Bacterial Expression of a Mitochondrial Cytochrome *c*. Trimethylation of Lys72 in Yeast *iso*-1-Cytochrome *c* and the Alkaline Conformational Transition[†]

W. Brent R. Pollock,[‡,§] Federico I. Rosell,[‡] Mark B. Twitchett,[‡] Mark E. Dumont,[||] and A. Grant Mauk*[,‡]

Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, and Department of Biochemistry, University of Rochester, Rochester, New York 14627

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ABSTRACT: *Saccharomyces cerevisiae iso*-1-cytochrome *c* has been expressed in *Escherichia coli* by coexpression of the genes encoding the cytochrome (*CYC1*) and yeast cytochrome *c* heme lyase (*CYC3*). Construction of this expression system involved cloning the two genes in parallel into the vector pUC18 to give the plasmid pBPCYC1(wt)/3. Transcription was directed by two promoters, *Lac* and *Trc*, that were located upstream from *CYC1*. Both proteins were expressed in the cytoplasm of *E. coli* cells harboring the plasmid. Semianaerobic cultures grown in a fermentor produced 15 mg of recombinant *iso*-1-cytochrome *c* per liter of culture. Attempts to increase production by addition of IPTG suppressed the number of copies of the *CYC1* gene within the population. Wild-type *iso*-1-cytochrome *c* expressed with pBPCYC1(wt)/3 in *E. coli* was compared to the same protein expressed in yeast. At neutral pH, the two proteins exhibit indistinguishable spectroscopic and physical (T_m, E_m') characteristics. However, electrospray mass spectrometry revealed that the lysyl residue at position 72 is not trimethylated by *E. coli* as it is by *S. cerevisiae*. Interestingly, the pK_a of the alkaline transition of the protein expressed in *E. coli* is ∼0.6 pK_a unit lower than that observed for the cytochrome expressed in yeast (8.5−8.7). ^{1}H NMR spectroscopy of the bacterially expressed cytochrome collected at high pH revealed the presence of a third alkaline conformer that is not observed in the corresponding spectrum of the cytochrome expressed in yeast. These observations suggest that Lys72 can serve as an axial ligand to the heme iron of alkaline *iso*-1-ferricytochrome *c* if it is not modified posttranscriptionally to trimethyllysine.

The conventional method for producing wild-type and variant forms of recombinant mitochondrial cytochromes *c* relies on the use of the yeast *Saccharomyces cerevisiae* as the host organism. This organism has been used for both homologous (*1*) and heterologous expression (*2*−*4*) of cytochromes with varying degrees of success. A characteristic of this system is that the strain of yeast normally employed must be devoid of both *iso*-1- and *iso*-2-cytochromes *c*. Selective pressure for the yeast expressing the recombinant cytochrome is applied on the basis of their ability to grow on nonfermentable carbon sources such as glycerol or lactate. As a result, only cytochrome variants that are sufficiently functional in respiration to support yeast growth can be prepared from this system. An advantage of this approach is that it ensures that the recombinant protein is not contaminated with the endogenous cytochromes that may be difficult to remove by available purification techniques. To investigate the properties of physiologically nonfunctional cytochromes *c*, investigators have developed semisynthesis methods (e.g., *5*, *6*). Alternatively, Lu et al. (*7*) have described the expression of the nonfunctional Met80Ala *iso*-1-cytochrome *c* variant in yeast through use of a plasmid that also contained the gene coding for the Leu58His variant of the protein. This latter variant supported cell growth and thereby permitted the concurrent expression of the nonfunctional axial ligand variant. Separation of the wild-type and variant cytochromes was facilitated by selective retention of the Leu58His variant on a Zn-chelate resin.

Expression of cytochrome *c* in *Escherichia coli* is an attractive alternative because bacterial growth is significantly faster than that of yeast, expression of recombinant proteins is often greater than achieved in yeast, physiologically nonfunctional variants could be expressed, and facile isotopic enrichment might be achieved. Previous success in heterologous expression of *c*-type cytochromes in *E. coli* has been variable (*8*−*16*) and uniformly less efficient than observed for expression of other heme proteins (*17*−*26*). The difficulties involved in bacterial expression of cytochrome *c* are presumably attributable to the more complex posttranscriptional processing required by this protein. The current model of *c*-type cytochrome biosynthesis involves several steps: (i) cytoplasmic synthesis of the apo-protein; (ii) excision of the *N*-terminal Met; (iii) export of the heme binding site -C-X-X-C-H- across a membrane; and (iv) covalent attachment of the heme (in the form of α-thioether bonds between the sulfhydryl groups of the protein heme binding site and the vinyl groups of the heme) to produce the native protein. In mitochondria, this last step is known to require the presence of cytochrome *c* heme lyase (*27*). Despite extensive genetic work (*28*−*34*), an enzyme ho-

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[‡] University of British Columbia.

[§] Recipient of an NSERC Postdoctoral Fellowship.

[||] University of Rochester.

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mologous to this heme lyase has yet to be identified in prokaryotes.

