reduced glutathione for this activity. D26N and D26L Trx are not as dependent on an exogenous thiol for cleavage of the mixed disulfide. Still, in a reaction between Trx and a misfolded protein, if the deprotonation of Cys35 is slowed enough, a mixed disulfide could be cleaved by exogenous thiol. This mechanism suggests that the redox equilibrium may not be achieved between enzyme and substrate. Then, the measured $E^{o\prime}$ would be a *kinetic* reduction potential rather than a thermodynamic one. Because equilibrium is achieved between NADPH and D26N or D26L Trx in the TR assay, the deprotonation of Cys35 occurs frequently enough that the thermodynamic reduction potential prevails.

The functional role of Asp26, or its equivalent (Figure 1-2), in catalysis by dithiol-disulfide oxidoreductases has not previously been examined. Catalysis of dithiol-disulfide exchange reactions by DsbA and PDI will also proceed through Steps (**I-IV**) in Figure 4-5. The reduction potentials of the active-site disulfides may determine the importance of general acid/base catalysis in these enzymes. Additionally, the different pK_a's of the first cysteine in the active-sites of DsbA (Nelson & Creighton, 1994), PDI (Hawkins & Freedman, 1991), and different Trxs (Forman-Kay *et al.*, 1992; Qin *et al.*, 1996) will perturb the Asp(Glu) pK_a to different extents. Thus, differences in the pK_a of this functional group in different dithiol-dsulfide oxidoreductases may modulate its ability to act as a general acid or base.

Figure 4-1 The functional groups of the active-site of reduced *E. coli* Trx (Jeng *et al.*, 1994). This figure was created using MOLSCRIPT v1.2 (Kraulis, 1991).

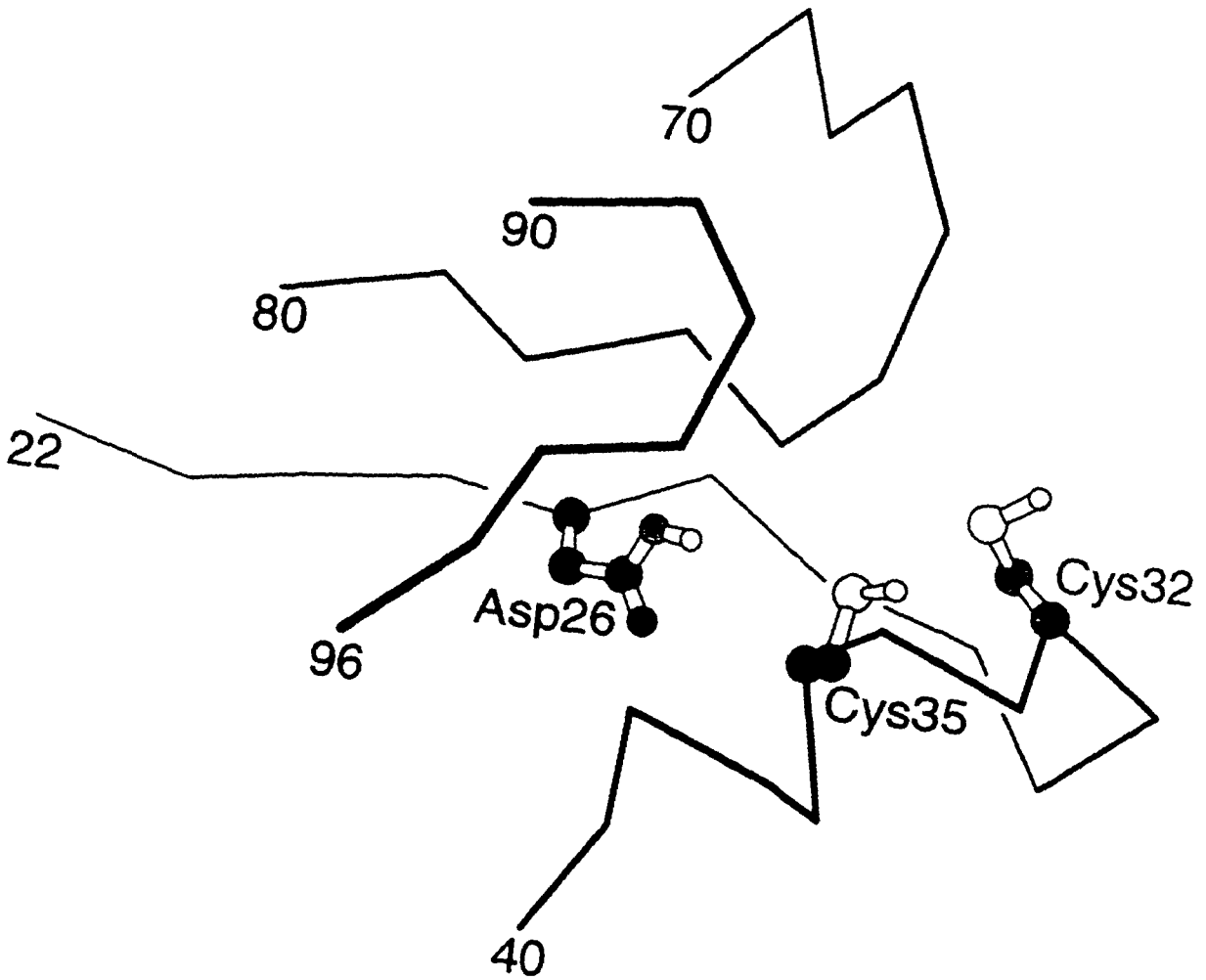

70
90
80
22
Asp26
96
Cys32
Cys35
40

Figure 4-2 The reduction of wild-type, D26N and D26L Trx by thioredoxin reductase. The inset is a magnification of the wild-type data to show the approach to equilibrium. Arrowheads indicate the addition of NADPH. Assays were performed at 298 K in 0.1 M Tris-HCl buffer, pH 7.0 containing 1.0 mM EDTA.

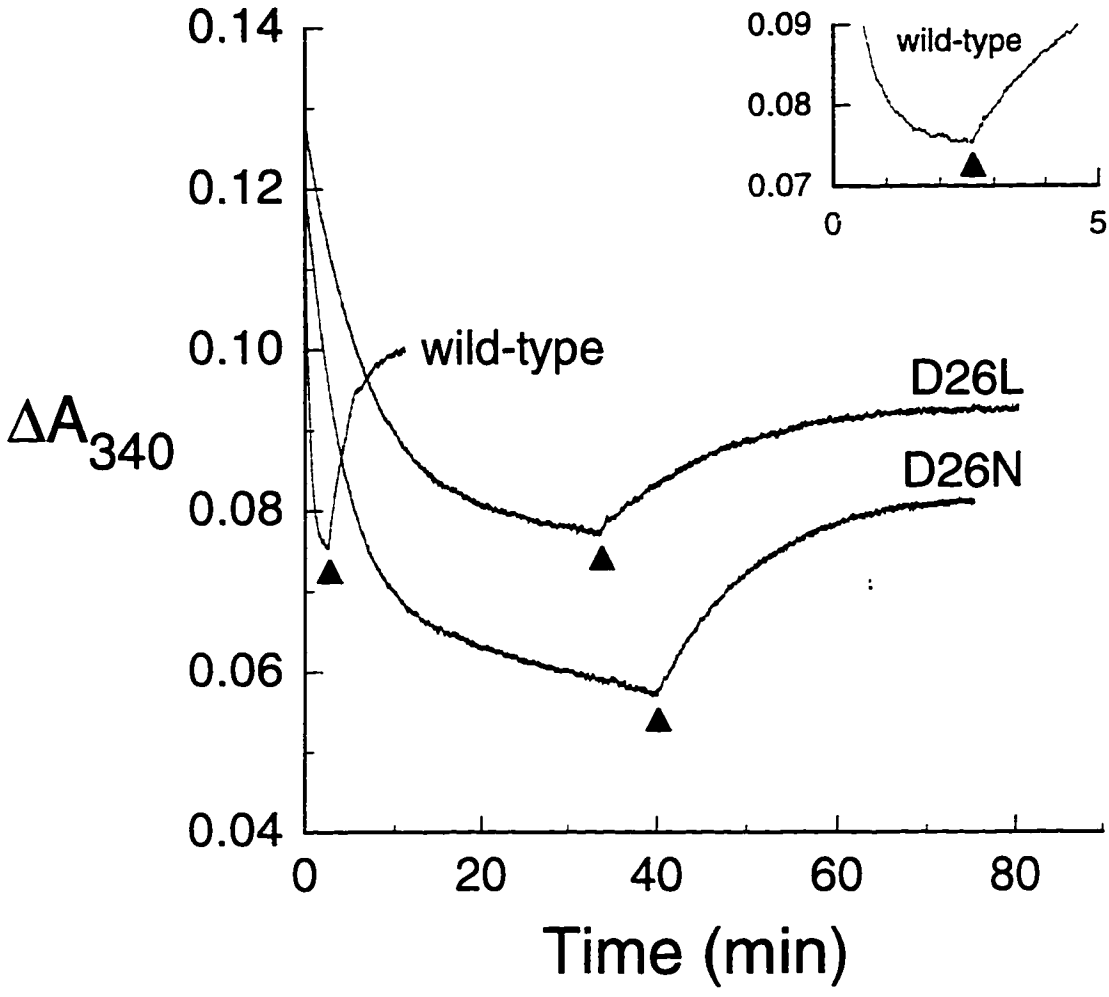

0.14
0.12
0.10
0.08
0.06
0.04
ΔA340
0
20
40
60
80
Time (min)
wild-type
D26L
D26N
0.09
0.08
0.07
0
5
wild-type

Figure 4-3 The reduction of DTNB by reduced Trx followed by stopped-flow visible spectroscopy. Assays were performed at 295 K in 0.05 M potassium phosphate buffer, pH 7.0, containing 1.0 mM EDTA.

