Characterization of a New Dihemic c_4-Type Cytochrome Isolated from *Thiobacillus ferrooxidans*

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ABSTRACT: A new soluble *c*-type cytochrome has been purified to homogeneity from the acidophilic proteobacterium *Thiobacillus ferrooxidans* BRGM. It is characterized by an α-peak wavelength of 552 nm, a molecular mass of 26 567 Da (as determined by mass spectroscopy) and a pI value of 8. Optical redox titrations at pH 4.0 revealed the presence of two distinguishable redox species with an E_m of 510 mV and an E_m of 430 ± 20 mV. EPR spectra recorded for this heme protein demonstrated the presence of stoichiometric amounts of two low-spin hemes with a g_z of 3.08 (510 mV species) and a g_z of 3.22 (430 mV species). Modifications of the physicochemical properties of the cytochrome were observed on complex formation with the blue copper protein rusticyanin, another soluble electron carrier in the genus *Thiobacillus*. N-Terminal sequencing yielded the polypeptide sequence up to the 50th residue. The determined sequence was found to be present (at 100% amino acid identity) in the (unfinished) genome of *T. ferrooxidans* ATCC 23270, and the corresponding full-length protein turned out to be surprisingly similar (34.5% amino acid identity) to another c_4-type diheme protein from *T. ferrooxidans* BRGM [Cavazza, C., et al. (1996) *Eur. J. Biochem. 242*, 308−314], the gene of which is also present (at 97% amino acid identity) in the *T. ferrooxidans* ATCC 23270 genome. The physicochemical properties and sequence characteristics of both c_4 cytochromes present in the same bacteria are compared, and the functional role of this new diheme protein in the iron(II)-oxidizing electron transport chain in the genus *Thiobacillus* is discussed.

Extremophilic microorganisms, i.e., species capable of growth in extreme environmental conditions such as high temperatures, high salt concentrations, or extreme pH values, have attracted considerable attention during recent years due both to their impact on the questions of the possible origins and limits of life and to their biotechnological potential.

The bioenergetic metabolism of the ore-leaching acidophilic proteobacterium *Thiobacillus ferrooxidans* involves an electron transfer chain coupling of iron(II) (a substrate with a high redox potential) at the external cell wall surface to reduction of the terminal electron acceptor, molecular oxygen (*1*, *2*). Most of the metalloproteins involved in the respiratory chain are acid-stable proteins with comparatively high redox potentials (*3*−*13*).

Several redox proteins that potentially participate in iron oxidation have been isolated from *T. ferrooxidans*: a blue copper protein, rusticyanin (RCy)[1] (*3*, *14*−*17*), a high-potential iron−sulfur protein (*5*), a membrane-bound cytochrome oxidase (*6*−*8*), a partially purified iron:rusticyanin oxidoreductase (*9*), a soluble and membrane-bound cytochrome *c* (*16*−*19*), and a soluble cytochrome c_4 (*10*). Rusticyanin accounts for up to 5% of the total soluble protein in *T. ferrooxidans* and has been studied in detail (*14*−*17*). We have characterized a cytochrome c_4 (M_r = 21 000), and the N-terminal sequence is identical to those of soluble cytochromes c_{552} (s) and membrane-bound c_{552} (m) described by Yamanaka et al. (*20*). Recently, we described a strong interaction between two proteins localized in the periplasmic space (pH ∼3), i.e., rusticyanin and cytochrome c_4 (M_r = 21 000) (*21*). The docking between rusticyanin and cytochrome c_4 was observed to induce a p*K* shift of the pH-dependent redox properties of RCy, resulting in a substantial drop in redox potential in the complex at low pH values. This modification of the redox properties of RCy induced by complex formation strongly facilitates electron transfer from RCy to cytochrome c_4 (M_r = 21 000). Electron transfer from Fe^{2+} to cytochrome c_4 is therefore catalyzed by rusticyanin, and the electron donation from RCy to cytochrome c_4 represents an individual segment of the overall physiological electron transfer chain (*21*).

In the work presented here, the purification, biochemical characterization, and preliminary features of a novel *c*-type cytochrome from *T. ferrooxidans* (strain BRGM) are described.

