

The CXC Motif: A Functional Mimic of Protein Disulfide Isomerase[†]Kenneth J. Woycechowsky^{‡,§} and Ronald T. Raines^{*,‡,||}

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ABSTRACT: Protein disulfide isomerase (PDI) utilizes the active site sequence Cys-Gly-His-Cys (CGHC; $E^{\circ\prime} = -180$ mV) to effect thiol–disulfide interchange during oxidative protein folding. Here, the Cys-Gly-Cys-NH₂ (CGC) peptide is shown to have a disulfide reduction potential ($E^{\circ\prime} = -167$ mV) that is close to that of PDI. This peptide has a thiol acid dissociation constant ($pK_a = 8.7$) that is lower than that of glutathione. These attributes endow the CGC peptide with substantial disulfide isomerization activity. *Escherichia coli* thioredoxin (Trx) utilizes the active site sequence Cys-Gly-Pro-Cys (CGPC; $E^{\circ\prime} = -270$ mV) to effect disulfide reduction. Removal of the proline residue from the Trx active site yields a CGC active site with a greatly destabilized disulfide bond ($E^{\circ\prime} \geq -200$ mV). The $\Delta P34$ variant retains high conformational stability and remains a substrate for thioredoxin reductase. In contrast to the reduced form of the wild-type enzyme, the reduced form of $\Delta P34$ Trx has disulfide isomerization activity, which is 25-fold greater than that of the CGC peptide. Thus, the rational *deletion* of an active site residue can bestow a new and desirable function upon an enzyme. Moreover, a CXC motif, in both a peptide and a protein, provides functional mimicry of PDI.

PDI¹ (EC 5.3.4.1 (1–3)) is the most efficient known catalyst of oxidative protein folding (4). A thiol–disulfide oxidoreductase with an archetypal active site motif: Cys-Xaa-Xaa-Cys (CXXC, where X is any amino acid), PDI catalyzes the formation, reduction, and isomerization of disulfide bonds in a protein substrate (5). The product contains the most stable arrangement of disulfide bonds in a particular redox environment (6).

Oxidoreductases with CXXC motifs vary in their disulfide bond stability, subcellular localization, and biological function (7–9). For example, PDI, a resident of the endoplasmic reticulum, has a CGHC active site with $E^{\circ\prime} = -180$ mV (10) and is essential for catalysis of disulfide isomerization in *Saccharomyces cerevisiae* (5). *Escherichia coli* Trx has a CGPC active site (11) with $E^{\circ\prime} = -270$ mV (12) and functions as a cytosolic reductant (13). DsbA has a CPHC

active site with $E^{\circ\prime} = -122$ mV (14) and catalyzes the oxidation of proteins that have been secreted to the *E. coli* periplasm (15). These three enzymes are believed to be of similar tertiary structure (16–18). Yet, their active site disulfide bonds differ by up to 148 mV (or 6.8 kcal/mol) in stability (Table 1).

The identity of the –XX– residues within a particular CXXC active site can make a large contribution to its $E^{\circ\prime}$ value (14, 19). For example, Trx variants with CGHC and CPHC active sites (resembling those of PDI and DsbA, respectively) have an active site disulfide bond that is destabilized by 35 and 75 mV (1.6 and 3.5 kcal/mol), respectively (19, 20). CGHC Trx also possesses greater functional resemblance to PDI than does wild-type Trx. In vitro, CGHC Trx is 10-fold more active than the wild-type enzyme at catalyzing native disulfide formation in RNase A (21). In vivo, CGHC Trx, but not the wild-type enzyme, can complement a PDI deficiency (22).

Removing the protein structural context can change the stability of a CXXC disulfide (Table 1). For example, the stability of the active site disulfide bond in CGPC (that is, wild-type) Trx decreases by 75 mV (3.5 kcal/mol) upon denaturation in urea (23). The disulfide bond in the octapeptide W–CGPC–KHI is destabilized by 70 mV (3.2 kcal/mol) relative to that in native CGPC Trx (24). Such CXXC-containing peptides have a narrow range of disulfide bond stabilities, with $E^{\circ\prime}$ values typically in the range of –200 to –230 mV (24, 25). Thus, small CXXC-containing peptides are generally poor mimics of CXXC-containing proteins.

Varying the number of residues between two cysteines yields peptides with a broad range of $E^{\circ\prime}$ values. Of such C(X)_nC peptides with $0 \leq n \leq 5$, those with only one intervening amino acid tend to have the least stable disulfide bond (25). Here, we report the synthesis and characterization

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¹ Abbreviations: BMC, (\pm)-*trans*-1,2-bis(mercaptoacetamido)cyclohexane; BPTI, bovine pancreatic trypsin inhibitor; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NADP⁺, oxidized β -nicotinamide adenine dinucleotide phosphate; NADPH, reduced β -nicotinamide adenine dinucleotide phosphate; PDI, protein disulfide isomerase; RNase A, bovine pancreatic ribonuclease (unglycosylated); sRNase A, bovine pancreatic ribonuclease (unglycosylated) with scrambled disulfide bonds; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TCEP, tris-(2-carboxyethyl)phosphine; TR, thioredoxin reductase; Tris, tris(2-aminoethyl)amine; Trx, thioredoxin.