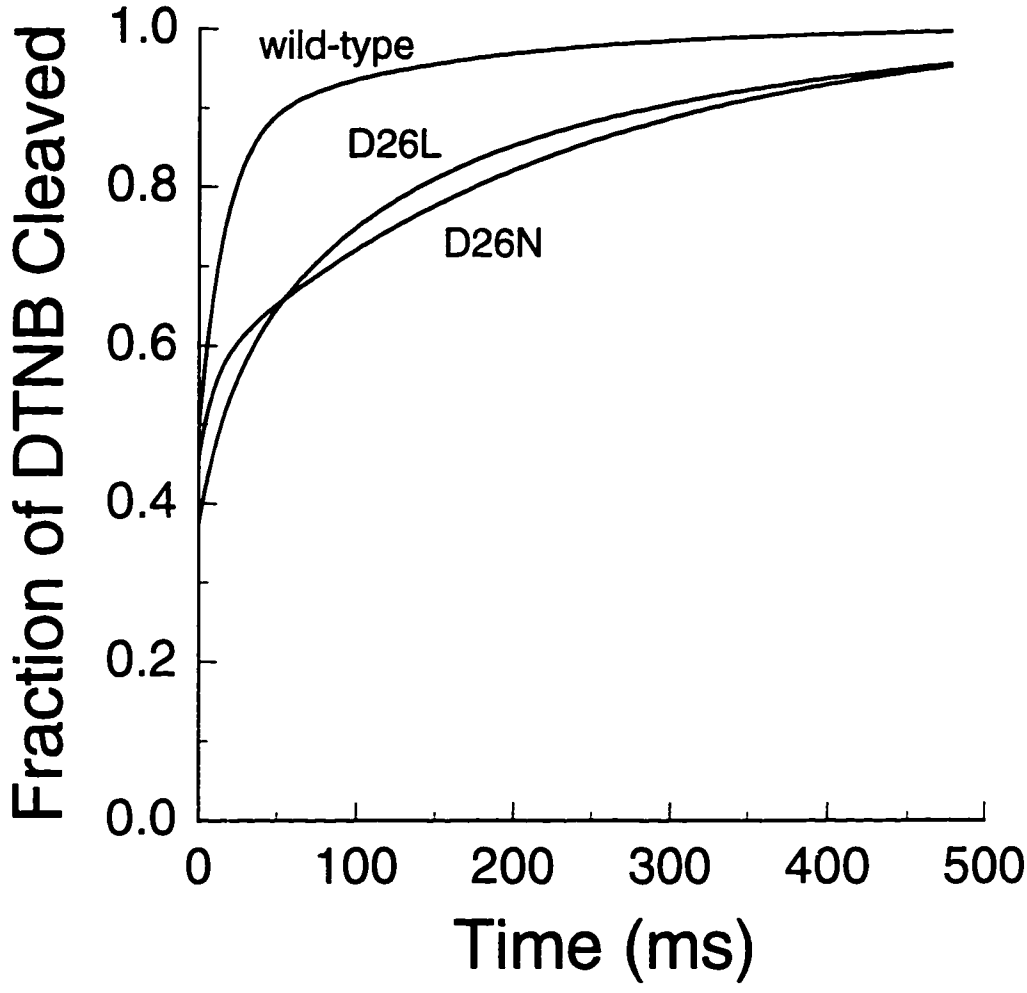

Fraction of DTNB Cleaved
1.0
0.8
0.6
0.4
0.2
0.0
wild-type
D26L
D26N
0
100
200
300
400
500
Time (ms)

Figure 4-4 The reduction of DTNB by reduced Trx in the presence of 10 mM Tris. Assays were performed at 295 K in 0.05 M potassium phosphate buffer, pH 7.0, containing 1.0 mM EDTA and 0.01 M Tris free acid.

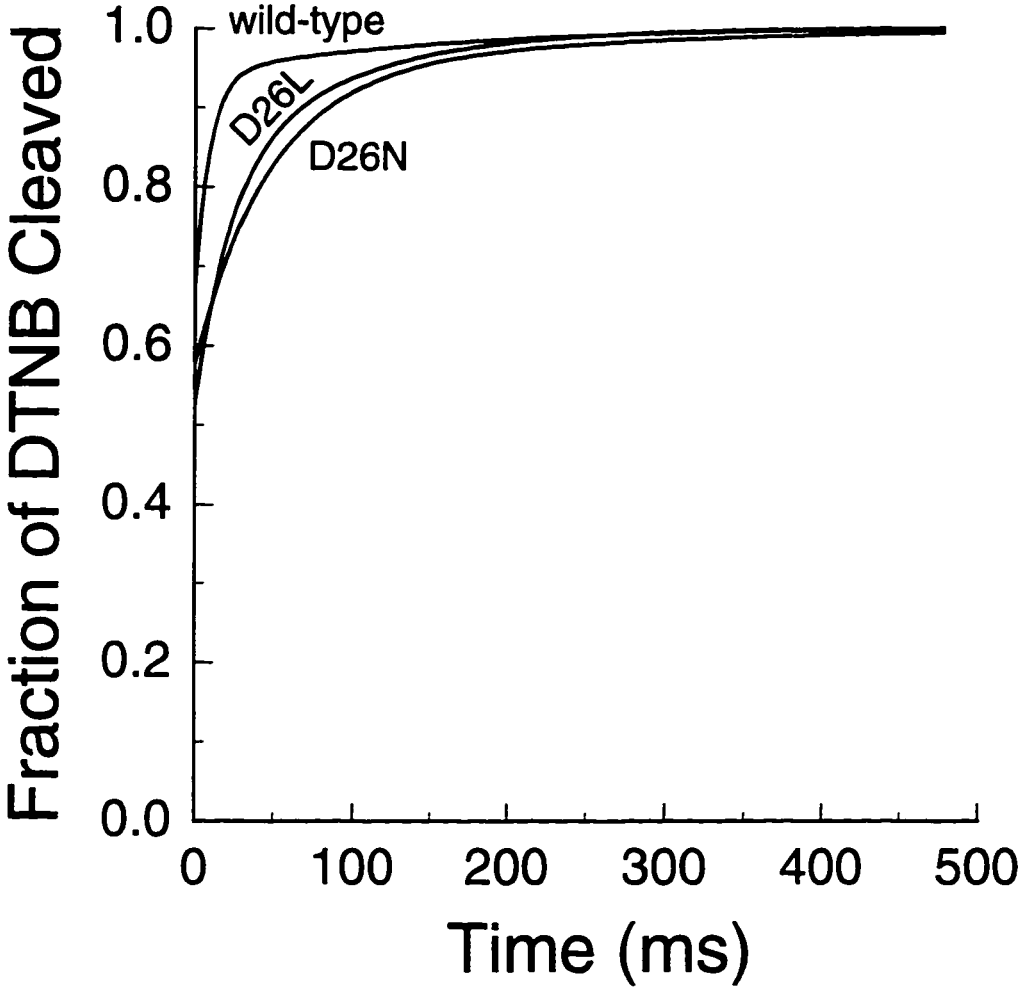
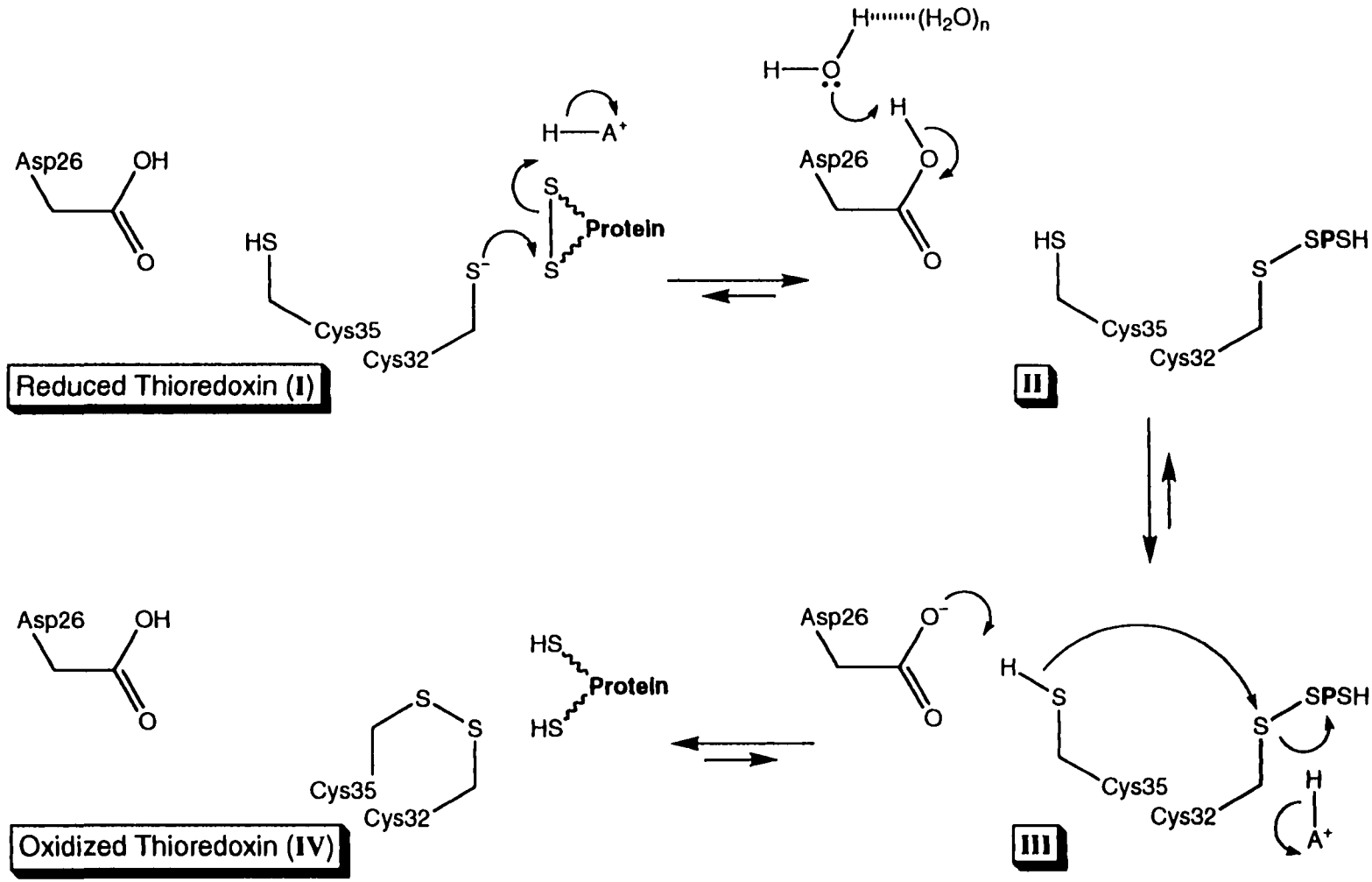

Fraction of DTNB Cleaved
1.0
0.8
0.6
0.4
0.2
0.0
wild-type
D26L
D26N
0
100
200
300
400
500
Time (ms)

Figure 4-5 The role of the active-site functional groups in the reactions catalyzed by Trx. AH^+ is any solute or solvent molecule than can donate and accept protons.