To characterize this cytochrome in more detail, we determined its biochemical properties as well as the nature of its heme iron ligands and compared them to those of the other *c*-type cytochromes described previously in *T. ferrooxidans*. Furthermore, the kinetic properties of the electron transfer reaction between the new cytochrome and possible redox partners were studied. The probable involvement of this electron carrier in the iron respiratory electron transport

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[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; TMBZ, 3,3′,5,5′-tetramethylbenzidine; RCy, rusticyanin.

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chain of *T. ferrooxidans* and the detailed nature of its functional role are discussed.

MATERIALS AND METHODS

Microorganism and the Culture Growth Procedure. T. ferrooxidans (strain BRGM) was kindly supplied by D. Morin (Bureau des Recherches Géologiques et Minières, Orléans, France). It was isolated from drainage water at the Salsigne sulfur mine (France), and was grown at pH 1.6 in 9 K Silverman and Lundgreen medium (*22*) supplemented with $CuSO_4 \cdot 5H_2O$ at a concentration of 1.6 mM. Large-scale cultivation of the organism was performed in 300 L of the medium described above with a homemade polypropylene fermenter. Typical yields were in the range of 80 mg of cell material per liter of culture.

Optical Absorption Spectra. Visible and ultraviolet absorption spectra of the protein were determined with a Beckman DU 7500 spectrophotometer. Molar extinction coefficients at the absorption maxima were obtained from these spectra using protein concentrations based on amino acid analysis data.

Isoelectric Point Measurements. The isoelectric point of the protein was determined by performing isoelectric focusing using a Phast System apparatus from Pharmacia LKB Biotechnology (*23*). Phast Gels IEF 3-9 were used with a Pharmacia broad-range pI calibration kit containing proteins with different isoelectric points ranging from 3 to 10.

Molecular Mass Determination. The molecular mass of the protein was determined by performing sodium dodecyl sulfate−polyacrylamide gel electrophoresis under reducing conditions on a Pharmacia PhastSystem with PhastGel 8 to 25% polyacrylamide and PhastGel SDS buffer strips. Proteins and hemoproteins were stained with Coomassie blue and tetramethylbenzidine, respectively (*24*).

MALDI-MS was performed on a reflectron time-of-flight mass spectrometer equipped with delayed extraction (Voyager DE-RP, Perceptive Biosystems Inc.). To evaluate their respective response in MALDI-MS, analyses were performed separately for solutions of holo- and apoprotein. Subsequently, these solutions were mixed in suitable proportions, and the resulting mixture was analyzed. In each case, 0.7 μL of the sample 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).

Amino Acid Analysis and Protein Sequencing. For the amino acid analysis, protein samples were hydrolyzed in 200 μL of 6 M HCl at 110 °C for 24 and 72 h in sealed vacuum tubes and then analyzed with a Beckman amino acid analyzer (System 6300). Heme was removed using Ambler's method (*25*), and the resulting apoprotein was isolated by gel filtration on Sephadex G-25 column in 5% (v/v) formic acid. Sequence determinations were carried out for the apoprotein with an Applied Biosystems gas-phase sequenator (models 470 and 473 A). Quantitative determinations were performed on the phenylthiohydantoin derivatives by high-pressure liquid chromatography (Waters Associates, Inc.) monitored by a data and chromatography control station (Waters 840).

Preliminary sequence data from *T. ferrooxidans* ATCC 23270 were obtained from The Institute for Genomic Research website at http://www.tigr.org.

Analysis of Heme Content. The total number of heme units was determined using the pyridine ferrohemochromogene test. A known mass of the protein (determined by hydrolyzing an aliquot of protein solution and performing quantitative amino acid analysis) was added to an aqueous alkaline solution (0.0075 M NaOH/25% pyridine) and reduced by adding a few crystals of sodium dithionite, and the heme content was determined from the pyridine ferrohemochrome spectrum, using the millimolar absorbance coefficient of 29.1 at 550 nm with the cytochrome derivative (*26*).

Redox Titration and EPR Spectroscopy. Optical redox titrations were performed on a Kontron Uvikon 932 spectrophotometer according to the method of Dutton (*27*) at pH 4.5 and 20 °C in 10 mM ammonium acetate. The following redox mediators were present at a concentration of 2 μM: ferrocene monocarboxylic acid, ferrocene dicarboxylic acid, *p*-benzoquinone, and potassium ferricyanide. The purified cytochrome *c* (7.5 μM) and an equimolar mixture (8 μM each) of cytochrome and rusticyanin were titrated.

