Modulating the Redox Potential and Acid Stability of Rusticyanin by Site-Directed Mutagenesis of Ser86

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ABSTRACT: The expression of rusticyanin in *Escherichia coli* and a number of mutants for Ser86 is reported. Mutations of Ser86 to Asn, Asp, Gln, and Leu were undertaken as this is an Asn residue in other structurally characterized cupredoxins, and it has been suggested that this may be partly responsible for the high redox potential (680 mV) and extreme acid stability of rusticyanin. N-Terminal sequence analysis, together with other biochemical and spectrochemical characterization, shows that the recombinant wild-type protein is indistinguishable from native rusticyanin. All four mutants retain the rhombic nature of the EPR spectra and a significant absorption maximum at ∼450 nm, thus confirming that the overall geometry of the Cu ligands is essentially maintained. The oxidized form of all four mutants is less acid stable than the wild-type protein, although the detailed mechanism of lability varies. Ser86Leu readily loses copper as the pH is reduced from 4.0, but the protein does not denature. A significant proportion (∼30%) of Ser86Gln is denatured at lower pH values, whereas Ser86Asn and Ser86Asp are stable as the reduced (Cu^{I}) protein. The redox potential also varies by ∼110 mV (590−702 mV) upon these single point mutations, thus providing direct experimental support to the idea that this residue is at least in part responsible for the acid stability and the highest redox potential of rusticyanin in the cupredoxin family.

Rusticyanin is a monomeric type-1 blue copper protein of 155 amino acids with extreme acid stability and redox potential. The protein is thought to be a principal component in the iron respiratory electron transport chain of the Gram-negative bacterium *Thiobacillus ferrooxidans* (*1*, *2*), with concentrations reaching up to 5% of the total cell protein. Although its position in the respiratory electron transport chain is unclear, it is believed to mediate electron transfer between a c-type cytochrome (iron:rusticyanin oxidoreductase) and a membrane-bound terminal oxidase.

The Cu I/II redox couple (∼680 mV) (*3*−*6*) is the highest in the family of single type 1 copper proteins, also known as cupredoxins. The lowest value is 185 mV for stellacyanin, with azurins and plastocyanins having more typical values of 305−395 mV (*7*, *8*). The crystallographically characterized blue copper proteins share a common fold of a β-barrel (or β-sandwich) and have a basic framework of copper ligation by a histidine, cysteine, and methionine from the C-terminal end of the protein and a second histidine nearer the N-terminal. The methionine is a weak ligand with a long methionyl sulfur−copper bond (∼3.1 Å in azurin and 2.9 Å in plastocyanin). The lack of this methionine in stellacyanin has been thought to be responsible for its lowest redox potential within the group. Azurins have an additional axial interaction (∼3.1 Å) from the carbonyl oxygen of Gly45 (a residue next to the one of the ligating His) (*9*−*11*). The azurins from *Pseudomonas aeruginosa* and *Alcaligenes denitrificans* have been extensively studied by site-directed mutagenesis (*12*−*15*). However, there have been no mutations of azurins that have enhanced the redox potential close to that of rusticyanin.

That presence of a second histidine ligating the copper in rusticyanin was debated until recently (*2*, *16*) and was settled only when EXAFS (*17*), NMR (*18*), and crystallographic (*19*, *20*) studies provided unambiguous evidence for coordination of two histidines with His85 being the second histidine ligand. In fact, the copper site ligands and their geometry in rusticyanin is very similar to other blue copper proteins, and copper is coordinated in a distorted trigonal planar geometry with the three strong planar ligands His85$N^{\delta 1}$, Cys138S^{γ}, and His143$N^{\delta 1}$ and a relatively weaker ligand Met148S^{δ} in an axial position (*19*, *20*).

The crystallographic structures of rusticyanin have revealed several differences relative to other small blue copper proteins in the vicinity of copper ligands which may determine the higher redox potential and the acid stability of the copper site. We have previously suggested that Ser86, rather than conserved Asn as found in all other structurally characterized single blue copper proteins, may provide some protection for the metal in highly acidic media (*17*). We note that Asn is also fully conserved in copper containing nitrite reductase (*21*). In umecyanin, for which no structure exists, the sequence alignment suggests that Asn is replaced by an Asp (*22*). In azurin, mutation of Asn to Leu results in an increase of redox potential by 110 mV (*14*). Site-specific mutations combined with spectroscopic and struc-

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tural studies is the most rational approach for addressing these issues. To this end, we have recently isolated the structural gene[1] for rusticyanin from *T. ferrooxidans* (*24*) and now report the expression together with biochemical and spectrochemical characterization of the recombinant wild-type protein and a series of Ser86 mutants in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *E. coli* strains XL-2 Blue and BL21 (DE3) were used in this study. The method of Nishimura et al. (*25*) was used for transformation of XL-2 blue while electroporation was used for BL21 (DE3). Plasmids were obtained from Novagen (pET21D), Statagene (Bluescript), and Promega (pGem Z).

