SUPPLEMENTAL DATA

SUPPLEMENTAL FIGURE LEGENDS

Fig. S1. Redox potential and pK_a measurements. (A-D) Redox potentals of DsbA and its mutants, DsbG and its mutants, DsbC and its mutants, and thioredoxin mutants CPHC and I75T/CPHC were measured in GSH-GSSG redox buffer. The redox states of DsbA and its mutants, DsbG and DsbG T200L, were followed by the detection of fluorescence emission at 330 nm (excitation at 295 nm). The redox status of DsbC and its mutants was examined by reverse phase HPLC. The two thioredoxin mutants had reduced and oxidized peaks that could not be fully separated from the reduced peak of cDsbD in HPLC analysis and thus could not be measured using cDsbD as a reference. DsbA (■), DsbA V150T (□), DsbG (●) and DsbG T200V (○), thioredoxin CPHC (▲) and I75T/CPHC (△) , and DsbC (◆) and DsbC T182V (◇). (E-F) Redox potentials of thioredoxin and mutant I75T were analyzed by determining the equilibrium between thioredoxin and the C-terminal domain of DsbD (cDsbD) using reverse-phase HPLC. (G-J) Determination of the pK_a of the nucleophilic cysteine in DsbA (■), DsbA V150T (□), DsbG (●) and DsbG T200V (○), thioredoxin (▲) and thioredoxin I75T (△) , and DsbC (◆) and DsbC T182V (◇). The pH dependence of the thiolate-specific absorbance signal ($S = (A_{240}/A_{280})_{reduced}/(A_{240}/A_{280})_{oxidized}$) was fit according to the Henderson-Hasselbach equation (the oxidized proteins were used as a reference). For DsbA V150T, no significant change in pK_a was detected. This finding is inconsistent with the observation that this mutant is substantially more oxidizing than wild-type DsbA. We note that it may not be possible to accurately measure pK_a values below about 3.5 due to the previously noted interference of protein denaturation at low pH values (1).

Fig. S2. *In vitro* reductase and isomerase activity assays for DsbA and DsbC *cis*Pro minus 1 variants. (A) Reductase activity was assayed using insulin and DTT as substrates for DsbC. (B) Refolding of scRNaseA was carried out by incubating the scrambled RNase A enzyme (40 μM) in 100 mM sodium phosphate/NaOH, pH 7.0, 1 mM EDTA, 10 mM DTT and in the presence of 10 μM DsbA(●) or DsbA V150T(○) and DsbC(■) or DsbC T182V (□). Folded RNase was used as a positive control (▲). The cleavage of cCMP by native RNase A was followed spectroscopically at 296 nm. (C) Control isomerase assay in the absence of any disulfide oxidoreductase catalyst with scrambled hirudin as a substrate. Equal molar quantities of GSH and scrambled hirudin were incubated for various times, as indicated in the figure. The reactions were then acid quenched and analyzed by reverse-phase HPLC on a Vydac™218TP54 C18 column. N and R indicate native and reduced hirudin, respectively.

Fig. S3. Characterization of DsbA-DsbB, thioredoxin-thioredoxin reductase and DsbG-DsbD interactions. (A) *In vitro* measurement of K_m and k_{cat} for DsbB-mediated catalysis of DsbA and V150T oxidation using the multi-turnover assay. Traces 1-3 and 4-6 represent the three repeated experimental trials for DsbA and V150T, respectively. The traces were used to derive a V versus [S] plot (shown in Fig. 3D) using the enzyme-monitored

turnover method. (B) Thioredoxin reductase based thioredoxin and I75T of insulin reduction activity were measured using NADPH as the reducing source. The inset panel shows insulin reduction protein in the presence of increasing thioredoxin reductase concentrations. (C) Reduction of 1 μM oxidized DsbG and its variants by 1 μM cDsbD when 10 nM nDsbD was added to the system.

