Gene Synthesis, High-Level Expression, and Mutagenesis of *Thiobacillus ferrooxidans* Rusticyanin: His 85 Is a Ligand to the Blue Copper Center[†]

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ABSTRACT: An artificial gene of the blue copper protein rusticyanin from *Thiobacillus ferrooxidans* was constructed from eight overlapping oligonucleotides in a recursive "one-pot" polymerase chain reaction. The gene was placed behind the T7/*lac*O_R promoter of pET24a and expressed in *Escherichia coli* as a soluble protein. A purification scheme involving a pH titration step, cation-exchange chromatography, and reverse-phase HPLC separation provided yields of the apoprotein ranging from 70 to 100 mg/L of cell culture; reconstitution with Cu(II) is quantitative at pH 3.4-5.5. The redox reactions and the electronic absorption and EPR spectra of the recombinant Cu(II)-rusticyanin and NMR spectra of the reduced holoprotein are indistinguishable from those of the protein derived from *T. ferrooxidans*. Rusticyanin possesses the phylogenetically conserved carboxy-terminal loop of three copper ligands (Cys 138, His 143, and Met 148), but the identity of the fourth ligand was not clear from sequence homology to other blue copper proteins. To address this question directly, we have prepared two site-specific mutants where two of the proposed ligands, Asp 73 and His 85, have been replaced with alanine. The Asp73Ala mutant retained the electronic properties of the wild-type blue copper center (absorption maxima at 452, 597, and 750 nm), whereas the His85Ala variant gave rise to a green type 1 copper protein (absorption maxima at 455 and 618 nm). The ability of the His85Ala mutant to bind exogenous ligands such as chloride ions is consistent with the idea that a cavity has been introduced at the copper site; the ^{15}N HSQC spectrum of the chloro derivative of the mutant indicates that the mutation at position 85 does not perturb the overall fold of the protein. These results, together with those of a recent NMR study [Hunt, A. H., Toy-Palmer, A., Assa-Munt, N., Cavanagh, J., Blake, R. C., II, & Dyson, H. J. (1994) *J. Mol. Biol. 244*, 370-384], firmly establish that rusticyanin has the classical "blue-copper" coordination set of $CysHis_2Met$. The availability of a high-level bacterial expression system will allow us to address the source of the unusual acid stability and redox properties of the protein through combination of site-directed mutagenesis experiments and structural analyses.

Thiobacillus ferrooxidans is an acidophilic sulfur bacterium that can obtain its energy for the assimilation of CO_2 from the aerobic oxidation of Fe(II) (Ingledew, 1982). A key component of the iron-dependent energy-harvesting mechanism in this organism is rusticyanin (Cox & Boxer, 1978), a small mononuclear copper protein (16 551 Da) that mediates the transfer of electrons in the periplasm from Fe(II) and an as-yet uncharacterized *c*-type cytochrome (termed iron:rusticyanin oxidoreductase) to the membrane-bound terminal oxidase. The importance of rusticyanin in this process is indicated by its *in vivo* concentration, which may reach 5% of the total cell protein.

The copper site in rusticyanin exhibits the properties of a type 1 ("blue copper") center. It is characterized by a strong electronic absorption at 597 nm ($\epsilon = 2240\ M^{-1}\ cm^{-1}$) and an EPR[1] signal with a small $A_{\parallel}$ hyperfine coupling constant ($42 \times 10^{-4}\ cm^{-1}$) and rhombic symmetry (Cox et al., 1978). Despite having an electronic structure strikingly similar to those of a number of small blue copper proteins (Solomon et al., 1992), rusticyanin has certain properties that are quite unlike other members of this class of copper proteins. For example, the protein and the copper coordination shell are stable under extreme acidic conditions (pH 0.5-6.5). Both electronic absorption and EPR spectra of the oxidized protein remain largely unchanged over this pH range. The Cu(II/I) midpoint potential (+680 mV vs NHE, pH 2.0) (Lappin et al., 1985; Ingledew & Cobley, 1980; Blake et al., 1991b; Haladjian & Bruschi, 1993) is considerably higher than those of most other blue copper proteins (+185 to +370 mV) and exhibits a small pH dependence between pH 1.0 and 5.0. This value is second only to that of the type 1 copper center in *Polyporus versicolor* laccase (+785 mV vs NHE), a multinuclear copper protein that catalyzes the four-electron oxidation of phenol derivatives from molecular oxygen (Reinhammar, 1972).

The nature of the ligation to the copper in rusticyanin has attracted a great deal of interest because of its unusual properties. No crystal or solution structure is yet available for rusticyanin, although crystals have been obtained (Djebli et al., 1992) and sequential assignments preparatory to the calculation of a solution structure have been made (Hunt et al., 1994; A. Toy-Palmer and H. J. Dyson, unpublished

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[1] Abbreviations: NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; NHE, normal hydrogen electrode; HSQC, homonuclear single-quantum coherence; PCR, polymerase chain reaction; HPLC, high-performance liquid chromatography.

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          BamHI  NdeI                                   EcoNI
  Amp1      |      |                                      |
5'  gagtctgatatcggatcccatatg  3'
5'  GAGTCTGATATCGGATCCCATATGGGTACCCTGGACACTACCTGGAAAGAAGCTACCCTGCCGCAGGTTAAAGCTATGCTGGAAAAAGATACC  3'       120
                                                                         3'  TCGATACGACCTTTTTCTATGGCCGTTTCAAAGACCGCTATGACATTGG
                      MetGlyThrLeuAspThrThrTrpLysGluAlaThrLeuProGlnValLysAlaMetLeuGluLysAspThrGlyLysValSerGlyAspThrValThr 32
                         1
             BsaAI            ScaI                   BstBI                                        HhaI    AccI
               |                |                      |                                            |       |
         5'  CACGTAGTTGCTGCTGCAGTACTGCCAGGCTTCCCGTTCCCATCCTTCGAAGTACACGACAAGAAAAACCCGACTCTGG  3'                240
ATGAGGCCATTTTGACAAGTGCATCAACGACGACGTCA  5'                          3'  CTGTTCTTTTTGGGCTGAGACCTCTAGGGTAGTCCGCGTTGACAT
TyrSerGlyLysThrValHisValValAlaAlaAlaValLeuProGlyPheProPheProSerPheGluValHisAspLysLysAsnProThrLeuGluIleProAlaGlyAlaThrVal 72

             Amp3                                  AvaII                  ClaI
      5'  aacaccaacaagggttttggtcac  3'                |                       |
      5'  AACACCAACAAGGGTTTTGGTCACTCTTTCGATATCACTAAAAAGGGTCCGCCATACGCAGTTATGCCGGTTATCGATCCAAT  3'              360
CTGCATTGGAAGTAATTGTGGTTGTTCCCAAAACCAG  5'                            3'AATACGGCCAATAGCTAGGTTAGCATCGACCGTGACCAAAGAGG
 Amp2 -> 3'  aattgtggttgttcccaaaaccag  5'
AspValThrPheIleAsnThrAsnLysGlyPheGlyHisSerPheAspIleThrLysLysGlyProProTyrAlaValMetProValIleAspProIleValAlaGlyThrGlyPheSer 112