Although *c*-type heme attachment may not require enzymatic catalysis in all cases (e.g., *35*, *36*), biosynthetic and structural considerations suggest that successful expression of native *iso*-1-cytochrome *c* requires coexpression of C*c*HL[1] that is encoded by *CYC3* (*27*). In *E. coli*, any native C*c*HL activity is expected to reside in the periplasm (*31*, *37*). Accordingly, all known bacterial genes for *c*-type cytochromes encode an apo-protein that carries an *N*-terminal sequence that directs export of the heme-binding domain across the cytoplasmic membrane. Because the recombinant *CYC1* does not encode a signal peptide for bacterial export, the apo-protein probably remains in the cytoplasm where it is largely inaccessible to any endogenous C*c*HL. Alternatively, heme attachment would have to take place in the cytoplasm in an uncatalyzed manner as seen with the Asn57Cys variant of cytochrome b_5 (*35*). When two other cytochromes, c_{550} from *Paracoccus denitrificans* and c_{552} from *Hydrogenobacter thermophilus*, were examined for expression in the cytoplasm of *E. coli* (*13*), only cytochrome c_{552} was recovered as the holo-protein. Presumably, apocytochrome c_{552} binds hemin with high affinity and facilitates its attachment in the absence of a heme lyase. For these reasons, the present work evaluates the efficacy of expressing the mitochondrial yeast *iso*-1-cytochrome *c* in *E. coli* through coexpression of the genes encoding the cytochrome and C*c*HL. The cytochrome produced by this system has then been characterized by a combination of spectroscopic and physical methods.

EXPERIMENTAL PROCEDURES

Molecular Genetics Procedures. Recombinant DNA manipulations were carried out essentially as described by Sambrook et al. (*38*). Restriction and DNA modifying enzymes were purchased from Pharmacia Biotech, Promega Corp., Gibco BRL, or Sigma-Aldrich Canada Ltd. Mutagenesis products were analyzed by DNA sequencing using a Sequenase V.2.0.1 kit and [α^{35}S]dATP (Amersham Life Sciences Inc.). 5′-Phosphorylated DNA primers were synthesized by the NAPS Unit at the University of British Columbia. A *Bam*HI linker made by annealing two copies of the oligonucleotide (5′-CGGATCCG-3′), and *E. coli* strain HB2151 were gifts from Professor M. Smith.

Construction of pBPCYC1(wt)/3. The *CYC1* gene used here encodes a Thr at position 102 instead of the wild-type Cys residue to limit autoreduction of the ferricytochrome and to avoid protein dimerization through disulfide bond formation (*39*). This gene was mutated in the phagemid pING4 (*40*) with the primer 5′-GCCTTGAATTCAGCCATGGTTAATTTAGTGTGTG-3′ by the method of Kunkel and co-workers (*41*). This mutation introduced an *Nco*I site encompassing the initiation ATG and mutated the gene to encode for Ala in place of Thr at position −5 (vertebrate cytochrome numbering scheme). The mutated phagemid was digested with *Eco*RV, and the *Bam*HI linker was inserted by blunt-end ligation to produce pINGBP3E-B. After digestion of pINGBP3E-B with *Nco*I and *Bam*HI, the 350 bp fragment which contained the *CYC1* gene was subcloned into the same sites of pCE820 (*42*) to produce the plasmid pCECYC1E-B. A 1.2 kbp *Hin*dIII−*Bam*HI fragment containing the *CYC3* gene was isolated from pER530/2 (*43*) and subcloned into the *Hin*dIII/*Bam*HI sites of pCECYC1E-B to yield pCECYC1(T-5A)/3. After digestion with *Hin*dIII and *Eco*RV, the fragment containing both the *CYC1* and *CYC3* genes was subcloned into the *Hin*dIII−*Sma*I fragment of pUC18 to generate pBPCYC1(T-5A)/3 (Figure 1). The Thr−5Ala mutation in pBPCYC1(T-5A)/3 was reverted by the method of Kunkel (*42*) with the primer 5′-TGAATTCAGTCATGGTTCT-3′ and M13CYC1(T-5A) as the template DNA. The reverted *CYC1* gene in M13CYC1(wt) was subcloned into pBPCYC1(T-5A)/3 using the *Sac*I/*Bam*HI restriction sites. A variation of pBPCYC1(wt)/3, denoted as pBPCYC1(wt)/CYC3 (Figure 1), contains an additional *lac* promotor between *CYC*1 and *CYC*3.

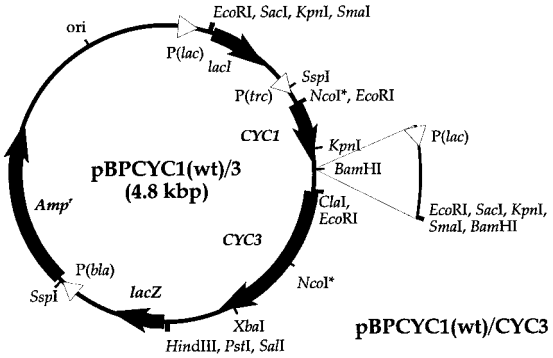

FIGURE 1: Physical map of pBPCYC1(wt)/3 for the expression of yeast *iso*-1-cytochrome *c* in bacteria. The genes encoding cytochrome *c* (*CYC1*), C*c*HL (*CYC3*), and the *β*-lactamase (*Amp*^{r}) are shown as the solid arrows. Promoters are denoted as the open triangles pointing in the direction of transcription. Selected restriction endonuclease sites are shown in sequence following the direction of gene transcription. The *Nco*I restriction site at the beginning of *CYC1* is not present in the plasmid encoding the wild-type protein. pBPCYC1(wt)/CYC3 is essentially the same as pBPCYC1(wt)/3 with an additional *lac* promoter as illustrated.