Fig. S4. Root-mean-square difference between the structures of wild-type thioredoxin and I75T. Shown here is a plot of rmsd for c alpha carbon atoms between the two structures versus the residue number of the primary sequence.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Sequence alignment

In order to determine the conservation of the residues in the CXXC and the *cis*-proline loop in these proteins, we aimed to analyze proteins from genomes that are as divergent as possible. However, we wanted to avoid comparing proteins that evolved over different evolutionary timeframes. Thioredoxin and glutaredoxin, for instance, are present in eukaryotes, bacteria and archaea, and therefore have been evolving for at least 3.8 billion years (2), whereas DsbC and DsbG are restricted to proteobacteria and have probably been evolving for at least ~0.5 billion years (3). Thus, we restricted our comparison to genomes that contain an orthologue to DsbC. We used our previous alignment of all species-specific DsbC sequences available in GenBank(3) to obtain the list of organisms that contain DsbC. We then obtained the sequence of the individual thioredoxin, DsbA, DsbG and glutaredoxin orthologues present in these individual genomes using BLAST by searching with the *E. coli* homologue.

Plasmid construction and site-directed mutagenesis

The coding DNA sequence of the thioredoxin gene *trxA*, the N-domain of *dsbD* (G63D), and the thioredoxin reductase gene *trxB* were amplified from the genome of *E. coli* ER1821 (New England Biolabs) by PCR using the primers described in the supplemental materials and ligated into pQE60 (Qiagen) that had been cut with NcoI and BglII. Other constructs were from our lab strain collection. Point mutations were introduced into the WT *dsbA* gene in pXK22, *dsbG* in pAH232, and *trxA* in pR122 using a Stratagene Quick Change kit (Stratagene, La Jolla, CA) and appropriate mutagenic primers, and PCR was performed as directed. The product was digested with Dpn I, precipitated using Pelletpaint® according to the protocol supplied by the manufacturer (Novagen) (Dpn I), and resuspended in ~10 μl ddH_2O. Five microliters of product was transformed into XL10-gold competent cells (Stratagene). DNA was extracted from the cells and all point mutations were verified by DNA sequencing. The coding DNA sequence of DsbC was PCR amplified from *E. coli* genomic DNA and cloned into the pET42b vector (Novagen, Madison, WI) using *Nde*I and *Xho*I restriction sites.

Protein purification, reduction, oxidation, concentration determination,

WT DsbA and mutants were overexpressed in XK31 cells (BL21 *dsbA*::kan). His-tagged versions of WT DsbG, DsbC and their mutants were overexpressed in BL21 cells. WT thioredoxin and mutants, nDsbD and cDsbD, and thioredoxin reductase were overexpressed

in M15 cells. WT DsbA and mutants were first purified from periplasmic extract using anion exchange (5 ml Q Sepharose Fast Flow). WT DsbG, DsbC and their mutants and WT thioredoxin and its mutants were first purified using a 6-His tag and nickel chromatography. Then, DsbA, DsbA V150T, DsbG, DsbG T200L, and thioredoxin I75T were further purified by size-exclusion using Superdex 75 chromatography. DsbC was further purified by size exclusion on Superdex S-200. All proteins were >95% pure as determined by Coomassie staining of SDS-PAGE gels. Scrambled hirudin and DsbB, prepared as previously described (4,5), were kindly provided by Shu Quan and Timothy Tapley, respectively.

DsbA, DsbG, thioredoxin, and their mutants were oxidized by incubation in 20 mM oxidized glutathione for 2 h at 4°C. DsbC and its mutant were oxidized with 1.7 mM copper(II)[1,10-phenanthroline]. DsbA, DsbG, thioredoxin, DsbC and their mutants and cDsbD were reduced by incubation with 10 mM DTT for 1 h at 4°C. The reactions were then desalted on NAP-5 columns (GE Healthcare) and the protein fractions were collected. Protein concentrations were determined by absorbance at 280 nm using extinction coefficients of 23,045 and 22,920 $cm^{-1}M^{-1}$, respectively, for reduced and oxidized DsbA; 44,015 and 43,890 $cm^{-1}M^{-1}$, respectively, for reduced and oxidized DsbG; 13,980, 14,105 $cm^{-1}M^{-1}$, respectively, for reduced and oxidized thioredoxin; and 17,670 and 17,420 $cm^{-1}M^{-1}$, respectively, for reduced and oxidized DsbC. Values for nDsbD, cDsbD and thioredoxin reductase were 20,340, 8,370, and 19,000 $cm^{-1}M^{-1}$, respectively. These extinction coefficients were calculated using ExPASy (http://ca.expasy.org/).

