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Effects of Substitutions in the CXXC Active Site Motif of the Extracytoplasmic Thioredoxin ResA

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Running Title: Biophysical and functional effects of substitutions at the active site of ResA

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ABSTRACT: The thiol-disulfide oxidoreductase ResA from *Bacillus subtilis* fulfils a reductive role in cytochrome *c* maturation. The pK\textsubscript{a} values of the CEPC active site cysteines of ResA are unusual for thioredoxin-like proteins, in that they are both high (> 8), and within half a unit of each other. To determine the contribution of the inter-cysteine dipeptide of ResA to its redox and acid-base properties, three variants (CPPC, CEHC and CPHC) were generated representing a stepwise conversion to the active site sequence of the high potential DsbA protein from *Escherichia coli*. The substitutions resulted in large decreases in the pK\textsubscript{a} values of both the active site cysteines: in CPHC (DsbA-type) ResA, pK\textsubscript{a} values of -2.5 were measured for both cysteine residues. Increases in midpoint reduction potentials were also observed, although these were comparatively small: CPHC (DsbA-type) ResA exhibited an increase of +40 mV compared to the wild-type protein. Unfolding studies revealed that, despite the observed differences in the properties of the reduced proteins, changes in stability were largely confined to the oxidised state. High resolution structures of two of the variants (CEHC and CPHC ResA) in their reduced states were determined and are discussed in terms of the observed changes in properties. Finally, the *in vivo* functional properties of CEHC ResA are shown to be significantly affected compared to those of the wild-type protein.

INTRODUCTION

Thiol-disulfide oxidoreductases (TDORs) occur in most cell types where they function in the control of the redox state of cysteine residue side chains. Many TDORs are structurally highly related and contain the active site motif CXXC, in which the two cysteine thiols redox cycle between reduced (di-thiol) and oxidized (disulfide) forms. The mid-point reduction potential (E\textsubscript{m}) varies considerably amongst TDORs, from the highly reducing archetypal TDOR thioredoxin (E\textsubscript{m} = -270 mV at pH 7) [1, 2] to the relatively oxidizing periplasmic TDOR DsbA of *Escherichia coli* (E\textsubscript{m} = -89 – -110 mV at pH 7) [3, 4]. Understanding the basis for this variation between structurally highly similar protein active sites has been the focus for much research. It has been demonstrated clearly that there exists an intrinsic connection between the acidity of the N-terminal active site cysteine and the reduction potential, such that a low pK\textsubscript{a} value corresponds to a (relatively) high reduction potential [4-7]. The pK\textsubscript{a} value for the N-terminal active site cysteine of thioredoxin is in the range 6.7 – 7.5 [8, 9], while that for *E. coli* DsbA is 3.2-3.5 [10], consistent with the respectively low and high reduction potentials of these two proteins.

There is considerable evidence to show that the XX dipeptide of the CXXC active site plays a significant role in determining the physico-chemical properties of the flanking thiols. Studies of thioredoxin and DsbA, in particular, have revealed that the replacement of the native XX sequence with the dipeptide found in other TDORs results in significant changes in acid-base and redox properties, such that they become more similar to those of the protein in which the dipeptide is found naturally [10, 11]. This effect is not dependent on the XX residues in isolation, as studies using short peptides with the CXXC motifs of TDOR proteins all have a reduction potential of ~ –200 mV [12]. Rather, a network of interactions between atoms of the active site and surrounding residues affect the acid-base and redox properties of the thiols. Thus, although substitution of the dipeptide has a significant effect, the variant usually does not achieve the pK\textsubscript{a} value and reduction potential of the model.

We are interested in the extra-cytoplasmic TDOR ResA, which is a component of the cytochrome *c* maturation (CCM) apparatus of *Bacillus subtilis* [13-15]. Together with ResB and ResC [16], and along with more generally functioning proteins (e.g. CcdA, which is involved in electron transfer across the cytoplasmic membrane [17, 18]), ResA constitutes a pathway for CCM (termed System II) that is conserved in a wide variety of other bacteria and even in plant chloroplasts [19]. Although it has not yet been shown directly, evidence indicates that ResA functions to reduce the cysteines of apo *c*-type cytochromes prior to attachment of the heme group [14, 15], via ResB and/or ResC. ResA is tethered to the membrane by a single transmembrane helix with a soluble domain located on the outer surface of the cell [13]. The high resolution X-ray structure of the soluble domain shows that it adopts the characteristic thioredoxin fold with two insertions: an N-terminal \(\beta\)-hairpin and a strand and helix inserted between

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strand β2 and helix α3 [14]. The active site of ResA, consisting of the sequence CEPC, is located at the N-terminus of helix α1, see Fig. 1.

In thioredoxin and DsbA, only the solvent-exposed N-terminal active site cysteine is available for reaction with alkylating reagents [20, 21]. Despite exhibiting the usual high structural similarity to other TDORs (such that the N-terminal cysteine residue (Cys74) is significantly more solvent exposed than Cys77), both cysteines of ResA are available to react with alkylating reagents [15]. Furthermore, the pKₐ values of the two cysteines are similar and both > 8 (Cys74 ~8.8 and Cys77 ~8.2). Recently, it was demonstrated that Glu80, located close to the active site, significantly affects the acid-base properties of the active site cysteines, such that in a E80Q variant, the pKₐ values of both cysteines decreases by approximately one unit [15].

To gain further insight into the unusual acid-base properties of the ResA active site, and specifically to determine the influence of the inter-cysteine dipeptide, the wild-type active site sequence of ResA (referred to as CEPC (wild-type) ResA) was altered stepwise to that of DsbA (CPHC (DsbA-type) ResA). We report redox, pKₐ, stability and structural studies that demonstrate that these substitutions result in very significant increases in acidity (decrease in pKₐ values) of both active site thiols, while changes in the reduction midpoint potential are comparatively small. Despite a significant effect on the acid-base properties of the reduced variant proteins, conformational stability changes are, in general, confined to the oxidised state. For one of the variants (CEHC ResA), we demonstrate that changes in active site properties have a substantial effect on the in vivo activity of the protein.