Plasmid pBPCYC1(wt) was constructed by religation of the 3.6 kbp *Bam*HI−*Sal*I fragment of pBpCYC1(wt)/3 following end-filling. The *CYC3*-containing *Bam*HI−*Hin*dIII fragment (1.3 kbp) from pCECYC1(T-5A)/3 was subcloned into the same restriction sites of the 2.65 kbp fragment from pUC18 to yield the plasmid pBPCYC3.

The Tml72Ala variant was generated with the phagemid pBTR1 (*44*) by the method of Deng and Nickoloff (*45*) with *E. coli* BMH71-18 *mut*S (5Prime-3Prime, Inc.). One synthetic oligonucleotide (5′-CAGGAATATATTTCGCTGGGTTAG-3′) introduced the Lys72Ala mutation while a second (5′-CCTTGAATTCAGTCATGGATATATCT-3′) reverted the Thr5Ala mutation and removed the unique *Nco*I site at the beginning of the *CYC1* gene.

Cultivation of Bacteria. Medium scale cultures were initiated with a single, freshly transformed colony of *E. coli* that served as the inoculum for 3 mL of superbroth (*38*) supplemented with 100 mg/L ampicillin and glycerol to 0.1% (v/v) (SBamp). After incubation overnight at 37 °C with vigorous shaking (300 rpm, New Brunswick Model Innova 4300 platform shaker), 400 μL was used to inoculate 50 mL of SBamp in a 250 mL flask. This culture was incubated for 12−15 h, and a minimum of 10 mL was used to inoculate

[1] Abbreviations: CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; C*c*HL, cytochrome *c* heme lyase; E_m', midpoint potential; IPTG, isopropyl *β*-D-galactopyranoside; kbp, kilobase pair; SHE, standard hydrogen electrode; T_m, melting temperature; Tml, trimethyllysine.

1.5 L of SBamp in a 2 L flask. These flasks were incubated for 36−48 h before harvesting the cells by centrifugation (Sorvall GS3 rotor, 8000 rpm, 20 min). For large-scale cultures, a single 12−15 h colony was used to inoculate 100 mL of YTamp (*38*) in a 250 mL flask. This culture was incubated for 8 h and then diluted 100-fold with SBamp in a Chemap FZ3000 fermentor. The resulting culture was incubated at 37 °C for 48 h before harvesting the *E. coli* with a Sharples continuous-flow centrifuge.

Isolation of Recombinant Cytochromes c. Cell pastes were incubated at room temperature in lysis buffer (50 mM Tris, 1 mM EDTA, pH 8, with 3 g/L lysozyme and a few crystals of DNase I and RNase A). Cell pastes from large-scale fermentation cultures (10 L) were frozen prior to treatment with lysis buffer. The mixture of cell paste and lysis buffer was stirred continuously until smooth, cooled on ice, and passed once through a precooled French press (1500 psi). This lysate was centrifuged (Sorvall GS3 rotor, 8000 rpm, 30 min), and the pellet was washed with lysis buffer. Centrifugation and pellet washing were repeated to minimize the pink color of the pellet. $(NH_4)_2SO_4$ was added to a concentration of 326 g/L of pooled supernatant fluid while stirring on ice. Following recentrifugation, the supernatant fluid was dialyzed (Spectrapor, 6000−8000 M_r cutoff) overnight in either 10 (medium-scale) or 40 L (large-scale) of buffer A (46 mM sodium phosphate, pH 6.8) to reduce the resistivity of the solution below 7000 $\mu\Omega^{-1}$ cm^{-1}. The resulting dialysate was cleared by centrifugation prior to application to a column of CM-Sepharose CL-6B (2.5 × 6 cm) (Pharmacia) equilibrated with buffer A. After the column was loaded, it was washed with 1 volume of buffer A followed by 1 volume of buffer B (A plus 75 mM NaCl). The recombinant cytochrome was eluted with buffer C (A plus 250 mM NaCl) and exchanged into buffer A by repeated ultrafiltration with a Centriprep-10 unit (Amicon). Samples were oxidized with NH_4[(dipicolinato)$_2$Co] (*46*) immediately prior to the final purification by cation exchange chromatography with a Pharmacia Mono-S HR 10/10 column equilibrated in 20 mM sodium phosphate buffer, pH 7.0. The protein was eluted with a linear NaCl gradient of 1 mM/mL around 300 mM. Fractions with an *R*-value ($A_{409.5}/A_{280}$) >4.5 were pooled, concentrated, and exchanged into buffer A prior to flash-freezing in liquid nitrogen and storage at −80 °C.

SDS−PAGE and Western Blotting. SDS−PAGE and trans-blotting of 15% (w/v) gels were performed with a BRL Mini-Gel apparatus and an ECL detection system (Amersham). Preparation of periplasmic and spheroplast fractions was carried out as previously described (*47*). The antiserum against C*c*HL has been reported previously (*48*). Antiserum directed against native cytochrome *c* was a gift from Professor Hans Bosshard.