Isomerase activity assay

Scrambled RNase A as substrate

To determine the *in vitro* isomerase activity of DsbA V150T and DsbC T182V, we utilized the scrambled RNase A (scRNase A) refolding assay. Reduced denatured RNase A (0.5 mg/ml) was incubated in 50 mM Tris-HCl, pH 8.5, and 6 M GdmCl for at least 3 days in the dark at room temperature to prepare scRNase A (6). The randomly reoxidized RNase A was concentrated and, after the solution was acidified, the oxidation of disulfide bonds was confirmed using Ellman's assay. Reshuffling of scRNase A (40 µM) was carried out by incubation in 100 mM phosphoric acid-NaOH, pH 7, 1 mM EDTA, with 10 µM oxidized DsbC, DsbCT182V, DsbA or DsbA V150T (DsbA, DsbC and mutants were oxidized with 1.7 mM copper(II)[1,10-phenanthroline]). The reactions were initiated by the addition of DTT to a final concentration of 10 µM. As a positive control, we carried out an additional reaction using folded RNase A without any Dsb protein. The assay was performed at 25 ˚C, and samples were withdrawn at several time points and assayed for RNase A activity by monitoring cCMP hydrolysis spectrophotometrically at 296 nm for 2.5 min. The fraction of native RNase A (in %) was plotted against incubation time.

Kinetics of DsbG, and variants interaction with DsbD

The reduction of oxidized DsbG and variants by a stoichiometric amount of reduced cDsbD and a catalytic amount of nDsbD was peformed according to Collet et al. (7). One micromolar oxidized DsbG and 1 µM reduced cDsbD were mixed at room temperature (no change in fluorescence), and 10 nM nDsbD was added to initiate the reaction. Tryptophan excitation

was monitored at 295 nm, and emission was monitored at 330 nm in a Hitachi F-4800 spectrofluorometer.

SUPPLEMENTAL TABLES

Table S1. Strains and plasmids used in the study

Strains	**Relevant genotype**
BL21	*E. coli* B *F– ompT hsdSB*(rB – mB–) *gal dcm* (DE3)
XK31	BL21 *DsbA::Kan*
DHB4	F' *lac-pro lacIQ/*[*ara-leu*]7697 *araD139 lacX74 galE galK rpsL phoR* (*phoA*) *Pvu*II *malF3 thi*
AH435	DHB4 *trxA null*
M15 (pREP4)	Nal^S str^S rif^S *thi lac ara gal mtl F* *recA*$^+$ *uvr*$^+$ *lon*$^+$ [pREP4 Kan^R]
Plasmids	**Vector and insert**
pXK22	pET11a-DsbA
pAH232	pET28a-DsbG (His tagged)
pR122	pQE-Trx (His tagged)
pR130	pQE-nDsbD (G63D) (His tagged)
pJFC2	pQE-cDsbD (His tagged)
pR319	pQE-TrxR (His tagged)

Table S2. Redox potential of thioredoxin I75 mutants (mV)

Hydrophobic				Polar					
Large side chain		Small side chain		Not charged		Aromatic		Charged	
I	−271±1	**A**	−256±0	**Q**	−247±1	**Y**	−221±1	**K**	−242±1
L	−270±1			**S**	−235±1	**W**	−219±4	**R**	−240±0
M	−262±1			**N**	−231±1			**H**	−258±1
F	−259±4			**T**	−226±1			**E**	−246±1
V	−257±1							**D**	−245±1
No side chain effect				**G**			−250±0		

