Cytochromes c_{555} from the Hyperthermophilic Bacterium *Aquifex aeolicus*. 2. Heterologous Production of Soluble Cytochrome c_{555}^{s} and Investigation of the Role of Methionine Residues

Corinne Aubert,*,‡ Françoise Guerlesquin,‡ Pierre Bianco,‡ Gisèle Leroy,‡ Pascale Tron,‡ Karl-Otto Stetter,§ and Mireille Bruschi‡

Laboratoire de Bioénergétique et Ingénierie des Protéines (UPR9036), CNRS-IBSM, 31 chemin Joseph Aiguier, 13402 Marseille Cedex 20, France, and Lehrstuhl für Mikrobiologie und Archaeenzentrum, Universität Regensburg, D-8400 Regensburg, Germany

Received June 11, 2001; Revised Manuscript Received August 13, 2001

ABSTRACT: The *cycB2* gene encoding the soluble cytochrome c_{555}^{s} from *Aquifex aeolicus*, an hyperthermophilic organism, has been cloned and expressed using *Escherichia coli* as the host organism. The cytochrome was successfully produced in the periplasm of an *E. coli* strain coexpressing the *ccmABCDEFGH* genes involved in the cytochrome *c* maturation process. Comparison of native and recombinant cytochrome c_{555}^{s} shows that both proteins are indistinguishable in terms of spectroscopic and physicochemical properties. Since two different methionine residues are present in the sequence stretch usually providing the sixth ligand to the heme iron, site-directed mutagenesis has been performed in order to identify the methionine serving as the axial ligand. Two single mutations were introduced, leading to the replacement of each methionine by a histidine residue. Characterization of both mutants, M78H and M84H cytochromes c_{555}^{s}, using biochemical and biophysical techniques has been carried out. The M84H mutant exhibits spectral features identical to those of native cytochrome. Its redox midpoint potential is decreased by 40 mV. By contrast, substitution of methionine 78 by a histidine residue strongly alters the structural and physicochemical properties of the molecule which exhibits characteristics of His/His iron coordination type rather than His/Met. These results allow us to identify methionine 78 as the sixth ligand of cytochrome c_{555}^{s} heme iron. Preliminary results on the thermostability of the native and mutant cytochromes c_{555} are also reported.

Thermophilic microorganisms are defined as having an optimum growth temperature between 40 and 70 °C whereas hyperthermophilic species grow at higher temperatures, up to about 110 °C. Proteins isolated so far from such organisms show enhanced thermostability. Recently, it has been proposed that the factors involved in the stabilization of proteins to very high temperature (close to 100 °C) are different from those usually involved in thermostable proteins in terms of number of ion pairs, number of cavities, polarity of the exposed surface, and secondary structure composition (*1*). Moreover, it appears that the structural parameters differ according to the protein family, which employ different structural devices to adapt their function to very high temperature (*1*, *2*).

Class I *c*-type cytochromes are widespread in a variety of metabolisms, and the structural properties of these molecules have been extensively studied in order to identify the factors responsible for the stabilization of this molecule at temperatures close to 60 °C (*3*−*6*). The study of a homologous cytochrome *c* from hyperthermophilic organisms should be helpful for understanding the structural parameters involved in hyperthermostability of this protein.

Aquifex aeolicus is a hydrogen-oxidizing, microaerophilic, and obligatory chemolithoautotrophic bacterium (*7*). It is considered to be the earliest diverging organism of the domain of the Bacteria, and it is one of the most hyperthermophilic bacteria known to date (*8*). Analysis of its complete genomic DNA sequence reveals several putative genes encoding for *c*-type cytochromes. Two of them (ORF Aq792 and ORF Aq1550) apparently encode a membrane-bound cytochrome c_{555}^{m} (166 amino acid residues) and a soluble cytochrome c_{555}^{s} (104 residues), respectively. Sequence comparison of both molecules shows a remarkably high similarity (85% identity and 89% homology) between the C-terminal 87 amino acid residues of the membrane-bound cytochrome and the soluble cytochrome [see accompanying article (*40*)].

In this work, our interest is focused on the structural properties of the soluble monohemic cytochrome c_{555}^{s} from *A. aeolicus*. The primary structure presents significant homology (>30% identity and >50% homology) to the cytochromes from mesophilic organisms (*Pseudomonas* and *Nitrosomonas europaea*) and thermophilic bacteria (*Hydrogenobacter thermophilus*). The heme binding motif of the class I cytochromes *c*, involving two cysteine residues in the consensus sequence C-X-Y-C-H covalently bound to the vinyl groups of the porphyrin, is conserved. The

* To whom correspondence should be addressed. Tel: +33 491 16 45 50. Fax: +33 491 16 45 78. E-mail: aubert@ibsm.cnrs-mrs.fr.

‡ Laboratoire de Bioénergétique et Ingénierie des Protéines (UPR9036), CNRS-IBSM.

§ Lehrstuhl für Mikrobiologie und Archaeenzentrum, Universität Regensburg.

10.1021/bi011202q CCC: $20.00
Published on Web 10/13/2001

Table 1: Bacterial Strains and DNA Vectors

strains or vector	genotype, comments, and/or references
E. coli TG1	K12, Δ(*lac-pro*), *supE, thi, hsdD5* (F′, *traD36, proA*$^{+}$*B*$^{+}$, *lac I*^{q}, *lacZ*Δ*M15*)
E. coli MC1061	*AraD139* Δ(*ara-leu*)*7969* Δ(*lac*)*l74galU galK hsdR2*($r_k^-m_k^+$) *mcrB1 rpsL* (Str^r)
E. coli MC1061(pEC86)	MC1061 transformed with pEC86 that contains the *ccm* genes (*39*)
Aquifex aeolicus	VF5 (*7*)
LITMUS 38 c_{555}	contains the *cycB2* gene on a 547 bp *Eco*RI−*Pst*I insert in phagemid vector LITMUS 38 (Bio-Labs)
pJF119EH	*tacP, rrnB, ApR*, Δ[*Xma*III−*Bam*HI, 0.5 kb], Ω[*lacI*^{Q}, *Hin*dIII, 1.2 kb], Δ[polylinker M13mp18], Ω[polylinker M13mp48] (*14*)
pJF119c_{555}	contains the *cycB2* gene on a 410 bp *Eco*RI−*Pst*I insert in pJF119EH
pJF119M78H	pJF119c_{555} containing *cyc*B2M78H that carries the mutation of methionine 78 in histidine
pJF119M84H	pJF119c_{555} containing *cyc*B2M84H that carries the mutation of methionine 84 in histidine

heme iron is hexacoordinated, adopting a low-spin form in both the oxidized and the reduced states. The two axial ligands at the fifth and sixth coordination position of the heme iron atom are an imidazole nitrogen atom of a histidine residue and a sulfur atom of a methionine residue, respectively. Whereas the histidine fifth ligand is always located in the consensus sequence of the heme binding motif, the identification of the sixth ligand residue is not possible from the primary structure alone, but it is generally found within the C-terminal part of the molecule in the class I cytochromes *c*.

