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The C*XX*C Motif Is More than a Redox Rheostat*[S]

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The C*XX*C active-site motif of thiol-disulfide oxidoreductases is thought to act as a redox rheostat, the sequence of which determines its reduction potential and functional properties. We tested this idea by selecting for mutants of the C*XX*C motif in a reducing oxidoreductase (thioredoxin) that complement null mutants of a very oxidizing oxidoreductase, DsbA. We found that altering the C*XX*C motif affected not only the reduction potential of the protein, but also its ability to function as a disulfide isomerase and also impacted its interaction with folding protein substrates and reoxidants. It is surprising that nearly all of our thioredoxin mutants had increased activity in disulfide isomerization *in vitro* and *in vivo*. Our results indicate that the C*XX*C motif has the remarkable ability to confer a large number of very specific properties on thioredoxin-related proteins.

Thiol-disulfide oxidoreductases are found in all living organisms. They catalyze the oxidation of protein thiols and the reduction and isomerization of disulfide bonds in proteins. They are thus critical for protein-folding reactions. Thiol-disulfide oxidoreductases present in the periplasmic space act as oxidases or isomerases, whereas those in the cytoplasm perform mainly reductive steps. The C*XX*C active site of these thioredoxin-related proteins is essential for their activity (1–3). The sequence of the *XX* dipeptide located between the cysteines in the active-site motif is very important in controlling the redox properties of the protein in which it is found (4, 5), so much so that it has been termed a redox rheostat (6). Several groups have shown that it is possible to alter the redox properties of these oxidoreductases by mutating the *XX* dipeptide in the C*XX*C motif (7–9). When changing the C*XX*C motif of one oxidoreductase to the sequence of another, the function of the original enzyme may be substantially altered. In some cases, the modified protein is even able to perform some of the activities of the protein from which the *XX* sequence was derived (7–9). The effect these mutations have on the protein's activity has been generally attributed to the effect they have on the redox potential of the protein (7, 9, 10). We decided to test this hypothesis by selecting for mutants of the C*XX*C motif in the reducing protein thioredoxin that allow it to complement the very oxidizing protein DsbA.

The formation of disulfide bonds in *Escherichia coli* requires DsbA (11). DsbA oxidizes proteins secreted into the periplasm by rapidly exchanging its disulfide with reduced pairs of cysteines present in substrate proteins (12). DsbA is kept in the oxidized state by the membrane protein DsbB (13). DsbA has the highest redox potential known ($E'_0 = -121$ mV) among members of the thioredoxin-related thiol-disulfide oxidoreductases. This is thought to be important for the its ability to rapidly oxidize substrate proteins and to keep the periplasm in an oxidized state (14). This high redox potential is understood in terms of the electrostatic forces in the vicinity of the disulfide CPHC active-site motif (14, 15). Although the forces behind the redox potential of DsbA have been well studied, the *in vivo* importance of this extremely high redox potential is not entirely clear. DsbA variants carrying mutations of the dipeptide in the C*XX*C active site can still function like wild-type DsbA in supporting normal cellular processes despite their less oxidizing redox potentials (14, 16). In addition, no clear relationship exists between the redox potential of DsbA mutants and their ability to complement DsbA-null strains (14).

When mutated, other members of the thioredoxin superfamily are capable of at least partially complementing *dsbA* function when secreted into the periplasm. Jonda *et al.* (17) tested four thioredoxin C*XX*C variants (harboring the *XX* dipeptides from the active sites of DsbA, protein-disulfide isomerase, glutaredoxin, and thioredoxin reductase) for their ability to restore motility to a *dsbA*$^-$ strain. They found that the DsbA-, protein-disulfide isomerase-, and glutaredoxin-type sequence variants could partially complement *dsbA*$^-$. Whereas thioredoxin is found nearly universally (18), DsbA is restricted mainly to the γ- and β-subgroups of proteobacteria, a clade that arose ~2.2 billion years ago (19), suggesting that thioredoxin and DsbA may have diverged at about this time. DsbA and thioredoxin share overall only 10% sequence identity (20). Thus, it is surprising that relatively minor alterations in the thioredoxin sequence can allow it to partially complement the very distantly related protein DsbA.

The redox potential of thioredoxin is 160 mV lower than that of DsbA (E'_0 of thioredoxin $= -270$ mV) (7), and this is thought to contribute to its ability to keep the cytoplasmic protein cysteines reduced (21). Previously, the ability of thioredoxin mutants to rescue *dsbA*$^-$ mutants was attributed mainly to their increased redox potentials, which bring them closer to the extremely oxidizing redox potential of DsbA (5). However, it should be noted that DsbC, the *in vivo* function of which is an

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5.

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TABLE 1
Strains and plasmids used

Strain or plasmid	Genotype or relevant characteristics	Source
Strain		
ER1821	F$^-$ *glnV44 e14*$^-$ (*McrA*$^-$) *rfbD1 relA1 endA1 spoT1 thi-1* Δ(*mcrC-mrr*)*114*::*IS10*	New England Biolabs
JP120	ER 1821 *dsbA*::*kan1, zih12*::*Tn10*	Lab collection
IS13	JP120, pssTRX	This study
JP373	ER1821, pKK233-2	Lab collection
WP591	DHB4, Δ*trxA*	Ref. 21
BW25113 *dsbC*::*kan*	*rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1 dsbC*::*kan*	Keio collection
Plasmid		
pssTRX	pASK40-ss-trxA, thioredoxin exported via DsbA signal sequence	Ref. 17
pKK233-2	Cloning vector, pBR322 origin, Amp^R	GE Healthcare

isomerase, is nearly as oxidizing as DsbA. The redox potential of DsbC is −129 mV (22), whereas that of DsbA is −121 mV (12); yet, in its function as an isomerase, DsbC reduces incorrect disulfide bonds, whereas DsbA oxidizes disulfide bonds. Thus, there are clearly more properties affecting the function of disulfide oxidoreductases than their redox potentials.

Previous studies on the role of the C*XX*C motif in the functional properties of thioredoxin-like proteins have typically limited the approach to changing thioredoxin family members' active-site sequences so that they precisely match those of other family members and then determining the protein's properties. We decided to take a much more systematic approach of randomly mutating the C*XX*C motif and then selecting active mutants to determine which features of the C*XX*C motif are involved in allowing thioredoxin to complement DsbA. With this approach, we hoped to gain insight into the key features of this widely conserved motif. We also hoped to further understand what makes DsbA such an effective disulfide catalyst and to explore the functional similarities between thioredoxin and DsbA. The powerful selections available for DsbA activity and the wealth of information on thioredoxin provide an ideal situation to systematically investigate the relationships between the active-site sequence, the redox potential, and the *in vivo* function of DsbA as an oxidase.

In a process similar to natural selection, we made random alterations in the C*XX*C motif of *E. coli* thioredoxin and then applied selective pressure on cells, demanding a *dsbA*$^+$ phenotype. We found that the sequence of the C*XX*C motif has the remarkable ability to control not just the redox potential of thioredoxin-related proteins, but also their ability to isomerize disulfides and to interact with their reoxidants and folding proteins. It is surprising that many of our complementing thioredoxin mutants were even more efficient than wild-type DsbA in disulfide isomerization.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—The strains and plasmids used in this study are listed in Table 1.