Table S3. Data collection and refinement statistics

	Thioredoxin (I75T)
Data collection	
Wavelength	1.5418 Å
Space group	C2
Unit cell (Å)	a = 58.8 Å, b = 39.4 Å, c = 90.6 Å, β = 96.6°
Resolution (Å)	2.0
Completeness (%)	97.0 (94.7)
I / σ	20.7 (5.1)
R_{merge} (%)	5.9 (24.9)
Redundancy	3.2
Unique reflections	13,739
Refinement	
R_{work}/ R_{free}	25.1/29.0
Rmsd, bonds (Å)	0.007
Rmsd, angles (°)	1.038
Mean B ($Å^2$)	26.2

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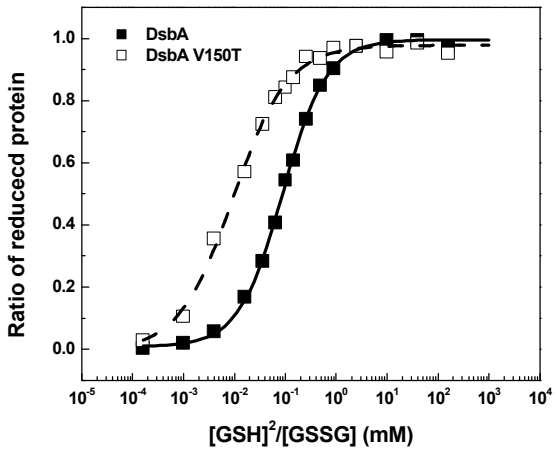
1. **Grauschopf, U., Winther, JR, Korber, P, Zander, T, Dallinger, P, Bardwell, JC. (1995)** ***Cell*** **83(6), 947-955**
2. **Hahn, J. P. H. (1986)** ***System Applied Microbiology (Archaebacteria '85 Proceedings)*** **7 178-183**
3. **Hiniker, A., Ren, G, Heras, B, Zheng, Y, Laurinec, S, Jobson, RW, Stuckey, JA, Martin, JL, Bardwell JC. (2007)** ***Proc Natl Acad Sci U S A.*** **104(28), 11670-11675**
4. **Quan, S., Schneider, I, Pan, J, Von Hacht, A, Bardwell, JC. (2007)** ***J Biol Chem.*** **282(39), 28823-28233**
5. **Tapley, T., Eichner, T, Gleiter, S, Ballou, DP, Bardwell, JC. (2007)** ***J Biol Chem.*** **282(14), 10263-10271**
6. **Hillson, D. A., Lambert, N., Freedman, R.B. (1984)** ***Methods Enzymol.*** **107, 281-294**
7. **Collet, J., Riemer, J, Bader, MW, Bardwell, JC. (2002)** ***J Biol Chem.*** **277(30), 26886-26892**

SUPPLEMENTAL FIGURES

FIG. S1.

A



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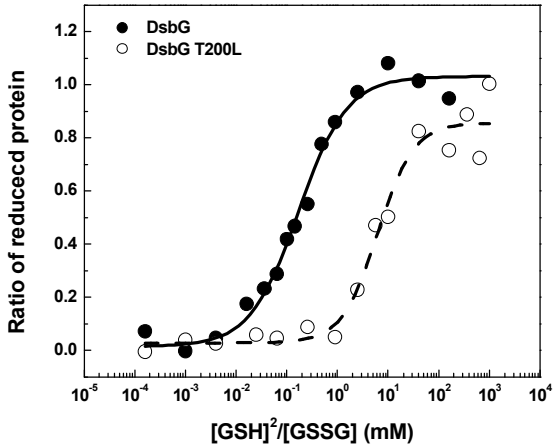
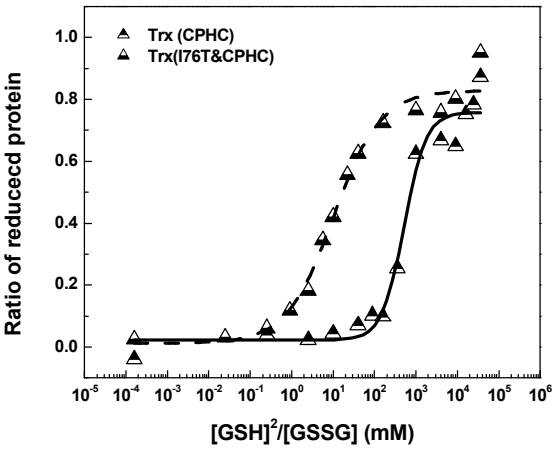


