SUPPLEMENTAL DATA

CONTROL OF PERIPLASMIC INTERDOMAIN THIOL:DISULFIDE EXCHANGE IN THE TRANSMEMBRANE OXIDOREDUCTASE DSBD*

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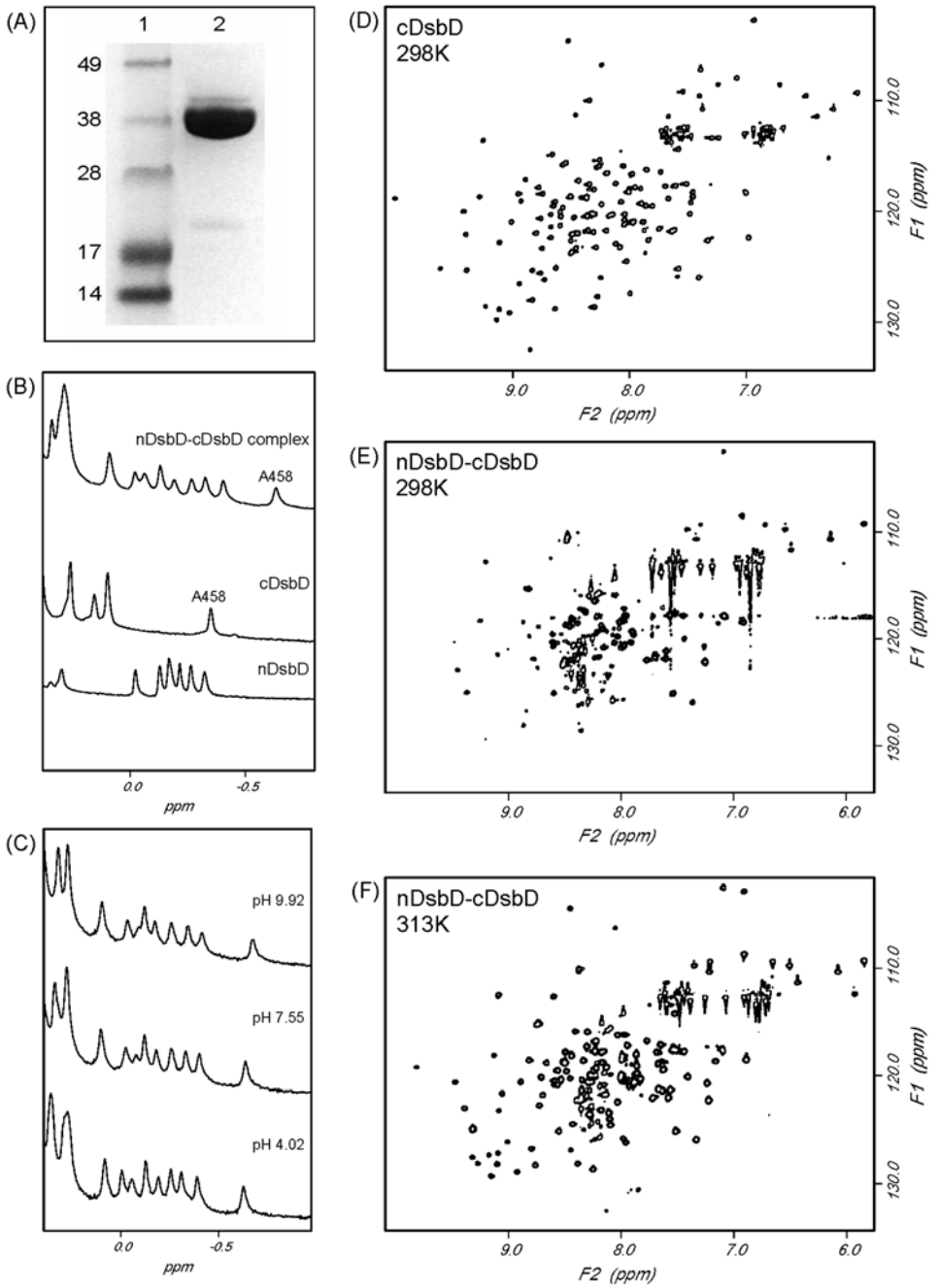
Additional Experimental Procedures

