Differences between the electronic environments of reduced and oxidized *Escherichia coli* DsbA inferred from heteronuclear magnetic resonance spectroscopy

JOEL COUPRIE, M. LYNDSEY REMEROWSKI, ARNAUD BAILLEUL, MARIE COURÇON,
NICOLAS GILLES, ERIC QUÉMÉNEUR, AND NADEGE JAMIN

CEA-DSV-DIEP, 91191 Gif-sur-Yvette Cedex, France

(RECEIVED January 7, 1998; ACCEPTED May 27, 1998)

Abstract

DsbA is the strongest protein disulfide oxidant yet known and is involved in catalyzing protein folding in the bacterial periplasm. Its strong oxidizing power has been attributed to the lowered pK_a of its reactive active site cysteine and to the difference in thermodynamic stability between the oxidized and the reduced form. However, no structural data are available for the reduced state. Therefore, an NMR study of DsbA in its two redox states was undertaken. We report here the backbone $^1H^N$, ^{15}N , $^{13}C^\alpha$, ^{13}CO , $^1H^\alpha$, and $^{13}C^\beta$ NMR assignments for both oxidized and reduced *Escherichia coli* DsbA (189 residues). Ninety-nine percent of the frequencies were assigned using a combination of triple (1H - ^{13}C - ^{15}N) and double resonance (1H - ^{15}N or 1H - ^{13}C) experiments. Secondary structures were established using the CSI (Chemical Shift Index) method, NOE connectivity patterns, $^3J_{HNH^\alpha}$ and amide proton exchange data. Comparison of chemical shifts for both forms reveals four regions of the protein, which undergo some changes in the electronic environment. These regions are around the active site (residues 26 to 43), around His60 and Pro151, and also around Gln97. Both the number and the amplitude of observed chemical shift variations are more substantial in DsbA than in *E. coli* thioredoxin. Large $^{13}C^\alpha$ chemical shift variations for residues of the active site and residues Phe28, Tyr34, Phe36, Ile42, Ser43, and Lys98 suggest that the backbone conformation of these residues is affected upon reduction.

Keywords: 3D NMR; chemical shifts; DsbA; resonance assignments; thiol-disulfide oxidoreductases; thioredoxin; triple resonance.

The formation of disulfide bonds is essential for the folding and stability of secretory proteins. It often constitutes a rate-limiting step in their folding pathways (Creighton, 1992), and thiol-disulfide oxidoreductases are present in appropriate cellular compartments as catalysts for oxidation or isomerization reactions (Freedman, 1995). The periplasm of *Escherichia coli* contains several factors forming the Dsb system, which help the disulfide-bonded proteins to reach their native conformation (Bardwell, 1994; Missiakas & Raina, 1997). Among them, DsbA (21 kDa) is particularly studied as it displays the strongest oxidizing properties yet characterized (Wunderlich & Glockshuber, 1993; Zapun et al., 1993) for a protein bearing the Cys-Xaa-Xaa-Cys motif typical for thioredoxin-like oxidoreductases (Chivers et al., 1996, 1997). Like other members of this family, the enzyme has two redox states, a reduced state E-(SH)₂ and an oxidized one E-S₂.

Biochemical studies have identified two features that might account predominantly for the mechanism of DsbA: (1) the extremely low pK_a of the thiol group of the N-terminal Cys30 in reduced DsbA, i.e., 3.5 (Nelson & Creighton, 1994), makes it highly nucleophilic and prone to leave its disulfide, and (2) the reduced enzyme is more stable toward denaturation than both the oxidized one (Wunderlich et al., 1993; Zapun et al., 1993) and the mixed disulfide intermediate with thiol-containing substrates (Zapun et al., 1993, 1994; Nelson & Creighton, 1994). The difference in stability of the two redox states has been suggested to arise either from an energetically unfavorable conformation of the oxidized form (Wunderlich et al., 1993), or from interactions that stabilize the thiol groups in the reduced form (Zapun et al., 1994). The crystal structure of oxidized DsbA was determined at 2 Å resolution (Martin et al., 1993), and recently refined to 1.7 Å (Guddat et al., 1997b). Despite very low sequence similarity with thioredoxin, a thioredoxin-like domain was found. An additional inserted 76 residues domain, composed essentially of α -helices, covers a part of the active site. While the conformations of the

Reprint requests to: Nadege Jamin, CEA-INSTN-CIE, 91191 Gif-sur-Yvette Cedex, France; e-mail: jamin@instndir.cea.fr.

residues that form the active site of oxidized DsbA and thioredoxin are the same, the presence of this extra domain leads to a different environment of the N-terminal active site Cys in the two proteins. In the absence of any structural information for reduced DsbA, a model for the stabilization of the Cys30 thiolate by electrostatic interactions in the reduced state was proposed from the structure of the oxidized enzyme (Gane et al., 1995). This model suggests that much of the redox potential difference between DsbA and thioredoxin arises from an altered stabilization of the N-terminal reactive thiolate. The fact that the disulfide stability alone might control the oxidizing power of DsbA was suggested after the drawing of a well-balanced thermodynamic cycle from analyses of the variation of free energies of unfolding on reduced and oxidized forms (Wunderlich et al., 1993; Zapun et al., 1993) and from the analysis of site-directed mutants in the Cys-Xaa-Xaa-Cys sequence (Grauschopf et al., 1995). The structural analysis of three His32 mutants of DsbA suggested that the critical role of this residue in the oxidizing power and the relative instability of the protein in its oxidized form was most likely electrostatic in nature (Guddat et al., 1997a). However, site-directed mutants made in order to probe the electrostatic model revealed that neither the kink of the active site helix, nor charged residues in its vicinity had a significant effect on the enzyme redox potential (Hennecke et al., 1997; Jacobi et al., 1997). Furthermore, the relationship between redox potential, pK_a of Cys30 and $\Delta\Delta G_{ox/red}$ proved difficult to satisfy using biochemical experiments.

To gain deeper insight into the molecular basis of the functional differences of these enzymes, and to compare in detail their structure and dynamics in both the reduced and oxidized states, we undertook an NMR study of DsbA in solution. High-resolution solution structures of oxidized and reduced *E. coli* glutaredoxin (Sodano et al., 1991; Xia et al., 1992), *E. coli* thioredoxin (Jeng et al., 1994), and human thioredoxin (Qin et al., 1994; Weichsel et al., 1996) have revealed that only minor structural differences exist between the two redox states of these enzymes and are localized within and around the active site. However, the active site of DsbA displays a fundamental difference from other related proteins due to its additional B domain, which might contribute significantly to the enzyme activity and dynamics. In this paper, we present the first step of this study, i.e., the NMR assignments of the peptide backbone resonances (1H , ^{13}C , and ^{15}N) and a comparison of the backbone chemical shifts of the oxidized and reduced forms, as the chemical shifts are very sensitive probes of small electronic environment differences.

Results

Two-dimensional ^{15}N - 1H chemical shifts correlation map

Two-dimensional 1H - ^{15}N HSQC (Heteronuclear Single Quantum Correlation) experiments, correlating the backbone and side-chain amide protons ($^1H^N$) to their attached ^{15}N , were recorded for both oxidized and reduced DsbA. A superimposition of these spectra is shown in Figure 1. Although most of the cross-peaks are clearly resolved, some are composed of two or three overlapping resonances: 23 and 18% of $^1H^N$ - ^{15}N connectivities are superposed in the two-dimensional 1H - ^{15}N HSQC spectra of reduced and oxidized samples, respectively (see Table 2 for a complete listing of the superpositions). Numerous residues (43/181) present significant $^1H^N$ and/or ^{15}N chemical shift variations between the two redox states (i.e., $\Delta\delta^1H^N > 0.1$ ppm; $\Delta\delta^{15}N > 0.3$ ppm). As a

result, some overlapped resonances observed in one oxidation state of DsbA were resolved in the other state, thus facilitating assignments. Twenty-one $^1H^N$ - ^{15}N connectivities are superposed in both oxidation states.

Finally, 179 of the expected 181 $^1H^N$ - ^{15}N backbone frequency pairs were assigned. The two missing pairs correspond to residues Gln2 and His32. No correlation between $^1H^N$ and ^{15}N could be found for these residues in the triple resonance experiments involving $^1H^N$ - ^{15}N polarization transfer steps, presumably either because the amide protons undergo rapid hydrogen exchange with the solvent or because of conformational exchange processes. His32 is, with Pro31, one of the two residues localized between the two active site cysteines. The amide protons of residues of the active site of *E. coli* thioredoxin and of the PDI-a domain are also apparently exchanging rapidly with the solvent (Chandrasekhar et al., 1994; Kemmink et al., 1995). The amide proton of Gly33, one residue of the active site of *E. coli* thioredoxin, was particularly difficult to observe (Chandrasekhar et al., 1991), while the amide protons of Gly37 and His38, residues of the active site of the PDI-a domain, were not observed. It has been suggested that strong electrostatic interactions in the active site region of thiol-disulfide oxidoreductases could alter the pK_a values of NH groups, resulting in fast hydrogen exchange of these amide protons with the solvent (Kemmink et al., 1995).

Main-chain sequential assignments

Triple and double resonance experiments were recorded to obtain $^1H^N$, ^{15}N , $^{13}C^\alpha$, $^1H^\alpha$, $^{13}C^\beta$, and ^{13}CO frequencies for all residues, and to connect the frequencies of residue i to those of residue $i + 1$. The list of these experiments and of the detected correlations is reported in Table 1.