C



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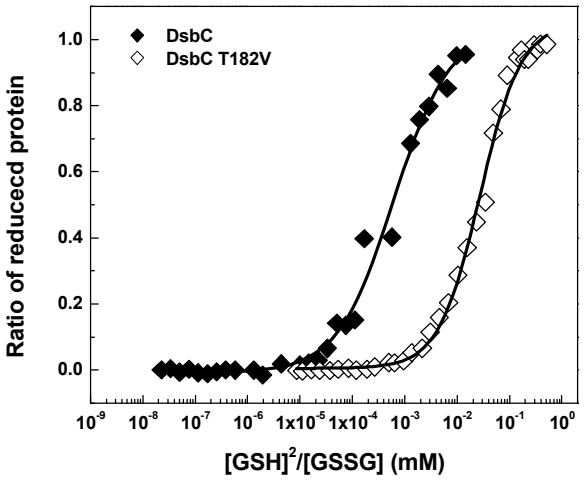
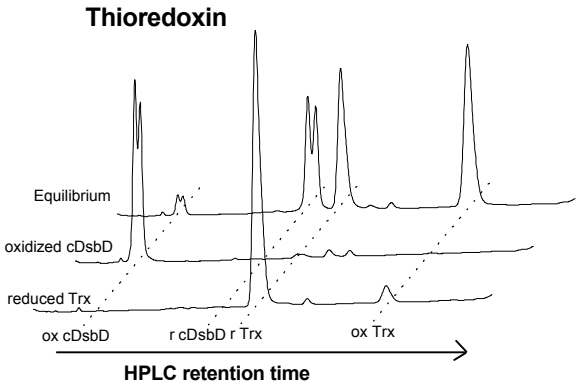
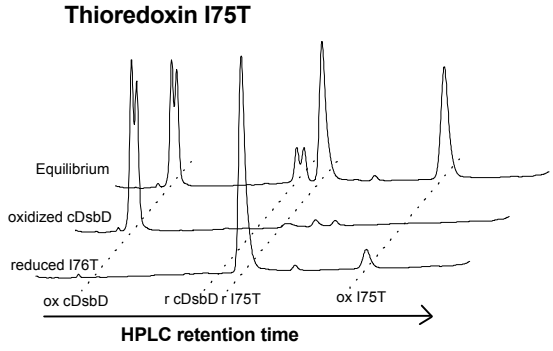

DsbC
DsbC T182V
Ratio of reducecd protein
1.0
0.8
0.6
0.4
0.2
0.0
10^-9 10^-8 10^-7 10^-6 1x10^-5 1x10^-4 10^-3 10^-2 10^-1 10^0
[GSH]^2/[GSSG] (mM)

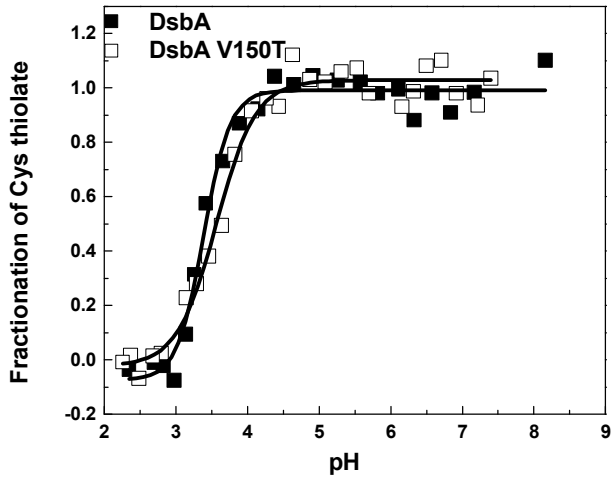
E


Thioredoxin
Equilibrium
oxidized cDsbD
reduced Trx
ox cDsbD
r cDsbD r Trx
ox Trx
HPLC retention time