EPR spectra were recorded on a Bruker ER300e X-band spectrometer fitted with an Oxford Instruments liquid He cryostat. Complete oxidation of the sample was achieved by adding an excess of potassium hexachloroiridate followed by removal of the oxidant via a passage through a PD-10 column. The sample contained 100 μM cytochrome *c* in 50 mM ammonium acetate (pH 4.8).

Multiple sequence alignments were performed using ClustalX (*28*), and prediction of secondary structure was carried out using the pSAAM package (A. R. Crofts, 1992, pSAAM for Windows, a program for protein sequence analysis and modeling, University of Illinois, Urbana, IL). Phylogenetic relationships were calculated using a maximum-likelihood approach (ClustalX).

RESULTS

Purification and Biochemical Characterization of the Studied Cytochrome. All purification steps were performed at 4 °C and pH 4.8. Cytochrome *c*, rusticyanin, and two additional proteins were purified by successive separation steps on carboxymethylcellulose, S-Sepharose, and mono S columns as described by Cavazza et al. (*10*). Rusticyanin and cytochrome c_4 (M_r = 21 000) were eluted from carboxymethylcellulose (CMC waters) with 220 mM ammonium acetate, 1 mM PMSF, and 0.1% Zwittergent (pH 4.8). Subsequently, a new *c*-type cytochrome which we called "cytochrome c_4 (M_r = 26 000)" with reference to its physicochemical properties (see below) eluted at 300 mM ammonium acetate [1 mM PMSF and 0.1% Zwittergent (pH 4.8)]. This fraction was dialyzed against 0.001 N sulfuric acid and loaded onto an S-Sepharose (HiLoad 16/10 Pharmacia) column [equilibrated with 50 mM ammonium acetate (pH 4.8)]. The fraction containing cytochrome c_4 (M_r = 26 000) eluted at 180 mM ammonium acetate (pH 4.8). After dialysis, the respective fraction was loaded onto a mono S column (HR/S/Pharmacia) equilibrated with 50 mM ammonium acetate (pH 4.8). Cytochrome c_4 (M_r = 26 000) eluted at 800 mM ammonium acetate and was found to be pure. SDS−PAGE analysis and TMBZ coloration tests showed that this heme protein was different from cytochrome c_4 (M_r = 21 000). It was determined to be a basic protein with a pI of about 8.

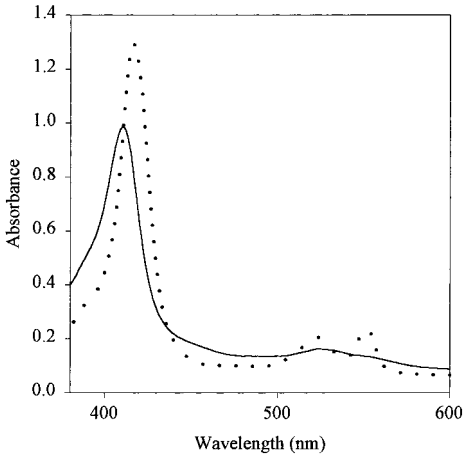

FIGURE 1: Visible spectrum of purified *T. ferrooxidans* BRGM cytochrome c_4 (M_r = 26 000) in the oxidized state (—) and reduced by dithionite (···).

Six milligrams of cytochrome c_4 (M_r = 26 000), 200 mg of pure rusticyanin, and 9 mg of pure cytochrome c_4 (M_r = 21 000) were obtained from 100 g of bacteria (wet weight).

The apparent molecular mass deduced from SDS−PAGE analysis was about 29 kDa. Mass spectra of this sample demonstrated that cytochrome c_4 (M_r = 26 000) is a monomeric protein with a molecular mass of 26 567 Da.