The sequential assignment was started by picking cross-peaks in a two-dimensional 1H - ^{15}N HSQC spectrum of the reduced form of DsbA, to obtain the $^1H^N(i)$ and $^{15}N(i)$ resonance frequencies. The three-dimensional CT-HNCO experiment was then performed to separate overlapping peaks and to identify backbone $^1H^N(i)$, $^{15}N(i)$, and $^{13}CO(i - 1)$ correlations. Because of its high sensitivity and the absence of overlapping resonances in the spectrum (Table 1), the HNCO experiment was used as reference and each peak was assumed to correspond to one residue's spin system. HNCA, HN(CO)CA, CBCA(CO)NH, and (HCA)CO(CAN)NH experiments were used to determine and connect $^{13}C^\alpha(i)$, $^{13}C^\alpha(i - 1)$, $^{13}C^\beta(i - 1)$, and $^{13}CO(i)$ frequencies to $^1H^N(i)$ - $^{15}N(i)$ backbone frequency pairs. Then, using the HCACBCO experiment, $^1H^\alpha(i)$ and $^{13}C^\beta(i)$ frequencies were correlated to $^1H^N(i)$ - $^{15}N(i)$ backbone frequency pairs by fitting peak frequencies to $^{13}C^\alpha(i)$ and $^{13}CO(i)$ frequencies identified in the experiments mentioned previously. At this stage of the assignments, linking of adjacent residues was achieved by matching intraresidue ^{13}CO , $^{13}C^\alpha$, and $^{13}C^\beta$ chemical shifts of residue i with corresponding sequential ^{13}CO , $^{13}C^\alpha$, and $^{13}C^\beta$ chemical shifts of residue $(i + 1)$. A tolerance (in ppm) of about 60–70% of the point-to-point resolution (in Hertz/point) was used to match $^{13}C(i)$ and $^{13}C(i - 1)$ frequencies.

In the next assignment stage, Gly, Ala, Ser, and Thr were identified by their characteristic $^{13}C^\alpha$ and $^{13}C^\beta$ chemical shifts and used as starting points in the sequential assignment. The neighboring information, combined with the residue typing based on $^{13}C^\alpha$ and $^{13}C^\beta$ chemical shifts (Grzesiek & Bax, 1993b), then allowed placement of stretches of sequentially assigned resonances in the amino acid sequence. Using this procedure, sequential assignments

Remaining difficulties in the assignments for the reduced form essentially involved residues around the active site, i.e., Glu24 to Cys33 and Ile42 to Asn45. Therefore, the variations of ^1H N, ^{15}N ,

Table 1. Acquisition parameters and detected correlations for the three-dimensional NMR experiments recorded on uniformly enriched reduced and oxidized samples

Experiment	Carrier (ppm)	Mixing time (ms)	Data points	Scans/inc.	Spectral width (Hz)	% of detected correlations/expected correlations			% of overlapped correlations
						Correlation	Reduced	Oxidized	
¹⁵ N-TOCSY-HSQC	¹ H 4.71 ¹⁵ N 11 8.7	24	t1 224 (¹ H) t2 80 (¹⁵ N)	8	ω1 9,090 (¹ H) ω2 2,020 (¹⁵ N) ω3 5,050 (¹ H)				
¹⁵ N-NOESY-HSQC	¹ H 4.71 ¹⁵ N 11 8.7	120	t1 224 (¹ H) t2 80 (¹⁵ N)	8	ω1 9,090 (¹ H) ω2 2,020 (¹⁵ N) ω3 5,050 (¹ H)				
¹⁵ N-HMQC-NOESY-HSQC	¹ H 4.71 ¹⁵ N 11 8.7	120	t1 80 t2 80	8	ω1 2,020 (¹⁵ N) ω2 2,020 (¹⁵ N) ω3 5,050 (¹ H)				
HNHA	¹ H 4.71 ¹⁵ N 11 7.5		t1 34 (¹⁵ N) t2 104 (¹ H)	24	ω1 2,000 (¹⁵ N) ω2 5,200 (¹ H) ω3 8,500 (¹ H)	¹ H _(i) - ¹⁵ N _(i) -Hα _(i)	87.1	90.7	10
CT-HNCO	¹ H 4.71 ¹³ C 1 75.9 ¹⁵ N 11 7.5		t1 64 (¹⁵ N) t2 126 (¹³ C)	16	ω1 2,000 (¹⁵ N) ω2 2,500 (¹³ C) ω3 5,000 (¹ H)	¹ H _(i) - ¹⁵ N _(i) - ¹³ CO _(i-1)	98.3	98.9	0.0
CT-HN(CO)CA	¹ H 4.71 ¹³ C 57.7 ¹⁵ N 11 7.5		t1 64 (¹⁵ N) t2 104 (¹³ C)	16	ω1 2,000 (¹⁵ N) ω2 5,500 (¹³ C) ω3 5,000 (¹ H)	¹ H _(i) - ¹⁵ N _(i) - ¹³ Cα _(i-1)	95.0		2.3
HNCA	¹ H 4.71 ¹³ C 57.7 ¹⁵ N 11 7.5		t1 64 (¹⁵ N) t2 120 (¹³ C)	16	ω1 2,000 (¹⁵ N) ω2 5,500 (¹³ C) ω3 5,000 (¹ H)	¹ H _(i) - ¹⁵ N _(i) - ¹³ Cα _(i-1) ¹ H _(i) - ¹⁵ N _(i) - ¹³ Cα _(i)	97.8 95.6	97.8 95.0	19
CBCA(CO)NH	¹ H 4.71 ¹³ C 43.7 ¹⁵ N 11 7.5		t1 122 (¹³ C) t2 64 (¹⁵ N)	32	ω1 9,300 (¹³ C) ω2 2,000 (¹⁵ N) ω3 5,000 (¹ H)	¹⁵ N _(i) - ¹³ Cα _(i-1) ¹ H _(i) - ¹⁵ N _(i) - ¹³ Cβ _(i-1)	98.3 96.4	98.3 97.6	7.5
(HCA)CO(CAN)NH	¹ H 4.71 ¹³ C 1 75.9 ¹⁵ N 11 7.5		t1 96 (¹³ C) t2 64 (¹⁵ N)	48	ω1 2,500 (¹³ C) ω2 2,000 (¹⁵ N) ω3 5,000 (¹ H)	¹⁵ N _(i) - ¹³ CO _(i-1) ¹ H _(i) - ¹⁵ N _(i) - ¹³ CO _(i)	96.7 77.3	94.5 74.6	16
HCACBCO	¹ H 4.71 ¹³ Cαβ 43.7 ¹³ CO 1 77.5		t1 104 (¹³ Cαβ) t2 128 (¹³ CO)	16	ω1 9,300 (¹³ Cαβ) ω2 2,500 (¹³ CO) ω3 1,800 (¹ H)	Hα _(i) -Cα _(i) - ¹³ CO _(i) Hα _(i) -Cβ _(i) - ¹³ CO _(i)	92.0 90.9	96.6 95.5	11

Overlapping resonances in the two-dimensional ¹H^N-¹⁵N correlation map:

For both oxidized and reduced DsbA: Ile102 and Arg109; Leu50 and Glu121; Asp107 and Val111; Ala 115, Ala124, and Ala143; Lys132 and Val182; Asp172 and Ala179; Val130 and Thr181; Lys47 and Asn162; Glu4 and Val16; Ala75 and Met171.

For the reduced state of DsbA: Cys30 and Lys188; Gln35 and Gln138; Asp44 and Asp71; Val78, Val96, and Ala141; Tyr122 and Ala152; Leu40 and Ser106; Glu52 and Met153; Val90 and Gln177; Phe26 and Asp180; Phe25 and His60.

For the oxidized state of DsbA: Val39 and Met64; Val54 and Lys70; Lys14 and Lys87; Thr73 and Val96; Leu68 and Tyr184; His60 and Ala105.

Fig. 2. Strips along the ^{13}C axes of the (A) (a) HNCO, (b) (HCA)CO(CAN)NH; (B) HNCA; (C) (a) CBCA(CO)NH, (b) HCACBCO spectra showing sequential connectivities for residues Ser27 to Cys30 (left and right panels for the reduced and oxidized samples, respectively).

Table 2. ^1H , ^{13}C , and ^{15}N backbone and $^{13}\text{C}^\beta$ chemical shifts (in ppm) for oxidized and reduced DsbA