Construction of DsbD plasmids– The plasmid pDzc1, described in previous work (1), was used to express ^{15}N-labelled wild-type cDsbD bearing a C-terminal His_6-tag. A single-cysteine variant (C464A) originating from pDz1a, also described previously (1), was produced by site-directed mutagenesis (QuikChange, Qiagen) using oligonucleotides 5'-CGACTGGTGCGTCGCCGCCAAAGAGTTTGAG-3' and 5'-CTCAAACTCTTTGGCGGCGACGCACCAGTCG-3'. The plasmid produced, pDzc5, expresses C464A-cDsbD with a thrombin cleavage site preceding the C-terminal His_6-tag.

To generate the full-length construct in which the N-terminal and C-terminal domains of DsbD could be cleaved from the transmembrane domain (thrombin-cleavable DsbD), two inverse PCRs were conducted. The template for the first reaction was full-length DsbD bearing a C464A mutation cloned into the *Eco*RI and *Afe*I sites of pTTQ18 (2). The first PCR used oligonucleotides 5'-cggcaccag gccAAAGGGCAATTGCGCGGTGGGCTG-3' (upper case bases are homologous to DsbD, lower case bases encode the thrombin cleavage site) plus 5'-cgcggcagcggcTCCGCGCTCTGGGCGTTGTTGATC-3' to introduce the thrombin cleavage site between the N-terminal domain and the transmembrane domain. This product was self-ligated and the resulting plasmid acted as the template for the second PCR using oligonucleotides 5'-cggcaccaggccAGTTTGCGCGGTATGCGTCGCACC-3' plus 5'- cgcggcagcg gcCAGACGCATCTCAACTTTACACAA-3' which was self-ligated to introduce the thrombin cleavage site between the transmembrane domain and the C-terminal domain. The resulting plasmid was named pDsbd4.