Electronic Absorption Spectroscopy. UV−visible absorption spectra were recorded at 25 °C with a Cary Model 3E spectrophotometer fitted with a thermostated circulating water bath. Molar absorption coefficients were determined by the pyridine hemochrome method (*49*) with protein concentrations of ∼10 μM. Spectrophotometric pH titrations were performed in 0.1 M NaCl as described previously (*50*). The T_m (20 mM sodium phosphate, pH 7.0) was determined with a water-jacketted cylindrical 200 μL quartz cuvette (1 mm path length) and a Jasco Model J-720 spectropolarimeter as described previously (*51*). A thermal gradient (50 °C/h) was applied with a computer-controlled Neslab RTE111 water bath. Each melting curve was smoothed, and the first derivative was calculated with the functions available in the Jasco software package.

Cyclic Voltammetry. Midpoint reduction potentials were determined by direct electrochemistry at an edge-oriented pyrolytic graphite electrode as described by Rafferty et al. (*52*) (sweep rate, 20 mV/s). Potentials and sweep rates were controlled with an Ursar Electronics potentiostat (Oxford) under computer control. The calomel reference electrode (Radiometer K401) was standardized to −239.2 mV vs SHE against quinhydrone at the ionic strength, temperature, and pH used for potential measurements.

Electron Paramagnetic Resonance Spectroscopy. X-band EPR spectra were obtained with a Bruker Model ESP 300E spectrometer that was equipped with an HP5352 frequency counter, an Oxford Instruments liquid helium cryostat, and an Oxford Instruments Model ITC4 temperature controller. Spectra (9.45 GHz) were collected at 10 K with a microwave power of 0.99 mW. The modulation frequency and amplitude were 100 kHz and 0.51 mT, respectively. Protein samples (∼250 μL, 1.5 mM) were prepared in 50 mM KCl at pH 7.0 and 50% glycerol. After measurement of the spectra at pH 7, the samples were thawed, CAPS buffer was added (∼20 μL of a 200 mM solution), and the pH was adjusted to 9.5 and 10.5 with NaOH (1 M). A Radiometer Model PHM84 pH meter fitted with an Aldrich microcombination electrode (no. Z11,343−3) was used to measure pH.

Mass Spectrometry. Solutions of chromatographically purified cytochromes were prepared in water (∼1 mg/mL) for analysis with a triple quadrupole mass spectrometer (API III MS/MS system; PE-Sciex) equipped with a pneumatically assisted electrospray interface (*53*).

^{1}H NMR Spectroscopy. Protein solutions (∼2−3 mM) were prepared in a buffer (pH* 7.0; pH* values are pH meter readings that are uncorrected for the isotope effect) comprised of deuterated sodium phosphate (25 mM) and sodium borate (25 mM) by repeated centrifugal ultrafiltration (Centricon-10, Amicon). Concentrated NaOD was used to titrate the protein solutions to ∼9.3. ^{1}H NMR spectra were recorded at 25 and 45 °C with a Bruker MSL-200 spectrometer operating at 200 MHz, which was equipped with a Bruker VT-100 temperature controller. The resulting spectra represent 10 000 transients of 38.5 kHz spectral width collected with a superWeft pulse sequence (*54*) and a recycle delay of 220 ms. Each spectrum is the result of an 8K point transform apodized with a 90° phase-shifted sine curve. Chemical shifts are referenced with respect to the residual HOD resonance. Assignments of the heme 3- and 8-methyl resonances were obtained through magnetization transfer experiments at 45 °C in which the resonances of the native isoforms were selectively presaturated for 650 ms to invert the magnetization.

RESULTS

Expression of Cytochrome c and Cytochrome c Heme Lyase in E. coli. SDS−PAGE analysis of *E. coli* HB2151 cells bearing the plasmid pBPCYC3 revealed high levels of the expected 30 kDa band that was reactive with the antiserum raised against C*c*HL (*48*). On the other hand, no holocytochrome *c* was detected in *E. coli* cells bearing the

gene for cytochrome *c* in the absence of a gene for C*c*HL (NM554 [pCECYC1E-B]) regardless of the scale of the culture, aeration level, or supplementation with $NaNO_3$ (25 mM) or $NaNO_2$ (5 mM) (data not shown). Samples prepared from both exponential and stationary phase cultures were analyzed by Western blotting with antiserum raised against cytochrome *c*. A faint signal that comigrated with the cytochrome *c* standard was discernible only in the exponential phase samples. This band may represent apo-cytochrome as the native cytochrome could not be recovered from *E. coli* cells containing only the plasmid with *CYC1*.

A variety of expression conditions was investigated to optimize the yield of cytochrome *c*. *E. coli* HB2151 bearing the plasmid pBPCYC1(T-5A)/3 yielded approximately 1.5 mg/L of protein from a medium-scale (1.5 L) culture. Western blots of periplasmic and spheroplast fractions of JM101 [pBPCYC1(T-5A)/3] cultures indicated that expression of the holoprotein was cytoplasmic. The inclusion of an additional *lac* promotor immediately before the *CYC3* gene [pBPCYC1(T-5A)/CYC3] did not improve the yield, which suggested that *CYC3* gene expression is not the limiting factor in holo-cytochrome *c* production. In contrast, reversion of the Thr−5Ala mutation improved the yield 5-fold (7.5 mg/L) in medium-scale cultures. This improvement was enhanced by growth in the fermentor (10 L) to yield 15 mg/L. Presumably, the Thr−5Ala substitution affected the expression and/or the stability of either the apo- or the holo-cytochrome *c*. Again, inclusion of a *lac* promotor in front of *CYC3* [pBPCYC1(wt)/CYC3] failed to improve the yield further but resulted in the deletion of a 1 kbp fragment. The deletion product retained the *Nco*I and *Xba*I restriction sites within *CYC3* (Figure 1), which suggested that the *CYC1* region had been deleted. The high incidence of deletion was attributed to the tandem repeats of the *lac* promoter region because the plasmid pBPCYC1(wt)/3 showed no detectable deletion after 3 days of growth. Furthermore, the deletion of this fragment was enhanced by and was directly related to the concentration of IPTG in the culture.