Reduced Thioredoxin (I)
Asp26
OH
O
HS
Cys35
Cys32
S⁻
S
S
Protein
H—A⁺
H
O
H·····(H₂O)ₙ
Asp26
O
H
O
II
HS
Cys35
Cys32
S
SPSH
III
Asp26
O⁻
O
H
S
Cys35
Cys32
S
SPSH
H
A⁺
Oxidized Thioredoxin (IV)
Asp26
OH
O
S
S
Cys35
Cys32
HS
Protein
HS

Chapter 5

The CXXC Motif—A Rheostat in the Active Site

ABSTRACT

The CXXC motif of dithiol-disulfide reductases is essential for their catalysis of redox reactions. Altering the XX residues of these enzymes can perturb the reduction potential of the active-site disulfide bond. This perturbation is manifested as a change in catalytic activity in vivo. There is a correlation between the pK_a of the first cysteine residue of the motif and the reduction potential ($E^{\circ\prime}$)of the active-site disulfide. The lower the value of pK_a the higher the value of $E^{\circ\prime}$. Analysis of CXXC mutants of the *Escherichia coli* proteins DsbA and thioredoxin (Trx) reveals a variation in the strength of this correlation. There is an absolute correlation between ΔpK_a and $\Delta E^{\circ\prime}$ for DsbA but not for Trx. A formal analysis of the Nernst equation reveals that reduction potential contains pH-dependent and pH-independent components. Indeed, the difference between the reduction potential of DsbA and Trx cannot be explained based just on the difference in the pK_a of the first cysteine residue. Available biochemical and structural data for CXXC-containing enzymes, reveals no single factor that is responsible pH-independent component of reduction potential. The cumulative effect of several small differences in interactions in the active-site of the reduced and oxidized forms of these proteins can account for differences in their catalytic activity. The malleability of the CXXC motif endows disulfide oxidoreductases with diverse roles in biology.

INTRODUCTION

Disulfide oxidoreductases are prevalent enzymes that catalyze the oxidation of protein thiols, and reduction and isomerization of protein disulfide bonds. The Cys-Xaa-Xaa-Cys (CXXC) motif of disulfide oxidoreductases is essential for their catalysis of redox reactions. This motif can be highly reducing [as in *Escherichia coli* thioredoxin, Trx; $E^{\circ\prime} = -0.27$ V (Moore *et al.*, 1964)], quite oxidizing [*E. coli* DsbA; $E^{\circ\prime} = -0.09$ to -0.11 V (Wunderlich & Glockshuber, 1993; Grauschopf *et al.*, 1995)], or of intermediate reduction potential [protein disulfide isomerase, PDI; $E^{\circ\prime} = -0.18$ V (Lundström & Holmgren, 1993)]. Altering the XX residues of Trx (Krause *et al.*, 1991; Chivers *et al.*, 1996), DsbA (Grauschopf *et al.*, 1995), or T_4 glutaredoxin [Grx; $E^{\circ\prime} = -0.23$ V (Joelson *et al.*, 1990)] perturbs the reduction potential of the disulfide bond of each enzyme.[1] For the mutant Trx's and DsbA's the change in $E^{\circ\prime}$ is manifested as a change in catalytic activity *in vivo* (Grauschopf *et al.*, 1995; Chivers *et al.*, 1996).

The effect of changing the XX residues in Trx and DsbA is opposite—Trx becomes a less efficient reductant but DsbA becomes a less efficient oxidant. These changes in $E^{\circ\prime}$ are accompanied by a change in the pK_a of the N-terminal cysteine residue (CXXC) of the motif (Hereafter, the first and second cysteines of the motif will be referred to as Cys_N and Cys_C, respectively.) In each mutant, a lower pK_a of Cys_N corresponds to an elevated $E^{\circ\prime}$. Analysis of the mutant DsbA's shows that the pK_1 alone can modulate $E^{\circ\prime}$. In contrast, analysis of the mutant Trx's shows that pK_1 is not the sole determinant of $E^{\circ\prime}$. What then are the elements that determine $E^{\circ\prime}$? This chapter is an investigation of the factors that modulate the reduction potential of the CXXC motif.

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1. To calibrate these differences in $E^{\circ\prime}$, consider that $\Delta E^{\circ\prime} = 0.03$ V corresponds to a 10-fold change in equilibrium constant near room temperature.

COMPONENTS OF $E^{\circ\prime}$

The general equilibrium for a reduction half-reaction is $\text{ox} + \text{n}e^- \rightleftarrows \text{red}$.

When this equilibrium also involves protons, the reduction potential is pH-dependent. Additionally, functional groups with different pK_a's in the reduced and oxidized molecules will also contribute to this pH-dependence. Thus, an enzyme that has a CXXC motif has (at least) two additional acid – base equilibria present in its reduced form. The reduced enzyme must be considered a population of dithiol, thiolate-thiol, thiol-thiolate, and dithiolate forms. The relative free energies of these forms, determined by their cysteine (Cys_N and Cys_C) pK_a's, give rise to the pH-dependence of the reduction potential.

The reduction potential for the half-reaction $\text{PS}_2 + 2\text{H}^+ + 2e^- \rightleftarrows \text{P(SH)}_2$ is given by a form of the Nernst equation:

$$E = E^\circ - \frac{RT}{nF}\ln\left(\frac{[\text{P(SH)}_2]}{[\text{PS}_2][H^+]^2}\right) \tag{5-1}$$

A pH-dependent term can be extracted from the Nernst equation by using the formal (*i.e.*, total) concentrations of the reduced [$\text{F}_{\text{P(SH)}_2}$] and oxidized ($\text{F}_{\text{PS}_2}$) molecules. This term accounts for the acid – base equilibria present in the oxidized or reduced molecules. For example, the fraction (α_0) of fully protonated dithiol species is:

$$\alpha_0 = \frac{[\text{P(SH)}_2]}{\text{F}_{\text{P(SH)}_2}} = \frac{[\text{H}^+]^2}{\left([\text{H}^+]^2 + [\text{H}^+]K_1 + [\text{H}^+]K_2 + K_1K_2\right)} \tag{5-2}$$

where K_1 and K_2 are the acid dissociation constants corresponding to the pK_a's of Cys_N and Cys_C respectively. The Nernst equation then becomes:

$$E = E^{\circ} - \frac{RT}{nF}\ln\left(\frac{\alpha_o F_{P(SH)_2}}{\left[H^+\right]^2 F_{PS_2}}\right) \tag{5-3}$$

Substitution for α_o yields:

$$E = E^{\circ} - \underline{\frac{RT}{nF}\ln\left(\frac{1}{\left[H^+\right]^2 + \left[H^+\right]K_1 + \left[H^+\right]K_2 + K_1K_2}\right)} - \frac{RT}{nF}\ln\left(\frac{F_{P(SH)_2}}{F_{PS_2}}\right) \tag{5-4}$$

The two underlined terms in eq 5-4 describe the formal potential of the reaction (Swift, 1939), which is defined here as E_F:

$$E_F = E^{\circ} - \frac{RT}{nF}\ln\left(\frac{1}{\left[H^+\right]^2 + \left[H^+\right]K_1 + \left[H^+\right]K_2 + K_1K_2}\right) \tag{5-5}$$

If $F_{P(SH)_2} = F_{PS_2}$, then $E = E_F$. This condition is met by both PDI in its natural environment [which is the endoplasmic reticulum of eukaryotic cells (Hwang *et al.*, 1992)] and Trx in its natural environment [which is the cytosol of *E. coli* (Gilbert, 1990)]. At pH 7, E_F, is defined as $E^{\circ\prime}$.

Eq 5-5 has two terms. The first term is simply E°, which reports on the intrinsic tendency of a disulfide bond to suffer reduction. The second term depends on the values of pH, K_1, and K_2. This term shows that E_F depends on pK_a and pH, and that $E^{\circ\prime}$ depends on pK_a.

The effects of thiol pK_1 and pH on E_F for three disulfide oxidoreductases is shown explicitly in Figure 5-1. To highlight the pH-dependency of E_F, the value of E° in Figure 5-1

was assumed to be zero for each enzyme. The breaks in the curves correspond to pK_1 and pK_2. Once the pH is above pK_2, the value of E_F is no longer pH-dependent.

According to the analysis used to derive Figure 5-1, the E_F values of Trx and PDI are nearly identical at pH 7.0. Yet, experiments show that $E^{\circ\prime}$ for Trx and PDI differ by 0.090 V (Holmgren, 1984; Lundström & Holmgren, 1993). Although the difference between DsbA and PDI is close to the value expected from experiment (~0.090 V) (Lundström & Holmgren, 1993; Wunderlich & Glockshuber, 1993; Grauschopf *et al.*, 1995), Trx is again in discord. Thus, the assumption used to derive Figure 5-1 must be incorrect—the value of E° must differ from that for PDI and DsbA.

The relationship between $E^{\circ\prime}$ and thiol pK_a can be thought of in another way. The values of $E^{\circ\prime}$ for DsbA and Trx differ by ~0.18 V. Near room temperature, this difference corresponds to a ΔG° of ~8.4 kcal/mol. The values of pK_1 for DsbA and Trx differ by ~4. Near room temperature, this difference corresponds to a ΔG° of ~5.6 kcal/mol. An aspect of protein structure other than pK_1 must therefore provide an additional ~2.8 kcal/mol of stability to the reduced form of DsbA or the oxidzed form of Trx.

CHANGING pK_a AND E°

A correlation between E_F and thiol pK_a's is provided by eq 5-5. This correlation should also be evident whenever E° is constant, and it has been observed in structurally related organic molecules (Szajewski & Whitesides, 1980; Burns & Whitesides, 1990). Wild-type and mutant CXXC-containing enzymes can in theory also be analyzed in this manner. At a minimum, the size (14 atoms) and composition ($C_9N_3S_2$) of the ring containing the disulfide bond remains constant.

A plot of pK_1 versus $E°$ for both wild-type and mutants of DsbA and Trx is shown in Figure 5-2. The value of $E°$ (=0.17 V) for wild-type DsbA has not been affected by mutations to the CXXC motif. Thus, the change in the E_F of DsbA imposed by mutating its CXXC motif is due solely to a change in pK_1. The relationship between pK_1 and $E°'$ for DsbA is $\Delta E°' = -0.0285\ \text{V} \times \Delta pK_1$.

The same plot for wild-type and mutant Trx's reveals a breakdown in this correlation. Although the pool of mutant Trx's is smaller, the change in $E°$ is greater. Thus, some property other than pK_1 is changing E_F in the mutant Trx's.