Cloning and Site-Directed Mutagenesis. The gene was amplified from the original Bluescript plasmid (*24*) using Tli DNA polymerase (Promega) and primers 1 and 2. Following purification initiation and termination, codons were added using primers 3 and 4 with Taq DNA polymerase: primer 3, 5′CGCGCCATGGGTACATTGGATACT 3′; primer 4, 5′CGCGAAGCTTACTTAACAACGATCTTG 3′.

The modified gene was then purified, treated with DNA polymerase Klenow fragment and T4 polynucleotide kinase (*26*), and blunt end cloned into *Sma*I cut Bluescript. The plasmids were isolated using the Wizard miniprep system (Promega) with slight modifications and cut with *Nco*I and *Bam*HI prior to ligating into the pET21d expression vector. Site-directed mutagenesis was performed using the PCR-based method of Higuchi et al. (*27*). The resulting mutants were cloned into pMCS-3 (a modified pGEM 3Z vector containing an *Nco*I site). Sequences were verified, using the Thermo Sequenase II kit (USB, Amersham), prior to ligating into pET21d. The primers used to introduce the mutations are given below.

Oligonucleotides were synthesized on an Applied Biosystems 391 DNA synthesizer PCR Mate: primer 1, 5′GGTA-CATTGGATACTACATGG 3′; primer 2, 5′CTTAACAAC-GATCTTGCCGAA 3′, 5′GGT CAT AAT TTT GAC ATCA 3′, 3′AAG CCA GTA TTA AAA CT 5′ Asn.

The underlined codons were replaced by CTT/GAA, GAT/CTA, and CAA/GTT for leucine, aspartic acid, and glutamine, respectively.

Cell Culture and Expression. For the initial screening experiments, expression of the protein from the pET21d plasmid in BL21 (DE3) was performed according to the manufacturers instructions. Subsequently, for large-scale preparations, 500 mL of LB medium containing ampicillin (200 μg/mL) was inoculated with 2 μL of a glycerol stock and grown overnight at 37 °C prior to induction with IPTG (1 mM) in the same medium with the further addition of ampicillin (200 μg/mL). These cultures were harvested after 5−6 h at 37 °C and stored in H_2SO_4 (10 mM) or acetate buffer (pH 4.0, 100 mM) at −20 °C prior to extraction.

SDS−PAGE and Western Blot Analyses. Cells were harvested by centrifugation and resuspended in loading buffer: Tris-HCl (pH 6.8, 62.5 mM), urea (4 M), 2% SDS, and 10% glycerol. It should be noted that, at high concentrations of both *T. ferrooxidans* and recombinant rusticyanin, reaction with the reducing agent in the loading buffer can occur producing higher molecular weight aggregates. SDS−PAGE was performed using standard conditions on either 15% gels or gradient gels from 5 to 30% acrylamide with prestained molecular weight markers (GIBCO BRL) using an Atto (Genetic Research Instrumentation) electrophoresis system (10 × 13.5 cm plates) or the Pharmacia Phast system with 20% gels. Standard methods were used for Western blots (*28*) using Atto equipment and the product detected using rabbit polyclonal antibodies, sheep anti-rabbit polyclonal antibodies labeled with alkaline phosphatase, and x-phosphate/nitro blue tetrazolium chloride (Boehringer Mannheim).

Protein Purification. *E. coli* BL21 (DE3) cells from 1 to 3 L of culture were resuspended in H_2SO_4 (10 mM) and sonicated (MSE Soniprep 150). The disrupted cells were then centrifuged at 15000*g* for 10 min to remove cell debris and the supernatant adjusted to 60% saturation with respect to $(NH_4)_2SO_4$. The supernatant was left on ice for 60 min and then centrifuged at 39000*g* for 15 min. The supernatant from this was adjusted to 100% with respect to $(NH_4)_2SO_4$ left on ice for a further 60 min before centrifuging at 39000*g*. The resulting pellet was resuspended in sodium acetate buffer (pH 4.0, 100 mM) and dialyzed overnight against the same buffer at 4 °C. Copper sulfate was added to a final concentration of 5−20 mM, resulting in the characteristic blue color of rusticyanin developing over 5 min. Routine purification was accomplished using a SP−Sepharose fast-flow column, loading in acetate buffer (pH 4.0, 100 mM) and eluting with a NaCl gradient (0.25−0.45 M). The main (and only blue) peak coelutes with nonrecombinant rusticyanin from *T. ferrooxidans*, at 350 mM NaCl. At this stage, the protein shows one contaminating band on SDS−PAGE and has an $R_{280/592}$ of 7−8 (wild-type protein). The protein is further purified on a Superdex 200 prep-grade gel filtration column. The protein was found to be homogeneous by SDS−PAGE and has an $R_{280/592}$ of 6.2−6.6 (wild-type protein).

The introduction of amino acid substitutions into the protein altered the behavior in the standard extraction procedure such that minor modifications were required. The Ser86Asn mutant formed a dimer when treated with $(NH_4)_2SO_4$, which could not subsequently be dissociated to the monomer. Therefore, the supernatant from the sonication step was concentrated directly in H_2SO_4 (10 mM) using Centricon-10 (Amicon), dialyzed against sodium acetate buffer (pH 4.0, 100 mM), and then purified as before. The majority of the protein produced in the Ser86Leu, Gln, and Asp mutants was found in the cell debris after sonication, presumably as inclusion bodies. Consequently, the cell debris we treated with urea (5 M) in acetate buffer (pH 4.0, 100 mM), centrifuged at 15000*g* concentrated in centricon-10 filters and then subjected to ion exchange and gel filtration chromatography as before.