Alignment of DsbA Sequences—To identify homologs of DsbA in different bacteria, the NCBI non-redundant data base was searched with the *E. coli* K12 DsbA protein sequence (GenBank^{TM} accession number CAA56736) using the PSI-BLAST algorithm with a threshold of 0.005. We used four rounds of iterations. DsbA family members can be distinguished from other thioredoxin family members because they contain an α-helical structural motif absent in other thioredoxin-related proteins such as DsbC, DsbD, DsbG, and thioredoxin (23). All sequences were checked to verify that they contain sequences homologous to this α-helical domain. When sequences were available from multiple strains, one set of sequence data were chosen to represent that species. For paralogs existing in the same organism, we chose the sequence most homologous to the *E. coli* DsbA query sequence based on BLAST E values. In this way, nearly all of the sequences chosen are likely to be sequences of DsbA orthologs. Final alignment of the active-site dipeptide sequences of DsbA homologs was done based on the sequences from 101 genera and 181 species.

Construction of a Thioredoxin Mutant Library—The thioredoxin plasmid pssTRX with the DsbA signal sequence for periplasmic expression was a gift from Dr. R. Glockshuber. It was constructed by replacing the DsbA sequence in plasmid pDsbA3 with the thioredoxin sequence at NheI and BamHI restriction sites (17). This plasmid was randomly mutated with the QuikChange multisite-directed mutagenesis kit (Stratagene) to obtain mutations of the dipeptide in the C*XX*C motif of thioredoxin. The primer used to construct the mutant collection has six degenerate nucleotides for the dipeptide (5′-TCGATTTCTGGGCAGAGTGGTGCNNNNNNTGCAAA-ATGATCGCCCCGATT-′3). A thermal cycling reaction was performed as directed, and the product was digested with DpnI and precipitated using the DNA Pellet Paint Co-Precipitant (Novagen). The precipitated DNA was electroporated into 50 μl of *E. coli* JP120 electrocompetent cells. Transformants were plated on LB plates supplemented with 200 μg/ml ampicillin and incubated at 37 °C for 14 h. Colonies obtained after electroporation were replicated using sterile filter papers (Whatman) onto LB plates supplemented with 15 μM $CdCl_2$ and 200 μg/ml ampicillin at 37 °C for 18 h. Strains were classified by their resistance to cadmium. The plasmids that conferred cadmium resistance in *dsbA*$^-$ strains were isolated via a miniprep kit (Promega Corp.), and the *trxA* genes encoded by those plasmids were sequenced.

Spot Titers for Cadmium Resistance and Copper Resistance—Spot titers were measured to quantify the relative cadmium or copper resistance caused by the dipeptide change at the active site. Briefly, mid-log phase cells ($A_{600} \approx 1$) were serially diluted in sterile 170 mM NaCl solution. 2 μl of each dilution was plated onto LB plates supplemented with 200 μg/ml ampicillin and $CdCl_2$ at 7.5, 10, or 15 μM or onto brain heart infusion plates containing 5 mM $CuCl_2$. After 18 h at 37 °C, the growth of each thioredoxin mutant was compared, and the cadmium or copper

resistance of each of the thioredoxin mutants was ranked 1–7, with higher values indicating increased resistance.

Motility Assay—The motility assay was performed in M9 minimal soft agar plates (0.2%) supplemented with 18 amino acids (excluding cysteine and methionine), 0.4% glycerol, 2 μg/ml nicotinamide, 0.2 μg/ml riboflavin B_2, 2 μg/ml thiamine B_1, 2 μg/ml biotin, 1 mM $MgSO_4$, and the appropriate antibiotic. Mid-log phase liquid cultures were diluted based on the absorbance at 600 nm to normalized cell density for 1 *A* unit. 2 μl of the cells was then inoculated into the center of the motility plate. After 20 h of incubation at 37 °C, the diameter of the swarm was measured.

Protein Purification—Thioredoxin variants were expressed and purified in the periplasm of *trxA*$^-$ strain WP591 as described previously (5, 17). Protein concentrations were determined by absorbance at 280 nm using extinction coefficients of 13,980 cm^{-1} M^{-1} for reduced wild-type thioredoxin and 14105 cm^{-1} M^{-1} for oxidized wild-type thioredoxin. The extinction coefficients for thioredoxin mutants were calculated using the on-line program ProtParam (ca.expasy.org/tools/protparam.html) (24).

4-Acetoamido-4′-maleimidylstilbene 2,2′-Disulfonate Trapping—To examine the redox states of the thioredoxin mutants, acid-precipitated proteins were solubilized in buffered SDS solution containing 4-acetoamido-4′-maleimidylstilbene 2,2′-disulfonate as described previously (25). The samples were incubated in the dark at 37 °C for 1 h. Alkylation was stopped by the addition of nonreducing SDS loading buffer and analyzed by electrophoresis and Western blotting.

Stopped-flow Kinetics Study of the Interaction with DsbB—Stopped-flow absorbance measurements were performed on a Hi-Tech Scientific SF61 instrument (1.0-cm path length) in single-mixing mode. The typical reaction contained ~100 μM freshly reduced thioredoxin variants, 10 μM DsbB, and 200 μM ubiquinone-1 (coenzyme Q_1; Sigma). Thioredoxin variants and a DsbB/coenzyme Q_1 mixture were incubated in 50 mM sodium phosphate, 300 mM NaCl, and 0.04% dodecyl maltoside (pH 8.0) at 10 °C before mixing. The absorbance after mixing was recorded at 510 nm. One data set contained three to four successive shots, and data for each trace were simulated separately using Program A (developed by Chung-Yen Chiu, Rong Chang, and Joel Dinverno under the direction of David P. Ballou, University of Michigan) (26) based on the Marquardt-Levenberg nonlinear fit algorithm. The resulting data were further fit by SigmaPlot using the Michaelis-Menten equation to give the K_m and V_{max} values.

Redox Potential Measurement—Proteins with redox potentials that were to be measured were incubated with the γ-domain of DsbD, a protein with a known redox potential of −235 mV (27). At equilibrium, different protein species were separated by reverse-phase HPLC,[2] and the equilibrium constant (K_{eq}) of the reaction and the standard redox potential (E'_0) of the protein were determined as described (28). For the mutants with reduced and oxidized peaks that could not be fully separated from either the reduced or oxidized peak of the γ-domain of DsbD, the equilibrium constants with glutathione were measured as described (5). A value of −240 mV was used for the standard redox potential of glutathione at pH 7.0 to calculate the standard redox potentials of the thioredoxin variants (29).

Oxidative Folding of Hirudin—24 μM reduced hirudin and 200 μM oxidized glutathione were incubated in 100 mM sodium phosphate containing 1 mM EDTA (pH 7.0) at 25 °C. Oxidized thioredoxin variants were added at a catalytic concentration of 1 μM to initiate each folding reaction. Aliquots of 120 μl were removed after different reaction times and quenched with 15 μl of formic acid and 15 μl of acetonitrile. Hirudin folding intermediates were separated by reverse-phase HPLC on a C_{18} column at 55 °C in a 19–25% acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. The absorbance was recorded at 220 nm.

