Insight into Molecular Stability and Physiological Properties of the Diheme Cytochrome CYC_{41} from the Acidophilic Bacterium *Acidithiobacillus ferrooxidans*

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ABSTRACT: The *cyc1* gene encoding the soluble dihemic cytochrome *c* CYC_{41} from *Acidithiobacillus ferrooxidans*, an acidophilic organism, has been cloned and expressed in *Escherichia coli* as the host organism. The cytochrome was successfully produced and folded only in fermentative conditions: this allowed us to determine the molecular basis of the heme insertion at extreme pH. Point mutations at two sequence positions (E121 and Y63) were introduced near the two hemes in order to assign individual redox potentials to the hemes and to identify the interaction sites with the redox partners, rusticyanin and cytochrome oxidase. Characterization of mutants E121A, Y63A, and Y63F CYC_{41} with biochemical and biophysical techniques were carried out. Substitution of tyrosine 63 by phenylalanine alters the environment of heme B. This result indicates that heme B has the lower redox potential. Interaction studies with the two physiological partners indicate that CYC_{41} functions as an electron wire between RCy and cytochrome oxidase. A specific glutamate residue (E121) located near heme A is directly involved in the interaction with RCy. A docking analysis of CYC_{41}, RCy, and cytochrome oxidase allowed us to propose a model for the complex in agreement with our experimental data.

The acidophilic bacterium *Acidithiobacillus ferrooxidans* is a mesophilic chimiolithotrophic, gamma-proteobacterium that lives at pH 2. It is found in mine drainage and coal wastes and other acidic environments. It is used for industrial recovery of metals. The study of this microorganism is interesting because of its ore-leaching capacity and for a better understanding of its adaptation to such extreme environments. It can obtain all its energy and electron requirements from the oxidation of various forms of reduced sulfur and ferrous iron. It can also reduce sulfur anaerobically, using electrons derived from hydrogen, and it can fix nitrogen and carbon dioxide (*1*, *2*). Several soluble or membrane-bound metalloproteins potentially involved in the electron chain oxidizing ferrous to ferric iron have been isolated and characterized: a type I blue copper protein, the rusticyanin (RCy[1]) (*3*−*6*), two diheme cytochromes c_4 (CYC_{41} and CYC_{42}) (*7*, *8*), a further cytochrome *c* (46 kDa) (*9*, *10*), a HiPIP (*11*), a *bc*1 complex (*12*−*16*), and a cytochrome *c* oxidase (*16*−*20*).

Despite substantial efforts, the electron transfer pathway from Fe^{2+} to O_2 has not been completely elucidated, and several models have been proposed (*2*, *3*, *8*, *10*, *21*−*24*). Genetic and biochemical analyses suggest that at least two *c*-type cytochromes, the rusticyanin and an aa_3-type cytochrome oxidase, are involved in iron oxidation. It has been demonstrated that all seven genes encoding the structural proteins corresponding to these last four redox components are located in a single operon and are cotranscribed (*22*, *23*). Our previous studies showed that the cytochrome c_4 CYC_{41} can interact with the blue copper protein rusticyanine forming a complex. Formation of this complex decreases the electrochemical potential of rusticyanin from +590 mV to +490 mV, allowing efficient electron transfer to the cytochrome (*24*). Spectrophotometric and electrochemical data for the rusticyanin:cytochrome c_4 CYC_{41} complex suggested a critical role of a surface-exposed histidine ligand to the copper (His 143) in the redox tuning effect of rusticyanin. Recently, we have determined the structure of cytochrome CYC_{41} and an interaction model between the two redox proteins has been proposed (*25*). This docking model for the rusticyanin:cytochrome CYC_{41} complex suggested a surface-exposed glutamic acid residue (Glu 121) of cytochrome CYC_{41} to be important in complex formation and electron transfer. Kinetic and spectroscopic data corroborated this model for the CYC_{41}:RCy complex. These experiments have demonstrated that interaction of cations like copper with CYC_{41} quenches the electron transfer from iron to the cytochrome via rusticyanin. We have therefore proposed that the surface where CYC_{41} interacts with RCy is formed by the area surrounding the edge of heme A and involving the exposed E121 residue.

Cytochrome CYC_{41} is also involved in electron transfer to cytochrome oxidase, an inner-membrane protein, that is the physiological terminal oxidase. Two models have been

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[1] Abbreviations: *E. coli*, *Escherichia coli*; kb, kilobase(s); PCR, polymerase chain reaction; RU, resonance units; EPR, electron paramagnetic resonance; CD, circular dichroism; SPR, surface plasmon resonance; RCy, rusticyanin; CYC_{41}, cytochrome c_4 [21 kDa]; CYC_{42}, cytochrome c_4 [26 kDa]; wt, wild-type; Rec, recombinant.

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postulated to describe this last step of the electron chain (*25*). The first one stipulates that CYC_{41} functions as an electron wire, with one heme accepting electrons from the RCy and the other handing them on to cytochrome oxidase. The second model views the diheme CYC_{41} as a two-electron storage device and proposes that both reduction and reoxidation of CYC_{41} proceed through the same heme. We have overexpressed CYC_{41} in *Escherichia coli* and performed site-directed mutagenesis in the vicinity of the two hemes in order to (i) attribute the redox potentials to the individual heme, (ii) test the interaction of the CYC_{41} with its two partners, (iii) determine the interaction site, and decide between these models. A docking model of the rusticyanin:cytochrome CYC_{41}−cytochrome *c* oxidase complex was performed in order to identify the key residues on the CYC_{41} cytochrome involved in the electron transfer with the cytochrome oxidase. The most promising residue, Tyr 63, was targeted for mutational studies. The molecular basis of the heme insertion at extreme acidic pH is also investigated.

EXPERIMENTAL PROCEDURES

Strains, Vectors, and Media. We used the *E. coli* MC1061 strain transformed with pEC86 containing the cytochrome *c* maturation *ccm* genes (*26*−*28*). Growth of this *E. coli* strain in aerobic and fermentative conditions was carried out in LB medium (*28*), supplemented with the antibiotics ampicillin and chloramphenicol at a final concentration of 0.27 mM and 31 μM, respectively.

Cells from *Acidithiobacillus ferrooxidans*, kindly supplied by Dr. D. Morin (Bureau des Recherches Géologiques et Minières, Orléans, France), were used for genomic DNA preparation. This bacterium was isolated from drainage water at the Salsigne sulfur mine (France). It was grown at pH 1.6 in 9 K Silverman and Lundgreen medium supplemented with 1.6 mM $CuSO_4 \cdot 5H_2O$. Large-scale cultivation of the organism was performed in 300 L of the above medium with a homemade polypropylene fermenter. Typical yields were 80 mg/L.

Biochemical Reagents. All restriction enzymes were obtained from Appligene. PCR was carried out using Taq polymerase from Promega. Cloning oligonucleotides and DNA sequencing were purchased from PROLIGO primers and probes and Genome Express, respectively.

Cloning of the cyc1 Gene. The *cyc1* gene that encodes cytochrome CYC_{41} was amplified from *A. ferrooxidans* by PCR using oligonucleotides Nter2.*cyc1* (5′-AATTCATGACGACATACTTAAGCCAAGAC-3′) and Cter2.*cyc1* (5′-GCAAGCTTTTCAGCGATGAAGATGAAAGATAAGCCGC-3′), which introduced an *Eco*RI site immediately upstream from the start codon and a *Hin*dIII site downstream from the stop codon. The 693 pb obtained by PCR was doubly digested by *Eco*RI−*Hin*dIII and subcloned into pJF119EH, cut with the same enzymes, to obtain $pJF119_{CYC1}$. The sequence of the insert in the resulting plasmid was verified.