                                                                                         HaeIII
                                                                                           |
          5'  AAGTTCGGTTACACCGACTTCACTTGGCACCCGACTGCAGGTACCTATTACTACGTTTGTCAGATTCCGGGCCACG  3'                   480
GGCCATGGCTTTCTGCCATTCAAGCCAATGTGGCTGAAGT  5'                         3'  ACAGTCTAAGGCCCGGTGCGTCGATGACCGTACAAACCGTTCTAG
ProValProLysAspGlyLysPheGlyTyrThrAspPheThrTrpHisProThrAlaGlyThrTyrTyrTyrValCysGlnIleProGlyHisAlaAlaThrGlyMetPheGlyLysIle 152

        EcoRI BamHI
          |     |
Amp4 ->  3' cttaagcctaggttaagagccaca  5'
CAACATTTTATTCTTAAGCCTAGGTTAAGAGCCACA  5'
ValValLysOch                        516
     155
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FIGURE 1: Sequences of the eight overlapping oligonucleotides employed to construct a 516-bp linear DNA consisting of the rusticyanin gene and flanking cloning sites. The sequences of the short primers (lower case) used for amplification are also indicated. The protein sequence using the three-letter code (Och, ochre stop codon) is shown below the coding region of the DNA.

results). Structures are available for a number of other blue copper proteins, including plastocyanin (Guss & Freeman, 1983; Collyer et al., 1990; Bagby et al., 1994; Redinbo et al., 1993; Moore et al., 1988, 1991), azurin (Adman & Jensen, 1981; Baker, 1988; Nar et al., 1991), pseudoazurin (Adman et al., 1989), cucumber blue protein (Guss et al., 1988), and amicyanin (Romero et al., 1994; Kalverda et al., 1994). Sequence comparison of rusticyanin with these proteins (Ronk et al., 1991; Yano et al., 1991; Nunzi et al., 1993) revealed a C-terminal sequence that is homologous with the highly conserved sequence (Tyr-X_{2-3}-Cys-X_{1-2}-Pro-X_{0-1}-His-X_{2-4}-Met) found in other blue copper proteins. This sequence encompasses three (underlined) of the four side-chain copper ligands. However, because of the poor homology in the rest of the sequence, several candidates (Asp 73, His 39, His 57, His 85, or a methionine) have been proposed for the fourth ligand in rusticyanin. EXAFS (Holt et al., 1990) and metal-substitution studies (Strong et al., 1994) of the protein support an N-ligated residue, presumably a histidine, and recent NMR studies suggest His 85 as a ligand to the copper (Hunt et al., 1994).

An understanding of the extent to which the protein fold and the coordination environment contribute to the acid stability and high reduction potential of the copper coordination site in rusticyanin requires knowledge of the protein structure combined with site-directed mutagenesis experiments. This article describes an important first step toward this goal: the design and synthesis of an artificial gene for rusticyanin and the development of an *Escherichia coli* expression system with a suitable purification scheme. A very rapid and highly efficient procedure for synthesizing a gene of any given size using the polymerase chain reaction technology (PCR) (Saiki et al., 1988; Innis et al., 1990) has been devised for this purpose. In addition, we present the results of initial mutagenesis experiments aimed at probing the identity of the copper ligands. The availability of an overexpression system will also facilitate our ongoing efforts to obtain a high-resolution structure of this copper protein through multidimensional NMR techniques.

MATERIALS AND METHODS

Materials. The oligonucleotides were synthesized on a 0.2-μmol scale using an Applied Biosystems 380B instrument. Oligonucleotides longer than 40 bases were prepared with the dimethoxytrityl groups and purified through OPC cartridges (Applied Biosystems, Inc.). Shorter oligonucleotides were detritylated by acid treatment and purified by passing through NAP-25 gel filtration columns (Pharmacia). pET24a vector and *E. coli* BL21(DE3) (*hsdS gal* [λc*I*ts857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene*1*]) strain were purchased from Novagen. Restriction enzymes and DNA ligases were obtained from New England Biolabs. *Thermus aquaticus* (*Taq*) DNA polymerase was obtained from Boehringer Mannheim. All buffers were prepared from analytical grade reagents.

Gene Design and Assembly. The sequence of the synthetic rusticyanin gene was designed with the aid of DNA Master software (Division of Biology, California Institute of Technology). Eight overlapping oligonucleotides of length ranging from 76 to 93 bases (Figure 1) were selected to construct the synthetic gene using a modification of the recursive PCR strategy (Prodromou & Pearl, 1992). All PCR reactions were carried out on either a Perkin-Elmer Cetus DNA Thermal Cycler 480 or an Ericomp Twin Block system; optimum reaction conditions were determined with the use of the Invitrogen 16-buffer system. The first PCR contained 4 pmol each of the eight oligonucleotides, 2.5 units of *Taq* DNA polymerase, and 0.25 mM of each deoxynucleotide triphosphate (dNTP) in 100 μL volume. The solution was buffered in 60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, and 2.5 mM $MgCl_2$, pH 8.5 (22 °C). The reaction involved a hot start at 80 °C followed by 30 cycles of 30 s at 95 °C, 30 s at 59 °C, and 1 min 10 s at 72 °C. The reaction was completed with a 5 min incubation at 72 °C. Ten microliters of this reaction mixture was transferred to a second PCR tube containing two 24-base primers (100 pmol each) representing the outermost 5′ sequences of both strands (Figure 1, Amp1 and Amp4). The final 100-μL mixture also contained 2.5 units of the polymerase and 0.25 mM of each dNTP in 60 mM Tris-HCl, 15 mM $(NH_4)_2SO_4$, and 2 mM $MgCl_2$, pH 9.0. The thermal cycling reaction was similar to the previous one except for an annealing temperature of 60 °C. The PCR products were desalted using Millipore 30-kDa MWCO spin filter units and cloned into the high-copy pCRII plasmid (Invitrogen). The ligation products were used to transform *E. coli* XL1-Blue (*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi*

*relA*1 *lac*$^-$ F′[*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(*tet*^{r})]), which was spread on Luria-Bertani (LB) plates supplemented with 50 μg/mL kanamycin, 0.4 mM isopropyl β-D-thiogalactopyranoside (IPTG), 20 μg/mL 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). Plasmids were isolated from the white colonies and screened for the PCR inserts by restriction analysis. The inserts were sequenced on an automated Applied Biosystems 373A instrument using dye termination chemistry.

Expression and Protein Purification. The rusticyanin gene was excised from the hybrid pCRII vector with *Nde*I and *Bam*HI and cloned into the appropriate site of pET24a. The plasmid was amplified in *E. coli* XL1-Blue for sequence verification and used to transform *E. coli* BL21(DE3) strain. The transformed cells were grown at 37 °C in 1 L of LB broth supplemented with 50 μg/mL kanamycin to an OD_{600} 1−1.5, at which time 1 mL of 1 M IPTG was added. The cells were incubated for another 5 h at the same temperature.