Table 1: Values of E° (mV) for CXXC Motifs in Trx, PDI, DsbA, and Related Peptides

enzyme	-XX-	native ^a	unfolded	CXXC peptide
Trx	-GP-	-270 ^b	-195 ^c	-200 ^d
PDI	-GH-	-180 ^e	ND ^f	-220 ^d
DsbA	-PH-	-122 ^g	-200 ^g	ND ^f

^a Relative to $E^{\circ} = -252$ mV for glutathione (30). ^b Data from ref (12). ^c Data from ref (23). ^d Data from ref (24). ^e Data from ref (10). ^f ND, not determined. ^g Data from ref (14).

of the tripeptide Cys-Gly-Cys-NH₂ (CGC). The disulfide E° of the CGC peptide is similar to that of the active site of PDI, and its first thiol pK_a is less than that of the tripeptide glutathione (γ -Glu-Cys-Gly), which is a natural redox reagent. Moreover, the CGC peptide is an efficient catalyst of disulfide isomerization. Then, we create a variant of Trx with a CGC active site. Unlike wild-type Trx, the variant has considerable disulfide isomerization activity. These data demonstrate that functional mimicry of PDI does not need to rely on structural mimicry, and the data reveal a new motif for the catalysis of oxidative protein folding.

MATERIALS AND METHODS

General. Commercial chemicals and biochemicals were of reagent grade or better and were used without further purification. Manipulations of DNA and *E. coli* were carried out by standard protocols (26, 27). Trx concentrations were determined by ultraviolet spectroscopy, using $\epsilon = 13\,700$ M⁻¹ cm⁻¹ at 280 nm (20). Thiol concentrations were determined likewise after reaction with DTNB using $\epsilon = 13\,600$ M⁻¹ cm⁻¹ at 412 nm (28).

Instrumentation. The absorbance of ultraviolet and visible light was measured with a Cary model 3 spectrophotometer. Peptide synthesis was performed with an Applied Biosystems 432A peptide synthesizer. HPLC was performed with a Waters system equipped with either a Dynamax preparative C18 column or a Vydac analytical C18 column.

Synthesis of the CGC Peptide. The tripeptide CGC was synthesized on an amide resin (25 μ mol scale). The two thiols of cysteine were protected with trityl groups. The peptide was deprotected and cleaved from the resin with trifluoroacetic acid/1,2-ethanedithiol/water (95:2.5:2.5). The resin was removed by filtration, and the filtrate was concentrated under reduced pressure. The CGC peptide was purified by precipitation in ice-cold ether (10 mL), dissolution of the resulting white solid in aqueous acetic acid (1% w/v), and reversed-phase HPLC to yield CGC (2.9 mg; 41%). The peptide was found to be pure by electrospray ionization mass spectrometry (m/z calculated, 281; found, 281).

Reduction Potential of the CGC Peptide. The stability of the cyclic disulfide formed by the CGC peptide was evaluated as described previously (29). Briefly, thiol-disulfide equilibria were established between the dithiol form of CGC (370 μ M) and the β -hydroxyethyl disulfide (810 μ M) and analyzed by HPLC. The equilibrium constant was determined by integration of the peaks corresponding to reduced and oxidized β -hydroxyethyl disulfide. The reduction potential of the CGC disulfide was calculated from the equilibrium constant by using the Nernst equation and $E_{\beta\text{-hydroxyethyl disulfide}}^{\circ} = -260$ mV (30). The value reported is the mean (\pm SE) of three measurements.

Thiol pK_a Values of the CGC Peptide. Sulfhydryl groups undergo a large increase in A_{238} upon deprotonation (31). Thiol titration curves were obtained by measuring A_{238} as a function of pH for a series of solutions containing reduced CGC (63 μ M) in 0.10 M potassium phosphate buffers. Data were analyzed using a double titration model as described previously (29).