Residue	Oxidized DsbA						Reduced DsbA					
	$^1\text{H}^\text{N}$	^{15}N	$^{13}\text{C}^\alpha$	$^{13}\text{C}^\beta$	^{13}CO	$^1\text{H}^\alpha$	$^1\text{H}^\text{N}$	^{15}N	$^{13}\text{C}^\alpha$	$^{13}\text{C}^\beta$	^{13}CO	$^1\text{H}^\alpha$
Ala1			51.5	19.3	173.2	4.00			51.5	19.2	173.1	4.00
Gln2			56.1	29.4	174.6	4.07			56.0	29.4	174.5	4.06
Tyr3	7.49	119.5	56.8	39.8	175.5	4.59	7.48	119.4	56.8	39.9	175.4	4.58
Glu4	8.85	122.4	55.1	33.5	174.6	4.68	8.85	122.4	55.0	33.6	174.5	4.68
Asp5	8.34	125.2	55.2	41.1	177.1	3.38	8.33	125.1	55.1	41.1	177.1	3.37
Gly6	8.86	116.1	44.9		173.5	3.54, 4.42	8.86	116.0	44.9		173.4	3.50, 4.42
Lys7	8.01	121.2	56.8	31.8	175.4	4.64	8.02	121.2	56.9	31.8	175.3	4.63
Gln8	8.36	115.2	59.5	27.0	174.1	4.21	8.36	115.2	59.5	27.1	174.0	4.20
Tyr9	7.45	111.3	55.2	40.9	172.3	5.26	7.45	111.1	55.2	41.0	172.2	5.26
Thr10	9.40	111.8	60.3	71.4	174.5	4.96	9.41	111.6	60.3	71.4	174.4	4.96
Thr11	8.91	120.9	63.0	69.3	175.2	5.06	8.90	120.8	62.9	69.2	175.2	5.07
Leu12	8.63	128.6	55.7	41.7	177.3	4.27	8.66	128.7	55.8	41.7	177.2	4.26
Glu13	8.51	122.4	58.6	29.8	176.7	4.04	8.51	122.3	58.7	29.8	176.6	4.04
Lys14	7.68	117.0	52.4	33.0	172.7	4.85	7.68	117.0	52.3	33.0	172.5	4.85
Pro15			62.3	32.6	176.3	4.56			62.3	32.6	176.3	4.56
Val16	8.89	122.5	61.5	32.5	176.0	4.04	8.88	122.5	61.5	32.6	175.9	4.04
Ala17	8.42	131.4	52.5	18.6	178.9	4.31	8.41	131.4	52.5	18.7	178.8	4.31
Gly18	8.63	109.8	45.7		174.2	3.83, 4.70	8.63	109.7	45.7		174.1	3.84, 4.73
Ala19	7.32	122.0	50.4	17.1	175.3	4.53	7.32	121.9	50.4	17.1	175.2	4.53
Pro20			62.1	31.8	175.6	4.44			62.2	31.9	175.6	4.43
Gln21	8.32	121.3	60.2	29.5	176.6	4.11	8.33	121.2	60.3	29.6	176.6	4.10
Val22	7.62	115.6	62.7	33.5	173.0	4.57	7.58	115.4	62.8	33.5	172.9	4.57
Leu23	8.74	128.8	52.8	46.2	173.5	5.08	8.78	128.7	52.7	46.3	173.4	5.12
Glu24	9.00	127.6	53.4	33.0	174.8	5.69	8.99	128.0	52.9	33.0	174.6	5.76
Phe25	9.06	121.8	56.4	43.8	174.8	6.05	9.00	122.5	56.4	43.7	175.0	6.03
Phe26	8.82	118.5	55.1	43.3	170.6	4.78	8.72	118.1	55.1	42.7	170.4	5.09
Ser27	6.48	108.7	54.8	64.5	177.0	3.91	7.51	110.9	55.0	65.3	177.1	4.11
Phe28	11.19	130.6	62.9	38.5	176.3	4.42	11.26	129.0	62.3	38.7	176.3	4.30
Phe29	8.60	116.0	59.1	40.0	175.1	4.38	8.50	114.9	59.3	41.0	175.5	4.28
Cys30	7.10	119.5	52.4	45.4	174.0	5.03	7.11	122.5	56.7	31.6	176.9	4.90
Pro31			65.5	32.0	180.5	5.07			64.3	31.9	180.5	5.13
His32			58.4	29.7	177.2	4.60			58.4	28.8	179.3	4.76
Cys33	8.84	116.7	63.4	33.5	175.1	4.52	10.31	129.5	64.4	29.5	176.2	4.22
Tyr34	7.58	122.3	60.4	37.6	177.2	2.95	8.19	120.7	62.0	37.5	177.3	2.89
Gln35	7.67	118.1	58.7	27.9	178.3	3.75	7.70	118.4	58.8	28.2	178.0	3.81
Phe36	8.40	120.5	60.9	39.3	175.8	4.00	8.46	120.9	61.6	40.8	175.5	3.91
Glu37	7.40	114.1	57.9	31.1	177.0	4.37	7.98	113.4	57.7	31.6	177.1	4.23
Glu38	7.84	112.2	55.7	29.4	175.6	4.05	7.79	111.4	55.3	28.8	175.6	3.93
Val39	6.86	116.5	64.2	32.2	176.7	4.01	6.71	116.6	64.2	32.2	176.7	4.02
Leu40	7.94	114.9	55.2	42.1	176.8	4.14	8.18	115.2	55.0	42.4	176.8	4.15
His41	6.23	109.8	55.5	25.6	176.8	4.52	6.10	109.2	55.3	25.7	176.9	4.47
Ile42	8.37	119.3	65.0	35.2	178.0	3.32	8.42	118.6	65.7	35.0	178.1	3.28
Ser43	7.64	113.6	62.7	61.2	176.2	3.86	7.12	111.7	62.0	61.4	176.5	4.18
Asp44	7.92	120.8	57.5	40.9	178.5	4.39	7.88	121.6	57.6	40.8	178.5	4.41
Asn45	7.74	117.1	57.4	39.6	177.4	4.34	7.68	117.5	57.4	39.6	177.4	4.33
Val46	8.52	119.7	66.7	31.4	178.0	3.54	8.50	119.6	66.9	31.4	178.0	3.52
Lys47	8.23	118.2	60.2	32.5	178.9	3.85	8.20	118.0	60.3	32.6	178.7	3.85
Lys48	7.26	114.9	58.3	32.8	177.4	4.17	7.23	114.9	58.3	32.9	177.3	4.17
Lys49	7.28	116.2	55.4	34.1	176.0	4.44	7.29	116.1	55.4	34.3	175.9	4.44
Leu50	7.05	119.7	53.3	40.9	174.9	4.41	7.03	119.7	53.3	40.7	174.9	4.38
Pro51			62.1	31.8	176.1	4.50			62.2	31.9	175.9	4.50
Glu52	8.32	120.7	58.2	29.4	177.7	4.07	8.31	120.7	58.1	29.4	177.6	4.07
Gly53	8.57	111.2	44.9		174.1	3.74, 4.21	8.59	111.2	45.0		173.9	3.70, 4.24
Val54	7.49	121.5	62.3	31.6	174.4	4.12	7.55	121.7	62.4	31.7	174.3	4.13
Lys55	8.05	126.4	54.7	34.6	175.4	4.59	8.03	126.3	54.6	34.8	175.3	4.59
Met56	8.66	122.0	54.7	34.6	175.1	4.96	8.70	122.2	54.4	34.7	175.0	5.01
Thr57	8.68	124.3	62.9	70.4	171.8	4.39	8.71	124.7	62.8	70.5	171.6	4.39
Lys58	8.97	126.1	54.6	35.7	175.3	5.45	9.02	125.3	54.3	35.7	175.2	5.43
Tyr59	9.25	123.6	51.9	40.5	173.2	5.68	9.42	123.5	52.0	40.4	173.0	5.67
His60	9.09	123.0	56.2	30.4	174.9	4.18	9.03	122.6	56.1	30.4	175.0	4.25

(continued)

Table 2. Continued

Residue	Oxidized DsbA						Reduced DsbA					
	$^1\text{H}^{\text{N}}$	^{15}N	$^{13}\text{C}^{\alpha}$	$^{13}\text{C}^{\beta}$	^{13}CO	$^1\text{H}^{\alpha}$	$^1\text{H}^{\text{N}}$	^{15}N	$^{13}\text{C}^{\alpha}$	$^{13}\text{C}^{\beta}$	^{13}CO	$^1\text{H}^{\alpha}$
Val61	6.13	111.8	58.2	33.8	176.0	4.62	6.15	111.9	58.1	33.9	175.7	4.56
Asn62	8.99	117.5	52.5	39.0	177.9	4.56	8.82	117.3	52.2	39.0	177.7	4.58
Phe63	6.30	111.4	58.0	37.4	174.8	4.62	6.37	111.9	57.8	37.6	174.8	4.60
Met64	6.85	116.5	55.0	35.4	175.9	4.75	6.93	116.4	54.9	35.4	175.7	4.68
Gly65	8.41	109.1	44.0		175.3	4.71	8.32	108.6	43.8		175.3	4.70
Gly66	8.29	110.1	45.5		176.7	3.73, 3.98	8.30	110.3	45.6		176.6	3.71, 3.92
Asp67	8.93	127.2	57.8	39.8	178.2	4.42	8.93	127.3	57.7	39.8	178.1	4.42
Leu68	7.94	119.6	56.7	42.0	179.2	4.50	7.94	119.8	56.8	41.8	179.1	4.49
Gly69	7.50	106.5	48.1		176.3	4.03, 4.27	7.46	106.2	48.0		176.1	4.06, 4.14
Lys70	7.45	121.2	59.7	31.6	179.7	4.32	7.41	121.2	59.7	31.7	179.8	4.32
Asp71	7.82	121.5	58.3	40.6	179.3	4.82	7.87	121.5	58.3	40.4	179.2	4.84
Leu72	8.71	120.0	57.8	42.4	178.4	4.22	8.72	120.2	57.6	42.2	178.4	4.23
Thr73	7.85	118.8	67.0	68.5	176.4	4.01	7.91	118.8	67.2	68.7	176.3	4.01
Gln74	7.77	124.3	59.5	25.9	177.4	3.67	7.80	124.3	59.5	26.1	177.4	3.67
Ala75	9.04	122.5	54.9	19.6	179.6	4.18	9.07	122.6	54.9	19.6	179.5	4.18
Trp76	8.97	120.0	59.0	29.6	177.6	4.91	9.02	120.1	59.0	29.6	177.6	4.92
Ala77	7.66	119.7	55.2	20.0	178.9	4.07	7.67	119.7	55.3	20.0	178.8	4.07
Val78	8.02	119.1	67.4	30.1	177.2	3.26	8.01	119.0	67.3	30.1	177.2	3.26
Ala79	8.55	120.7	55.0	17.5	179.7	4.03	8.59	120.7	55.0	17.5	179.7	4.04
Met80	8.08	115.6	58.6	34.4	179.3	4.03	8.09	115.6	58.6	34.5	179.2	4.04
Ala81	8.56	123.3	54.9	17.8	180.0	4.03	8.55	123.4	54.8	17.8	179.9	4.03
Leu82	8.26	113.4	54.7	42.9	177.5	4.42	8.26	113.3	54.6	42.8	177.4	4.41
Gly83	7.64	110.2	47.1		176.8	4.02, 4.34	7.65	109.9	47.1		176.8	4.00, 4.32
Val84	8.04	108.5	59.5	31.6	176.6	4.94	8.06	108.4	59.4	31.6	176.5	4.93
Glu85	10.24	125.5	61.6	29.4	179.2	3.55	10.13	125.3	61.5	29.6	179.0	3.54
Asp86	8.81	116.0	56.3	39.7	177.4	4.63	8.78	115.7	56.2	39.8	177.3	4.64
Lys87	7.70	116.8	57.3	34.0	177.9	4.50	7.72	116.7	57.4	34.0	177.8	4.49
Val88	6.96	103.9	61.0	32.6	176.0	4.39	6.97	103.9	61.0	32.6	175.9	4.40
Thr89	7.56	119.6	69.4	68.8	175.0	4.17	7.53	119.4	69.5	68.9	174.8	4.19
Val90	8.85	119.4	68.8	28.7	175.9	4.13	8.72	119.1	68.7	28.6	175.7	4.13
Pro91			65.5	31.4	180.8	4.44			65.7	31.2	180.8	4.44
Leu92	7.65	119.1	58.2	40.0	177.8	3.97	7.61	119.3	58.1	39.9	177.6	3.99
Phe93	8.22	119.2	63.9	39.5	180.0	4.62	8.18	119.0	64.2	39.8	180.0	4.64
Glu94	9.09	115.8	59.7	29.6	180.0	4.01	8.98	115.0	59.4	29.7	180.1	4.01
Gly95	8.36	107.6	46.5		175.6	3.49, 4.28	8.40	107.6	46.4		175.8	3.51, 4.27
Val96	7.84	118.6	66.2	31.9	177.6	3.83	7.98	118.9	66.4	31.9	177.6	3.79
Gln97	8.10	112.4	58.3	30.6	176.8	4.42	7.87	111.8	58.7	31.1	176.7	4.52
Lys98	8.18	117.8	57.5	33.2	177.5	4.67	8.18	117.7	57.0	32.7	177.5	4.66
Thr99	8.41	107.6	62.1	68.4	175.4	4.32	8.47	107.7	62.3	68.4	175.4	4.33
Gln100	6.63	114.2	57.4	25.7	175.4	4.12	6.62	113.9	57.5	25.7	175.2	4.10
Thr101	7.96	106.1	61.2	68.8	175.2	4.26	7.95	105.8	61.1	68.7	175.1	4.25
Ile102	6.98	120.6	60.7	36.6	174.1	4.21	6.99	120.4	60.8	36.8	174.0	4.22
Arg103	9.39	125.6	55.2	32.3	175.5	4.63	9.41	125.6	55.2	32.2	175.4	4.63
Ser104	8.18	114.3	56.8	67.0	174.5	4.88	8.16	114.2	56.8	67.0	174.5	4.88
Ala105	9.08	123.2	55.2	16.8	180.7	3.98	9.07	123.1	55.2	16.8	180.6	3.98
Ser106	8.19	115.0	61.3	62.1	176.1	4.70	8.20	114.9	61.5	62.2	176.1	4.69
Asp107	7.29	119.8	56.9	42.7	178.8	4.47	7.30	119.8	56.9	42.7	178.7	4.46
Ile108	7.19	119.1	65.8	38.1	176.9	3.36	7.21	119.1	65.7	38.1	176.9	3.36
Arg109	7.02	120.3	59.3	29.4	177.8	2.86	7.03	120.3	59.3	29.5	177.7	2.85
Asp110	7.50	116.8	57.6	40.0	178.4	4.37	7.49	116.7	57.6	40.0	178.3	4.37
Val111	7.32	119.7	66.4	30.6	178.9	3.66	7.33	119.7	66.4	30.7	178.8	3.65
Phe112	7.54	120.0	61.9	38.3	177.8	3.93	7.56	120.0	62.0	38.3	177.7	3.93
Ile113	8.21	121.5	63.5	37.5	181.9	4.76	8.21	121.6	63.6	37.5	181.8	4.76
Asn114	8.37	119.9	55.5	38.1	176.1	4.55	8.36	119.8	55.5	38.1	176.0	4.54
Ala115	7.48	120.7	51.8	18.4	176.9	4.49	7.47	120.7	51.8	18.4	176.8	4.49
Gly116	7.71	105.0	45.1		174.0	3.72, 4.40	7.71	104.8	45.1		173.9	3.72, 4.38
Ile117	7.93	124.4	60.4	37.3	175.1	4.29	7.93	124.3	60.3	37.2	175.0	4.29
Lys118	8.53	125.3	56.7	32.6	179.1	4.23	8.54	125.3	56.8	32.7	179.1	4.23
Gly119	9.15	112.9	48.2	0.0	175.1	3.48, 3.89	9.15	112.8	48.2	0.0	175.0	3.43, 3.88

