MOLECULAR BASIS FOR SPECIFICITY OF THE EXTRA-CYTOPLASMIC THIOREDOXIN ResA
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Running Title: Basis for Specificity of the Thioredoxin ResA
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ResA, an extra-cytoplasmic thioredoxin from Bacillus subtilis, acts in cytochrome c maturation by reducing the disulfide bond present in apo-cytochromes prior to covalent attachment of heme. This reaction is (and has to be) specific, as broad substrate specificity would result in unproductive short-circuiting with the general oxidizing thioredoxin(s) present in the same compartment. Using mutational analysis and subsequent biochemical and structural characterization of active site variants, we show that reduced ResA displays unusually low reactivity at neutral pH, consistent with the observed high pK_a values >8 for both active site cysteines. Residue E80 is shown to play a key role in controlling the acid-base properties of the active site. A model in which substrate binding dramatically enhances the reactivity of the active site cysteines is proposed to account for the specificity of the protein. Such a substrate-mediated activation mechanism is likely to have wide relevance for extra-cytoplasmic thioredoxins.

Thiol-disulfide oxidoreductases (TDORs) comprise a large superfAMILY of proteins, members of which are present in all cells where they are involved in redox state management of protein cysteine thiols. Those in the cell cytoplasm, eg thioredoxin itself, are usually involved in maintaining protein cysteines in a reduced state (1), whereas those on the outside of the cytoplasmic membrane can be involved in generating disulfide bonds in protein and peptide substrates, eg E. coli DsbA and B. subtilis BdbD (2-5), or in removing disulfide bonds in protein substrates as part of post-translational modification processes, eg E. coli CcmG and B. subtilis StoA (6, 7). Some TDORs have an isomerase activity that enables them to rearrange disulfide bonds in protein/peptide substrates, eg E. coli DsbC (8, 9).

Many TDORs consist of a five stranded mixed β-sheet surrounded by four α-helices, an arrangement known as the thioredoxin fold, after the original member of the family (1, 10). The two cysteine thiols of the thioredoxin TDOR active site motif, CXXC (in which X denotes an unspecified amino acid residue) are positioned at the terminus of an α-helix and are correctly orientated to reversibly cycle between reduced (thiol) and oxidised (disulfide) forms. The redox potential of the disulfide/dithiol couple correlates with the function of the protein; low and high potential TDORs act to reduce and oxidise their substrates, respectively (eg (11, 12). ResA is an extra-cytoplasmic thioredoxin-like TDOR from Bacillus subtilis that is involved in cytochrome c maturation (13, 14). Bacteria exhibit one of two distinct maturation pathways (System I and II) (15, 16) and Gram-positive organisms such as B. subtilis have System II which, in this organism, consists of only three dedicated proteins, ResA, ResB and ResC (13, 17). Strains lacking ResA are deficient in c-type cytochromes. This is reversed in strains also lacking BdbD, a TDOR that catalyses formation of disulfide bonds in the extra-cytoplasmic compartment of B. subtilis, indicating that ResA is involved in reduction of the apo-cytochrome prior to covalent heme attachment (13). Soluble ResA (lacking its single transmembrane segment anchor) was found to have a low redox potential and low activity in the insulin assay, indicating that it functions specifically as a reductase in cytochrome c maturation (13).
High resolution structures of soluble ResA in both oxidation states revealed a typical thioredoxin fold, with an additional N-terminal β-hairpin and a strand/helix insertion between strand β2 and helix α3 (14). Upon reduction, several stabilizing interactions are broken in the vicinity of the active site, whereas only one hydrogen bond, between the C74 thiol and the backbone amide hydrogen of G71, is formed (note that the numbering of ResA residues in the original structure pdb file (1SU9) and paper (14) were in error by -1. The active site thiol residues are C74 and C77 rather than C73 and C76). Overall this results in a destabilization of the reduced structure, relative to the oxidised, consistent with its low redox potential. In reduced ResA, the thiol of C74 is significantly more solvent exposed than that of C77, as found in other TDOR structures, but the separation between the Sγ atoms is unusually long. Present only in the reduced structure is a cavity lined by mainly hydrophobic residues (F66, N68, P76, C77, F81, M84, P141, T143, G158, T159 and M160). At its base is E80, which is stabilised through hydrogen bonding interactions with T159 and a water molecule that is also hydrogen-bonded to the side chain of C77. The location of the cavity close to the active site led us to propose that it plays an important role in substrate recognition (14). Solution NMR experiments have recently confirmed that redox-related conformational changes occur in this region (18).

Here we present biochemical and structural studies of wild-type and variants of soluble ResA. By labelling with the environment-sensitive fluorescent probe badan and iodoacetate, we demonstrate that the thiol groups of C74 and C77 both have pKₐ values between 8 and 9. The unusually high pKₐ of C74 is consistent with the protein’s low reactivity at low to neutral pH values. In contrast to thioredoxin, at pH values above 8 both active site cysteines can be readily alkylated. Biochemical and structural studies of E80Q ResA reveal that E80 plays a key role in controlling the acid/base properties of the active site. The data support a model for reduced ResA in which E80 locks the active site thiols in an unreactive conformation, and substrate binding specifically unlocks the active site reactivity. Similar substrate-mediated activation mechanisms are likely to be relevant for many other extra-

Experimental Procedures

**Site-directed Mutagenesis and Protein Purification** – Strains, growth media and genetic methods used in this study were as previously described (13). Site directed mutagenesis was carried out using a whole-plasmid method (19), with pRAN10 as the template (13). Ndel/EcoRI fragments were cloned into pET21a giving pALR8 (E80Q), pALR5 (C74A) and pALR14 (C77A). For C74A/C77A in pET21a (pALR19), pALR5 was used as template. Mutations were confirmed by sequencing (MWG Biotech).