In these analyses, the pK_a of Cys_C in DsbA and Trx is assumed to be constant (pK_2 = 9.5). This assumption has little effect on our conclusions. A plot of ($E°' - E°$) vs pK_1 vs pK_2 at pH 7 can be derived from eq 5-5 and is shown in Figure 5-3. This plot reveals that if a thiol has pK_a » 7, then that pK_a has a negligible effect on $E°' - E°$ (and therefore on $E°'$ if $E°$ is constant). In other words, a thiol pK_a will have no significant effect on the formal potential when its value is at least one unit higher than the pH at which the formal potential is measured. Because the pK_a of Cys_C is changed little in the mutant Trx's (Chapter 3), this thiol almost certainly does not produce the observed effect on $E°$.

FACTORS AFFECTING $E°$

A quantity related to reduction potential is the effective concentration (EC) of one thiol relative to the other. This term describes the tendency of a dithiol-containing molecule to form an intramolecular disulfide bond relative to a reference monothiol-containing molecule that forms an intermolecular (*i.e.*, mixed) disulfide bond. In biological thiol – disulfide interchange reactions, glutathione (GSH) is used as the reference monothiol (Creighton, 1984; Lin & Kim,

1989; Gilbert, 1990). The relevant equilibria are $GSSG + 2H^+ + 2e^- \overset{K_{inter}}{\rightleftarrows} 2GSH$ and $PS_2 + 2H^+ + 2e^- \overset{K_{intra}}{\rightleftarrows} P(SH)_2$, and EC is defined by:

$$EC = \frac{K_{intra}}{K_{inter}} = \frac{[PS_2][GSH]^2}{[P(SH)_2][GSSG]} \tag{5-6}$$

Dithiols with a stronger tendency to form an intramolecular disulfide than that of two molecules of GSH to form an intermolecular disulfide have EC > 1. For the equilibria described by eq 5-6, EC = K_{ox} (Gilbert, 1990). The value of K_{ox} can be substituted into the Nernst equation to determine $E^{\circ\prime}$ for a dithiol when it is in equilibrium with GSH, with $E^{\circ\prime}$ for GSH = –0.252 V (Lees & Whitesides, 1993).

As part of an extensive effort to design strong organic dithiol reducing agents, Whitesides and coworkers used molecular mechanics calculations to reveal the factors that affect the EC of dithiols in molecules of similar structure and thiol pK_a (Burns & Whitesides, 1990). The three parameters found to be important were the dihedral angle of the disulfide bond (θ_{CSSC}), the two thiol bond angles ($\angle_{CCS}$), and van der Waal's interactions in the vicinity of the sulfur atoms. The importance of each of these factors varied from molecule to molecule, as did the chemical structure and disulfide ring size. Therefore, comparisons based on these parameters are valid only on related dithiols. Such a series of related thiols is shown in Figure 5-4. Because thiol pK_a's of the molecules in this series are equivalent (*i.e.*, the value of the pH-dependent term in eq 5-5 is the same for each molecule), conformational factors must change EC by altering the E°.

In the crystalline and solution structures of the oxidized (Katti *et al.*, 1990) and reduced (Jeng *et al.*, 1994) forms of Trx, oxidized DsbA (Martin *et al.*, 1993), and oxidized wild-type

and mutant Grx's (Eklund *et al.*, 1992), the disulfide dihedral angles (θ_{CSSC}) fall between 60° and 90°. This data set includes a mutant of Grx that affects $E^{\circ\prime}$ without a significant effect on θ_{CSSC} (Joelson *et al.*, 1990). Although differing dramatically in $E^{\circ\prime}$, oxidized DsbA and oxidized Trx have almost identical θ_{CSSC} (~80°). Molecular mechanics calculations have shown that there is little change in the relative energy of H_3CSSCH_3 for $60° < \theta_{CSSC} < 90°$ (Burns & Whitesides, 1990). Thus, the dihedral angle of these disulfide bonds is not a reliable indicator of $E^{\circ\prime}$. Additionally, virtually all structural protein disulfide bonds have θ_{CSSC} near 90° (Richardson, 1981; Thornton, 1981). The wide range of reduction potentials observed for these bonds (Gilbert, 1990) provides further evidence that θ_{CSSC} does not correlate with $E^{\circ\prime}$ in enzymes. Similarly, $\angle_{CCS}$ for Cys_N and Cys_C are almost identical for all three enzymes, ranging from 105° to 115°.

Analysis of the mainchain dihedral angles for the residues in the active site region of these enzymes show little correlation with $E^{\circ\prime}$ (Figure 5-5). The ϕ and ψ angles for X_1, X_2, and Cys_C are in the range expected for residues in an α-helical conformation. The ϕ and ψ angles for Cys_N and X_{-1} (which precedes Cys_N) are more dispersed, but do not correlate with $E^{\circ\prime}$. As with θ_{CSSC} and $\angle_{CCS}$, the ϕ and ψ angles of the CXXC motifs in Trx and DsbA are almost identical.

The value of $E^{\circ\prime}$ for a CXXC motif is not determined by the sequence of amino acid residues in the active site. Octapeptides corresponding to the active sites of Trx, PDI, Grx and thioredoxin reductase have $E^{\circ\prime} = -0.20 \pm 0.01$ V (Siedler *et al.*, 1993; Siedler *et al.*, 1994). Interactions arising from the three-dimensional fold of the enzyme must modulate E° of each CXXC motif.

The value of $E^{\circ\prime}$ is likely to result from noncovalent interactions between the CXXC motif and surrounding residues. Each atom of the 14-membered disulfide-containing rings of oxidized Trx, DsbA, and Grx make numerous contacts within a 3.25 Å radius. Differences in these interactions could alter E°, and hence $E^{\circ\prime}$. Yet, without knowledge of the structures of the

reduced and oxidized forms, a molecular rationalization of $E^{\circ\prime}$ is not possible. Even with both structures, an analysis would be limited to the pH at which the structures were determined.

EXPERIMENTAL DETERMINATION OF E°

The data presented above show that E° cannot be the same for all CXXC motifs. Can the value of E° be determined by experiments on a single enzyme? Eq 5-5 provides the pH-dependence of the formal potential for a reduction half-reaction. The pH-dependence of a complete reduction – oxidation reaction can be derived from eq 5-5. In this redox reaction, glutathione is often used as a reference thiol because its reduction potential and pH titration behavior are well characterized (Jung *et al.*, 1972; Rabenstein, 1973; Reuben & Bruice, 1976; Burns & Whitesides, 1990). The Nernst equation for a dithiol in equilibrium with glutathione is

$$E^{\circ\prime}_{\mathrm{P(SH)_2}} = E^{\circ\prime}_{\mathrm{GSH}} - \frac{RT}{nF}\ln K_{\mathrm{ox}} \tag{5-7}$$

In terms of formal concentrations, eq 5-7 is:

$$\ln K_{\mathrm{ox}} = \frac{nF}{RT}\left(E^{\circ}_{\mathrm{GSH}} - E^{\circ}_{\mathrm{P(SH)_2}}\right) + \ln\left(\frac{\left[\mathrm{H}^+\right]^2 + 2\left[\mathrm{H}^+\right]K_{\mathrm{GSH}} + \left(K_{\mathrm{GSH}}\right)^2}{\left[\mathrm{H}^+\right]^2 + \left[\mathrm{H}^+\right]K_1 + \left[\mathrm{H}^+\right]K_2 + K_1K_2}\right) \tag{5-8}$$

where $K_{\mathrm{ox}} = F_{\mathrm{PS_2}}F_{\mathrm{GSH}}^2/[F_{\mathrm{P(SH)_2}}F_{\mathrm{GSSG}}]$. The value of K_{ox} can be determined as a function of pH, and when $E^{\circ\prime}$ and all relevant pK_a values are known, a value of E° can be extracted from the equation. Creighton and coworkers have determined experimental values for K_{ox} over wide range of pH for both Trx and DsbA (Nelson & Creighton, 1994; Takahashi & Creighton,

1996). The results for Trx fit eq 5-8 only when pK_1 and pK_2 are close to 9. This value for pK_1 is greater by 2 units than that determined by direct measurement (Dyson *et al.*, 1991; Li *et al.*, 1993; Jeng *et al.*, 1995; Wilson *et al.*, 1995). Use of eq 5-8 assumes that the only additional equilibria in the reduced enzyme arises from the titration of the thiols of the CXXC motif. For example, the amino group of glutathione has a different pK_a in GSH and GSSG (Jung *et al.*, 1972). Such a pK_a, which differs in the reduced and oxidized species, requires the addition of terms to eq 5-8. There is much debate about the pK_a of Asp26 in the reduced and oxidized species of *E. coli* Trx (Wilson *et al.*, 1995; Jeng & Dyson, 1996). A change in the pK_a of Asp26 has indeed been observed upon oxidation of human thioredoxin (Qin *et al.*, 1996). Consideration of additional titrating groups is also important for DsbA and PDI because both contain a histidine residues within the CXXC motif. Thus, the experimental determination of K_{ox} therefore requires an accurate and thorough analysis of the titration of the reduced and oxidized species of the enzyme. Extending eq 5-8 to CXXC motifs is difficult because enzymes are complex molecules with many acid – base equilibria.

RELEVANCE TO BIOLOGICAL FUNCTION

Enzymes containing CXXC motifs appear to have evolved both divergently (PDI and Trx) and convergently (DsbA and Trx). The versatility of the CXXC motif has resulted in a family of enzymes that catalyze the same reactions, but with different efficiencies. Each enzyme has evolved in an environment (*e.g.*, the cytosol, periplasm, or endoplasmic reticulum) that imposes constraints for its optimization (Burbaum *et al.*, 1989). In addition, each enzyme has different substrates (*e.g.*, unfolded, misfolded, or folded proteins) and some of the enzymes are themselves substrates for different oxidases or reductases. Each enzyme is a solution to particular energetic problem.

The biological screen used to identify mutant DsbA's did not demand any correlations (Grauschopf *et al.*, 1995). Still, $E^{o\prime}$ is well-correlated to pK_1 in these mutant enzymes. Yet in a plot of $E^{o\prime}$ vs pK_1 (Figure 5-2), most of the mutant DsbA's are clustered within a narrow range. This grouping suggests that the structure of DsbA favors a limited range of $E^{o\prime}$ and pK_1 values. Further, both the reduced and the oxidized forms of the mutant DsbA's are more stable than are the two forms of wild-type DsbA (Grauschopf *et al.*, 1995). CXXC sequences of DsbA that lie outside the narrow pK_1, $E^{o\prime}$ range [PH (wild-type) and PP] must have some unique properties that destabilize either the oxidized or reduced species. This destabilization has apparently endowed wild-type DsbA with optimal biological activity.