(continued)

Table 2. Continued

Residue	Oxidized DsbA						Reduced DsbA					
	¹ H ^N	¹⁵ N	¹³ C ^α	¹³ C ^β	¹³ CO	¹ H ^α	¹ H ^N	¹⁵ N	¹³ C ^α	¹³ C ^β	¹³ CO	¹ H ^α
Glu120	9.24	117.0	59.7	28.7	179.9	4.27	9.25	117.0	59.6	28.8	179.8	4.27
Glu121	7.00	119.8	58.4	29.5	179.1	4.25	7.01	119.8	58.4	29.6	179.0	4.25
Tyr122	8.21	122.2	62.6	38.0	176.7	3.72	8.21	122.1	62.7	38.0	176.7	3.71
Asp123	8.67	118.7	57.3	39.5	178.7	4.28	8.67	118.6	57.3	39.5	178.6	4.28
Ala124	7.49	120.5	54.5	17.8	180.5	4.23	7.49	120.5	54.6	17.8	180.5	4.23
Ala125	7.73	119.7	54.9	18.1	181.0	4.09	7.75	119.6	54.7	18.2	181.0	4.09
Trp126	9.06	120.8	61.4	28.5	175.3	3.49	9.07	120.8	61.4	28.4	175.3	3.49
Asn127	6.86	109.9	53.0	40.1	174.8	4.80	6.88	109.8	53.0	40.1	174.7	4.80
Ser128	7.68	115.9	58.7	65.5	175.4	4.48	7.67	115.8	58.7	65.3	175.3	4.48
Phe129	8.93	123.3	61.4	38.4	178.5	4.29	8.93	123.7	61.3	38.5	178.4	4.29
Val130	8.16	119.2	66.0	31.7	178.0	3.72	8.16	119.1	65.9	31.7	177.9	3.71
Val131	7.09	121.6	67.2	31.3	177.0	3.53	7.10	121.5	67.2	31.3	176.9	3.52
Lys132	7.68	121.1	60.0	31.7	180.2	3.78	7.68	121.1	59.9	31.6	180.1	3.78
Ser133	8.14	114.8	61.4	62.5	176.8	4.18	8.15	114.7	61.2	62.5	176.7	4.18
Leu134	8.04	123.4	57.6	43.8	181.2	4.21	8.04	123.5	57.6	43.7	181.1	4.21
Val135	8.52	122.8	69.0	31.2	177.0	3.33	8.51	122.8	68.9	31.2	177.0	3.33
Ala136	7.35	120.8	55.0	17.4	180.7	4.23	7.33	120.8	55.2	17.4	180.7	4.23
Gln137	8.44	117.9	59.1	29.2	179.0	4.08	8.44	117.9	59.1	29.3	178.9	4.07
Gln138	7.72	118.7	59.9	30.7	179.5	3.54	7.71	118.6	60.0	30.5	179.4	3.54
Glu139	8.00	120.4	59.8	29.9	179.6	4.02	7.91	120.1	59.8	29.9	179.5	4.03
Lys140	8.52	121.4	58.8	32.5	177.7	4.12	8.52	121.4	58.9	32.6	177.7	4.12
Ala141	7.96	119.1	55.2	18.1	179.2	4.38	8.02	119.0	55.2	18.2	179.1	4.38
Ala142	7.15	116.5	54.5	17.7	179.0	3.57	7.11	116.6	54.4	17.7	178.9	3.54
Ala143	7.50	120.4	54.7	17.7	182.0	4.20	7.44	120.3	54.5	17.8	182.0	4.20
Asp144	8.93	120.4	57.3	40.3	178.2	4.42	8.92	120.4	57.4	40.3	178.2	4.42
Val145	7.00	108.3	60.3	30.8	174.2	4.23	7.05	108.2	60.5	30.8	174.2	4.21
Gln146	7.59	117.9	56.1	26.0	175.4	3.75	7.58	117.8	56.0	26.1	175.3	3.73
Leu147	7.43	117.8	56.4	43.0	178.1	3.94	7.43	117.8	56.3	43.0	178.1	3.92
Arg148	8.98	123.4	55.1	31.6	175.8	4.25	9.00	123.7	55.1	31.6	175.8	4.31
Gly149	7.09	105.6	45.0		171.0	4.01, 4.30	7.08	105.4	45.0		170.8	4.00, 4.30
Val150	8.04	109.2	57.9	35.0	173.1	4.48	8.02	109.1	58.0	34.7	172.6	4.43
Pro151			62.1	35.0	175.8	4.91			62.0	34.3	176.2	4.92
Ala152	8.12	122.0	51.8	25.0	174.9	4.84	8.16	122.3	51.4	25.1	175.0	4.82
Met153	8.41	120.9	55.1	36.9	173.3	5.89	8.31	120.3	55.1	36.9	173.3	5.88
Phe154	9.55	123.7	56.1	43.6	176.4	5.86	9.55	123.2	56.0	43.6	176.5	5.83
Val155	9.40	122.4	60.7	34.7	175.9	5.22	9.44	122.5	60.8	34.9	175.8	5.22
Asn156	10.21	126.8	54.6	37.4	174.2	4.70	10.25	127.0	54.5	37.4	174.1	4.69
Gly157	9.26	105.0	46.4		172.5	3.36, 4.21	9.26	104.8	46.4		172.4	3.35, 4.23
Lys158	7.77	114.2	57.1	37.1	174.3	4.55	7.78	114.1	57.2	37.1	174.2	4.55
Tyr159	7.83	116.9	55.2	43.1	174.6	5.88	7.85	116.9	55.2	43.0	174.5	5.82
Gln160	9.45	124.3	53.5	30.6	175.6	4.80	9.48	124.4	53.5	30.8	175.4	4.80
Leu161	8.18	130.2	54.6	40.5	177.3	4.06	8.19	130.7	54.1	40.1	177.2	4.04
Asn162	8.26	118.2	49.9	38.5	172.5	5.54	8.23	118.0	49.8	38.5	172.3	5.53
Pro163			64.8	32.1	177.6	4.81			64.8	32.2	177.5	4.81
Gln164	7.85	114.5	58.0	28.0	177.0	4.26	7.83	114.3	57.9	28.0	177.0	4.25
Gly165	7.83	106.6	44.7		173.7	3.74, 4.35	7.81	106.3	44.7		173.7	3.73, 4.36
Met166	7.25	118.8	54.7	34.4	174.4	4.68	7.25	118.8	54.7	34.5	174.3	4.68
Asp167	8.50	120.6	54.4	40.5	175.7	4.66	8.50	120.4	54.2	40.4	175.6	4.66
Thr168	7.70	111.9	60.5	68.5	175.6	4.20	7.70	111.9	60.6	68.6	175.5	4.20
Ser169	8.56	117.9	61.2	63.3	174.4	4.18	8.55	117.7	61.1	63.2	174.4	4.17
Asn170	7.21	117.0	52.5	40.5	175.5	4.82	7.20	116.9	52.6	40.4	175.4	4.81
Met171	8.99	122.6	57.6	32.5	177.5	4.59	9.01	122.6	57.6	32.6	177.4	4.59
Asp172	8.07	119.4	57.6	40.0	179.2	4.51	8.07	119.4	57.6	40.1	179.2	4.50
Val173	7.64	120.8	65.8	31.8	177.7	3.79	7.64	120.7	65.7	31.9	177.7	3.79
Phe174	8.12	121.2	61.6	39.2	176.4	4.40	8.13	121.3	61.5	39.3	176.4	4.40
Val175	7.94	116.2	67.0	31.6	177.6	3.09	7.97	116.3	67.0	31.7	177.6	3.08
Gln176	7.25	116.5	58.5	28.0	178.1	4.02	7.24	116.3	58.4	28.0	178.0	4.02
Gln177	8.64	119.1	59.3	28.2	179.6	4.16	8.66	119.3	59.3	28.3	179.6	4.16
Tyr178	8.84	125.6	61.8	38.1	178.2	3.64	8.83	125.6	61.7	38.1	178.3	3.64
Ala179	8.09	119.3	55.6	18.0	179.4	4.03	8.06	119.3	55.6	18.0	179.4	4.04