Wild-type and variant soluble ResA proteins were purified as previously described (13), except that 20 mM Tris, pH 8.0 was used instead of sodium phosphate, and a Superdex S75 gel filtration column (GE Healthcare) was used in place of a Sephacryl S100 column. The single cysteine variants showed a tendency to form disulfide-linked dimers and so 1 mM DTT was included in the buffer during the gel filtration step.

**Thiol Reactivity Experiments** – All proteins were pre-reduced by incubating with 1 mM DTT at 4 °C overnight. DTT was removed by gel filtration. Under aerobic conditions, all variants remained reduced for at least 24 hours following removal of DTT. For fluorescence labelling experiments, ResA and variants (final conc. 1 µM) were added to a 13 µM badan solution (Molecular Probes) (20) and fluorescence spectra recorded between 400 - 600 nm (ex. wavelength 390 nm) at 25 °C using a Perkin Elmer LS55 fluorescence spectrophotometer. Badan was in either a mixed buffer system containing ammonia, potassium acetate, MES, MOPS and Tris (10 mM each) and 200 mM KCl (21), or single buffer solutions of 150 mM NaCl containing 50 mM MES (pH 5.5 - 7.0), Tris (pH 7.5 - 9.0) or CHES (pH 9.5 - 10.0). Equivalent results were obtained in each.

For carboxymethylation experiments, reduced protein (~40 µM) was incubated with 10 mM iodoacetate at room temperature. For kinetic experiments, 30 µl samples were removed at different time points and the reaction quenched by
the addition of 10 µl native PAGE loading buffer containing 200 mM DTT. Modified and unmodified proteins were separated using native PAGE run in 5 mM Tris-glycine buffer, pH 8.0. ESI-MS was conducted on badan-modified ResA as previously described for ResA (13).

**Determination of Cysteine Thiol \( \mathcal{p}K_a \) Values** – The reaction of cysteine side chains with alkylating reagents is well established and occurs only with the ionized thiolate anion (22). Measurement of the rate of alkyla tion as a function of pH can be used to determine the \( \mathcal{p}K_a \) values of protein cysteine thiol groups (21, 23, 24), where the observed rate constant is proportional to the extent of thiol deprotonation at a given pH value (21). Reaction of wild-type ResA and variants with badan were carried out under pseudo-first order conditions (see above). Fluorescence data were fitted to a single exponential function to obtain an observed, pseudo-first order rate constant (\( k_o \)). Where necessary, a double exponential fit was used and the rate constant for the initial reaction was taken as \( k_o \). \( \mathcal{p}K_a \) values were determined by plotting \( k_o \) values as a function of pH and fitting to an appropriate equation. For single \( \mathcal{p}K_a \) processes, Equation (1) was used:

\[
 k_o = \frac{k_{SSH} + k_{S} \cdot 10^{\mathcal{p}H - \mathcal{p}K_a}}{1 + 10^{\mathcal{p}H - \mathcal{p}K_a}}
\]

where \( k_{SSH} \) and \( k_S \) are the rate constants for the protonated and deprotonated forms, respectively (21). For two proton dissociation events, where the \( \mathcal{p}K_a \) values are well separated, Equation (2) was used:

\[
 k_o = \frac{k_{SSH} + k_{S} \cdot 10^{\mathcal{p}H - \mathcal{p}K_a} + k_{S} \cdot 10^{2\mathcal{p}H - 2\mathcal{p}K_a}}{1 + 10^{\mathcal{p}H - \mathcal{p}K_a} + 10^{2\mathcal{p}H - 2\mathcal{p}K_a}}
\]

Where \( k_{SSH} \), \( k_{S} \) and \( k_{S\cdot S} \) are the rate constants for the fully protonated, and singly and doubly deprotonated forms, respectively (25, 26). In cases where two proton dissociation events were cooperatively coupled, Equation (3) was used (see Supplemental Data):

\[
 k_o = \frac{k_{SSH} + k_{S\cdot S} \cdot 10^{2\mathcal{p}H - 2\mathcal{p}K_a}}{1 + 10^{2\mathcal{p}H - 2\mathcal{p}K_a}}
\]

Where \( \mathcal{p}K_a \) is the apparent average of the two \( \mathcal{p}K_a \) values (see Supplemental Data). For fitting titration data to three proton dissociation events where the latter two are cooperatively coupled, Equation (4) was used (see Supplemental Data):

\[
 k_o = \frac{k_{SSH} \cdot 10^{\mathcal{p}H - \mathcal{p}K_a} + k_{S\cdot S} \cdot 10^{2\mathcal{p}H - 2\mathcal{p}K_a}}{1 + 10^{\mathcal{p}H - \mathcal{p}K_a} + 10^{2\mathcal{p}H - 2\mathcal{p}K_a}}
\]

For the determination of the \( \mathcal{p}K_a \) of C77 in C74A ResA using iodoacetate, samples at pH values between 7.0 and 9.5 were taken after 24 hr incubation in iodoacetate and run on native PAGE. Stained modified and unmodified proteins were quantitated using imaging software. For the pH titration, fraction modified, \( f \), was converted to an observed relative rate constant, \( k_o \), using Equation (5):

\[
 k_o = -\ln(1 - f)/c
\]

where \( c \) is a constant that includes a time component. Rate constants were plotted against pH and fitted using the appropriate equation above. Attempts to determine \( \mathcal{p}K_a \) values using UV absorbance spectroscopy to monitor directly the concentration of cysteine thiolates (21) did not give reliable data. This is likely to be a consequence of the overlap of cysteine thiol and tyrosine hydroxyl deprotonation events (ResA has 6 tyrosine residues).