EXPERIMENTAL

Strains, growth media and genetic methods

Strains and plasmids used in this study are given in Table 1. E. coli strains were grown at 37 °C in LB [22], or on LA plates consisting of LB broth with 1.25 % (w/v) agar. Ampicillin, where appropriate, was used at a concentration of 100 mg/l. B. subtilis strains were grown at 37 °C in LB or nutrient sporulation medium with phosphate [23]. Strain LUL9 and derivatives were grown in the presence of 1 mM IPTG [13]. Antibiotics used for B. subtilis were erythromycin (1 mg/l) and chloramphenicol (5 mg/l). Molecular genetics techniques were used as described by Sambrook et al [22]. Plasmid DNA was isolated using commercial mini- or midiprep kits (Qiagen). Enzymes for DNA manipulation were purchased from Roche. All constructs created by using PCR were verified by DNA sequencing (MWG Biotech).

Site-directed mutagenesis and protein purification

Site-directed mutagenesis was carried out using a whole-plasmid method [24] using plasmid pRAN10, encoding the soluble domain of ResA, as the template [13] to generate pALR2 (E75P) and pALR3 (P76H). For the double mutant E75P/P76H, pALR2 was used as a template, generating pALR28. A full list of the oligonucleotides used as primers in this study is given in Table S1. The NdeI/EcoRI fragments of the generated plasmids were cloned into pET21a, giving pALR6 (encoding variant E75P, referred to hereafter as CPPC ResA), pALR7 (encoding variant P76H, referred to as CEHC ResA) and pALR31 (encoding variant E75P/P76H, referred to as CPHC (DsbA-type) ResA). Wild-type and variant soluble ResA proteins were generated and purified as previously described [15].

For in vivo studies, site-directed mutagenesis of wild-type resA was carried out as described above using pALR9 as template, generating plasmid pCHN5 (encoding CEHC ResA). The XbaI/HindIII fragment of pCHN5 was cloned into pVK48 to give plasmid pCHN6 (Table 1). B. subtilis LUL9 was transformed to chloramphenicol resistance with linearised pCHN6, generating strain LUN8. Integration of resA at amyE of the strain was initially determined by testing for amylase activity on TBAB plates containing 1% (w/v) starch using Lugol’s solution, and then confirmed by using PCR analysis. The presence of the mutation encoding the P76H substitution was verified by sequencing (MWG Biotech).

in vivo activity of CEHC ResA
TMPD staining of *B. subtilis* colonies was carried out as previously described [16]. The activity of cytochrome *c* oxidase *ca* was measured more quantitatively using a cytochrome *c* oxidase activity assay [25] using *B. subtilis* membranes purified as previously described [26]. Following the mixing in equal volume of membranes and reduced horse heart cytochrome *c* solution, absorbance changes at 540 and 550 nm were measured every 15 s using a Perkin Elmer Lambda 800 spectrophotometer. Absorbance difference (*A*550-*A*540) was plotted against time and the initial rate of cytochrome *c* oxidation determined from data beginning at 30 s after mixing. Total cytochrome *c* content of membranes was determined by SDS-PAGE/heme stain [27]. Membrane proteins (~100 µg per well) were separated by SDS-PAGE and the resulting gel was soaked in 10% TCA for 10 min, washed twice in water (each for 10 min), and stained for heme by the addition of a solution containing 1 mg/ml o-dianisidine-HCl, 0.7% (v/v) H2O2 in 0.1 M sodium citrate, pH 4.4, followed by incubation at room temp for 30 min. The reaction was stopped by washing the gel several times in water.

**Reduction potential determinations**

Isolated wild-type and variant ResA proteins (0.1 µM) were incubated for 1 hour at 25 °C with varying proportions of reduced and oxidised DTT in 50 mM potassium phosphate, pH 7.0. The concentration of oxidised DTT was fixed to 1 mM and the concentration of reduced DTT varied from 2.75 mM to 0.1 µM. Control experiments indicated that, in the absence of oxidised DTT, 0.1 µM DTT was sufficient to fully reduce pre-oxidised protein in 1 hour. Tryptophan fluorescence was measured using a Perkin Elmer LS55 luminescence spectrophotometer with an excitation wavelength of 290 nm. Midpoint potentials were found to be independent of the DTT concentration. In an alternative approach, varying concentrations of reduced and oxidised DTT were mixed to give a fixed total concentration of 1 mM. Again, essentially identical behaviour was observed, after compensation for the significant quenching of intensity caused by oxidised DTT. From the fluorescence data at 353 nm, midpoint reduction potentials were determined, as described previously [13]; further details are also given in *Supplementary data*.

**Determination of cysteine thiol pKα values**

Following the reaction of cysteine side chains with alkylating reagents is a well established method for determining the pKα values of protein cysteine thiol groups [15, 21, 28], where the observed rate constant is proportional to the extent of thiol deprotonation at a given pH [21]. Reaction of reduced wild-type ResA and variants with 6-bromoacetyl-2-dimethylaminonaphthalene (badan) were carried out under pseudo-first order conditions, as previously described [15]. pKα values were determined as previously described [15]; further details are also given in *Supplementary data*.

