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ARTICLE

Backbone and side chain ^{1}H, ^{15}N and ^{13}C assignments for a thiol-disulphide oxidoreductase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125

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Abstract Enzymes produced by psychrophilic organisms have successfully overcome the low temperature challenge and evolved to maintain high catalytic rates in their permanently cold environments. As an initial step in our attempt to elucidate the cold-adaptation strategies used by these enzymes we report here the ^{1}H, ^{15}N and ^{13}C assignments for the reduced form of a thiol-disulphide oxidoreductase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125.

Keywords Cold-adapted enzymes · DsbA · Reduced oxidoreductase · *Pseudoalteromonas haloplanktis* TAC125

Biological context

Habitats of permanently low temperatures have been successfully colonized by a wide variety of psychrophilic organisms which have been found to not only survive but to thrive in this environmental extreme (Feller and Gerday 2003). Clearly these cold-adapted organisms have successfully overcome the major obstacles inherent to life in the cold and effectively adapted their cellular components, both structural and functional, to the environment. Their strategy of adaptation must include maintenance of membrane fluidity, efficient transcription and translation rates, as well as effective nutrient transport and cryoprotection. A further essential component of this adaptation is the maintenance of an efficient enzyme activity (Collins et al. 2008; Feller and Gerday 2003). Indeed, enzymes produced by these organisms have been found to have successfully adapted to their environment and typically display high catalytic activity at low to moderate temperatures (Collins et al. 2008; Feller and Gerday 2003). As compared to their mesophilic and thermophilic homologs, their activity at low temperatures is in general much higher and their apparent optimal temperature is shifted towards lower temperatures while they are also typically characterized by a reduced thermal and chemical stability (Collins et al. 2003, 2008). Furthermore, while being much less well studied than mesophilic and thermophilic enzymes, comparative crystal structure analyses indicate that cold adapted enzymes are characterized by subtle enzyme specific structural modifications, with, in particular, a reduction in the number and/or strength of stabilizing interactions being reported. Presently it is hypothesized that these enzymes efficiently catalyze reactions at low temperatures through an inherent increased flexibility and thereby allowing for the molecular motions necessary for activity in their low thermal energy environment (Collins et al. 2008; Feller and Gerday 2003). Nevertheless the actual molecular basis for the adaptation is still only poorly understood and direct evidence of the proposed increased flexibility is scant, with previous attempts to demonstrate this leading to conflicting results (Collins et al. 2008).

In an attempt to better understand strategies of cold-adaptation we will determine the solution structure of the reduced form of a cold adapted thiol disulphide oxidoreductase (DsbA) which has been isolated from the Antarctic marine γ-proteobacterium *Pseudoalteromonas haloplanktis* TAC125 (Medigue et al. 2005). Thiol disulphide oxidoreductases (EC 1.8.4.-) catalyze the extracytoplasmic formation of disulphide bonds in newly synthesized

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proteins (Ito and Inaba 2008) and have been extensively studied at the biochemical and structural levels (Paxman et al. 2009; Schirra et al. 1998; Williams et al. 2009). A number of crystal and NMR structures of mesophilic homologs in the absence and presence of substrate have been reported while function and dynamics have also been examined (Horne et al. 2007; Paxman et al. 2009; Schirra et al. 1998; Williams et al. 2009). The availability of this in depth information for mesophilic enzymes homologous to the cold adapted protein of the present study should allow for a more comprehensive comparative analysis of the structure and dynamics of this and a better understanding of cold adaptation in this enzyme. Furthermore, while a number of crystal structures for cold adapted enzymes have been published, this is the first report of assignments for NMR structure determination for these enzymes and thereby should open up a new dimension in the study of cold adaptation.