Optical Spectra. UV−visible spectra of cytochrome c_4 (M_r = 26 000) are represented in Figure 1. These spectra show the presence of a Soret peak at 411 nm in the oxidized state and of γ, β, and α peaks at 417, 523, and 552 nm, respectively, in the dithionite-reduced form. The purity coefficient, expressed as the ratio between the absorbances at given wavelengths ($c = A^{red}_{552} - A^{red}_{570}/A^{ox}_{280}$), was found to be 1 for the purified protein. The extinction coefficient of reduced cytochrome at 552 nm was 46 000 M^{-1} cm^{-1}, and the α/β ratio of reduced cytochrome was found to be 1.2.

Cytochrome c_4 (M_r = 26 000) was oxidized by addition of an excess of hexachloroiridate in 50 mM glycine, and 200 mM ammonium sulfate (pH 2.7). The oxidized protein was then loaded onto a PD-10 column (Sephadex G-25), equilibrated in the same buffer, to remove the oxidant. The UV−visible spectrum was monitored in the presence of various concentrations of $FeSO_4$. In contrast to cytochrome c_4 (M_r = 21 000) isolated from the same species (*10*), cytochrome c_4 (M_r = 26 000) is completely reduced by 2 mM $FeSO_4$ (data not shown). The apparent dissociation constant deduced (in first approximation) from the experiment is around 0.5 mM.

The number of heme groups per molecule was determined by pyridine ferrohemochrome analysis, and a value of 2 was found.

Amino Acid Analysis. The amino acid composition of the protein was determined by performing amino acid analysis on the basis of the molecular mass of the protein (26 567 Da) (Table 1).

Automated Edman degradation of 1 nmol of the carboxymethylated protein yielded the amino-terminal sequence up to the 50th cycle. A search for this sequence in protein databases revealed no obvious homologous protein. This sequence stretch, however, could be retrieved from the unfinished genome of *T. ferrooxidans* ATCC 23270 (TIGR). The 50 N-terminal residues of our protein and the corresponding stretch of the TIGR sequence were found to be identical. The respective ORF in the *T. ferrooxidans* genome encodes a protein made up of an N-terminal (58-residue) stretch followed by a C-terminal domain that is very similar to cytochrome c_4 (M_r = 21 000) from *T. ferrooxidans* BRGM described earlier (*10*; see Figure 3). The gene for cytochrome c_4 (M_r = 21 000) is also present in the *T. ferrooxidans* ATCC 23270 genome (with 97% amino acid identity between the proteins in *T. ferrooxidans* BRGM and *T. ferrooxidans* ATCC 23270).

Table 1: Amino Acid Compositions of Cytochrome c_4 (M_r = 26 000) and Cytochrome c_4 (M_r = 21 000) from *T. ferrooxidans* BRGM[a]

amino acid	no. of cytochrome c_4 (M_r = 26 000) residues	no. of cytochrome c_4 (M_r = 21 000) residues
aspartic acid	15.2 (15)	15
threonine	17.9 (18)	8
serine	16.6 (17)	11
glutamic acid	19.1 (19)	19
proline	20.1 (20)	10
glycine	21.1 (21)	18
alanine	34.9 (35)	23
cysteine	3.6 (4)	4
valine	14.1 (14)	10
methionine	8.6 (9)	8
isoleucine	8.2 (8)	9
leucine	15.1 (15)	11
tyrosine	9.1 (9)	9
phenylalanine	5.7 (6)	4
histidine	5.2 (5)	7
lysine	11.3 (11)	10
arginine	9.9 (10)	8
tryptophan	ND	(1)
total no. of residues	236	185

[a] The values given for threonine and serine were obtained by extrapolation from the hydrolysis time.

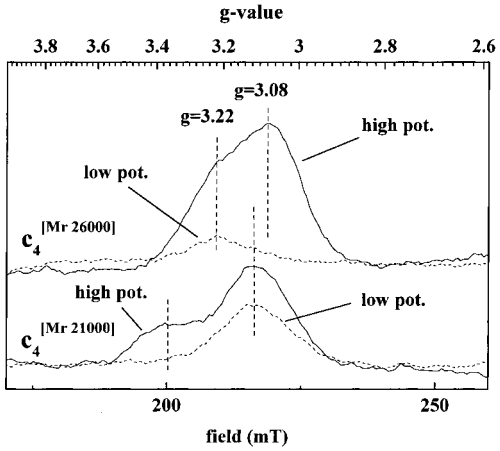

FIGURE 2: EPR spectra of the isolated cytochrome c_4 (M_r = 26 000) from *T. ferrooxidans* BRGM in the untreated state after purification (broken line) as well as in the hexachloroiridate-oxidized state (solid line). Cytochrome c_4 (M_r = 26 000) was present at a concentration of 100 μM in 50 mM ammonium acetate at pH 4.8. In the bottom part of the figure, spectra of cytochrome c_4 (M_r = 21 000) as previously published in ref *10* are shown for comparison. Instrument settings were as follows: temperature, 15 K; microwave frequency, 9.42 GHz; modulation amplitude, 3.2 mT; and microwave power, 6.3 mW.