Site-Directed Mutagenesis. To replace Glu 121 by Ala, and Tyr 63 by Ala and Phe, mutations of the *cyc1* gene were introduced by PCR (*29*). Oligonucleotides c4c+ (5′-CCTGCCTGTATGGCATGCCACGGATCG-3′) and c4g− (5′-CGATCCGTGGCATGCCATACAGGCAGG-3′) were used to replace Glu 121 by Ala. Plasmid $pJF119_{CYC41}$ was used as template in the PCR reaction. The mutated insert of 693 pb was then digested by *Eco*RI and *Hin*dIII and subcloned in pJF119EH previously cut with the same enzymes to obtain pJF119E121A. The mutation was checked by DNA sequencing. In the same way, oligonucleotides YA+ (5′-GAATGGCGAAATCGCCATGTGGCCCGTG-3′) and YA− (5′-CACGGGCCACATGGCGATTTCGCCATTC-3′) and oligonucleotides YF+ (5′-GAATGGCGAAATCTTTATGTGGCCGTG-3′) and YF− (5′-CACGGGCCACATAAAGATTTCGCCATTC-3′) were designed to replace Tyr-63 by Ala 63 or Phe 63, respectively. The same protocol as described above was used to obtain pJF119Y63A and pJF119Y63F.

Protein Overproduction and Purification. For purification of cytochromes, 100 L cultures of recombinant *E. coli* MC1061 were grown at 37 °C in aerobic and fermentative conditions until mid-log phase and then induced with 40 μM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Typical yields were in the range of 1.6 g of cell material per liter of culture in aerobic conditions, and 1.3 g in fermentative conditions. Cells were harvested by centrifugation at 3000*g* for 30 min. Periplasmic extraction was performed as described previously (*30*). After concentration on a JUMBOSEP STARTER KIT 10K (Pall Filtron) and dialysis against 20 mM ammonium acetate buffer pH 4.6, cytochromes CYC_{41} (Recombinant, E121A, Y63A and Y63F) were purified as described in (*7*).

Purification of Wild-Type Cytochromes c_4 CYC_{41} and CYC_{42}, Cytochrome [46 kDa], Rusticyanin, and Cytochrome Oxidase from A. ferrooxidans. Cytochromes CYC_{41}, CYC_{42}, and rusticyanin were purified as previously described (*7*, *8*). Cytochrome [46 kDa] has been purified as described in ref *9*. Cytochrome oxidase from *A. ferrooxidans* was kindly supplied by D. Lemesle-Meunier (Bioénergétique et Ingénérie des Protéines, CNRS, Marseilles, France).

Protein Sequencing and Molecular Mass Determination. N-Terminal determinations were performed with an Applied Biosystems A470 gas-phase sequencer. Quantitative determination of phenylthiohydantoin was done by high-pressure liquid chromatography (Waters) monitored by a data and chromatography control station (Waters model 840).

MALDI-MS was performed on a reflectron time-of-flight mass spectrometer equipped with delayed extraction (Voyager DE-RP, Perspective Biosystem Inc). The sample (0.7 μL) was directly mixed on the support with an equal volume of matrix (saturated solution of sinapinic acid in 40% acetonitrile and 60% water made 0.1% in trifluoroacetic acid).

EPR Spectrophotometry. EPR spectra were recorded on a Bruker ESP300e X-band spectrometer fitted with an Oxford instruments liquid helium cryostat and temperature control system at 15 K. These EPR conditions are partially saturating for the copper centers resulting in slight broadening of the individual lines while retaining the global aspect of the spectrum. Measuring at 15 K was chosen to simultaneously monitor heme and copper centers. The spectral region above $g = 2$ in Figure 3B was contaminated by adventitious copper. This signal of free copper was subtracted, and the respective full spectra are shown as Supporting Information. All experiments were performed with 10 μM concentrations of proteins in ammonium acetate 20 mM buffer, pH 4.6. Complete oxidations of the samples were achieved by addition of iridium chloride followed by removal of the oxidant via passage through a PD-10 column.

Electrochemical Technique. Cyclic voltammetry (CV) and square-wave voltammetry (SWV) measurements were carried out using an EG&G 273 potentiostat modulated by EG&G PAR M 270/250 software. The CV scan rate generally was 20 mV s^{-1} . SWV were obtained using 5 Hz as the square-wave frequency, 2 mV as the scan increment, and 25 mV as the pulse height amplitude. A conventional three-electrode system was used in 20 mM acetate ammonium pH 4.6 buffer. Measurements were made at room temperature and required the use of gold electrodes pretreated for 1 min with 1 mM bis(4-pyridyl) disulfide solution (*31*). The reference electrode was a Metrohm Ag/AgCl/satured NaCl electrode. The auxiliary electrode was a gold wire.

Circular Dichroism. CD spectra were recorded on a Jasco J-715 spectropolarimeter equipped with a Peltier-type temperature control system (model PTC-348WI) between 390 and 450 nm (Soret band) and 190−250 nm, at 0.2 nm/min at 25 °C and were averaged from three independent acquisitions. Mean ellipticity values per residue [θ] were calculated as $[\theta] = 3300m\Delta A/(lcn)$ where l (path length) = 0.1 cm, n = number of residues, m = molecular mass in daltons, and c = protein concentration in mg/mL. Protein concentration was 0.05 mg/mL or 1 mg/mL in ammonium acetate buffer pH 4.6 for experiments performed in the far-UV region and in the Soret band, respectively. Baselines of spectra were corrected by subtraction of the baseline corresponding to the spectrum of the buffer. The α-helical content was derived from the ellipticity at 220 nm as described in ref *32*.

Surface Plasmon Resonance (SPR) Binding Experiments. The interaction between rusticyanin and cytochrome CYC_{41} (wt, recombinant, E121A, Y63F) from *A. ferrooxidans* was investigated with a biomolecular interaction analysis biosensor based analytical system (BIAcore). All experiments were done at pH 4.6 to maintain the physiological ionization state of rusticyanin and cytochromes and to enable interaction between proteins. Rusticyanin was immobilized on a CM5 sensor chip (BIAcore) in 10 mM MES buffer (pH 6.5) by amine coupling using the amine coupling kit supplied by BIAcore. Rusticyanin injection resulted in 500 resonance units (1000 RU equivalent to a change of about 1 mg/mm^2 in surface protein concentration). A range of protein (analyte) concentration in ammonium acetate 100 mM pH 4.6 buffer was used to determine the apparent dissociation constant. In each run, 100 μL of the analyte was injected at 30 μL/min to record the association phase to the flow cells, after which 110 μL of buffer was injected at 30 μL/min to record the dissociation phase. Global fittings of the exponential curves (sensorgrams) giving both k_{on} and k_{off} values, and the response at the equilibrium (R_{eq}) in RU was performed using the BIAeval software. The kinetic data are interpreted on the basis of the simple binding model L + A → LA where L denotes mobile ligand and A immobilized receptor. Sensorgrams were fitted by the equation $r_0/(k_s)\{1 - \exp[-(k_s)(t - t_0)]\}$ where r_0/k_s corresponds to R_{eq}, the maximal signal at the equilibrium of the reaction. The response at equilibrium R_{eq} was plotted as a function of the analyte (cytochrome) concentration. The experimental points were fitted to the Langmuir equation $Y = Y_{max}[cyt]/(K_d + [cyt])$.

