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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

James Horne[1], Edward J. d'Auvergne[2], Murray Coles[3], Tony Velkov[1] Yanni Chin[1], William N. Charman[4], Richard Prankerd[4] Paul R. Gooley[2] and Martin J. Scanlon[1]*

[1]*Department of Medicinal Chemistry, Victorian College of Pharmacy, Monash University 381 Royal Parade, Parkville VIC 3052, Australia*

[2]*Department of Biochemistry and Molecular Biology University of Melbourne Melbourne, VIC 3010, Australia*

[3]*Department of Protein Evolution, Max-Planck-Institute for Developmental Biology, Spemannstr. 35 72076 Tuebingen, Germany*

[4]*Department of Pharmaceutics Victorian College of Pharmacy Monash University, 381 Royal Parade, Parkville, VIC 3052 Australia*

**Corresponding author*

We have determined the structure of the reduced form of the DsbA oxidoreductase from *Vibrio cholerae*. The reduced structure shows a high level of similarity to the crystal structure of the oxidized form and is typical of this class of enzyme containing a thioredoxin domain with an inserted α-helical domain. Proteolytic and thermal stability measurements show that the reduced form of DsbA is considerably more stable than the oxidized form. NMR relaxation data have been collected and analyzed using a model-free approach to probe the dynamics of the reduced and oxidized states of DsbA. Akaike's information criteria have been applied both in the selection of the model-free models and the diffusion tensors that describe the global motions of each redox form. Analysis of the dynamics reveals that the oxidized protein shows increased disorder on the pico- to nanosecond and micro- to millisecond timescale. Many significant changes in dynamics are located either close to the active site or at the insertion points between the domains. In addition, analysis of the diffusion data shows there is a clear difference in the degree of interdomain movement between oxidized and reduced DsbA with the oxidized form being the more rigid. Principal components analysis has been employed to indicate possible concerted movements in the DsbA structure, which suggests that the modeled interdomain motions affect the catalytic cleft of the enzyme. Taken together, these data provide compelling evidence of a role for dynamics in the catalytic cycle of DsbA.

Keywords: DsbA; dynamics; bacterial oxidoreductase; model-free; inter-domain motion

Introduction

The Dsb (disulfide bond-forming) family of proteins are oxidoreductase enzymes found within the periplasm of Gram-negative bacteria. The DsbA/B system is primarily responsible for the formation of new disulfide bonds in substrates within the periplasm. DsbA catalyzes the oxidation of a wide range of substrate proteins *via* an efficient thiol-disulfide transfer mechanism. Reduced DsbA, which is formed in the reaction, is re-oxidized by a cognate, membrane-bound partner, DsbB to complete the catalytic cycle. For substrates that contain more than one pair of cysteine residues, disulfides may be linked incorrectly, hence a second, complementary system exists to catalyze disulfide isomerisation; DsbC and its membrane-bound reductive partner DsbD.[1]

The prototypical DsbA enzyme is comprised of two domains (Figure 1) including a largely α-helical domain that inserts into a thioredoxin-like domain

Abbreviations used: NOE, nuclear Overhauser enhancement; HSQC, heteronuclear single quantum coherence; DSC, differential scanning calorimetry.

