Calculation of Cys 30 ΔpK_a's and oxidising power for DsbA mutants

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Abstract DsbA possesses a redox active disulphide, with the equilibrium strongly shifted towards the reduced form as compared to its structural homologue, thioredoxin. It is widely believed that the two amino acids that separate the active site cysteines play a crucial role in determining oxidising power within the thioredoxin family. Data concerning redox and pK_a properties for DsbA mutants in this region are available. Electrostatics calculations show reasonable agreement with the experimental data, and support the suggestion that amino acids outside of the CXXC active site sequence are as important in determining oxidising power within the thioredoxin family as are those within it.

Key words: Electrostatic interaction; Redox potential; Thioredoxin; Disulphide bridge; Molecular modelling

1. Introduction

DsbA is a periplasmic protein that catalyses the introduction of disulphide bonds [1,2]. The structure of the *Escherichia coli* enzyme, solved at 2 Å resolution [3], shows a thioredoxin-like domain plus a 76 residue insert that forms a predominantly α-helical domain. The region containing the active site disulphide superimposes almost precisely onto the homologous region of the 1.7 Å crystal structure of *E. coli* thioredoxin [4]. Whereas DsbA acts to make disulphide bonds, thioredoxin reduces them. This functional difference is reflected in the large separation in standard redox potentials, which are about −260 mV for thioredoxin and −100 mV for DsbA [5]. Establishing the molecular basis for these differences, in the context of homologous structures, has presented a challenge for experiment and theory. Since DsbA lies at the oxidising end of the thioredoxin family spectrum, this challenge is often stated as a search for the basis of the oxidising power of DsbA.

Experimental studies have gone a long way towards explaining the oxidising power of DsbA in terms of the stabilities of reduced and oxidised forms, and with reference to the pK_a of the active site thiolate (C30). It has been found that the reduced form is favoured by 16 to 22 kJ/mol over the disulphided form [6,7]. The pK_a of C30 in DsbA is about 3.5 [8], as compared to that of 6.7 for the homologous thiolate (C32) in thioredoxin [9]. It has been suggested that the oxidising power of DsbA could originate from the low C30 pK_a, through stabilisation of the reduced form [8]. The link between DsbA stability in reduced and oxidised forms, C30 pK_a (reduced form), and redox potential has been further investigated [10]. These properties were measured for DsbA mutants and wild-type (wt), and it was found that changes in C30 pK_a correlate closely with alterations in the relative stability of reduced and oxidised forms, and with redox potential. The mutations were contained within the two amino acids that separate the active site cysteines. Since protein properties vary significantly with changes in these two residues, it was concluded that they are critical in determining the oxidising power of DsbA [10]. Sub-groups within the thioredoxin family are characterised by the nature of these two residues, which are PH in DsbA and normally GP in thioredoxin. In comparison to the ΔpK_a between homologous thiolates in DsbA and thioredoxin of 3.2, the range of ΔpK_a between wt and mutant is 0.8–1.4 for all mutants other than PP, for which $\Delta pK_a = 3.3$ [10].

Modelling has also been used to study the oxidising power of DsbA. Finite difference (FD) electrostatics calculations have been used to study the ΔpK_a between C30 of DsbA and C32 of thioredoxin [11]. Such a difference calculation between structurally homologous systems is well suited to these methods, which solve the Poisson-Boltzmann (PB) equation. Based on obtaining a reasonable match between calculated and experimental C30/C32 ΔpK_a's, the calculations were analysed to give a molecular model for the difference in oxidising power between DsbA and thioredoxin. It was suggested that the identity of the two amino acids between the active site cysteines plays a role, along with the presence of the extra domain in DsbA, and differences in mainchain conformation within the common (thioredoxin-like) domain. These calculations suggest that differential stabilisation of a functionally homologous thiolate can be achieved in a variety of ways, not all of which are as clear-cut as sequence variation within two amino acids.

