Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11456–11459, December 1993
Biochemistry

Structurally engineered cytochromes with unusual ligand-binding properties: Expression of *Saccharomyces cerevisiae* Met-80 → Ala iso-1-cytochrome *c*

(site-directed mutagenesis/metal-affinity chromatography/protein engineering)

YI LU, DANILO R. CASIMIRO, KARA L. BREN, JOHN H. RICHARDS, AND HARRY B. GRAY

Beckman Institute, California Institute of Technology, Pasadena, CA 91125

Contributed by Harry B. Gray, August 23, 1993

ABSTRACT **A strategy has been developed to express and purify a recombinant, nonfunctional axial-ligand mutant of iso-1-cytochrome *c* (Met-80 → Ala) in *Saccharomyces cerevisiae* in quantities necessary for extensive biophysical characterization. It involves coexpressing in the same plasmid (YEp213) the nonfunctional gene with a functional gene copy for complementation in a selective medium. The functional gene encodes a product with an engineered metal-chelating dihistidine site (His-39 and Leu-58 → His) that enables efficient separation of the two isoforms by immobilized metal-affinity chromatography. The purified Met-80 → Ala protein possesses a binding site for dioxygen and other exogenous ligands. Absorption spectra of several derivatives of this mutant show striking similarities to those of corresponding derivatives of horseradish peroxidase, myoglobin, and cytochrome P450. The use of a dual-gene vector for cytochrome *c* expression together with metal-affinity separation opens the way for the engineering of variants with dramatically altered structural and catalytic properties.**

Cytochrome *c* (1, 2) has been a benchmark for studies of eukaryotic transcription (3), protein folding and stability (4), protein engineering (5), and long-range electron transfer (6). Because of the requirement of posttranslational heme attachment, the expression (either homologous or heterologous) of mitochondrial cytochromes *c* has been largely limited to eukaryotic organisms, the most favored of which is baker's yeast (*Saccharomyces cerevisiae*). Smith and coworkers (7) have utilized a multicopy plasmid (YEp) for overexpressing yeast iso-1-cytochrome *c* under the control of its natural promoter. The host cells are grown in nonfermentable carbon medium where cytochrome *c* expression is fully derepressed and a functional gene product is required for aerobic respiration. Other groups (8, 9) have used integrative vectors that result in a single-copy gene of cytochrome *c*. Having a chromosomal gene copy allows expression of the protein either in a rich or in a lactate medium.

While a great many cytochrome *c* mutants have been generated by recombinant DNA techniques, only those variants that retain significant wild-type electron transfer activity have been isolated in reasonable yields. With the exception of His-18 → Arg yeast cytochrome *c* (10), these exclude replacements of the axial ligands (His-18 and Met-80) or the cysteine residues (positions 14 and 17) that provide the covalent heme linkages (11). Thus, the development of a more general expression system for cytochrome *c* is of considerable interest; such a system would greatly expand the possible structural changes that could be incorporated into this paradigmatic protein.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

In this paper, we report an expression system using a YEp vector bearing both the target nonfunctional variant as well as the functional cytochrome *c* gene to support the growth of the yeast cells in medium containing a nonfermentable carbon source. To facilitate the purification of the functional protein from the target nonfunctional mutant protein, a dihistidine chelating site (His-39, Leu-58 → His) was engineered onto the surface of the functional protein so that the two isoforms could be separated by immobilized metal-affinity chromatography (12, 13). The initial target protein was chosen to be Met-80 → Ala iso-1-cytochrome *c* (M80A) for the following reasons. First, studies by Sherman *et al.* (14) have shown that yeast cells containing Met-80 mutant proteins (Met-80 → Arg, Thr, Ile) maintain certain levels of holoproteins (≈20% of native protein), and their *in vitro* levels of heme incorporation and mitochondrial transport also are similar to those of the native protein. Although no mutants were isolated, a result that has been attributed to instability of the proteins, the above experiments indicate that they can be made *in vivo*. Second, several semisynthetic Met-80 mutant proteins of horse heart cytochrome *c* have been prepared and shown to be stable species (15–17). Finally, replacing Met-80 with alanine creates a binding site for dioxygen (16, 17), which represents an important step in the engineering of artificial oxygenases (18).