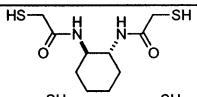
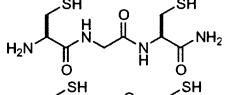
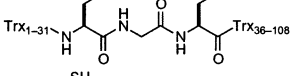
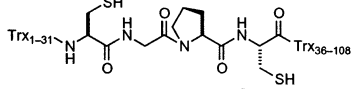
Disulfide Isomerization Activity. The activation of sRNase A, a fully oxidized protein containing predominantly nonnative disulfides, is indicative of disulfide isomerization (32). Only those RNase A molecules possessing all four native disulfides have significant ribonucleolytic activity. sRNase A was obtained by air oxidation of fully reduced RNase A (70 μ M) in 20 mM Tris-HCl buffer, pH 8.0, containing guanidine-HCl (6.0 M) and EDTA (1.0 mM) over a period of several days followed by exhaustive dialysis against 0.10 M Tris-HCl buffer, pH 7.6, containing EDTA (1.0 mM). This method produced RNase A with <0.1 mol thiol/mol protein and approximately 1% of the ribonucleolytic activity of native RNase A. The activation of sRNase A by a peptide or protein catalyst was monitored and analyzed as described previously (29).

Production and Purification of Wild-Type Trx and its Δ P34 Variant. *E. coli* Trx is a 108 residue (11.7 kDa) protein that acts as a cytosolic reductant for ribonucleotide reductase, Trx peroxidase, methionine sulfoxide reductase, and other proteins (13, 33). Trx effects the reduction of substrates through its active site, Cys32-Gly33-Pro34-Cys35. Plasmid pTRX directs the overexpression of wild-type Trx in *E. coli* (34). The codon for Pro34 was deleted from the *trx*A gene in plasmid pTRX by site-directed mutagenesis using oligonucleotide KW08 (5' GG GCA GAG TGG TGC GGT TGC AAA ATG ATC GCC CC 3') to yield plasmid p Δ P34TRX. Wild-type Trx and its Δ P34 variant were produced in *E. coli* BL21(DE3) cells that had been transformed with plasmid pTRX or p Δ P34TRX.

Wild-type Trx was purified in its oxidized form as described previously (34). Its reduced form was produced by the addition of DTT (15 mg, 0.10 mmol) to the oxidized protein (5 mg, 0.43 μ mol) in 1.0 mL of 0.10 M Tris-HCl buffer, pH 7.6, containing EDTA (1.0 mM). After the mixture was incubated overnight at 4 °C, reduced wild-type Trx was separated from DTT by gel filtration chromatography and found to contain 2.0 mol thiol/mol protein.

The reduced form of Δ P34 Trx was purified in the same manner as was the oxidized form of wild-type Trx, except that the cell lysis buffer contained DTT (0.10 M) to disrupt disulfide-bonded oligomers. To produce oxidized monomers of Δ P34 Trx, 50 μ L of a solution of DTNB (25 mM, 1.3-fold molar excess) was added to 55 mL of a solution of purified reduced protein (18 μ M) in 0.10 M potassium phosphate buffer, pH 7.6. The reaction mixture was stirred in the dark at room temperature for 1 h and then dialyzed at 4 °C against 4 L of 20 mM imidazole-HCl buffer, pH 7.6. The solution volume was decreased to <10 mL with a Vivaspin concentrator (5 kDa molecular mass cutoff; Vivascience AG, Hannover, Germany), and the protein was purified by anion exchange column chromatography as described previously (22). The protein eluted as two peaks, and neither contained detectable thiols. Both proteins were monomeric, as judged by SDS-PAGE. The protein in the first peak constituted 82% of the total protein and coeluted

Table 2: Properties of Dithiol Catalysts of Disulfide Isomerization

molecule	structure	pK _a	E ^{o'} (mV)	specific activity (U/mol) ^a
BMC ^b		8.3; 9.9	-240	56
CGC		8.7; 9.8	-167	132
ΔP34 Trx		ND ^c	≥-200	3300
wild-type Trx		7.5; ≥11 ^d	-270 ^e	0

^a One unit (U) of reduced catalyst activates 1 mmol of sRNase A per min in 0.10 M Tris-HCl buffer, pH 7.6, containing EDTA (1.0 mM). ^b Data from ref (29). ^c ND, not determined. ^d Value of pK_a = 7.5 is for the first microscopic pK_a of Cys32 (34). ^e Data from ref (12).

with wild-type Trx. This protein was used in all subsequent experiments. The protein in the second peak eluted later in the NaCl gradient, consistent with its arising from deamidation of an asparagine residue.

Conformational Stability of ΔP34 Trx. The value of T_m , which is the temperature at the midpoint of the thermal unfolding transition, provides a measure of the conformational stability of a protein. To assess the consequences of Pro34 deletion on conformational stability, T_m values were determined for both reduced and oxidized ΔP34 Trx. The decrease in A_{280} upon unfolding of Trx was used to report on the equilibrium between native and unfolded Trx. To obtain thermal unfolding curves, a solution of oxidized ΔP34 Trx was exchanged into 20 mM potassium phosphate buffer, pH 7.0, by using a Vivaspin concentrator. The A_{280} of the protein (1.6 mL of a 0.16 mg/mL solution) was recorded in 1 °C increments as the temperature was increased from 25 to 80 °C at 0.15 °C/min. Upon reaching 80 °C, a similar temperature scan was performed in the reverse direction, and the temperature-dependent change in A_{280} was found to be reversible for >90% of the protein. The thermal unfolding of reduced ΔP34 Trx was monitored in the same manner, except for the presence of the reducing agent TCEP (1.0 mM) in the buffer to protect the protein from air oxidation. A two state model was used to fit the data, and values of T_m were determined as described previously (35, 36).