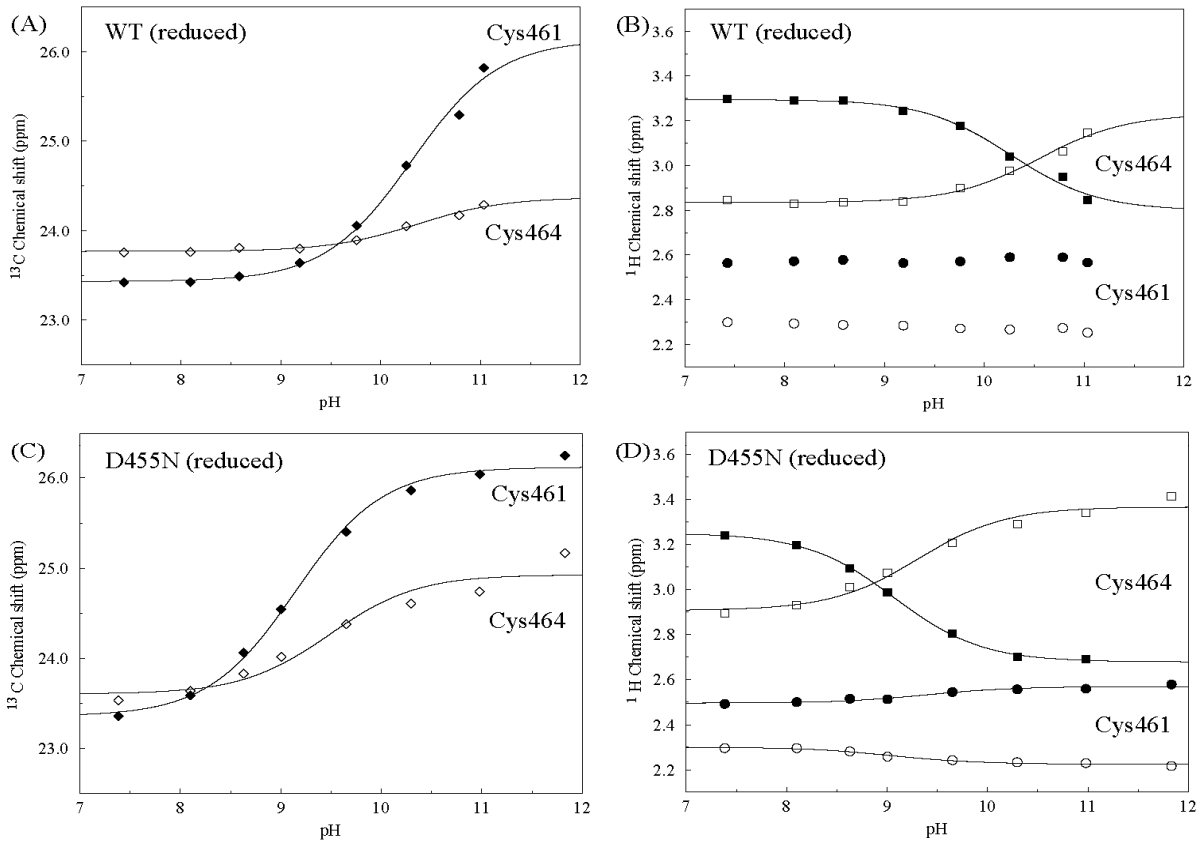
To produce the plasmid for expression of the N-terminal domain of DsbD with the PelB signal peptide replacing the endogenous signal peptide and with a C-terminal streptavidin tag, a series of PCRs was conducted. Each reaction used the specific product of the previous PCR as a template; the template for the first reaction was purified *E. coli* K12 genomic DNA. The following oligonucleotides were used for the sequential reactions: DM1 (5'-catggatactTTATTCGACGCGCCGGGACG-3'; bases homologous to DsbD are in upper case, those encoding the PelB signal peptide are in lower case and underlined) plus DM2 (5'-ggtggctccaagcgctAAAGGGCAATTGCGCGGTGG-3'; bases homologous to DsbD are in upper case, those encoding the streptavidin tag are in lower case and underlined), DM3 (5'-ggaagctttattttttcgaactgcgggtggctccaagcgct-3'; underlined bases encode the streptavidin tag, contains a full *Hin*dIII site) plus DM4 (5'-gctgcccagccggcgatggccatggatactTTATTCGA-3'; bases homologous to DsbD are in upper case, those encoding the PelB signal peptide are in lower case and underlined), DM5 (5'-ctgctgctggtctgctgctcctcgctgcccagccggcgatg-3'; bases encoding the PelB signal peptide are underlined) plus DM3 and finally DM6 (5'-gggaattcatgaaatacctgctgccgacggctgctgctggtctgctgc-3'; bases encode the PelB signal peptide are underlined, contains a full *Eco*RI site) plus DM3. This final product was digested with *Eco*RI and *Hin*dIII and cloned into *Eco*RI/*Hin*dIII digested pEG278 (a modified version of pEG276 (3)) and was named pDzn1. A single-cysteine variant originating from pDzn1 was produced using site-directed mutagenesis (QuikChange, Qiagen). pDzn2 encodes nDsbD with a C103A mutation and was produced using oligonucleotides 5'-GTCACCTACCAGGGCGCTGC-TGATGCCGGTTTC-3' plus 5'-GAAACCGGCATCAGCAGCGCCCTGGTAGGTGAC-3'.

DNA manipulations were conducted using standard methods. *Pwo* DNA polymerase (from *Pyrococcus woesei*) was used for all PCR reactions according to the suppliers instructions (Boehringer Mannheim Biochemicals) and all constructs were sequenced and confirmed to be correct prior to use. Oligonucleotides were synthesized by Sigma-Genosys.