This article presents electrostatics calculations to compare with the experimental data for DsbA mutants other than PP [10]. The agreement with experiment is discussed in terms of both the molecular basis of the oxidising power of DsbA, and the limits of such calculations in relation to conformational alterations. It will be argued that the PP mutant cannot adopt the same active site structure in detail as that common to *E. coli* thioredoxin and *E. coli* DsbA, so that its properties represent a deviation from the redox spectrum that links these two proteins. Elements which contribute significantly to this spectrum, and which lie outside of the active site sequence, are discussed.

2. Methods

2.1. DsbA wt and mutant structures

The basis for modelling with DsbA is the X-ray structure of the oxidised molecule at 2 Å resolution [3]. A model for the reduced structure has been used previously, based on breaking the disulphide (C30–C33) and torsioning C30 to maintain van der Waals (VdW) contact with C33 [11]. The calculated electrostatic stabilisation of

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the negatively charged C30 thiolate was found to be relatively insensitive to torsioning of the cysteine sidechain within the steric constraints of the surrounding protein. DsbA mutants involving the residues that lie between C30 and C33 have been constructed, and their pK_a's measured [10], (J. Bardwell, personal communication). These were modelled with the program QUANTA (Molecular Simulations Inc., 200 Fifth Avenue, Waltham, MA 02154, USA). Sidechains were rotated to avoid steric clashes, and ΔpK_a's were calculated as differences between wt and mutants, using previously described procedures [11]. All mutants other than PP could be accommodated without movement of the surrounding protein. The double proline mutant would require significant mainchain rearrangement, as the wt backbone torsion angles are not consistent with such a sequence. Since the modelling is based on homology to wt, without mainchain movement, the PP mutant has not been included in the comparison.

2.2. Electrostatics calculations

Computational analysis used FD solutions to the PB equation [12,13], implemented in the program FDCALC. A value of 3 was used for the protein relative dielectric (ε_p), within the normally accepted range of 2–4 [14]. Some calculations were carried out with $\varepsilon_p = 20$, to test the suggestion that higher ε_p may be more appropriate [15]. A solvent relative dielectric (ε_s) of 80, and a linear ion response for 0.15 M univalent ions were used in the FD calculations. Calculated results vary by only about 1% when the ionic strength is altered from 0.15 M to 0.2 M, which corresponds to experiment [10]. Partial charges were allocated from the GROMOS library [16]. Ionisable group charges are assigned to OD1 and OD2 of aspartic acid, to OE1 and OE2 of glutamic acid, to ND1 and NE2 of histidine, to NH1 and NH2 of arginine, to NZ of lysine, to the nitrogen of N-terminal groups, and to the oxygens of C-terminal groups. Estimates of the dependence on charge and atomic radii were made with the use of an alternative partial charge set from QUANTA/CHARMm, and through also using 2 VdW radii sets [17,18].

In pK_a calculations, the three electrostatic contributions ΔSE (self-energy), ΔDIP (fixed dipolar charges) and ΔIONIS (ionisable groups) are calculated for the change in ionisable group charge interactions that accompanies the transfer from free amino acid to protein. These calculations are not consistently accurate [15,19], and much of the problem appears to be associated with ionisable group solvent accessibility (SA) changes on transfer to the protein (unpublished work). Such calculations for the DsbA system are also likely to be complicated by a pH-dependent conformational change, by analogy with that involving homologous groups in thioredoxin [20]. In order to calculate ΔpK_a's to compare with experiment, the differences between wt and mutant are considered directly, rather than proceeding through the free C30 amino acid step (which cancels). For simplicity, the calculated contributions to ΔpK_a are considered as single differences, ΔSE, ΔDIP and ΔIONIS (taken directly between wt and mutant). By comparison with pK_a calculations, these ΔpK_a's are derived for very small changes in SA, and this is a key factor in providing more accuracy than is generally associated with pK_a computations. In place of a titration curve calculation, ΔIONIS is calculated with an assumed set of ionisable group charge states in the acidic range of the measured pK_a's. Following previous work [11], the only deviations from model pK_a's are assumed for H60, which is taken to be neutral based on hydrogen bonding in the crystal structure, and for E24, which is assumed to be one half negatively charged. Lysines, arginines and histidines (except H60) have a charge of +1, whilst aspartic and glutamic acids (other than E24) have a charge of −1. Excepting the contribution of H32, ΔIONIS is small, and does not contribute significantly to the ΔpK_a values.