Control experiments were done on sensorchip in the absence of immobilized rusticyanin.

Electron Transfer between Rusticyanin and Cytochrome CYC_{41}. The reduction rate of cytochrome CYC_{41} by the rusticyanin was determined by following changes in the cytochrome absorbance at 552 nm in a stopped-flow spectrophotometer (Hi-Tech Scientific) at room temperature. 6 μM cytochrome c_4 was oxidized by a stoichiometric amount of potassium hexachloroiridate(IV) and added to one syringe of the stopped-flow spectrophotometer. 20 μM rusticyanin and 5 mM ferrous chloride were added to the other. Results were analyzed with SigmaPlot.

Kinetics of Cytochrome Oxidation by Cytochrome Oxidase from A. ferrooxidans. Spectrophotometric measurements were performed on a Cary 50 bio spectrophotometer (Varian). The activity of cytochrome *c* oxidase was followed spectrophotometrically via the oxidation of cytochromes (CYC_{41}, CYC_{42}, Rec CYC_{41}, and mutants CYC_{41}) reduced by sodium ascorbate prior to the experiment. Measurements were performed with cytochrome concentrations ranging from 1 to 60 μM and with active cytochrome oxidase at a concentration of 0.048 μM in ammonium acetate 20 mM pH 4.6 buffer. The cytochrome oxidation rate was determined on the basis of the absorption band at 552 nm and by using the slope of the tangent drawn at the beginning of the recorded trace. Results were fitted to the Michaelis−Menten equation using SigmaPlot.

RESULTS

Heterologous Synthesis and Characterization of the CYC_{41} Cytochrome in E. coli. CYC_{41} was first synthesized in *E. coli* under aerobic and pH-controlled conditions. We used the strain MC1061 containing the plasmid pEC86 that carries the *c*-type cytochrome-maturation *ccm* genes and a controllable strong *tac* promoter, which improves production of cytochromes *c* (*26*, *28*, *33*). Purification gave 5 mg of pure recombinant cytochrome c_4 with a single band on SDS−polyacrylamide gels. The N-terminal sequence was identical to that of the native protein purified from *A. ferrooxidans*, showing that the signal sequence was correctly cleaved in *E. coli* during the transport of the protein into the periplasm. The UV−visible spectra of the recombinant protein showed the presence of a Soret band at 407 nm and a band at 620 nm in the oxidized state (Figure 1A). Typically, this band is observed in pentacoordinated hemes giving rise to a high-spin spectrum (*34*). In the dithionite-reduced form, this band disappeared and the α, β, and γ bands appeared at 552, 523, and 417 nm, respectively. In the reduced form, the Soret band of the recombinant enzyme differed from that obtained with the wild-type protein (Figure 1). CD spectra in the Soret absorption region, which reflect the asymmetric environment of the heme groups, showed corresponding differences from the wild-type protein in the untreated preparation as well as in the reduced state. The aerobically expressed recombinant protein showed a reduction of the negative Cotton effect (*35*−*37*), but the Soret spectrum was different from that of a partially unfolded cytochrome (Figure 2B). CD spectra in the far-UV region, which reflect the macrostate of the protein, showed characteristic α helix content with troughs at 222 and 208 nm (*38*). The similarity of the two CD spectra (wild-type/recombinant) demonstrates a global fold (secondary structure), which is similar for the recombinant and the wild-type cytochrome (Figure 2A, Table 1). The EPR spectra of recombinant cyt CYC_{41} (Figure 3) revealed modifications also in the region of heme g_z peaks. As compared to wild-type CYC_{41}, peak positions were shifted and the higher redox

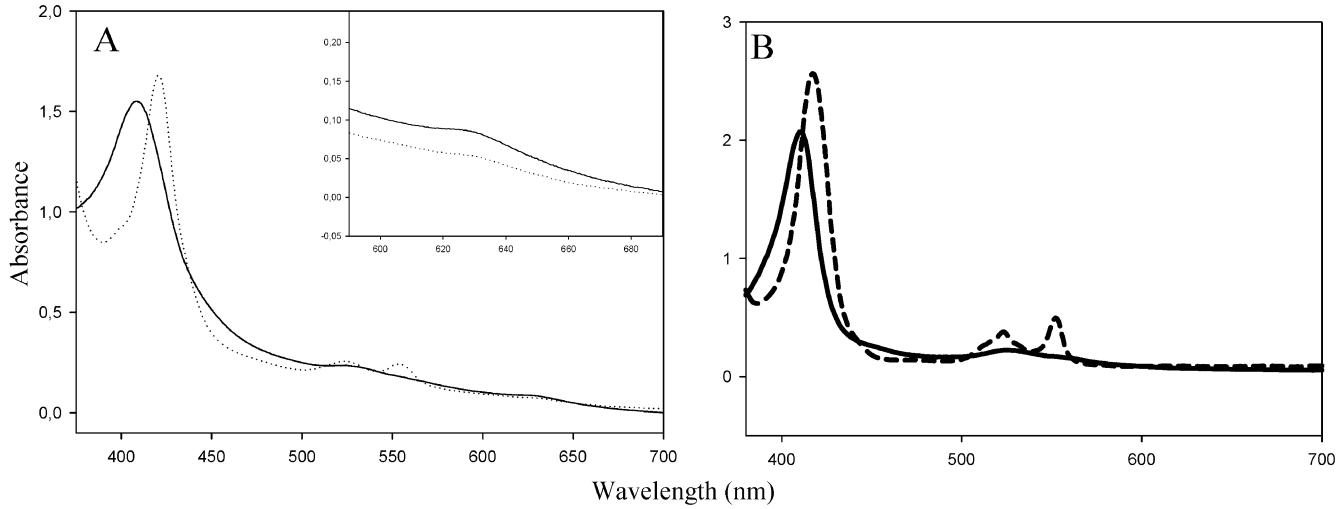

FIGURE 1: Visible spectrum of (A) purified recombinant of CYC_{41} in aerobic and pH regulated conditions and (B) wild-type CYC_{41} purified from *A. ferrooxidans*: (—) oxidized state; (- - -) reduced by dithionite. Inset: Magnification of the 620 nm band region.

potential component corresponded to the heme with the lower g_z value rather than the reverse. Denaturation experiments by heating or by treatment with guanidine hydrochloride and refolding under acidic conditions did not produce the native heme environment (data not shown). All these results showed a modification of the heme environment when the protein was expressed under conventional growth conditions for *E. coli*. Various cytochromes have been successfully overproduced in *E. coli* under aerobic conditions, however, and in the case of acidophilic proteins, the external pH and consequently the pH of the periplasm where heme maturation occurs are very important. In *E. coli*, periplasmic proteins are folded in a neutral environment with a pH around 7 (*39*). In the acidophilic bacterium *A. ferrooxidans*, periplasmic proteins have to fold at pH around 2. To test the role of the external pH on the folding of cytochrome CYC_{41} we grew *E. coli* strain MC1061 under fermentative conditions. Under these conditions, the pH at the end of growth falls to about 5.5 in line with an anaerobic acid regulation of the fermentation genes. At this pH, our previous studies did not detect modifications of the physicochemical properties of the wild-type cytochrome c_4 (*25*). Purification gave 3 mg of pure recombinant CYC_{41}, with a purity coefficient [$A553_{red} - A570_{red}/A280_{ox}$] of 1.1. Recombinant and wild-type cytochromes CYC_{41} have the same isoelectric point. N-terminal sequences (up to residue 20) were found to be identical to that of the native protein. The UV−visible, CD and EPR (Figures 2, 3) spectra as well as the redox properties are similar to those obtained with the wild-type cytochrome CYC_{41} (Table 1).