**Protein conformational stability measurements**

Wild-type ResA and variants were pre-reduced or oxidised by incubation overnight at 4 °C in 1 mM DTT or diamide, respectively [13]. Excess reductant/oxidant was removed using a desalting column (PD-10, GE Healthcare) equilibrated in 0.1 M Tris, 1 mM EDTA pH 8. For refolding, 20 µM protein was then incubated in 4 M guanidine in buffer (including 1 mM DTT for reduced protein) for 3 h at room temperature to allow complete unfolding prior to dilution. 1 µM native or denatured protein was incubated for 1 hour at 25 °C in solutions containing varying concentrations of guanidine hydrochloride in the same buffer. Incubation for longer time periods did not result in further changes in fluorescence intensity. Reduced samples included 1 mM DTT to prevent re-oxidation. Tryptophan fluorescence was measured at 25 °C using a Perkin Elmer LS55 luminescence spectrophotometer with an excitation wavelength of 290 nm. Fluorescence intensity at 353 nm was converted to fraction unfolded, *f*u, where fluorescence intensity in the absence of denaturant and at 3 M denaturant were taken as values for the native and denatured forms of the protein, respectively. *f*u was plotted against denaturant concentration and data were fitted to Equ. 1 which describes a two step unfolding process.
\[
\frac{f_a}{1 + \exp^{\frac{m[D]-\Delta G^0}{RT}}} = \frac{\exp^{m[D]-\Delta G^0/RT}}{1 + \exp^{m[D]-\Delta G^0/RT}}
\]

where $\Delta G^0$ is the Gibbs free energy for stabilisation at zero denaturant and $m$ describes the dependence of $\Delta G$ on the concentration of denaturant. Midpoints of unfolding ($D_{0.5}$) were calculated using Equ. 2.

$$D_{0.5} = -\frac{\Delta G^0}{m}$$

For unfolding and refolding at pH 6.5, experiments were repeated as above, except that 50 mM sodium phosphate, 1 mM EDTA, pH 6.5 was used as the buffer. Refolding experiments demonstrated that a fully reversible process was being followed in each case.

**Crystallization, data collection, structure determination and refinement**

Purified ResA variant proteins were buffered in 20 mM Mops (pH 7.0), 1 mM DTT at a concentration of 15 mg/ml prior to crystallisation using the sitting drop vapour diffusion technique. Crystals of CEHC and CPHC (DsbA-type) ResA were grown by equilibrating equal volumes (~1 µl) of protein solution and crystallisation reagent (22 – 30% (w/v) polyethylene glycol 4000, 0.2 M ammonium acetate, 0.1 M Mes pH 6.0 – 6.7) over an 800 µl reservoir of reagent alone. Crystals were cryo-protected in a solution identical to the crystallisation reagent supplemented with 20% (v/v) ethylene glycol.

X-ray diffraction experiments were performed on beam line ID23-1 of the ESRF. Full details of the structure determinations are given in Supplementary data. Briefly, a set of starting phases for each structure was obtained by molecular replacement using MOLREP [29] using a search probe derived from the wild-type structure of reduced ResA [14]. Model-building and refinement were conducted with COOT [30] and REFMAC [31]. Coordinates and associated structure factors were submitted to the Protein Data Bank (PDB entries: 3C73 (CEHC ResA); 3C71 (CPHC (DsbA-type) ResA)). Data collection and refinement statistics are given in Table S2.

**Other methods**

Western blot analysis was performed by transferring proteins separated by SDS-PAGE to polyvinylidene difluoride membranes using an electroblotter (Hoefer). Rabbit antiserum against ResA [13] was used as a primary antibody (at 1000-fold dilution), and horseradish peroxidase-conjugated donkey anti-rabbit serum as the secondary antibody (at 5000-fold dilution). Immunoreactive proteins were visualised using peroxidase reactive chemiluminescence detection system (GE Healthcare). Insulin reduction experiments [32] were performed using 0.11 mM insulin from bovine pancreas (Sigma) in 0.1 M sodium phosphate pH 7.0 containing 2 mM EDTA and 1 mM DTT. 20 µM pre-reduced ResA was added and the precipitation of insulin followed by recording the absorbance at 650 nm. All reactions were carried out at 25 °C using a Perkin-Elmer λ35 spectrophotometer. 1 µM thioredoxin from a *Spirulina* species (Sigma) was used as a positive control, while reaction in the absence of ResA or thioredoxin served as a negative control.

**RESULTS**

**Reduction potentials are increased in variant ResA proteins**

*B. subtilis* ResA contains only two cysteine residues. Reduction potentials were measured at pH 7.0 using the difference in tryptophan fluorescence observed for the oxidised and reduced proteins [13], see Fig. 2. The fit for wild-type ResA revealed a mid-point potential of ~256 mV (versus NHE), with $n = -2$. This is a different value to that previously reported by us (~340 mV at pH 7) under the same conditions [13]. Our earlier report was in error because the absorption due to oxidized DTT artificially reduced the observed fluorescence intensity, and therefore affected the Nernstian fit. The constant concentration of oxidized DTT used here was low and any effect due to absorption by this species was the same for all samples.
All the variants showed increases in reduction potential, with the largest change of +40 mV observed for CPHC (DsbA-type) ResA, see Fig. 2 and Table 2. For CPPC ResA, \( n = \approx 2 \), as expected, but for CEHC and CPHC (DsbA-type) ResA fits indicated the redox processes for these two variants are not simple two electron processes: in each case \( n = \approx 4 \). One explanation for this is that \( n \) two ResA molecules associate in a cooperatively coupled electron transfer process. However, we have not found evidence of a ResA-ResA association in either reduced or oxidised states (not shown). Another possibility is that DTT interacts in an unexpected way with these variants. However, structural data (see below) do not provide evidence for a stable interaction, and so the explanation for this remains to be established. Despite differences in the electron stoichiometry of the redox reactions, the mid-point potentials are clear, and indicate that the oxidising power increases in the order CEPC (wild-type) < CPPC < CEHC < CPHC (DsbA-type).