**Crystallization and Data Collection** – ResA proteins were concentrated to 12 - 17 mg/ml in 20 mM MOPS pH 7.0. Conditions for crystallization of ResA variants were screened by the sitting drop vapour diffusion method at 25 ºC, employing a 24 condition 2-dimensional grid screen (see Supplemental Data), where equal volumes of protein were mixed with well solution. Crystals were soaked in a cryoprotectant solution composed of 30% PEG 4,000, 0.1 M ammonium acetate, 0.1 M sodium citrate, 20% ethylene glycol for up to 5 minutes before freezing. For the high pH structure of ResA, crystals were grown at pH 5.6 and subsequently equilibrated in an equivalent cryoprotectant for which the MES component was replaced by 0.1 M CHES pH 9.25. X-ray diffraction datasets were collected on beam lines ID-29 and ID14-2 of the European Synchrotron Radiation Facility (Grenoble, France). Diffraction data was indexed and integrated with MOSFLM (27), and subsequently scaled using SCALA (28).
Data collection statistics for each dataset are presented in Table 1.

**Structure Determination and Refinement**

Structure determination was aided using programs of the CCP4 suite (29). Starting phases were obtained by molecular replacement using the program MOLREP (30). Efforts to minimize phase bias are described in [Supplemental Data](#). In each case, monomer A from the reduced structure of wild-type ResA (1SU9) was used as the initial searching model. Refinement of successful molecular replacement solutions was initiated using REFMAC (31) and refined further through alternate rounds of manual building in COOT (32) and restrained refinement in REFMAC. Completed structures were validated with the aid of PROCHECK (33) and SFCHECK (34) and the coordinates submitted to the RCSB Protein Data Bank along with appropriate structure factor files. Assigned PDB ID codes are: 2H1A (C74A); 2H19 (C77A); 2H1G (C74A/C77A); 2H1B (E80Q); 2H1D (high pH ResA). Refinement statistics for each structure are presented in Table 1. See [Supplemental Data](#) for further details of crystallization, data collection and structure refinement.

**Results and Discussion**

The Active Site Cysteines of Wild-type ResA have Unusual Reactivities – The alkylating reagent 6-bromoacetyl-2-dimethylaminonaphthalene (badan) forms a thioether bond with the side chain of cysteine residues, leading to a significant increase in its fluorescence intensity. The spectrum of unbound badan features a band at ~540 nm. On addition of ResA (at pH 8.5) the probe fluorescence showed a marked increase over time, with two bands developing at ~460 and 520 nm, see Fig. 1A. The probe fluorescence is highly sensitive to its environment, with emission maxima for the β-mercaptoethanol adduct ranging from ~550 nm in a polar solvent (eg water) to 450 nm in hydrophobic solvents (eg toluene) (20). From the crystal structure of reduced ResA, it is known that the C74 sulfur atom is reasonably exposed to the bulk solvent and so badan attached to this residue is expected to give a fluorescence signal near to that of the unbound probe. C77, which is substantially more buried in a hydrophobic pocket, is likely to give rise to the peak at 460 nm. Addition of badan to a cysteine-free C74A/C77A ResA double variant (at pH < 10) did not cause an increase in intensity, confirming the specificity of the probe for thiol groups.

Fluorescence at 450 and 540 nm was plotted against time (off-maxima wavelength data were used to minimise effects from peak overlap), at a range of pH values, see Fig. 1B and C. The initial increase and subsequent decrease of intensity observed at 450 nm at higher pH is most likely due to local unfolding caused by the addition of two labels at the active site (see [Supplemental Data](#)). At pH values above pH 9.5, significantly increased rates of modification were observed, suggesting that the protein undergoes unfolding in this pH range; this was confirmed by pH stability experiments (see [Supplemental Data](#)). Pseudo-first order rate constants were obtained from fits of the data at 540 and 450 nm and plotted against pH (for values below 9.5), Fig. 1D. Data at 450 nm, reporting on C77, fitted well to a single proton dissociation event, with a \( pK_a \) of 8.2 ± 0.13. The data at 540 nm, reporting on C74, could not be satisfactorily fitted to equations describing simple Henderson-Hasselbach titration behaviour (Equations (1) and (2)). The plot is sigmoidal, but the transition region of the curve is much steeper than expected. This implies that the titration behaviour of C74 is strongly cooperatively coupled to another ionizable residue which binds and releases protons simultaneously with C74. Attempts to fit the data to an equation describing interacting functional groups (35) indicated that the concentration of the two mono-protonated species must be negligible (see [Supplemental Data](#)). Thus, Equation (3) describing the cooperative binding of two protons, was employed, giving a good fit with an average apparent \( pK_a \) of 8.8 ± 0.2. We note that, because of the strong cooperativity observed, it may be more appropriate to consider an apparent overall equilibrium constant (\( K_{app} \)) for the two proton process, where, here, \( pK_{app} = 17.6 \). A similarly high \( pK_a \) value (>9) was recently reported for the solvent exposed active site cysteine of the C-terminal domain of DsbD (36).

ESI-MS spectra of ResA samples reacted with badan at pH 7 and pH 9 contained peaks at 15925 and 16350 Da, with a higher relative intensity of
the higher molecular weight peak at higher pH (not shown). These correspond to the unmodified protein (with the N-terminal methionine excised (13)) and doubly-labelled ResA, respectively, confirming the stoichiometry of the labelling reaction. The lack of singly modified protein could imply cooperativity in the alkylation reaction or simply that the singly modified protein is not detected. A native gel of ResA following reaction with iodoacetate, Fig. 2A, shows clearly that both cysteines react to give the doubly modified protein, and that this does not occur cooperatively.