Methods and experiments

The 187 amino acid cold-adapted thiol disulphide oxidoreductase was overexpressed at 18°C using the pET22b(+)/*E. coli* BL21(DE3) expression system and induction with 0.1 mM isopropyl-1-thio-β-galactaopyranoside. Overexpression of double (^{15}N, ^{13}C) and single (^{15}N) labeled enzyme was carried out using modified minimal medium (Lamosa et al. 2003) supplemented with ^{13}C glucose and/or $^{15}NH_4Cl$ where appropriate. Following 20 h induction at 18°C the overexpressed enzyme was released from the harvested cells by periplasmic extraction via osmotic shock with sucrose/EDTA. The enzyme was then purified using a combination of hydrophobic exchange chromatography on a Phenyl Sepharose column (20 mM Tris–HCL pH 8.0, 1 mM EDTA, 4 mM DTT, 1 M $(NH_4)_2SO_4$) and anion exchange chromatography on a DEAE-Sepharose Fast Flow column (10 mM MOPS pH 7.2, 1 mM EDTA and 4 mM DTT). Enzyme elution and purity were monitored with the insulin reduction assay and SDS-PAGE. The purified enzyme was reduced with a 100-fold excess of dithiothreitol and extraneous reducing agent was subsequently removed by gel filtration on a Superdex 75 column in 10 mM MOPS pH 7.0, 50 mM NaCl, 1 mM EDTA and 4 mM DTT. The residual DTT was then removed in an anaerobic chamber using a Hitrap desalting column, a final concentration of 10% D_2O and 0.001% sodium azide were added and the sample immediately loaded and sealed in a 5 mm NMR tube.

NMR data were acquired at 25°C on a Bruker AvanceII+ 800 MHz spectrometer equipped with a TXI-Z H-C/N/-D (5 mm) probe. Data were processed with Topspin 2.1 (Bruker Biospin) and analyzed in CARA 1.8.4. Backbone sequential assignments were obtained from ^1H–^{15}N HSQC and triple resonance HNCA, HNCO, HNCOCA, HNCOCACB, CBCANH, HBHACONH and HNCACO experiments. Side chain assignments were made from HCcH TOCSY, hCCH TOCSY, ^{15}N HSQC TOCSY and ^1H–^{13}C HSQC spectra while hCCH TOCSY aromatic, hbCBcgcd HD and hbCBcgcdceHE spectra were collected to aid in aromatic side chain assignments.

^{13}C chemical shift values were analyzed and recalibrated using the Checkshift program (Ginzinger et al. 2007). Secondary structural elements were predicted using the chemical shift indices (CSI) of C_α and C_β nuclei (Wishart and Sykes 1994). Protein backbone mobility was predicted using the random coil index (RCI) derived from backbone chemical shifts of C_α, C_β, CO, N and H_α nuclei using the web based program RCI (Berjanskii and Wishart 2005).

Assignments and data deposition

The ^1H–^{15}N HSQC of the reduced form of DsbA is shown in Fig. 1. All the expected resonances, with the exceptions of A1, N2 and T153, can be observed. The amide peak of N169 is weak but still observable in triple resonance experiments. The poor data for these residues may be due to amide proton exchange with the bulk solvent as RCI data (Fig. 2) suggests these to be located in flexible regions of the protein.

The sequential assignment is almost complete with 99% of the backbone resonances identified. Proline residues were assigned using the CA-1/CB-1 and HB-1/HA-1 resonances of the succeeding amino acid as obtained from the CBCACONH and HBHACONH spectra, respectively. The aliphatic side chains were assigned using the hCCH TOCSY and HCcH COSY spectra and verified using the ^{13}C HSQC. The extent of the assignment for the aliphatic side chains was 96% for protons and 93% for the ^{13}C resonances. For the side chain of the aromatic residues we used the hbCBcgcdHD and hbCBcgcdceHE experiments and were able to assign 90% of the aromatic protons.

A small number of groups with resonances far removed from the average values seen in the BMRB database are present. These are E24 (HG2/3), F26 (HE1/HE2), E44 (HB2/3), N56 (HB3/2), I92 (HG2), Q140 (HE22/21), Q174 (HE22/21) and L184 (HD2). Similar deviations from the average values have been observed for other members of the DsbA family in their reduced form. In particular, E24 and F26, are located close to the active site and an aromatic rich region which may be responsible for the observed unusual shift.

The chemical shift assignments of the reduced protein (Acronym: PshDsbA) have been deposited in the BioMagResBank (http://www.bmrb.wisc.edu) under the accession number 16689.