Stopped-flow fluorescence measurements were performed on a KinTek SF-2004 instrument in single-mixing mode. The typical reaction contained 0.5 μM oxidized protein (DsbA, thioredoxin, or its variants) and 2.5, 5, 7.5, or 10 μM reduced hirudin. The oxidized proteins and reduced hirudin were incubated in 100 mM sodium phosphate containing 1 mM EDTA (pH 7.0) at 25 °C before mixing. An excitation wavelength of 295 nm and a band-pass filter were used to monitor the fluorescence change of the proteins. Each stopped-flow trace was fit to a single exponential according to the following equation: $F = F_o + \Delta F(1 - e^{-kobs \cdot t})$, where F is the fluorescence emission at known times, F_o is the fluorescence emission of completely oxidized protein, and ΔF is the difference between the fluorescence emissions of completely reduced and oxidized proteins. k_{obs} is the peudo first-order rate constant. Different k_{obs} values were plotted against hirudin concentrations. The slope is the observed second-order rate constant of the reaction.

Isomerization of Scrambled Hirudin—21 μM scrambled hirudin and 21 μM freshly reduced protein (DsbC, DsbA, thioredoxin, or its variants) were incubated in isomerization buffer (20 mM sodium phosphate, 130 mM sodium chloride, and 0.13% polyethylene glycol 8000) at 25 °C. Aliquots of 120 μl were removed after different reaction times and quenched with 15 μl of formic acid and 15 μl of acetonitrile. Hirudin folding intermediates were separated by the same gradient as used in the oxidative folding assay.

RESULTS

Construction of a Thioredoxin Mutant Library—We decided to systematically explore the role played by the central *X* residues in the C*XX*C active-site motif of thiol-disulfide oxidoreductases. To do this, we randomly mutated this motif in the reducing protein thioredoxin and investigated which mutants complement a null mutant in the oxidizing protein DsbA. The plasmid pssTRX contains the coding sequences of thioredoxin fused to the export signal of DsbA (17). This plasmid was randomly mutated in the C*XX*C active site using multisite-directed mutagenesis. The resulting plasmid library was transformed into the *dsbA*$^-$ strain JP120. About 10,000 transformants were obtained on LB plates containing 200 μg/ml ampicillin. To judge the mutation frequency, 96 colonies were randomly chosen, and plasmid DNA was prepared and sequenced. The sequencing results showed that 40% of the clones contained mutated C*XX*C sequences with an almost

[2] The abbreviation used is: HPLC, high pressure liquid chromatography.

equal distribution of the four nucleotides A (36%), T (20%), G (24%), and C (20%) in the mutated sites, indicating that the mutagenesis was nearly random. Our aim in this mutagenesis was to obtain the vast majority of the 400 possible dipeptide combinations in the C*XX*C active site. Our library size of ~4000 and the almost equal distribution of nucleotides in the mutants make it likely that we achieved our aim.

Identification of Thioredoxin Mutants That Complement dsbA—All of the transformants generated from the random C*XX*C mutagenesis were replica-plated onto plates containing 15 μM $CdCl_2$ to select for a *dsbA*$^+$ phenotype. Cadmium is a toxic divalent metal ion (30). The high affinity of cadmium for protein thiol groups is believed to be the major biochemical basis of cadmium toxicity (31, 32). In bacteria, *dsbA*$^-$ strains contain a much higher content of free thiol groups than *dsbA*$^+$ strains. Cd^{2+} binds to these free thiol groups and inhibits their proper folding (33, 34). DsbA-null strains are especially Cd^{2+}-sensitive, failing to grow when exposed to 4 μM Cd^{2+}. In contrast, wild-type strains are resistant to up to 400 μM Cd^{2+}. JP120 transformed with plasmid encoding wild-type thioredoxin showed only residual growth at 15 μM Cd^{2+}. This provides a powerful selection for the *dsbA*$^+$ phenotype. By selecting for cadmium resistance in a *dsbA*$^-$ strain, we expected to recover thioredoxin mutants that could at least partially substitute for DsbA. We obtained 231 colonies resistant to 15 μM Cd^{2+} from the 4000 mutant colonies screened. Sequencing of the plasmids contained in these strains revealed 37 different combinations of amino acids present in the C*XX*C motif. We verified that the mutations present on the plasmids were sufficient for complementation of cadmium resistance by retransforming the mutant plasmid DNA into the *dsbA*$^-$ strain JP120. We classified the cadmium resistance of the various mutants by plating different dilutions of mid-log phase cultures onto LB plates supplemented with ampicillin and various $CdCl_2$ concentrations (Fig. 1) (data not shown). The mutants were rank-ordered from highest cadmium resistance to lowest. To verify that the cadmium resistance of these thioredoxin mutants reflects their ability to oxidize proteins, we tested cadmium-resistant isolates for their ability to restore motility, a phenotype characteristic of *dsb*$^+$ strains. To be motile, *E. coli* must properly assemble its bacterial flagella, and to do this, a critical disulfide in the flagellar component FlgI must be introduced. Thus, Δ*dsb* strains are non-motile (35). A good correlation exists ($R = 0.95$) between the extent of motility and the degree of cadmium resistance (supplemental Fig. 1). These results indicate that the thioredoxin mutants we obtained are capable of restoring at least two of the phenotypes disrupted in *dsbA*$^-$ strains.

Analysis of the Dipeptide Sequences in the CXXC Motif of Thioredoxin Mutants—We then analyzed the dipeptide sequences in the C*XX*C motif of the plasmids that confer a *dsbA*$^+$ phenotype (Fig. 2). We reasoned that the most cadmium-resistant mutants were likely to survive the selection most often. We found a reasonable correlation ($R = 0.7$) between the degree of cadmium resistance and the frequency at which mutants were obtained (supplemental Fig. 2). It is interesting that, for the more N-terminal position, the most frequently discovered amino acid is proline (65%), which is found ~16-fold

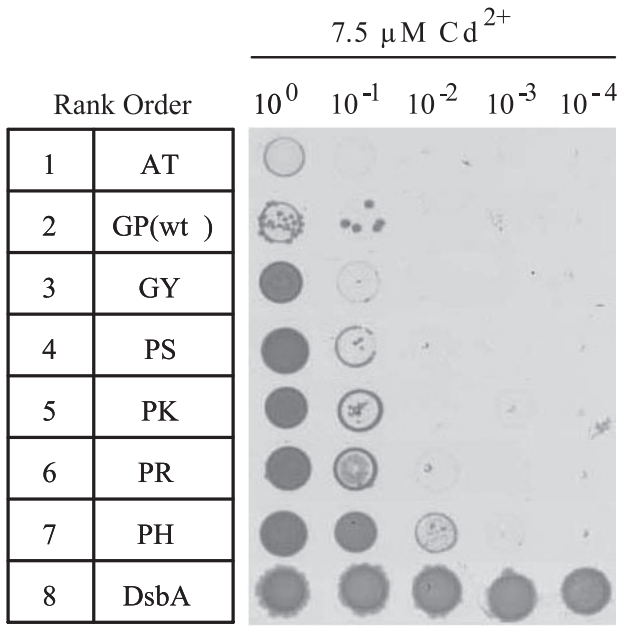

FIGURE 1. **Thioredoxin active-site mutants show various resistances to cadmium.** Cadmium resistance for each thioredoxin variant was ranked 1–7, from weak to strong, based on growth on LB plates containing ampicillin and 7.5, 10, and 15 μM Cd^{2+}. Only the 7.5 μM Cd^{2+} plate is shown. DsbA showed the highest cadmium resistance and ranked 8. Variants are identified by their *XX* dipeptide sequence within the C*XX*C active-site motif. AT is a DsbA-non-complementing thioredoxin mutant. *wt*, wild-type.