**Active site cysteine \( pK_a \) values in variant ResA proteins are significantly decreased**

ResA is highly unusual among thiol-disulfide oxidoreductases in two major respects. Firstly, both active site cysteines (not just the N-terminal cysteine) are available for reaction with alkylating reagents, and, secondly, both its active site cysteine thiol groups have high \( pK_a \) values, which are within approximately half a pH unit of each other, see Table 2. The \( pK_a \) values of active site cysteine thiols in each of the ResA variants were determined by measuring rates of reaction with the fluorescent probe 6-bromoacetyl-2-dimethylaminonaphthalene (badan) [15] as described in the **Experimental** section. The fluorescence due to badan-modified ResA is sensitive to the environment of the modified cysteine such that the more solvent exposed Cys74 fluoresces at 550 nm, while the more buried Cys74 can be followed at 440 nm.

Data for both active site cysteines of CPHC (DsbA-type) ResA fitted well to the Henderson-Hasselbalch equation (Equ. S2), see Fig. 3C, while that for CEHC, although somewhat noisy, also fitted reasonably to Equ. S2 (Fig. 3B). Data for Cys74 of CPPC ResA fitted well to Equ. S2, but that for Cys77 did not, see Fig. 3A. Like Cys74 in the wild-type protein, the slope of the data points is too steep to be adequately described by a single deprotonation event. A good fit was obtained with Equ. S3, which describes the tight cooperative coupling of two deprotonation events. This indicates that a similar type of cooperativity is observed between Cys77 and another, non-cysteine residue in this variant. As this type of cooperativity was not observed for either active site cysteine in variants lacking proline at position 2 of the inter-cysteine dipeptide, this residue may be important for mediating coupling.

In the wild-type protein, an additional, non-cooperative \( pK_a \) event was observed at pH \( \approx 6.5 \) [15]. This cannot be clearly distinguished in the variants but may still occur with a \( pK_a \) value shifted below the pH range of the experiment (measurements could not be made below pH 4.5 as the protein begins to unfold) [15]. In all cases, fits yielded \( pK_a \) values significantly lower than those of CEPC (wild-type) ResA, see Table 2. Very significant changes were observed in the variant CPPC, i.e. on substituting Glu75 with proline. Both active site cysteine \( pK_a \) values decreased by \( \approx 1.5 \) pH units. Somewhat less dramatic, but still significant, changes were observed for CEHC ResA. The effects were cumulative (and, for Cys77, essentially additive) in the double residue variant CPHC (DsbA-type) ResA, which exhibited \( pK_a \) values of 6.3 and 5.7 for Cys74 and Cys77, respectively.

**Active site dipeptide substitutions specifically destabilize the oxidised state of ResA**

The guanidine-induced unfolding/refolding of wild-type ResA and variants was measured in both reduced and oxidised states at pH 6.5 and 8.0. In the reduced protein, unfolding was monitored by following the quench of the tryptophan fluorescence intensity. Because the fluorescence within the oxidised protein was already quenched, unfolding resulted in an increase in fluorescence intensity until the intensity approached that of the unfolded reduced protein. Data were fitted to a simple two step unfolding model, as described in the **Experimental** section, see Fig. 4. The unfolding of CEPC (wild-type) ResA at pH 8 (Fig. 4A and B) revealed that the oxidised protein is 3.6 kJ mol\(^{-1}\) more stable than the reduced, i.e. \( \Delta G_{\text{oxygenated}} = -3.6 \text{ kJ mol}^{-1} \) (see Table 3), consistent with the reducing function of the protein.

At pH 6.5 the stability of the oxidised wild-type protein was similar to that at pH 8, but the stability of the reduced protein was significantly increased (\( \Delta G_{\text{oxygenated}} = +8.7 \text{ kJ mol}^{-1} \)), indicating an increase in the
reduction potential (relative to the potential of the denatured protein) at lower pH. This may be a consequence of there being very little deprotonation of the cysteine residues at pH 6.5, thereby decreasing the destabilising effect of thiolates at the active site. At lower pH, the m value for the oxidised state of the wild-type and variant proteins was significantly lower than that for the reduced state (see Table 3). Because the m value gives an indication of the change in solvent-accessible surface area upon unfolding, this indicates that the disulphide bond induces some residual structure in the denatured oxidised proteins.

When reduced, CPPC and CEHC ResA proteins exhibited similar stabilities to the reduced wild-type protein at both pH 6.5 and 8. In contrast, at both pH values, their oxidised forms were significantly less stable than the oxidised wild-type protein, see Fig. 4 for data at pH 8 (and Fig. S1 for data at pH 6.5) and Table 3. The effect was greater at pH 6.5, at which ΔG_m(red) = +10.4 and +19.1 kJ mol⁻¹ for CPPC and CEHC ResA, respectively. These increases are qualitatively consistent with the observed increases in midpoint reduction potentials (Fig. 2). In both variants m values were similar to those determined for the wild-type protein.

The reduced CPHC (DsbA-type) ResA also exhibited a similar stability to the wild-type protein at pH 8, but was somewhat less stable at pH 6.5. At pH 6.5 and 8 the oxidised CPHC (DsbA-type) protein exhibited markedly different behaviour. Unfolding did not follow a simple two step mechanism; there was an initial increase in tryptophan fluorescence resulting in an intensity greater than that observed for the unfolded reduced protein (see inset Fig 4B). The intensity subsequently decreased back to the level of the unfolded reduced protein. In all of the variants, the emission maximum shifted towards longer wavelength as the protein unfolded (not shown). For CPHC (DsbA-type) ResA, this was observed through the initial increase and subsequent decrease in intensity until both I_max and F_353 became constant, with values resembling those observed for the other oxidised variants. This indicated that the initial increase and subsequent decrease in F_353 represent two stages of unfolding (rather than further quenching of the emission from the unfolded protein). Other than to note that the oxidised protein began to unfold at lower concentrations of GdHCl, we did not attempt to analyse the unfolding data for oxidised CPHC (DsbA-type) any further (for example, in terms of three-state unfolding) because of the difficulty in making a meaningfully comparison of the results with those above for the other variants and the wild-type protein, which are derived from a two-state unfolding model.