Expression and Characterization of Mutants of CYC_{41}. Purification gave 1.5 mg of pure mutant cytochrome CYC_{41} E121A, Y63A, and Y63F. Mutant cytochromes CYC_{41} have the same isoelectric point as wild-type cytochrome CYC_{41} within experimental error. The N-terminal sequences (up to residue 15) were found to be identical to that of the native protein. Mutant cytochromes CYC_{41} were found to be pure with a purity coefficient [$A553_{red} - A570_{red}/A280_{ox}$] of 1.1. The UV−visible spectra showed no difference from that of the wild-type cytochrome CYC_{41}.

The reconstructed mass spectra of E121A and Y63F cytochromes CYC_{41} gave only one peak each, corresponding to molecular masses of 21131 Da for E121A and 21161 Da for Y63F, respectively, consistent with the molecular masses calculated from the E/A and Y/F replacement (Table 1).

Figure 2 shows the CD spectra in the Soret region of wild-type cytochrome c_4, its recombinant in *E. coli*, and its E121A, Y63A, and Y63F derivatives. The CD spectrum of E121A is almost identical, and therefore the expression in a neutrophilic bacterium under fermentative conditions as well as the mutation did not affect the native secondary structure of the protein and the local surroundings of the hemes. In contrast, mutation in the vicinity of heme B induced modifications even when the aromatic environment is conserved (Y/F). However, the integrity of the cytochrome is not affected and these mutants were used to assay the interaction with the redox partners.

Figure 3 shows a comparison of heme g_z peaks obtained on the wild-type protein as well as on the various *E. coli* expression products. As mentioned above, the recombinant protein purified from conventionally grown *E. coli* host cells (i.e. at neutral pH) yielded EPR characteristics drastically different from those of wild-type protein, whereas the recombinant protein recovered from fermenting *E. coli* showed the correct spectrum. The same was true for the E121A mutant, which yielded an EPR spectrum similar to that of the wild type, as well as the E_m values resembling those of the wild type (see below). Surprisingly, no g_z peaks could be discerned in the spectrum recorded on the Y63A mutant. This mutant, however, contained two heme groups as seen by optical and CD spectroscopy as well as by heme content analysis. It therefore seems likely that it exists in a range of conformations, thus broadening the individual g_z peak(s) beyond detection. The Y63F mutant, by contrast, yielded a well-resolved spectrum in the region of low-spin g_z peaks. Althoughs in this mutant the higher g_z value signal is unperturbed, the peak at the lower g_z value is shifted toward higher magnetic field. This indicates that the $g_z = 3.1$ line arises from heme B (as defined in the structure) and also demonstrates that in wild-type protein heme B has the lower redox midpoint potential.

The heme redox potentials in E121A, Y63A, and Y63F cytochromes were determined by electrochemical techniques. Redox-potential data, measured as SWV peak potentials, are

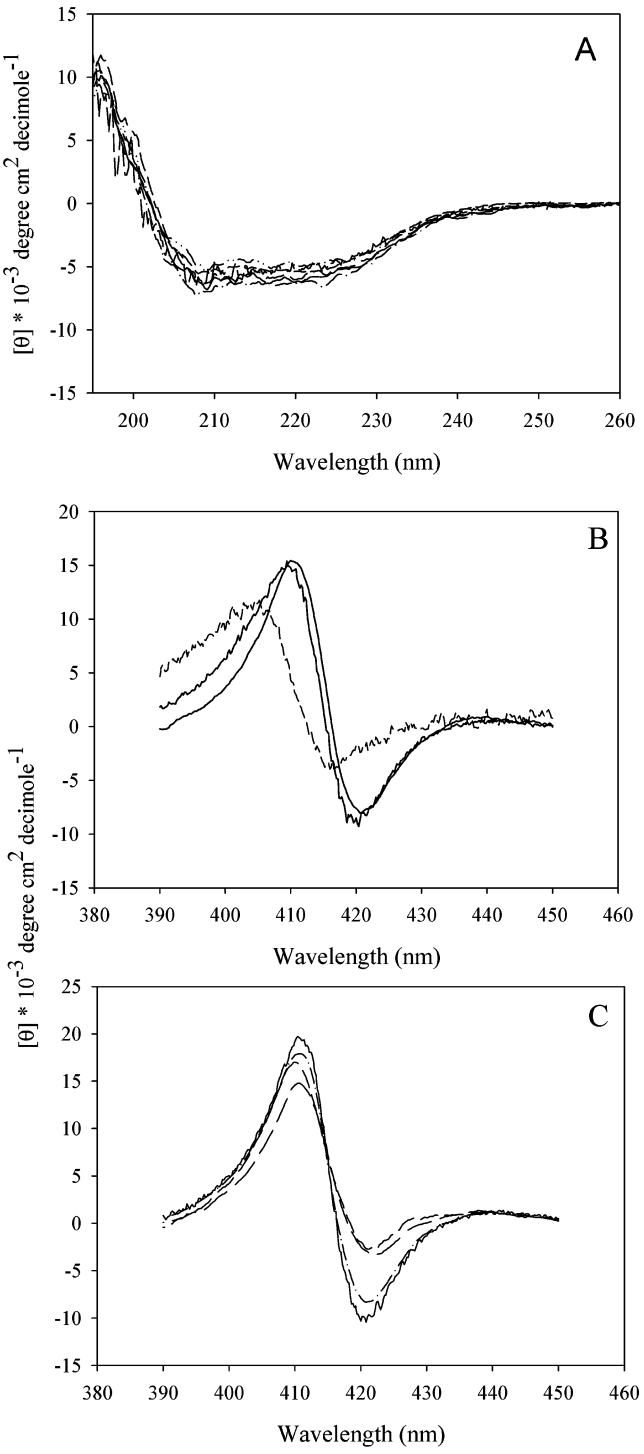

FIGURE 2: Circular dichroism of wild-type, recombinant, and mutant forms of *Acidithiobacillus ferrooxidans* CYC_{41} pH 4.6, 25 °C. (A) Far-UV spectra: (—) wild type; (- -) recombinant CYC_{41} obtained in aerobic and pH regulated conditions; (— • •) recombinant CYC_{41} obtained in fermentative conditions; (— •) E121A and (— —) Y63F mutated CYC_{41} in fermentative conditions. (B) Soret spectra of "as prep": (—) wild type; (- -) recombinant CYC_{41} obtained in aerobic and pH regulated conditions; (— • •) recombinant CYC_{41} obtained in fermentative conditions. (C) Soret spectra in reduced state of (—) wild type; (— •) E121A, (— —)Y63F, and (— —) Y63A mutated CYC_{41} obtained in fermentative conditions.

summarized in Table 1. The E121A mutant had the same electrochemical behavior as wild-type and recombinant CYC_{41}, with two electrochemical waves at +350 and +460 mV, respectively. The Y63A mutant gave a significantly weaker electrochemical signal than all other samples. Furthermore, the intensity of the lower potential redox transition was greatly reduced with respect to the higher potential one. In the SWV measurements, the lower potential transition was only visible as a shoulder and its exact value was therefore difficult to evaluate. Within the limits of precision the E_m values of both hemes do not deviate much from those of the wild type. However, taken together with the EPR results described above, the electrochemical data indicate a destabilization of the native structure and possibly the presence of conformational heterogeneity. In the Y63F mutant only one reversible electrochemical process is observed with an apparent midpoint potential of +410 mV. As the EPR data show that two distinct hemes are present, we interpret this wave as the superposition of two unresolved transitions. Furthermore the EPR results demonstrate that it is the lower potential (lower g value) heme that is affected by the Y63F substitution, and it seems likely, therefore, that the convoluted wave arises from a significantly upshifted potential of heme B (>60 mV) and a smaller decrease of the potential of heme A by about 30 mV. The removal of the partial negative charge induced by the Tyr to Phe substitution is in line with a higher redox potential of the neighboring heme.