MATERIALS AND METHODS

Site-Directed Mutagenesis and Molecular Cloning. Mutations in the yeast iso-1-cytochrome *c* (*cyc1*) gene were introduced by using standard oligonucleotide-directed mutagenesis protocols (19) and confirmed by dideoxynucleotide DNA sequencing (20). YEp213/Leu58His*cyc1* (F. H. Arnold) (13) was digested with *Sma* I and *Hin*dIII; the 1.1-kb fragment containing a functional cytochrome *c* gene (His39/Leu58His*cyc1*$^+$) was isolated by agarose gel electrophoresis and the silica bead adsorption-elution protocol (Qiagen, Chatsworth, CA). The fragment was cloned into the *Hin*dIII site of YEp213/Met80Ala*cyc1* using a phosphorylated *Hin*dIII linker (5′-CCCAAGCTTGGG-3′) from Stratagene.

Protein Expression and Purification. *S. cerevisiae* GM3C2 strain (*MATα leu2-3 leu2-112 trp1-1 his4-519 cyc1-1 cyp3-1*) (21) was transformed with the YEp213 hybrid vectors by electroporation (22). The mutant proteins were expressed by growing the cells to saturation on a glycerol-based medium (YPG = 1% Bacto yeast extract, 2% Bacto peptone, and 3% glycerol) at 30°C. Cell lysis and preliminary protein purification (i.e., ammonium sulfate precipitation, Sepharose CM52 ion-exchange chromatography) were according to established procedures (23). The ensuing chromatographic runs were carried out on an FPLC system from Pharmacia LKB.

Abbreviations: HRP, horseradish peroxidase; M80A, Met-80 → Ala iso-1-cytochrome *c*.

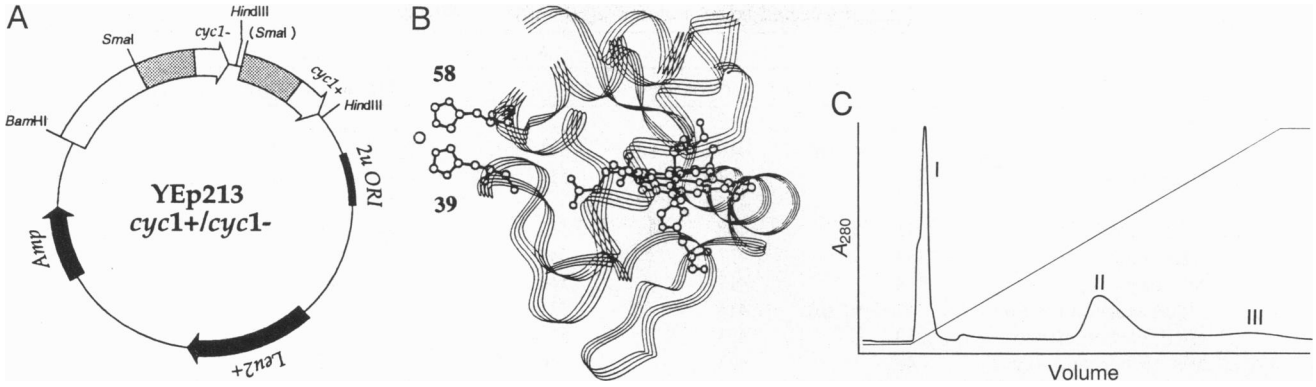

FIG. 1. (*A*) Vector construct for expression of Met-80 → Ala (*cyc1*$^-$) and Leu-58 → His (*cyc1*$^+$) cytochrome *c*. The shaded boxes contain the promoter and upstream activation sequences for *cyc1* transcription. *Leu2*$^+$ and ampicillin resistance markers also are present for plasmid selection in yeast and *Escherichia coli*, respectively. (*B*) Molecular model of Leu-58 → His cytochrome *c*, indicating the location of the surface dihistidine site for high-affinity binding to immobilized metal complexes. (*C*) Chromatogram showing the purification on a copper(II) iminodiacetate column of homogenized protein extracts containing the nonfunctional M80A (band I) and the functional His-39/Leu-58 → His proteins (band II, ferrous; band III, ferric form).

The protein extracts were loaded onto a 5-ml HiTrap chelating column (Pharmacia LKB) that was charged with 2.5 ml of 100 mM $CuSO_4$. The proteins were eluted with a 1–20 mM imidazole gradient in 50 mM sodium phosphate/500 mM NaCl, pH 7.0. The band containing the M80A mutant protein bound weakly to the column and was subsequently purified to homogeneity by ion-exchange chromatography using a Mono S HR 16/10 column (Pharmacia LKB) with a 0–1 M NaCl gradient in 50 mM sodium phosphate (pH 7.0).