ΔP34 Trx as a Substrate for TR. TR is a flavoprotein that catalyzes the reduction of the Trx active site disulfide by NADPH to form an active site dithiol and NADP⁺ (12, 33, 37). A perturbation to the overall fold and, in particular, the active site structure of a Trx variant can be ascertained by assaying the Trx variant as a substrate for TR (22, 38, 39). To assay ΔP34 Trx and the wild-type enzyme as substrates for TR, TR (1 μL of a 0.1 mg/mL solution from Sigma Chemical, St. Louis, MO) was added to 0.6 mL of 10 mM potassium phosphate buffer, pH 7.0, containing Trx (10 μM), NADPH (25 μM), and EDTA (1.0 mM). The decrease in A_{340} over time upon reduction of NADPH ($\epsilon = 6200 \text{ M}^{-1} \text{ cm}^{-1}$ (40)) was measured as described previously (22, 39).

Reduction Potential of ΔP34 Trx. The reduction potential of the active site disulfide in oxidized ΔP34 Trx was determined by measuring the equilibrium constant for the

TR-catalyzed oxidation of NADPH ($E^{\circ'} = -315 \text{ mV}$ (41)) as described previously (12, 22).

RESULTS

Design of the CGC Peptide. The simplest mechanism for catalysis of disulfide isomerization begins with the nucleophilic attack of a thiolate by the catalyst on a substrate disulfide to form a covalent catalyst-substrate complex (42, 43). Subsequent thiol-disulfide interchange within the substrate leads to native disulfides and, eventually, release of the catalyst. For a given dithiol, the fraction of molecules in the thiolate form under specified solution conditions (pH and E_{solution}) is governed by both the disulfide $E^{\circ'}$ and the thiol pK_a. CGC was chosen as a promising reagent for the isomerization of disulfide bonds for two reasons. First, the disulfide bond in CXC peptides is relatively unstable (25). Second, a favorable Coulombic interaction with the *N*-terminal amino group should stabilize a thiolate in the side chain of the *N*-terminal cysteine residue. Amidation of the *C*-terminal carboxyl group prevents an unfavorable Coulombic interaction with a thiolate. The tripeptide CGC (Table 2) was synthesized by standard solid phase methods. The HPLC-purified peptide was subjected to an analysis of its physicochemical properties (disulfide $E^{\circ'}$ and thiol pK_a) and disulfide isomerization activity.

Properties of the CGC Peptide. The equilibrium between dithiol, thiol-thiolate, and dithiolate in reduced CGC was monitored by measuring the A_{238} over a pH range that spans from mostly protonated to mostly unprotonated species (Figure 1A). A fit of the data to a double titration model gives thiol pK_a values of 8.7 and 9.8 (Table 2).

The reduction potential of the CGC disulfide was determined by measuring the equilibrium constant for its reduction of β-hydroxyethyl disulfide. By using HPLC analysis of thiol-disulfide interchange equilibria between CGC and β-hydroxyethyl disulfide, CGC was determined to have $E^{\circ'} = -167 \pm 1 \text{ mV}$ (Table 2). This value is similar to the $E^{\circ'}$ value of -169 mV reported for the YSR-CGC hexapeptide (25).

The disulfide isomerization activity of the dithiol CGC was assayed by monitoring its ability to increase the ribonucleolytic activity of sRNase A. Reduced CGC folds