In contrast, a biological selection was used to identify mutant Trx's that could replace PDI in a eukaryotic cell (Chivers *et al.*, 1996). The selection required a narrow range of pK_1 and $E^{o\prime}$ values, which were similar to those of PDI. Kinetic constraints could also have been important because the mutant Trx's must be reduced (or oxidized) by glutathione, which reacts slowly with wild-type Trx (Nikkola *et al.*, 1991). In addition, interactions with protein substrates may have required the production of favorable binding energies. Thus, the mutant Trx's may represent the few enzymes that meet all the challenges imposed by the *in vivo* selection.

The determinants of the reduction potential of a CXXC motif are complex, involving the titration of thiol and other groups as well as noncovalent interactions imposed by three-dimensional structure. The reduction potential is also malleable, as it can changed by pH or mutation. The ability of CXXC motifs to vary widely in their capacity to assist electron flow makes the motif a molecular rheostat, which can be set to a particular reduction potential to fit a particular need. The result is a family of disulfide oxidoreductases that play diverse roles in biology.

Figure 5-1 pH-Dependence of the formal reduction potential of a molecule with two titrating groups (*i.e.*, Cys_N and Cys_C) in its reduced form. The curves were calculated using eq 5-4 with $E^\circ = 0$ for each enzyme.

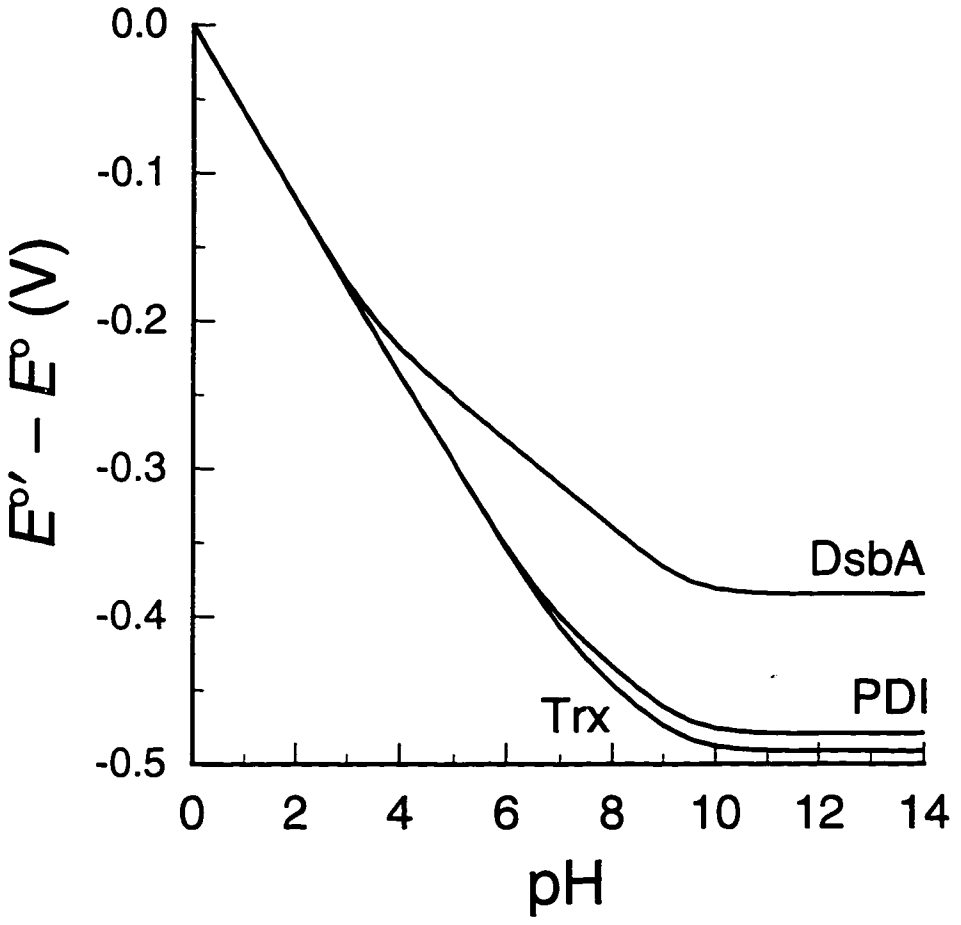

0.0
-0.1
-0.2
-0.3
-0.4
-0.5
E°' – E° (V)
0
2
4
6
8
10
12
14
pH
DsbA
PDI
Trx

Figure 5-2 Relationship between pK_1 and reduction potential (open symbols, $E^{\circ\prime}$; closed symbols, E°) for DsbA (circles) and Trx (triangles). DsbA data are from Grauschopf et al. (1995). Trx data are from Chapter 2 [also, Chivers et al. (1996)].

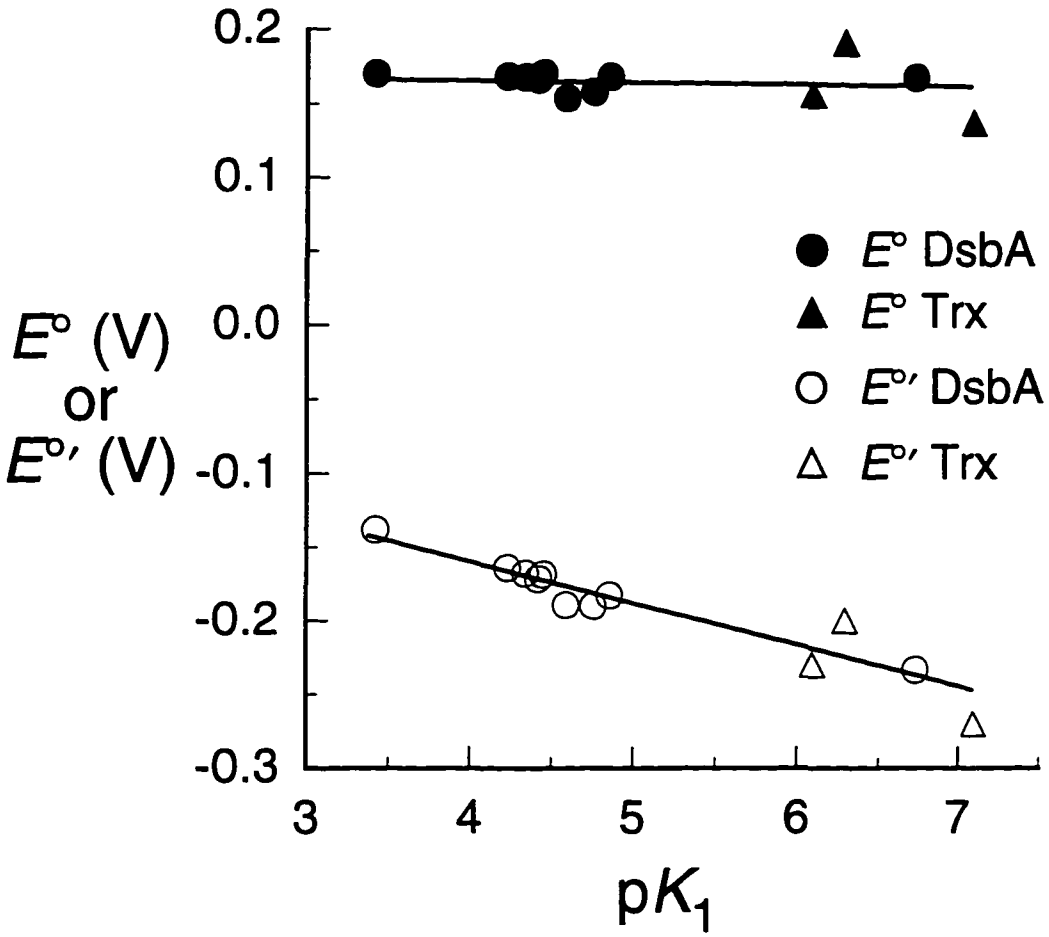

0.2
0.1
0.0
-0.1
-0.2
-0.3
E° (V)
or
E°′ (V)
E° DsbA
E° Trx
E°′ DsbA
E°′ Trx
3
4
5
6
7
pK1

Figure 5-3 Relationship between pK_1, pK_2, and $E^{\circ\prime} - E^{\circ}$. Surface is derived from eq 5-5 at pH 7.0. The shaded squares indicate the values of pK_1 and pK_2 observed for mutants of DsbA (Grauschopf *et al.*, 1995) and Trx (Chapter 3).