Protein Characterization. The purity and identity of the M80A protein were established by SDS/PAGE and amino acid sequencing. The holoprotein (0.7 mM solution in 70% formic acid) was incubated with a 130-fold excess of cyanogen bromide for 46 hr. The reaction mixture was exchanged by ultrafiltration to 100 mM NH_4HCO_3 (pH 8.0), lyophilized, and run through a Pharmacia ProRPC HR 5/10 column in order to isolate the 65–103 fragment. Sites of heme attachment (Cys-14 and Cys-17) and the mutation at position 80 were confirmed by NH_2-terminal sequencing of the holoprotein and of the 65–103 cyanogen bromide fragment, respectively. Sequences were confirmed via automated Edman degradation on an Applied Biosystems 473A sequencer.

Horseradish peroxidase (HRP) and horse heart myoglobin (Mb) were obtained from Sigma; *Pseudomonas putida* cytochrome $P450_{cam}$ (induced by growth on camphor; J. H. Dawson, University of South Carolina) was purified by T. Pascher (Beckman Institute, Pasadena, CA). The oxy and carbon monoxy forms of HRP were prepared according to D. Keilin and E. F. Hartree (24). The deoxy derivatives of both proteins were made under a nitrogen atmosphere by adding excess $Na_2S_2O_4$ to the protein solutions and running the solution through a Sephadex G-25 column. Exposing the deoxy form of the M80A protein to air results in the oxy form; and exposure of the protein to carbon monoxide yields the carbon monoxy derivative. The thiomethoxide derivative of the M80A protein was obtained by adding a 500-fold excess of $NaSCH_3$ to a ferric-M80A protein solution. The imidazole derivative of cyt $P450_{cam}$ was prepared according to Rux and Dawson (25). Electronic absorption spectra were measured at room temperature on a Cary 14 spectrophotometer. Extinction coefficients were determined by the pyridine hemochrome method (26).

Molecular Modeling. Energy-minimization calculations on His-39/Leu-58 → His cytochrome *c* were performed on the

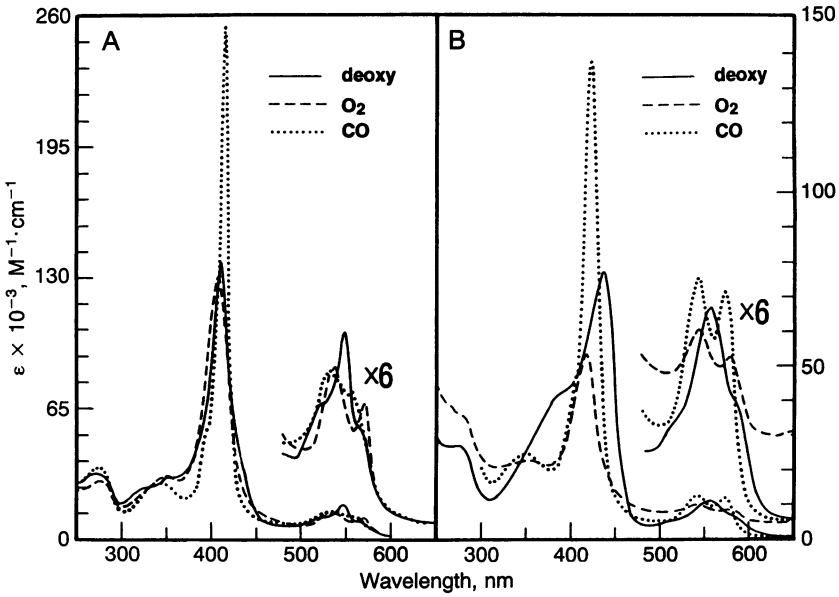

FIG. 2. Electronic absorption spectra of ferrous M80A derivatives (*A*) and analogous HRP species (*B*). The proteins were in 50 mM sodium phosphate buffer (pH 7.0).