Supplemental Figure S1

Supplemental Fig. S1. Characterization of the nDsbD-^{15}N-cDsbD complex. (A) SDS-PAGE was used to confirm the formation and purity of the nDsbD-^{15}N-cDsbD mixed-disulfide complex. Lane 1 shows molecular mass markers and lane 2 shows the purified nDsbD-^{15}N-cDsbD mixed-disulfide complex. (B) Upfield region of 950 MHz 1D ^{1}H NMR spectra of nDsbD, C464A-cDsbD and nDsbD-C464A-cDsbD at pH 6.5 and 313K. Peaks observed in this region of the spectrum arise from methyl groups which are located close to aromatic side chains in the protein structure. The spectrum of the complex is characterized by significantly broader peaks than the spectra of either individual domain. This suggests that the two domains behave in solution as a single globular entity and that significant independent motion of the two domains about the disulfide linkage does not occur; this is consistent with the X-ray structure of the complex which shows a well-defined interdomain interface (5). Several peaks in the spectrum of the complex are shifted relative to their positions in the spectra of the isolated domains indicating a change in the local environments of these methyl groups. For example, an upfield shift of ~0.28 ppm is observed for H^{β} of A458 from cDsbD. This shift is likely to arise from a change in the relative orientations of A458 and W460 in the nDsbD-cDsbD complex (1); W460 is located near to the interface between the two domains and is within 4 Å of Y71, Q101 and C109 of nDsbD (5). (C) Upfield region of 750 MHz 1D ^{1}H NMR spectra of nDsbD-C464A-cDsbD at 313K and pH values ranging from 4.02 to 9.92. The well-dispersed spectra and the presence of upfield shifted resonances indicate that even at pH values close to 10 both nDsbD and cDsbD retain their native folds. (D-F) ^{1}H-^{15}N HSQC spectra collected at 950 MHz for (D) the isolated ^{15}N-C464A-cDsbD domain at 298K, and the nDsbD-^{15}N-C464A-cDsbD complex at (E) 298K and (F) 313K; both samples are at pH 6.5 in 95%H_2O/5%$^{2}H_2O$.



Supplemental Fig. S2. Measurement of the pK_a value of C461 at 313K from the pH dependence of the $^{13}C^{\beta}$ and $^{1}H^{\beta}$ peaks from C461 and C464 in $^{13}C^{\beta}$-Cys-labelled wild-type and D455N cDsbD. (A) $^{13}C^{\beta}$ chemical shifts of C461 (♦) and C464 (◊) in reduced wild-type cDsbD. (B) $^{1}H^{\beta}$ chemical shifts of C461 (●,○) and C464 (■,□) in reduced wild-type cDsbD. (C) $^{13}C^{\beta}$ chemical shifts of C461 (♦) and C464 (◊) in reduced D455N cDsbD. (D) $^{1}H^{\beta}$ chemical shifts of C461 (●,○) and C464 (■,□) in reduced D455N cDsbD. The solid lines show the best fit to a single pK_a value for each dataset. The fitting procedure is described in Experimental Procedures and the fitted parameters are summarized in Table S2.

Supplemental Table S1. Results from the fitting* of the pH dependence of the chemical shifts of the ^{15}N and $^{1}H^{N}$ of Asp455 and Glu468 and $^{15}N^{\varepsilon}$ of Gln488 at 313K in oxidized and reduced cDsbD, in C464A-cDsbD and in the nDsbD-C464A-cDsbD complex. Where two pK_a fits are used the pK_a value assigned to D455 is underlined; the second pK_a value reflects titration of E468 or of C461 (higher value in C464A-cDsbD).

Asp455

	oxidized cDsbD		reduced cDsbD		C464A-cDsbD		nDsbD-C464A-cDsbD	
	(^{15}N)	($^{1}H^{N}$)	(^{15}N)	($^{1}H^{N}$)	(^{15}N)	($^{1}H^{N}$)	(^{15}N)	($^{1}H^{N}$)
pK_1	4.02	4.33	4.50	3.99	<u>7.14</u>	5.76	8.49	8.40
pK_2	<u>7.06</u>	<u>6.94</u>	<u>6.63</u>	<u>6.44</u>	8.70	<u>7.31</u>	------	------
δ_{HAH}	125.94	9.066	126.52	9.052	126.42	9.024	126.34	9.012
δ_{HA}-	126.29	9.008	126.83	8.977	127.54	8.965	128.07	8.803
δ_{A}2-	127.63	8.788	127.85	8.805	128.07	8.799	------	------