The cells were harvested by centrifugation (4000*g*, 15 min, 4 °C) and resuspended in 50 mL of 50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 20 mM β-mercaptoethanol, pH 7.5. 2.5 mL of 10 mg/mL hen lysozyme dissolved in the resuspension buffer and 1 mL of 100 mM phenylmethanesulfonyl fluoride (PMSF) dissolved in methanol were added to the cell suspension which, after gentle mixing, was allowed to sit on ice for 30 min. To the viscous suspension, 250 μL of 1 mg/mL DNaseI and 125 μL of 2 M $MgSO_4$ were added; the mixture was incubated on ice for another 30 min. Sonication of the ice-cold suspension using a Branson 4500 Sonicator lysed any remaining intact cells. The mixture was spun at 20000*g* for 20 min at 4 °C. The supernatant was reserved and the cell debris washed with 15 mL of the resuspension buffer. The resuspended cell debris was spun as before. The wash was pooled with the original supernatant and the pellet was discarded.

The pH of the supernatant was lowered to 6.0 by slowly adding 1 M HCl solution; the precipitate was separated by centrifugation and discarded. The supernatant was further acidified at 1 pH unit intervals to pH 4.0; at each step, the precipitate was separated and discarded. The buffer of the supernatant was exchanged to 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 5.5, using an Amicon ultrafiltration stirred cell fitted with a YM10 membrane. The sample was loaded into a 5-mL HiTrap SP cation-exchange column (Pharmacia) fitted to an FPLC system and fractionated by eluting with a 0−1 M NaCl gradient in 20 mM MES, pH 5.5. The fractions containing aporusticyanin were identified by SDS−PAGE analysis and the development of intense blue color upon addition of an aqueous solution of $CuSO_4$ to small aliquots of the fractions. These fractions were pooled and concentrated. The sample was loaded onto a Millipore Sep Pak C-18 column fitted to a Waters Prep LC4000 HPLC system and eluted with 5−95% acetonitrile gradient in 0.1% aqueous trifluoroacetic acid (TFA) solution. The fractions were examined for purity by isocratic HPLC analysis at 57.2% acetonitrile in 0.1% aqueous TFA solution. Pure fractions of rusticyanin were pooled and lyophilized. The apoprotein was dissolved in deaerated 1 mM H_2SO_4, pH 3.4, to 15 mg/mL concentration and reconstituted by slowly adding 10-fold molar excess of $CuSO_4$. The solution was purged with argon, sealed, and left overnight at room temperature. The extent of Cu(II) incorporation was evaluated on the basis of the A_{280}/A_{597} ratio [~7.01, for 1:1 Cu(II):protein ratio]. The molecular mass and purity of the recombinant protein samples were checked using pneumatically assisted electrospray mass spectrometry (Chait & Kent, 1992).

To prepare uniformly ^{15}N-labeled derivative of the protein, *E. coli* cells harboring the rusticyanin gene were used to inoculate 1 L of M9 minimal broth containing a labeled nitrogen source [2 g/L D-glucose, 1.5 g/L $(^{15}NH_4)_2SO_4$, 100 μM $CaCl_2$, 1 mM $MgCl_2$, 50 μg/mL kanamycin, 6.7 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 1× basal medium eagle vitamin solution (Gibco BRL)]. The cells were grown to an OD_{600} of 1.0−1.5 at 37 °C; at this point IPTG was added to a final concentration of 1 mM. One milliliter of 100 mg/mL rifampicin (United States Biochemical) dissolved in dimethyl sulfoxide was added 30 min after the addition of IPTG. The cells were harvested after 5 h, and the proteins were purified as before.

Protein Mutagenesis. PCR-mediated mutagenesis experiments (Higuchi et al., 1988) were performed using the two 24-mer outermost primers and the following sets of mutagenic oligonucleotides (mutated codons are underlined).

His85Ala

```
5'-           TTT GGT GCT TCT TTC GAT ATC ACT AAA AAG -3'
3'- CT  CCA   AAA CCA CGA AGA AAG CTA TAG TGA         -5'
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Asp73Ala

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5'-               GTA GCT GTA ACC TTC ATT AAC ACC AAC -3'
3'-  G  CGT   TGA CAT CGA CAT TGG AAG TAA TTG         -5'
```

The PCR products were cloned into pCRII, and the sequences were confirmed. The mutant rusticyanin genes were cloned into pET24a plasmid and expressed as before.

Spectroscopy. UV−visible absorption spectra of the recombinant wild-type and mutant rusticyanins were recorded on a Cary 14 dual-beam spectrophotometer modified and interfaced to a Compaq 386SX computer by On Line Instrument Systems, Inc.

EPR spectra of frozen protein samples were collected at 80 K using a Bruker ESP 300 spectrometer equipped with an ER41MR X-band microwave bridge and Scientific Instruments M9300 temperature indicator/controller. Parameters for recording the EPR spectra were typically 16−33 mT/min sweep rate, 1.04 mT modulation amplitude, 9.51 GHz frequency, and 100 μW incident microwave power.

Samples of ^{15}N-labeled wild-type Cu(I)−rusticyanin and Cu(I)−His85Ala variant at pH 3.2 and 3.8 were prepared for NMR spectroscopy as described previously (Hunt et al., 1994). $^1H-^{15}N$ HSQC spectra were recorded on a Bruker AMX500 spectrometer. Data were acquired using the TPPI−States method (Marion *et al.*, 1989), with 2048 points in ω_2 (spectral width 5000 Hz) and 256 transients in ω_1 (spectral width 2057 Hz). The data were processed using Felix 2.05 (Hare Research, Inc.).

Kinetics of Reduction of Recombinant Rusticyanin. Rapid-scan kinetic measurements were performed on the OLIS-RSM stopped-flow spectrophotometer described previously (Blake & Shute, 1994). Purified recombinant rusticyanin was introduced into one syringe of the spectrophotometer. A solution containing soluble iron, either as sulfatoiron(II) or as dicyanobis(1,10-phenanthroline)iron(II) and, when present, iron:rusticyanin oxidoreductase [prepared as described in Blake and Shute (1994)] was introduced into the other syringe. Standard reaction conditions after mixing were 15 μM rusticyanin, 0.2 M sulfate, pH 2.0, and iron:rusticyanin oxidoreductase, when present, 17 μg/mL. The

temperature of the driving syringe was maintained at 25 ± 1 °C by circulating water; room-temperature solutions were allowed to equilibrate for 10 min in the driving syringes. Reactions were initiated by rapidly mixing 0.14 mL of the solutions from each driving syringe. An operational bandwidth of 1.0 nm provided acceptable signal-to-noise characteristics between 400 and 700 nm.