(continued)

Table 2. Continued

Residue	Oxidized DsbA						Reduced DsbA					
	$^1\text{H}^{\text{N}}$	^{15}N	$^{13}\text{C}^{\alpha}$	$^{13}\text{C}^{\beta}$	^{13}CO	$^1\text{H}^{\alpha}$	$^1\text{H}^{\text{N}}$	^{15}N	$^{13}\text{C}^{\alpha}$	$^{13}\text{C}^{\beta}$	^{13}CO	$^1\text{H}^{\alpha}$
Asp180	8.71	118.2	57.4	40.0	179.3	4.51	8.73	118.2	57.5	39.9	179.2	4.50
Thr181	8.16	119.4	67.5	67.0	176.2	3.54	8.18	119.4	67.4	67.0	176.2	3.54
Val182	7.68	120.9	67.6	30.6	177.6	3.23	7.70	120.8	67.7	30.7	177.6	3.26
Lys183	7.74	120.1	60.3	32.1	178.2	3.70	7.74	120.0	60.4	32.0	178.2	3.69
Tyr184	7.90	119.6	60.5	38.0	177.7	4.06	7.89	119.5	60.6	37.9	177.7	4.07
Leu185	8.00	117.3	56.6	42.1	179.0	3.26	8.00	117.3	56.5	42.1	179.0	3.26
Ser186	8.32	114.4	61.9	62.4	175.4	3.89	8.32	114.3	61.9	62.5	175.3	3.88
Glu187	6.93	118.8	56.2	30.2	176.1	4.29	6.95	118.8	56.1	30.3	176.1	4.29
Lys188	7.12	122.4	56.6	32.3	175.7	4.06	7.13	122.3	56.7	32.4	175.6	4.04
Lys189	7.77	128.4	57.7	33.2	181.6	4.16	7.76	128.4	57.5	33.2	181.5	4.16

$^{13}\text{C}^{\alpha}$, $^{13}\text{C}^{\beta}$, and ^{13}CO chemical shifts upon oxidation were used to resolve overlapping resonances and confirm the assignments. $^1\text{H}^{\text{N}}(i)$, $^{15}\text{N}(i)$, $^{13}\text{CO}(i-1)$, $^{13}\text{C}^{\alpha}(i)$, $^{13}\text{C}^{\alpha}(i-1)$, and $^{13}\text{C}^{\beta}(i-1)$ resonances were assigned for the oxidized form using HNCO, HNCA, and CBCA(CO)NH spectra. The correspondence of these frequencies for a given residue in oxidized and reduced form was established for all residues, except residues undergoing large chemical shift deviations. The assignments were then completed for the oxidized form using HNCA and CBCA(CO)NH experiments benefiting from resolved resonances. (HCA)CO(CAN)NH and HCACBCO experiments recorded for the oxidized sample confirmed the assignments and yielded $^{13}\text{CO}(i)$, $^{13}\text{C}^{\beta}(i)$, and $^1\text{H}^{\alpha}(i)$ frequencies for the oxidized form. Slices of triple resonance spectra used for sequential assignments of residues 27 to 30 of oxidized and reduced DsbA are displayed in Figure 2.

$^{13}\text{C}^{\alpha}$, $^{13}\text{C}^{\beta}$, ^{13}CO , and $^1\text{H}^{\alpha}$ frequencies for residues Ala1, Gln2, His32, and for the seven prolines, were obtained from the HCACBCO experiment. At this stage of the assignment process, missing $^1\text{H}^{\alpha}$ frequencies remained for some residues, especially for the 13 Gly which were filtered out in the HCACBCO experiment. HNHA and ^{15}N -separated NOESY-HSQC spectra provided the missing $^1\text{H}^{\alpha}$ frequencies and constituted an additional check for $^1\text{H}^{\alpha}$ chemical shifts, especially when overlap occurred in the HCACOCB experiment. For Gly65, Thr73, Ser106, and Arg109 $^1\text{H}^{\alpha}$, tentative assignments were made using the ^{15}N -separated NOESY-HSQC as no correlation involving these $^1\text{H}^{\alpha}$ frequencies was found in the HCACOCB and HNHA experiments.

A list of the chemical shifts of $^1\text{H}^{\text{N}}$, ^{15}N , $^{13}\text{C}^{\alpha}$, $^1\text{H}^{\alpha}$, $^{13}\text{C}^{\beta}$, and ^{13}CO is given in Table 2.

Solution secondary structure of DsbA

The secondary structure of DsbA was deduced from analysis of secondary chemical shifts, sequential NOE connectivity patterns, $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ and amide proton exchange data (Fig. 3).

Using the nearly complete $^{13}\text{C}^{\alpha}$, $^1\text{H}^{\alpha}$, $^{13}\text{C}^{\beta}$, and ^{13}CO signal assignments, a consensus Chemical Shift Index (CSI) was calculated for both reduced and oxidized DsbA using the Chemical Shift Index program (Wishart et al., 1992; Wishart & Sykes, 1994). A strong correlation exists between the chemical shift of these nuclei and the secondary structure of the residue involved. The deviation of the chemical shift from random coil values allows prediction of

the secondary structure. The α -helices and β -strands identified by this method for oxidized DsbA are reported in Figure 3A.

Sequential and medium-range NOEs obtained from the three-dimensional ^{15}N -separated NOESY-HSQC also provide information about the secondary structure (Wüthrich, 1986). For α -helices, relatively strong $^1\text{H}^{\text{N}}(i)$ - $^1\text{H}^{\text{N}}(i+1)$ and weak $^1\text{H}^{\alpha}(i)$ - $^1\text{H}^{\text{N}}(i+1)$ NOEs are observed, as well as $^1\text{H}^{\alpha}(i)$ - $^1\text{H}^{\text{N}}(i+3)$ NOEs. In the case of β -strands, relatively weak $^1\text{H}^{\text{N}}(i)$ - $^1\text{H}^{\text{N}}(i+1)$ and strong $^1\text{H}^{\alpha}(i)$ - $^1\text{H}^{\text{N}}(i+1)$ NOEs are found but no $^1\text{H}^{\alpha}(i)$ - $^1\text{H}^{\text{N}}(i+3)$ NOEs. Interstrand long-range $^1\text{H}^{\alpha}$ - $^1\text{H}^{\text{N}}$ NOEs allow the determination of the topology of the β -sheet and help define the ends of the β -strands. The residues that make up the β -sheet region of oxidized DsbA and a summary of medium- and long-range inter-strand NOEs as well as hydrogen exchange data are shown in Figure 4. Slow exchange values are found for the hydrogen bonded amide protons of regular secondary structures. Finally, the $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ coupling constants are small in α -helices and large in β -strands, as they are correlated to the torsion angle Φ values (Karplus, 1959), and constitute an additional parameter used for the identification of the secondary structure elements.

Discussion

The solution secondary structure deduced from the NMR parameters (NOEs, $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$, amide proton exchange data, and CSI) agrees well with that determined by X-ray crystallography (Fig. 3A). Discrepancies between the CSI prediction and the X-ray structure were seen in the exact positions of the initiation and termination of some secondary structural elements, the length of helix $\alpha 3$ which is shortened by half, and the length of strand $\beta 4$ which is predicted to be three residues longer. The exact positions of the secondary structural elements differ by one or two residues in about half of the elements. Uncertainties of this nature are common and reflect the difficulties of defining precisely the ends of the secondary structural elements. In terms of the length of helix $\alpha 3$, CA, and CO CSI predict a helix for the C-terminal part of segment 85–98 (residues 89–98 and 91–98 for CA and CO CSI, respectively), but both the NOEs and $^3J_{\text{H}^{\text{N}}\text{H}^{\alpha}}$ indicate the presence of a helix for the segment 85–98 (Fig. 3A). The disagreement between the CSI-predicted and X-ray secondary structure for segment 85–90 could reflect the distortion of the N-terminal part of the helix $\alpha 3$ induced by Pro91 as seen in the X-ray structure. High

Fig. 3. Summary of the sequential and medium-range NOEs involving the $^1\text{H}^N$ and $^1\text{H}^\alpha$ protons, the amide exchange and $^3J_{HNH\alpha}$ and the consensus Chemical Shift Index (CSI) with the secondary structure derived from this index for (A) oxidized DsbA and (B) reduced DsbA. The thickness of the line reflects the strength of the NOEs. The open boxes represent potential sequential assignment NOEs that could not be assigned unambiguously due to resonance overlap. Amide protons still present 48 h after exchange to D₂O are indicated by closed circles, and intermediate exchange rates by open circles. For $^3J_{HNH\alpha}$, the thickness of the line reflects the amplitude of the coupling constant ($^3J_{HNH\alpha} < 5$ Hz, 5 Hz $< ^3J_{HNH\alpha} < 8$ Hz, $^3J_{HNH\alpha} > 8$ Hz). The secondary structure of oxidized DsbA (Martin et al., 1993) is represented on the bottom line according to the symbolism of PROCHECK (Laskowski et al., 1993): H, helix; E, sheet; G, 3_{10} -helix; T, hydrogen-bonded turn; S, bends. Lowercase letters refer to extensions of the aforementioned secondary structure. (Figure continues on next page.)

values of $^3J_{HNH\alpha}$ for residues Lys87 and Val88 are in agreement with Φ values, -99.3 and -103.0 , respectively, found in the X-ray structure. For strand β 4, the extension of the N-terminal part predicted by the CSI method agrees with the Φ and Ψ values reported for residues Gly149 and Val150 in the X-ray structure (Gly149: $\Phi = -164.3$, $\Psi = 156.9$; Val150: $\Phi = -136.3$, $\Psi = 157.1$). These values fall in the range of Φ and Ψ angle values found in β -strands. However, interstrand NOEs were observed only for residues 152 to 155 following Pro151, and Gly149 and Val150 NHs are in fast exchange with the solvent. Thus the initiation of strand β 4 probably follows Pro151, as seen in the X-ray structure. The remaining

part of the β -sheet has a global topology in good agreement with the crystallographic structure. Slow exchange was found for hydrogen bonded amide protons and identified interstrand NOEs correspond to short $^1\text{H}^\alpha$ - $^1\text{H}^N$ distances in the X-ray structure (Fig. 4). As expected, no significant secondary structure difference was found between oxidized and reduced DsbA (Fig. 3B).