Study of Rusticyanin−Cytochrome c_4 Complex by EPR. We have previously reported that complex formation between RCy and CYC_{41} alters optical and EPR parameters of the copper site in rusticyanin (*24*). Addition of a stoichiometric amount of oxidized cytochrome CYC_{41} to the sample containing rusticyanin resulted in dramatically modified EPR spectral properties (*24*), involving an increase in $g_{||}$ as well as modifications in apparent hyperfine parameters on all g values. These spectral modifications are rationalized by a redistribution of the unpaired electron density between the copper and its ligands upon complex formation (Figure 3). We have analyzed the EPR spectra of rusticyanin in the presence of recombinant CYC_{41}, E121A, and Y63F. Figure 3 (right panel) shows the high-field region of the $g_\perp$ transition of the RCy copper signal, which is strongly indicative of the presence or absence of interaction. Free RCy shows strong hyperfine lines in this region which collapse when RCy is engaged in the electron transfer complex with CYC_{41} (*24*). For CYC_{41} rec and the Y63F mutant, the absence of the hyperfine lines demonstrates that the RCy:CYC_{41} complex has been formed. By contrast, the presence of these hyperfine signals (i.e. similar to an unmodified free RCy spectrum) when RCy was mixed with the E121A mutant of CYC_{41} shows that this CYC_{41} mutant is not able to form a complex with Rcy in the same way as wild-type CYC_{41}. These results (a) demonstrate the loss of a specific interaction between the two proteins upon removal of the charge in position 121 on CYC_{41}, (b) corroborate the role of the negatively charged E121 in the electronic changes at the copper site (most probably, via affecting the pK value of the proton bound to the ϵ-nitrogen of the copper ligand His 183 (*24*)), and (c) argue against the presence of further interaction sites between CYC_{41} and rusticyanin inducing redox modifications.

Study of the Interaction between Rusticyanin and CYC_{41} by SPR. EPR experiments have suggested that E121 plays a role in forming the complex for electron transfer. We therefore performed SPR experiments to evaluate the implication of E121 in the complex formation between CYC_{41}

Table 1: Characteristics of Wild-Type, Recombinant, and Mutated CYC_{41}

	molecular mass (Da)	λ_{max} (nm) oxidized	λ_{max} (nm) reduced	CD λ_{max} (nm)	redox potential (mV)
WT	21188 ± 7	411	417, 523, 552	410, 420	+350 ± 10, +460 ± 10
Rec cyt c_4	21183 ± 7	411	417, 523, 552	410, 420	+355 ± 10, +460 ± 10
E121A	21131 ± 9	411	417, 523, 552	420.2, 410.2	+350 ± 10, +460 ± 10
Y63A	21096 ± 7	411	417, 523, 552	423, 410.6	+350 ± 10, +460 ± 10
Y63F	21161 ± 9	411	417, 523, 552	423, 410	+ 410 ± 10

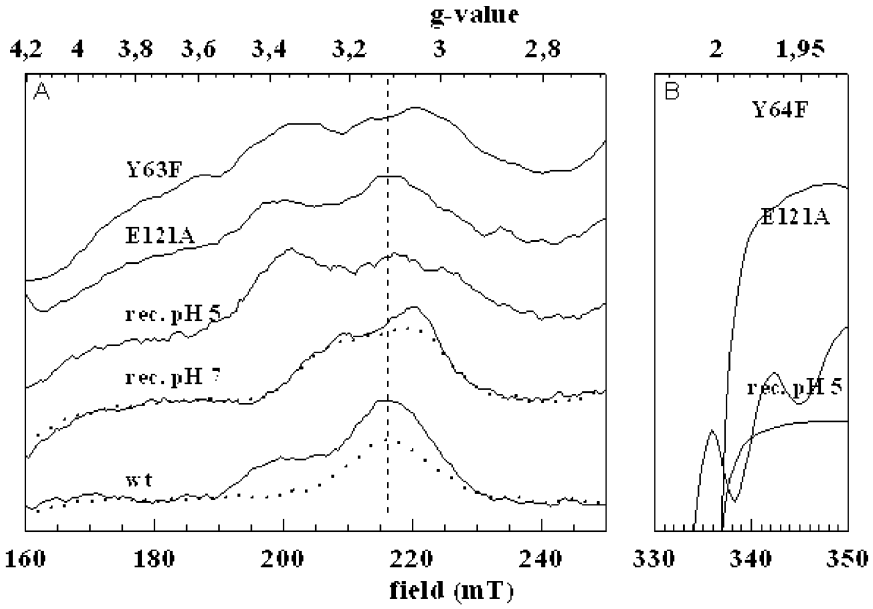

FIGURE 3: EPR spectra recorded on CYC_{41} cytochromes (A) and on the RCy:CYC_{41} complex (B) in the oxidized state. (A) The spectra have been obtained on wild-type (wt), recombinant protein expressed at pH 7 and at pH 5 (rec pH 7 and rec pH 5, respectively), the E121A and the Y64F mutants. The dotted lines correspond to the ascorbate reduced samples rec pH 7 and wt, respectively. (B) Spectra of oxidized RCy after addition of recombinant or mutated CYC_{41}. The spectra represent the high-field flank of RCy's $g_{||}$ where hyperfine lines characteristic of the absence/presence of the RCy:CYC_{41} complex are observed. Instrument settings: microwave frequency, 9.42 GHz; microwave power, 6.3 mW; modulation amplitude, 3.2 mT (left panel) or 1.6 mT (right panel); temperature, 15 K.

and rusticyanin. SPR studies allowed us to determine the apparent dissociation constant (K_d) of the complexes formed by rusticyanin and wild-type, recombinant, or mutated cytochrome c_4. Figure 4 shows binding isotherms, and Table 2 summarizes the apparent dissociation constants. Comparable apparent dissociation constants were obtained for the wild-type, recombinant, and Y63F cytochromes (0.5×10^{-6} M). A higher K_d (3.6×10^{-6} M) was measured for the E121A cytochrome c_4. These experiments show that interaction between rusticyanin and E121A is impaired and thus confirm the involvement of this residue not only in the redox modifications induced in rusticyanin upon complex formation but also in the association between the CYC_{41} and rusticyanin itself.