RESULTS

Design and Assembly of the Synthetic Gene. Three different amino acid sequences have been reported for *T. ferrooxidans* rusticyanin (Ronk et al., 1991; Yano et al., 1991; Nunzi et al., 1993); the synthetic gene was based on the primary sequence published by Ronk et al. (1991). The nucleotide sequence was designed with the following guidelines: (1) elimination of palindromic sequences that would lead to stable intramolecular hairpins; (2) low overall G+C content; (3) minimum sequence repeats; and (4) biased use of *E. coli* codons. The first two requirements are essential to ensure high-level transcription of the gene as well as to simplify direct sequencing of the gene, whereas the third criterion is intended to prevent mispriming during the thermal cycling reactions. It is known that in *E. coli* as well as in other organisms, the levels of tRNAs within an isoacceptor family can vary significantly and that the highly expressed genes utilize codons that correspond to those tRNAs present at high concentrations (Ikemura, 1981, 1982; Aota et al., 1988). Thus, a bias in the selection of codons in the synthetic rusticyanin gene is expected to enhance the yields of the expression product. However, in order not to compromise the first three design requirements, the two most commonly used *E. coli* codons were utilized for each amino acid (except Met and Trp), instead of just the best one. This also allows access to a larger tRNA pool during protein synthesis.

Figure 1 shows the optimized sequence of a 516 bp fragment containing the gene corresponding to the rusticyanin amino acid sequence, together with flanking cloning sites. The fragment has an overall G+C composition of only 49.4% and incorporates a number of unique restriction sites for rapid analysis of the synthetic gene. Ninety-five of the 150 codons, excluding AUG (Met) and UGG (Trp), represent the most commonly used codon for the given amino acid. The entire fragment is constructed from eight oligonucleotides which were designed to be of intermediate length (76–93 bases) in order to minimize errors and obtain reasonable yields during solid-phase syntheses. They overlap by 19–22 bases, providing melting temperatures between 62 and 66 °C which are sufficient for high primer specificity.

The initial PCR (Figure 2) involved the assembly of the full-length gene from the eight overlapping oligonucleotiodes present at very low concentrations (40 nM). An aliquot of this reaction mixture is then used for the next PCR reaction, in which the full-length gene is selectively amplified by two primers (1 μM) representing the outermost sequences of both DNA strands. These amplifying primers (Figure 1, Amp1 and Amp4) are short enough (24 bases) to ensure high chemical yield of the full-length oligonucleotide and, at the high concentrations employed in the PCR, prevent any nonspecific hybridization with another primer molecule or with the template. Analysis of the results of the second PCR by agarose gel electrophoresis revealed an intense band of the right size (Figure 3A). Digestion of the PCR product

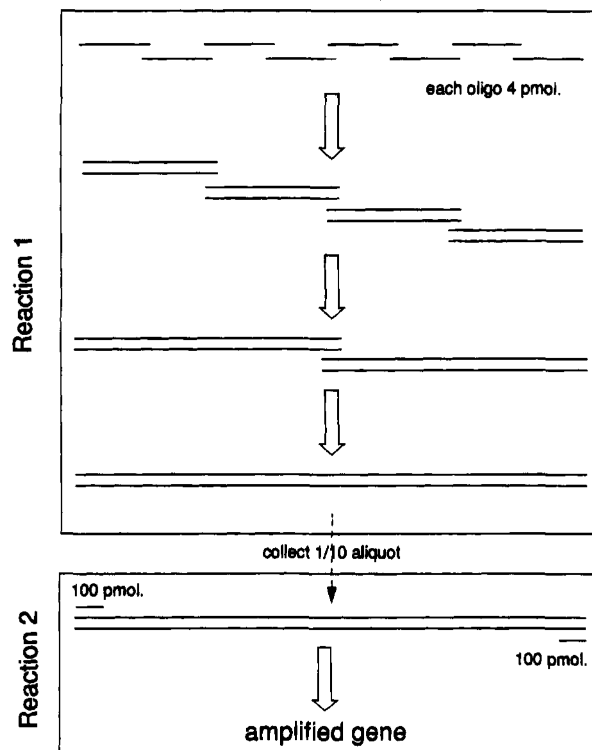

FIGURE 2: PCR-mediated synthesis of the rusticyanin gene. The amounts of the oligonucleotides in the 100-μL reaction mixtures are indicated. Outlined are the events that most efficiently lead to the amplified gene product. In the first cycle of the first reaction tube, a duplex is generated from each pair of adjacent oligonucleotides via polymerase-catalyzed extension from the 3′ ends. The succeeding cycles involve the stepwise integration of adjacent duplexes, ultimately leading to the fully assembled gene. The fragment is then amplified in the second PCR with the Amp1 and Amp4 primers.

with the enzymes cognate to the restriction sites that were built into the sequence suggests that the oligonucleotides have assembled in the intended orientation (not shown).

The PCR product was cloned into a linearized vector (pCRII) with overhanging thymine to complement the unprimed adenines at the 3′ ends of the PCR fragment (Clark, 1988). Hybrid plasmids from 22 clones were isolated and sequenced. While none of these clones gave a completely correct sequence for the 516 bp synthetic insert, two clones were found for which nonidentical halves (the contiguous 1–282 segment in one and 180–516 stretch in another) of the fragment have the proper sequences. To construct the full-length gene, the correct segment from each clone was PCR-amplified with one of two overlapping internal primers (Figure 1, Amp2 and Amp3) and one of the outermost oligonucleotides (Amp1 and Amp4, respectively). These fragments were then fused and amplified by PCR using Amp1 and Amp4. The product was cloned into the pCRII vector and its sequence confirmed.

Protein Expression. The gene was placed behind a hybrid T7/*lac*O_R regulatory sequence (Figure 3B) of pET24a plasmid and transformed into the lysogenic BL21(DE3) strain. The *lac* operator sequence ensures a low basal expression (i.e., prior to induction with IPTG) by physically blocking transcription initiation with a bound *lac* repressor (Dubendorff & Studier, 1991) (Figure 3C, lane 2). Addition of IPTG induces the production of the T7 RNA polymerase and concomitant dissociation of the *lac* repressor from the T7/*lac*O_R region. This leads to the expression of a protein which, on the gel, has an apparent molecular weight