A. aeolicus cytochrome c_{555}^{s} and homologous thermophilic or mesophilic *c*-type cytochromes differ in sequence in several positions. For example, the cytochromes c_{555} contain two methionine residues, M78 and M84, located at the C-terminal half of the protein. Comparison of mesophilic and thermophilic monohemic cytochrome *c* sequences [see Figure 1 in the accompanying article (*40*)] reveals that methionine 78 from the soluble cytochrome c_{555} can be aligned to the methionine ligand of cytochromes *c* in mesophilic organisms (*9*). By contrast, two methionine residues five amino acids apart are present in the sequence of cytochrome c_{552} from *Thermus thermophilus*, similar to methionines 78 and 84 in the *A. aeolicus* cytochrome c_{555}^{s} sequence [see Figure 1 in the accompanying article (*40*)]. Intriguingly, the second methionine residue is involved in coordination of the heme iron in *T. thermophilus* (*3*). To identify the sixth ligand of heme iron, we have studied the soluble *A. aeolicus* cytochrome c_{555}^{s} in detail. First, the gene encoding cytochrome c_{555}^{s} was cloned, and its heterologous expression in *Escherichia coli* was achieved. The spectroscopic and physicochemical properties of the recombinant holocytochrome were analyzed. Second, two cytochrome c_{555} single-site mutants were constructed in which methionines 78 and 84 were replaced by a histidine residue, respectively. Electrochemical and spectroscopic studies were carried out in order to establish which methionine serves as the sixth ligand.

EXPERIMENTAL PROCEDURES

Strains, Vectors, and Media. The bacterial strains and plasmids used are described in Table 1. Growth of *E. coli* strains was carried out in LB medium (*10*), supplemented with the appropriate antibiotic at a final concentration of 0.27 mM for ampicillin and 31 μM for chloramphenicol when necessary and with δ-aminolevulinic acid (0.1 mM) when specified.

Cells from *A. aeolicus* (VF5) were used for DNA genomic preparation.

Biochemical Reagents. All restriction enzymes were obtained from Appligene. PCR was carried out using PWO DNA polymerase from Roche. Cloning oligonucleotides and DNA sequencing were purchased from Genome Express.

Cloning of the cycB2 Gene. The ORF Aq1550 encoding cytochrome c_{555}^{s} was amplified from *A. aeolicus* genomic DNA by PCR[1] using oligonucleotides PromcycB2 (5′-cctgaattcatgaagaagctcc-3′) and CtercycB2 (5′-gcctgcagcgctg-gaaagttcaag-3′) which introduced an *Eco*RI site immediately upstream from the start codon and a *Pst*I site downstream from the stop codon. In the special case of expression of the *cycB2* gene using the *A. aeolicus* endogenous promoter, the oligonucleotide NtercycB2 (5′-gcgaattcgggcaaagggctataac-3′) was used instead of PromcycB2. The 410 bp obtained by PCR was double digested by *Eco*RI−*Pst*I and subcloned into pJF119EH, cut with the same enzymes to obtain pJF119c_{555}. The sequence of the insert in the resulting plasmid was verified by DNA sequencing.

Site-Directed Mutagenesis. To replace Met-78 and Met-84 by histidine residues, mutations of the *cycB2* gene were introduced by PCR.

Oligonucleotide M78H (5′-gctggggcttgtgtatggcttcttttgc-3′) was synthesized in order to replace Met-78 by His-78 (mutation is underlined). Plasmid pJF119c_{555} was used as template in the PCR reaction. The mutated insert of 410 bp was then digested by *Eco*RI and *Pst*I and subcloned in pJF119EH previously cut with the same enzymes to obtain pJF119M78H. The mutation was checked by DNA sequencing.

In the same way, oligonucleotide M84H (5′-cggagagac-cctttaagtgggtgagctggggc-3′) was designed in order to replace Met-84 by His-84. The same protocol as described above was used to obtain pJF119M84H.

Protein Overproduction and Purification. Cultures (10 mL) of recombinant *E. coli* grown to the stationary phase were centrifuged at 3000*g* for 30 min. The cell pellet was treated to extract the periplasmic proteins using the lysozyme method as described previously (*11*). For cytochrome purification, cultures (20 L) of recombinant *E. coli* were grown at 37 °C until mid-log phase and then induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were harvested by centrifugation and were disrupted by a French press in 10 mM Tris-HCl, pH 7.6, containing a protease inhibitor cocktail from Roche Diagnostic Co. The crude extract was immediately heated

[1] Abbreviations: *E. coli*, *Escherichia coli*, kb, kilobase(s); PCR, polymerase chain reaction; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance; cytochrome c_{555}^{s}, soluble cytochrome c_{555}; cytochrome c_{555}^{m}, membranous cytochrome c_{555}.

Table 2: Characteristics of Wild-Type and Recombinant Cytochromes c_{555}^s

	NH_2 terminus	molecular mass (Da)	λ_{max} (nm) oxidized	λ_{max} (nm) reduced	redox potential (mV)	p*K*
A. aeolicus cyt c_{555}	A-D-G-K-A	9861 (±7)	413 695	418 523 550/555	210 (±10)	$pK_{ox1} = 4$ (±0.4) $pK_{ox2} = 8.4$ (±0.1) $pK_{red} = 5.2$ (±0.2)
A. aeolicus cyt c_{555} expressed in *E. coli*	A-D-G-K-A- and I-F-Q-Q-K	9861 (±7) 9425 (±7)	413 695	418 523 550/555	221 (±10)	$pK_{ox1} = 3.7$ (±0.4) $pK_{ox2} = 8.1$ (±0.1) $pK_{red} = 4.9$ (±0.2)

to 80 °C for 40 min. Following ultracentrifugation at 40000*g* for 2 h, the overexpressed protein was found in the supernatant. The soluble fraction was loaded onto a column of DEAE-cellulose (Whatman DE52) equilibrated with 10 mM Tris-HCl (pH 7.6). The cytochrome-containing fraction was not retained on the column. This fraction was concentrated using Centricon concentrators (Amicon) with YM-3 membranes and was then applied onto a hydroxyapatite (Bio-Rad) column equilibrated with 10 mM Tris-HCl (pH 7.6). Two fractions of pure cytochrome c_{555}^s were obtained: one eluting at 10 mM phosphate buffer (pH 7.6) and the other at 200 mM phosphate buffer (pH 7.6). The same purification protocol was applied to obtain M78H and M84H cytochromes c_{555}^s. The purity of the sample was analyzed by SDS−polyacrylamide gel electrophoresis (PhastSystem, Pharmacia).