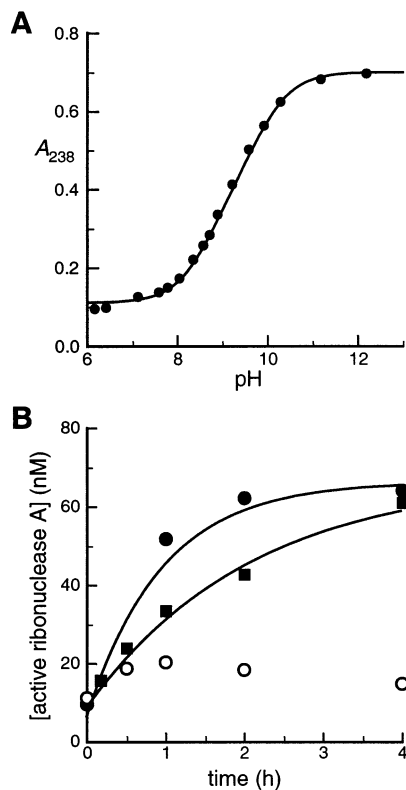


FIGURE 1: Characterization of CGC peptide. (A) Titration of CGC by ultraviolet spectroscopy. Titrations were performed in 0.10 M potassium phosphate buffers at 25 °C. Values of pK_a were obtained from a fit of the data to a double titration model (29) and are listed in Table 2. (B) Time-course of the activation of scrambled ribonuclease A by reduced CGC (0.5 mM, ●); BMC (0.5 mM, ■); and glutathione (1.0 mM, ○). Assays were performed at 30 °C in 50 mM Tris-HCl buffer, pH 7.6. Specific activities of CGC and BMC were determined from a fit of the data to a single exponential (29) and are listed in Table 2.

sRNase A more efficiently than does either reduced glutathione (which is a monothiol commonly used for protein folding in vitro (44, 45)) or BMC (Vectrase-P, which is a dithiol known to be an efficient catalyst of protein folding (29, 46)) (Figure 1B). The specific activity of CGC is 2.2-fold higher than that of BMC (Table 2).

Design of Δ P34 Trx. Having characterized the physicochemical properties and disulfide isomerization activity of the CGC peptide (Table 2), we next explored the consequences of inserting this sequence of three amino acids into the context of a folded protein, specifically Trx. The CGPC active site of wild-type Trx has $E^{\circ} = -270$ mV (20, 47–49). The high stability of its active site disulfide limits the effectiveness of Trx as a catalyst of native disulfide formation. Trx variants with destabilized disulfide bonds do, however, show enhanced catalysis (21, 22). Given the instability of CXC relative to CXXC disulfides in peptides (25), we hypothesized that deletion of the active site proline residue in wild-type (CGPC) Trx would increase catalytic activity.

Properties of Δ P34 Trx. Δ P34 Trx was produced by heterologous overexpression in *E. coli* of the mutant *trxA* gene (34). Gel filtration chromatography of cell lysates gave multiple peaks containing Δ P34 Trx. SDS-PAGE indicated that the majority of the Δ P34 Trx formed disulfide-linked oligomers. After addition of DTT to the cell lysis buffer,

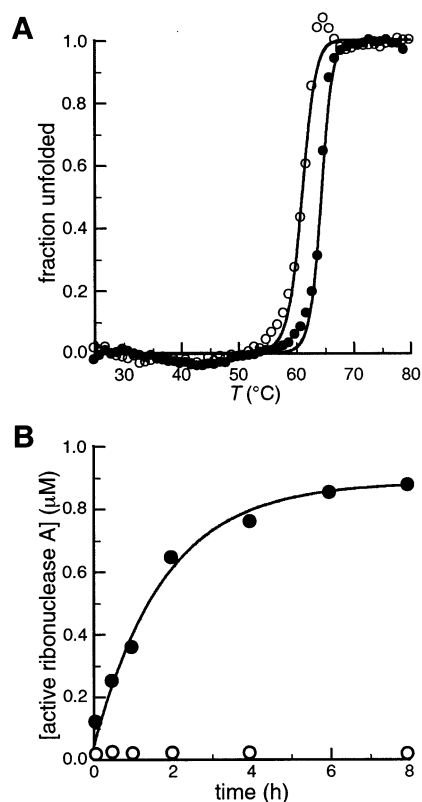


FIGURE 2: Characterization of Δ P34 Trx. (A) Thermal unfolding of oxidized (○) and reduced (●) Δ P34 Trx. Experiments were performed in 20 mM potassium phosphate buffer, pH 7.0, containing TCEP (oxidized Δ P34 Trx, 0 mM; reduced Δ P34 Trx, 1.0 mM). Values of T_m were 61 (oxidized Δ P34 Trx) and 65 °C (reduced Δ P34 Trx). (B) Time-course of the activation of scrambled ribonuclease A by reduced Δ P34 Trx (0.14 mM, ●) and reduced wild-type Trx (0.10 mM, ○). Assays were performed at 30 °C in 50 mM Tris-HCl buffer, pH 7.6. The specific activity of Δ P34 Trx was determined from a fit of the data to a single exponential (29) and is listed in Table 2.

Δ P34 Trx eluted from the gel filtration column as a monomer in a single peak and was fully reduced (2 mol thiol/mol Trx). The yield of purified protein was typically 0.10 g/L of culture. Oxidation of a dilute solution of Δ P34 Trx by DTNB produced fully oxidized, predominantly monomeric protein, which was easily purified by anion exchange chromatography. No attempt was made to remove endogenous wild-type Trx from the samples of the Δ P34 variant. Any such contamination, which was estimated to be <0.1% (34), would have had no significant effect on the interpretation of the results reported herein.