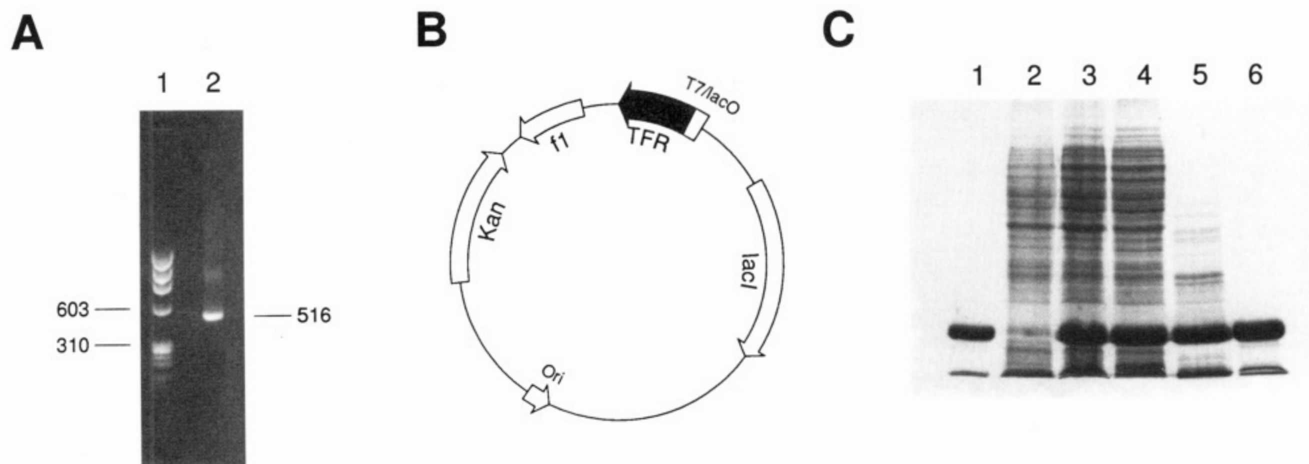

FIGURE 3: (A) Agarose gel analysis of the PCR products (lane 2) from the synthesis of the rusticyanin gene. The MW ladder in lane 1 consists of *Hae*III digests of ϕX174 DNA (New England Biolabs). (B) Map of the pET24a/TFR (TFR, *T. ferrooxidans* rusticyanin) expression vector. The plasmid contains the kanamycin resistance gene for plasmid selection and the T7/*lac*O_R sequence to control expression of TFR. (C) SDS–PAGE analyses of the protein product during expression and subsequent purification. (Lane 1) Rusticyanin extracted from *Thiobacillus*. (Lane 2) Total lysate of *E. coli* BL21(DE3) harboring pET24a/TFR vector before IPTG induction. (Lane 3) Total lysate of BL21(DE3) harboring pET24a/TFR vector after 5 h of induction at 37 °C. (Lane 4) Soluble fraction of the cell lysate of lane 3. (Lane 5) Remaining soluble components after acidification to pH 4. (Lane 6) Rusticyanin after further fractionation by cation-exchange and reverse-phase chromatography.

comparable to that of rusticyanin derived from *Thiobacillus* (Figure 3C, lanes 1 and 3).

The recombinant protein is localized in the soluble fraction of the cell lysate (Figure 3C, lane 4). Because of the intrinsic acid stability of rusticyanin, a preliminary step in which the supernatant is acidified to pH 4 causes a significant fraction of the contaminating components to precipitate (Figure 3C, lane 5). The rusticyanin in the supernatant is further purified by cation-exchange and reverse-phase chromatography. The purity and identity of the protein were confirmed by SDS–PAGE (Figure 3C, lane 6) and electrospray mass spectrometry. The mass spectrum revealed a single component with a molecular mass of 16 554 ± 3 Da corresponding to the isotopically corrected mass of the mature 155 amino acid apoprotein (i.e., after posttranslational cleavage of the initiation methionine residue). Yields of the purified apoprotein ranged from 70 to 100 mg/L of cell culture. Reconstitution with Cu(II) is quantitative at pH 3.4–5.5.

Characterization of the Wild-Type Recombinant Rusticyanin. The electronic absorption (Figure 4A) and EPR spectra (Figure 5A) of the recombinant wild-type protein are indistinguishable from those of the protein extracted from *T. ferrooxidans* (Cox et al., 1978), indicating that the ligands and their geometry around the copper are preserved. ^{15}N HSQC NMR spectra of a uniformly ^{15}N-labeled sample of the recombinant Cu(II)–rusticyanin were collected at two different pHs, 3.2 and 3.8; the amide chemical shifts show no significant changes between these two pHs. The spectrum at pH 3.8 (Figure 6A) is virtually identical to that obtained for the natural material from *T. ferrooxidans* at pH 3.4 (Hunt et al., 1994). This result is a strong indication that the rusticyanins from the two sources have identical tertiary structures, since the chemical shifts, particularly of the amide protons, are extremely sensitive to even slight changes in local environment.

The electron-transfer kinetic properties of the purified recombinant rusticyanin are indistinguishable from those of the native protein from *T. ferrooxidans*. Second-order rate constants for the reduction of recombinant rusticyanin by excess sulfatoiron(II) and dicyanobis(1,10-phenanthroline)-iron(II) are 2.5 M^{-1} s^{-1} and 0.03 μM^{-1} s^{-1}, respectively (data not shown). These values compare favorably with those of 2.3 M^{-1} s^{-1} (Blake & Shute, 1987) and 0.1 μM^{-1} s^{-1} (Blake et al., 1991a), respectively, for the corresponding reactions of the native protein. An even greater indication of functional identity between the two rusticyanins is shown by the results of kinetic studies with an iron:rusticyanin oxidoreductase prepared from cell-free extracts of *T. ferrooxidans* (Figure 7). As for the native protein (Blake & Shute, 1994), the iron-dependent reduction of the recombinant rusticyanin is greatly accelerated in the presence of catalytic quantities of the oxidoreductase; the data points of Figure 7 show results obtained with the recombinant protein, while the corresponding solid curve represents data obtained previously with protein isolated from the natural host. The close correspondence between the two suggests that the protein–protein interactions of the iron:rusticyanin oxidoreductase with rusticyanin obtained from the two different sources are the same.

Characterization of the Mutant Rusticyanins. The yields of both Asp73Ala and His85Ala mutant apoproteins are comparable to that of the wild-type rusticyanin; both are expressed in soluble forms with acid stabilities similar to that of the wild-type apoprotein. The mutations were confirmed by gene sequencing and ion-spray mass spectral analysis of the expression products (Asp73Ala: 16 508 ± 3 Da; His85Ala: 16 485 ± 3 Da). Addition of Cu(II) to the Asp73Ala mutant gives rise to a blue protein with a visible absorption spectrum identical to that of the wild-type protein, indicating that the coordination sphere of the copper(II) has not been perturbed by the substitution at Asp 73. This observation confirms results of EXAFS (Holt et al., 1990) and metal-substitution studies (Strong et al., 1994), which indicate that the copper has no oxygen-containing ligands.