Protein Sequencing. NH_2-terminal sequence determinations were performed with an Applied Biosystems A470 gas-phase sequencer. Quantitative determination of phenylthiohydantoin derivatives was done by high-pressure liquid chromatography (Waters) monitored by a data and chromatography control station (Waters Model 840).

Molecular Mass Determination. 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).

Ultraviolet and Visible Absorption Spectra. Spectra were recorded using a Kontron (model UV 922) spectrophotometer. Samples used for thermostability experiments contained 20 μM cytochrome in 50 mM Hepes, pH 7.6.

NMR Measurements. Samples of native, recombinant, and mutant cytochromes c_{555}^s were prepared in 0.1 M phosphate buffer, pH 7.6, containing 10% D_2O. The protein concentration was 0.1 mM. The 1D-NMR experiments were carried out on a Bruker Avance DRX 500, at 296 K, with the water line preirradiated.

Electrochemistry. See accompanying article (*40*). The protein concentration was 0.1 mM.

EPR Spectroscopy. See accompanying article (*40*). All experiments were performed with 0.1 mM cytochrome in 0.1 M phosphate buffer, pH 7.6.

RESULTS

Cloning and Heterologous Expression of the cycB2 Gene. The genome of *A. aeolicus* contains an open reading frame (ORF Aq1550) which apparently encodes for the soluble cytochrome c_{555}^s described in the accompanying article (*40*). The nucleotide sequence upstream from the first methionine codon contains elements similar to the *E. coli* σ^{70} consensus promoter sequence (*12*) including −10 (TATAAG) located 22 bases upstream from the methionine and −35 (TTGAAA) located 28 bases upstream from the TATAAG sequence. A nondegenerated ribosome binding site (AGGAGG) is found only 7 bases upstream from the first methionine codon.

PCR primers (NtercycB2 and CtercycB2) were designed to amplify the transcription unit and the coding sequence of the *cycB2* gene. Expression of the *cycB2* gene with its own promoter in an *E. coli* strain (MC1061), coexpressing the *ccmABCDEFGH* genes involved in cytochrome *c* maturation (*13*), has led to detectable amounts of cytochrome c_{555}^s in the periplasmic fraction. The production level of cytochrome reached was approximately 1 mg/L of culture calculated using the millimolar absorption for the α-band in the reduced state at 555 nm (21 mM^{-1} cm^{-1}).

To increase cytochrome production, we used a controllable strong *tac* promoter (*14*). The coding region of the *cycB2* gene was amplified by PCR using PromcycB2 and CtercycB2 primers. This fragment was ligated into the pJF119EH vector to allow expression of the *cycB2* gene from a strong IPTG-inducible *tac* promoter. This strategy led to the production of 10 times more periplasmic cytochrome c_{555}^s than was found with the endogenous *cycB2* promoter.

Characterization of Recombinant Cytochrome c_{555}^s. Pure cytochrome c_{555}^s (4 mg) was obtained at the final step for 1 L of growth medium. Two bands of cytochromes were estimated pure from the SDS−polyacrylamide gel. The NH_2-terminal sequence of the minority fraction was identical to that of the native protein purified from *A. aeolicus* [see accompanying article (*40*)], showing that the signal sequence was correctly cleaved in *E. coli* during the transport of the protein to the periplasm. This result is consistent with the values obtained from the mass spectrometry (9861 Da) for the native and the recombinant form. For the main fraction, the obtained molecular mass of 9425 Da was consistent with the loss of five amino acid residues (A-D-G-K-A) at the NH_2 terminus (Table 2). The truncated form accounts for about 75% of the total produced cytochrome c_{555}^s. It was characterized by spectroscopic techniques (Table 2) and compared to the native cytochrome. The "truncated" recombinant protein and native cytochrome exhibit the same bands in UV−visible absorption spectra in both redox states, identical redox potential, and a similar pH dependence of ionizable groups near the heme (Table 2). The EPR spectrum obtained on the recombinant protein was also identical to that of native protein [see accompanying article (*40*)].

1D-NMR was used to study the heme environment of native and recombinant protein. Figure 1 shows the downfield-shifted methyl proton resonances of the heme and the high-field-shifted methyl resonances of the axial ligand

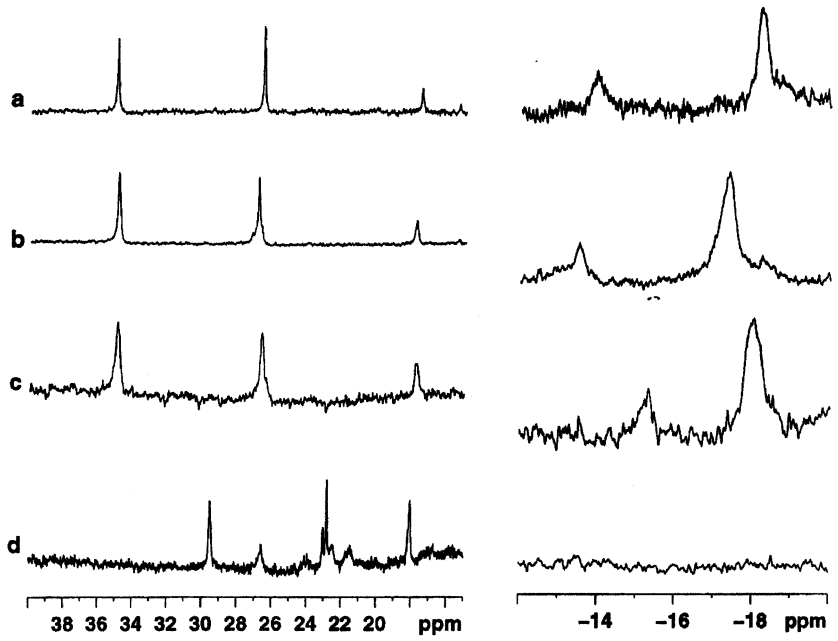

FIGURE 1: Paramagnetically shifted ^{1}H-NMR spectra of wild-type (a), recombinant (b), M84H (c), and M78H (d) ferricytochromes c_{555}^s.

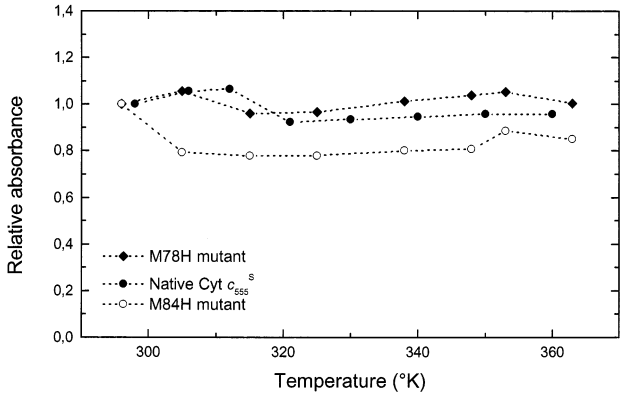

FIGURE 2: Thermal stability of wild-type (●), M84H (○), and M78H (◆) cytochromes c_{555}^s in the oxidized state as monitored by the Soret band. The samples contained 20 μM cytochrome in 50 mM Hepes, pH 7.4.