Optical Redox Titration. In the absence of rusticyanin, two distinct redox waves could be discerned with E_m values of 510 ± 20 and 430 ± 20 mV. In the presence of a stoichiometric amount of rusticyanin, a modification of the

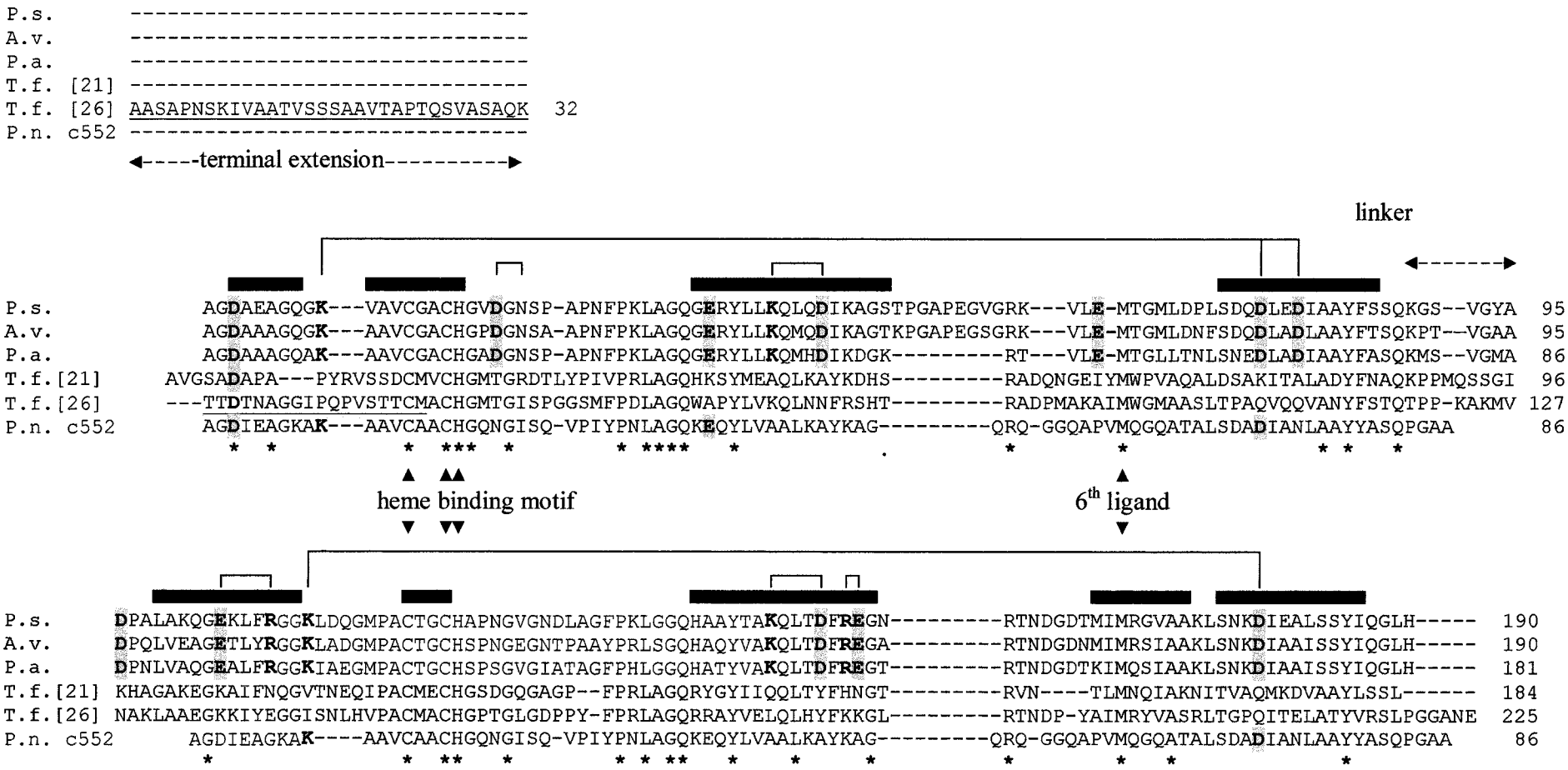

FIGURE 3: Multiple sequence alignment of c_4-type cytochromes from *P. stutzeri* (P.s.), *A. vinelandii* (A.v.), *P. aeruginosa* (P.a.), *T. ferrooxidans* ATCC 23270 (T.f.) [[21] and [26] denote cytochromes c_4 (M_r = 21 000) and c_4 (M_r = 26 000), respectively] and *Pseudomonas nautica* (P.n.). α-Helical regions as seen in the three-dimensional structure of the P.s. cytochrome c_4 are indicated by black bars above the aligned sequences. Glutamate and aspartate residues which are conserved in all of the neutrophilic cytochromes c_4 are highlighted by a gray background. Structurally important salt bridges seen in the P.s. cytochrome and conserved in all neutrophilic cytochromes c_4 are indicated by thin lines above the alignment, and the corresponding residues are marked in bold. Asterisks below the aligned sequences denote fully conserved residues. N-Terminal sequencing of the purified protein yielded the underlined amino acid residues in cytochrome c_4 (M_r = 26 000) from *T. ferrooxidans* BRGM.

redox titration curves was observed that could be analyzed as a shift of E_m values to 575 and 465 mV, respectively. No modification of the redox midpoint potential of rusticyanin was observed.

EPR Spectroscopy. Figure 2 shows the EPR spectra in the region of heme g_z peaks recorded on untreated (dashed line) and on fully oxidized (continuous line) cytochrome c_4 (M_r = 26 000) from *T. ferrooxidans* BRGM.

The untreated sample featured a weak peak at $g = 3.22$. The hexachloroiridate-oxidized cytochrome, however, had a prominent peak at $g = 3.08$ and a pronounced shoulder at the position of the g_z line of the untreated sample. Application of the semiempirical correction for g value-dependent line intensities reported by deVries et al. (*29*) yielded a stoichiometry of 