Light microscopy of *E. coli* cells bearing the genes for both the cytochrome and C*c*HL (HB2151 [pBPCYC1(wt)/3]) revealed inclusion bodies that were readily visible at 400-fold magnification. Cells bearing only the gene for the cytochrome (HB2151 [pBPCYC1]) did not exhibit inclusion bodies even following induction by IPTG. On the other hand, bacteria with the gene for C*c*HL (HB2151 [pBPCYC3]) formed inclusion bodies following induction by IPTG. As a result, we assume that the inclusion bodies formed by the complete system (HB2151 [pBPCYC1(wt)/3]) result from overproduction of C*c*HL.

Mass Spectrometry. In addition to covalent attachment of heme to the apo-protein, the other posttranscriptional modification that yeast *iso*-1-cytochrome *c* undergoes is the trimethylation of Lys72 (*55*). This reaction occurs before translocation of the apo-protein from the cytoplasm to the intermembranal space of mitochondria (*56*). Because our expression system does not include the *S. cerevisiae* methyltransferase that is responsible for this modification, it was expected that Lys72 would remain unmodified during expression of the yeast cytochrome in *E. coli*. Electrospray mass spectrometry of the protein purified from *E. coli* confirmed this expectation. In addition, this analysis indicated that the initiator Met residue was removed from the cytochrome obtained from *E. coli* [12 663(2) amu] as is true for the cytochrome expressed in yeast [12 705(1) amu].

Electronic Absorption Spectroscopy. Recombinant ferricytochrome *c* purified from *E. coli* NM554 [pBPCYC1(T-5A)/3] or HB2151 [pBPCYC1(wt)/CYC3] had an $A_{409.5}/A_{280}$ ratio typical of the wild-type protein obtained from the *S. cerevisiae* GM3C-2 [pING4] system (∼4.5), and molar absorptivities were comparable to those of the cytochrome from yeast. The absorbance maximum at 695 nm that is characteristic of axial coordination by Met80 in ferricytochrome *c* (*57*) was also present.

The alkaline conformational change of ferricytochrome (*58*) expressed in *E. coli* was studied by titrating the protein with NaOH solution from pH 6 to pH 10 and monitoring the resulting change in the visible spectrum. A nonlinear least-squares fit of the dependence of A_{695} on pH to a single proton titration indicated that the alkaline pK_a of the bacterially expressed cytochrome [7.95(2)] is significantly lower than that of the wild-type cytochrome expressed in yeast (8.5−8.7; *50, 59*). The pH-dependent spectroscopic changes were reversible. Similar analysis of the Tml72Ala variant expressed in bacteria established that substitution of Lys72 with an amino acid residue that cannot coordinate to the heme iron restores the pK_a for the alkaline transition to the value observed for the wild-type protein [8.59(2)]. This finding is consistent with coordination of Lys72 to the heme iron at alkaline pH.

The thermal stability of recombinant ferricytochromes was evaluated by observing the change in ellipticity at 222 nm as a function of temperature. The T_m values observed for the wild-type protein expressed in yeast [56.0(5) °C] and *E. coli* [56.5(5) °C] were within experimental error of each other. From this result, we conclude that trimethylation of Lys72 has no effect on the stability of the ferricytochrome.

Cyclic Voltammetry. The midpoint reduction potentials (E_m') observed for the wild-type cytochrome expressed in yeast [282(2) mV, $n = 60.3$ mV] and in *E. coli* [283(2) mV, $n = 61.1$ mV] established that posttranslational modification of Lys72 has no effect on the oxidation−reduction equilibrium of the protein. The dependence of $v^{1/2}$ on I_{PC} (not shown) was linear for sweep rates <500 mV/s for both proteins.

Electron Paramagnetic Resonance Spectroscopy. At pH 7.0, the X-band EPR spectra (10 K) of the cytochromes expressed in yeast and *E. coli* are indistinguishable from each other (Figure 2A,B). The spectra of both proteins exhibit g_z values of 3.07. However, as the solution pH is increased to 9.5, some differences in the two proteins are revealed. For the cytochrome expressed in yeast, the g_z signal at 3.07, which corresponds to the native state III conformation (*58*, *59*, *61*), decreases in intensity, and two low-spin species with g_z values of 3.36 and 3.56 (Figure 2C) appear. These new signals correspond to the two alkaline conformers of the cytochrome expressed in yeast (*59*). Increasing the pH of the cytochrome expressed in *E. coli* gives rise to two signals with $g_z = 3.20$ and 3.42 and a shoulder at 3.56 (Figure 2D) at the expense of the signal with $g_z = 3.07$. As the pH of this sample is increased to pH 10.5, the signal resulting from coordination of Lys73 to the heme iron (*59*) increases in intensity (Figure 2E). At pH 10.5, the shoulder at 3.56 is nearly absent from the spectrum of the protein expressed in