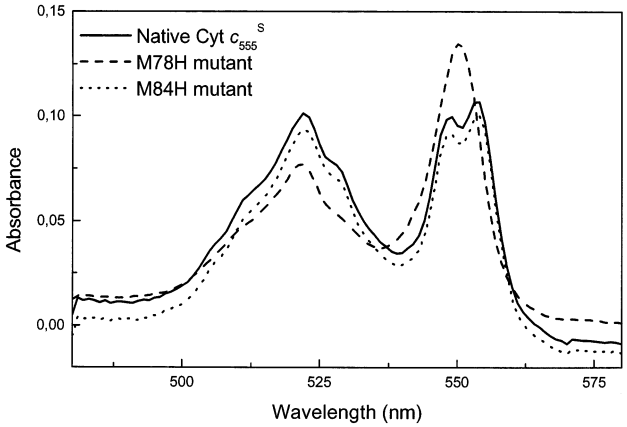

FIGURE 3: Visible absorption spectra from 450 to 600 nm of sodium dithionite-reduced native (—), M78H (- - -), and M84H (···) cytochromes c_{555}^s showing the α- and β-band region.

methionine from native (a) and recombinant (b) ferricytochrome c_{555}^s. The similarity of the two NMR spectra shows that the heme is correctly bound to the protein and that the recombinant cytochrome is well folded with respect to minor changes probably correlated to the five deleted residues.

These results showed that cytochrome c_{555}^s expressed in *E. coli* is structurally similar to the native protein apart from the loss of the five amino acid residues at the NH_2-terminal end.

Substitutions of Met-78 and Met-84. Two mutants of cytochromes c_{555}^s (M78H and M84H) were overproduced in *E. coli*, in a manner similar to that for the recombinant protein, and purified with the same ratio (about 4 mg/L) as the recombinant cytochrome c_{555}^s. The NH_2-terminal sequences of the mutants indicate that both proteins correspond to the truncated form of the recombinant cytochrome.

Stability against Thermal Denaturation. Optical changes can be used to follow the unfolding of cytochromes *c*. Figure 2 shows the amplitude of the Soret band of the M78H and M84H mutants and native cytochromes from 293 K up to 360 K, 360 K being the optimal growth temperature for *A. aeolicus*. The visible spectra of all three cytochromes are constant throughout this range of temperatures. Unfortunately, our experimental equipment did not allow to study the stability of native and mutant cytochromes at even higher temperature. A difference in stability between mutant and native cytochromes at $T > 360$ K can therefore not be ruled out.

Characterization of the M84H Mutant. Optical properties (Figure 3 and Table 3) and EPR spectra (Figure 4) of native and M84H cytochromes c_{555}^s were found to be very similar.

Figure 5 shows the upfield-shifted ^{1}H-NMR spectra of reduced native (a) and M84H mutant (b) cytochromes. The resonance at −3.21 ppm indicates that a methionine residue is the sixth ligand in the M84H cytochrome. Moreover, the similarity of the two ^{1}H-NMR spectra of native and M84H mutant ferricytochromes indicates that this mutation does not affect the environment of the protein.

Substitution of methionine 84 by a histidine residue results in a decrease in redox potential value of about 40 mV (Table 3). Three p*K* values, $pK_{ox1} = 3.7 \pm 0.4$, $pK_{red} = 4.9 \pm 0.2$, and $pK_{ox2} = 8.1 \pm 0.1$, have been found

Table 3: Determination of Spectral Features and Physicochemical Properties of Native, M78H, and M84H Cytochromes c_{555}^{s}

	λ_{max} (nm)		redox potential (mV)
	oxidized	reduced	
native cyt c_{555}	413	418	+221 (±10)
	695	523	
		550/555	
cyt c_{555} M78H	408	417	−168 (±10)
		523	
		550	
cyt c_{555} M84H	413	418	+181 (±10)
	695	523	
		550/555	

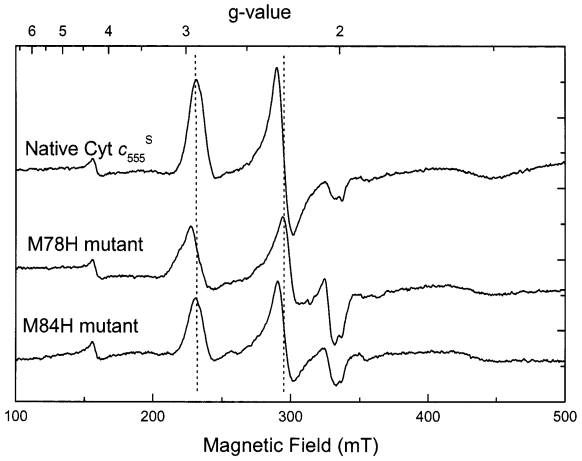

FIGURE 4: EPR spectra of native (top), M78H (middle), and M84H (bottom) ferricytochromes c_{555}^{s}.

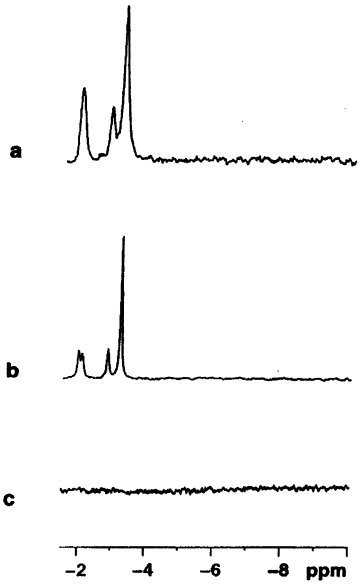

FIGURE 5: Upfield-shifted ^{1}H-NMR resonances for reduced native (a), M84H (b), and M78H (c) cytochromes c_{555}^{s}.

in the pH dependence of the redox midpoint potential in native cytochrome c_{555}^{s} (Figure 6). The E_m vs pH curve of the M84H mutant (Figure 6) also shows three pK values, all of which are slightly higher than in the native protein (pK_{ox1} = 5.1 ± 0.16, pK_{red} = 6.2 ± 0.1, and pK_{ox2} = 8.6 ± 0.1).

