NMR Solution Structure of a Cold-Adapted Thiol-Disulphide Oxidoreductase

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Life in the Cold

➲ Permanently low temperature habitats have been successfully colonised by a wide variety of psychrophilic organisms which not only survive, but thrive, in this environment.

➲ Life at low temperatures requires a multitude of adaptations, both structural and functional, at all levels within the cells.

➲ Enzymes produced by cold-adapted organisms have successfully overcome the low temperature challenge and maintain efficient catalytic rates at low temperatures with, in addition, a reduced stability also being commonly reported.

➲ Presently it is believed that this low temperature adaptation is brought about by an increase in the protein flexibility which can also lead to the observed reduced stability.

➲ The proposed increased flexibility is a difficult parameter to demonstrate and as yet unequivocal direct experimental evidence of this is lacking.

➲ Presently 23 3D-structures of cold-adapted enzymes are known and all have been obtained by X-ray crystallography.

Objectives

- To determine the solution structure of a cold adapted enzyme.
- To carry out a comparative biochemical and structural characterisation of homologous cold adapted and mesophilc enzymes.
- To obtain a better understanding of the molecular basis of cold adaptation.

This is the first report of an NMR structure for a cold-adapted enzyme and should open up a new dimension in the study of cold adaptation. The potential power of NMR to monitor both local and global motions over a large range of time scales should allow for a better understanding of the role of dynamics in protein adaptation to temperature.

The model protein for the study: a cold adapted DsbA (PshDsbAp)

DsbA: Thiol-Disulphide Oxidoreductase (EC 1.8.4.-).
DsbA catalyses the extracytoplasmic formation of disulphide bonds in newly synthesised proteins.
Catalyses a thiol disulphide exchange reaction during which substrate is oxidised and DsbA is itself reduced. Reduced DsbA is then re-oxidised by the enzyme DsbB.
Cold adapted DsbA isolated from the Antarctic marine bacterium *Pseudoalteromonas haloplanktis* TAC125[1]
PshDsbAp UniProtKB/TrEMBL Accession code: Q3ILM4 187 amino acid / 20804 Da protein.

Overproduction and Purification

- Recombinant PshDsbAp production at 18°C using the pET22b(+)/*E. coli* BL21(DE3) expression system.
- Protein purifcation: periplasmic extraction
  hydrophobic exchange (Phenyl Sepharose)
  anion exchange (DEAE-Sepharose FF)
  gel filtration (Superdex 75)
- The mesophilc homolog (VcDsbAm) from *Vibrio cholerae* was produced and purified as previously described[2].
- Purified DsbAs were reduced with 100-fold excess of DTT or oxidised with 1.5 mM copper phenanthroline, these agents were subsequently removed by gel filtration.

Thermal Unfolding

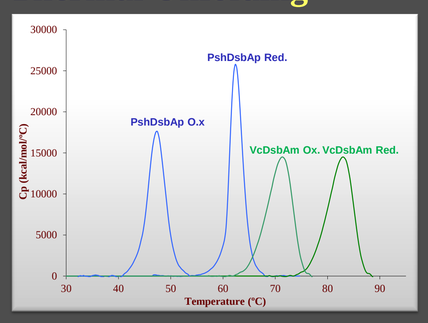

Fig. 1: Protein unfolding as monitored by DSC at a scan rate of 1°C/minute on a Microcal MCS-DSC apparatus. 35µM of each protein was used. Baseline subtracted data have been normalised for protien concentration. Both oxidised (Ox.) and reduced (Red.) forms of the psychrophilic (PshDsbAp) and mesophilic (VcDsbAm) enzymes are shown.

- Cold adapted enzyme is less thermally stable than its mesophilic homolog.
- Oxidised state is less stable than reduced state for both enzymes studied.

Irreversible Thermal Inactivation

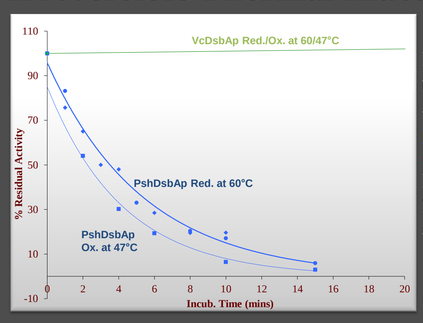

Fig. 2: Irreversible protein unfolding for the reduced (Red.) and oxidised (Ox.) states of the psychrophilic (PshDsbAp) and mesophilic (VcDsbAm) DsbAs. Residual activity was measured at 25°C using the insulin reduction assay.