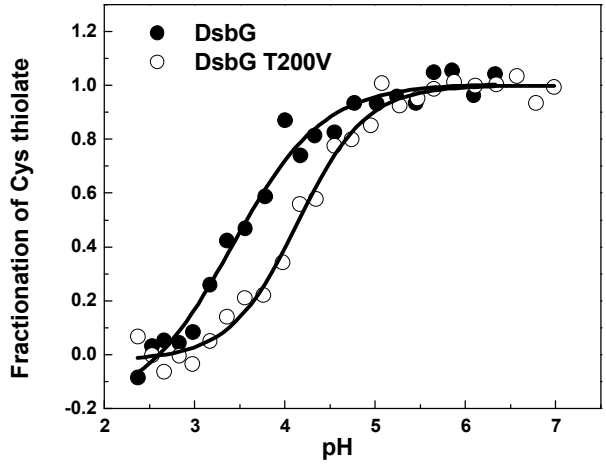
F


Thioredoxin I75T
Equilibrium
oxidized cDsbD
reduced I76T
ox cDsbD
r cDsbD r I75T
ox I75T
HPLC retention time

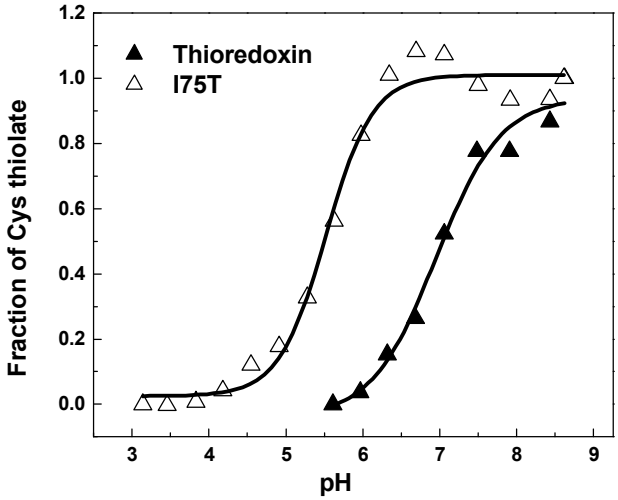
G


DsbA
DsbA V150T
Fractionation of Cys thiolate
pH

H


DsbG
DsbG T200V
Fractionation of Cys thiolate
pH

I


Thioredoxin
I75T
Fraction of Cys thiolate
pH

J

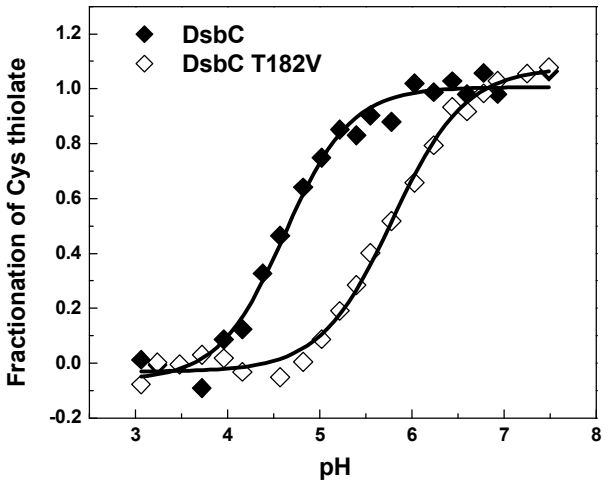
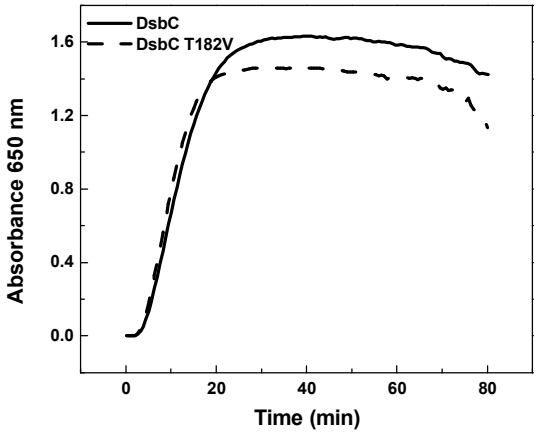