Characterization of the M78H Mutant. Optical properties of the M78H mutant (Figure 3) show significant shifts in the UV−visible part of the absorption spectrum of M78H

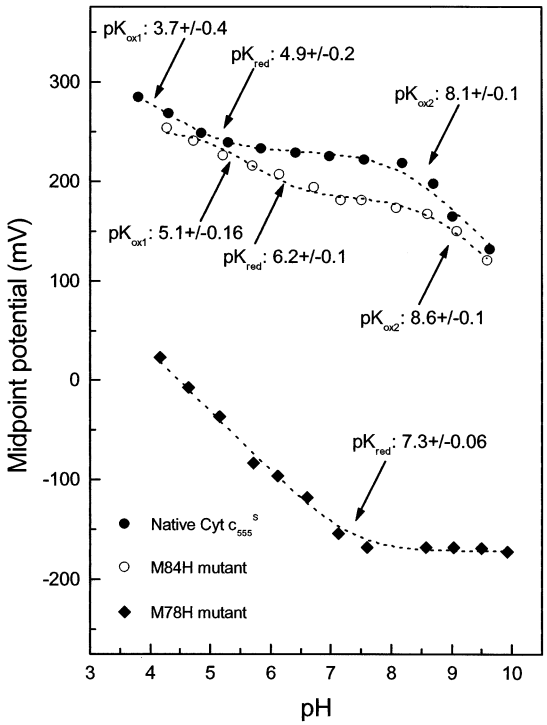

FIGURE 6: Variation of redox potential versus pH for wild-type (●), M84H (○), and M78H (◆) cytochromes c_{555}^{s}. pK_{ox1}, pK_{red}, and pK_{ox2} were obtained from experimental data fitted using relevant Nersnt equations derived from the method of Clark (*16*).

cytochrome c_{555}^{s}. In the spectrum of the oxidized M78H mutant, the Soret band is blue shifted, and the disappearance of the weak 695 nm band corresponding to the methionine as axial ligand is observed. In the reduced state, the main change concerns the loss of the split of the α-band, which is characteristic for native cytochrome c_{555}^{s}. The optical bands characteristic for five-coordinated heme are not observed in this mutant, indicating that a sixth ligand coordinates the heme iron. Accordingly, M78H cytochrome c_{555}^{s} exhibits a rhombic EPR spectrum (Figure 4) typical for a low-spin heme but with significant g value shifts (2.95, 2.23, and 1.52) as compared to the values obtained for the native and M84H mutant cytochromes (2.89, 2.287, and 1.52). A low-field shoulder on the g_z peak of this mutant furthermore indicates that the sample does not contain a homogeneous population of molecules. However, heterogeneity is not observed in the NMR spectra, indicating that the differing populations observed in EPR are probably due to low temperature (*15*). Figure 5 shows the ^{1}H-NMR spectra of reduced native (a) and M78H mutant (c) cytochromes. The resonance at −3.21 ppm assigned to the methyl protons of the axial methionine in the native and M84H mutant proteins disappears in the M78H mutant. Moreover, in the NMR spectrum of the oxidized form (Figure 1), large chemical shifts of the heme methyl resonances are observed, which confirms that the heme environment of this cytochrome is definitively different from that of the recombinant cytochrome. Electrochemical studies of the M78H mutant show a drastic decrease in the redox midpoint potential by about 390 mV (Table 3). The value of E'_0 = −168 mV is in the range of potentials observed for bihistidinyl ligated *c*-type cytochromes (*9*). The M78H mutant exhibits a pH dependence of redox potential which is described by only one pK of 7.3 in the reduced

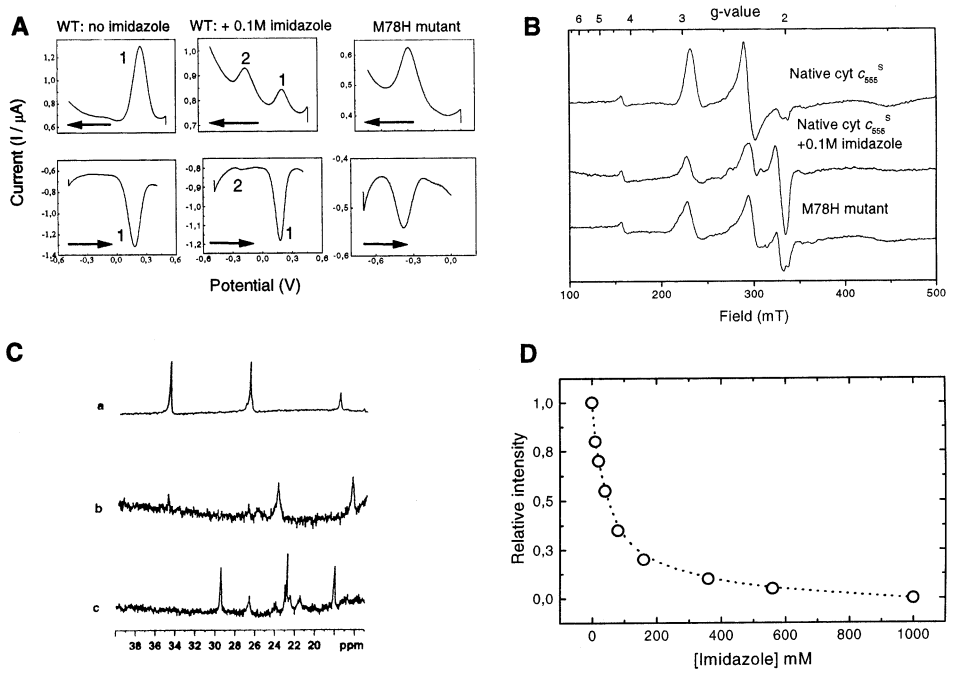

FIGURE 7: (A) Square-wave voltammograms of native cytochromes c_{555}^s in the absence (left) and in the presence (middle) of 0.1 M imidazole and of the M78H mutant (right). Potentials are versus Ag/AgCl. Positive scans were performed from $E_i = -500$ mV to $E_f = +400$ mV for wild type and from $E_i = -700$ mV to $E_f = 0$ mV for M78H. Negative scans were performed from $E_i = +400$ mV to $E_f = -500$ mV for wild type and from $E_i = 0$ mV to $E_f = -700$ mV for M78H. (B) EPR spectra of native cytochrome c_{555}^s in the absence of imidazole (top), native cytochrome in the presence of 0.1 M imidazole (middle), and the M78H mutant (bottom). (C) Paramagnetically shifted ^{1}H-NMR spectra of native ferricytochrome c_{555}^s in the absence (a) and in the presence (b) of 0.1 M imidazole and of the M78H mutant (c). (D) Relative intensity of the methyl proton of the methionine sixth ligand from the ^{1}H-NMR spectrum of wild-type ferricytochrome c_{555}^s at various concentrations of imidazole.

state (Figure 6). The potential is pH invariant over the pH range 8−10. Below pH 7, a linear E_m vs pH dependence is observed with a slope of about −60 mV/pH unit, which is characteristic of the involvement of one dissociable proton in the reduced state of the protein during redox transition (*16*). The loss of the p*K* between 8 and 9 on the oxidized form of M78H mutant generally observed in the wild-type protein, correlated to the axial methionine coordination in type I cytochromes *c*, should result from the substitution of Met-78 by His-78 at the sixth position.