Reconstitution of His85Ala with Cu(II) results in the appearance of a light green colored solution (Figure 4B, dashed line). In the absence of stabilizing anions, the copper site in this mutant, presumably an aquo form, is relatively unstable; a sample at pH 5.5 will typically lose close to 30% of the signal at 618 nm overnight, presumably by loss of

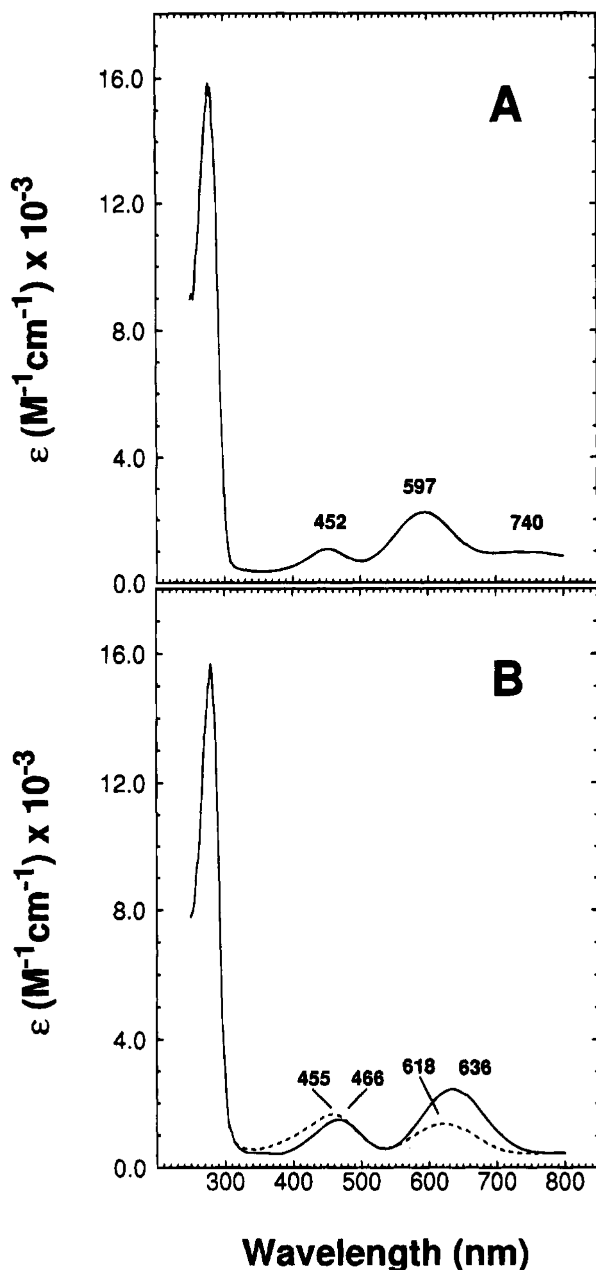

FIGURE 4: Electronic absorption spectra of the recombinant rusticyanins in 10 mM H_2SO_4, pH 3.5, at room temperature. (A) Recombinant wild-type Cu(II)−rusticyanin. Molar extinction coefficients ϵ at 452, 597, and 740 nm are ~1060, 2240, and 960 M^{-1} cm^{-1}, respectively. (B) Cu(II)−His85Ala rusticyanin in the absence (dashed) or presence (solid) of 100 mM NaCl. The ϵ values at the observed maxima, 455, 466, 618, and 636 nm, are ~1650, 1500, 1360, and 2400 M^{-1} cm^{-1}, respectively.

the copper ion from the protein. Titration of a solution of the mutant protein with NaCl causes the color to intensify with concomitant red-shifts in both visible bands (Figure 4B, solid line). Unlike the putative aquo form, the chloro derivative of the Cu(II)−His85Ala protein is extremely stable toward autoreduction and/or metal loss. According to the UV−visible absorption spectrum, the copper site in this derivative remains largely intact between pH 2.0 and 6.8, but subtle differences in the positions and intensities of the two visible absorption bands are observed over this pH range; at higher pH, the protein begins to precipitate. The X-band EPR spectrum of the chloro derivative (Figure 5B) shows a $A_{\parallel}$ (56 × 10^{-4} cm^{-1}) well within the range of values for type 1 sites (Solomon et al., 1992) and a reduction in rhombicity in the $g_{\perp}$ region relative to the wild-type spectrum.

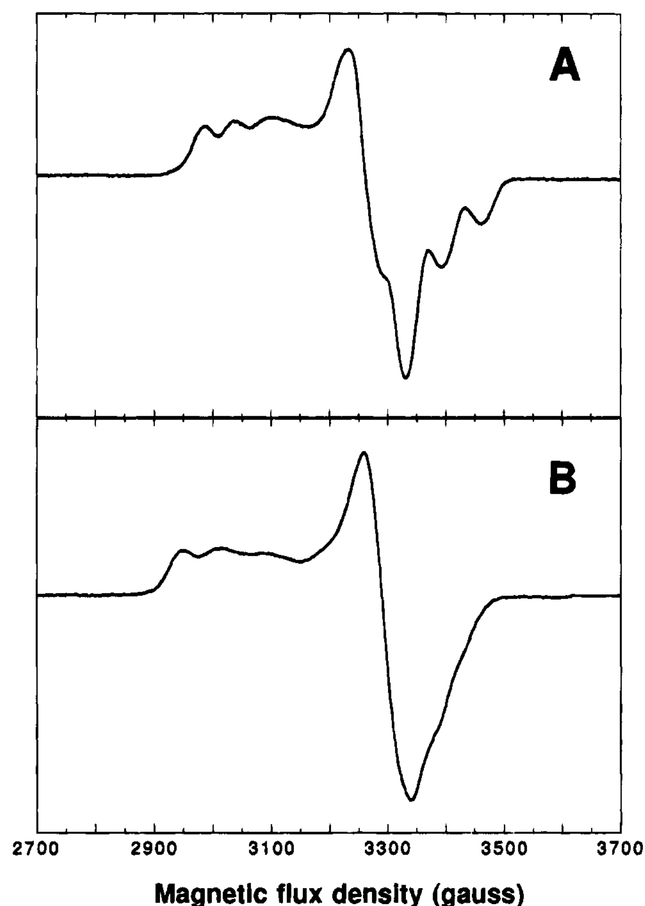

FIGURE 5: X-band EPR spectra of frozen 2 mM solutions (80 K, 9.51 GHz frequency) of the (A) recombinant wild-type Cu(II)−rusticyanin in 0.01 M H_2SO_4, pH 3.0, and (B) Cu(II)−His85Ala rusticyanin in 0.01 M H_2SO_4 and 100 mM NaCl, pH 3.0.