Electron Transfer between CYC_{41} and Rusticyanin. Formation of the complex between RCy and CYC_{41}, as observed by EPR and SPR, is indeed strongly affected by the E121A mutation, whereas Y63F does not induce any perturbations. We have therefore studied the effect of the corresponding mutations on the electron transfer reaction itself. As described previously (*24*), electron transfer between Fe(II) and CYC_{41} is dependent on the presence of RCy. This electron transfer reaction was inhibited by cations. Figure 5 shows the effect of the mutation in the vicinity of heme A (E121A) on the CYC_{41} reduction by RCy and Fe(II), as monitored by stopped-flow spectroscopy. The first obvious result of this experiment is that recombinant CYC_{41} is reduced with

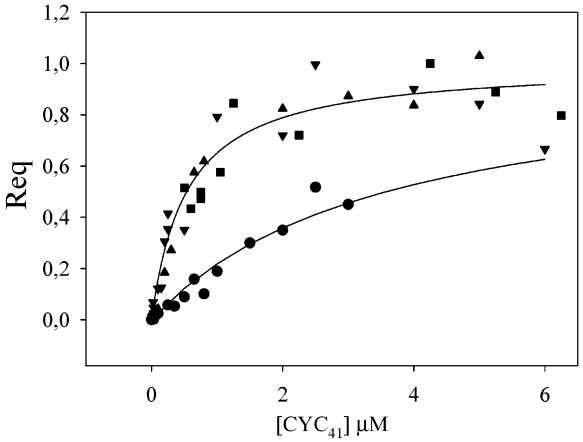

FIGURE 4: SPR binding experiments. The response at equilibrium R_{eq} (in resonance units (RU)) was reported as a function of the analyte concentration. The errors on the estimated parameters (R_{eq}) were less than 10% in all cases. The experimental points were fitted to a hyperbola $R_{eq} = R_{max}([cyt])/(K_d + [cyt])$. Scatchard plots ($R_{eq}$/[cyt] versus R_{eq}) were also used, and the slope gave the same estimations (data not shown). RCy was used as a ligand, and CYC_{41} (▼), recombinant CYC_{41} (▲), E121A CYC_{41} (●), and Y63F CYC_{41}- (■) were used as analyte.

kinetics similar to those of wild-type CYC_{41}. This result confirms the similarity in the heme environments of the two proteins. A similar result was obtained with the Y63F mutant. As shown above, heme B is not implicated in the interaction

Table 2: Thermodynamic Parameters of the RCy/CYC_{41} Complex and Kinetic Parameters of the CYC_{41}/Cytochrome Oxidase Complex by Surface Plasmon Resonance and Steady State Kinetics, Respectively

	CYC_{41}/RCy	CYC_{41}/cytochrome oxidase	
	K_D (μM)	K_m (μM)	k_{cat} (s^{-1})
CYC_{41}			
wt	0.53 ± 0.07	6.26 ± 0.4	11.57 ± 2.2
rec	0.62 ± 0.13	6 ± 0.8	11 ± 1.8
E121A	3.58 ± 0.19	8.21 ± 2	11.25 ± 2.3
Y63F	0.68 ± 0.16		1.6 ± 1
CYC_{42}		15.04 ± 5	1.21 ± 0.24

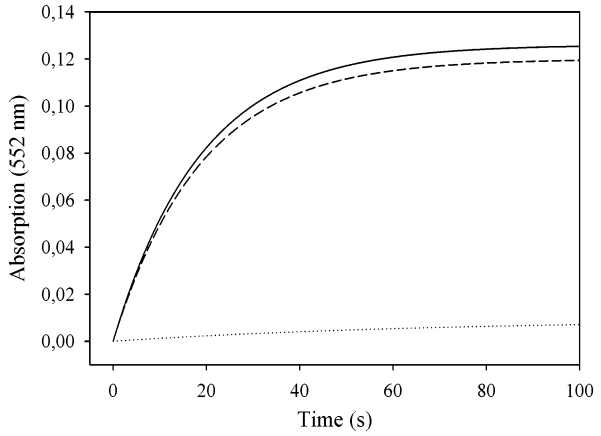

FIGURE 5: Time course of the absorbance changes at 552 nm after mixing of oxidized wt CYC_{41} (—), recombinant CYC_{41} (− −), or E121A mutated CYC_{41} (•••) with RCy and ferrous iron. The reaction was monitored in the stopped-flow spectrophotometer as described in Experimental Procedures. Final concentrations after mixing were as follows: 20 μM rusticyanin, 5 mM ferrous iron, 3 μM CYC_{41}.

with RCy and mutation in its vicinity does not affect the electron transfer. In contrast, the E121A mutation indeed resulted in pronounced alterations of CYC_{41} reduction kinetics.

Study of the Electron Transfer between CYC_{41} and Cytochrome Oxidase from A. ferrooxidans. We have tested the capacity of the different cytochromes *c* from *A. ferrooxidans* to interact with and transfer electrons to cytochrome oxidase. To assay the specificity of the electron transfer, experiments were performed with CYC_{41} (+350 mV, +460 mV), CYC_{42} (+430 mV, +505 mV), and cytochrome *c* [46 kDa] from *A. ferrooxidans*, which is encoded by a gene in the same operon as CYC_{41} and cytochrome oxidase (*23*). Results with CYC_{42} showed that this further c_4-type cytochrome could not be oxidized by cytochrome oxidase, in spite of its high sequence similarity to CYC_{41}. Similarly, no electron transfer was detectable between cytochrome *c* [46 kDa] and cytochrome oxidase. CYC_{41}, by contrast, was competent in electron transfer toward cytochrome oxidase. To determine the interaction site between CYC_{41} and cytochrome oxidase, we measured the oxidation of CYC_{41}, recombinant CYC_{41}, and the two mutants E121A and Y63F by *A. ferrooxidans* cytochrome oxidase. Steady-state kinetics with wild-type and recombinant CYC_{41} were first recorded. Both cytochromes showed the same enzymatic properties (Figure 6). The corresponding enzymatic parameters are summarized in Table 2. The screening of all our CYC_{41}s shows that the E121A mutation does not affect the reaction. By contrast, mutating the Y63 residue has a drastic effect on the electron transfer between CYC_{41} and cytochrome oxidase. In all cases, the turnover is extremely slow compared to the parameters obtained in other organisms. However, we found kinetic parameters similar to those described by Yamanaka T. et al. (*17*). The cytochrome *c* oxidase activity of the whole cells measured with a Clark electrode in the presence of 100 μM of horse heart cytochrome *c* was also very slow, around 40 nmol of O_2 min^{-1} mg^{-1} (*16*).

DISCUSSION

Problems in Expressing an Acid-Adapted Protein in a Neutrophilic Host. The interaction site between RCy, CYC_{41}, and cytochrome oxidase from *A. ferrooxidans* was investigated by site-directed mutagenesis, with the aim to answer the question whether c_4-diheme cytochromes serve as buffers for storing two electrons or whether they play the role of an electron wire connecting the donating and accepting redox proteins.