Protein Characterization. Homogeneous recombinant rusticyanin from the gel filtration column was dried in a spinvac (Appligene) prior to N-terminal sequencing on an Applied Biosystems Procise Sequencer. Electronic absorption spectra were taken on a Perkin-Elmer λ 16 double beam spectrometer. Wavelength accuracy was calibrated using holium oxide filters: accuracy ±1 nm, precision ≤±0.5 nm. X-band EPR spectra of native rusticyanin and the Ser86 mutants were recorded on a JEOL RE2X spectrometer equipped with a variable temperature liquid nitrogen cryostat.

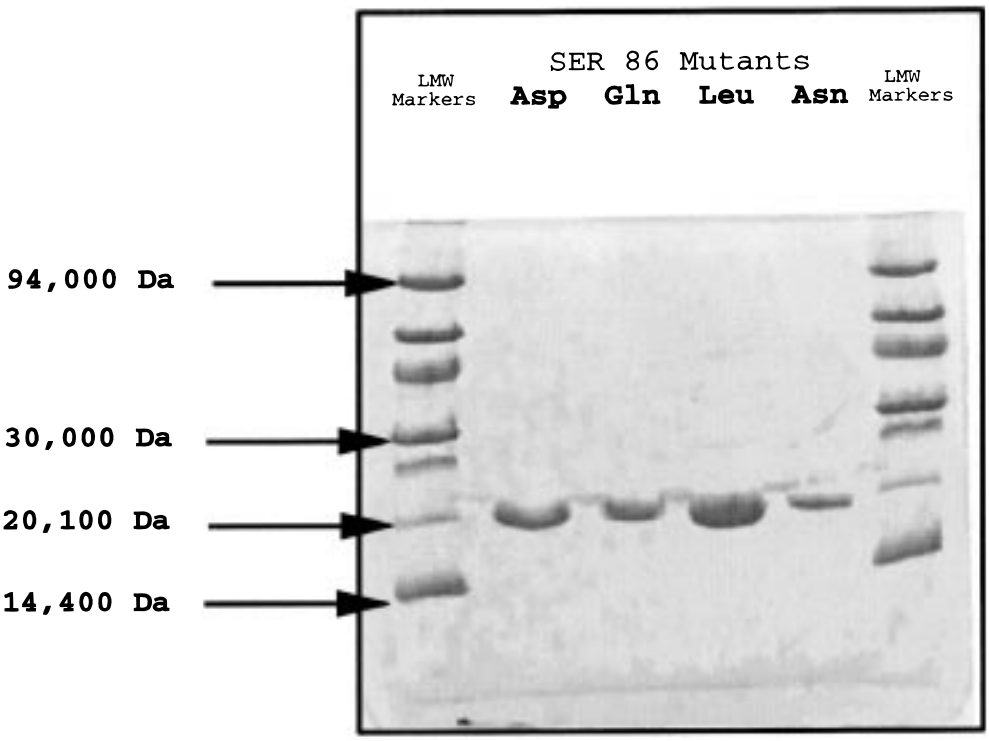

FIGURE 1: SDS−PAGE of purified mutants showing them to be highly homogeneous.

Other conditions were as follows: microwave frequencies, 9.128−9.142 GHz; power, 10 mW; temperature, 123 K; modulation amplitude, 1−0.5 mT. EPR simulations were produced using the in-house computer program E (R. W. Strange, available on request) using the intermediate or strong field approximation (*29*, *30*). For both UV−vis and EPR, protein was in acetate buffer (pH 4.0, 100 mM).

The midpoint redox potential of the copper center of the recombinant wild-type protein and mutants was determined by redox potentiometry. Redox titrations were performed according to Ingledew and Colby (*3*). In each case, at least two measurements were performed giving an estimated internal error of ±5 mV. The solutions were poised at approximately 800 mV with K_2IrCl_6 in alanine sulfate buffer (pH 3.2) at 25 °C with FeCl (100 μM) as mediator. Electrodes were calibrated against 2,6-dichlorophenol indophenol. Reduction of proteins was achieved with ascorbic acid.

The M_r was determined by electrospray mass spectroscopy using a FISONS VG platform.

pH Stability Tests. Protein samples at approximately 1.6 mg/mL were fully oxidized with K_2IrCl_6 in acetate buffer (pH 4.0, 100 mM). Following removal of excess oxidizing agent with PD-10 columns (Pharmacia) the absorbance at 600 nm was monitored for 60 min at 6 min intervals. The pH of the solution was then lowered to pH 3.2 by adding a predetermined volume of HCl (1.2 N) and the absorbance monitored. This procedure was repeated at pH 2.5, 1.9, and 1.5. In a separate experiment, the pH was set at 1.5 for 1 h after which the solution was exchanged for acetate buffer (pH 4.0, 100 mM). The samples were monitored for 30 min, then 2.5 μL of oxidant was added and monitored for another 60 min. Copper sulfate (5 μL, 20 mM) was then added and the response followed for a further 30 min, and finally a further 2.5 μL of oxidant was added.