1:0.94 for the two hemes at $g = 3.22$ and 3.08, respectively. This finding corroborated the conclusion that the studied hemoprotein is in fact a diheme cytochrome resembling the previously described cytochrome c_4 from the same organism. A comparison of the EPR spectral features of these two distinct diheme cytochromes (Figure 2) shows that the spectral differences between the two individual hemes are less pronounced in the heme protein described here than in the previously studied cytochrome c_4 (M_r = 21 000) (*10*).

In addition, whereas in cytochrome c_4 (M_r = 21 000) the higher redox component corresponded to the heme with the higher g value, the reverse is observed for the novel diheme cytochrome. This further demonstrates that there is no straightforward correlation between the g value and redox midpoint potential, as already discussed previously (*10*).

Stimulated by our observations of interaction-induced alterations of EPR spectra upon complex formation between cytochrome c_4 (M_r = 21 000) and RCy (*21*) and by the results described above [showing that addition of a stoichiometric amount of RCy to cytochrome c_4 (M_r = 26 000) altered the E_m values of the hemes], we recorded EPR spectra of a 1:1 complex between the two redox proteins. No spectral modifications of the copper signals of RCy could be observed (not shown). Nor were the EPR spectral features of the heme centers shifted by the presence of RCy. However, a slight (about 10%) reduction of the $g = 3.08$ heme in the presence of RCy and of a slight excess of hexachloroiridate was seen. This finding is in line with the observed upshift by more than 60 mV of the E_m value of the more oxidizing heme component.

DISCUSSION

The results shown above demonstrated that the 26 kDa heme protein purified from *T. ferrooxidans* BRGM is a diheme cytochrome. From its amino acid sequence, this protein was identified as a c_4-type cytochrome containing a long N-terminal extension when compared to five amino acid sequences of cytochromes c_4 reported previously (Figure 3).