The structures of reduced CEHC and CPHC (DsbA-type) ResA reveal important changes at the active site

To investigate further the consequences of substituting residues of the dipeptide motif, we endeavoured to crystallize each of the ResA variant proteins. While we were not able to obtain well-diffracting crystals of the CPPC mutant, both the CEHC and CPHC (DsbA-type) variant proteins were crystallized (in their reduced states) and their structures solved (Table S2). Crystals of CEHC ResA were isomorphous to those previously reported for the wild-type (reduced) form of ResA, contained two molecules per asymmetric unit and belonged to space group P2₁2₁2₁. In contrast, crystals of CPHC (DsbA-type) ResA belonged to space group P6₅ and contained only a single molecule per asymmetric unit. In both cases, each individual ResA monomer was generally very similar to the wild-type structure, with structural differences largely confined to the active site (Fig. 5).

The internuclear distance between reduced cysteine sulfurs were found, in general, to be shorter in the CEHC and CPHC (DsbA-type) variants than for the wild-type protein (4.59 Å and 4.39 Å for wild-type; 4.56 Å and 4.17 Å for CEHC; 4.01 Å for CPHC (DsbA-type)) and the distance to the buried and highly conserved Glu80 carboxyl group (from the Cys77 sulfur) was also found to be variable (6.24 Å and 5.22 Å for wild-type; 6.24 Å and 4.87 Å for CEHC; 6.00 Å for CPHC (DsbA-type)).

In the case of the CEHC variant, the active site clearly has a great deal of flexibility that was not observed in the wild-type structure. In the chain denoted “A”, both Glu75 and His76 were observed to adopt at least two alternative conformations (Fig. 5A left panel) while, in chain B, a further conformation of His76 was observed that is distinct from either of those seen in chain A (Fig. 5B right panel). Such flexibility may well result from the lack of a proline residue between the two cysteines which would otherwise limit the available conformational freedom of the protein backbone.
In the CPHC (DsbA-type) variant (Fig. 5B), there was no evidence of conformational flexibility, probably because of the presence of proline between the cysteines; the fact that the proline is located at position 1 of the disulfide motif, rather than position 2 as in the wild-type protein (CEPC), suggests that rigidity of the CXXC motif can be conferred by the presence of proline at either position of the motif.

The side-chain position of His76 in the CPHC (DsbA-type) and CEHC structures is of significant interest; in both chain B of the CEHC variant and chain A of the CPHC (DsbA-type) variant, the His76 side chain was bent 'backwards' into a cavity on the surface of ResA. This surface cavity has previously been proposed to be a potential binding site for the conserved histidine of target apo-cytochrome CXXCH motifs that would allow ResA to specifically recognise apo-cytochromes c, and thus reduce them in preference to other potential substrates [14]. In the CPHC (DsbA-type) structure, the interaction of His76 with the surface cavity was quite weak, with the histidine side chain docked within the groove above Glu80. In the CEHC structure, however, His76 permeated much deeper within the cavity, with the Nε(H) atom of the His76 side chain forming a strong hydrogen bond to the carboxyl oxygen of Glu80 (2.24 Å) (see Fig. S2). Although the interactions observed here are clearly not physiologically relevant, they do mimic the proposed interaction between the CXXCH motif histidine of apo-cytochrome c and ResA. The observations show that the cavity can accommodate a histidine side chain, and that the glutamate carboxyl is able to receive a hydrogen bond from a histidine residue.

A ResA variant with significantly different properties remains partially active in vivo

LUL9, a strain of *B. subtilis* in which the native resA gene is disrupted, is deficient in c-type cytochromes [13]. The integration, at the amyE locus of the *B. subtilis* chromosome, of resA under the control of the Pspec promoter (strain LUN1) was found to restore the CCM capacity of the cells, as judged by their ability to oxidise TMPD, an artificial substrate of cytochrome c oxidase [16], see Fig. 6A. Integration at amyE of LUL9 of a mutant resA encoding the CEHC ResA variant (strain LUN8) also resulted in TMPD oxidase activity, but to an apparently lesser extent. To investigate this in more quantitative terms, a cytochrome c oxidase activity assay was utilised. *B. subtilis* 1A1 membranes containing wild-type ResA gave a cytochrome c oxidase activity of 83 ±4 nM min⁻¹ mg⁻¹ (of membrane protein), while in membranes from LUL9 activity was at background level, 5 ±4 nM min⁻¹ mg⁻¹, i.e. 10-20 fold lower. LUN1 membranes had wild-type activity (82 ±6 nM min⁻¹ mg⁻¹), while LUN8 membranes (containing CEHC ResA) showed only ~40% of wild-type activity (35 ±10 nM min⁻¹ mg⁻¹), consistent with the TMPD staining data.

The TMPD oxidation and cytochrome c oxidase activity assays are probes of one specific c-type cytochrome, CtaC (subunit II of cytochrome c oxidase). To determine the entire cytochrome c content of membranes, heme staining of an SDS-PAGE gel was performed, see Fig. 6B. The gel confirmed that LUL9 lacks the c-type cytochromes present in 1A1. LUN1 membranes contained all c-type cytochromes at essentially wild-type levels, while for LUN8 (containing CEHC ResA), bands due to all of the c-type cytochromes are present, but are clearly less intense than those observed for wild-type membranes. Western blot using anti-ResA serum confirmed that wild-type ResA and CEHC ResA are present at wild-type levels in LUN1 and LUN8, respectively (Fig. 6C). In combination, these data show that the decreased pKα values, increased reduction potential, and increased conformational flexibility associated with the active site of CEHC ResA result in a reduced degree of CCM, but do not completely inhibit the in vivo activity of the protein.