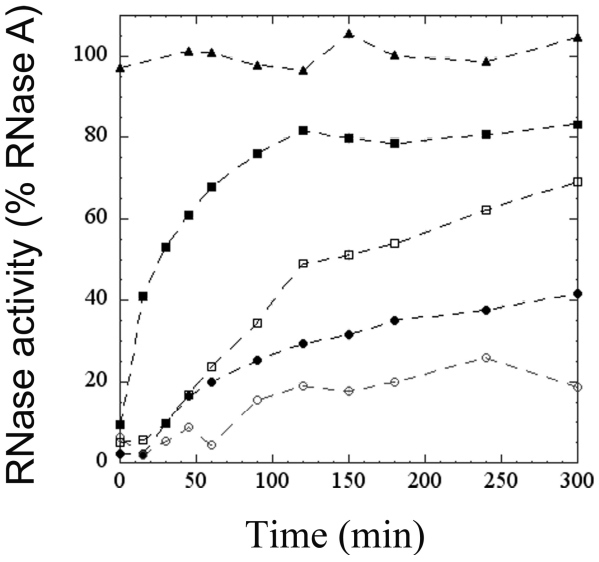

DsbC
DsbC T182V
Fractionation of Cys thiolate
pH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
3
4
5
6
7
8

FIG. S2

A


DsbC
DsbC T182V
Absorbance 650 nm
1.6
1.2
0.8
0.4
0.0
0
20
40
60
80
Time (min)

B


RNase activity (% RNase A)
100
80
60
40
20
0
0
50
100
150
200
250
300
Time (min)

C

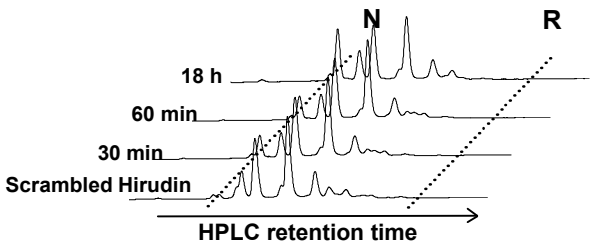
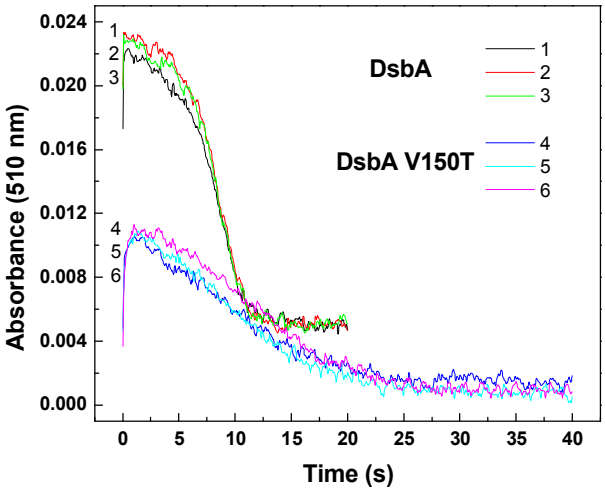
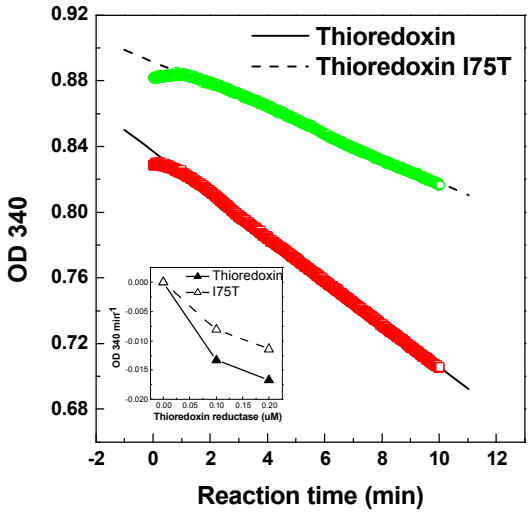