- Higher rate of thermal inactivation for cold-adapted DsbA as compared to its mesophilic homolog.
- No thermal inactivation observed for Red. or Ox. mesophilic DsbA at temperatures investigated.

NMR Structure Determination of Reduced PshDsbAp

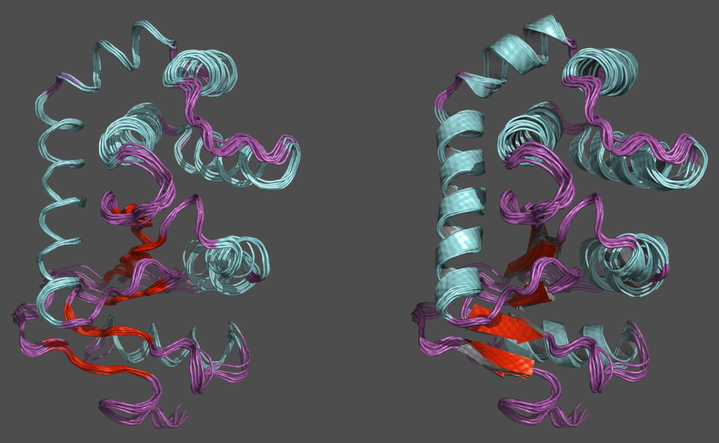

Fig. 3 Solution structures of the reduced state of the cold adapted DsbA; an ensemble of the 10 best structures are shown. NMR data were acquired at 25°C on a Bruker Avance+ 800 MHz spectrometer. 1.5 mM protein at pH 7 was used. A large variety of 2D and triple resonance spectra were collected for backbone side chain and aromatic assignments[3]. 2D and 3D NOESYs allowed for structure calculation with UNIO-ATNOS/CANDID and CYANA as well as manual picking of NOEs. Water refinement was carried with CNS1.1 following the RECOORD NMR structure refinement protocol.

Table I: Assignment Report

Class of Spins analysed : all atoms	found	missing	complete
1H shifts :	1120	54	95.400%
1Haro shifts :	85	14	85.859%
1Hali shifts :	1035	40	96.279%
Assignments separated by AtomType			
13C shifts :	841	101	89.278%
13Caro shifts :	52	65	44.444%
13Cali shifts :	789	36	95.636%
15N shifts :	199	39	83.613%
All shifts :	2160	194	91.759%

Table II: Statistics for protein structure determination

Number of residues	187 (1–187)
Molecular weight [Da]	20804.46
Number of models	20
Target function [Å²]	32.75 +/– 0.58 (31.32..33.68)
Setup-given RMSD range	4–187
– Backbone RMSD [A]	0.43 +/– 0.10 (0.30..0.71)
– Heavy atom RMSD [A]	0.74 +/– 0.07 (0.66..0.96)
Optimal RMSD range	1–187
– Backbone RMSD [A]	0.44 +/– 0.10 (0.30..0.72)
– Heavy atom RMSD [A]	0.75 +/– 0.07 (0.66..0.96)
NOE restraints [#]	4412
– intraresidual (|i–j|=0)	863 (19.56%)
– sequential (|i–j|=1)	1205 (27.31%)
– medium-range (1<|i–j|<5)	1089 (24.68%)
– long-range (|i–j| >4)	1255 (28.45%)
NOE restraints per residue	23.59
RMS NOE restraint violation [A]	0.0263
Dihedral restraints [#]	1005
RMS dihedral restraint violation [°]	4.4347
Ramachandran statistics	
– most favoured [%]	77.01
– additionally allowed [%]	21.57
– generously allowed [%]	1.24
– disallowed [%]	0.18