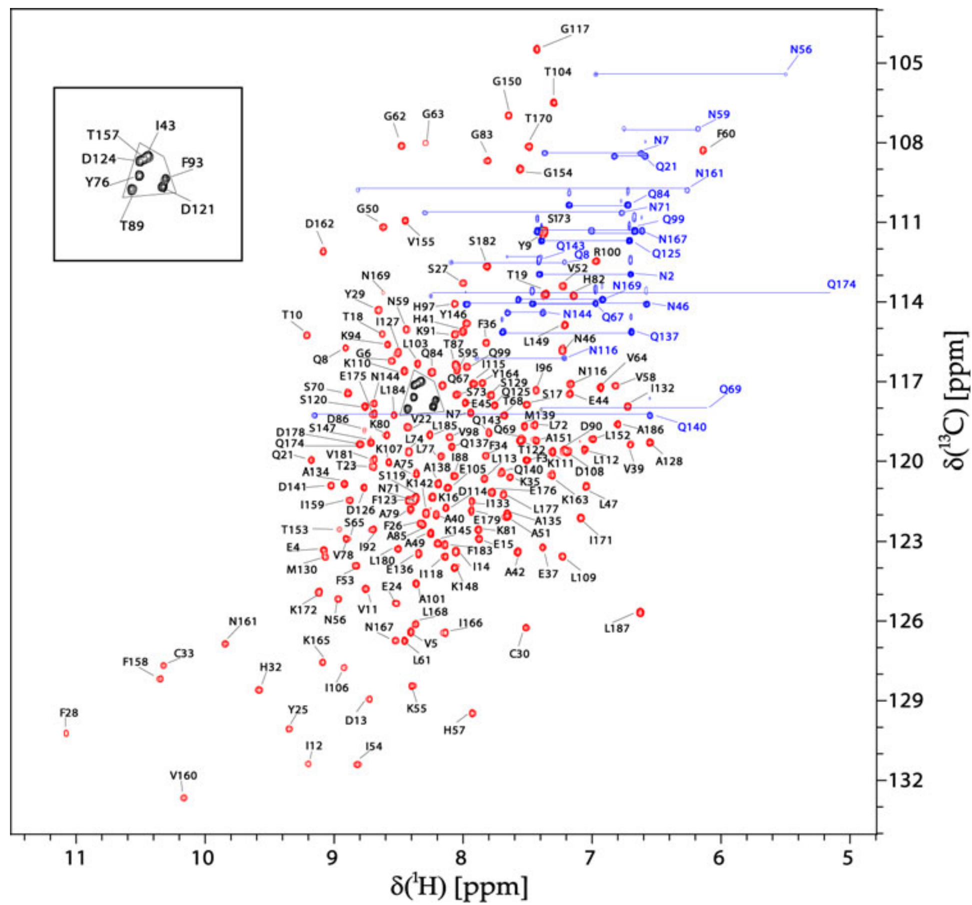

Fig. 1 ^{15}N–^{1}H HSQC spectrum at 25°C for the reduced form of the thiol disulphide oxidoreductase from *Pseudoalteromonas haloplanktis* TAC125. Backbone and side chain amides are shown with the resonances of the geminal protons of the side chain amides (glutamine and asparagine residues) linked by *horizontal lines*

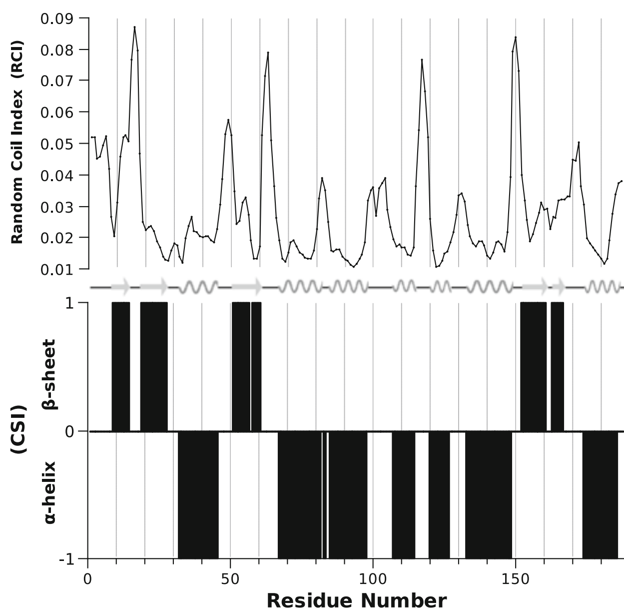

Fig. 2 Predicted secondary structure of PshDsbA based on the Chemical Shift Index (CSI). Random coil chemical shifts (RCI values) are also included as an indicator of loop regions or unordered segments

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