Glu468

	oxidized cDsbD		reduced cDsbD		C464A-cDsbD		nDsbD-C464A-cDsbD	
	(^{15}N)	($^{1}H^{N}$)	(^{15}N)	($^{1}H^{N}$)	(^{15}N)	($^{1}H^{N}$)	(^{15}N)	($^{1}H^{N}$)
pK_1	5.12	6.87	5.15	6.46	5.90	<u>7.45</u>	6.46	7.14
pK_2	<u>6.95</u>	-------	<u>6.53</u>	-------	<u>7.48</u>	9.27	<u>8.60</u>	<u>8.51</u>
δ_{HAH}	116.58	7.280	116.50	7.451	117.40	7.400	116.89	7.661
δ_{HA}-	117.09	7.464	117.36	7.632	118.06	7.580	117.71	7.714
δ_{A}2-	120.14		120.15	-------	120.23	7.774	120.29	7.889

Gln488

	oxidized cDsbD	reduced cDsbD	C464A-cDsbD	nDsbD-C464A-cDsbD
	($^{15}N^{\varepsilon}$)	($^{15}N^{\varepsilon}$)	($^{15}N^{\varepsilon}$)	($^{15}N^{\varepsilon}$)
pK_1	4.19	4.38	5.50	5.46
pK_2	<u>6.97</u>	<u>6.46</u>	<u>7.48</u>	<u>8.55</u>
δ_{HAH}	111.46	112.30	111.61	111.98
δ_{HA}-	112.87	113.51	113.39	112.67
δ_{A}2-	117.61	117.56	118.15	117.69

*pK_a values were determined from $^{1}H^{N}$, or ^{15}N chemical shifts measured as a function of pH. The titration data were fitted to either one- or two-pK_a curves (4) using in-house software. For titrations described by a single pK_a value the chemical shift at each pH is defined as:

$$\delta = \delta_{HA} - [(\delta_{HA} - \delta_{A}-) / (1 + 10^{(pKa - pH)})].$$

For titrations described by two pK_a values the chemical shift at each pH is defined as:

$$\delta = ((\delta_{HAH})[H^{+}]^2 + (\delta_{HA}-)K_1[H^{+}] + (\delta_{A2}-) K_1K_2) / ([H^{+}]^2 + K_1[H^{+}] + K_1K_2).$$

Supplemental Table S2. Results from the fitting* of the pH dependence of the chemical shifts of the $^{13}C^{\beta}$ and $^{1}H^{\beta}$ of C461 and C464 at 313K in reduced wild-type and D455N cDsbD. Where two pK_a fits are used the pK_a value assigned to C461 is underlined; the second pK_a value reflects titration of C464.

WT (reduced)

	C461			C464		
	($^{13}C^{\beta}$)	($H^{\beta1}$)**	($H^{\beta2}$)**	($^{13}C^{\beta}$)	($H^{\beta1}$)	($H^{\beta2}$)
pK_1	10.31			10.36	10.27	10.56
δ_{HA}	23.43			23.77	3.295	2.835
δ_{A}-	26.13			24.37	2.799	3.231

D455N (reduced)

	C461			C464		
	($^{13}C^{\beta}$)	($H^{\beta1}$)	($H^{\beta2}$)	($^{13}C^{\beta}$)	($H^{\beta1}$)	($H^{\beta2}$)
pK_1	9.14	9.39	9.04	9.49	9.07	9.31
δ_{HA}	23.36	2.496	2.300	23.60	3.249	2.906
δ_{A}-	26.12	2.569	2.225	24.93	2.679	3.366

*pK_a values were determined from $^{13}C^{\beta}$ or $^{1}H^{\beta}$ chemical shifts measured as a function of pH. The titration data were fitted to one-pK_a curves (4) using in-house software. The chemical shift at each pH is defined as:

$\delta = \delta_{HA} - [(\delta_{HA} - \delta_{A}-) / (1 + 10^{(pKa - pH)})]$.

** These resonances did not show significant pH-dependent shifts in WT cDsbD.

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