3. Results and discussion

3.1. pK_a changes in DsbA mutants

Table 1 shows experimental [10], (J. Bardwell, personal communication), and calculated ΔpK_a's for 8 mutants of DsbA with amino acid changes at residues 31 and 32, with the PP mutant excluded from this comparison which is based on conserved mainchain structure. For calculations with the GROMOS partial charge set and the first VdW radii set [17], 5 out of 8 calculated values match the measurements, within experimental error. Calculations with the QUANTA/CHARMm partial charge set, for which 5 out of 8 again match within experimental error, differ mostly for those mutants where P31 is altered. Changing a proline to a non-proline residue involves a larger alteration in partial charges for the GROMOS set than for the QUANTA/CHARMm set, with the result that the ΔpK_a's for such a mutation tend to be larger for GROMOS partial charges. For the mutants which agree well with experiment, the GROMOS and QUANTA/CHARMm charge set values tend to surround the experimental values, and it is not possible to determine a preference. Changing to the second VdW radii set [18] shows a variation of up to 15% in ΔpK_a (calculations not shown). Clearly the detailed results depend on the choice of partial charge and VdW radii sets. The degree of error associated with this choice is just fractions of a pK_a unit for the DsbA mutants, and does not obscure the overall success of the dielectric continuum model in giving the direction and overall size of the set of ΔpK_a's.

Another source of model error arises from the use of just one modelled conformation for each mutant. A small degree of variation with sidechain rotamer choice was found, and it is unlikely that better agreement with experiment (for most of the mutants in Table 1) could reasonably be expected without a substantially improved algorithm for including conformational heterogeneity. The worst agreement is found for the TR mutant. The arginine sidechain was modelled as predominantly extended, and it is possible that failure to include hydrophobic packing against the protein could have contributed to this error. Discrepancies of less than half a pK_a unit are comparable to thermal energy at physiological temperatures, giving another indication that more accurate modelling will require sensitive techniques for handling conformational variation.

Whilst the ranking of mutants within this relatively small range of ΔpK_a's is not well reproduced, it is possible to investigate the molecular basis for the overall sign and size of the ΔpK_a's. Table 2 shows the ΔpK_a's calculated with the GROMOS partial charge set, along with the major contributions. These are the removal of H32, which is common to all mutants, and ΔSE/ΔDIP. The calculated ΔpK_a contribution of H32 is 0.77, which is independent of partial charge set and does not vary significantly between the two VdW radii sets. It is reasonable to assume that H32 will be protonated at the acidic pH of the C30 pK_a measurements. A change in redox potential of 35 mV was measured for the P34H mutation in thioredoxin [21], which would correspond to a ΔpK_a of about 0.6 if redox potential is directly related to thiolate pK_a [10]. Table 2 shows that the calculated ΔpK_a's mostly consist of the positive H32 contribution plus another positive result gained from adding ΔSE and ΔDIP. If the range of calculated H32 contribution is correct, then the experimental data in Table 1 also support the suggestion of an additional positive contribution to ΔpK_a.

The net positive contribution from ΔSE+ΔDIP comes from three general sources. First, the GROMOS partial charge set gives a contribution of about +0.5 to ΔDIP from the replacement of the P31 with a non-proline residue containing the peptide NH group. As discussed, this effect is much smaller with the QUANTA/CHARMm partial charge set, and it is therefore not clear how large a contribution P31 makes to the oxidising power of DsbA. The charge disposition (com-

Table 1. Experimental and calculated ΔpK_a's for DsbA mutants

Mutant	Experiment	GROMOS	QUANTA/ CHARMm
SV	0.81 (0.23)	1.11	0.87
SF	0.92 (0.23)	1.07	0.83
PL	1.00 (0.28)	0.85	0.84
ST	1.03 (0.24)	0.79	0.43
QL	1.17 (0.21)	1.28	1.01
TR	1.34 (0.23)	0.76	0.46
LT	1.44 (0.21)	1.64	1.30
PS	1.45 (0.23)	0.96	0.82