In thioredoxin, only the first cysteine residue of the CXXC motif can be modified in the folded protein by iodoacetate (37). The $pK_a$ value for this cysteine is in the range 6.7 – 7.5, significantly lower than the typical value of ~8.5 – 9.0 observed for cysteine residues, while that of the second cysteine is estimated to be $>$9 (25, 35, 37, 38). This large separation of $pK_a$ values is consistent with the close proximity of the two thiol groups, indicating that the ionisation of one significantly influences that of the other. The cysteine thiols of thioredoxin may even share a proton after the deprotonation of the N-terminal cysteine thiol (25). The wide separation of active site thiol $pK_a$ values appears to be a general feature of TDORs that act with low specificity (21, 39).

One of the most striking features of the structure of reduced ResA is the atypically large separation of ~4.5 Å between the cysteine thiols (compared to the 3.7 – 3.9 Å separation in reduced thioredoxin (40, 41) and 3.5 Å in reduced DsbA (42)). Thus, the two cysteines should have significantly less effect on each other in terms of their acid/base properties and their resulting reactivities. Data indicate that this is the case and ResA has very different properties to thioredoxin and related TDORs: both cysteine thiols are reactive to alkylating reagents; the $pK_a$ values for the two cysteine residues of the active site CXXC motif are both above 8 and within ~0.5 units of each other; and, the second cysteine residue at the active site has a lower $pK_a$ than the first.

**Structural Analyses of Active Site Cysteine Variants of ResA** – To investigate the properties of the active site thiols individually, C74A and C77A single variants of ResA were generated and isolated, and their crystal structures solved, along with that of the C74A/C77A double variant (see Table 1). Cysteine variants crystallised in space group P2$_1$2$_1$2$_1$, with two molecules per asymmetric unit and were isomorphous with previously reported crystals of reduced wild type ResA. Fig. 3D - F depict the active sites of C77A, C74A and C74A/C77A ResA, respectively. Beyond the point of mutation itself, the variants do not show any significant conformational deviations from the wild type structure (Fig. 3A). The cysteine variant structures show that any biochemical differences observed are not due to inconsequential structural rearrangements and thus provide a valuable control for the interpretation of biochemical data derived from these proteins.

The Reactivities of ResA Active Site Cysteines are not Inter-Dependent – Reaction of C77A with badan caused an increase in fluorescence at 500 nm (Fig. 4A), indicating that the environment of the probe at C74 is slightly more hydrophobic in this variant compared to wild-type. C74A exhibited a band at 443 nm, similar to that observed for C77 in the wild-type protein (Fig. 4D). The difference in solvent exposure of the two cysteines results in badan-labelled C77 exhibiting an intensity 8-fold greater than that for C74. These intensities are also, respectively, 16- and 2-fold greater than the equivalent bands in the wild-type protein spectrum (note that this is not apparent from Figs. 4A and D because a smaller emission slit width was employed for the variant proteins). Thus, significant self-quenching occurs within the doubly-labelled wild-type protein. Plots of 500 and 450 nm intensity against time (Figs. 4B and E) show that C74 and C77 exhibit behaviour similar to the wild-type protein. As pH increases, local and then global unfolding occurs, as observed for the wild-type protein (see Supplemental Data).

Pseudo-first order rate constants for C77A and C74A were obtained from the data and plotted against pH, see Figs. 4C and F, respectively. As for the wild-type C74 data, attempts to fit each data set to a one proton dissociation process did not give good fits (see Fig. 4F), but Equation (3), describing two cooperatively coupled proton dissociation processes, gave a significantly improved fit in the latter part of the plot. However, the low pH data are still not well simulated. The best fits (particularly for the less
noisy C74A data) were obtained by fitting each data set to Equation (4), which describes, in addition to two cooperatively coupled proton dissociation processes, a lower pH dissociation event. This gave a pK_a value of 6.5 ± 0.6 for the initial proton dissociation event (for both plots), which we ascribe to a non-cysteine residue. This dissociation event also very likely occurs in the wild-type protein; the inclusion of this process in the fitting of the wild-type ResA data gave equivalent/improved fits (not shown). In addition to the initial pK_a of ~6.5, fits of the single cysteine variant data gave average apparent pK_a values of 8.48 ± 0.08 and 8.36 ± 0.05, respectively, for C74 and C77 (corresponding to pK_{app} values of 17 and 16.7, respectively). Data from pH stability studies also indicate the presence of a dissociable proton between pH 6.5 – 7.0 (see Supplemental Data).

To verify the reactivities of the active site cysteines, wild-type ResA and both single cysteine variants were reacted with Ellman’s reagent. This demonstrated similar rates of reaction for each protein (see Supplemental Data). To verify pK_a values determined with badan-labelling, the pK_a of C77 in C74A ResA was determined using iodoacetate at pH values between 7.0 and 9.5 (note that the reactivity of the individual residues in the wild-type protein cannot be followed separately using this method and so individual pK_a values in the wild-type protein could not be measured). Reaction products at different pH values were run on a native gel, Fig. 2B, and observed rate constants (see Experimental Procedures) were plotted as a function of pH, Fig. 2C. At pH 9.5 and above, a significant increase in rate was observed (not shown) consistent with protein unfolding at high pH. The shape of the plot is very similar to the equivalent plot for the reaction with badan (see inset), and the data were fitted to the same three proton dissociation process, giving an average apparent pK_a value for C77 of 8.3 ± 0.1 (pK_{app} = 16.6).