Δ P34 Trx tends to form disulfide-linked oligomers when purified in the absence of DTT. In contrast, wild-type Trx is recovered exclusively as oxidized monomers when produced and purified similarly. The tendency of Δ P34 Trx to form oxidized oligomers suggests that the deletion of Pro34 destabilizes the active site disulfide. This deletion could also disrupt the Trx structural scaffold. We used both thermodynamic and functional assays to search for deleterious effects on the structure and stability of Δ P34 Trx.

To discern any destabilization to the Trx tertiary structure caused by deletion of Pro34, we examined the thermal denaturation behavior of Δ P34 Trx. Both reduced and oxidized Δ P34 Trx show reversible thermal unfolding behavior (Figure 2A), and both redox states of Δ P34 Trx

are less stable than are the corresponding redox form of wild-type Trx (50). The reduced and oxidized forms of Δ P34 Trx have T_m values of 65 and 61 °C, respectively; the reduced and oxidized forms of wild-type Trx have T_m values of 73 and 85 °C (50), respectively. Interestingly, the oxidized form of Δ P34 Trx is slightly less stable than is the reduced form. The increase in conformational stability upon disulfide reduction indicates that the disulfide destabilizes the tertiary structure of Δ P34 Trx, in marked contrast to the increased stability imparted by the disulfide bond in wild-type Trx (50). The thermal denaturation data also indicate that while deletion of Pro34 causes significant destabilization of both redox states, Δ P34 Trx still maintains high conformational stability (Figure 2A).

We used the rate of decrease in A_{340} upon oxidation of NADPH to report on the ability of a Trx variant to act as a substrate for TR and thus, indirectly, on the structural similarity between the Δ P34 Trx and the wild-type enzyme. The rate of TR-catalyzed NADPH oxidation was 3.9-fold lower with Δ P34 Trx than with wild-type Trx. This only slight decrease provides a further indication that any structural perturbation incurred upon deletion of Pro34 is small.

The TR assay could in theory be used to estimate the equilibrium constant for reduction of Δ P34 Trx by NADPH.² Unfortunately, NADPH reduces Δ P34 Trx beyond the detection limit of this assay (that is, the decrease in A_{340} upon Δ P34 Trx reduction is complete to within the noise of the spectrophotometer). Attempts to shift the equilibrium by the addition of excess NADP⁺ resulted in no detectable NADPH formation. Although this assay failed to establish an $E^{\circ'}$ value for Δ P34 Trx, it did provide a lower limit of $E^{\circ'} \geq -200$ mV. Thus, the active site disulfide of Δ P34 Trx is markedly destabilized relative to that of wild-type Trx, which has $E^{\circ'} = -270$ mV (20).

The sRNase A assay was performed to assess the disulfide isomerization activity of reduced wild-type Trx and its Δ P34 variant. Wild-type Trx did not activate sRNase A to a detectable extent (Figure 2B). In contrast, reduced Δ P34 Trx was an efficient catalyst of sRNase A activation. Incorporation into the Trx scaffold enhances the specific activity of the CGC sequence by 25-fold, relative to the simple tripeptide (Table 2).

DISCUSSION

Comparison of the CXC and CXXC Motifs. The rational design of catalysts of protein folding is an important and ongoing challenge. Enzymes, such as PDI, that catalyze oxidative protein folding employ CXXC active sites (43, 51). This active site motif imparts a great deal of functional flexibility. Variations in the sequence of $-XX-$ residues give rise to significant changes in both disulfide $E^{\circ'}$ and thiol pK_a , thereby allowing the active site to be tuned to suit a particular biological function (8). For example, Trx and DsbA are two *E. coli* CXXC-containing proteins with high structural similarity. Yet, the CPHC active site of DsbA has a

disulfide $E^{\circ'}$ that is 148 mV more positive and a thiol pK_a that is four units lower than that of the CGPC active site in Trx. Such physicochemical differences are important for the proper function of a CXXC-containing protein in its cellular environment (8, 9).

The disulfide $E^{\circ'}$ and thiol pK_a of a CXXC motif can be varied by changing the $-XX-$ residues. Most attempts at tuning the physicochemical properties of CXXC motifs have relied on swapping one natural $-XX-$ sequence for another (14, 19) or creating random libraries of $-XX-$ sequences (7, 22, 52). Only one attempt has been made to examine the functional consequences of altering the number of intervening amino acids in a CXXC motif (38).