Analysis of Imidazole Effect on the Axial Ligand of Native Cytochrome c_{555}^s. To definitively conclude on the sixth ligand in the M78H mutant, EPR, NMR, and electrochemical experiments have been performed in the presence of imidazole. It has been known for several decades that in cytochrome *c* the bond between the heme iron and the sulfur of the axial methionine is significantly weaker in the oxidized state than in the reduced state of the heme (*17*, *18*). Thus, exogenous ligands such as imidazole or cyanide are sometimes able to replace the methionine ligand in the oxidized protein (*19*).

Figure 7A compares the square-wave voltammetry of the native cytochrome in the absence and in the presence of 100 mM imidazole with that of the M78H mutant. Addition of imidazole induces the apparition of a new peak (peak 2 in Figure 7A) at a lower redox potential, −168 mV, close to the value obtained for the M78H mutant (Figure 7A, right panel). This peak is mainly present when the potential is swept from the oxidized state to the reduced state of the heme (see Figure 7A in the presence of imidazole). This peak is attributed to the coordination of the exogenous imidazole to the heme iron. Values in the same order of magnitude were equally reported for exogenous imidazole binding to the oxidized state of horse heart cytochrome *c* ($E'_0 = -178$ vs +48 mV for native protein; *20*) as well as yeast iso-1 cytochrome ($E'_0 = -126$ vs +269 mV for native protein; *21*). When the cytochrome changes from the reduced state to the oxidized state (Figure 7A, middle panel, bottom), peak 1 at +220 mV corresponding to the native form with methionine as the sixth axial ligand is mainly observed (left panel, bottom, peak 1). The corresponding cyclic voltammograms obtained in the presence of imidazole have been shown to be strongly dependent on sweep rate (data not shown). In particular, a quasi-reversible couple not observed at low scan rate (20 mV·s^{-1}) is detected at high sweep rate (1 V·s^{-1}), at $E_{pc} = -258$ mV and $E_{pa} = -120$ mV. It is qualitatively consistent with an E_rC_i coupled electrochemical reaction (*22*) as observed in previous work on cytochrome *c* (*21*, *23*). This implies that the less stable reduced imidazole-ligated form converts to the reduced methionine-ligated form. The ligand-exchange rate constant has been estimated to be 3 ± 1 s^{-1}.

EPR spectra obtained from native ferricytochrome c_{555}^s in the presence of 100 mM imidazole exhibit *g* values shifted similar to those of the M78H mutant (Figure 7B). In the ^{1}H-NMR spectra the presence of imidazole induces large chemicals shifts of the heme methyl proton resonances in the oxidized form (Figure 7C) and the disappearance of the signal at −18 ppm assigned to the methionine axial ligand. The values of 23.91 and 16.45 ppm obtained are, however, different from those observed in the NMR spectrum of M78H mutant (29.5, 22.82, and 18.06 ppm), suggesting a specific environment due to the heme iron coordination by the exogenous imidazole. The titration of the effect of imidazole

on the NMR spectrum of native ferricytochrome c_{555}^{s} is shown in Figure 7D. The intensity of the methyl line of the sixth ligand (methionine 78) is observed at various concentrations of imidazole. The decrease of the intensity demonstrates the dissociation of the sixth ligand, which is in agreement with the low-field part of the NMR spectrum of the oxidized cytochrome. However, the EPR spectra indicate that the sixth heme iron coordination is filled. The low-field part of the ferricytochrome c_{555}^{s} NMR spectrum is definitively different from that of the M84H mutant, indicating that in these conditions imidazole replaces Met-78 and not Met-84.

In conclusion, the presence of imidazole in native cytochrome mimics the ligation pattern of the M78H mutant, further corroborating the assignment of histidine 78 as the sixth ligand in the M78H mutant.

DISCUSSION

The unusual presence of two conserved methionine residues at the C-terminal part of the cytochrome c_{555}^{s} was investigated by site-directed mutagenesis, with the aim to give evidence of the axial ligands of the heme iron and to quantify the role of these two residues in the protein.

A prerequisite of such studies is that the gene encoding the protein of interest must be expressed and correctly matured in a host organism. The organism usually chosen for heterologous production of foreign proteins is *E. coli*. The maturation of soluble cytochromes necessitates complex posttranslational events to covalently bind the heme cofactor in the periplasmic space (*13*). Unfortunately, attempts of heterologous production of cytochromes *c* from various mesophilic organisms in *E. coli* have produced only a few successful examples (*11*, *24*−*27*). In the vast majority of such works, either the recombinant protein exhibited properties different from those of the native cytochrome or obtained yields were too low for biophysical experiments. In the special case of heterologous production of cytochromes *c* from the thermophilic organisms *T. thermophilus* and *H. thermophilus*, maturation was shown to be very unusual because it occurs in the cytoplasm of *E. coli* rather than the periplasm (*28*, *29*). This property is unique to the thermophilic cytochromes *c* and seems to be due to the spontaneous cytoplasmic attachment of the heme to the reduced apoprotein (*28*, *29*). However, similar to what has been reported for cytochromes of mesophilic organisms, only a small fraction of recombinant protein exhibited structural features identical to those of the native protein (*29*, *30*).

Finally, the best results for heterologous production of cytochromes *c* were obtained by coexpressing in *E. coli* the *ccmABCDEFGH* genes implied in the cytochrome *c* maturation process (*13*, *31*, *32*). To our knowledge, this study constitutes the first example of overproduction of hyperthermophilic class I *c*-type cytochrome.

Analysis of the *cycB2* gene encoding cytochrome c_{555}^{s} reveals putative transcription and translation signals which fit the σ_{70} *E. coli* consensus promoter sequences well. This observation was confirmed by the ability of cytochrome c_{555}^{s} to be overproduced in *E. coli* from its endogenous promoter. The presence of transcription signals downstream and upstream from the gene coding region strongly suggests that the gene is monocistronic as is the case for the gene encoding cytochrome c_{552} from *H. thermophilus* (*33*). The situation is opposite to that found for the *cycA* gene encoding cytochrome c_{552} from *T. thermophilus*, which seems to be in the same operon as a gene encoding an ABC transporter (*29*). However, these two latter cytochromes seem to function as electron donors to terminal oxidases.