more frequently than the 4% frequency expected by chance (Fig. 2). It is worth noting that proline is found in the corresponding position of *E. coli* DsbA and in many DsbA homologs (76%) (Table 2). Thus, there seems to be a very strong bias toward proline at the N-terminal position. For the more C-terminal position in the C*XX*C motif, we obtained basic (His, Lys, and Arg) or aromatic (Tyr and Phe) amino acids most frequently (51 and 32%, respectively). His, Tyr, and Phe are found 9-, 9-, and 4-fold more frequently, respectively, than expected by chance. The expected frequency for arginine is high because it is specified by six codons; however, the observed frequency is still 1.7-fold higher than expected by chance (Fig. 2). These observations clearly indicate the preference for basic or aromatic amino acids at the more C-terminal position and are consistent with the frequencies observed in naturally occurring DsbA homologs (79% for basic and 13% for aromatic amino acids at the more C-terminal position) (Table 2). Note that all of these expected frequencies are what one calculates for the N- and C-terminal positions independently. The observed frequencies for the dipeptide sequences of the complementing mutants are on average 18-fold higher than the dipeptide frequencies expected by chance (supplemental Fig. 2).

The mutant that most frequently survived the cadmium selection has the CPHC motif, observed in 58 of 231 clones (25%), which is exactly the same C*XX*C sequence most frequently found in DsbA homologs in evolution. The expected frequency of this dipeptide in unselected clones is 218-fold lower. The short CPHC motif, which was optimized by evolution to work in DsbA, also seems to be optimized in conferring DsbA-like properties on thioredoxin, a protein that shares only 10% sequence identity with DsbA and is separated from DsbA

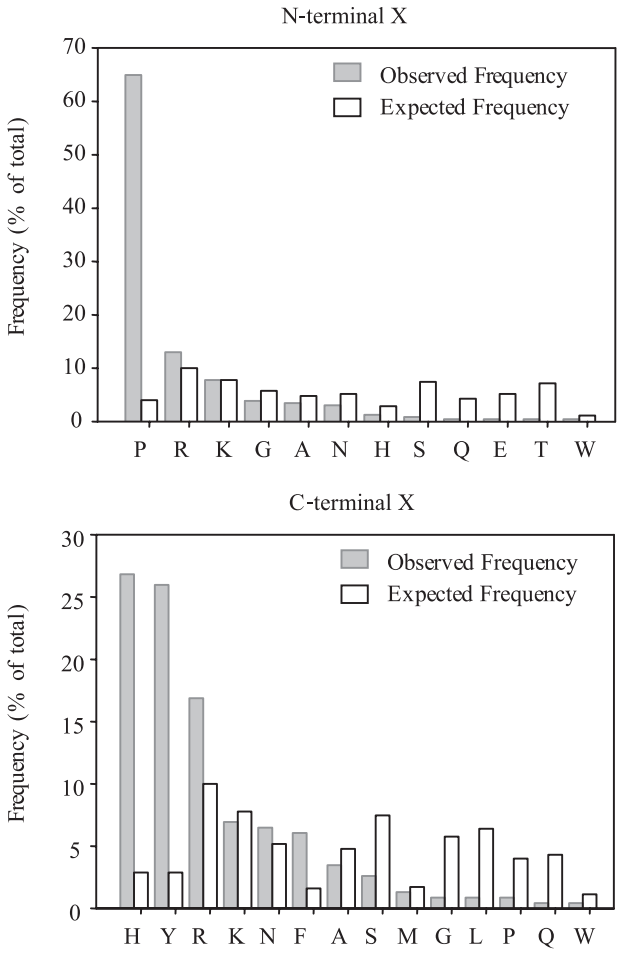

FIGURE 2. **DsbA-complementing thioredoxin mutant *CXXC* active-site motif with the sequence Cys-Pro-basic (or aromatic) residue-Cys.** 12 amino acids were obtained at the more N-terminal position (*upper panel*), and 14 were obtained at the more C-terminal position (*lower panel*). Frequencies are given as a percent of the total. The expected frequency of each amino acid was calculated by adding together the frequencies of each of the natural codons of that amino acid. These codon frequencies were calculated by multiplying the abundance of each single nucleotide (A, 36%; T, 20%; G, 24%; and C, 20%) that was observed in unselected mutant sequences.

TABLE 2

Analysis of dipeptide sequences in the *CXXC* motif of DsbA and thioredoxin mutants

The frequencies of occurrence for each of the amino acids at the N- and C-terminal positions in the *CXXC* motif of DsbA homologs from 181 species are compared with the frequencies for our laboratory-evolved thioredoxin mutants showing resistance to 15 μM cadmium. The frequency for each amino acid is presented as a percentage, with the amino acids listed in the order with which they were found in the laboratory-evolved thioredoxin mutants.

	Nature-evolved DsbA homologs	Lab-evolved thioredoxin mutants
	%	
Amino acid at N-terminal position in *CXXC*		
Pro	76	65
Arg		13
Lys		8
Gly	5	4
Ala	0.5	3
Asn		3
Ser	6	1
His	1	1
Gln	1	0.4
Glu	1	0.4
Thr	1	0.4
Trp		0.4
Ile	5	
Val	3	
Leu	1	
Amino acid at C-terminal position in *CXXC*		
His	79	27
Tyr	8	26
Arg		17
Lys		7
Phe	5	6
Asn	3	6
Ala	4	3
Ser		3
Pro	0.5	1
Met		1
Gly		1
Leu		1
Trp	0.5	0.4
Gln		0.4

by ~2 billion years of evolution (23). We find the apparent portability of this CPHC motif remarkable.

Kinetics of Oxidation of Thioredoxin Mutants by DsbB in Vitro—One trivial reason for our mutants' increased ability to complement the *dsbA*$^+$ phenotype might be an increased expression level or an increased fraction of the oxidized form. This was not the case, as quantitative Western blots showed that the expression levels of the different thioredoxin mutants were almost identical to each other and to the level of expression observed for the wild type (0.53–1.1-fold of the wild type). 4-Acetoamido-4′-maleimidylstilbene 2,2′-disulfonate trapping showed that all of the thioredoxin mutants were in the oxidized state.

Our mutants are DsbB-dependent *in vivo* as judged by their inability to allow bacterial motility in a *dsbB*$^-$ strain (data not shown), suggesting that they are functioning by replacing DsbA in the periplasm. It is possible that our thioredoxin mutants are active in oxidizing proteins because they can be effectively oxidized by DsbB. To test this idea, we evaluated the oxidation efficiency of different thioredoxin mutants by DsbB using the enzyme-monitored turnover method described by Gibson *et al.* (36). This method utilizes the time course of the decay of an enzyme intermediate upon reaction with an excess of substrate to determine K_{cat} and apparent K_m values. A purple charge transfer complex intermediate characterized by a strong absorbance peak at 510 nm is formed during the oxidation of DsbA by DsbB (37, 38). Our experiments revealed that wild-type thioredoxin and its *CXXC* variants also induced an absorbance peak at 510 nm, which almost certainly corresponds to a very similar intermediate as is seen between DsbB and DsbA. We monitored the decay of absorbance at 510 nm and used these data to derive V_{max} and K_m values for both wild-type DsbA and wild-type thioredoxin and a large number of our thioredoxin mutants (supplemental Fig. 3 and Table 3). A K_m of 14 ± 0.7 μM and a V_{max} of 0.75 ± 0.01 s^{-1} were obtained for the oxidation of wild-type thioredoxin by DsbB, similar to the previously published K_m of 20 ± 7 μM (17). It is surprising that these values are very similar to those of DsbA for DsbB (8 ± 1 μM). This implies that wild-type thioredoxin interacts with DsbB almost as efficiently as does DsbA. This reduced the likelihood that our mutants can achieve their increased activity by increasing their catalytic efficiency with DsbB. Indeed, within the group of active thioredoxin mutants, the correlation between *in vivo* motility values and their catalytic efficiency

TABLE 3

Parameters for DsbA and the thioredoxin variants

The results are shown for DsbA, wild-type thioredoxin, and 19 thioredoxin active-site variants studied by phenotypical and biochemical assays. PH to AY are the DsbA-complementing thioredoxin mutants. A value of 0 for observed frequency indicates that the mutants were non-complementing. In the pool of DsbA-non-complementing mutants, AT and TA were not measurably oxidized by DsbB, so the K_m and V_{max} values could not be determined. ND, not determined; wt, wild-type.