RESULTS

Expression and Purification of Rusticyanin from E. coli. The pET21d plasmids containing the rusticyanin gene (designated pROC) were used to transform either XL-2 Blue or BL21 (DE3) *E. coli*. A number of clones were then selected and treated with IPTG to induce protein expression. Analysis by SDS−PAGE and Western blotting showed that the product had a similar electrophoretic mobility and antigenicity to native (nonrecombinant) rusticyanin. The expressed protein also shows identical chromatographic behavior to nonrecombinant protein. The SDS−PAGE showed a single band for all of the mutants (Figure 1) demonstrating high purity for these mutants. The levels of recombinant rusticyanin produced are of the order of 50 mg/L as estimated from the molar extinction coefficient (*31*).

Characterization of Recombinant Wild-Type Rusticyanin. The electronic absorption spectrum of the recombinant (wild-type) rusticyanin has maxima at 280, 452, 592, and 740 nm and is indistinguishable from native rusticyanin (Figure 2a). The observed ratio of 280:592 nm and 452:592 nm are identical for the two proteins. The EPR spectra of wild-type and native proteins were simulated using identical parameters (Table 1, Figure 2b), which suggests that the copper-site coordination and geometry are fully preserved in the expressed protein. The spectra are rhombic and also exhibit the small hyperfine splitting in the g_{II} region, which is typical for type 1 copper centers. The g and A values obtained from the simulation differ from those obtained by Cox et al. ($g_z = 2.229$, $g_y = 2.064$, $g_x = 2.019$, $A_z = 4.5$ mT, $A_y = 2.0$ mT, $A_x = 6.5$ mT) (*32*), whose parameters did not give the best fit to the experimental data for both nonrecombinant and recombinant wild-type rusticyanin.

N-terminal sequencing of the recombinant wild-type rusticyanin gave a sequence identical to that reported for rusticyanin (*2*): GTLDTTWKEATLPQVKAMLE. The