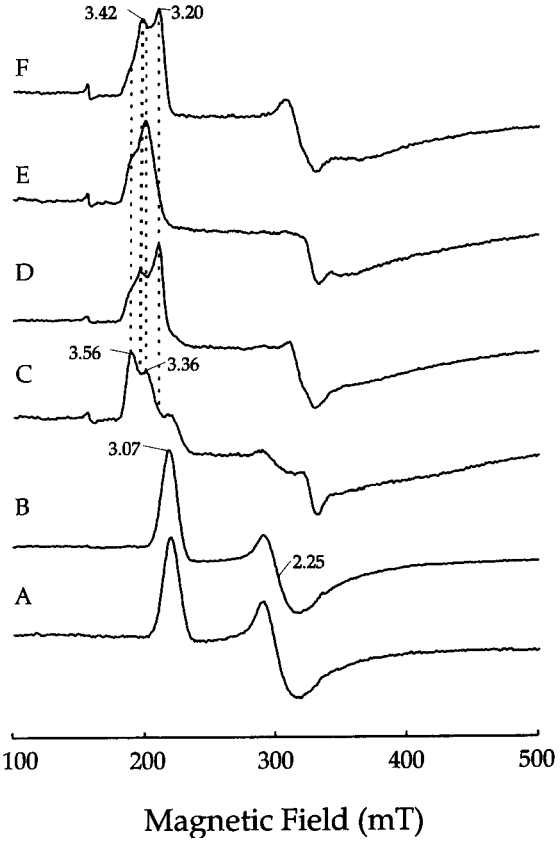

FIGURE 2: X-band EPR spectra (10 K) of recombinant yeast *iso*-1-ferricytochromes *c*: (A) cytochrome expressed in yeast, pH 7.0; (B) cytochrome expressed in *E. coli*, pH 7.0; (C) cytochrome expressed in yeast, pH 9.5; (D) cytochrome expressed in *E. coli*, pH 9.5; (E) cytochrome expressed in yeast, pH 10.5; (F) cytochrome expressed in *E. coli*, pH 10.5.

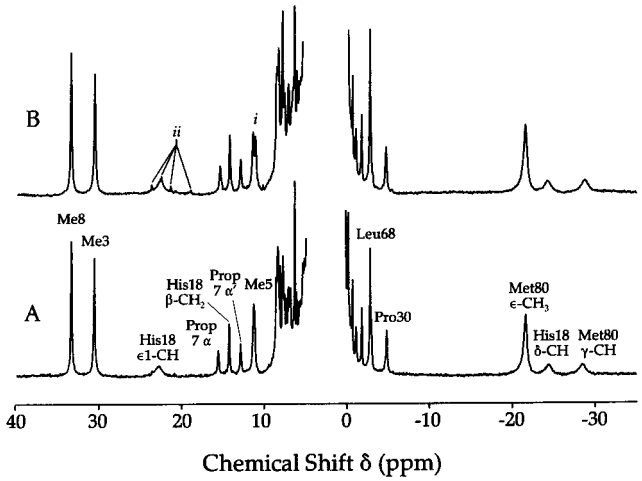

FIGURE 3: ^{1}H NMR spectra of recombinant ferricytochromes (pH 7, 45 °C): (A) cytochrome expressed in yeast and (B) cytochrome expressed in *E. coli*. Assignments are according to the literature (*62*, *63*). The resonance assigned to the heme 5-methyl group (i) and the resonances of the alkaline conformers that appear at 45 °C (ii) are indicated.

E. coli while the other two signals are of comparable magnitude (Figure 2F).

^{1}H NMR Spectroscopy. The ^{1}H NMR spectra of ferricytochrome *c* expressed in *E. coli* and in yeast are highly similar to each other at pH 7.0 (Figure 3). In particular, the signals corresponding to the axial His and Met ligands are identical. However, at 45 °C, the resonance of the heme