CXXC variant	Observed frequency	Motility (% of *dsbA*$^+$ strain)	Cadmium resistance (rank order)	Copper resistance (rank order)	E'_0	K_m	V_{max}	V_{max}/K_m	k_2	Native hirudin at 2 h
	%				*mV*	μM	s^{-1}	$s^{-1}\,\mu M^{-1}$	$M^{-1}\,s^{-1}$	%
DsbA		100	8		−121	8	0.31	0.041	2.0×10^6	3.8
DsbA-complementing mutants										
PH	25	45	7	4	−210	22	1.03	0.049	7.6×10^5	50.5
PY	5.6	39	7	6	−197	7	0.09	0.014	ND	40.0
PF	0.4	32	6	6	−197	4	0.07	0.016	ND	49.3
PR	14	27	6	3	−203	121	2.19	0.018	2.4×10^5	12.1
PK	6	24	5	3	−207	121	2.09	0.018	ND	15.0
PS	2.6	21	4	6	−219	11	0.23	0.022	2.8×10^5	18.4
RY	7.4	21	5	7	−215	5	0.21	0.036	ND	98.3
PM	1.3	18	4	3	−216	21	0.41	0.020	ND	9.5
GY	0.4	18	3	6	−220	4	0.15	0.035	ND	57.0
GW	0.4	18	3	6	−220	7	0.21	0.033	ND	40.0
WR	0.4	17	3	3	−215	105	4.80	0.041	ND	10.7
KY	4.8	16	5	7	−214	10	0.51	0.051	1.6×10^6	79.1
NY	1.3	14	3	6	−214	6	0.21	0.033	ND	53.2
AY	3	14	3	7	−219	6	0.22	0.039	ND	47.0
Non-complementing mutants										
RS	0	7	2	3	−225	43	0.92	0.022	ND	18.4
TA	0	6	1	1	−236				ND	1.0
GP(wt)	0	5	2	6	−270	14	0.74	0.048	9.0×10^4	6.7
AT	0	5	1	3	−224				3.4×10^4	1.7
GG	0	5	1	3	−234	68	1.07	0.016	ND	6.1
HE	0	3	1	6	−229	28	0.35	0.013	ND	5.4

with DsbB is very poor. For simplicity, we refer to our thioredoxin mutants using only the dipeptide sequence of the active site; thus, the mutant with the CPHC sequence is referred to as "PH." We do note, however, that the PH mutant, which has the best motility, does have a much better catalytic efficiency, *i.e.* a higher V_{max}/K_m value than almost all of the other mutants.

Notably, we tested the interaction of DsbB and five non-complementing control mutants (RS, TA, AT, GG, and HE). No *in vitro* interaction could be detected between DsbB and the AT or TA mutant. Thus, the inactivity of 40% of our mutants might be connected to their inability to interact with DsbB (Table 3). In contrast, all of the motile mutants tested were able to interact with DsbB to some degree. Another trend we observed is that all the mutants with aromatic amino acids at the more C-terminal position had lower K_m values (Table 3), whereas some of the mutants with a basic amino acid in this position had large K_m values. This observation may indicate that the aromatic amino acid-containing mutants have a stronger tendency to form a substrate-enzyme complex with DsbB. It is intriguing that aromatic residues are also common in nature; they are found in 13% of DsbA homologs and are present in almost 100% of DsbC sequences. DsbC is the principal disulfide isomerase of *E. coli* (39). We concluded from these experiments that the ability to be reoxidized by DsbB is necessary but not sufficient for thioredoxin mutants to be able to complement DsbA.

Measuring the Redox Potentials of Thioredoxin Mutants—One of the most prevalent hypotheses in the disulfide catalyst field is that the redox potential determines the function of thioredoxin-related thiol-disulfide oxidoreductases (4, 7, 9). Thus, we determined the standard redox potentials of our thioredoxin mutants (Table 3). We found that the complementing mutants all showed an increased redox potential (average increase of 58 mV). On its own, this would help confirm that an increased redox potential is key in the ability of mutants to complement DsbA. However, in an important control experiment, we found that the five non-complementing control mutants also exhibited an increased redox potential. Although these were uniformly less oxidizing, the difference between the two groups was very small (Table 3). We observed a correlation between motility and redox potential for the complementing mutants (supplemental Fig. 4). The more oxidizing the mutant, the better it is at complementing. The PH mutant is the most DsbA-like phenotypically and, not coincidentally, has the most DsbA-like sequence. However, with a redox potential of −210 mV, it is not the most oxidizing of the complementing mutants, which range from −220 to −197 mV. Notably, it is only slightly more oxidizing than the non-complementing mutants, the redox potentials of which range from −236 to −224 mV, or the poorly complementing mutants such as AY (−219 mV) and NY (−214 mV). Thus, although a more oxidizing redox potential may be a prerequisite for allowing thioredoxin to functionally replace DsbA, it is clearly not the complete story.

Thioredoxin Mutants Have Enhanced Protein Oxidation and Isomerase Activity in Vitro and in Vivo—To obtain a more direct measure of the *in vivo* function of our thioredoxin mutants, we decided to examine how efficiently our thioredoxin mutants could oxidize substrate proteins *in vitro*. We chose hirudin as a model substrate; hirudin is a 65-residue protein with three disulfide bonds in the native state and is a well established model substrate for DsbA (40, 41). This protein has a well studied disulfide-linked *in vitro* folding pathway and is commercially available, and the folding intermediates can be easily separated by HPLC. This is in contrast to the few known *E. coli* substrates for DsbC (such as RNase I), which have none of these properties. To determine the rate at which our mutants transferred their disulfide to hirudin, we followed their increase