However, information about changes occurring upon formation or breakage of the disulfide bond can be obtained by comparison of backbone chemical shifts for oxidized and reduced DsbA, because chemical shifts are very sensitive probes of electronic environment changes. Changes in polypeptide backbone conformation

Fig. 3. Continued.

are the major determinants for variation in ^{13}C chemical shifts, while changes in the hydrogen bonding and long-range electrostatic interactions will be reflected in ^{15}N chemical shift variations. Ring current effects, originating from the circulation of delocalized π electrons around the aromatic rings, will mostly affect the proton NMR spectrum (Oldfield, 1995).

Figure 5 reports the $^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, $^{13}\text{C}^\beta$, ^{15}N , $^1\text{H}^\text{N}$ chemical shift differences between oxidized and reduced DsbA. Comparison of these five difference plots highlights four regions of the amino acid sequence more or less perturbed by the reduction of the active site disulfide bridge.

The largest variations are observed for residues 26 to 43, i.e., residues of the active site and its vicinity (C-terminal part of β_2 to N-terminal part of α_1'). The largest variations in ^{13}C chemical shifts are observed for Cys30: absolute values of 4.3 ppm for $^{13}\text{C}^\alpha$, 13.8 ppm for $^{13}\text{C}^\beta$, and 2.9 ppm for ^{13}CO . Smaller variations in chemical shifts are observed for Cys33: absolute values of 0.8 ppm for $^{13}\text{C}^\alpha$, 4.1 ppm for $^{13}\text{C}^\beta$, and 1.0 ppm for ^{13}CO . The differences between the chemical shift variations of the two cysteines are

related to the different chemical states of the sulfur atoms. These variations can be compared with large chemical shift variations for residues around the active site involving Tyr34 ($\Delta\delta^{13}\text{C}^\alpha\text{I} = 1.6$ ppm), Phe36 ($\Delta\delta^{13}\text{C}^\beta\text{I} = 1.5$ ppm), or His32 ($\Delta\delta^{13}\text{COI} = 2.0$ ppm).

Significant variations in $^1\text{H}^\alpha$ chemical shifts concern Ser43, Phe26, Cys33, and Ser27 (absolute chemical shift variations of 0.32, 0.31, 0.30, and 0.2 ppm, respectively). Variations in ring current effects due to proximity of aromatic residues (proximity of His60, Phe26, and Phe28 for Ser27) may account for the large variation observed for the $^1\text{H}^\alpha$ chemical shift of Ser27. For Ser43, this variation could be due to putative changes of charges from residues Glu24, Lys58, and/or Lys47. The largest $^1\text{H}^\text{N}$ and ^{15}N chemical shift variations are observed for Cys33: absolute values of 1.47 ppm for $^1\text{H}^\text{N}$ and 12.8 ppm for ^{15}N . Much smaller variations in $^1\text{H}^\text{N}$ and ^{15}N chemical shifts are found for Cys30: absolute values of 0.01 and 3.0 ppm, respectively. Changes in the hydrogen bond pattern are expected upon reduction for Cys33 as its amide proton exhibits a significant increase in its exchange rate with the

Fig. 4. Topology of the five-stranded β -sheet of oxidized DsbA as deduced from NMR data. Interstrand medium- and long-range NOE connectivities are indicated by blue arrows. Dashed arrows indicate ambiguous connectivities. Slow exchanging amide protons are represented in bold type. Red dashed lines indicate hydrogen bonds as deduced from the X-ray structure.

solvent. This could reflect the disruption of the hydrogen bond between $^1\text{H}^N$ Cys33 and ^{13}CO Cys30 observed in the X-ray structure.

The three other sequence segments, for which smaller chemical shift perturbations occur (absolute chemical shift variations greater than 0.1 ppm for ^1H and 0.3 ppm for ^{13}C and ^{15}N), are around His60, i.e., residues 58 to 62, around Gln97, i.e., Glu94, Gln97, and Lys98 and around Pro151, i.e., residues 150 to 154.

The comparison of structural differences between the two redox states of DsbA and thioredoxin could yield important clues to explain the different oxidizing powers of these two oxidoreductases. For thioredoxin, the disulfide bond stabilizes the folded conformation in contrast to DsbA. Changes in the chemical shifts upon reduction of the disulfide bridge of thioredoxin have been published (Dyson et al., 1989; Chandrasekhar et al., 1991, 1994). Differences in the ^1H , ^{15}N , and ^{13}C chemical shifts between the oxidized and reduced forms were observed mainly for residues close to the active site. The high resolution solution structure of the two forms showed subtle conformational changes in the active site region together with a correlation between $^{13}\text{C}^\alpha$ chemical shift differences and backbone conformational changes (Jeng et al., 1994). Figure 6 highlights residues for which significant chemical shift variations have been observed between oxidized and reduced forms for DsbA and thioredoxin. Homologous residues of the thioredoxin domain of DsbA exhibit larger variations in chemical shifts between the oxidized and reduced forms. Furthermore, for DsbA, more residues present significant chemical shift variations, i.e., residues following the active site (residues 38 to 43) in the helix $\alpha 1'$ and residues 153–154 in the strand $\beta 4$. The $^{13}\text{C}^\alpha$ chemical shift variations observed for residues of the active site and for residues 28, 34, 36, 42, 43, and 98 could be correlated with backbone conformational changes, as such conformational changes were

observed for thioredoxin residues with $^{13}\text{C}^\alpha$ chemical shift variations larger than 0.5 ppm upon reduction (Jeng et al., 1994). According to a previously established model (Gane et al., 1995), the difference in redox potential between DsbA and thioredoxin might involve in particular residues 37–43 in the thioredoxin domain and Gln97 in the additional domain of DsbA (Gane et al., 1995). Due to a difference in the relative orientation of the common α -helix on which the active site is situated, residues 37 to 43 of DsbA are in a different conformation than their counterparts in thioredoxin. These residues were shown by NMR to differ significantly in their electronic environment between the reduced and oxidized state of DsbA, unlike the homologous residues in thioredoxin. It is worth noting that Gln97 and its neighboring residues (in particular, Glu94 and Lys98) are the only residues of the additional domain which undergo large chemical shift variations upon reduction of the disulfide bridge.

Conclusions

We have reported nearly complete backbone and $^{13}\text{C}^\beta$ assignments of oxidized and reduced DsbA using a combination of triple resonance experiments. The secondary structure of both forms was deduced from the analysis of NOE connectivities, coupling constants, amide proton exchange data, and Chemical Shift Index (CSI). Comparison of oxidized and reduced ^1H , ^{15}N , and ^{13}C chemical shifts for DsbA reveals four regions of the protein that undergo significant chemical shift variations: around the active site (residues 26 to 43), around His60, Pro151 (a loop close to the active site), and around Gln97. Thus, the two redox states of DsbA differ in their electronic environments in these regions. These differences are larger for DsbA than for *E. coli* thioredoxin for homologous residues. Furthermore, in contrast to thioredoxin, the helix $\alpha 1'$ (residues 38 to 43) exhibits large chemical shift variations. The electronic environment of the C-terminal part of helix $\alpha 3$ (i.e., Glu94 to Lys98), located in the B domain, is also altered. Finally, large $^{13}\text{C}^\alpha$ chemical shift variations could reflect backbone conformational changes in the active site region and for residues 42, 43, and 98. It will be of great interest to correlate these variations in the electronic environment with conformational changes.

Materials and methods

Sample preparation and chemicals

All chemicals were from Sigma Chemical Co. (St. Louis, Missouri) unless otherwise specified. DsbA was expressed in an overproducing strain of *E. coli* K12 transformed with the plasmid pPB 2190 (Belin, 1996). The labeled proteins were obtained by growing the recombinant strain in different labeled media. $u\text{-}^{13}\text{C}$ -/ ^{15}N -DsbA was produced in a rich labeled medium prepared from $u\text{-}^{13}\text{C}$ -/ ^{15}N *Spirulina maxima* cyanobacteria and, $u\text{-}^{15}\text{N}$ -DsbA in modified M9 (6 g/L NaH_2PO_4 ; 3 g/L KH_2PO_4 ; 0.7 g/L $(^{15}\text{NH}_4)_2\text{SO}_4$ (Boehringer, Mannheim, Germany); 0.5 g/L NaCl; 4 g/L glucose). Ampicillin was added to both media at a concentration of 150 mg/L. DsbA expression was induced by addition of 2 mM isopropyl- β -D-thiogalactoside at an absorbance of 0.8 (600 nm). The cells were then grown for 5 h at 37°C, harvested by centrifugation ($5,500 \times g$, 30 min, 4°C) and resuspended in a 30 mM Tris buffer containing 20% sucrose, 7 μM lysozyme, 10 mM EDTA (Merck, Darmstadt, Germany), 12,000 units/L Polymyxin-B, 1 μM pepstatin, 0.3 μM aprotinin, and 10 μM leupeptin, to release

Fig. 5. Chemical shift differences between oxidized and reduced DsbA. (A) $^{13}\text{C}\alpha$; (B) $^{13}\text{C}\beta$; (C) ^{13}CO ; (D) $^1\text{H}\alpha$; (E) $^1\text{H}\text{N}$; (F) ^{15}N . (*) Residues for which the chemical shift difference is larger than the maximum value displayed. The largest chemical shift differences are 13.8 ppm for Cys30 $^{13}\text{C}\beta$ and -12.8 ppm for Cys33 ^{15}N .

the periplasmic proteins. After 1 h at 4 °C, the suspension containing the spheroplasts was centrifuged at $18,000 \times g$, 4 °C, for 1 h and the supernatant was treated with Benzon nuclease (Merck), 1 mM MgCl_2 at 4 °C overnight. After precipitation of the proteins with ammonium sulfate to 80% (51.6 g/100 mL) saturation at 4 °C and centrifugation ($18,000 \times g$, 1 h, 4 °C), DsbA was purified as previously described using DEAE Sephacel (Pharmacia, France) and Phenyl Sepharose (Pharmacia) columns (Wunderlich & Glockshuber, 1993; Zapun et al., 1993). Yields of 130 mg of ^{15}N -/ ^{13}C -labeled DsbA per liter of u - ^{13}C - ^{15}N labeled medium and 16 mg of ^{15}N -labeled DsbA per liter of M9 were obtained after purification. Both purity and iso-

tope enrichments were determined to be about 99% by SDS-PAGE and mass spectrometry.