Experimental ΔpK_a's [10], (J. Bardwell, personal communication) are taken relative to a wt pK_a of 3.42 (0.18), and sequence of PH. Values in parentheses give the reported experimental error. Calculated values are quoted for the GROMOS and QUANTA/CHARMm partial charge sets. Listed amino acids are residues 31 and 32 that lie between the active site cysteines, 30 and 33. Mutants are ranked according to the experimental values.

pared with non-proline) probably has some effect, but not more than 0.5 of a pK_a unit. Second, the location of hydroxyl groups introduced in the mutants affects ΔDIP, but generally to a small degree since the OH–thiolate interactions are outside of hydrogen-bonding range and solvent exposed. Third, the effect of changing the dielectric boundary is to modify accumulated interactions with the thiolate. This boundary alteration also changes ΔSE, but in the opposite sense. In general terms, an increase in SA will decrease the (mostly) favourable thiolate–partial charge interactions, whilst also decreasing the unfavourable self-energy penalty for charge burial. It is this last effect that plays a large part in the compensation of ΔSE and ΔDIP, where mutant thiolates tend to be more stable in self-energy terms (negative ΔSE), but less stable in partial charge interactions (positive ΔDIP). This is consistent with the listed changes in SA between wt and mutants, which are mostly positive.

The calculations suggest that the molecular factors underlying the experimental ΔpK_a's are H32 removal, possibly some contribution from charge disposition for P31 or non-P31, relatively small contributions for introduced sidechain hydroxyls, and a sizeable effect associated with dielectric boundary alteration, reflected in SA changes. This last term acts to modify the interactions which have evolved to stabilise the C30 thiolate, and it appears that generally a slight opening of the active site gives a small reduction in the stabilisation, and contributes to move the mutant C30 pK_a's further away from wt than would H32 removal alone. The detailed ranking of these effects is not accurate in comparison with experiment, and such discrepancy again lies in the area of conformational and other modelling error. The contributions listed in Table 2 suggest that mutations can have electrostatic effects where direct charge alteration is not involved. The energetics of dielectric boundary movement is significant, although there is some compensation between self-energy and charge–charge interactions. These observations are conducted at one end of the spectrum of SA changes in which overall pK_a calculations operate, and give some indication of the problems associated with calculating the energetics of a process that involves substantial charge burial.

It may be expected that the DsbA C30 thiolate system will be a severe test for the model which uses $\varepsilon_p = 20$ [15], since low solvent exposure places emphasis on the protein dielectric. With regard to the active site peptide mutants of DsbA this is indeed the case, with $\varepsilon_p = 20$ calculations substantially underestimating experimental values. For example, comparing experiment and calculations with the GROMOS partial charge set, the following results (listed as mutant–experiment/$\varepsilon_p = 3/\varepsilon_p = 20$) are obtained: PL — 1.00, 0.85, 0.49; SV — 0.81, 1.11, 0.43; QL — 1.17, 1.28, 0.51; LT — 1.44, 1.64, 0.55. It is clearly important to use low ε_p to provide a reasonable estimate of thiolate interactions in DsbA. Such discussion also argues against the use of simple Coulomb calculations based on a uniform high dielectric, in this case. Additionally, the effect of incorporating the $\varepsilon_p/\varepsilon_s$ boundary in the PB solution is seen in the ΔpK_a contributions that arise from dielectric boundary movement in the mutants.