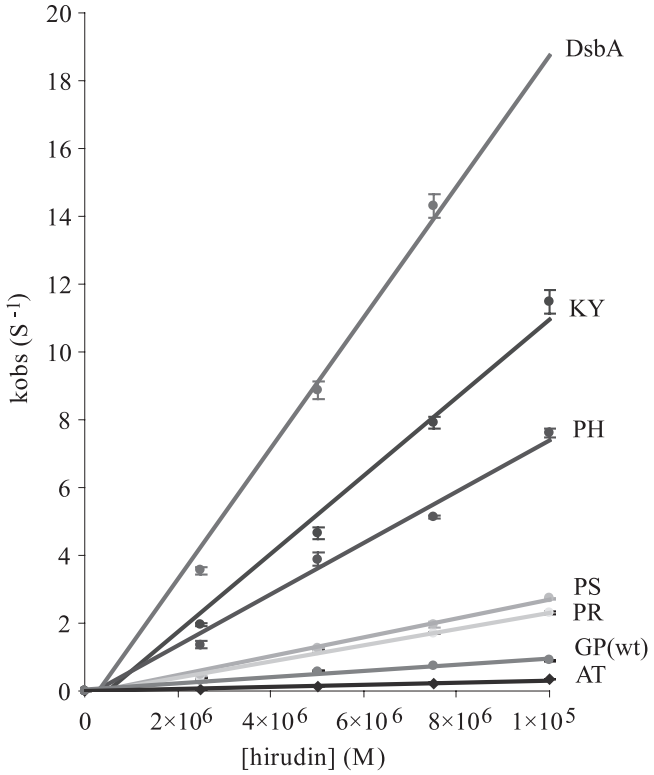

FIGURE 3. **Kinetics of the oxidation of reduced hirudin by DsbA, thioredoxin, and its variants.** The second-order rate constants (k_2; M^{-1} s^{-1}) for the interaction of reduced hirudin and DsbA, thioredoxin, or its mutants were obtained by fitting the observed pseudo first-order rate constants (k_{obs}; s^{-1}) against hirudin concentrations (M). The slopes are the apparent second-order rate constants for the reaction, and the values are as follows: DsbA, 2.0×10^6 M^{-1} s^{-1}; KY mutant, 1.0×10^6 M^{-1} s^{-1}; PH mutant, 7.6×10^5 M^{-1} s^{-1}; PS mutant, 2.8×10^5 M^{-1} s^{-1}; PR mutant, 2.4×10^5 M^{-1} s^{-1}; thioredoxin (*GP(wt)*, where *wt* is wild-type), 9.0×10^4 M^{-1} s^{-1}; and AT mutant, 3.4×10^4 M^{-1} s^{-1}.

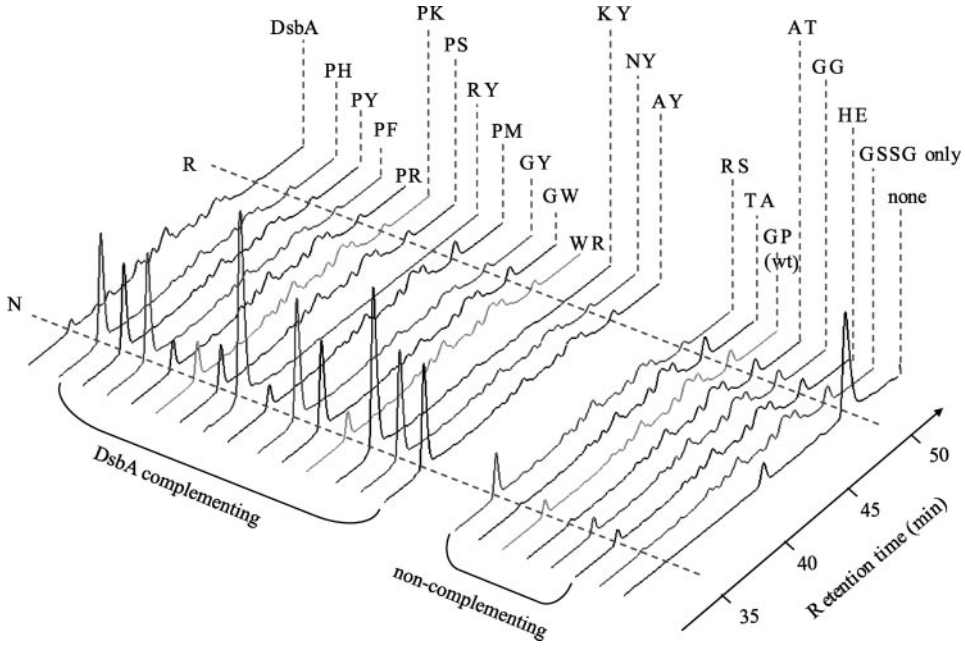

FIGURE 4. **DsbA-complementing thioredoxin mutants fold reduced hirudin efficiently.** 2 h after initiation, hirudin folding intermediates were separated by reverse-phase HPLC. The absorbance traces are aligned on the same time scale. Each trace is labeled by the catalyst added or by the name of the control. From left to right, the traces follow the order of decreased motility of the strains containing the corresponding thioredoxin variants or DsbA. The *dashed lines* indicate fully reduced (*R*) and native (*N*) hirudin.

in fluorescence that accompanied their incubation with reduced hirudin. A disulfide in the active site of thioredoxin acts to quench its tryptophan fluorescence (42). The kinetics of the reaction are shown in Fig. 3, with the second-order rate constants for the oxidation of reduced hirudin by DsbA, thioredoxin, and its variants provided in the figure legend. The complementing mutants were 3–11-fold faster at oxidizing hirudin compared with wild-type thioredoxin. A good correlation exists between these rate constants and their ability to restore motility *in vivo* (supplemental Fig. 5), strongly suggesting that an increased ability to oxidize proteins is crucial to their enhanced *in vivo* activity. One of the mutants (KY) was actually nearly as fast as the very rapid kinetics of DsbA. DsbA is known for its extremely fast disulfide exchange reaction rates, which are ~3 orders of magnitude above the known values for mono- and dithiols (12). DsbA is also much more reactive than thioredoxin; dithiothreitol reduces DsbA ~1000 times faster than it does thioredoxin, for instance (43). We find it noteworthy that some of our mutants approached the very rapid disulfide exchange kinetics of DsbA.

We then tested whether our mutants are more efficient than wild-type thioredoxin in the overall oxidative folding of hirudin, a process that involves both disulfide oxidation and isomerization steps. We were very pleased to find that all of the DsbA-complementing mutants exhibited an enhanced ability to oxidatively refold reduced hirudin. They not only were more efficient compared with wild-type thioredoxin, but (a real surprise) were also faster compared with wild-type DsbA, showing complete refolding of hirudin at time points where wild-type DsbA was just getting started (Fig. 4 and Table 3). We find this surprising because DsbA has presumably been optimized for the oxidative folding of proteins over millions of years of evolution, yet by changing just two residues in a related protein, we appear to have been able to isolate a mutant protein that is superior to DsbA in the oxidative folding of at least one protein.

The oxidative refolding of reduced hirudin involves two processes, an initial oxidation reaction, followed by isomerization of incorrect disulfides to generate native hirudin. Our thioredoxin mutants were better than DsbA in the overall oxidative folding of hirudin and not quite as good as DsbA in the initial oxidation step, leading us to predict that they had isomerization ability superior to that of DsbA.