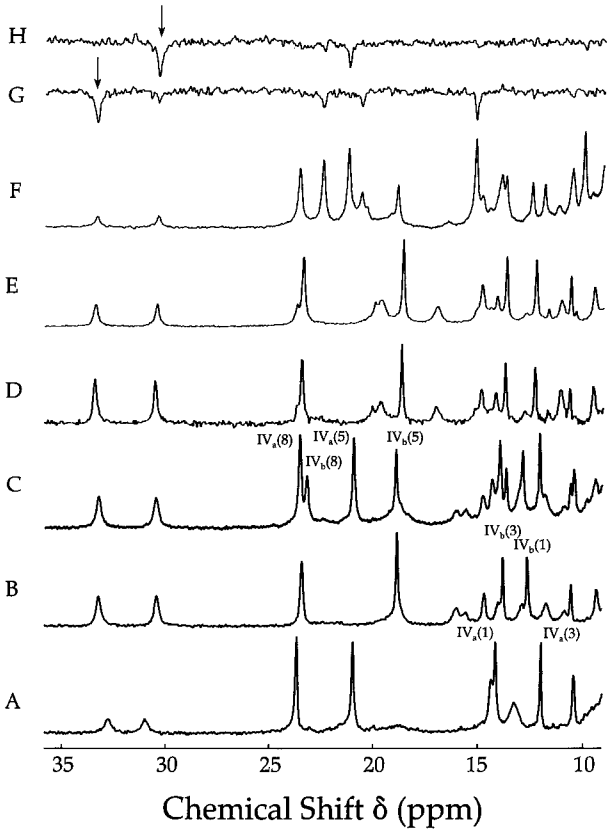

FIGURE 4: Downfield ^{1}H NMR spectra of the yeast *iso*-1-ferricytochromes *c* expressed in yeast and in *E. coli* (p^2H ~9.3 and 45 °C): (A) Lys79Ala variant expressed in yeast; (B) Lys73Ala variant expressed in yeast; (C) wild-type cytochrome expressed in yeast; (D) Tml72Ala variant expressed in yeast; (E) Lys72Ala variant expressed in *E. coli*; (F) wild-type cytochrome expressed in *E. coli*. The difference spectra obtained from inversion transfer experiments in which the heme 8-methyl (G) and the heme 3-methyl (H) groups were selectively inverted (emphasized with an arrow) are also shown. The assignments are as described in Rosell et al. (*59*) where "IV" refers to the alkaline conformers [state IV of Theorell and Åkesson (*58*)], the letters indicate which lysyl residue is bound to the heme iron atom [(a) Lys73-bound; (b) Lys79-bound)], and the number in parentheses refers to the heme methyl group.

5-methyl group (10.8; *62*, *63*) is different for the two proteins. At this temperature, this resonance is a singlet occurring at 11.2 ppm in the spectrum of the protein expressed in yeast, while the same resonance is a doublet in the spectrum of the protein derived from bacterial expression. Furthermore, under these conditions, weak features observed at 23.6, 22.4, 21.3, and 18.9 ppm in the spectrum of cytochrome prepared from bacteria are shown in Figure 4 to correspond to resonances of the alkaline conformers. The high temperature used to measure the spectra exerts a detectable influence over the lower pK_a of the protein expressed in *E. coli* relative to that expressed in yeast (*64*). The resonances of the nine methyl protons of Tml72 (3.31 ppm; *2*, *63*) should not be present in the spectrum of the cytochrome expressed in bacteria, but this region is poorly resolved under the conditions used in this work.

The region between 10 and 25 ppm is diagnostic of the alkaline conformers of cytochrome *c* (*59*, *61*, *65*). It is in this region where the differences between the yeast- and the *E. coli*-derived proteins are best illustrated at pH > 9. While the cytochrome from yeast expression (Figure 4C) consists

of a mixture of the Lys73- and Lys79-bound alkaline conformers under these conditions (Figure 4A,B, respectively), the situation for the protein from *E. coli* is more complex (Figure 4F). On the basis of results from magnetization transfer experiments in which the resonance of the heme 8-methyl group in the native protein was selectively inverted (Figure 4G), it appears that this ferricytochrome exhibits an equilibrium of three alkaline species under these conditions. Two signals of approximately equal intensity (22.5 and 20.7 ppm) correspond to the heme 8-methyl groups of the Lys73- and Lys79-bound forms that are shifted upfield.

We tentatively assign the third signal (15.2 ppm) to a new alkaline conformer in which Lys72 is coordinated to the heme iron. Three observations indicate that this new conformer is more stable at lower pH than is the case for the conformers with either Lys73 or Lys79 coordinated. First, the pK_a for the alkaline transition of the ferricytochrome expressed in *E. coli* is 0.6 pK_a unit lower than that of the same protein expressed in yeast. As this pK_a represents the weighted average of the pK_as for formation of all three alkaline conformers, the pK_a for formation of the Lys72-bound conformer is probably <7.9. Second, as stated above, the Lys72Ala variant expressed in *E. coli* restores the pK_a for this transition and the ^{1}H NMR spectrum to those of the wild-type protein and the Lys72Ala variant expressed in yeast. Third, the magnetization transfer observed in Figure 4G is most intense for the third alkaline conformer that appears to correspond to the Lys72-bound state. This greater intensity implies greater abundance of this conformer under these experimental conditions. Although the resonance at 15.2 ppm could correspond to some other group of protons experiencing NOE interactions with the heme 8-methyl group, this possibility is inconsistent with elimination of this signal when Lys72 is replaced with an alanyl residue (Figure 4E). Definitive characterization of the properties of this conformer will require analysis of the Lys73Ala/Lys79Ala double variant expressed in *E. coli*.

DISCUSSION

Expression of CYC1 and CYC3 in E. coli. The present results demonstrate that functional C*c*HL can be expressed as a cytoplasmic protein in *E. coli* and that expression of native *iso*-1-cytochrome *c* in this host requires coexpression of this enzyme. This observation supports the hypothesis that no native C*c*HL that is able to interact with yeast mitochondrial cytochrome *c* occurs in the cytoplasm of *E. coli* (*31*). Although expression of native cytochrome c_{552} from *H. thermophilus* as a cytoplasmic protein in *E. coli* (*37*) appears to be inconsistent with this conclusion, it seems plausible that unlike mitochondrial apo-cytochromes (*66*−*68*), the apo-protein of this thermally stable cytochrome possesses sufficient secondary and/or tertiary structure to bind heme covalently in the absence of the heme lyase. This interpretation is consistent with our previous conclusions from demonstration of spontaneous formation of a thioether bond between heme and the Asn57Cys variant of cytochrome b_5 expressed in *E. coli* (*35*). Mitochondrial apo-cytochrome *c* is incapable of binding heme covalently in the absence of C*c*HL (*69*), consistent with our results for *E. coli* NM554 [pCECYC1E-B].