Two distinct c_4 cytochromes with differing electrochemical properties are therefore present in *T. ferrooxidans*.

Structure Prediction and Phylogenetic Analysis. Figure 3 shows a multiple alignment of the two *Thiobacillus* proteins with three previously studied c_4-type cytochromes from *Pseudomonas stutzeri* (*Ps*), *Azotobacter vinelandii* (*Av*), and *Pseudomonas aeruginosa* (*Pa*) as well as with cytochrome c_{552} from *Pseudomonas nautica*, a dimerizing monoheme cytochrome that is very homologous to cytochrome c_4 (*30*). An outstanding characteristic of cytochrome c_4 (M_r = 26 000) in *Thiobacillus* is the presence of an N-terminal extension of about 30 residues.

Within the c_4 domains of the two *Thiobacillus* cytochromes, α-helical stretches were predicted in regions roughly equivalent to those where helices are present in the crystal structure of the *Ps* protein (*31*). We therefore expect the global three-dimensional structure of both *Thiobacillus* c_4 cytochromes to be close to that of the *Ps* protein. Intriguingly, the degree of sequence homology between the two *Thiobacillus* proteins is much higher that of one of both to any other c_4 cytochrome. This observation is substantiated by the phylogenetic relationship between c_4 proteins predicted by maximum likelihood algorithms (not shown). Both *Thiobacillus* cytochromes cluster together and are well-separated from the other members of the c_4 group. It therefore appears likely that the two *Thiobacillus* c_4 cytochromes arise from a gene duplication event within the *Thiobacillus* lineage rather than descending from two distinct c_4 cytochromes already present in other proteobacteria. We note that c_4-like diheme cytochromes (including flavocytochrome *c* from *Chromatium vinosum*) have so far only been described in proteobacteria. However, genes encoding diheme cytochromes exhibiting sequences moderately similar to that of the c_4 group can be detected in *Heliobacillus mobilis* and *Deinococcus radiodurans*. If these proteins should turn out to be structurally related to the c_4 group, this class of diheme proteins may actually be evolutionarily quite ancient.

Adaptation to Acidic Conditions. The analysis of the three-dimensional structure of cytochrome c_4 from *P. stutzeri* showed the presence of numerous structure-stabilizing salt bridges involving the negatively charged residues aspartate and glutamate (*31*). Most of these interactions stabilize α-helices, and some of them are involved in maintaining the global structure by forming interhelix links (see the annotations in Figure 3).

The free forms of aspartate and glutamate exhibit p*K* values in the vicinity of pH 4. Unless significant p*K* shifts are induced by the protein environment, these residues are therefore likely to be in the protonated, i.e., neutral form at the pH values of a typical *T. ferrooxidans* growth medium (pH <2). As shown in Figure 3, all but one of the conserved Glu and Asp residues participating in stabilizing salt bridges as indicated above the aligned sequences are indeed absent from both *Thiobacillus* c_4 cytochromes. The total number of aspartate and glutamate residues is also strongly reduced (from 32, 19, and 17 in *Ps*, *Av*, and *Pa*, respectively, to 14 and 10 in the M_r = 21 000 and M_r = 26 000 proteins from *Thiobacillus*). The acidic conditions therefore seem to strongly hamper the use of salt bridges as structure-stabilizing elements. The alignment shown in Figure 3 does not suggest straightforward substitutes employed by *Thiobacillus* to compensate for this loss in stability. Only the detailed analysis of the three-dimensional-structure of one of the c_4 cytochromes from *Thiobacillus* will clarify whether these salt bridge interactions have been replaced by hydrogen-bonding, hydrophobic, or even other interactions. Attempts to crystallize cytochrome c_4 (M_r = 21 000) from *T. ferrooxidans* BRGM are presently underway in our laboratory.