Solution Structure

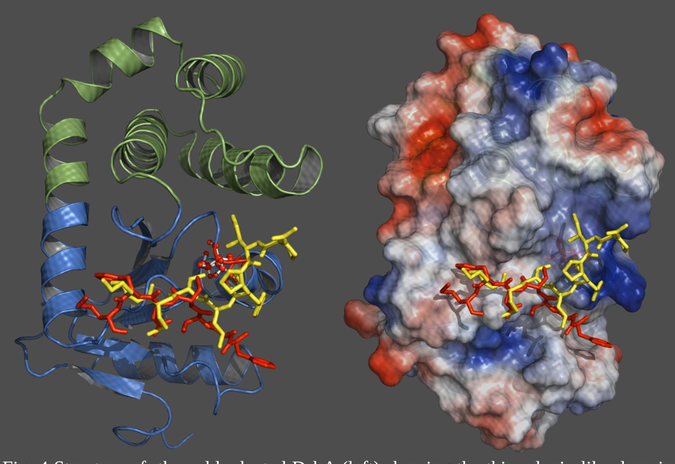

Fig. 4 Structure of the cold-adapted DsbA (left) showing the thioredoxin-like domain in blue and the α-helical domain in green. Peptide substrate (yellow) and the re-oxidising loop of DsbB (red) were overlayed onto the PshDsbAp structure by alignment with the *E. coli* DsbA structural complexes DsbA-peptide (pdb: 3DKS) and DsbA-DsbB (pdb: 2ZUP). The redox active catalytic site cysteines of PshDsbAp are shown as ball and stick. The surface representation on the right shows positive surfaces in blue and negative surfaces in red. All figures were prepared in Pymol 1.3.

- The reduced cold-adapted PshDsbAp shows a typical DsbA structure consisting of two domains.
- A helical domain (4 α-helices) is inserted into a thioredoxin like fold (central 5 stranded β-sheet flanked by 3 α-helices).
- The active site catalytic residues (Cys30-Pro31-His32-Cys33) are located at a break in the first α-helix in the thioredoxin-like domain.
- Substrate peptide binds at the interdomain interface while DsbB binds within a closely located hydrophobic groove.

Structural Comparison

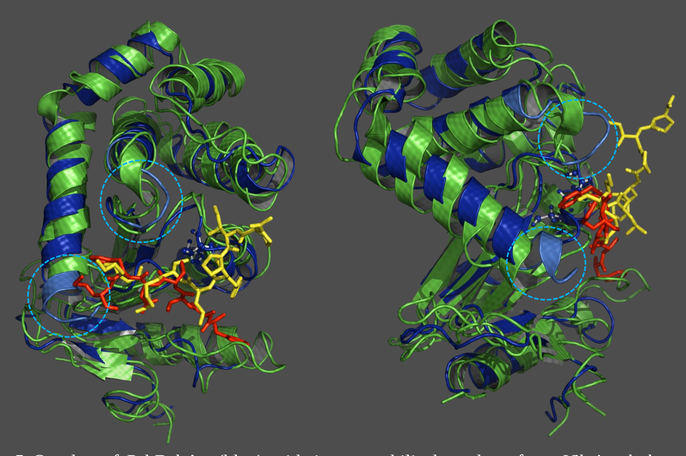

Fig. 5 Overlay of PshDsbAp (blue) with its mesophilic homologs from *Vibrio cholerae* (pdb: 2IJY) and *E. coli* (pdb: 1A23). Both mesophiles are shown in green. The two insertions in the cold-adapted DsbA are displayed in light blue and are circled.

- Structural comparisons of PshDsbAp with its mesophilic homologs indicate that it is distinguished by the presence of two insertions.
- One insertion of 3 residues occurs in an inter-domain loop which is believed to be important in substrate binding and inter-domain movement.
- The second insertion of 4 residues occurs at the interface of the C-terminal end of the long backbone α-helix and at the start of a long loop believed to be important in catalytic activity.
- Sequence alignments indicate that the only other DsbA sequences containing insertions in both these regions are from marine psychrophilic/psychrotrophic bacteria.

Conclusions

➲ We report here the first NMR structure of a cold adapted enzyme.
➲ The cold-adapted DsbA is characterised by a reduced thermal stability as compared to its mesophilic homolog.
➲ PshDsbAp is a two-domain protein with an overall architecture and fold very similar to previously described DsbAs.
➲ Short 3 to 4 amino acid insertions in two critical inter-domain regions may play central roles in adaptation to low temperatures in thiol-disulphide oxidoreductases.

Future Studies

➲ In-depth structural comparison with its mesophilic homologs, in particular comparing the number and strength of stabilising interactions.
➲ Development of an activity assay to evaluate and compare the thermal dependence of activity of this enzyme with that of its mesophilic homolog.
➲ Comparative dynamics using NMR.

References

1. Medigue C et al. (2005) Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. Genome Res 15:1325-1335.
2. Scanlon MJ (2007) Probing the flexibility of the DsbA oxidoreductase from *Vibrio cholerae*--a ^{15}N - ^{1}H heteronuclear NMR relaxation analysis of oxidized and reduced forms of DsbA. JMB 371:703-716.
3. Collins T et al. (2010) Backbone and side chain ^{1}H, ^{15}N and ^{13}C assignments for a thiol-disulphide oxidoreductase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125, Biomol NMR Assign. Online.