N
R
18 h
60 min
30 min
Scrambled Hirudin
HPLC retention time

FIG. S3

A


Absorbance (510 nm)
Time (s)
DsbA
DsbA V150T
1
2
3
4
5
6

B


OD 340
Reaction time (min)
Thioredoxin
Thioredoxin I75T
Thioredoxin
I75T
OD 340 min-1
Thioredoxin reductase (uM)

C

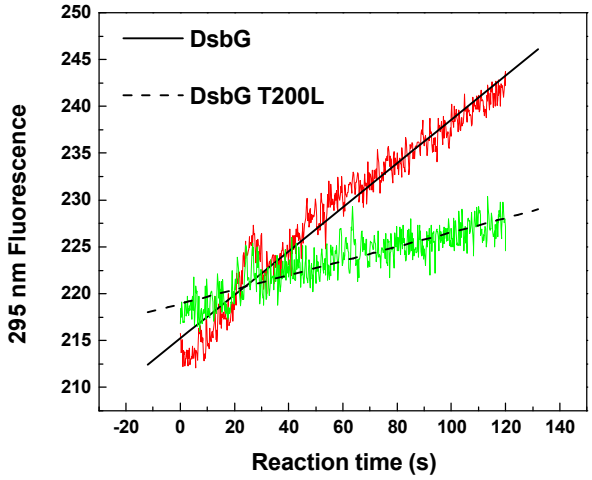
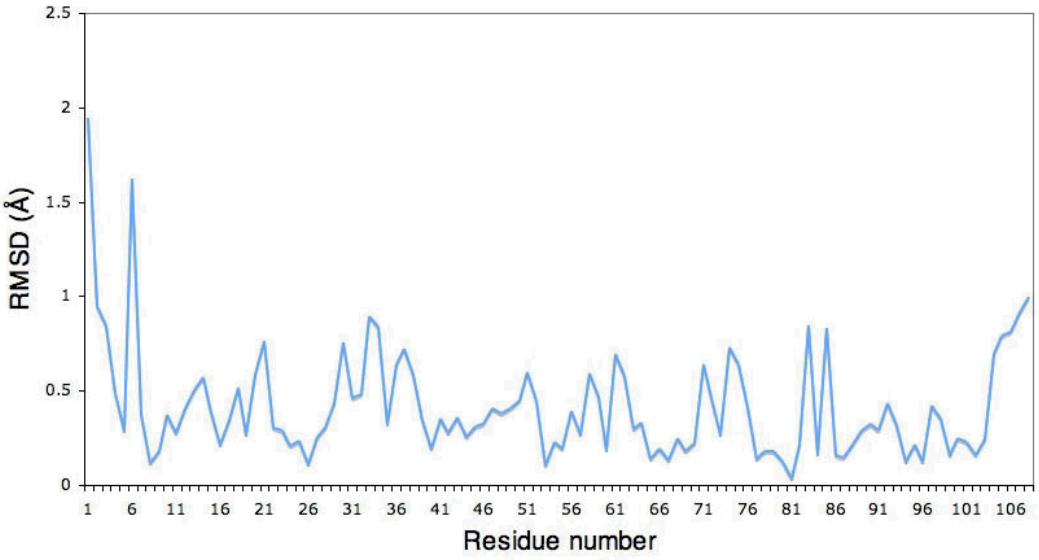

295 nm Fluorescence
Reaction time (s)
DsbG
DsbG T200L

Fig. S4


RMSD (Å)
2.5
2
1.5
1
0.5
0
1
6
11
16
21
26
31
36
41
46
51
56
61
66
71
76
81
86
91
96
101
106
Residue number