Thus, in both single cysteine variants, the deprotonation of the remaining cysteine is cooperatively coupled to another, non-cysteine residue. In the wild-type protein, only the deprotonation of C74 is coupled in this way. Thus, in the C74A variant, the coupling interaction that is normally between C74 and the non-cysteine residue is replaced by a similar interaction between C77 and the non-cysteine residue. The identity of the coupled, non-cysteine residue is unknown but we presume that it is the same residue in wild-type ResA (coupled to C74) and in each variant (coupled to C74 and C77, respectively). The residue must be spatially close to the active site in order to have a significant effect on the rate of modification, but cannot be so close that its ionization inhibits that of the cysteine residue(s). There are very few ionizable residues close to the active site that are not entirely solvent exposed (and therefore unlikely to have an unusual pK_a or be cooperatively coupled to C74). One such residue is E105; this partially buried residue is ~7.4 Å away from C74 (and ~8.7 Å away from C77) and is also conserved or conservatively substituted in ResA/CcmG homologues (by sequence and structural alignment). A pK_a value > 8 would be very unusual for a glutamate residue side chain; one possibility is that its deprotonation at lower pH is inhibited and that this is somehow relieved by the deprotonation of C74. This would be consistent with the very tight cooperativity observed, but further investigation is required to test this possible coupling mechanism.

In summary, the data from single cysteine variants demonstrate that the cysteine pK_a values are not inter-dependent; removal of one or the other of the cysteines has only a small effect on the pK_a of the remaining cysteine thiol. This is consistent with the large separation between the cysteine thiols of reduced ResA.

Structural Analysis of ResA at high pH – We have previously reported crystal structures of ResA in which the protein was crystallised at pH 5.6 (14). The pK_a data presented above clearly indicate that the reduced structure represents the protonated, dithiol, form of the protein. To investigate potential structural rearrangements linked to deprotonation of the active site cysteines, we have determined a 2.4 Å crystal structure of DTT-reduced ResA at pH 9.25 (Table 1; see Experimental Procedures). Overall, the structures determined at pH 9.25 and pH 5.6 are extremely similar (compare Fig. 3A and B); individual ResA monomers of the high pH and low pH forms can be superposed with an average root mean square deviation (r.m.s.d.) of just 0.24 Å for Cα positions and 0.52 Å for all atoms. The arrangement of residues in the active site is also extremely similar. Thus,
crystallography does not reveal any significant structural rearrangements upon moving from a low to high pH medium. While the lack of significant structural rearrangements that might stabilize the thiolate form is consistent with the low potential of the protein, we cannot rule out that some structural changes might occur in solution but are not observed in the crystalline form of the protein, due, for example, to crystal packing effects. Also, given the resolution limits, and although we believe it likely, we cannot be absolutely certain that the high pH structure represents the dithiolate form of ResA.

**E80 plays a Key Role in Controlling the Reactivities of the Active Site Cysteines** – Adjacent to the active site of reduced ResA (in thiol and thiolate forms) is a hydrophobic cavity, which is proposed to be important for the interaction of ResA with its substrates (14, 18). To test the possibility that the presumed negative charge associated with E80, which is located at the base of the cavity, is an important determinant for the unusually high pKₐ values of the active site thiols, a E80Q variant was constructed and isolated. The variant was found to be significantly less stable than wild-type protein at high pH (see Supplemental Data). Reaction with badan revealed that the reactivities of the two cysteines are an order of magnitude greater than for the wild-type protein, and also much better resolved: C74 (monitored though the fluorescence intensity at 550 nm) reacted much more rapidly than C77 (at 440 nm), see Fig. 5A. Although the crystal structure (see below) shows that the protein adopts the wild-type fold, the decreased stability of the variant could indicate greater flexibility around the cysteines. Consistent with this is the shift in the C77 peak maximum to ~470 nm. In order to follow C74, reactions were carried out at lower protein concentration and temperature (10 °C). Kinetic runs (Fig. 5B and C) were fitted to obtain pseudo-first order rate constants (kₒ) and the data for C77 were plotted as a function of pH (Fig. 5D). The data fitted well to a single proton dissociation event, indicating that the removal of E80 results in the loss of the low pKₐ (= ~6.5) proton dissociation event observed for the wild-type protein and the single cysteine variants. This is supported by the unfolding data for E80Q (Supplemental Data) which also indicates the loss of the titratable group with a pKₐ of ~6.5. We conclude that this group is the carboxylate side chain of E80. This is, in general, rather high for a glutamate side chain, but perhaps not for one that is buried in a hydrophobic environment (55). The pKₐ value for C77 obtained from the fit is 7.4 ± 0.1, ie approximately one log unit lower than in the wild-type protein. Due to the lower stability of the protein to high pH, we were unable to obtain sufficient data points in the high pH region to determine the pKₐ of C74. Nevertheless, the reactivity data indicate strongly that the pKₐ of C74 is significantly lower than in the wild-type protein (and we estimate it to be <8).

We conclude that E80 plays a key role in modulating the reactivity of the protein. It has been noted previously that the equivalent of E80 in CcmG (E86) is located close to the position of a conserved aspartate in thioredoxin (D26 in the *E. coli* protein). This is believed to deprotonate the second cysteine thiol of the active site, thus facilitating resolution of mixed disulfide intermediates (43, 44). This raises the possibility that E80 serves a similar function in ResA/CcmG homologues (45). D26A thioredoxin, however, was found to have significantly lower activity and an increased pKₐ value associated with its N-terminal active site thiol (46). Our data indicate that the negative charge associated with E80 inhibits deprotonation of the cysteine residues, thus elevating their pKₐ values. Hence, it is unlikely that E80 has the same function as D26 in thioredoxin. Consistent with this is the observation that substitution of E86 in CcmG did not affect cytochrome c maturation (45).