Small peptides can serve as templates for the design of new protein folding reagents. The desirable features of this approach include the ease of peptide synthesis, chemical variety of the natural amino acids, and ability to incorporate useful sequences into proteins by using the genetic code. Short CXXC-containing peptides (7–10 residues) with sequences that correspond to natural oxidoreductases have been shown to catalyze native disulfide formation in RNase A (53). The activities of these peptides correlate qualitatively with their disulfide $E^{\circ'}$ values. Peptides with less negative (that is, more PDI-like) $E^{\circ'}$ values tend to have higher activities. Backbone cyclization destabilizes the disulfide bond of a CXXC peptide still further (54, 55), but cyclic peptides cannot be incorporated into proteins by using the genetic code.

Rather than mimic structure, that is, the amino acid sequence and disulfide loop size of PDI, we sought to mimic *function*, in particular, the values of disulfide $E^{\circ'}$ and thiol pK_a . CXC peptides can form 11-membered disulfide-bonded rings with $E^{\circ'}$ values that are typically 30–40 mV (1.4–1.8 kcal/mol) less stable than those of CXXC peptides (53, 56). The structural basis for the relative instability of CXC disulfide-bonded rings relative to CXXC disulfide-bonded rings is not entirely clear. For cycloalkanes, 11-membered rings have higher strain energy than do 14-membered rings (57). This higher strain probably results from unfavorable transannular steric interactions in 11-membered rings that are relieved by enlarging the ring to 14 atoms (58). Furthermore, a 14-membered ring in a CXXC peptide could have more favorable transannular hydrogen bonding than an 11-membered ring in a CXC peptide (53).

Oxidized CXC sequences are rare in nature. The few known examples include CSC in Mengo encephalomyelitis virus coat protein (59), CDC in *Bacillus* Ak.1 protease (60), and CTC in the *E. coli* chaperone Hsp33 (61). The last protein is particularly interesting because its CXC motif is thought to act as a redox switch for chaperone function. Recently, a CXC motif was also found in the thiol oxidase Erv2p, a yeast flavoprotein (62). The mechanism of this enzyme is thought to involve a disulfide relay, with a CGC sequence near the C terminus shuttling electrons from substrate proteins to a CGEC sequence adjacent to the flavin. Apparently, the enzyme takes advantage of a relatively strained CXC disulfide to effect efficient oxidation.

The CXC motif is common in cysteine-rich peptides such as endothelins, sarafotoxins, and bee and scorpion venom toxins. The bioactive forms of these peptides do not, however, contain an oxidized CXC motif. Indeed, the CXC sequence could be a means of decreasing the number of

² The fluorescence spectra of the reduced and oxidized forms of Δ P34 Trx were not distinguishable, as had been observed with a Trx variant containing a CAC active site (38). Accordingly, fluorescence spectroscopy could not be used to determine the value of $E^{\circ'}$ for Δ P34 Trx.

accessible disulfide-bonded isomers (63), as nonnative CXC disulfides are likely to be less stable than the native disulfides.

Attributes of the CGC Peptide. The tripeptide CGC has disulfide $E^{\circ} = -167$ mV (Table 2). This stability is close to that of the PDI active site ($E^{\circ} = -180$ mV (10)). In addition, the formation of a thiolate in reduced CGC is stabilized by Coulombic interactions. The CGC peptide has thiol $pK_a = 8.7$ (Table 2), which is 0.3 units less than that of glutathione (thiol $pK_a = 9.0$ (29)). This difference results in a 2-fold increase in thiolate concentration for the CGC peptide near neutral pH.

The CGC peptide is able to use its favorable disulfide E° and thiol pK_a to fold sRNase A. At the same total thiol concentration, the protein folding activity of reduced CGC is superior to that of the dithiol BMC as well as the monothiol glutathione. The E° for CGC is approximately 73 mV greater than that for BMC ($E^{\circ} = -240$ mV (29)). The relative instability of its disulfide bond is likely to underlie the superior activity of CGC. BMC is more likely to form a cyclic disulfide, releasing a partially reduced substrate. Protein folding would then require the reoxidation of this reduced substrate by oxidized BMC. CGC could rely less on the reduction–reoxidation pathway because of its more favorable E° . Also, oxidized CGC is a better oxidant of reduced substrates than is oxidized BMC. The higher activity of CGC relative to BMC, despite its higher thiol pK_a , underscores the importance of disulfide E° for catalysis of disulfide isomerization.

Attributes of Δ P34 Trx. The disulfide isomerization activity of the CGC peptide is unlikely to benefit from noncovalent interactions. This peptide is a minimal unit for catalysis of disulfide isomerization and likely uses only covalent catalysis. In contrast, oxidoreductases can take advantage of both covalent and noncovalent interactions in the catalysis of disulfide isomerization.