In a model recently proposed for how ResA and other extra-cytoplasmic TDORs achieve specificity, the unusually high pKα values of the active site thiols was proposed to be important for preventing the protein from acting as a general reductase (which would lead to a futile redox cycle involving the general extra-cytoplasmic oxidase BdBd) [15]. According to the model, the reduced pKα values of the active site cysteines of CEHC ResA would be expected to lead to a loss of specificity. The properties exhibited by CEHC ResA in vivo could be consistent with this, as the true substrates would be in competition with other oxidised extra-cytoplasmic proteins for the electrons available from ResA. To test the general reductase activity of the CEHC variant, its activity in the insulin assay was investigated, see Fig. 7. Although it was much less active than that of a thioredoxin control sample (which was present at 20-fold lower concentration in the assay), the CEHC variant exhibited a significantly enhanced activity.
The connection between pK_interchange reactions [4, 36, 37] and on a Nernstian analysis [7] have been proposed which simulate well higher the reduction potential. Distinct theoretical models based on the rate constants for thiol-disulfide

For example, *E. coli* thioredoxin, in which the active site (CPHC) resulted in a decrease in pK from 7.1 to 6.1 [11], and for DsbA, a change of the active site (CPHC) to that resembling thioredoxin (CGPC) increased the pK values of both cysteine residues were observed; the largest of these, for the variant in which Glu-Pro was replaced with Pro-His (to generate a DsbA-type active site motif), were decreases of ~2.5 pH units for both Cys74 and Cys77, resulting in pK values of 6.2 and 5.7, respectively.

These data are in agreement with previous studies of other TDORs that have revealed a similar relationship between the XX dipeptide and pK properties of the active site cysteine residues [11, 34, 35]. For example, *E. coli* thioredoxin, in which the active site (CGPC) was changed to resemble that of DsbA (CPHC) resulted in a decrease in pK from 7.1 to 6.1 [11], and for DsbA, a change of the active site (CPHC) to that resembling thioredoxin (CGPC) increased the pK of the N-terminal cysteine from 3.3 to 6.2 [10].

Previous studies have revealed a clear relationship between the pK values of the active site cysteines and their redox properties, such that the lower the pK value of the N-terminal cysteine, the higher the reduction potential. Distinct theoretical models based on the rate constants for thiol-disulfide interchange reactions [4, 36, 37] and on a Nernstian analysis [7] have been proposed which simulate well the connection between pK values and redox properties. The redox properties of the ResA variants reported here, in which the largest shift, observed for the CPHC (DsbA-type) variant, was only +40 mV, do not appear to be well correlated with the large changes in pK values. These changes are significantly less than those observed for both thioredoxin and DsbA, in which the changes in pK values (as described above) were correlated with a decrease of ~75 mV and an increase of > 90 mV, respectively [10, 11]. However, it is important to note that the well established linear correlation between pK values and reduction potential becomes much shallower when the pK values of the cysteines are higher than the pH at which the reduction potential is measured [11]. A plot of pK as a function of reduction potential for ResA and variants is shown in Fig. 8, illustrating a shallow, but essentially linear correlation (gradient = -15.4 ±3 mV per pK unit). As far as we are aware, this is the first plot of this type in which the pK values of both active site cysteines are shown to be affected and is consistent with previous studies of thioredoxin, in which reduction potential and pK data could only be modelled by assuming that the N- and C-cysteine pK values were the same [11]. This implies that changes in the dipeptide of thioredoxin significantly affect the pK values of both cysteines, as directly demonstrated here for ResA.

Can any conclusions be drawn about why the dipeptide changes alter the acid-base properties in the way observed? Structural data presented here for CEHC and CPHC (DsbA-type) ResA proteins confirmed that the overall fold of the protein was not significantly affected by changes of the active site dipeptide. However, changes were observed in the vicinity of the active site. CEHC ResA, in which Pro76 is replaced with a histidine residue, exhibited significant flexibility, whereas the structures of CEPC (wild-type) and CPHC (DsbA-type) did not, and we conclude that the proline at either position 1 or 2 of the XX dipeptide is important for imparting structural rigidity on the active site loop.

In the wild-type protein, the negatively charged solvent-exposed side chain of Glu75 (located at position 1 of the XX dipeptide) is expected, through electrostatic effects, to destabilise the thiolate form of cysteine relative to its neutral thiol form (and therefore increase pK values). In CEHC ResA, Glu75 is salt-bridged to Lys78 or Lys79 at close distance (< 2.8 Å) in two of its three observed conformations, and so its effect on the cysteine pK values is likely to be diminished. In CPPC ResA (for which a structure is not available), the replacement of Glu75 with proline should stabilise the thiolate form of cysteine and,
because proline at position 1 promotes a favourable interaction with the α-helix [38], should further stabilise the thiolate form of (at least) the N-terminal cysteine. The data reported here are consistent with these observations and clearly indicate an important role for Glu75 in elevating the pK_a values in the wild-type protein.

In thioredoxin and several other known and putative low potential TDORs (including ResA), the proline at position 2 of the dipeptide is believed to play an important, if unclear, role in destabilising (relative to DsbA) the cysteine thiolate form [35]. Replacement of this proline of ResA should, therefore, lead to stabilization of active site thiolates. Furthermore, the histidine residue at position 2 of the active site of PDI domain a interacts electrostatically with the N-terminal cysteine thiolate [34]. Thus, substituting proline with histidine at position 2 should stabilise the thiolate form of (at least) the N-terminal cysteine. In addition, our structural data revealed that His76 interacts with Glu80 (which has been shown to significantly affect pK_a values of the active site cysteines), and this interaction likely reduces the effect of Glu80 on the pK_a values of cysteines. Also noteworthy is the gradual decrease in the (average) inter-cysteine sulfur to sulfur distance in CEHC and CPHC (DsbA-type) ResA compared to the wild-type protein. We previously proposed that the long separation between the cysteine thiol groups is important for their relatively independent acid-base chemistries, and that a significant shortening might be expected to result in a drop in the pK_a of the N-terminal cysteine pK_a and a concomitant increase in the C-terminal cysteine pK_a value [15]. The shortening observed here is clearly not sufficient to cause the pK_a values to diverge (they remain within ~0.5 pH units of one another); nevertheless it is of interest that the general trend of pK_a decreases mirrors the drop in the inter-sulfur distance.