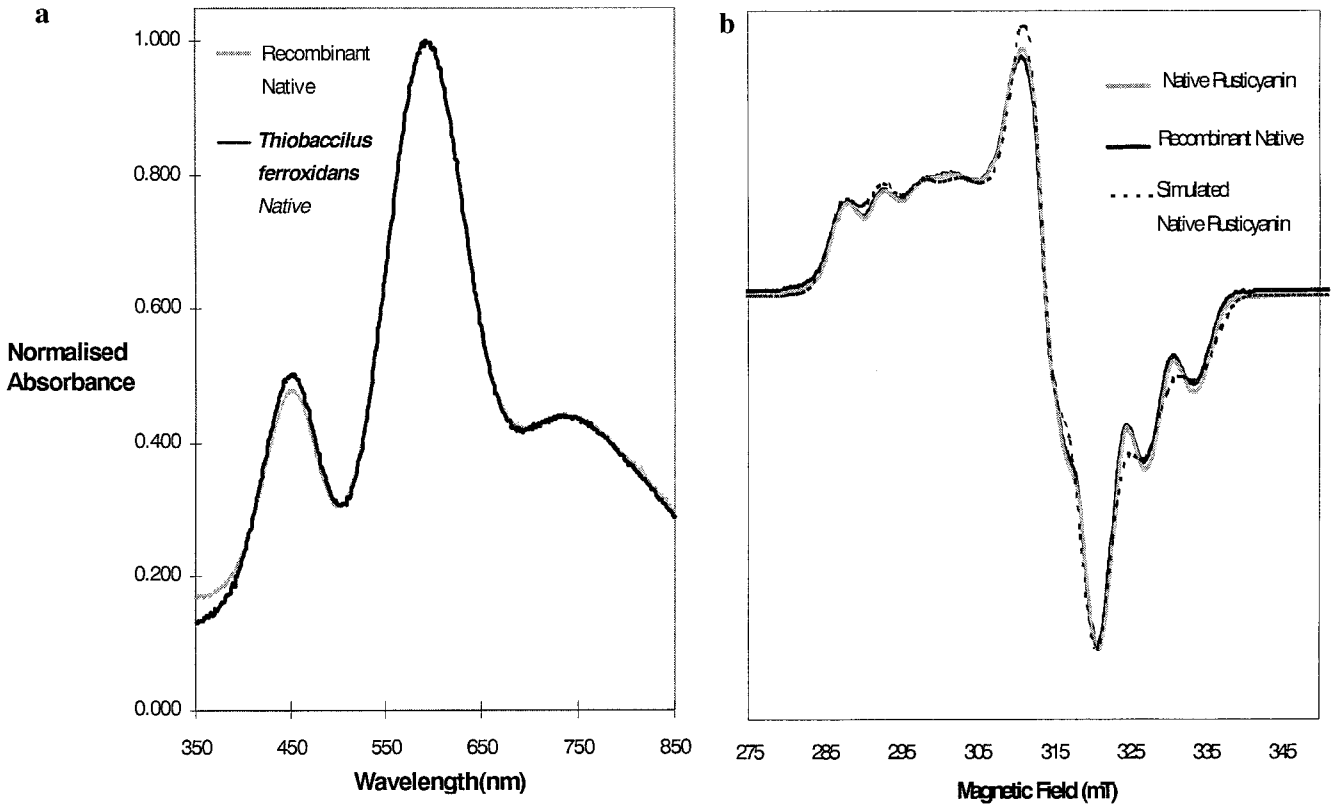

FIGURE 2: The visible (a) and EPR (b) spectra of *T. ferrooxidans* and *E. coli* recombinant rusticyanin. EPR simulation is also included.

Table 1

	Recombinant Native	Ser86Asn	Ser86Asp	Ser86Gln	Ser86Leu
λ_{max} (nm)	449	456	458	454	448
λ_{max} (nm)	592	599	600	601	600
A(∼600/∼450)	2.08	3.06	3.58	2.79	2.49
			Redox Potential		
(mV)	667	590	623	664	702
			EPR Parameters (Hyperfine Couplings in milliTesla)		
g_x	2.015	2.022	2.014	2.016	2.016
g_y	2.052	2.054	2.049	2.046	2.051
g_z	2.211	2.211	2.200	2.214	2.224
A_x	6.50	6.50	6.50	6.50	6.50
A_y	1.30	1.15	1.30	0.80	1.15
A_z	5.00	4.50	4.90	4.90	4.58

electrospray mass spectrum showed two main peaks, at 16 550.3 ± 2 and 16 612.5 ± 2 Da. The lower mass corresponds to the correct amino acid sequence and demonstrates that the protein is processed correctly. The higher mass constitutes approximately 40% of the mass distribution and corresponds to the copper-loaded form. Observation of the copper-loaded form in the gas phase was somewhat unexpected as no such band has been seen for azurins (*7*). The retention of Cu by 40% of the mass is further evidence for the very robust nature of the Cu site in this highly stable protein. The midpoint redox potential of the copper center of the recombinant wild-type protein was determined to be 667 ± 5 mV at pH 3.2 (Table 1), in good agreement with the reported value of 680 mV (*3*−*6*) for the nonrecombinant protein.

Characterization of Ser86 Mutants. Optical and EPR spectra for four mutants of Ser86 are shown in Figure 3. The main optical, redox potential, EPR parameters are summarized in Table 1. All the mutants exhibit an intense absorption at ∼600 nm, which is characteristic of type 1 copper sites, and an additional band at ∼450 nm, which is observed for wild-type rusticyanin and some other blue copper proteins. The mutants absorption maxima at ∼590 and ∼450 nm are all red-shifted compared to those of the wild-type, except for Ser86Leu in which the ∼450 nm band is effectively unchanged in position. The optical spectrum for the Ser86Leu mutant most closely resembles the wild-type. The 450 nm band exhibits the most variability in the mutants, with Ser86Leu showing the strongest and Ser86Asp the weakest with the A_{590}/A_{450} ratios ranging 2.5−3.6.

The EPR spectra of the Ser86 mutants were simulated using the parameters shown in Table 1. In all cases, the patterns of g and A values obtained were similar to those of wild-type rusticyanin. The EPR spectra are all rhombic with small hyperfine coupling in the g_{II} region (A_{II} ranging 4.58−5.00 mT). The hyperfine lines in the g_{II} region are not equally well resolved, which may be due to so-called g strain which has been observed for the EPR spectra of other blue copper proteins (*33*, *34*). This effect is particularly evident for Ser86Leu and Ser86Asn and is reflected as reduction of the corresponding A_z, to ca. 4.5−4.6 mT, together with some small changes in the g values. In all cases, the hyperfine coupling A_y is unresolved so that the values used for the simulations should be taken as upper limits. The rhombicity of the mutants EPR spectra, measured in terms of the splitting between g_x and g_y, are slightly decreased for Ser86Gln (0.030) and Ser86Asn (0.032) relative to native rusticyanin (0.037). Ser86Gln also exhibits the smallest hyperfine coupling along g_y. The EPR spectrum of Ser86Asp is most like the wild-type protein. These small differences are indicative of small perturbation of the Cu site and is most