3.2. The oxidising power of DsbA

In terms of understanding the oxidising power of DsbA, and how this may be further manipulated, the current study emphasises that the amino acids at the active site are not only important for what charge they present, but also for how they form a low dielectric region around the thiolate that intensifies interactions from other parts of the protein. It will be interesting to calculate ΔpK_a for the PP mutant when the structure is determined experimentally. In the sense that DsbA has evolved to stabilise the C30 thiolate, it is not surprising that disruptions to the active site structure will interfere with this stabilisation, and give a large positive ΔpK_a (3.3 for the PP mutant [10]). An analysis of the likely conformational effect of the PP mutant has been made. Starting with the mainchain conformation common to *E. coli* DsbA and *E. coli* thioredoxin, a CB (P31)–CD (P32) contact distance of 3.0 Å is observed. This loop, which forms the amino terminus of an α-helix, can accommodate these prolines separately but must alter to accept the PP mutant. Model-building suggests that the close contact can be alleviated within a loop that maintains a helical turn, and in which the largest change is an increase in the solvent exposure of the C30 sidechain, with accompanying movement of M64. This proposed conformational change would substantially reduce the C30 thiolate stabilisation associated with the ΔDIP term, and would be consistent with the large experimental ΔpK_a. Modelling also suggests that the equivalent change in thioredoxin would have a smaller effect on the C32 pK_a, since ΔDIP is smaller [11], and since there is no residue directly equivalent to M64 of DsbA. Indeed, the PP variant occurs in thioredoxins from *Arabidopsis thaliana* [23], without appearing to cause a large change in functional properties.

Non-PP mutants are assumed to adopt homologous active site structures to *E. coli* DsbA and *E. coli* thioredoxin. The maximum ΔpK_a for these mutants is 1.5, less than half the difference of 3.2 between DsbA/C30 and thioredoxin/C32 pK_a's. This observation is consistent with the suggestion [11]

Table 2. Contributions to calculated ΔpK_a's for DsbA mutants

Mutant	Total	% ΔSA	H32	ΔSE	ΔDIP
SV	1.11	1.2	0.77	−0.33	0.70
SF	1.07	2.2	0.77	−0.94	1.26
PL	0.85	−0.4	0.77	−0.56	0.61
ST	0.79	0.7	0.77	−0.39	0.39
QL	1.28	0.9	0.77	−0.26	0.79
TR	0.76	1.7	0.77	−0.58	0.91
LT	1.64	0.8	0.77	−0.42	1.28
PS	0.96	0.4	0.77	−0.12	0.32

All columns are listed in pK_a units, except for that labelled % ΔSA, which gives the fractional solvent accessibility [22].

that the sum of many interactions (rather than just those within the disulphide sequence) provides the oxidising power of DsbA in comparison with thioredoxin. A hypothetical DsbA mutant with the thioredoxin sequence of GP gives a calculated ΔpK_a of 1.6 (using the GROMOS partial charge set), again indicating the presence of DsbA/thioredoxin differences in the addition to the active site sequences. An nmr study of *E. coli* thioredoxin has been used to suggest that the low C32 pK_a is largely due to the close approach of the C35 thiol [24]. The current discussion emphasises that it is equally important to look outside of the CXXC sequences to account for pK_a and redox effects in this family of proteins.

The agreement between experiment and computation, demonstrated in this article for the sign and overall size of ΔpK_a's, gives confidence that the model is identifying the molecular detail that determines thiolate pK_a and oxidising power. Mutant calculations emphasise the effect of H32 (up to 0.8 pK_a units), and raise the possibility of a contribution of up to 0.5 pK_a unit for P31/non-P31, although the size of this effect depends on the partial charge set. Variation of ΔpK_a across mutants which have common contributions from these two sources is partly due to sidechain hydroxyl interactions, and more significantly to changes in SA at the active site, that alter the balance of charge interactions. It has been suggested [11] that around half of the DsbA/thioredoxin difference arises from two identifiable sources outside of the active site peptide. These are the DsbA Q97 sidechain, and the mainchain region 37–43 in DsbA. Residue Q97 lies in the extra domain, relative to thioredoxin. The overall effect of the extra domain is difficult to assess since it depends on the detail of the link between the extra module and the thioredoxin-like domain, around residues 62–64. The mainchain segment 37–43 in DsbA is associated with an α-helical insert that is absent in thioredoxin.