Sequence analysis of the *cycB2* gene shows that the protein is synthesized as a precursor of 104 amino acid residues. This forms differs from the purified cytochrome c_{555}^{s} [see the accompanying article (*40*)] in the NH_2-terminal sequence, which included 17 additional amino acid residues. Since this extra amino-terminal sequence has the typical features of a signal peptide used for export of proteins to the periplasm in other known apocytochromes (*9*), we decided to keep the endogenous signal sequence to target the cytochrome to the periplasm. Our results show that recombinant cytochrome c_{555}^{s} is correctly addressed to the *E. coli* periplasm but a major fraction of the mature protein contains an unusual NH_2 terminus shortened by 5 amino acid residues. A minority fraction contains an NH_2 terminus identical to that of the mature native cytochrome. Computer analysis of the apoprotein primary sequence predicts, however, only one cleavage site for the peptidase between residues 17 and 18, which is in agreement with the NH_2-terminal sequence of the mature protein isolated from *A. aeolicus*. On the basis of the predicted secondary structure of cytochrome c_{555}^{s} [Figure 2, accompanying paper (*40*)], one might expect that the first five residues are not involved in the N-terminal helix of the protein and are then accessible to *E. coli* proteases. This is consistent with the identical structural properties observed for wild-type and recombinant cytochromes despite loss of the first five residues in the recombinant cytochrome. It is noteworthy that heterologous production of cytochrome c_{552} from *T. thermophilus* with the help of the *ccm* maturation genes also induced a loss of 2 amino acid residues from its N-terminus (*30*). The authors, in this case, attributed the error in the signal peptide cleavage to an amino acid mutation introduced in the sequence signal that modified the cleavage site.

The yield of cytochrome c_{555}^{s} overproduction obtained by our method allows protein engineering studies. Spectroscopic and physicochemical properties suggest that the M84H mutant has the same folding as native cytochrome and the respective mutation does not induce drastic structural changes. In contrast, replacement of the methionine residue by a histidine residue in position 78 results in spectacular changes in terms of spectroscopic and biophysical properties. A combination of spectroscopic experiments (optical, EPR, ^{1}H-NMR) allows us to demonstrate convincingly that H78 is the sixth ligand of the heme iron whatever the oxidation−reduction state of the M78H mutant and that methionine 78 is the axial ligand of wild-type cytochrome c_{555}.

Extensive studies on the influence of the axial ligand on redox potential have been reported (*9*, *34*, *35*). It is clear that the electron donor−acceptor potential of the axial ligand influences the E_m value. Histidine, which is a good electron donor, tends to stabilize the oxidized state and results in low redox potentials. In contrast, the methionine which is a good electron acceptor favors the reduced state and is correlated to high redox potentials. Our data are in agreement with this model since substitution of axial methionine by histidine shifts the redox potential by 380 mV to more negative values.

Moore and Pettigrew (*9*) estimated that sulfur versus histidine ligation should account for a redox potential difference of 160 mV. The drop of redox potential value reported here is significantly more pronounced than has been predicted. Moreover, to our knowledge, such a strong drop in redox midpoint potential induced by the change of an endogenous axial ligand is extremely rare. Substitution of the methionine axial ligand by histidine in semisynthetic horse heart cytochrome *c* or in cytochrome c_{551} from *Pseudomonas* gave rise to a 200 mV decrease of redox potential (*35*, *36*). In the opposite direction, replacement of histidine by methionine in cytochrome c_3 led to an increase of redox potential values by about 250 mV (*34*). In yeast Phe82His iso-1 cytochrome, however, a drop of 377 mV is attributed to His-His coordination, with His/His coordination predominating in the oxidized state and a further shift of the redox potential away from wild type resulting from the kinetics of the axial ligand exchange (*21*). Intramolecular rearrangements in the vicinity of the heme are a further factor modulating redox properties. In regard to a slight modification of the redox potential value (221 to 181 mV) for the M84H mutation, similar effects of mutating a residue near the coordinating methionine have been discussed by Rafferty et al. (*37*) to be due to the perturbation of the heme crevice and the nearby coordinating axial methionine ligand. In the present work the M84H mutation does not change the axial ligand, which is still Met-78, but affects the redox potential of the molecule. Such minor variations in redox potential value are frequently observed in cytochromes *c* when single mutations are performed close to the heme of the protein and are probably due to perturbations of the hydrogen-bonding network of the heme pocket. In the M78H mutant a combination of the axial ligand replacement and a perturbation of the hydrogen-bonding network may account for the exceptional strong decrease in redox potential. The determination of the three-dimensional structures of both native and mutant cytochromes may help to clarify the mutual importance of these factors.

Proteins from hyperthermophilic organisms are important tools in biochemistry. The molecular determinants of their thermostability are, however, not yet well understood. Recently, genomic and structural approaches have been used to identify changes in the frequencies of particular amino acids from hyperthermophilic and mesophilic organisms (*38*). These global studies are valuable but must be combined to mutational studies in order to determine the putative effect of each amino acid residue substitution on the protein thermostability. Because of the large number of known primary sequences of cytochromes *c* from mesophilic, thermophilic, and now hyperthermophilic organisms, their small size and the large number of resolved three-dimensional structures render class I *c*-type cytochromes an excellent model for identifying the origins of thermostability at the molecular level.

Preliminary experiments performed on the thermal stability of mutant cytochromes indicate that substitutions of methionine residues do not affect the stability of the molecule up to 90 °C. It is, however, clear that this study constitutes only the first step in the investigation of the involvement of both methionine residues in the hyperthermostability of the cytochrome c_{555}^s. We are now working actively to adapt all of our spectroscopic and biophysical techniques to perform experiments at the physiological temperature of *A. aeolicus* (about 90 °C). A detailed study of thermal denaturation of native and mutant cytochromes using circular dichroism and differential scanning calorimetry is also in progress.

One striking feature is the presence of another methionine (Met-84) in addition to the sixth ligand methionine (Met-78) in both cytochromes c_{555} from *A. aeolicus*. Methionine 84 is positioned in a region of extensive primary sequence differences between mesophilic and hyperthermophilic cytochromes *c*. The structure prediction suggests that this residue is located in a loop only observed in cytochromes c_{555} and cytochrome c_{552} from *T. thermophilus* [see Figure 1 in the accompanying article (*40*)]. One may speculate that the presence of this supplementary loop would be correlated to the hyperthermostability of the cytochrome. Our interest will now be focused on the putative contribution of this loop to the stability of the protein and more generally on the factors responsible for hyperthermostability of cytochromes c_{555}^s.

This study constitutes the first example of successful heterologous production of a hyperthermophile class I *c*-type cytochrome. It is a prerequisite step for understanding the function of this molecule and the factors responsible for its thermal stabilization. Site-directed mutagenesis of both methionine residues constitutes a first investigation of these factors.

ACKNOWLEDGMENT

We thank L. Thöny-Meyer for providing us with the *E. coli* MC1061(pEC86) strain; M. Bauzan and R. Toci for large-scale cultures; J. Bonicel for mass spectrum determination, W. Nitschke for recording the EPR spectra and for helpful discussions; O. Bornet for recording the ^{1}H-NMR spectra; and M. T. Giudici-Orticoni, F. Baymann, and A. Dolla for critical reading of the manuscript.