**Structural Insights into the Interaction of ResA with its Substrate(s)** – The crystal structure of E80Q in the reduced state was determined at 1.95 Å (see Table 1 and Fig. 3C). The structure not only confirms that the variant is correctly folded in a manner analogous to the wild type reduced protein, but also reveals several interesting intermolecular contacts, not previously seen in other ResA crystal structures. Some of these contacts occur close to the active site and appear to induce conformational changes to the active site cysteines. We have therefore explored the possibility that these contacts, while not physiological, may nonetheless give structural insight into the interaction of ResA with its redox partners.
The structure of E80Q was determined for a monoclinic crystal with 4 monomers per asymmetric unit (chains A, B, C, D; see Table 1). The active sites of monomers B and D are essentially identical to the wild-type structure with no unusual conformations imparted by the replacement of E80 by glutamine. The two remaining monomers show differences.

In monomer A, the side chain of the more solvent-exposed cysteine (C74) adopts two alternative conformations and a significant kink is observed in the active site helix (Fig. 6A). This does not appear to result directly from the E80Q substitution (since monomers B and D have single cysteine conformations); rather, we attribute this behaviour to a crystal contact with monomer D.

The most striking feature of this contact is the insertion of a side chain (Q130) from monomer D into the hydrophobic cavity of monomer A. We and others have previously shown by X-ray crystallography and NMR spectroscopy that the cavity may provide a means for ResA to specifically recognise the conserved histidine of the CXXCH motif (14, 18, 47). While the intermolecular interaction between these two ResA monomers does not confirm the cavity as being important for physiological interactions with apo-cytochrome substrates, it does prove that a single residue side chain can be accommodated within the cavity and that such interactions may lead to subtle conformational changes at the active site. The importance of the cavity for substrate binding was also recently proposed by Colbert and colleagues (18) on the basis of NMR chemical shift data resulting from the addition of a mimetic apo-cytochrome c peptide to ResA.

Monomer C of the E80Q structure is also subject to a crystal contact in the vicinity of the active site (Fig 6B). The sulfur-to-sulfur distance in this monomer is just 3.3 Å, the shortest distance observed for any ResA structure. Monomer C contacts a symmetry-related monomer A via L140 (immediately preceding cis-P141) and W73. The backbone of L140 is able to form a pair of hydrogen bonds with the side chain of N112 (from monomer A) by virtue of the cis conformation of P141 which orientsates the L140 carbonyl oxygen away from the interior of the protein and W73 forms a hydrophobic interaction with I108 (from monomer A). The mode by which these contacts induce conformational changes in the cysteine positions is unclear, but contact with the cis-proline and W73 appears to be important; P141 is invariant among thioredoxin-like proteins (in the cis conformation) and W73 is only occasionally substituted by other large aromatics.

It is interesting to note that the pair of hydrogen bonds formed between the backbone atoms of L140 (in monomer C) and the side chain of N112 (in monomer A) closely mimic hydrogen bonds that are universally conserved among structures of TDORs in complex with their substrates. For example, the crystal structure of E. coli CcmG in a mixed disulfide with the N-terminal domain of DsbD (48) revealed hydrogen bonds between the backbone carbonyl of CcmG A143 (equivalent to L140 in ResA) and the backbone amide nitrogen of DsbD C109, and another between the backbone amide nitrogen of A143 and the backbone carbonyl of C109. Similar pairs of hydrogen bonds are also apparent in other structures of mixed disulfide complexes (49-51) even though very few direct intermolecular hydrogen bonds are observed in such complexes. The importance of the cis-proline for TDOR:substrate interactions is further demonstrated by studies of E. coli DsbA proline mutants which are observed to accumulate mixed disulfide complexes with substrates (52) and modelling studies of the interaction of glutathione with glutaredoxin (53). We conclude that the cis-proline appears to play a critical role in the reactivity of TDORs, and that interactions similar to those observed here are likely to be important for ResA:apo-cytochrome c interactions.

The Mechanism of ResA Specificity – The data reported here lead to the following proposal for how ResA achieves specificity. In the absence of an apo-cytochrome c substrate molecule, the high pKₐ values of the ResA active site thiols, resulting at least in part from their interaction with E80, ensure that ResA is unreactive towards non-specific substrates. ResA must become activated in some way, and we propose that this occurs through binding of a substrate molecule. Substrate binding would result in hydrogen bonding interactions, most likely including a reciprocal pair between the backbone carbonyl and amide nitrogen of L140 and the N-terminal cysteine in the CXXCH motif of the apo-cytochrome. We suggest that this orientsates the substrate molecule and facilitates the docking of the histidine residue...
side chain (of the apo-cytochrome CXXCH motif) into the hydrophobic surface cavity, resulting in a likely hydrogen bond interaction between it and E80 at the base of the cavity. This would mask, at least partially, the effect of E80 on the acid/base properties of the active site, thus bringing the $pK_a$ values of the active site thiols into the physiological range. Structural changes, resulting from substrate binding in the cavity, and other protein-protein interactions involving the L140-cis-P141 loop and W73 are likely to considerably reduce the separation between the $S_\alpha$ atoms of the active site cysteines, causing the $pK_a$ values to separate significantly (25). In this form, ResA, with one cysteine now in its thiolate form, is primed for nucleophilic attack on the disulfide bond of the substrate. Experiments designed to further test this model are in progress.