A prerequisite to such studies is that the gene encoding the acid-stable protein must be expressed and correctly matured in the host organism. Cytochrome *c* is unique among heme proteins in having hemes covalently attached to the polypeptide chain (*34*). During the last decade, the specific cellular apparatus in bacteria for covalent attachment of a heme to the cytochrome *c* polypeptide has been elucidated (*27*, *40*−*42*). This knowledge substantially facilitated heterologous expression of *c*-type cytochromes. Various cytochromes (including multiheme and thermo/hyperthermostable representatives) are now produced, and mutagenesis studies as well as structural analysis have been made possible (*43*). Due to the generation of a pH gradient in *A. ferrooxidans*, the cytoplasmic space is close to neutrality and the heterologous expression of cytoplasmic proteins from *A. ferrooxidans* in *E. coli* is straightforward. Few metalloproteins from acidophilic organisms have been produced by heterologous expression in *E. coli*, and expression of CYC_{41} from *A. ferrooxidans* is, to our knowledge, the first example of overproduction of an acid-stable c_4-type cytochrome. Only one periplasmic protein, i.e. rusticyanin, a blue copper protein, has previously been successfully expressed heterologically in *E. coli* as an apoprotein (*44*). In the case of RCy, copper was inserted after expression by incubating the apoprotein with copper sulfate at pH 4. A few years ago, the *iro* gene encoding a HiPIP-protein was cloned, but its expression in *E. coli* host cells failed (*45*). The difficulty to obtain recombinant HiPIP from *A. ferrooxidans* was probably due to problems of cluster insertion or an instability of the [4Fe-4S] cluster at the pH used. To our knowledge, only the Rieske protein from the thermoacidophilic crenarchaeon *Sulfolobus acidocaldarius* has been expressed in holoprotein form in *E. coli* at pH 7 and 40 °C (*46*). In this case, the iron−sulfur cluster was correctly inserted and the protein displayed the typical spectroscopic properties of a respiratory Rieske protein. Apart from resisting acidic conditions, the *Sulfolobus* protein is also hyperthermostable. It is likely that the more rigid structure required to confer stability at high temperatures is precisely what permitted expression of this acid-stable iron−sulfur protein at neutral pH.

When the gene encoding CYC_{41} was expressed in *E. coli* under aerobic conditions, recombinant protein was obtained in sufficient quantities for physicochemical studies, and it

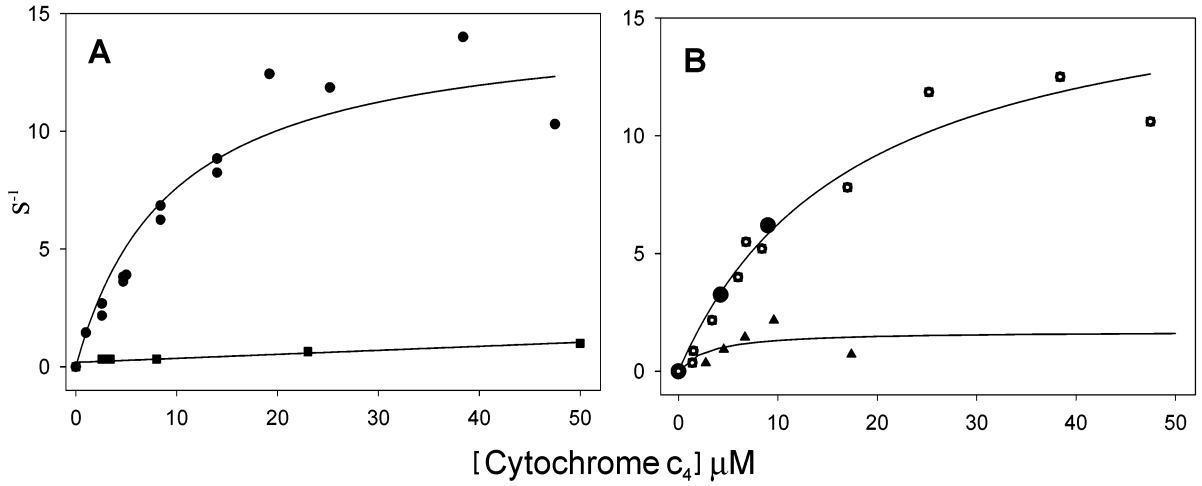

FIGURE 6: Steady-state kinetics of electron transfer between cytochrome c_4 and cytochrome oxidase. (A) Kinetic oxidation of CYC_{41} (●) and CYC_{42} (■) by the cytochrome oxidase from *A. ferrooxidans*. (B) Kinetic oxidation of recombinant CYC_{41} (●), E121A (■), and Y63F (▲) mutated CYC_{41} by the cytochrome oxidase from *A. ferrooxidans*. The experimental points were fitted to the Michaelis−Menten equation (solid line).

was matured and folded properly. However, major differences remain in the heme environment, as shown by all the spectroscopic data detailed above. Various studies have demonstrated that modifications of the heme axial ligands or their immediate environment alters the electron density of the heme macrocycles, and leads to drastic changes in the stability and the redox potential of the cytochromes (*47*). In the present case, most probably, the modifications are not drastic but residues that add or remove hydrogen bonds to the heme propionates lead to alteration of the electron density of the heme macrocycles and consequently affect its spectra.

The structure of CYC_{41} has been solved (*25*). Various residues were seen to participate in stabilization of the hemes by direct interaction (Cys16, Cys 19, His 20, Pro 33, Leu 35, and Met 64 for heme B, and Cys 119, Cys 122, His 123, Pro 134, Leu 136, Leu 147, and Met 161 for heme A) or engaged in hydrogen bonds in the heme vicinity (Arg 55, Tyr 50, and Asp 57 for heme B, and Arg 156 for heme A; Gln 38, Tyr 42, Gln 46, Gln 139, and Tyr 143 surrounding the two hemes). All these residues are conserved in cytochrome c_4 and not involved in the properties leading to acidostability. An inspection of the 3D structure shows residues in a loop involved in the heme B stabilization: Tyr 42, Tyr 50, Arg 55, and Asp 57. Since Tyr 50 is involved in a hydrogen bond with the Arg 57 residue and located near Met 64, the sixth ligand to heme B, modification of the protonation state, induced when CYC_{41} was expressed under aerobic conditions at a pH near neutrality, may have induced a shift of this residue toward Met 64 and thus a loss of the heme bond. In this case heme B became pentacoordinated (band 620 nm). Heme A seems to be less sensitive to the protonation state of the residues, which may be explained by the involvement of fewer residues (Tyr 143, Arg 156) in the heme stabilization.

Substitution of Y63 Affects Heme B. Intramolecular rearrangements in the vicinity of the heme are a further factor modulating redox properties. With regard to modification of redox potential in the Y63A and Y63F mutations, similar effects induced by mutating a residue near the coordinating methionine have been discussed by various authors (*28*, *47*−*49*) to be due to the perturbation of the heme crevice and the axial ligand. Lunntz et al. replaced Tyr in rat cytochrome *c* by Phe and observed that the variant protein was similar to the wild-type, with respect to its spectra and enzymatic electron transfer properties, but exhibited a 35 mV lower redox potential (*50*). It was proposed that the loss of the hydroxyl group prevents fixation of an internal water molecule involved in stabilization of the structure. In the present work, Y63A or Y63F mutations do not change the axial ligand but modulate the redox potential of CYC_{41} by affecting the Met64−heme iron axial bond strength and/or by the absence of the partial charge on the tyrosine. This hypothesis is corroborated by the CD results. The circular dichroism spectra of Y63A and Y63F show that the Cotton effect is blue-shifted by 3 nm, indicating a less packed heme crevice (*51*), and that it becomes less intense, indicating that the mutation affects the strength of the Met64−Fe bond. Inspection of the 3D structure shows that replacement of the Tyr residue by a Phe induces a slight increase in the heme solvent accessibility, and may also result in displacement of a water molecule located between two tyrosine residues, Y63 and Y29, thus leading to a destabilization of heme B.