The catalytic efficiency of DsbA was assessed by the oxidative refolding of reduced, denatured RNase A assay as previously described (Akiyama et al., 1992). The protocol slightly differed for the preparation of Ribonuclease A, which was denatured at 20 mg/mL in 0.1 M Tris-acetate, pH 8.0, 0.2 M DTT, 6 M guanidine chloride (GdnCl), then dialyzed overnight against 0.1 M acetic acid, pH 3.5, 3 M GdnCl to remove DTT. A specific activity of $0.018 \mu\text{M RNaseA} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ DsbA was measured (data not shown), demonstrating that DsbA was in a folded, active form.

Fig. 6. Ribbon diagram of (A) oxidized *E. coli* DsbA (1FVK; Guddat et al., 1997) and (B) oxidized *E. coli* thioredoxin (2TRX; Katti et al., 1990) generated with the MOLMOL program (Koradi et al., 1996). The active site Cys are displayed in yellow. Residues that undergo large chemical shift variations (^{15}N and ^{13}C chemical shifts larger than 0.3 ppm and/or ^1H chemical shifts larger than 0.2 ppm) are colored in red.

DsbA was further concentrated by ultrafiltration using an Amicon YM-10 membrane and Centricon-10 microconcentrators (Millipore, Bedford, Massachusetts) and the buffer was changed by successive dilutions in 20 mM phosphate, pH 5.8, containing 0.02% NaN_3 . NMR samples were 90% $\text{H}_2\text{O}/10\%$ D_2O , 0.4 mM TSPd4, 2 mM DsbA, and 20 mM deuterated DTT (Isotec, Miamisburg, Ohio) for the reduced form. Except for the presence of DTT, the same buffer preparation was used for oxidized and reduced samples.

The oxidation state of DsbA under NMR conditions was checked by Ellman's assay in 6 M GdnCl after elimination of the DTT by HPLC on a Fast Desalting column HR 10/10 (Pharmacia), flow rate 1.5 mL/min, mobile phase: 0.1 M Tris-HCl, pH 7.5, 0.2 M KCl, 1 mM EDTA. A value of $13,600\text{ cm}^{-1}\cdot\text{M}^{-1}$ for the extinction coefficient at 412 nm was used (Riddles et al., 1983). The protein appeared to be fully reduced in the presence of a 10-fold excess of DTT and fully oxidized in the absence of DTT. The degradation of the samples was also tested by incubation of small amounts of 2 mM oxidized and reduced DsbA at 37 and 45 °C, pH 5.8 for 8 and 15 days. No proteolysis was observed by SDS-PAGE, and no variation in the secondary structure was noted at either temperature as evidenced by far UV-CD experiments.

NMR spectroscopy

All experiments were performed at 37 °C on a Bruker AMX-600 or DRX-600 using a triple-resonance 5-mm inverse probe with a self-shielding z-gradient.

^{15}N - ^1H heteronuclear NMR spectroscopy

Two-dimensional ^{15}N - ^1H HSQC were acquired with the pulse sequence described by others (Grzesiek & Bax, 1993a). The fol-

lowing three-dimensional experiments were recorded with the 2 mM oxidized and reduced ^{15}N -labeled DsbA: a three-dimensional ^{15}N -separated NOESY-HSQC (Marion et al., 1989; Fesik & Zuiderweg, 1990) experiment with NOE mixing time of 120 ms, and a three-dimensional HNHA experiment (Vuister & Bax, 1993; Kuboniwa et al., 1994). To resolve NOE cross-peaks of degenerate amide protons, a three-dimensional ^{15}N -separated HMQC-NOESY-HSQC (Frenquiel et al., 1990; Ikura et al., 1990) with a mixing time of 120 ms was recorded. A three-dimensional ^{15}N -separated TOCSY-HSQC (Marion et al., 1989; Fesik & Zuiderweg, 1990) experiment with a mixing time of 24 ms using a dipsi-2 mixing sequence (Shaka et al., 1988) was recorded only for the reduced sample.

For the evaluation of the NH-solvent exchange rates, "fast" HSQC spectra (Grzesiek & Bax, 1993a), matrix size $128 \times 1,024$, were collected at various times over 3 weeks (every 10 min for the first 4 h) using freshly prepared lyophilized samples of protein dissolved in 99.9% D_2O .

Six two-dimensional water-flip-back constant-time HMQC-J spectra were recorded for different $\text{H}^{\text{N}}\text{-H}^{\alpha}$ J dephasing periods (45, 60, 80, 100, 120, and 140 ms) to estimate $\text{H}^{\text{N}}\text{-H}^{\alpha}$ J from the dephasing time duration where the signal intensity changes sign (Kuboniwa et al., 1994).

In all ^{15}N edited experiments, the water was suppressed using the water flip-back scheme (Grzesiek & Bax, 1993a).

^1H - ^{15}N - ^{13}C triple-resonance NMR spectroscopy

For the reduced and oxidized ^{15}N -/ ^{13}C -labeled DsbA samples, the HNCA (Kay et al., 1990), CT-HNCO (Grzesiek & Bax, 1992a), CBCA(CO)NH (Grzesiek & Bax, 1992b), (HCA)CO(CAN)NH (Löhr & Rüterjans, 1995), and HCACBCO (Kay, 1993) experi-

ments were collected as described previously, except that HNCA, CT-HNCO, and CBCA(CO)NH pulse sequences use WATERGATE (Piotto et al., 1992) as the water suppression scheme.

For the reduced ^{15}N -/ ^{13}C -labeled DsbA sample, the CT-HNCOCA experiment was also recorded as described previously (Grzesiek & Bax, 1992a) with WATERGATE as the water suppression scheme.

Quadrature detection in the indirectly detected dimensions was obtained using TPPI except for (HCA)CO(CAN)NH, HNHA, and CT-HNCOCA (States-TPPI).

Heteronuclear decoupling during proton acquisition was achieved using GARP-1 (globally optimized alternating-phase rectangular pulse 1) phase-modulated irradiation (Shaka et al., 1985).

Additional parameters concerning the acquisition of the three-dimensional NMR experiments are given in Table 1.

Data were processed using the software package FELIX and the peak assignments were obtained using the ASSIGN module of this package (Biosym Technologies, San Diego, California). In general, processing included multiplication of the free induction decays by a shifted sine-squared apodization function, zero-filling to enhance resolution, linear back prediction of one data point in the indirectly acquired dimensions to obtain zero phase corrections and Fourier transformation. When appropriate, processing also included linear prediction of additional points in the indirectly acquired dimensions to enhance resolution, or FLATT baseline correction (Güntert & Wüthrich, 1992). After Fourier transformation of the three time domains, the final data sets consisted of 512 real points for the ^1H dimension, 256 real points for the ^{13}C dimension, and 128 real points for the ^{15}N dimension.

^1H chemical shifts were referenced using internal TSP as a standard. ^{15}N and ^{13}C chemical shifts were referenced indirectly to liquid NH_3 , at 0 ppm and to TSP at 0 ppm, respectively, using the $^{15}\text{N}/^1\text{H}$ frequency ratio (Live et al., 1984; Wishart et al., 1995) or the $^{13}\text{C}/^1\text{H}$ frequency ratio (Bax & Subramanian, 1986; Wishart et al., 1995).

Acknowledgments

This work was partly supported by grant #Bio4 CT960436 from the Biotechnology program of the European Commission and the TMR program of the European Commission (LSF at the Frankfurt University Centre of Biomolecular NMR).