**Significance of Substrate-Mediated Activation among TDORs** – The requirement for specificity is a common one among TDORs on the outside of the cytoplasmic membrane, raising the question of whether related or quite distinct mechanisms have evolved to prevent unproductive short circuiting of thiol-disulfide oxidative and reductive branches. Here we have identified E80 in ResA as a key residue for preventing reaction of the protein with non-cognate substrates. Amino acid residue sequence alignments show that E80 is not conserved in cytoplasmic TDORs, but is highly conserved among reductive branch extracytoplasmic TDORs, including many that have no role in cytochrome $c$ maturation (see Supplemental Data). In contrast, residues that form the near-active site hydrophobic cavity of ResA are generally well conserved only in Gram-positive TDORs involved in System II cytochrome $c$ maturation. Thus, while it is likely that the latter residues are important for binding apo-cytochrome $c$ substrates, the primary role of E80 appears to be to control active site reactivity. This glutamate residue is likely to be similarly important in other reductive branch TDORs, where it may function as part of substrate-mediated activation mechanisms similar to that proposed here for ResA.

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**Footnotes**

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2The abbreviations used are: badan, 6-bromoacetyl-2-dimethylaminonaphthalene; CHES, 2-(cyclohexylamino)ethanesulfonate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; ESI-MS, electrospray ionisation mass spectrometry; MES, 2-(N-morpholino)ethanesulfonate; MOPS, 3-(N-morpholino)propanesulfonate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).
Figure Legends

**Figure 1. Reaction of ResA with badan.** Panel (A) shows the time-dependent increase in the badan fluorescence emission intensity upon reaction with ResA at pH 8.5. 1 µM protein was added to 13 µM badan in a mixed buffer system (see Experimental Procedures) at 25 °C. Fluorescence intensity at 450 nm (B) and 540 nm (C) was plotted as a function of time for reactions at selected pH values, as indicated. Note that data were recorded over a longer time period than shown. The plots were fitted to obtain an observed, pseudo-first order rate constant, $k_o$. Panel (D) shows a plot of $k_o$ as a function of pH. Filled squares indicate data at 450 nm, corresponding to C77, and open circles indicate data at 540 nm. Solid lines show fits to Equation (1) for C77 and Equation (3) for C74.

**Figure 2. Reaction of ResA and C74A ResA with iodoacetate.** Panel (A) shows non-denaturing PAGE of ResA at pH 8.0 following reaction of the protein (40 µM) with 10 mM iodoacetate in 20 mM Tris pH 8.8. Reactions were quenched with 50 mM DTT before loading 10 µl on to the gel. Time points as indicated. Panel (B) shows similar non-denaturing PAGE of C74A ResA with iodoacetate in a mixed buffer system after 24 h; pH as indicated. Band intensity was used to estimate a rate constant, $k_o$, for the reaction (see Experimental Procedures). Panel (C) shows a plot of $k_o$ (left hand abscissa, filled squares) as a function of pH. There are insufficient data points to obtain a very reliable fit, but because the data has the same form as the data from badan labelling of C74A ResA (right hand abscissa, open circles), the iodoacetate data were fitted to Equation (4) (solid line).

**Figure 3. Crystal structures of wild type ResA determined at pH 9.25 and four ResA variants.** Panel (A) shows the active site region of wild-type ResA (1SU9) in sticks representation to aid comparison with the variant structures. Panels (B) – (F) show the active site regions of each variant in sticks representation with relevant $2mF_o-dF_c$ electron density map contoured at 1σ. Carbon, nitrogen, oxygen and sulfur atoms are shown in white, blue, red and yellow, respectively. Panels correspond to: (B) Wild-type ResA determined at pH 9.25; (C) E80Q ResA; (D) C77A ResA; (E) C74A ResA; and, (F) C74A/C77A ResA. All structure figures were rendered with Pymol ((54) www.pymol.org) and edited with GIMP (www.gimp.org).

**Figure 4. Reaction of ResA variants with badan.** Panels (A) – (C) present data for C77A ResA (reporting on C74) and panels (D) – (F) present data for C74A ResA (reporting on C77). (A) and (D), time-dependent increase in the badan fluorescence spectrum upon reaction with variant proteins at pH 9.25. 1 µM protein was added to 13 µM badan in a mixed buffer system (see Experimental Procedures) at 25 °C. (B) and (E), fluorescence intensity at 440 nm and 550 nm, respectively, as a function of time at selected pH values, as indicated. The plots were fitted to obtain an observed, pseudo-first order rate constant, $k_o$. (C) and (F) shows plots of $k_o$ as a function of pH for C77A and C74A ResA, respectively. The solid lines show fits to Equation (4). Panel (F) also shows fits of the same data to Equation (1) (dashed line) and Equation (3) (dotted line).

**Figure 5. Reactivity of E80Q ResA.** Panel (A) shows time-dependent increases in the badan (13 µM) fluorescence emission intensity upon reaction with E80Q ResA (1 µM) at pH 7.2 in a mixed buffer system at 25 °C. Panels (B) and (C) show fluorescence intensity at 440 nm and 550 nm, respectively, as a function of time for reactions at pH 6.2, 6.7, 7.2 and 7.7. Data at 440 nm (reporting on C77) were obtained with 1 µM E80Q ResA at 25 °C, while data at 550 nm (reporting on C74) were obtained with 0.25 µM E80Q ResA and 3.25 µM badan at 10 °C. The plots were fitted to obtain an observed, pseudo-first order rate constant, $k_o$. Panel (D) shows a plot of $k_o$ for data at 440 nm (C77) as a function of pH. The solid lines show a fit to Equation (1). Data at 550 nm (C74) could not be fitted because the protein begins to unfold before the titration end point.
Figure 6. Intermolecular contacts between E80Q monomers that may mimic the interaction between ResA and its redox partners. (A) Intermolecular contact between chain A (light blue) and chain D (grey) in the 1.95 Å structure of E80Q ResA. The side chain of Q130 has become inserted into the hydrophobic cavity found only in the reduced form of ResA. (B) Intermolecular contact between the active site of chain C (green) and a symmetry related monomer of chain A (aubergine). The interaction gives rise to a significant conformational change in the position of the active site cysteines reducing the distance between the $S_\gamma$ atoms by 25%.
Table 1. Data Collection and Structure Refinement Statistics