Proteins within the thioredoxin family, including protein disulphide isomerase (PDI) and glutaredoxin, are unlikely to use exclusively those features identified here to modify redox potential. It has been shown that redox potential alteration is directly related to changes in thiolate pK_a [10], and electrostatic calculations suggest that such changes incorporate effects inside and outside of the active site. Whilst some features are repeated, such as an equivalent histidine to H32 (DsbA) in PDI, it can be expected that evolution will find several routes to pK_a modification. However, it is likely that all systems that act to increase oxidising power by reducing the thiolate pK_a substantially below that of free cysteine, will do so by charge burial. This is a key factor in intensifying interactions between the negatively-charged thiolate and amino acids around the active site. Given the extent of these interactions, it is unlikely that simply writing down the active site sequence will be sufficient to describe in full the oxidising properties of proteins within the thioredoxin family.

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References

[1] Bardwell, J.C.A. and Beckwith, J. (1993) Cell 74, 769–771.
[2] Bardwell, J.C.A. (1994) Mol. Microbiol. 14, 199–205.
[3] Martin, J.L., Bardwell, J.C.A. and Kuriyan, J. (1993) Nature 365, 464–468.
[4] Katti, S.K., LeMaster, D.M. and Eklund, H. (1990) J. Mol. Biol. 212, 167–184.
[5] Freedman, R.B., Hirst, T.R. and Tuite, M.F. (1994) Trends Biochem. Sci. 19, 331–336.
[6] Zapun, A., Bardwell, J.C.A. and Creighton, T.E. (1993) Biochemistry 32, 5083–5092.
[7] Wunderlich, M., Jaenicke, R. and Glockshuber, R. (1993) J. Mol. Biol. 233, 559–566.
[8] Nelson, J.W. and Creighton, T.E. (1994) Biochemistry 33, 5974–5983.
[9] Kallis, G.-B. and Holmgren, A. (1980) J. Biol. Chem. 255, 10261–10265.
[10] Grauschopf, U., Winther, J.R., Korber, P., Zander, T., Dallinger, P. and Bardwell, J.C.A. (1995) Cell, 83, 947–955.
[11] Gane, P.J., Freedman, R.B. and Warwicker, J. (1995) J. Mol. Biol. 249, 376–387.
[12] Warwicker, J. and Watson, H.C. (1982) J. Mol. Biol. 157, 671–679.
[13] Warwicker, J. (1986) J. Theoret. Biol. 121, 199–210.
[14] Sharp, K.A. and Honig, B. (1990) Annu. Rev. Biophys. Chem. 19, 301–332.
[15] Antosiewicz, J., McCammon, J.A. and Gilson, M.K. (1994) J. Mol. Biol. 238, 415–436.
[16] van Gunsteren, W.F. and Berendsen, H.J.C. (1987) GROMOS Manual, BIOMOS Biomolecular Software, Laboratory of Physical Chemistry, University of Groningen.
[17] McCammon, J.A., Wolynes, P.G. and Karplus, M. (1979) Biochemistry 18, 927–942.
[18] Handschumacher, M. and Richards, F.M. (1983) ACCESS program, Yale University, New Haven, CT, USA.
[19] You, T.J. and Bashford, D. (1995) Biophys. J. 69, 1721–1733.
[20] Langsetmo, K., Fuchs, J.A., Woodward, C. and Sharp, K.A. (1991) Biochemistry 30, 7609–7614.
[21] Lundstrom, J., Krause, G. and Holmgren, A. (1992) J. Biol. Chem. 267, 9047–9052.
[22] Lee, B.K. and Richards, F.M. (1971) J. Mol. Biol. 55, 379–400.
[23] Rivera-Madrid, R., Mestres, D., Marinho, P., Jacquot, J.-P., Decottignies, P., Miginiac-Maslow, M. and Meyer, Y. (1995) Proc. Natl. Acad. Sci. USA 92, 5620–5624.
[24] Jeng, M.-F., Holmgren, A. and Dyson, H.J. (1995) Biochemistry 34, 10101–10105.