Attribution of Redox Potentials to Individual Hemes. A better understanding of the electron transfer route through CYC_{41} requires an attribution of redox potentials to the individual hemes in the structure. In principle, the rhombicity of the paramagnetic centers, arising mainly from the nature and conformation of the axial ligands, may help to identify the parent hemes in the structure. As pointed out previously (*25*), the specific structure of CYC_{41} shows only marginal differences in axial ligand conformation for both hemes thus precluding an attribution to g_z values and correspondingly redox midpoint potentials to the individual hemes. From circumstantial evidence, it was concluded that heme A was probably the lower potential heme. The results obtained on the Y63 mutants invalidate this hypothesis and clearly show that heme B has the lower redox potential. This finding has repercussions on the detailed sequence of electron transfer steps from RCy to CYC_{41}. In contrast to what has been assumed previously, the electron entering CYC_{41} via heme A will continue to reside on this heme rather than proceed toward heme B in an intramolecular electron transfer process.

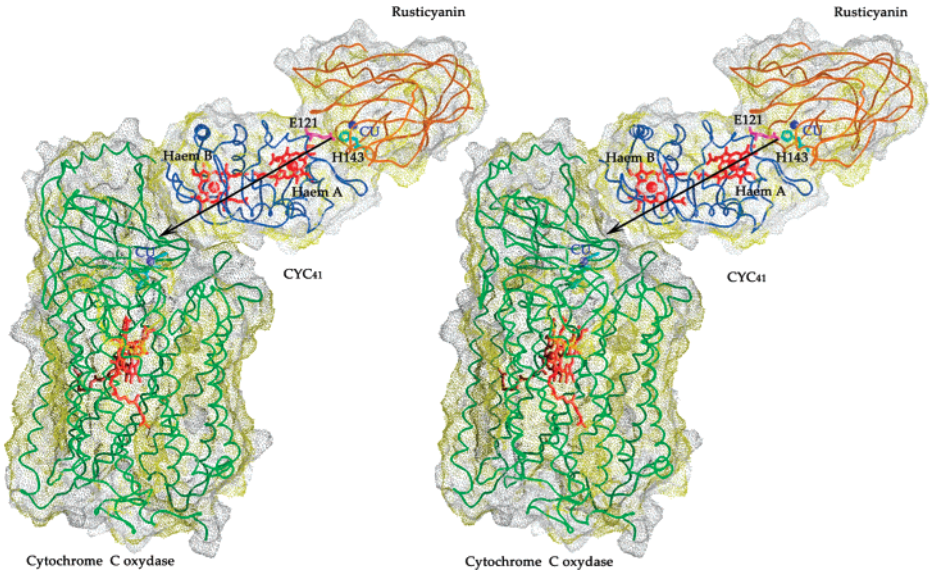

FIGURE 7: Stereoview of the model of rusticyanin/CYC_{41}/cytochrome *c* oxidase interaction. The molecular surface of the cytochrome *c* oxidase (1AR1 in green), CYC_{41} (in blue), and RCy (in orange) model was obtained using GRASP. Hydrophobic surfaces are colored in yellow. The copper ions are marked in blue. The black arrow shows the direction of electron transport from RCy to the cytochrome *c* oxidase membrane protein.

Only when the physiological electron acceptor is available at heme B (see below) will electron transfer take place, involving an uphill electron transfer step through heme B toward the ultimate electron sink on the physiological acceptor.

Interaction Sites on CYC_{41} for Its Physiological Electron Donor and Acceptor. A specific heme in CYC_{41} from *A. ferrooxidans* has been proposed as direct electron acceptor from its physiological redox partner RCy via the resolution of the 3D structure of CYC_{41}. By directed mutagenesis, we have demonstrated the role of glutamate 121, a residue close to heme A, in the interaction with RCy. E121 is involved in deprotonation of histidine 143 on RCy inducing a 100 mV decrease of its redox potential. SPR experiments have demonstrated the role of this residue in the interaction with the redox partner. The electron transfer which requires the specific interaction of the two residues was strongly affected by the replacement of E121 by alanine. No modification in the interaction between CYC_{41} and RCy was detected with the Y63F or Y63A mutants. This result corroborates the model proposing a unique interaction site on CYC_{41} close to heme A. To settle the question whether this protein functions as an electron wire, one heme acting as the entrance and the other as the exit site for electron transfer, we have tested the electron transfer between CYC_{41} wild-type and mutants and cytochrome *c* oxidase, its presumable physiological electron acceptor. The four *cox* genes encoding the subunits of an aa_3 oxidase are in a locus containing four other genes: *cyc2*, which encodes a high molecular weight cytochrome (cyt [46 kDa]); *cyc1*, which encodes CYC_{41}; *rus*, which encodes RCy; and ORF 1, which encodes a putative periplasmic protein of unknown function (23). The cotranscription of seven genes encoding redox proteins suggests that all these proteins are involved in the same electron transfer chain.

Wild-type CYC_{41} and the E121A mutant can both transfer electrons to cytochrome *c* oxidase with comparable efficiencies. The presence of the charge on the E121 residue thus is not necessary for the interaction between CYC_{41} and cytochrome oxidase to occur. This, of course, does not formally exclude heme A as the site of electron transfer toward cytochrome oxidase. More suggestively, mutation of Y63 strongly affects electron transfer. This residue is located in the hydrophobic area surrounding heme B, and as this hydrophobic region is conserved in the two available structures of c_4-type cytochromes, it is tempting to speculate that its corresponds to the interaction surface between cytochromes c_4 and cytochrome *c* oxidase. Electron transfer between cytochrome *c* oxidase and its various physiological partners has been under extensive investigation during recent years (*52*−*58*). The alignments between the COX II subunit from *A. ferrooxidans* and subunit II from *Paracoccus denitrificans* or from *Thermus thermophilus* show higher similarity with cytochrome oxidase from *P. denitrificans* (40%). Most residues described to be involved in the interaction and electron transfer are conserved, i.e. D178, W121, D159; E126 being replaced by an S in the COX II subunit of *A. ferrooxidans*. Association of two biological macromolecules is a fundamental biological phenomenon, and molecular docking is an appropriate tool to predict the geometry of a complex from the atom coordinates of its uncomplexed constituents in order to study and visualize their physical interaction. We used the HEX software to dock the CYC41 molecule on the cytochrome oxidase (from *P. denitrificans*, PDB: 1AR1) in an orientation allowing electron transfer to occur between the CYC_{41} heme B and the oxidase (*59*, *60*). The resulting buried area involves 1920 $Å^2$ of the solvent accessible surface (Figure 7). The inspection of the 3D docking model highlights a series of residues on the cytochrome CYC_{41} side as potentially involved in the interaction and located near the COX II subunit conserved residues, i.e. V 12, D 15, Y 29 (N-terminus loop) and I 62, Y 63, P 66 (second helix). This observation seems to be confirmed by our experimental results on the Y 63 mutants which strongly affect electron transfer. These residues are also conserved in the CYC_{43} cytochrome from *A. ferrooxidans* (*25*), which has never been detected in our growth conditions. Since this bacterium also contains four types of

terminal oxidases with a degree of expression dependent on the growth conditions (*16*), we suggest that the interaction between CYC_{43} and cytochrome oxidase occurs in the same way as the model proposed in this paper.

In summary, identification of opposite reaction sites on CYC_{41} for its electron transfer partners, i.e. reduction from RCy via heme A and reoxidation by cytochrome oxidase through heme B, demonstrates that the raison d'être of the two hemes is to form an electron wire rather than to provide two redox centers for charge storage.

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SUPPORTING INFORMATION AVAILABLE

EPR spectra recorded on the RCy:CYC_{41} complexes. This material is available free of charge via the Internet at http://pubs.acs.org.

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