REFERENCES

1. Szilagyi, A., and Zavodsky, P. (2000) *Structure 8*, 493−504.
2. Fujii, T., Hata, Y., Wakagi, T., Tanaka, N., and Oshima, T. (1996) *Nat. Struct. Biol. 3*, 834−837.
3. Than, M. N., Hof, P., Huber, R., Bourenkov, G. P., Bartunik, H. D., Buse, G., and Soulimane, T. (1997) *J. Mol. Biol. 271*, 629−644.
4. Hasegawa, J., Yoshida, T., Yamazaki, T., Sambongi, Y., Yu, Y., Igarashi, Y., Kodama, T., Yamazaki, K.-I., Kyogoku, Y., and Kobayashi, Y. (1998) *Biochemistry 37*, 9641−9649.
5. Hasegawa, J., Shimahara, H., Yamazaki, T., Mizutani, M., Uchiyama, S., Arai, H., Ishii, M., Kobayashi, Y., Ferguson, S. J., Kobayashi, Y., and Igarashi, Y. (1999) *J. Biol. Chem. 274*, 37533−37537.
6. Hasegawa, J., Uchiyama, S., Tanimoto, Y., Mizutani, M., Kobayashi, Y., Sambongi, Y., and Igarashi, Y. (2000) *J. Biol. Chem. 275*, 37824−37828.
7. Huber, R., Wilharm, T., Huber, D., Trincone, A., Burggraf, S., König, H., Rachel, R., Rockinger, I., Fricke, H., and Stetter, K. O. (1992) *Syst. Appl. Microbiol. 15*, 340−351.
8. Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Graham, D. E., Overbeek, R., Snead, M. A., Keller, M., Aujay, M., Huber, R., Feldman, R. A., Short, J. M., Olsen, G. J., and Swanson, R. V. (1998) *Nature 392*, 353−358.
9. Moore, R. G., and Pettigrew, G. W. (1990) in *Cytochromes c. Evolutionary, Structural and Physicochemical Aspects*, Springer-Verlag, Berlin.
10. Maniatis, T., Fritsh, E., and Sambrook, J. (1982) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

11. Pollock, W. B. R., and Voordouw, G. (1994) *Microbiology 140*, 879−887.
12. McClure, W. R. (1985) *Annu. Rev. Biochem. 54*, 171−204.
13. Thöny-Meyer, L. (2000) *Biochim. Biophys. Acta 1459*, 316−324.
14. Fürste, J. P., Pansegrau, W., Frank, R., Blöcker, H., Scholz, P., Bagdasarian, M., and Lanka, E. (1986) *Gene 48*, 119−131.
15. Yometani, T., and Anni, H. (1987) *J. Biol. Chem. 262*, 9547−9554.
16. Clark, W. M. (1960) in *Oxidation reduction potential of organic systems*, Williams and Wilkins, Baltimore, MD.
17. Margoliash, E., and Schejter, A. (1966) *Adv. Protein Chem. 21*, 126−213.
18. George, P., and Schejter, A. (1964) *J. Biol. Chem. 239*, 1504−1508.
19. Schejter, A., and Aviram, I. (1969) *Biochemistry 8*, 149−153.
20. Liu, G., Shao, W., Zhu, S., and Tang, W. (1995) *J. Inorg. Biochem. 60*, 123−131.
21. Feinberg, B. A., Liu, X., Ryan, M. D., Scheijter, A., Zhang, C., and Margoliash, E. (1998) *Biochemistry 37*, 13091−13101.
22. Nicholson, R. S., and Shain, I. (1964) *Anal. Chem. 36*, 706−723.
23. Barker, P. D., and Mauk, A. G. (1992) *J. Am. Chem. Soc. 114*, 3619−3624.
24. Grisshammer, R., Oeckl, C., and Michel, H. (1991) *Biochim. Biophys. Acta 1088*, 183−190.
25. Ubbink, M., Van Beeumen, J., and Canters, G. W. (1992) *J. Bacteriol. 174*, 3707−3714.
26. Bott, M., Thöny-Meyer, L., Loferer, H., Rossbach, S., Tully, R. E., Keister, D., Appleby, C. A., and Hennecke, H. (1995) *J. Bacteriol. 177*, 2214−2217.
27. Sambogi, Y., Stoll, R., and Ferguson, S. J. (1996) *Mol. Microbiol. 19*, 1193−1204.
28. Sambongi, Y., and Ferguson, S. J. (1994) *FEBS Lett. 340*, 65−79.
29. Keightley, J. A., Sanders, D., Todaro, T. R., Pastuszyn, A., and Fee, J. A. (1998) *J. Biol. Chem. 273*, 12006−12016.
30. Fee, J. A., Chen, Y., Todaro, T. R., Bren, K. L. Patel, K. M., Hill, M. G., Gomez-Moran, E., Loehr, T. M., Ai, J., Thöny-Meyer, L., Williams, P. A., Stura, E., Sridhar, V., and McRee, D. E. (2000) *Protein Sci. 9*, 2074−2084.
31. Grove, J., Tanapongpipat, S., Thomas, G., Griffiths, L., Crooke, H., and Cole, J. (1996) *Mol. Microbiol. 19*, 467−481.
32. Herbaud, M.-L., Aubert, C., Durand, M.-C., Guerlesquin, F., Thöny-Meyer, L., and Dolla, A. (2000) *Biochim. Biophys. Acta 1481*, 18−24.
33. Sanbongi, Y., Yang, J.-H., Igarashi, Y., and Kodama, T. (1991) *Eur. J. Biochem. 198*, 7−12.
34. Dolla, A., Florens, L., Bianco, P., Haladjian, J., Voordouw, G., Forest, E., Wall, J., Guerlesquin, F., and Bruschi, M. (1994) *J. Biol. Chem. 269*, 6340−6346.
35. Miller, G. T., Zhang, B., Hardman, J. K., and Timkovich, R. (2000) *Biochemistry 39*, 9010−9017.
36. Raphaël, A. L., and Gray, H. B. (1991) *J. Am. Chem. Soc. 113*, 1038−1040.
37. Rafferty, S. P., Pearce, L. L., Barker, P. D., Guillemette J. G., Kay, C. M., Smith M., and Mauk, A. G. (1990) *Biochemistry 29*, 9365−9369.
38. Cambillau, C., and Claverie, J. M. (2000) *J. Biol. Chem. 275*, 32383−32836.
39. Arslan, E., Schulz, H., Zuffery, R., Zünkler, L., and Thöny-Meyer, L. (1998) *Biochem. Biophys. Res. Commun. 251*, 744−747.
40. Baymann, F., Tron, P., Schoepp-Cothenet, B., Aubert, C., Bianco, P., Stetter, K.-O., Nitschke, W., and Schütz, M. (2001) *Biochemistry 40*, 13681−13689.

BI011202Q