The ResA variants all exhibited significant changes in stability compared to the wild-type protein. However, where we might have predicted that the reduced state would become stabilized and the oxidized state largely unaffected, in fact, it was the oxidised states of the variants that, in general, exhibited significant decreases in stability, while the stabilities of the reduced variants were largely unaffected. Similar observations have been reported for thioredoxin and DsbA variants [10, 11]. Observations for ResA could be a consequence of competing effects in the reduced state: while the substitutions introduced result in a stabilisation of the thiolate form (and hence likely stabilise the reduced state), and therefore reduce the pK_a, they may result in other, non-favourable interactions that reduce the stability of the reduced state. For example, the replacement of solvent exposed Glu75 with the hydrophobic side chain of proline is likely to be destabilising. Thus, although the substitutions have specific effects on the cysteine pK_a values, the overall effect on the conformational stability is essentially neutral. In the oxidised state, the stabilising effects due to the substitutions are much less significant (because there are no thiolate groups), while the destabilising effects are still present, leading to a decrease in the conformational stability.

A key question arising from the observed changes in acid-base and redox properties of the ResA variants is whether or not they have an effect on in vivo activity. Studies of Bradyrhizobium japonicum TlpA, a TDOR involved in the biogenesis of cytochrome aa_3, revealed that the substitution of proline at the CVPC active site with histidine caused a significant increase in the midpoint potential, but surprisingly did not affect the in vivo activity of the protein [39]. Here, the equivalent replacement of proline at position 2 of the ResA active site dipeptide with histidine (CEHC ResA) resulted in a ~60% decrease in CCM activity. Thus, changes in acid-base and redox properties of the active site cysteine thiols and conformational flexibility at the active site motif, as described here, result in significant effects in vivo.

The decrease in CCM activity could result from the variant ResA having an insufficiently low midpoint potential to efficiently drive substrate reduction and/or not being able to interact optimally with the presumed apo-cytochrome c substrates. In connection with the latter, the ability of His76 to fold back into the hydrophobic cavity and H-bond to Glu80 may inhibit the interaction of ResA with its substrates. Finally, the decreased pK_a values of the active site cysteines are likely to increase the reactivity of the protein towards non-substrate molecules, as demonstrated in vitro via the insulin assay (Fig. 7). Thus, it is likely that the decrease in CCM activity is due to a combination of effects that increase the ability of non-specific interactions to compete with those of true substrates.
ACKNOWLEDGEMENTS
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ABBREVIATIONS
Abbreviations used: badan, 6-bromoacetyl-2-dimethylaminonaphthalene; CCM, cytochrome c maturation; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; IPTG, isopropyl-β-D-thiogalactoside; LB, Luria-Bertani; Mes, 2-(N-morpholino)ethanesulfonate; Mops, 3-morpholinopropanesulfonate; rmsd, root mean square deviation; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TMPD, N,N',N'-tetramethyl-p-phenylenediamine; TDOR, thiol-disulfide oxidoreductases; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.
FIGURE LEGENDS

Figure 1. The active site of wild-type *B. subtilis* ResA in reduced and oxidised states. Active site regions of wild-type ResA in (A) reduced (1SU9) and (B) oxidised (1ST9) states in sticks representation. Carbon, nitrogen, oxygen and sulfur atoms are shown in white, blue, red and yellow, respectively. Each figure was rendered with Pymol ([40]; www.pymol.org) and edited with GIMP (www.gimp.org).

Figure 2. Redox titrations of wild-type ResA and variants. 0.1 µM protein was incubated with varying ratios of oxidised and reduced DTT at 25 °C in 50 mM potassium phosphate, pH 7.0. Oxidised DTT concentration was fixed at 1 mM to compensate for fluorescence quenching effects. Fraction of reduced ResA was calculated from the fluorescence intensity as described in the Experimental section. Data are shown for wild-type (CEPC) ResA (squares), CPPC (circles), CEHC (triangles) and CPHC (DsbA-type) (inverted triangles). Lines show fits to Equ. S1.

Figure 3. $pK_a$ plots for ResA variants. Plots show pseudo-first order rate constants obtained for the reaction of badan with the cysteine thiols of (A) CPPC, (B) CEHC and (C) CPHC (DsbA-type) ResA variants at 25 °C in a mixed buffer system (see the Experimental section). Filled symbols show data recorded for Cys74 (recorded at 550 nm), and open symbols correspond to Cys77 (recorded at 440 nm). Lines show fits to Equ. S2 or S3, as described in the main text.

Figure 4. Unfolding profiles of wild-type ResA and variants. 1 µM pre-reduced and pre-oxidised protein was incubated in various concentrations of guanidine hydrochloride at 25 °C. Fraction unfolded was calculated from fluorescence intensity, as described in the Experimental section. (A) and (B) show data from reduced and oxidised protein, respectively, in 0.1 M Tris, 1 mM EDTA, pH 8.0. Data and fits to Equ. 1 are shown for wild-type (CEPC) ResA (squares, thick solid line), CPPC (circles, thin solid line), CEHC (triangles, thick dotted line) and CPHC (DsbA-type) (inverted triangles, thin dotted line). Filled and open symbols correspond to data from unfolding and refolding experiments, respectively. Diamonds in (B) show wild-type data from (A) to aid comparison. The inset of (B) shows relative fluorescence intensities for oxidised (hexagons) and reduced (inverted triangles) CPHC (DsbA-type) ResA, indicating clearly that the oxidised protein does not exhibit simple two state unfolding thermodynamics. 1 mM DTT was included with reduced protein to prevent re-oxidation.