Using scrambled hirudin as a substrate, we indeed found that our mutants could generate native hirudin from a disulfide-scrambled hirudin substrate faster than DsbA could (Fig. 5*A*). Two of those tested (KY and PH) were very efficient, at least as effective as DsbC, the principal disulfide isomerase of *E. coli* (Fig. 5*A*). This was surprising

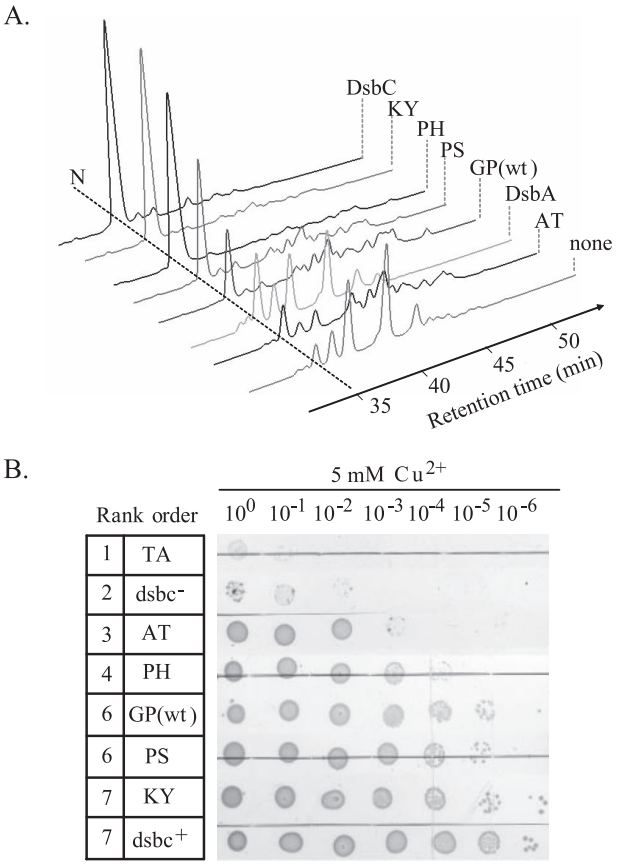

FIGURE 5. **Thioredoxin active-site variants function as isomerases *in vitro* and *in vivo*.** *A*, 5 min after initiation, hirudin folding intermediates were separated by reverse-phase HPLC. *N*, native hirudin. DsbA-complementing thioredoxin mutants folded scrambled hirudin more efficiently than did DsbA; some were even comparable with DsbC. The non-complementing AT mutant was worse than wild-type (*wt*) thioredoxin. *B*, thioredoxin mutants, as well as the wild type, conferred various copper resistances to a *dsbC*$^-$ strain. Copper resistance for each thioredoxin C*XX*C variant was ranked 1–7, from weak to strong. Variants are identified by their *XX* dipeptide sequence within the C*XX*C active-site motif.

because the phenotype we selected for is the ability to complement DsbA (an oxidase) and not DsbC (an isomerase). We note that wild-type thioredoxin did show some isomerase activity *in vivo* and *in vitro*, as has been reported previously (44, 45). We note that scrambled hirudin is a very heterogeneous substrate, consisting of at least eight different species that are separable by HPLC (Fig. 5*A*). The thioredoxin mutants are apparently capable of accelerating the isomerization of these different species, strongly implying that these mutants are active in the isomerization of a variety of substrates.

To determine whether our mutants also have an improved ability to isomerize protein disulfides *in vivo*, we tested if they could complement the copper sensitivity of null mutants in DsbC. Plasmids containing thioredoxin active-site variants were transformed to the *dsbC*-null strain BW25113 *dsbC*::*kan*. Copper can catalyze the formation of incorrect disulfides, including disulfide-linked oligomers, which DsbC appears to be able to resolve (39). Eight of 14 of the active mutants we tested were either as copper-resistant as *dsbC*$^+$ strains or nearly as copper-resistant. This was in contrast to the five inactive mutants, where all but one was no more copper-resistant than the empty vector (which had a copper resistance rank of 3) (Fig. 5*B* and Table 3) (data not shown). Our mutants seem to act by increasing their ability to both oxidize and isomerize disulfides in proteins.

The mutants that seemed to show the best isomerase activity *in vivo* and *in vitro* are those with an aromatic or histidine amino acid in the C-terminal position of the C*XX*C motif (Table 3). (The side chain of histidine can also be considered aromatic, as it meets the electron rule of aromatic amino acids in one of its protonation states.) Previous work on the *in vivo* folding of a multi-disulfide protein in *E. coli* by DsbC mutants with a randomized C*XX*C central dipeptide suggested a strong preference for hydrophobic and particularly aromatic amino acids at the C-terminal position (16). In this study, we found a higher average rate of hirudin refolding for mutants with aromatic amino acids in the active site than for those with basic amino acids. This result supports our hypothesis that the aromatic amino acid-containing active-site dipeptide mutants of thioredoxin may function as disulfide isomerases.

DISCUSSION

In a process similar to natural selection, we identified amino acid combinations of the dipeptide in the C*XX*C motif of thioredoxin mutants that complement the oxidizing thiol-disulfide oxidoreductase DsbA. We have shown that this C*XX*C motif is a major factor in determining the function of the protein in ways that go well beyond simple changes in the redox potential. Our results closely recapitulate evolution. A strong preference was found for proline at the more N-terminal position of the motif and a basic or aromatic amino acid at the more C-terminal position, very similar to what is observed for natural DsbA sequences. The C*XX*C sequence that best complemented DsbA deletion mutants was CPHC, exactly the same sequence that is most commonly found in natural DsbA proteins. This implies that the CPHC motif is optimal for conferring DsbA-like properties on either DsbA or thioredoxin, two proteins that share only 10% sequence identity.

The spectrum of the C*XX*C sequences found in our laboratory-evolved thioredoxins is slightly broader than that found in evolution. In natural DsbA proteins, the only positively charged residue observed is histidine, whereas in our laboratory-evolved DsbA-like thioredoxins, the preference for positively charged residues is slightly more relaxed (His, 27%; Arg, 17%; and Lys, 7%). In our laboratory-evolved mutants, a positively charged residue was observed in the more N-terminal position 20% of the time in the absence of a positively charged residue in the C-terminal position, possibly indicating that an N-terminal positively charged residue may substitute for a C-terminal one.

In DsbA, the positively charged His^{32} residue is known to act to form a salt bridge with the thiolate anion of Cys^{30} (46). This stabilizes the reduced form of DsbA and thus provides some of the thermodynamic driving force behind the oxidizing power of DsbA (14, 15). Our observation that thioredoxin mutants frequently possess a positively charged residue at the C- or N-terminal position is further evidence that this electrostatic interaction is important in determining the functional properties of the thioredoxin-related proteins. The aromatic amino acids we found may also act by stabilizing the N-terminal thiolate anion, in this case by a sulfur-aromatic ring interaction (47, 48). The

π-electron cloud on the faces of the aromatic ring is known to interact strongly with cations (49), so the ring edges are relatively positively charged and capable of interacting with negatively charged thiolate ions. This type of reaction is common in proteins containing thiol groups (50, 51).

Our results suggest that proline is strongly favored as the N-terminal amino acid in the C*XX*C motif. This was unexpected, as we know of no prior evidence implicating the importance of proline at this position. We reason that proline at the more N-terminal position may be important due to effects on the local conformation near the active site. The CPHC active-site motif for DsbA is located at the N terminus of the α1-helix, and proline tends to break helices. The positive dipole that occurs at the N-terminal end of the α1-helix is thought to contribute to the oxidizing power of DsbA (52, 53). The interaction between the N-terminal thiolate anion in the C*XX*C motif and the α-helix dipole appears to be very sensitive to the microenvironment at the helix terminus (54, 55). The precise positions of the proline may be important in breaking the helix and in generating the appropriate geometry for such an interaction.