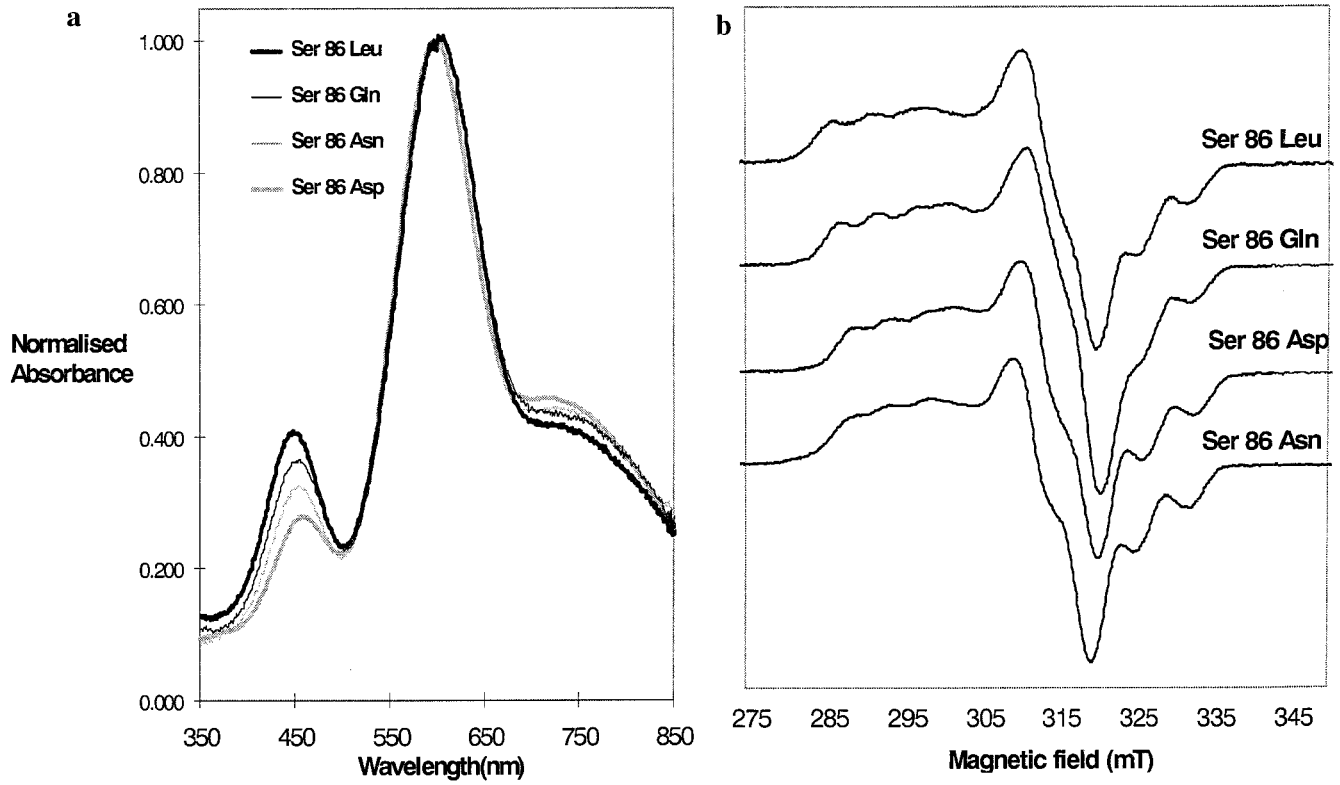

FIGURE 3: The visible (a) and EPR (b) spectra of the Ser86 Asn/Asp/Leu/Gln mutants.

likely to arise from subtle changes in the hydrogen bonding as a result of the mutation (see below).

The redox potential of the mutants is comparable to that of the wild-type rusticyanin with the Asn mutant exhibiting the lowest redox potential of 590 mV and Leu mutant showing the highest (702 mV) potential. Thus, a change of ~110 mV is observed among the mutants. However, the redox potential of 590 mV is still some 200 mV higher than plastocyanin even when Ser86 is mutated to the fully conserved residue Asn. We note here that plastocyanin has the next highest redox potential known for single Cu proteins (*35*, *36*) and where also no axial carbonyl oxygen interaction exists.

pH Stability. Recombinant wild-type rusticyanin is barely affected at any pH tested, retaining 81% of the original absorbance at pH 1.5 (Table 2a), which upon reoxidation at pH 4.0 recovers (Table 2b) to the original value, indicating no loss of copper or denaturation. In contrast, all the mutants display reduced stability (by varying degrees) with respect to copper oxidation state, copper loss, and denaturation. Thus, Ser86Asn, Ser86Asp, and Ser86Gln are relatively stable at pH 2.5 (92, 94, and 90%, respectively, remain oxidized after 60 min) and can be fully reoxidized. Ser86Leu was readily reduced however, with 32% reduction at pH 2.5 after 60 min and complete loss after several hours. As most of the blue color is recovered upon addition of copper at pH 4.0, the original loss of color with Ser86Leu is mainly due to the loss of copper rather than reduction or denaturation. At pH 1.9 and 1.5, Ser86Gln and Asn are affected to a greater degree than Ser86Asp and the recovery of Ser86Gln was considerably less than that of the others (Table 2b), indicating loss of approximately one-third of the protein (or the Cu-binding site) by denaturation. In the case of Ser86Asn, 20% of the protein is not recovered.

Table 2

(a) Relative Stability of Recombinant Rusticyanin (WT) Compared to the Ser86 Mutants[a]

pH	WT	Leu	Asp	Asn	Gln
4	100	100	100	100	100
3.2	97	85	95	87	97
2.5	98	68	92	94	90
1.9	86	3	83	64	66
1.5	81	0	66	58	36

(b) Recovery of Original Absorbance from pH 1.5

WT	Leu	Asp	Asn	Gln	
81	0	66	58	36	after 60 min at pH 1.5
95	14	72	59	31	exchange of buffer to pH 4.0
100	17	74	59	25	upon reoxidation
100	72	78	69	40	plus copper
100	86	85	79	64	further oxidation

[a] Percentage of original absorbance at 592 nm.