|                      | ResA E80Q | ResA C74A | ResA C74A C77A | ResA pH 9.25 | ResA C77A |
|----------------------|-----------|-----------|----------------|--------------|-----------|
| **Data Collection Statistics** |           |           |                |              |           |
| Beam line            | ESRF ID14-2 | ESRF ID-29 | ESRF ID-29 | ESRF ID-29 | ESRF ID-29 | ESRF ID-29 | ESRF ID-29 | ESRF ID-29 | ESRF ID-29 | ESRF ID-29 |
| Wavelength (Å)       | 0.93400   | 0.97950   | 1.0688        | 1.0688       | 0.97620   |
| Resolution (Å)       | 30-1.950 (2.060-1.950) | 40-2.4 (2.53-2.4) | 37-3.1 (3.27-3.1) | 40-2.6 (2.74-2.60) | 55-2.0 (2.11-2.0) |
| Unique Reflections a | 39,655 (5,730) | 12,619 (1,807) | 5,722 (761) | 9,636 (1,405) | 21,619 (3,095) |
| Space Group          | P2₁       | P2₁,2,₁   | P2₁,2,₁       | P2₁,2,₁     | P2₁,2,₁   |
| Cell Parameters      | a = 64.0, b = 46.5, c = 46.9, d = 59.6, e = 47.0, f = 59.5, g = 47.3, h = 59.8, i = 47.2, j = 59.4, k = 47.2, l = 59.4, m = 95.1, n = 105.4 | c = 109.8 | c = 109.6 | 110.1 | 110.5 |
| Multiplicity a       | 4.1 (3.8) | 6.9 (7.2) | 5.5 (5.4) | 3.5 (3.5) | 5.2 (3.5) |
| R<sub>sym</sub> a    | 0.063 (0.148) | 0.136 (0.350) | 0.127 (0.296) | 0.094 (0.247) | 0.115 (0.396) |
| completeness         | 99.9 (100.0) | 100.0 (100.0) | 96.6 (91.9) | 97.2 (98.6) | 99.6 (99.2) |
| Multiplicity a       | 4.1 (3.8) | 6.9 (7.2) | 5.5 (5.4) | 3.5 (3.5) | 5.2 (3.5) |
| R<sub>sym</sub> a    | 0.063 (0.148) | 0.136 (0.350) | 0.127 (0.296) | 0.094 (0.247) | 0.115 (0.396) |
| completeness         | 99.9 (100.0) | 100.0 (100.0) | 96.6 (91.9) | 97.2 (98.6) | 99.6 (99.2) |
| R<sub>ave</sub> b    | 0.1507 [0.176] | 0.1765 [0.207] | 0.1853 [0.238] | 0.1953 [0.231] | 0.1809 [0.202] |
| R<sub>free</sub> b   | 0.2058 [0.227] | 0.2480 [0.279] | 0.2629 [0.302] | 0.2749 [0.299] | 0.2368 [0.247] |
| ResA Monomers per Asymmetric Unit | 4 | 2 | 2 | 2 | 2 |
| R.m.s.Bond Length a (Å) | 0.014 | 0.019 | 0.024 | 0.019 | 0.016 |
| R.m.s.Angle a (°) | 1.420 | 1.93 | 2.044 | 1.829 | 1.597 |
| Number of Atoms | 5082 | 2354 | 2166 | 2206 | 2425 |
| Number of Waters | 577 | 135 | 13 | 45 | 166 |
| Ethylene Glycol (x7), Acetate (x1) | None | Ethylene Glycol (x2) | Ethylene Glycol (x2) | Ethylene Glycol (x2) |
| Cruickshank DPI Free d | 0.1501 | 0.2691 | 0.5338 | 0.3633 | 0.1750 (0.1662) |
| Correlation Coefficient | 0.9545 | 0.9381 | 0.9194 | 0.9214 | 0.9390 |
| NCS restraints used in refinement | No | Yes | Yes | Yes | No |
| Ramachandran Plot Statistics (% residues) | 94.7 | 94.1 | 89.3 | 94.4 | 94.9 |
| Favoured Region | 94.7 | 94.1 | 89.3 | 94.4 | 94.9 |
| Additionally Allowed | 5.3 | 5.5 | 10.3 | 5.6 | 5.1 |
| Generously Allowed | 0 | 0.4 | 0.4 | 0 | 0 |
| Disallowed | 0 | 0 | 0 | 0 | 0 |

- Parentheses indicate the highest resolution bin.
- R factor values are quoted from REFMAC; Values in braces are quoted from SFCHECK; R<sub>free</sub> is calculated using 5% of the data (see Supplemental Data).
- Root mean square deviation from ‘ideal’ geometry.
- Cruickshank Dispersion Precision Index calculated with the free set of reflections.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Molecular basis for specificity of the extra-cytoplasmic thioredoxin ResA
Allison Lewin, Allister Crow, Arthur Oubrie and Nick E. Le Brun

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