The efficiency of selected thioredoxin mutants was studied *in vitro*; the interaction with their oxidant DsbB and the substrate protein hirudin and the redox potentials were also determined. All DsbA-complementing thioredoxin mutants had increased redox potentials, as expected, because an increased redox potential ensures that the thioredoxin mutant will pass the threshold to be an effective oxidant. However, all five non-complementing mutants tested also exhibited increased redox potentials, strongly suggesting that an increased redox potential is not sufficient for making thioredoxin DsbA-like in function. That both complementing and non-complementing mutants have a redox potential more oxidizing than that of thioredoxin is, upon reflection, not surprising; the function of thioredoxin in the cell is to reduce disulfides, so its sequence is likely to be optimized for this function. Random mutagenesis usually leads to a decline in a protein's structure and function and thus, in the case of thioredoxin, will be expected to move thioredoxin's redox potential in a less reducing direction, toward the reducing power present in a minimally structured peptide. Consistent with this, the redox potentials of our mutants are similar to the -230 mV value observed for a CAAC motif in an α-helical peptide free in solution (56). Thus, a more oxidizing redox potential, although probably necessary for thioredoxin mutants to complement DsbA, cannot be the only requirement.

The interactions of DsbA with substrate proteins and the reoxidant DsbB are important components of the DsbA catalytic cycle. It is surprising that our data did not establish a correlation between the efficiency of interaction with DsbB and the ability to complement DsbA. In fact, wild-type thioredoxin interacts with DsbB almost as efficiently as does DsbA, indicating that interacting with DsbB may not be the rate-limiting step in the catalytic cycle. However, we did observe that more rapid interaction with DsbB does help to improve the catalytic efficiency of the reoxidation reaction. This was seen for the CPHC mutant; its relatively high efficiency of interaction with DsbB compensates for its only moderately oxidizing redox potential, making it a very strong DsbA-complementing mutant. Although it is not a rate-determining step, elimination of this step abolishes the whole catalytic cycle, resulting in the inability of several mutants to complement DsbA (AT and TA mutants).

We found that all of the DsbA-complementing mutants exhibited an enhanced ability to oxidatively refold reduced hirudin compared with wild-type thioredoxin. It is surprising that we found that the majority of the DsbA-complementing mutants were better at isomerizing scrambled hirudin than was DsbA and had isomerase activities *in vivo*. The observation that our mutants selected on the basis of complementing DsbA function *in vivo* had increased isomerase activity raises the interesting possibility that DsbA has more isomerase function *in vivo* than previously thought. Although most of the *in vivo* and *in vitro* work on DsbA has emphasized its oxidase activity, it does exhibit measurable levels of isomerase activity *in vitro* (57). Copper(II) apparently acts as a nonspecific oxidant *in vivo*, generating large numbers of nonspecific disulfides, including disulfide-linked dimers and multimers (39). Mutants of the gene for DsbC, which is thought to be the principal isomerase of *E. coli*, are copper-sensitive, presumably because *dsbC*$^-$ strains are deficient in their ability to reshuffle these incorrect disulfides. It is interesting to note, however, that *dsbA*$^-$*dsbC*$^-$ double mutant strains are even more copper-sensitive than *dsbC*$^-$ strains, implying that DsbA and DsbC cooperate in correcting the incorrect disulfides generated by copper (39). Our finding that thioredoxin mutants that rescue DsbA-null mutant phenotypes show enhanced isomerase activity is consistent with the notion that one of the properties that they are rescuing is isomerase activity. A number of the mutants selected on the basis of DsbA complementation appear by our tests to have thiol disulfide activity *in vitro* as efficient as that of DsbA and isomerase activity *in vitro* as efficient as that of DsbC. If one thioredoxin-related protein can "do it all," then why has evolution chosen to evolve both a DsbA-based oxidation pathway and a DsbC-based isomerization pathway? We do not have a clear answer to this question, but in general, multigene families are thought to have evolved to address questions of substrate specificity. We note that, in the *in vivo* tests for oxidation and isomerization, our thioredoxin mutants, although showing considerable activity, were not as efficient as wild-type *E. coli*. We also note that nature has chosen to give wild-type DsbA an extremely oxidizing redox potential of -121 mV. Although the C*XX*C motif is important in determining the redox potential of thioredoxin-related proteins, it is very unlikely to be the sole determinant. Thus, mutations in thioredoxin that are restricted to altering this motif, as ours were, are unlikely to be able to achieve the full oxidizing power of DsbA.

Despite almost 45 years of work on protein-disulfide isomerases, surprisingly little is known about the *in vivo* requirements for disulfide isomerization. It is known that dimerization enhances disulfide isomerization activity (58, 59). During purification, different thioredoxin mutants eluted at approximately the same position as wild-type thioredoxin (monomer) on the gel filtration column, indicating that it is unlikely that our thioredoxin mutants are dimers. It also seems likely that the redox potential of the isomerase is important for its function. The mechanism of isomerization requires that the isomerase functions as both an acceptor and a donor of disulfides, thus,

a priori, one would expect the redox potential of the isomerase to be finely balanced so that it can both accept and donate disulfides. Consistent with this notion, our thioredoxin mutants do have a redox potential intermediate between the strongly oxidizing redox potential of DsbA and the relatively reducing redox potential of thioredoxin, and the redox potential does seem to be important in their mechanism.

A selection for mutants of thioredoxin that complement null mutants of protein-disulfide isomerase performed in yeast yielded mutants with a redox potential more oxidizing than that of the original thioredoxin (9); however, it now appears that sulfhydryl oxidation, not disulfide isomerization, is the principal function of protein-disulfide isomerase in yeast (60). This probably explains why more oxidizing mutants were obtained using this selection. It is interesting to note that, in the previous selection, as well as in ours, only a very limited range of mutants that allowed complementation were obtained. It is interesting that it was also found that an aromatic residue (in the previous case, tryptophan) works well to cause thioredoxin to have isomerase activity *in vivo*. These results suggest that there are very specific sequences for good isomerase function that go beyond having the appropriate redox potential.

Overall, we conclude that at least three properties are required to allow a thioredoxin mutant to complement DsbA: an increased redox potential, the ability to be efficiently oxidized by DsbB, and the ability to oxidize substrate proteins. An increased redox potential seems to be the prerequisite. On the other hand, the other two properties, if missing, will abolish the ability to complement DsbA. The two properties sometimes can even compensate for relatively "low" redox potential and make the mutant protein very active *in vivo*. The total efficiency as an oxidant is a good combination of the three aspects.

Our work further establishes the vital importance of the *CXXC* residues in determining the functional properties of thiol-disulfide oxidoreductases of the thioredoxin superfamily. Remarkably, the short motif, which was optimized by evolution to work in DsbA, also seems to be optimized in conferring DsbA-like properties on thioredoxin, a protein separated from DsbA by >2 billion years of evolution. The ability of two amino acids to confer properties of one enzyme or another shows the great importance of these residues. In contrast to current thinking, this importance is not limited to the effect these changes have on the redox potential of the protein, but our work shows that these residues have important effects on interactions with both folding proteins and the reoxidant DsbB and on the ability of the protein to participate in disulfide isomerization reactions. We were surprised to find that thioredoxin mutants selected for their ability to complement the oxidizing thiol-disulfide oxidoreductase DsbA turned out to be much more efficient in isomerizing disulfides than was DsbA; some were even as efficient as DsbC, the principal disulfide isomerase of *E. coli*.

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