1H, 13C and 15N resonance assignments for the oxidized and reduced states of the N-terminal domain of DsbD from Escherichia coli

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Abstract Viability and pathogenicity of Gram-negative bacteria is linked to the cytochrome c maturation and the oxidative protein folding systems in the periplasm. The transmembrane reductant conductor DsbD is a unique protein which provides the necessary reducing power to both systems through thiol-disulfide exchange reactions in a complex network of protein–protein interactions. The N-terminal domain of DsbD (nDsbD) is the delivery point of the reducing power originating from cytoplasmic thioredoxin to a variety of periplasmic partners. Here we report 1H, 13C and 15N assignments for resonances of nDsbD in its oxidized and reduced states. These assignments provide the starting point for detailed investigations of the interactions of nDsbD with its protein partners.

Keywords Escherichia coli DsbD · NMR resonance assignments · Thiol-disulfide exchange reaction · Oxidation/reduction · Oxidoreductase

Biological Context

In Gram-negative bacteria a large number of extracytoplasmic proteins contain disulfide bonds which are essential for their stability and function (Heras et al. 2009; Riemer et al. 2009). Disulfide bond formation is catalyzed by the Disulfide bond (Dsb) system, comprising five proteins (DsbA-G) located in the periplasm and the inner membrane of the bacterial cell (Porat et al. 2004; Messens and Collet 2006). This system is involved in the formation of disulfide bonds and the isomerization of wrongly formed disulfides. DsbD plays a central role in oxidative folding. It transfers reducing power across the cytoplasmic membrane to DsbC allowing the isomerization of wrongly formed disulfide bonds that leads to correct protein folding (Fabianek et al. 2000; Gleiter and Bardwell 2008). DsbD is also required for the covalent attachment of heme to c-type cytochromes, which are key proteins for the viability of virtually all organisms as they are widely involved in respiratory processes (Thony-Meyer 2002). In c-type cytochromes heme is covalently attached to the CXXCH motif of the apocytochrome via two thioether bonds; the cysteines of this conserved motif need to be in a reduced thiol form (Reid et al. 2001). DsbD is vital for two processes of widespread significance in bacterial life and essential for the survival and virulence of numerous pathogenic organisms. Therefore it is considered a novel target for the development of therapeutics against bacterial infection (Heras et al. 2009).

DsbD consists of three domains, a central transmembrane domain (tmDsbD) flanked by two globular periplasmic domains, the N-terminal (nDsbD) and the C-terminal (cDsbD) domains. Each domain of the protein contains a pair of conserved cysteine residues which are essential for function as the transfer of reductant occurs through a series of thiol-disulfide exchange reactions involving these residues (Katzen and Beckwith 2000). Electrons are transferred from cytoplasmic thioredoxin to tmDsbD, to cDsbD and finally to nDsbD (Gordon et al. 2000; Katzen and Beckwith 2000; Collet et al. 2002; Stirnimann et al. 2006). nDsbD is the branching point for DsbD-transferred reductant. It acts as a hub which interacts with a variety of protein partners (cDsbD, DsbC, DsbG, CcmG) and thus controls the redox state of the periplasm (Stirnimann et al. 2006). nDsbD is a 16 kDa, globular domain with an immunoglobulin fold, a
structural feature not otherwise described for a redox-active protein (Goulding et al. 2002; Haebel et al. 2002). The cysteine pair of this domain (C103 and C109) is located in an unusual CXX2C motif and is shielded from solvent exposure by the so-called “cap-loop” residues (residues 66–72) which adopt a more open conformation upon complex formation with the partner proteins (Stirmimmann et al. 2006).

X-ray structures for oxidized and reduced nDsbD (Goulding et al. 2002; Haebel et al. 2002; Mavridou et al. 2011) as well as for its covalent mixed disulfide complexes with partner proteins (cDsbD, cDsbD and CcmG) (Haebel et al. 2002; Rozhkova et al. 2004; Stirmimmann et al. 2005) have been determined. We have shown in previous studies that NMR can provide important insights into the control of the specificity and reactivity of cDsbD that cannot be obtained from X-ray structures alone (Bushell et al. 2002; Mavridou et al. 2007, 2009, 2011). We now wish to extend these NMR studies to understand how the specificity and reactivity of nDsbD are controlled and to investigate the network of protein–protein interactions of nDsbD, at the residue-specific level. Here we report 1H, 13C and 15N assignments for backbone and sidechain resonances of oxidized and reduced nDsbD.

Methods and experiments

Protein expression and purification

*Escherichia coli* BL21 (DE3) competent cells were transformed with the expression vector pET22b(+) (Novagen) containing the gene encoding residues L2-V132 of full length *E. coli* DsbD (N-terminal domain of the protein, nDsbD); the extra residues MDT at the N-terminus and GLVPR at the C-terminus of the protein are a result of the cloning process and the cleavage of a C-terminal His6-tag; assignments for these non-native residues are not reported. Isotopically 15N- and 13C/15N-labelled samples were produced using M9 minimal medium enriched with 15NH4Cl and 13C glucose (Cambridge Isotope Laboratories) and supplemented with 1 mM thiamine. Bacteria were grown in volumes of 500 ml to an OD600 of 1.1 in 2 l flasks in the presence of 100 µg.ml−1 carbenicillin from overnight starter cultures (grown at 30°C on Luria–Bertani broth) before addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After overnight incubation the cells were harvested, sphaeroplasted as described (Ausubel et al. 1989), except that EDTA was omitted, and the periplasmic fraction was applied to 10 ml of Fast Flow Chelating Sepharose (Amersham Biosciences) charged with Ni2+. The column was washed with 50 mM Tris–HCl, 150 mM NaCl, 20 mM imidazole, pH 7.5, and the bound protein was eluted with 50 mM Tris–HCl, 150 mM NaCl, 200 mM imidazole, pH 7.5, according to the manufacturer’s instructions. The protein was cleaved using the Sigma Thrombin CleanCleave Kit according to the manufacturer’s instructions.