DISCUSSION

The gene encoding rusticyanin has been successfully subcloned into the pET21d vector and expressed at a high level in *E. coli* BL21(DE3). Spectroscopic, N-terminal sequence analysis and electrospray mass measurements have confirmed the identity of the recombinant material. A similar system has been recently used to express rusticyanin from a synthetic gene which has been optimized for *E. coli* codon usage (*37*). The reported levels of purified protein from this system at between 70 and 100 mg/L of culture are similar to those reported here (ca. 50 mg/L), indicating that codon usage does not significantly affect the level of expression.

The rhombic nature of the EPR spectra, high redox potentials, and intense bands at ~450 and ~600 nm are retained by all four mutants, suggesting that the overall

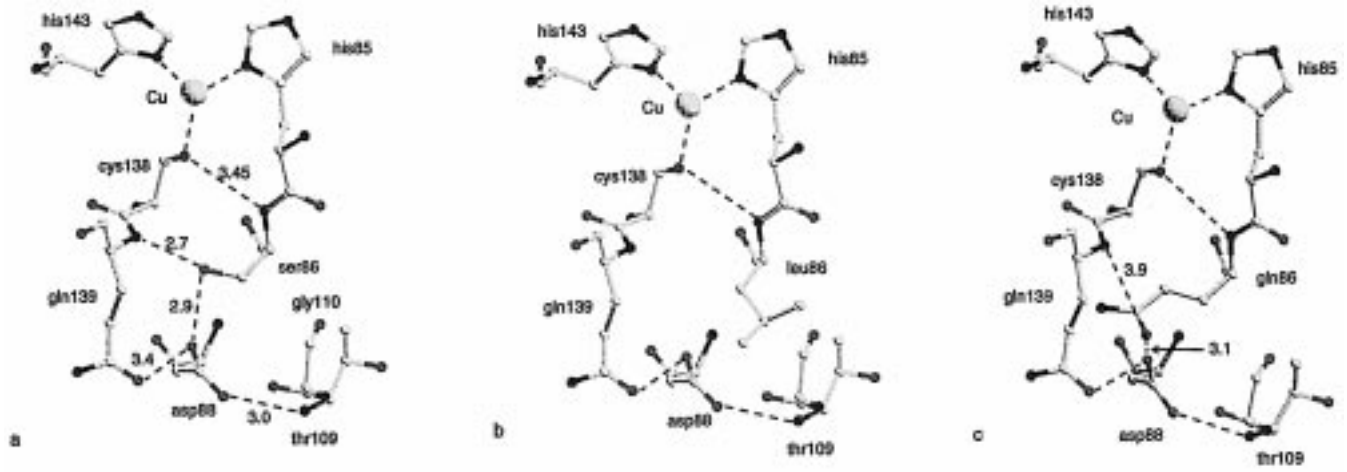

FIGURE 4: The structure around residue 86 of *T. ferrooxidans* rusticyanin [PDB ID 1a8z (*20*)]. Panel a is the observed structure while panels b and c are modeled structures based on panel a; see text for further details. (a) The wild-type protein. The side-chain oxygen of Ser86 forms hydrogen bonds with the backbone nitrogen of Gln139 (2.7 Å) and the side-chain oxygen of Gln86 (2.9 Å). (b) Ser86Leu showing the most probable rotamer. All three suggested rotamers adopt similar positions. (c) Ser86Gln. Although not the most probable rotamer on statistical grounds, this rotamer preserves the hydrogen bond between the side chains of Asp88 and Gln86 in a analogous manner to the wild-type protein. The distance between the side-chain oxygen of Gln86, and the backbone nitrogen of Gln139 (3.9 Å) is probably too long to form a significant hydrogen bond.

geometry of the Cu site is essentially maintained. The small differences observed in both the optical and EPR spectral features suggest that only subtle changes occur in the copper-ligands geometry.

In the type 1 copper optical spectra, the band at ~600 nm arises from a charge-transfer transition from S_{cys} to copper. The band at ~450 nm is also believed to be associated with a separate cysteine−copper charge-transfer transition, although a recent paper has assigned it differently (*38*). It has been previously suggested (*13*, *39*−*40*) that in type 1 copper centers there is a correlation between the presence of an intense band at ~450 nm and the occurrence of a rhombic EPR signal. Several explanations for the rhombicity of the EPR signal and the intense 450 nm band have been offered including involving a larger displacement of copper atom with respect to the three strong ligands (His, His, Cys) and a strengthening of the Cu−X axial ligand bond (*15*, *39*). Dodd et al. (*41*) have recently observed that a type 1 copper site containing a short Cu−S_{met} bond (~2.62 Å), in the blue nitrite reductase from *Alcaligenes xylosoxidans*, is not associated with either a rhombic type 1 EPR signal or a significant band at ~450 nm. In fact, a comparison of the crystal structures of a green nitrite reductase from *Alcaligenes faecalis* (*42*) and the more recent 2.1 Å structure of the blue nitrite reductase from *A. xylosoxidans* (*21*) reveals one main difference; namely the His_c-Cu-Met angle is 115° in the blue vs 131° in the green nitrite reductase (*21*). For both of these, displacement of the Cu atom with respect to the strong ligands is essentially the same (~0.5 Å) and is close to rusticyanin [0.4 Å (*20*)]. We note that the His_c-Cu-Met angle is ~90° in azurin, 110° in pseudoazurin, and 105° in rusticyanin (*19*, *20*). Thus, differences in optical properties can arise from a number of small interlinked and complex structural differences which effectively result in changes in the orbital overlap of S_{cys} and Cu (*43*).