Figure 5. The active site of variants CEHC and CPHC (DsbA-type) ResA in the reduced state. Active site regions of (A) CEHC (PDB code: 3C73) and (B) CPHC (DsbA-type) (3C71) ResA variants in sticks representation with relevant $2mFo-DF$ electron density map contoured at 1 $\sigma$. Nitrogen, oxygen and sulfur atoms are shown in blue, red and yellow, respectively. Panels in (A) correspond to CEHC ResA chain A (left panel) and chain B (right panel) of the asymmetric unit cell. Relevant residues are labelled; asterisks indicate substituted residues.

Figure 6. *In vivo* activity of CEHC ResA. (A) TMPD-staining of bacterial colonies; dark colour indicates cytochrome *c* oxidase activity. Strain 1A1 is wild-type *B. subtilis*, LUL9 is a ResA-deficient strain, LUN1 is a derivative of LUL9 with wild-type *resA* integrated at *amyE*, LUN8 is a similar derivative but with *resA* encoding CEHC ResA. (B) SDS-PAGE gel of *B. subtilis* membranes stained for covalently bound heme. Cytochromes indicated are CtaC (subunit II of cytochrome *caaa*), QcrC (subunit *c* of the cytochrome *bc* complex) and CccA/B (cytochrome *c550* and cytochrome *c551* which are not resolved). We note that a band at ~22 kDa due to QcrB (an unusual *b*-type cytochrome of the *bc* complex that binds heme covalently [41]) has previously been observed by $^{14}$C-labelling/autoradiography. It is not clearly detected here by heme staining, and we have generally found this to be variable [42]. (C) Western blot of membrane preparations from the above strains probed with ResA antiserum.
**Figure 7. General reductase activity of CEHC ResA.** Measurement of scattering at 650 nm due to the precipitation of insulin caused by reduction of the native, soluble oxidised form. Pre-reduced wild-type ResA (20 µM final concentration), CEHC ResA (20 µM), thioredoxin (Trx) (1 µM) in 0.1 M sodium phosphate pH 7.0 were added to insulin (110 µM) in the same buffer containing 2 mM EDTA and 1 mM DTT, as indicated. The addition of buffer only served as a negative control.

**Figure 8. Plot of reduction potential as a function of active site cysteine thiol pKₐ values.** Data for Cys74 and Cys77 in *B. subtilis* wild-type ResA and variants are shown in filled and open circles, respectively. A linear fit of the data is drawn in.
Table 1. Strains and plasmids used in this study.

<table>
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<th>Strains and plasmids</th>
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<th>Origin /reference</th>
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<td>pDH32-derivative containing Pspac lacI; ApR CmR</td>
<td>Dr V. Chary</td>
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</table>

* ApR, CmR, EmR indicate resistance to ampicillin, chloramphenicol and erythromycin, respectively.
* An arrow indicates transformation of the strain with DNA.
* BGSC; Bacillus Genetic Stock Center, Ohio, USA.
* Temple University School of Medicine.
<table>
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<th>ResA active site</th>
<th>pKₐ value Cys 74</th>
<th>pKₐ value Cys 77</th>
<th>ΔpKₐ Other</th>
<th>Midpoint reduction potential, Eₘ (mV)</th>
<th>Number of electrons (n)</th>
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<td>CEPC (wild-type)</td>
<td>8.8 ± 0.2</td>
<td>8.2 ± 0.1</td>
<td>-</td>
<td>-256 ± 1</td>
<td>2.2 ± 0.1</td>
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<td>CPPC</td>
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<td>6.6 ± 0.1</td>
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<td>1.9 ± 0.2</td>
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<td>5.7 ± 0.1</td>
<td>-2.5</td>
<td>-216 ± 3</td>
<td>4.0 ± 0.4</td>
</tr>
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ΔpKₐ values report differences between the variants and wild-type ResA.

b Measured at 25 °C, pH 7 versus NHE.

c note that this pKₐ is an average of two coupled pKₐ values.
Table 3. Free energies of stabilisation of oxidised and reduced wild-type ResA and active site dipeptide variants at 25 °C.

<table>
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<tr>
<th>ResA active site</th>
<th>Midpoint of transition (D_{0.5}, M Gdn HCl)</th>
<th>Cooperativity (m, kJ mol^{-1} M^{-1})</th>
<th>ΔG_{stab} (kJ mol^{-1})</th>
<th>ΔΔG_{ox/red} (kJ mol^{-1})</th>
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<tr>
<td>CEPC (Wild-type)</td>
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<tr>
<td>reduced</td>
<td>1.47</td>
<td>1.34</td>
<td>21.7 ± 2.8</td>
<td>14.9 ± 0.7</td>
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<td>1.71</td>
<td>1.49</td>
<td>13.5 ± 1.4</td>
<td>15.8 ± 2.0</td>
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<tr>
<td>CPPC</td>
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<tr>
<td>reduced</td>
<td>1.36</td>
<td>1.26</td>
<td>21.0 ± 4.0</td>
<td>15.1 ± 1.0</td>
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<td>1.01</td>
<td>12.8 ± 0.7</td>
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<tr>
<td>CEHC (DsbA-type)</td>
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<tr>
<td>reduced</td>
<td>1.45</td>
<td>1.31</td>
<td>23.1 ± 2.8</td>
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<td>oxidised</td>
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<td>1.27</td>
<td>18.1 ± 1.5</td>
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* ΔΔG_{ox/red} indicates the difference between the conformational stabilities of the oxidised and reduced proteins, i.e. ΔG_{ox} - ΔG_{red}.