The copper site of the reduced form of the chloro derivative of the mutant is apparently less stable than in the oxidized form, possibly due to an imbalance of charges in the region of the copper site. The ^{15}N HSQC spectrum of chloro derivative of Cu(I)−His85Ala rusticyanin shows evidence of some conformational heterogeneity in solution at pH 3.2; raising the pH from 3.2 to 3.8 reduces this heterogeneity. The ^{15}N HSQC spectrum of the chloro derivative of the His85Ala mutant is shown in Figure 6B. A comparison of the wild-type and mutant spectra (Figure 6A,B) reveals that a number of the strong cross peaks in the mutant spectrum are unchanged from the wild-type, while the remaining cross peaks frequently, though not always, show evidence of heterogeneity. A preliminary mapping of the unchanged strong cross peaks onto the folded structure of rusticyanin previously obtained by NMR (Hunt et al., 1994) reveals a consistent pattern: if these resonances are in fact the same between mutant and wild-type (which must be confirmed by a more complete analysis of the NMR spectra of the mutant), then they belong to residues distant from the copper site. Most strikingly, the unchanged cross peaks are present both in β-strands and loops and in the helical segment, an indication that the heterogeneity that causes the weakening and multiplicity of the remaining cross peaks in the spectrum is a local phenomenon in the region of the copper site, and that the molecule as a whole is correctly folded into the same structure as the wild-type protein. Further examination of the NMR spectra of the His85Ala mutant will require the establishment of solution conditions where the heterogeneity at the active site is

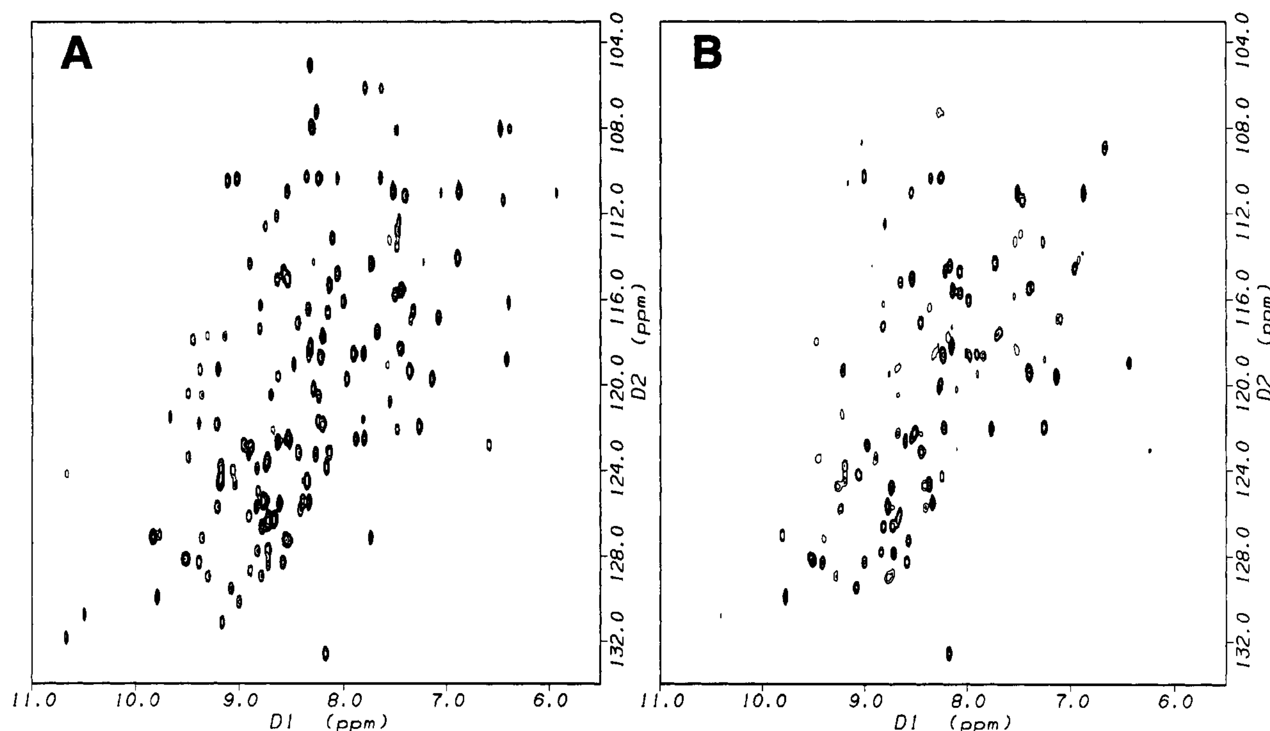

FIGURE 6: 500-MHz 2D ^{15}NH HSQC of (A) 1.5 mM recombinant wild-type Cu(I)−rusticyanin in 1 mM H_2SO_4 and 5 mM ascorbic acid, pH 3.8, and (B) 1.8 mM Cu(I)−His85Ala rusticyanin in 1 mM H_2SO_4, 100 mM NaCl, and 5 mM ascorbic acid, pH 3.8.

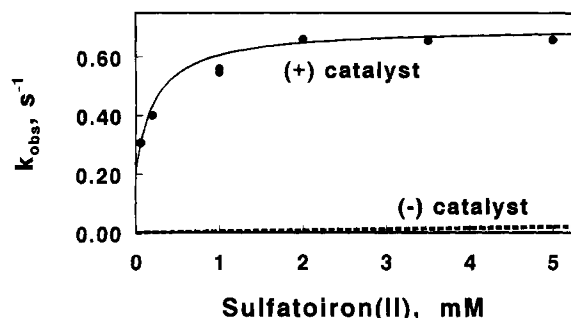

FIGURE 7: Dependence of the pseudo-first-order rate constant for the reduction of recombinant rusticyanin on the concentration of sulfatoiron(II) in the presence [(+) catalyst] and absence [(−) catalyst] of iron:rusticyanin oxidoreductase. The data (circles) in the presence of catalyst were obtained with the recombinant protein, while the associated solid curve represents corresponding kinetic data obtained with the wild-type rusticyanin isolated from *T. ferrooxidans* (Blake & Shute, 1994).

minimized. It appears likely that the heterogeneity in the NMR spectrum arises from changes (slow on the NMR time scale) in copper ligation and/or from loss of the copper ion under these conditions. Work is continuing on the NMR characterization of this protein.

DISCUSSION

Attempts have been made to clone the rusticyanin gene from *T. ferrooxidans*, but this has been largely unsuccessful (R. C. Blake II, unpublished results). The exceptionally long generation times and obligately autotrophic and acidophilic metabolism of the organism contribute to the difficulties of this task. In addition, such a cloned gene would most likely have unusual codon frequencies (Salazar et al., 1989; Rawlings et al., 1991) widely different from those utilized by organisms such as *E. coli* that are commonly used in laboratory expression systems, resulting in low yields of expressed protein. We therefore designed an artificial gene for rusticyanin under high stringency, optimizing the sequence for the codon usage of *E. coli*.

The gene was assembled from eight long, overlapping oligonucleotides using an optimized version of the recursive PCR method (Prodromou & Pearl, 1992) performed in two steps involving assembly of the gene from all eight oligonucleotides present at extremely low concentrations, followed by a second reaction where the assembled fragment is amplified by two short primers representing the 5′ outermost sequences of the double-stranded fragment. This procedure differs from the original protocol of Prodromou and Pearl (1992), where the two long flanking oligonucleotides used to synthesize the gene are likewise employed for fragment amplification. High concentrations of long oligonucleotides are avoided during amplification as these can lead to mispriming events that may not be eliminated at temperatures <72 °C. In contrast, specific hybridization of shorter primers can be controlled within the working temperature range; these primers can also be synthesized with better yields and higher purity. Synthesis of the rusticyanin gene using the modified recursive PCR procedure resulted in a single amplified DNA product of the correct size, attesting to the success of the experimental design.