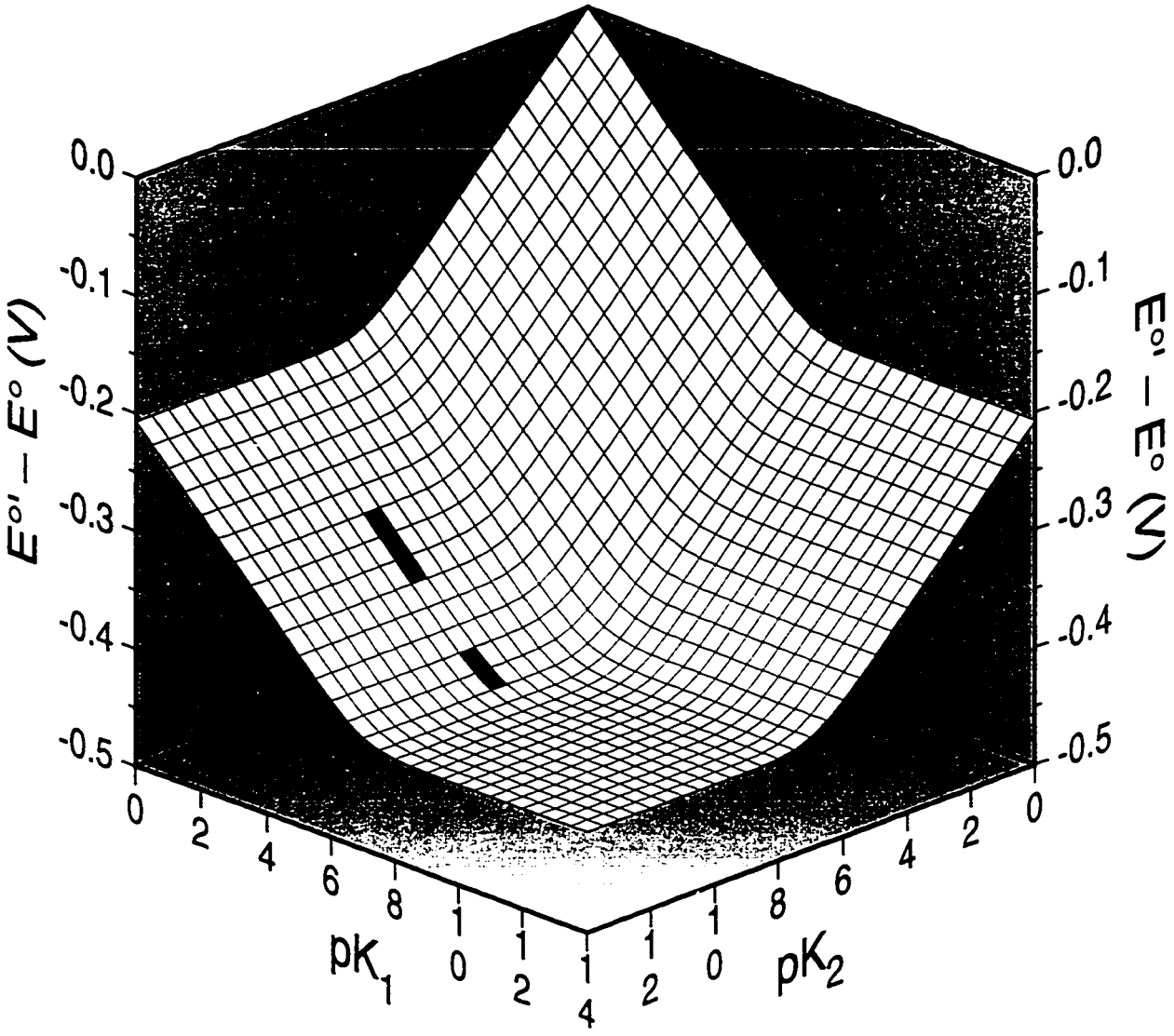
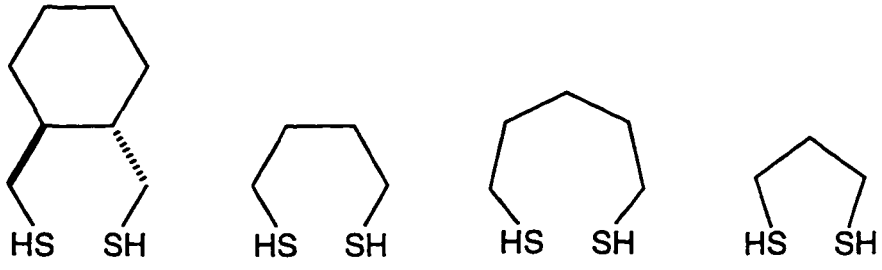
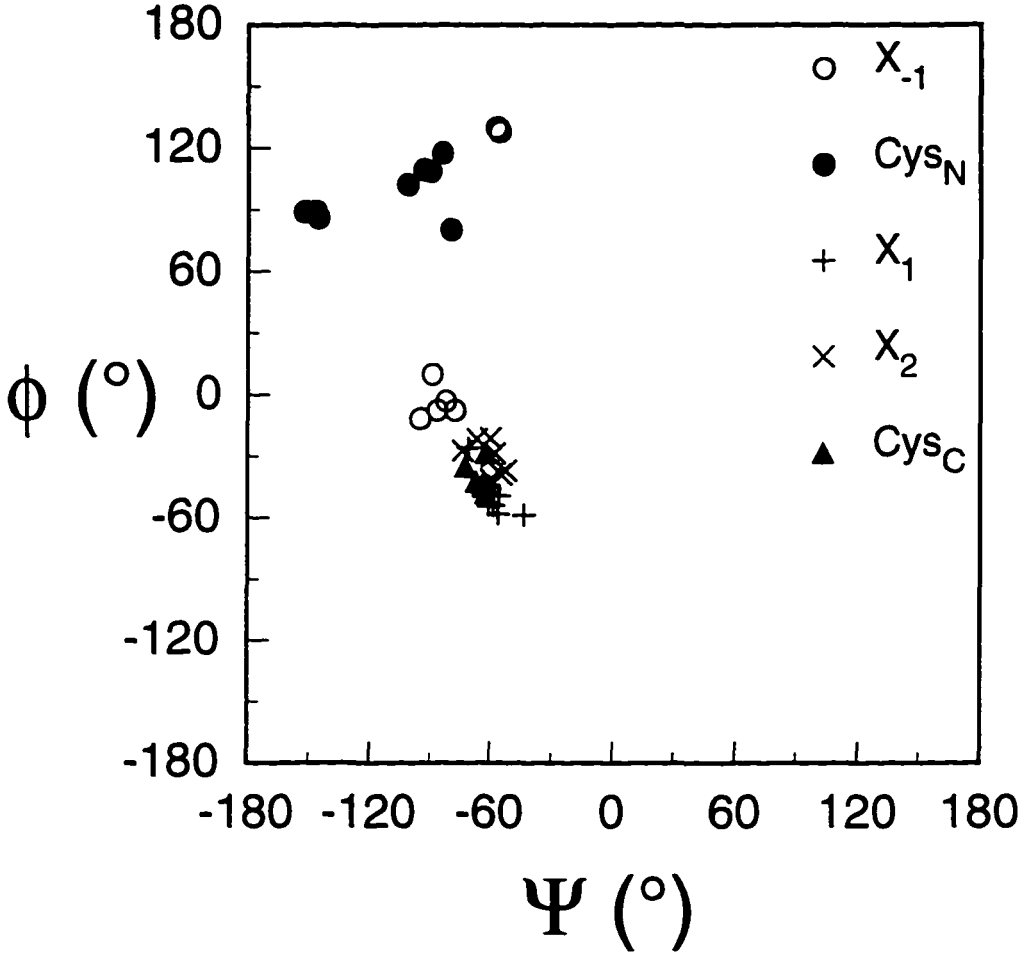

0.0
-0.1
-0.2
-0.3
-0.4
-0.5
E°' – E° (V)
0
2
4
6
8
10
12
14
pK1
0
2
4
6
8
10
12
pK2

Figure 5-4 EC and $E^{\circ\prime}$ values of similar organic dithiols. Data are from Lees and Whitesides (1993)



EC (M)	1500	180	77	2.3
$E^{\circ\prime}$ (V)	− 0.35	− 0.32	− 0.28	− 0.27

Figure 5-5 Mainchain dihedral angles for residues in CXXC motifs. Values were determined using the program MIDAS PLUS (Ferrin *et al.*, 1988) and PDB files 1AAZ (Eklund *et al.*, 1992), 1ABA (Eklund *et al.*, 1992), 1DSB (Martin *et al.*, 1993), 1THX (Saarinen *et al.*, 1995), 2TRX (Katti *et al.*, 1990), and 1XOA (Jeng *et al.*, 1994).


180
120
60
0
-60
-120
-180
φ (°)
-180 -120 -60 0 60 120 180
Ψ (°)
X-1
CysN
X1
X2
CysC

Appendix

Derivation of Equations

DERIVATION OF FRACTION THIOLATE

Chapter 2 of this Thesis reports a correlation between the growth rate of *pdi1Δ S. cerevisiae* complemented with CXXC mutants of Trx and fraction thiolate. Fraction thiolate depends on two properties of the Trx molecule: its reduction potential ($E^{o\prime}$), which determines how much of the protein is in the reduced form under particular redox conditions (E), and the pK_a of the first thiol, which determines how much of the reduced protein is present as the catalytically active thiolate form. The derivation of an equation to determine fraction thiolate is presented below:

$$P_{total} = PS_2 + P(SH)_2 = 1 \tag{A-1}$$

and

$$P(SH)_2 = P_{SH}^{SH} + P_{SH}^{S^-} + P_{S^-}^{SH} + P_{S^-}^{S^-} \tag{A-2}$$

The Nernst equation, eq A-3, can be solved for P(SH)2:

$$E = E^{o\prime} - \frac{RT}{nF} \ln \frac{[P(SH)_2]}{[PS_2]} \tag{A-3}$$

$$e^{\left[\left(E - E^{o\prime}{}_{P(SH)_2}\right)\cdot\left(-\frac{nF}{RT}\right)\right]} = \frac{[P(SH)_2]}{[PS_2]} \tag{A-4}$$

Substitution using eq A-1 yields:

$$e^{\left(\left(E - E^{o\prime}{}_{P(SH)_2}\right)\cdot\left(-\frac{nF}{RT}\right)\right)} * \left(1 - [P(SH)_2]\right) = [P(SH)_2] \tag{A-5}$$

$$\frac{e^{\left(\left(E-E^{o\prime}_{P(SH)_2}\right)\cdot\left(-\frac{nF}{RT}\right)\right)}}{1+e^{\left(\left(E-E^{o\prime}_{P(SH)_2}\right)\cdot\left(-\frac{nF}{RT}\right)\right)}}=\left[P(SH)_2\right] \tag{A-6}$$

Upon simplification eq A-6 becomes:

$$\frac{1}{1+e^{\left(\left(E-E^{o\prime}_{P(SH)_2}\right)\cdot\left(\frac{nF}{RT}\right)\right)}}=\left[P(SH)_2\right] \tag{A-7}$$

Eq A-7 describes the amount of protein in the reduced form ($P(SH)_2$). The amount of $P(SH)_2$ present in the the thiolate form can be determined using eq A-7, eq A-2, and the Henderson-Hasselbalch equation, eq A-9. Eq A-2 can be simplified to eq A-8 at pH 7, when the only significant forms of the protein are the doubly protonated (dithiol) and singly (thiolate-thiol) deprotonated species.

$$P(SH)_2 = P^{SH}_{SH} + P^{S^-}_{SH} \tag{A-8}$$

$$pH = pK_1 + \log\frac{\left[P^{S^-}_{SH}\right]}{\left[P^{SH}_{SH}\right]} \tag{A-9}$$

$$\left[P^{S^-}_{SH}\right] = 10^{(pH-pK_1)} * \left[P^{SH}_{SH}\right] \tag{A-10}$$

Substitution using eq A-8 yields:

$$\left[P^{S^-}_{SH}\right] = \frac{1}{10^{(pK_1-pH)}} * \left(\left[P(SH)_2\right] - \left[P^{S^-}_{SH}\right]\right) \tag{A-11}$$

Substitution of [P(SH)2] in eq A-11 using eq A-7 yields:

$$\left[P_{SH}^{S^-}\right] = \left(\frac{1}{1+10^{(pK_1-pH)}}\right) * \left(\frac{1}{1+e^{\left(\left(E-E^{\circ\prime}_{P(SH)_2}\right)\cdot\left(\frac{nF}{RT}\right)\right)}}\right) \qquad \text{(A-12)}$$

Figure A-1 shows the fraction thiolate for a protein when it is located in the endoplasmic reticulum (pH 7, $E = -0.180$ V).