Redox Potentials. The E_m values of the two heme groups in *T. ferrooxidans* BRGM cytochrome c_4 (M_r = 26000) have

been determined as 510 and 430 mV. Complex formation with RCy renders these potentials even more positive (575 and 465 mV, respectively). To our knowledge, these are the most oxidizing redox potentials observed in *c*-type cytochromes so far. The previously studied cytochrome c_4 (M_r = 21 000) exhibited E_m values of 385 and 480 mV. For comparison, the redox midpoint potentials observed in cytochrome c_4 from *Ps* are 190 and 300 mV. The high redox potentials in *Thiobacillus* are in line with a functioning of this cytochrome in the downhill electron transport from the Fe(II)−Fe(III) couple toward reduction of molecular oxygen. An elucidation of the molecular details resulting in the unusually high E_m values must await determination of the structure of the *Thiobacillus* cytochromes. A conspicuous characteristic of c_4 (M_r = 26 000) is the strong disequilibrium between Glu/Asp and Lys/Arg residues (10:17) as compared to cytochrome c_4 (M_r = 21 000) (14:17) or to cytochrome c_4 from *Ps* (21:19). Although a fraction of the Glu and Asp residues may be neutral if their p*K* values are not significantly downshifted, this disequilibrium suggests a particularly high positive electrostatic potential on cytochrome c_4 (M_r = 26 000). Alternatively (or additionally), the N-terminal extension might wrap around the c_4 core, decreasing the extent of heme exposure and consequently raising the redox midpoint potential.

Function. To gain information concerning the functional role of cytochrome c_4 (M_r = 26 000) in Fe^{2+} oxidation, we have studied its reduction by Fe(II) as well as the effect of the presence of RCy on the biophysical properties of the cytochrome. Cytochrome c_4 (M_r = 26 000) was found to be reduced by Fe^{2+}, whereas no reduction of cytochrome c_4 (M_r = 21 000) could be observed at the same Fe^{2+} concentrations. The dependence of the rate on concentration exhibited a half-maximal effect at about 0.5 mM. The apparent mean K_m value for $FeSO_4$ measured on whole cells is in the range of about 0.25 mM (*9*). Therefore, the value obtained on the isolated cytochrome c_4 (M_r = 26 000) is compatible with a functional role of cytochrome c_4 (M_r = 26 000) in the initial step of iron oxidation. Recently (*21*), we have observed a decrease in the redox midpoint potentiel of RCy by more than 100 mV during complex formation with its electron transfer partner cytochrome c_4 (M_r = 21 000). Formation of a complex between RCy and cytochrome c_4 (M_r = 26 000), on the other hand, modified the redox potential of the cytochrome (+60 mV, see above).

Previously, an iron:rusticyanin oxidoreductase activity has been demonstrated by Blake and Shute (*9*), in a partially purified sample. In a recent paper, Appia-Ayme et al. (*33*) have argued that the 46 kDa cytochrome *c* encoded by *cyc2* corresponds to this iron:RCy oxidoreductase. Their conclusions, however, solely rely on the presence of this cytochrome in a transcriptional unit also containing RCy, cytochrome c_4 (M_r = 21 000), and cytochrome oxidase.

In contrast, on the basis of our results, we tentatively suggest that cytochrome c_4 (M_r = 26 000) represents the iron:rusticyanin oxidoreductase discussed by Blake and Shute (*9*) and, thus, that it is the primary cellular oxidant of ferrous ions in the iron respiratory electron transport chain in *T. ferrooxidans*.

Diheme versus Monoheme Cytochromes in Thiobacillus. Diheme c_4-type cytochromes have so far been found in several proteobacteria and are in most cases believed to participate in electron donation to the terminal aerobic oxidases of these species. To our knowledge, *T. ferrooxidans* is the only organism reported so far that harbors two different representatives of this family of diheme cytochromes. This raises the question of the structural and functional characteristics favoring the presence of c_4-type diheme over the use of "standard" monoheme cytochromes in *T. ferrooxidans*. Possible explanations comprise the following scenarios. (a) The diheme cytochromes act as electron wires in ternary complexes allowing for structural localization of electron transfer pathways as opposed to diffusional and/or collisional electron transfer. (b) The compact structure of c_4-type cytochromes facilitates stabilization to cope with the extremely acidic pH values of the medium. (c) Electrostatic interaction between the individual hemes as well as the reduced solvent accessibility of the redox centers might help to induce the high redox potentials required by the energetic constraints of electron transport in the genus *Thiobacillus*. The elucidation of three-dimensional structures of the two *Thiobacillus* c_4-type cytochromes may provide hints for understanding the preference of this species for cytochromes of the diheme type.

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