The yield of recombinant cytochrome obtained from *E. coli* in the current work is similar to that obtained for *b*-type cytochromes (*22*, *26*), yeast cytochrome *c* peroxidase (*70*), and myoglobin (*21*, *71*). This level of expression represents an increase of about 1 order of magnitude over the levels reported for heterologous expression of other *c*-type cytochromes in either bacteria (*12*) or yeast (*7*, *52*). Although IPTG enhances expression of recombinant *H. thermophilus* cytochrome c_{552} (*72*), IPTG did not increase the level of cytochrome expression in the current work but suppressed the copy number of the *CYC1* gene within the culture. The basis for possible toxicity of *CYC1* overexpression is unknown.

Consequences of Lys72 Trimethylation. Mass spectrometry and ^{1}H NMR spectroscopy establish that the Lys72 residue of native recombinant *iso*-1-cytochrome *c* obtained from *E. coli* is not trimethylated. This result was expected because the gene coding for expression of the methyltransferase that is responsible for the trimethylation of Lys72 in yeast (*56*) was not included in our *E. coli* expression system. Evidently neither of the two *E. coli* strains possesses a methylase that can catalyze this reaction under the growth conditions used in this work. It also appears that Tml72 is not required for interaction of the apo-protein with C*c*HL, so it is possible that the small amount of unmethylated cytochrome *c* observed in yeast mitochondria results from import of the unmethylated apo-protein rather than demethylation following import (*73*).

The cytochrome *c* produced by this bacterial expression system is suitable for physical studies provided that the functional effects of the Tml72Lys replacement at alkaline pH are accounted for. Near neutrality, the electronic absorption, EPR, and ^{1}H NMR spectra, reduction potentials, and thermal stabilities of the ferricytochromes produced in yeast and *E. coli* are indistinguishable from each other. However, the pK_a for the alkaline conformational transition of the cytochrome expressed in the bacterium is ∼0.6 pK_a unit lower than that for the cytochrome obtained from yeast. These findings differ from those reported previously (*74*, *75*) in which methylation of Lys72 was found to have no effect on this pK_a and to decrease the T_m by 5 °C. These apparently contradictory results may be related to differences in experimental conditions. In previous work (*74*, *75*), thermal stability was determined in sodium phosphate buffer (μ = 0.1 M, pH 6.5), and the alkaline transition was studied in Tris-HCl buffer (50 mM, pH 7.0, unspecified temperature). In any event, the influence of trimethylation on the alkaline conformation change observed in the current work by electronic spectroscopy, EPR spectroscopy, and ^{1}H NMR spectroscopy combined with related studies of the Lys72Ala variant expressed in *E. coli* provides compelling evidence that in yeast ferricytochrome *c*, unmodified Lys72 is capable of coordinating to the heme iron atom at alkaline pH to generate a third alkaline conformer (state IV).

This conclusion suggests that a role of Lys72 trimethylation in yeast cytochrome *c* is to impede the apparently facile alkaline transition of the native state III to the Lys72-bound alkaline state IV conformer. Some precedent for this possibility is suggested by studies of a chemically modified derivative of horse heart cytochrome *c* in which a β-thiopropionyl (TP) group was introduced specifically at Lys72 (*76*). This modified cytochrome was reported to exhibit a conformer in which the TP-Lys72 coordinates to the heme iron. Interestingly, in rice cytochrome *c*, both Lys72 and

Lys86 are trimethylated (*77*, *78*). In previous work, we have found that replacement of Lys86 with alanine in the yeast cytochrome has no effect on the alkaline conformational equilibrium (*59*), but it remains to be determined whether an unmodified Lys86 in the rice cytochrome could coordinate to the heme iron atom at alkaline pH.

As mentioned previously (*59*), the ^{1}H NMR spectrum of alkaline horse heart ferricytochrome *c* indicates the formation of just two alkaline (state IV) conformers (*65*) despite the presence of Lys72, Lys73, and Lys79 in this protein. Evidently, one of these residues is unable to coordinate the heme iron of the horse heart protein at alkaline pH. In view of the limited structural differences between the yeast and horse heart cytochromes, the inability of one of these lysyl residues to coordinate to the heme iron of the horse heart protein at alkaline pH is currently inexplicable. Understanding the structural origin of this difference between the two species of cytochrome should provide important insight into the factors that dictate the pH-linked conformational dynamics of this protein. Moreover, in view of previous findings that Lys72 is important in the interaction of cytochrome *c* with other electron-transfer proteins (e.g., *79*−*81*), it may be of interest to consider the effect of Lys72 trimethylation on interactions with cytochrome *c* peroxidase, cytochrome *c* oxidase, and flavocytochrome b_2 in greater detail.

Development of a bacterial expression system for yeast cytochrome *c* provides a facile means of preparing cytochrome variants that are incapable of supporting respiration as required by the most widely used yeast expression system. Initial experiments in expression of the Met80Ala variant of the yeast cytochrome have established that this nonfunctional form of the protein can, in fact, be expressed with the bacterial system although preliminary yields [2−3 mg/L of a large-scale culture (10 L)] are not as high as those obtained for the wild-type protein (*82*). While this paper was under review, Fetrow and colleagues (*83*) reported the preparation of isotopically enriched yeast *iso*-1-cytochrome *c* from *S. cerevisiae*, but the expression level was quite low. With further work, it should be possible to identify fermentation conditions that permit use of the bacterial system described here for more efficient production of isotopically enriched mitochondrial cytochromes.

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