DERIVATION OF THE pH-DEPENDENCE OF REDUCTION POTENTIAL

Chapter 5 of this Thesis describes the relationship between thiol pK_a and disulfide $E^{\circ\prime}$ This section of the appendix provides a complete derivation of eq 5-5 and eq 5-8. Disulfide oxidoreductases have a pH-dependent reduction potential because of the presence of two cysteine residues in the reduced molecule. These cysteines do not titrate in the oxidized molecule because they are in a disulfide bond. The reduction-oxidation equilibrium shown below must account for these titrating groups.

$$PS_2 + 2e^- + 2H^+ \xleftrightarrow{E^{\circ\prime}} P(SH)_2 \qquad \text{(A-13)}$$

The concentrations of the oxidized (PS_2) and reduced ($P(SH)_2$) forms of a CXXC-containing protein are fully described by their formal concentrations. Thus, the formal concentration $P(SH)_2$ (that is, $F_{P(SH)_2}$) will contain pH-dependent terms.

$$F_{P(SH)_2} = P_{SH}^{SH} + P_{SH}^{S^-} + P_{S^-}^{SH} + P_{S^-}^{S^-} \qquad \text{(A-14)}$$

Eq A-14 can be defined by $P(SH)_2$ using the acid-dissociation equilibria for the thiol protons, eqs A-15 and A-16:

$$K_1 = \frac{\left[P_{SH}^{S^-}\right]\left[H^+\right]}{\left[P_{SH}^{SH}\right]} \tag{A-15}$$

$$K_2 = \frac{\left[P_{S^-}^{SH}\right]\left[H^+\right]}{\left[P_{SH}^{SH}\right]} \tag{A-16}$$

$$F_{P(SH)_2} = \left[P(SH)_2\right] + \frac{K_1\left[P(SH)_2\right]}{\left[H^+\right]} + \frac{K_2\left[P(SH)_2\right]}{\left[H^+\right]} + \frac{K_1K_2\left[P(SH)_2\right]}{\left[H^+\right]^2} \tag{A-17}$$

Simplification of eq A-18 yields:

$$F_{P(SH)_2} = \left[P(SH)_2\right]\left(1 + \frac{K_1}{\left[H^+\right]} + \frac{K_2}{\left[H^+\right]} + \frac{K_1K_2}{\left[H^+\right]^2}\right) \tag{A-18}$$

The fraction of $P(SH)_2$ is given by:

$$\alpha_0 = \frac{\left[P(SH)_2\right]}{F_{P(SH)_2}} = \frac{\left[H^+\right]^2}{\left[H^+\right]^2 + \left[H^+\right]K_1 + \left[H^+\right]K_2 + K_1K_2} \tag{A-19}$$

or

$$\left[P(SH)_2\right] = \alpha_0 F_{P(SH)_2} = \frac{\left[H^+\right]^2 F_{P(SH)_2}}{\left[H^+\right]^2 + \left[H^+\right]K_1 + \left[H^+\right]K_2 + K_1K_2} \tag{A-20}$$

Eq A-20 can be substituted into the Nernst equation (eq A-3) to determine the formal potential for eq A-13.

$$E = E^{\circ} - \frac{RT}{nF}\ln\left(\frac{[P(SH)_2]}{[PS_2][H^+]^2}\right)$$
$$= E^{\circ} - \frac{RT}{nF}\ln\left(\frac{\dfrac{[H^+]^2 F_{P(SH)_2}}{[H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2}}{F_{PS_2}[H^+]^2}\right) \qquad \text{(A-21)}$$

Simplification and rearrangement yields eq A-22:

$$E = \underline{E^{\circ} - \frac{RT}{nF}\ln\left(\frac{1}{[H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2}\right)} - \frac{RT}{nF}\ln\left(\frac{F_{P(SH)_2}}{F_{PS_2}}\right) \qquad \text{(A-22)}$$

Eq A-22 shows the pH-dependence of the reduction potential of CXXC-containing proteins. The underlined term is defined as $E^{\circ\prime}$ at pH 7. This derivation has assumed that the thiols are the only groups that titrate differently in the reduced species, thus F_{PS2} is $[PS_2]$. Figure 5-1 shows the pH-dependence of the formal potential for three different CXXC-containing proteins.

The pH-dependence of a full reduction-oxidation reaction, such as the K_{ox} equilibrium (eq A-23), must account for the formal concentration of all molecules in the reaction:

$$K_{ox} = \frac{[PS_2][GSH]^2}{[P(SH)_2][GSSG]} \qquad \text{(A-23)}$$

At equilibrium, K_{ox} can be substituted into the Nernst equation.

$$E^{\circ\prime}_{P(SH)_2} = E^{\circ\prime}_{GSH} - \frac{RT}{nF}\ln\left(\frac{[PS_2][GSH]^2}{[P(SH)_2][GSSG]}\right) \quad \text{(A-24)}$$

Considering only the thiol titrations in the reduced species, eq A-24 becomes:

$$E = E^{\circ} - \frac{RT}{nF}\ln\left(\frac{[H^+]^2 + 2[H^+]K_{GSH} + K_{GSH}{}^2}{[H^+]^2 + [H^+]K_1 + [H^+]K_2 + K_1K_2}\right) - \frac{RT}{nF}\ln\left(\frac{F_{PS_2}F_{GSH}{}^2}{F_{P(SH)_2}F_{GSSG}}\right) \quad \text{(A-25)}$$

Additional terms will appear in numerator or denominator for every functional group that does not have the same pK_a in the reduced and oxidized molecules. Eq A-25 also becomes complex if the titrating groups exhibit microscopic pK_a's.

Eq A-25 does not accurately describe the reduction-oxidation equilibrium between *E. coli* thioredoxin and glutathione. The amino group of glutathione has different pK_a in the reduced (~9.5) and oxidized (~8.9) forms (Jung *et al.*, 1972). Further, in reduced glutathione the thiol and amino groups exhibit microscopic pK_a's. From the data presented in Chapter 3, reduced Trx contains groups with microscopic pK_a's (Cys32 and Asp26). Because the pK_a of Asp26 is different in the oxidized and reduced forms of Trx, the formal concentration of oxidized must include a pH-dependent term.

The various equilibria pertinent to the oxidation-reduction equilibrium between *E. coli* thioredoxin and glutathione are presented in Figure A-2. The formal concentrations of each of the reactants (fully protonated forms) using these equilibria are:

$$\alpha_{GSSG} = \frac{[GSSGNH_3^+]}{F_{GSSG}} = \frac{[H^+]}{[H^+] + K_{GSSG}} \quad \text{(A-26)}$$

$$\alpha_{TrxS_2} = \frac{[TrxS_2CO2H]}{F_{TrxS_2}} = \frac{[H^+]}{[H^+] + K_{CO2Hox}} \quad \text{(A-27)}$$

$$\alpha_{\mathrm{Trx(SH)_2}} = \frac{[\mathrm{Trx(SH)_2}]}{\mathrm{F}_{\mathrm{Trx(SH)_2}}}$$

$$= \frac{[\mathrm{H}^+]^3}{[\mathrm{H}^+]^3 + [\mathrm{H}^+]^2 K_{32\mathrm{SH}} + [\mathrm{H}^+]^2 K_{\mathrm{CO2H}} + [\mathrm{H}^+] K_{32\mathrm{SH}} K_{\mathrm{CO2H^{\bullet}}} + K_{32\mathrm{SH}} K_{\mathrm{CO2H^{\bullet}}} K_{35\mathrm{SH}}} \tag{A-28}$$

$$\alpha_{\mathrm{GSH}} = \frac{[\mathrm{GSH}]}{\mathrm{F}_{\mathrm{GSH}}} = \frac{[\mathrm{H}^+]^2}{[\mathrm{H}^+]^2 + [\mathrm{H}^+] K_{\mathrm{GSH}} + [\mathrm{H}^+] K_{\mathrm{NH}} + K_{\mathrm{GSH}} K_{\mathrm{NH^{\bullet}}}} \tag{A-29}$$

Substituting of eqs A-26 through A-29 into eq A-24 followed by simplification yields:

$$E = E^{\circ} - \frac{RT}{nF} \ln \left(\frac{\left([\mathrm{H}^+]^3 + [\mathrm{H}^+]^2 K_{32\mathrm{SH}} + [\mathrm{H}^+]^2 K_{\mathrm{CO2H}} + [\mathrm{H}^+] K_{32\mathrm{SH}} K_{\mathrm{CO2H^{\bullet}}} + K_{32\mathrm{SH}} K_{\mathrm{CO2H^{\bullet}}} K_{32\mathrm{SH}}\right)}{\left([\mathrm{H}^+]^2 + [\mathrm{H}^+] K_{\mathrm{GSH}} + [\mathrm{H}^+] K_{\mathrm{NH}} + K_{\mathrm{GSH}} K_{\mathrm{NH^{\bullet}}}\right)^2} \times \left(\frac{\left([\mathrm{H}^+] + K_{\mathrm{GSSG}}\right)\left([\mathrm{H}^+]\right)}{\left([\mathrm{H}^+] + K_{\mathrm{CO2Hox}}\right)} \right) \right) - \frac{RT}{nF} \ln \left(\frac{\mathrm{F}_{\mathrm{TrxS_2}} \mathrm{F}_{\mathrm{GSH}}^{\ 2}}{\mathrm{F}_{\mathrm{Trx(SH)_2}} \mathrm{F}_{\mathrm{GSSG}}} \right) \tag{A-30}$$

Solving eq A-30 for K_{OX} yields:

$$K_{ox} = \exp^{\left(\left(\frac{nF}{RT}\right)\left(E^{\circ}_{GSH} - E^{\circ}_{Trx}\right)\right)} - \left(\frac{\left([H^+]^3 + [H^+]^2 K_{32SH} + [H^+]^2 K_{CO2H} + [H^+] K_{32SH} K_{CO2H*} + K_{32SH} K_{CO2H*} K_{35SH}\right)}{\left([H^+]^2 + [H^+] K_{GSH} + [H^+] K_{NH} + K_{GSH} K_{NH*}\right)^2} \times \left(\frac{\left([H^+] + K_{GSSG}\right)\left([H^+]\right)}{\left([H^+] + K_{CO2Hox}\right)} \right) \right)$$

(A-31)

A plot of K_{ox} vs pH for Trx is shown in Figure A-2. The pK_a and E° values for this plot are: pK_{GSSG}, 8.9 (Jung *et al.*, 1972); K_{CO2Hox}, 7.5 (Dyson *et al.*, 1991; Langsetmo *et al.*, 1991); K_{GSH}, 8.72 (Reuben & Bruice, 1976); K_{NH}, 8.9 (Jung *et al.*, 1972); K_{NH*}, 9.5 (Reuben & Bruice, 1976); K_{32SH}, 7.5; K_{CO2H}, 7.5; K_{CO2H*}, 9.5; K_{32SH}, 11.0; E°_{GSH}, 0.414 V; and E°_{Trx} , 0.396 V. The absolute values of the reduction potentials is unimportant, it is the difference between these two values that affects K_{ox}. The magnitude of this difference determines the position of the K_{ox} curve on the y-axis. The pH-dependent term in eq A-31 determines the shape of the curve.