The purified nDsbD samples contain a mixture of oxidized (with a C103–C109 disulfide) and reduced (without a C103–C109 disulfide) protein. Complete oxidation or reduction of the disulfide bond in nDsbD, prior to NMR experiments, was carried out as follows. 5, 5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was used to oxidize the C103–C109 disulfide bond. 10 mM DTNB was added to the sample and the mixture was incubated at 27°C for 30 min. The C103–C109 disulfide bond in nDsbD samples was reduced using 10 mM dithiothreitol (DTT); complete reduction requires exposure to DTT for at least 12 h (Mavridou et al. 2011).

All constructs were subjected to SDS-PAGE and electrospray ionization mass spectrometry (ES-MS), which was performed using a Micromass Bio-Q II-ZS triple quadrupole mass spectrometer (10 µl protein samples in 1:1 water: acetonitrile, 1% formic acid at a concentration of 20 pmol µl−1 were injected into the electrospray source at a flow rate of 10 µl min−1) to confirm that the proteins were pure and of the expected masses.

NMR spectroscopy

Assignments for oxidized and reduced nDsbD were obtained using uniformly 15N- or 13C/15N-labelled samples of 0.5–1 mM nDsbD in 95% H2O/5% D2O at pH 6.5. All NMR spectra were acquired at 298 K using either a home-built 750 MHz spectrometer which is controlled with GE/Omega software and is equipped with a home-built triple-resonance pulsed-field-gradient probehead or a Bruker Avance 500 MHz spectrometer with a Cryoprofile, equipped with a TCI CryoProbe. Sequential assignments were carried out initially using 15N-labelled nDsbD and 3D 15N-edited TOCSY-HSQC, NOESY-HSQC and HSQC-NOESY-HSQC experiments collected at 750 MHz; analysis of these spectra resulted in nearly complete assignment of the 1H, 13C and 15N resonances for both oxidized and reduced nDsbD. The sequential assignments obtained from the 15N-labelled sample were confirmed with a 3D HNCA experiment collected at 750 MHz and further 13C and 1H assignments were obtained using 3D HNCO, (H)CC(CO)NH and HCC-TOCSY experiments, collected at 750 MHz, and 3D CBCA(CO)NH, HBHA(CBCACO)NH and HN(CA)CO experiments, collected at 500 MHz.

Extent of assignments and data deposition

Figure 1a shows the 1H–15N HSQC spectrum of reduced nDsbD. 1H, 13C and 15N backbone resonances for all
non-proline residues (L2 to V132 of the native sequence) except E67 have been assigned. In the case of oxidized nDsbD, \(^{1}H\) and \(^{15}N\) assignments were not obtained for E67, C103, C109 and Y110; it is likely that exchange broadening, due to isomerization of the C103–C109 disulfide bond, is responsible for the absence of peaks corresponding to C103, C109 and Y110. \(^{15}N\) assignments for the 9 proline residues have not been obtained in reduced or oxidized nDsbD. A total of 99.3% of the \(^{1}H_a\), 99.0% of the \(^{1}H_b\), 99.2% of the \(^{13}C_a\), 98.4% of the \(^{13}C_b\) and 96.9%
of the $^{13}$C resonances were assigned for reduced nDsbD. For the oxidized state, 97.1% of the $^1$H, 96.2% of the $^1$H/$^1$B, 97.7% of the $^{13}$C, 94.4% of the $^{13}$C/$^1$B and 94.7% of the $^{13}$C resonances were assigned. In addition, 60.4% and 45.5% of the remaining sidechain $^1$H and $^{13}$C were assigned for reduced nDsbD, while 52.7% and 42.7% of the remaining sidechain $^1$H and $^{13}$C were assigned for oxidized nDsbD. Most of the missing sidechain assignments correspond to proline and aromatic residues. For reduced nDsbD, 85.9% and 73.0% of the non-proline/non-aromatic sidechain $^1$H and $^{13}$C were assigned. For oxidized nDsbD, 76.7% and 71.1% of the remaining non-proline/non-aromatic sidechain $^1$H and $^{13}$C were assigned. The $^1$H-$^{15}$N HSQC spectra of oxidized and reduced nDsbD are overlaid in Fig. 1b and residues showing a combined chemical shift difference ($\Delta\delta_{comb} = [\Delta\delta_{HN} + 0.1|\Delta\delta_{15N}|]^{1/2}$) of greater than 0.2 ppm are labelled. The combined chemical shift difference is plotted as a function of amino acid sequence. The red dashed line indicates a combined shift change of greater than 0.2 ppm; these residues are labelled in Fig. 1b.

The chemical shift assignments for reduced and oxidized nDsbD have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under the accession numbers 17830 and 17831, respectively.

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