Insertion of a CGC module into a stable protein scaffold enhances its disulfide isomerization activity. Trx has an intrinsic affinity for proteins (13, 33). Although Trx acts as a cellular reductant, it can also catalyze native disulfide formation using its CGPC active site (47–49). Yet, its catalytic efficiency is low because of the stabilization imparted to its active site disulfide by the protein scaffold, and efficient folding requires a large excess of Trx. Deletion of the active site proline residue gives an active site sequence of CGC, which has a destabilized disulfide and much improved disulfide isomerization activity (Figure 2B; Table 2).

Despite its higher activity, Δ P34 Trx is still a less active catalyst than is PDI. Using literature data (64), the activity of PDI can be estimated to be ca. 50 000 U/mol with a sRNase A substrate under conditions similar to those used herein. This value is 15-fold higher than the activity of Δ P34 Trx (Figure 2B; Table 2). This difference in activity could arise from the quaternary structure of PDI (65). The two isolated catalytic domains of PDI, each of which is homologous to Trx, have 7–12-fold lower activity with a sRNase A substrate than does full length PDI, even though their E° values remain unchanged (66). Thus, Δ P34 Trx appears to possess a level of disulfide isomerization activity that is similar to that of the isolated catalytic domains of PDI.

The CGPC disulfide is intrinsically more stable than the CGC disulfide. Still, the Trx scaffold adds even more to the stability of the CGPC disulfide than to the CGC disulfide (Tables 1 and 2). Indeed, there is no evidence that the Trx scaffold provides any additional stabilization to the CGC disulfide. In the context of the Trx active site, the CGC disulfide is ≥ 70 mV (≥ 3.2 kcal/mol) less stable than is the CGPC disulfide (Table 2).

Despite the large destabilization to its active site disulfide, Δ P34 Trx suffers only a modest, 3.9-fold decrease in activity as a substrate for TR. Similarly, a Trx variant with a CAC active site sequence showed a 2.1-fold decrease in k_{cat} as a TR substrate (38). In comparison, small molecule disulfides, such as L-cystine or oxidized lipoamide, are extremely poor TR substrates (67), indicating that the tertiary structure of Trx contributes $> 10^3$ -fold to its activity as a substrate for TR. Indeed, the structure of a crystalline TR–Trx complex shows an extensive protein–protein interface flanking the active site (68). Notably, Trp31 of Trx projects into a hydrophobic pocket in TR, and this interaction is likely to be an important determinant of substrate specificity. The high activity of Δ P34 Trx as a substrate for TR suggests that deletion of Pro34 does not have a large effect on the structure of Trx, particularly near its active site.

In wild-type Trx, the link between active site disulfide formation and protein stability is well-established (23). For example, the T_m of wild-type Trx decreases by 12 °C upon disulfide reduction (50). In contrast, the stability of the Δ P34 Trx increases by 4 °C upon disulfide reduction (Figure 2A), indicating that the CGC disulfide actually destabilizes the protein scaffold (and vice versa).

Reduced Δ P34 Trx has a much greater disulfide isomerization activity than does wild-type Trx. Indeed, fully reduced wild-type Trx showed no detectable activation of sRNase A (Figure 2B). Protein folding with wild-type Trx requires a large excess of the oxidized form (47), as wild-type Trx is more likely to cause substrate reduction rather than isomerization. The much greater activity of the Δ P34 variant highlights the importance of E° for catalysis of disulfide isomerization. Deletion of Pro34 creates a Trx active site with a more favorable E° value and thus endows Trx with a new and useful property. We know of no other enzyme in which the deletion of an active site residue has such a marked benefit.

Prospectus. Small molecule redox reagents can be useful for oxidative protein folding in vitro because they tend to be inexpensive and easily separable from the target protein. The CGC motif is a promising component of redox buffers for protein folding. Recently, a 19 residue peptide corresponding to the functional element of α A-crystallin was reported to have chaperone activity (69), suggesting that peptides can be endowed with affinity for unfolded proteins. The fusion of CGC to such a peptide could enhance further its protein folding activity.

In vivo, many proteins are synthesized and fold as precursor forms that are cleaved by a protease after folding (70). In vitro, the precursor of BPTI (pro-BPTI), which contains three disulfide bonds in its native state, folds approximately 3-fold faster and with a 2-fold higher yield of native protein than does mature BPTI (71). The prosequence of BPTI contains a single cysteine residue that is required for this rate enhancement. Furthermore, a single

cysteine residue tethered to the C terminus of mature BPTI through an artificial (Ser-Gly-Gly)₃ linker provides rate and yield enhancements similar to those seen with pro-BPTI. Although the prosequence is not necessarily important for the physiological folding of BPTI (72), these results suggest a general strategy for more efficient protein folding. Specifically, a CGC sequence could be attached to the terminus of a protein via a linker containing a protease recognition site. Such a proprotein should fold more quickly and in higher yield. Cleavage of the prosequence would yield native protein. Such intramolecular catalysis by a CGC motif could provide a new and useful tool for efficient protein production.

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