Figure A-1 The relationship between cysteine pK_a and the reduction potential of a CXXC motif.

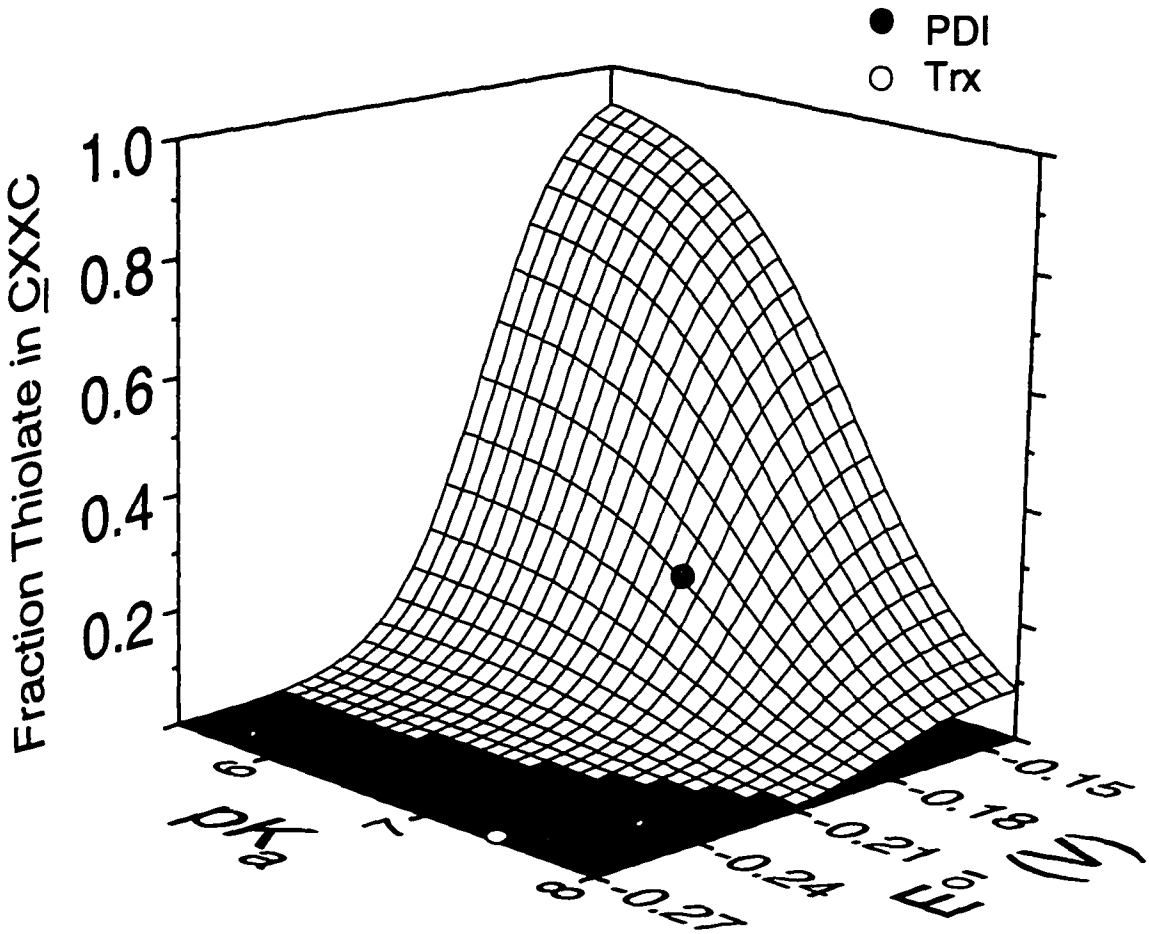
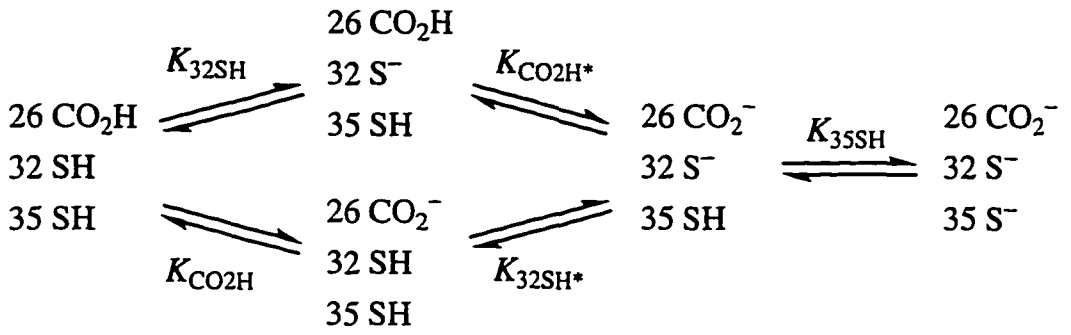
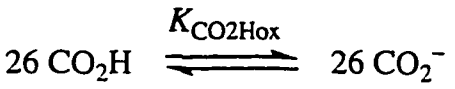

PDI
Trx
Fraction Thiolate in CXXC
1.0
0.8
0.6
0.4
0.2
pKa
-0.15
-0.18
-0.21
-0.24
-0.27

Figure A-2 Acid-dissociation equilibria of glutathione and thioredoxin used to determine K_{ox}.

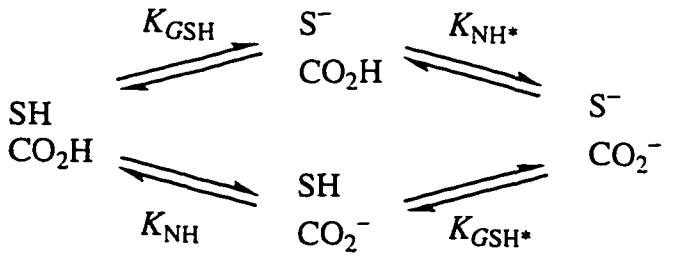
$Trx(SH)_2$:



$TrxS_2$:



GSH:



GSSG:

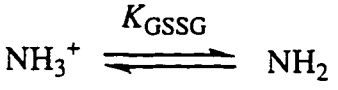
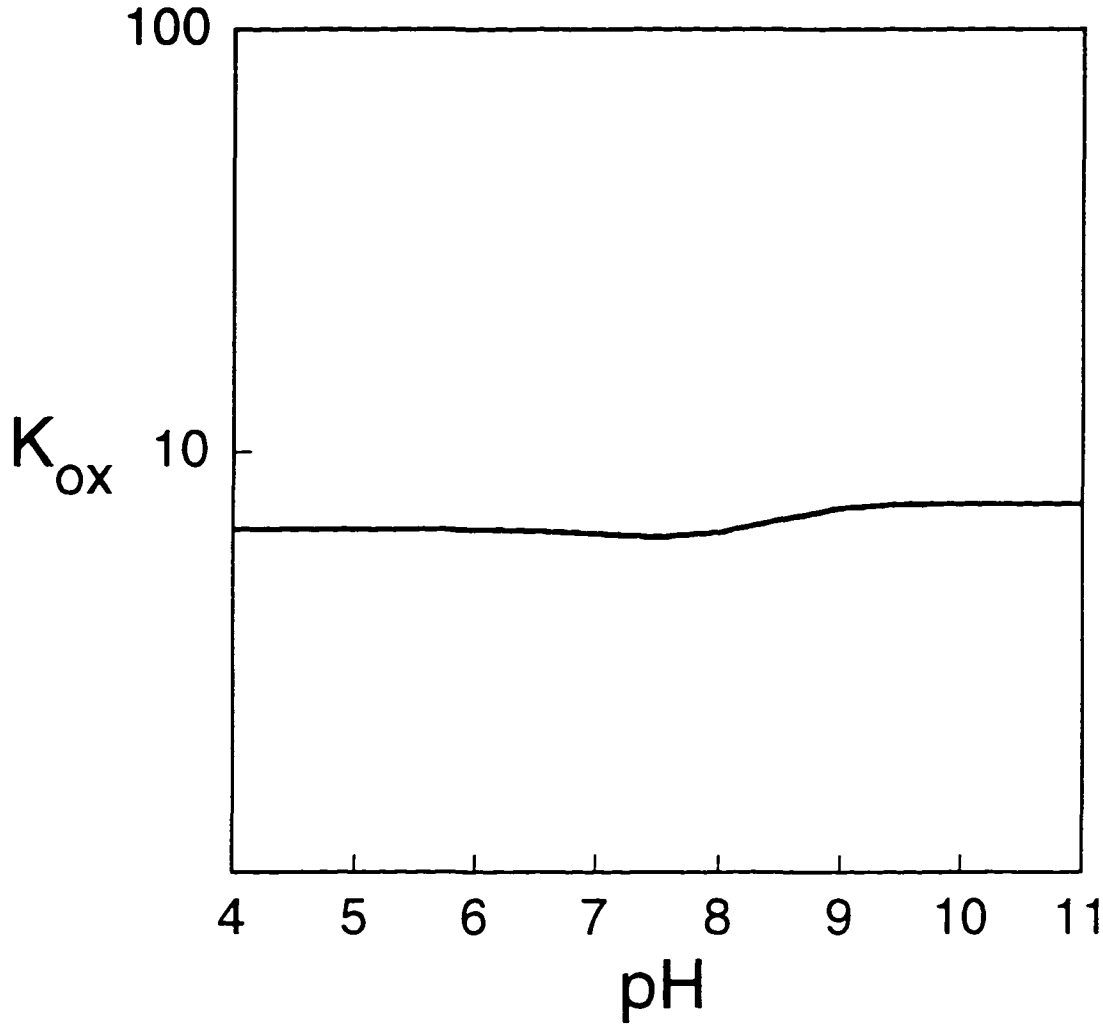


Figure A-3 K_{ox} vs pH for *E. coli* thioredoxin accounting for microscopic pK_a's.


100
10
K_{ox}
4
5
6
7
8
9
10
11
pH

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