E-mail address of the corresponding author: martin.scanlon@vcp.monash.edu.au

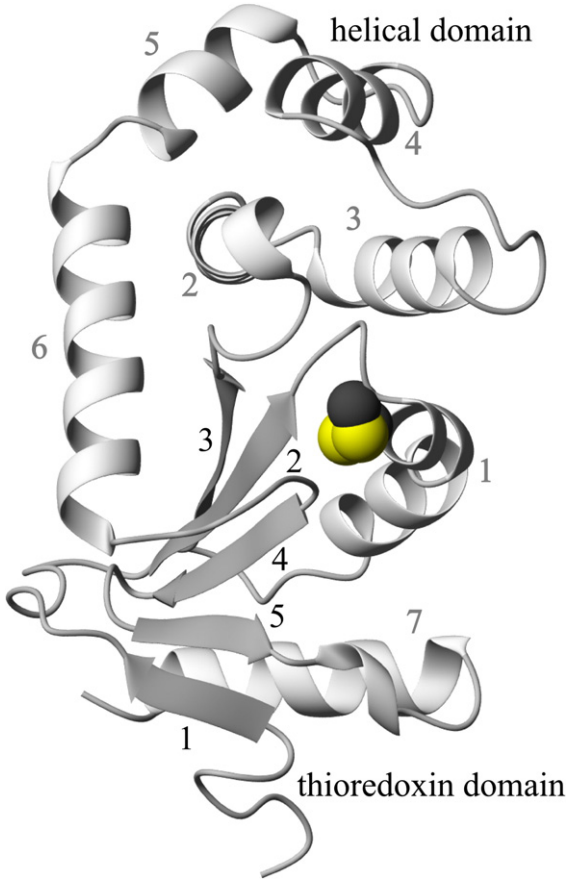

Figure 1. Cartoon of the crystal structure 1BED of oxidized VcDsbA. Secondary structure elements are numbered sequentially from the N terminus. Black numbers denote helices and grey numbers denote strands. The side-chain atoms of C30 and C33 are shown in CPK representation.

at the end of a long helix (residues 143–148 in *Escherichia coli* DsbA (*Ec*DsbA);[2] residue numbering conforms to the *E. coli* protein sequence unless otherwise noted) and a loop between strand 3 of the thioredoxin domain and helix 2 in the helical domain (residues 58–62). The relative orientation of the two domains can vary through simple rotations around the insertion points. By inference from thioredoxin-substrate crystal structures,[3] binding of peptide substrate is predicted at a groove between helix 1 and helix 7 within the thioredoxin domain. The relative size of this groove is affected by variations in the interdomain angle. Among the various DsbA enzyme structures solved using crystallographic and NMR methods there is considerable variation in the observed interdomain angle.[4–6] However, for the *Ec*DsbA there is as much variation in the interdomain angle within different crystal forms of the oxidized protein as there is between oxidized and reduced forms, and the significance of the changes in domain orientation is not clear.

The active site of DsbA lies in a cleft at the interface of the two domains and comprises a highly conserved primary sequence motif C-P-X-C at the N-terminal end of helix 1. In oxidized DsbA, a disulfide bond links C30 and C33. In the reduced form of *Ec*DsbA a sulfhydryl at C33 and a thiolate anion at C30 remain after release of the oxidized product post catalysis. Recent observations with a mutant C33A form of *Ec*DsbA,[7] indicate the possibility of *cis-trans* proline isomerization within the C-P-X-C motif common to many DsbAs. However, mutation of the proline in this motif of the *Ec*DsbA does not diminish the activity of the enzyme,[8] suggesting that this isomerism is not a requirement for catalysis.

Proteolysis experiments with *Ec*DsbA indicated marked differences for the reduced and oxidized forms of DsbA in relative susceptibility to protease digestion.[9] Oxidized DsbA was found to be cleaved more readily than reduced DsbA. This was interpreted as an indication of possible higher degree of flexibility in the oxidized form, which may be important for accommodating substrate interactions, the more stable reduced form perhaps driving the release of the oxidized product.

Thus, there are several lines of evidence suggesting that there may be dynamic differences between the oxidized and reduced forms of DsbA. However, *Ec*DsbA is the only protein for which the structure of the reduced form of the protein has been determined. As such, it is unclear if the changes in domain orientation are a general feature of this class of enzymes or if this observation is unique to *Ec*DsbA. Furthermore, to date there has been no direct measurement of protein dynamics to complement observations that have been inferred from the available static structures of DsbA. Here, we present the structure of the reduced form of *Vibrio cholerae* DsbA (*Vc*DsbA) as well as proteolytic data, thermal stability data and measurements of backbone heteronuclear ^{15}N T_1, T_2 and $\{^1H\}$-^{15}N steady-state NOEs for both reduced and oxidized forms of *Vc*DsbA. Model-free analyses of the NMR relaxation data demonstrate clear differences in the dynamic properties of the two oxidation states, both of local motions and global movements of the thioredoxin and helical domains. We show that interdomain motions for reduced and oxidized *Vc*DsbA, while sharing broadly similar mechanics, are clearly different in amplitude and that local motions, both on the pico- to nanosecond and micro- to millisecond timescale, are also different. We present a principal components analysis of potential modes of interdomain motions to supplement our discussion of the importance of these dynamic processes in the catalytic cycle of DsbA enzymes.

Results

Stability of *Vc*DsbA redox forms

Figure 2 shows a comparison of HPLC traces for oxidized and reduced *Vc*DsbA after incubation for