References

- Akiyama Y, Kamitani S, Kusakawa N, Ito K. 1992. In vitro catalysis of oxidative folding of disulfide-bonded proteins by the *Escherichia coli* DsbA (ppfa) gene product. *J Biol Chem* 267:22440–22445.
- Bardwell JC. 1994. Building bridges: Disulfide bond formation in the cell. *Mol Microbiol* 14:199–205.
- Bax A, Subramanian SJ. 1986. Sensitivity-enhanced two-dimensional heteronuclear shift correlation NMR spectroscopy. *J Magn Reson* 67:565–569.
- Belin P. 1996. La formation des ponts disulfure des phosphatases dans l'espace périplasmique de *Escherichia coli*: Analyse du rôle du système Dsb [Ph.D.]. Paris: University of Paris VI.
- Chandrasekhar K, Campbell AP, Jeng MF, Holmgren A, Dyson HJ. 1994. Effect of disulfide bridge formation on the NMR spectrum of a protein: Studies on oxidized and reduced *Escherichia coli* thioredoxin. *J Biomol NMR* 4:411–432.
- Chandrasekhar K, Krause G, Holmgren A, Dyson HJ. 1991. Assignments of the ^{15}N NMR spectra of reduced and oxidized *Escherichia coli* thioredoxin. *FEBS Lett* 284:178–183.
- Chivers PT, Laboissière MCA, Raines RT. 1996. The CXXC motif: Imperatives for the formation of native disulfide bonds in the cell. *EMBO J* 15:2659–2667.
- Chivers PT, Prehoda KE, Raines RT. 1997. The CXXC motif: A rheostat in the active site. *Biochemistry* 36:4061–4066.
- Creighton TE. 1992. Folding pathways determined using disulfide bonds. In: Creighton TE, ed. *Protein folding*. New York: W.H. Freeman & Co. pp 301–351.
- Dyson HJ, Holmgren A, Wright PE. 1989. Assignment of the proton NMR spectrum of reduced and oxidized thioredoxin: Sequence-specific assignments, secondary structure, and global fold. *Biochemistry* 28:7074–7087.
- Fesik SW, Zuiderweg ERP. 1990. Heteronuclear three-dimensional NMR spectroscopy of isotopically labelled biological macromolecules. *Q Rev Biophys* 23:97–131.
- Freedman RB. 1995. The formation of protein disulphide bonds. *Curr Opin Struct Biol* 5:85–91.
- Frenquiel T, Bauer C, Carr MD, Birdsall B, Feeney J. 1990. HMQC-NOESY-HMQC, a three-dimensional NMR experiment which allows detection of nuclear Overhauser effects between protons with overlapping signals. *J Magn Reson* 90:420–425.
- Gane PJ, Freedman RB, Warwicker J. 1995. A molecular model for the redox potential difference between thioredoxin and DsbA, based on electrostatics calculations. *J Mol Biol* 249:376–387.
- Grauschopf U, Winther JR, Korber P, Zander T, Dallinger P, Bardwell JCA. 1995. Why is DsbA such an oxidizing disulfide catalyst? *Cell* 83:947–955.
- Grzesiek S, Bax A. 1992a. Improved 3D triple-resonance NMR techniques applied to a 31 kDa protein. *J Magn Reson* 96:432–440.
- Grzesiek S, Bax A. 1992b. Correlating backbone amide and side chain resonances in larger proteins by multiple relayed triple resonance NMR. *J Am Chem Soc* 114:6291–6293.
- Grzesiek S, Bax A. 1993a. The importance of not saturating H_2O in protein NMR. Applications to sensitivity enhancement and NOE measurements. *J Am Chem Soc* 115:12593–12594.
- Grzesiek S, Bax A. 1993b. Amino acid type determination in the sequential assignment procedure of uniformly $^{13}\text{C}/^{15}\text{N}$ -enriched proteins. *J Biomol NMR* 3:185–204.
- Guddat LW, Bardwell JCA, Glockshuber R, Huber-Wunderlich M, Zander T, Martin JL. 1997a. Structural analysis of three His32 mutants of DsbA: Support for an electrostatic role of His32 in DsbA stability. *Protein Sci* 6:1893–1900.
- Guddat LW, Bardwell JCA, Zander T, Martin JL. 1997b. The uncharged surface features surrounding the active site of *Escherichia coli* DsbA are conserved and are implicated in peptide binding. *Protein Sci* 6:1148–1156.
- Güntert P, Wüthrich K. 1992. FLATT—A new procedure for high-quality baseline correction of multidimensional NMR spectra. *J Magn Reson* 96:403–407.
- Hennecke J, Spleiss C, Glockshuber R. 1997. Influence of acidic residues and the kink in the active-site helix on the properties of the disulfide oxidoreductase DsbA. *J Biol Chem* 272:189–195.
- Ikura M, Bax A, Clore GM, Gronenborn AM. 1990. Detection of nuclear Overhauser effects between degenerate amide proton resonances by heteronuclear three-dimensional nuclear magnetic resonance spectroscopy. *J Am Chem Soc* 112:9020–9022.
- Jacobi A, Huber-Wunderlich M, Hennecke J, Glockshuber R. 1997. Elimination of all charged residues in the vicinity of the active-site helix of the disulfide oxidoreductase DsbA. *J Biol Chem* 272:21692–21699.
- Jeng MF, Campbell AP, Begley T, Holmgren A, Case DA, Wright PE, Dyson HJ. 1994. High-resolution solution structures of oxidized and reduced *Escherichia coli* thioredoxin. *Structure* 2:853–868.
- Karplus M. 1959. Contact electron-spin coupling of nuclear magnetic moments. *J Chem Phys* 30:11–15.
- Katti SK, Le Master DM, Eklund H. 1990. Crystal structure of thioredoxin from *Escherichia coli* at 1.68 Å resolution. *J Mol Biol* 212:167–184.
- Kay LE. 1993. Pulsed-field gradient-enhanced three-dimensional NMR experiment for correlating $^{13}\text{C}\alpha/\beta$, ^{13}C , and $^1\text{H}\alpha$ chemical shifts in uniformly ^{13}C -labeled proteins dissolved in H_2O . *J Am Chem Soc* 115:2055–2057.
- Kay LE, Ikura M, Tschudin R, Bax A. 1990. Three-dimensional triple-resonance NMR spectroscopy of isotopically enriched proteins. *J Magn Reson* 89:496–514.
- Kemmink J, Darby NJ, Dijkstra K, Scheek RM, Creighton TE. 1995. Nuclear magnetic resonance characterization of the N-terminal thioredoxin-like domain of protein disulfide isomerase. *Protein Sci* 4:2587–2593.
- Koradi R, Billeter M, Wüthrich K. 1996. MOLMOL: A program for display and analysis of macromolecular structures. *J Mol Graphics* 14:51–55.
- Kuboniwa H, Grzesiek S, Delaglio F, Bax A. 1994. Measurement of $\text{H}^{\text{N}}\text{H}^{\alpha}$ J couplings in calcium-free calmodulin using new 2D and 3D water-flip-back methods. *J Biomol NMR* 4:871–878.
- Laskowski RA, MacArthur MW, Moss DS, Thornton JM. 1993. PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr* 26:283–290.
- Live DH, Davis DG, Agosta WC, Cowburn D. 1984. Long range hydrogen bond mediated effects in peptides: ^{15}N NMR study of gramicidin S in water and organic solvents. *J Am Chem Soc* 106:1939–1941.

- Löhr F, Rüterjans H. 1995. A new triple-resonance experiment for the sequential assignment of backbone resonances in proteins. *J Biomol NMR* 6:189–197.
- Marion D, Driscoll PC, Kay LE, Wingfield PT, Bax A, Gronenborn AM, Clore GM. 1989. Overcoming the overlap problem in the assignment of ^1H NMR spectra of larger proteins by use of three-dimensional heteronuclear ^1H - ^{15}N Hartmann-Hahn-multiple quantum coherence and nuclear Overhauser-multiple quantum coherence spectroscopy: Application to interleukin 1 β . *Biochemistry* 28:6150–6156.
- Martin JL, Bardwell JCA, Kuriyan J. 1993. Crystal structure of the DsbA protein required for disulphide bond formation *in vivo*. *Nature* 365:464–468.
- Missiakas D, Raina S. 1997. Protein folding in the bacterial periplasm. *J Bacteriol* 179:2465–2471.
- Nelson JW, Creighton TE. 1994. Reactivity and ionisation of the active site cysteine residues of DsbA, a protein required for disulfide bond formation *in vivo*. *Biochemistry* 33:5974–5983.
- Oldfield E. 1995. Chemical shifts and three-dimensional protein structures. *J Biomol NMR* 5:217–225.
- Piotto M, Saudek V, Sklenar V. 1992. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J Biomol NMR* 2:661–665.
- Qin J, Clore GM, Gronenborn AM. 1994. The high-resolution three-dimensional solution structures of the oxidized and reduced states of human thioredoxin. *Structure* 2:503–522.
- Riddles PW, Blakeley RL, Zerner B. 1983. Reassessment of Ellman's reagent. *Methods Enzymol* 91:49–60.
- Shaka AJ, Barker PB, Freeman R. 1985. Computer-optimized decoupling scheme for wideband applications and low-level operation. *J Magn Reson* 64:547–552.
- Shaka AJ, Lee CJ, Pines A. 1988. Iterative schemes for bilinear operators; application to spin decoupling. *J Magn Reson* 77:274–293.
- Sodano P, Xia TH, Bushweller JH, Björnberg O, Holmgren A, Billeter M, Wüthrich K. 1991. Sequence-specific ^1H n.m.r. assignments and determination of the three dimensional structure of reduced *Escherichia coli* glutaredoxin. *J Mol Biol* 221:1311–1324.
- Vuister GW, Bax A. 1993. Quantitative J correlation: A new approach for measuring homonuclear three-bond J ($\text{H}^{\alpha}\text{H}^{\alpha\alpha}$) coupling constants in ^{15}N -enriched proteins. *J Am Chem Soc* 115:7772–7777.
- Weichsel A, Gasdaska JR, Powis G, Montfort WR. 1996. Crystal structures of reduced, oxidized, and mutated human thioredoxins: Evidence for a regulatory homodimer. *Structure* 4:735–751.
- Wishart DS, Bigam CG, Yao J, Abildgaard F, Dyson HJ, Oldfield E, Markley JL, Sykes BD. 1995. ^1H , ^{13}C and ^{15}N chemical shift referencing in biomolecular NMR. *J Biomol NMR* 6:135–140.
- Wishart DS, Sykes BD. 1994. The ^{13}C chemical-shift index: A simple method for the identification of protein secondary structure using ^{13}C chemical-shift data. *J Biomol NMR* 4:171–180.
- Wishart DS, Sykes BD, Richards FM. 1992. The chemical shift index: A fast and simple method for the assignment of protein secondary structure through NMR spectroscopy. *Biochemistry* 31:1647–1651.
- Wunderlich M, Glockshuber R. 1993. Redox properties of protein disulfide isomerase (DsbA) from *Escherichia coli*. *Protein Sci* 2:717–726.
- Wunderlich M, Jaenicke R, Glockshuber R. 1993. The redox properties of protein disulfide isomerase (DsbA) of *Escherichia coli* result from a tense conformation of its oxidized form. *J Mol Biol* 233:559–566.
- Wüthrich K. 1986. *NMR of proteins and nucleic acids*. New York: John Wiley & Sons, Inc.
- Xia TH, Bushweller JH, Sodano P, Billeter M, Björnberg O, Holmgren A, Wüthrich K. 1992. NMR structure of oxidized *Escherichia coli* glutaredoxin: Comparison with reduced *E. coli* glutaredoxin and functionally related proteins. *Protein Sci* 1:310–321.
- Zapun A, Bardwell JCA, Creighton TE. 1993. The reactive and destabilizing disulfide bond of DsbA, a protein required for protein disulfide bond formation *in vivo*. *Biochemistry* 32:5083–5092.
- Zapun A, Cooper L, Creighton TE. 1994. Replacement of the active-site cysteine residues of DsbA, a protein required for disulfide bond formation *in vivo*. *Biochemistry* 33:1907–1914.