Even though the optical and EPR spectra of the Ser86Gln show some differences, the redox potential for this mutant is closest to the native protein. The redox potentials observed for the mutants show a change of 112 mV (590−702 mV), providing direct experimental evidence for the view that Ser86 plays a significant role in giving the copper site of rusticyanin the highest redox potential in the family of single copper protein. The lowest potential exhibited by the Ser86Asn mutant is only 140 mV higher than the Asn46Leu mutant of azurin but is still 200 mV higher than any known naturally occurring single Cu protein. Thus, the unusually high redox potential of rusticyanin can only be in part determined by the nature of this residue; additional factors must be operative.

The mutants retain a high degree of acid stability, and apart from Ser86Leu, substantial differences are not observed at pH ≥2.5. The data indicate that the rapid reduction of Ser86Leu at pH 1.5 is mainly due to the lability of the copper site, rather than denaturation of the protein (or copper site) or reduction of copper. At low pH, Ser86Gln and Asn are affected, considerably resulting in a loss of protein (and/or Cu binding site) of 30 and 20%, respectively.

Current explanations of the factors contributing to the extreme acid stability and high redox potential shown by rusticyanin favor the hypothesis that the hydrophobic nature of the copper site affords protection from protonation (*17*, *19*). Furthermore, the substitution of conserved asparagine to serine in rusticyanin has been suggested to provide greater stability by creating a tighter copper cavity via relatively shorter hydrogen bonds (*17*). It has been noted before that, unlike Ser, Asn tends to make rather long-range interactions which cross-link more distant parts of the polypeptide chain (*44*). Even though there is no substitute for crystallographic structure determination, it is possible to gain some insight into the possible structural consequences of these mutations by *modeling* the mutations using the side-chain rotamer library (*45*) and crystallographic structure of native rusticyanin (*20*). The side-chain oxygen of Ser86 forms a tight (2.73 Å) hydrogen bond to the backbone nitrogen of Gln139 [Figure 4a (*20*)]. Additionally, the backbone nitrogen of Ser86 hydrogen bonds to the copper ligand $S138_{Cys}$. All three rotamers for Ser86Leu suggested that even if chemistry were permitting, Leu would be unable to adopt a conforma-

tion capable of forming a hydrogen bond to the backbone nitrogen of Gln139 (see Figure 4b). The greater acid lability of the copper observed for this mutant (loss of copper) is consistent with the reduced hydrogen bonding. The increase in redox potential of this mutant to 700 mV is consistent with the increase observed for the analogous mutation in azurin (*14*). The most likely rotamers for the other three mutants (Asp, Asn, Gln) would also be unable to form a hydrogen bond to the backbone nitrogen of Gln139. However, there are possible rotamers of Asp and Asn that could form a very tight (~2.6 Å) hydrogen bond to the backbone nitrogen of Gln139. These two mutants have lower redox potentials than the wild-type protein and the acid stability of the copper site is reduced. Interestingly, the most likely rotamer for Ser86Gln suggests a possible hydrogen bond (3.0 Å) between Gln86NE and Gln139OE. However, in the wild-type protein, Gln139NE already forms part of this hydrogen bond network and this bond would need to be broken to form the suggested bond in the mutant. A less likely rotamer can form a satisfactory (3.3 Å) hydrogen bond to the backbone nitrogen of Gln139 without significantly disrupting the rest of the hydrogen bond network. Although the redox potential of the Ser86Gln mutant is virtually the same as that for the wild-type, the acid stability of the protein is significantly affected resulting in both reduction of the copper site and inability to take up copper (probably due to unfolding). The Cu site may be disrupted once the pH is reduced due to the bulkier nature of the Gln ligand, with the effect being less pronounced in the case Asn mutant.

CONCLUSIONS

It has been demonstrated by site-directed mutagenesis that Ser86 is an important substitution in rusticyanin and is in part responsible for its greater acid stability and very high redox potential. It is also clear that redox potential of the blue Cu site can be significantly altered without much effect on the spectroscopic properties which are largely determined by the coordinating ligands and their geometry to Cu. Thus, factors outside the immediate coordination sphere of Cu influence its redox potential. The amenability of rusticyanin for site-directed mutagenesis should allow structure−function studies of these factors as well as help in identifying the critical hydrophobic residue(s) close to the copper site.

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