Rusticyanin is expressed in a stable soluble form in *E. coli*, and a purification scheme involving minimal number of steps provides excellent yields of the apoprotein. Cu(II) reconstitution is typically complete at pH $\leq$ 5.5. The recombinant holoprotein exhibits the same copper electronic structure, pH behavior, and NMR spectrum as the *Thiobacillus*-derived protein. The similarities in the kinetic rates of reduction by inorganic complexes and *T. ferrooxidans* iron:rusticyanin oxidoreductase between rusticyanins from both sources suggest that the recombinant protein has the same microscopic electron-transfer properties (e.g., reduction potential, reorganization energies) (Marcus & Sutin, 1985; Winkler & Gray, 1992) and offers the same surface interac-

tions for the recognition of its redox partners as the protein from the natural host.

It has been suggested from several spectroscopic properties of the oxidized rusticyanin (i.e., high A_{452}/A_{597} ratio and rhombic EPR signal) that the copper in this protein is coordinated to four ligands in a nearly tetrahedral arrangement (Solomon et al., 1992). Three possible ligands (Cys 138, His 143, and Met 148) were identified from sequence alignments with other small blue copper proteins (Ronk et al., 1991; Yano et al., 1991). To identify the fourth ligand in rusticyanin, each of two proposed residues, Asp 73 and His 85, was replaced with alanine. An alanine substitution removes the ligating side chain while retaining the stereochemistry at the C^{α}. Results of these mutagenesis experiments strongly support coordination of His85 to the copper, consistent with the conclusions of earlier NMR assignments (Hunt et al., 1994). Compared to the wild-type protein, the Cu(II)-reconstituted His85Ala mutant has a reduced absorption at 618 nm, causing the protein to appear green. Presumably, a water molecule replaces His 85 in the coordination shell. Similar results were observed when either one of the two histidine copper ligands (His 46 and His 117) in azurin is replaced with glycine (den Blaauwen et al., 1991; den Blaauwen & Canters, 1993; van Pouderoyan & Canters, 1993).

The copper site in the His85Ala protein shows a binding affinity for chloride ion, indicated by changes in the visible spectrum upon addition of NaCl. This observation suggests that a cavity has been created in the copper site by the mutation. In contrast, the copper electronic structures of the wild-type protein and Asp73Ala variant are not altered by the addition of salt. Both visible bands of the chloro derivative of His85Ala protein are strongly red-shifted (466, 636 nm) from those of the wild-type protein (452, 597 nm). These shifts can be rationalized in terms of the relative strengths of the respective ligand fields. The strong 452- and 597-nm bands of the wild-type rusticyanin parallel those observed in mutant superoxide dismutases (Lu et al., 1993; Han et al., 1993) and azurins (den Blaauwen et al., 1993); in these proteins, the high- and low-energy bands have been assigned to pseudo-$\sigma \rightarrow d(x^2-y^2)$ and $\pi p \rightarrow d(x^2-y^2)$ Cys-to-Cu(II) charge transitions, respectively. By replacing a histidine in rusticyanin with a weaker ligand such as a chloride ion, the d−d spacings are reduced (Owen & Booker, 1991); this lowers the energy level of the acceptor metal orbital relative to the Cys orbitals and reduces the associated transition energies.

The EPR spectrum of the chloro derivative reveals a type 1 copper site with reduced rhombicity, probably indicative of the lowered symmetry and greater freedom of movement of the copper as a result of the loss of the histidine ligand and the formation of a cavity in the protein. The identity of the ligand that replaces His 85 in the mutant is not obvious, and it appears likely from the heterogeneity of the HSQC cross peaks in the vicinity of the active site that a number of ligands are utilized. These could include a water molecule, a chloride ion, or a ligand from the protein. For example, there are three additional histidine residues in rusticyanin. However, the fold of the molecule (Hunt et al., 1994) indicates that these are all located far from the copper site. The similarity of the NMR spectra of the mutant and wild-type proteins for parts of the molecule distant from the copper site indicates that there is no large-scale disruption of the folded structure of the protein such as might occur upon coordination with one of the other histidine residues. Although the copper appears to remain type 1, the changes in the visible absorption spectrum probably indicate that the fourth ligand in the mutant is probably not a well-coordinated histidine imidazole group.

Attempts to recover a blue copper spectrum for the His85Ala mutant by adding imidazole to either the putative aquo or chloro derivative at pH 5.5 were unsuccessful; slow titration with the ligand only led to metal loss from the protein site. This result could arise from a "cavity" size that is insufficient to accommodate the ligand and/or inaccessibility of the site from the surface. To date, the reconstitution of cavities at protein metal sites (e.g., azurin, myoglobin, cytochrome *c* peroxidase) with imidazole have been demonstrated only when the histidine ligand has been replaced with a glycine rather than an alanine (Barrick, 1994; McRee et al., 1994; den Blaauwen et al., 1991; den Blaauwen & Canters, 1993; van Pouderoyan & Canters, 1993). This is not surprising since the volume of an alanine methyl group together with an imidazole molecule in a nonbonding interaction exceeds that of a histidine side chain. Derivatives of His85Ala with other small exogenous ligands are currently being explored.

What is the source of the high acid stability of the copper site in rusticyanin? It appears that unusual copper coordination can be ruled out. Our studies establish that rusticyanin has the classical "blue copper" coordination set of $CysHis_2$-Met. The electronic properties of other blue copper proteins such as pseudoazurin and cucumber basic blue protein are nearly identical to those of rusticyanin, yet these proteins are stable only at pHs above 4.5. Furthermore, alteration of a ligand (His85Ala) gives rise to a copper site that remains relatively stable under acidic conditions. The most likely reason for the acid stability of the copper site in rusticyanin is sequestration of the site from solvent by the polypeptide chain of the protein. The global fold of rusticyanin, identified by NMR, has shown a preponderance of loops containing hydrophobic residues in the vicinity of the copper site; such loops could sequester the copper site from the solvent environment and protect the histidine ligands from protonation. Shielding of the copper site in rusticyanin may also be responsible for its remarkably high reduction potential, due to the exclusion of solvent molecules, which preferentially stabilize the oxidized state (net charge +1) by dipole interactions. The effects of solvent and protein dipoles on the reduction potentials of metalloproteins have been illustrated in a number of site-directed mutants of azurin (Pascher et al., 1993) and cytochrome *c* (Louie et al., 1988; Rafferty et al., 1990) and have recently been subject to rigorous theoretical analyses (Langen et al., 1992a,b).

As a result of the work described in this paper, it is now possible to examine directly and systematically the unique properties of rusticyanin. The synthesis of a gene and development of a high-level bacterial expression system open the way to the design and construction of structural variants that will probe the environment around the copper site as well as provide a route to the sizeable amounts of isotopically labeled materials necessary for NMR analyses.

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