Table 1. Electronic absorption data for selected heme proteins

Protein	λ_{max}, nm						
	δ	Soret (γ)	Visible (α, β)/near-IR				
Ferrous derivatives							
M80A, deoxy	327 (354, sh)	410 (437, sh)	521 (sh)	548	564 (sh)		
HRP, deoxy	382	437	519 (sh)	558	589 (sh)		
Mb, deoxy		435		559			
M80A, oxy	351	408	537		570		
HRP, oxy	354	417	544		578		
Mb, oxy	348	417	544		580		
M80A, carbon monoxy	345 (394, sh)	414	531		554		
HRP, carbon monoxy	350	423	542		572		
Mb, carbon monoxy	346	423	540		579		
Ferric derivatives							
M80A (pH 3.5)		398	494			624	
HRP		403	498			641	
Mb		408	505			633	
M80A (pH 7.0)	357	406	536		566	626	
HRP (alkaline)		414	542		576	640	
Mb (alkaline)	358	411	539		585		
M80A, thiomethoxide	355	416	535		566	648	763
Cyt P450, imidazole	358	425	542		574	638	753
M80C*	355	416	540		570	635	734

Sh, shoulder.
*Semisynthetic Met-80 → Cys horse heart cytochrome *c* (15).

1.23-Å structure of yeast iso-1-cytochrome *c* (27) using BIOGRAF version 3.0 (Molecular Simulations, Burlington, MA).

RESULTS AND DISCUSSION

In the plasmid construct shown in Fig. 1*A*, identical copies of the natural promoter region of *cyc1* direct the independent expression of the two cytochrome *c* genes. The functional cytochrome *c* contains a surface dihistidine site (His-39, Leu-58 → His; Fig. 1*B*) that endows it with a high affinity for immobilized metal complexes. Transformation of *S. cerevisiae* GM3C2 (21) (strain deficient in both cytochrome *c* isozymes) with a YEp213 vector containing the M80A gene (*cyc1*$^-$) alone did not show any cell growth on a nonfermentable carbon medium (YPG), whereas cells harboring the *cyc1*$^+$/*cyc1*$^-$ vector grew as well as those containing only the *cyc1*$^+$ vector.

Cupric iminodiacetate chromatography of the protein extract (Fig. 1*C*) revealed three heme-containing bands, two of which (II and III) were retained strongly on the column and later identified to be the chelating wild-type protein in different oxidation states ($Fe^{3+/2+}$). The third band (I) bound weakly to the column and was further fractionated by cation-exchange chromatography. The UV/visible spectrum of purified M80A matches that of the oxyferrous form of the analogous horse heart mutant prepared by semisynthesis (16, 17). The total amount of cytochromes *c* purified from this

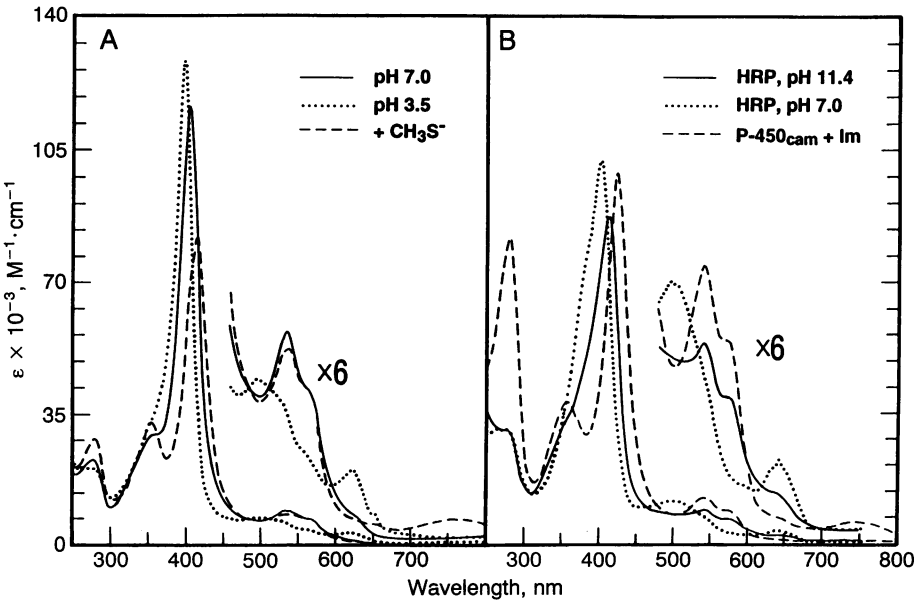

FIG. 3. Electronic absorption spectra of ferric M80A derivatives (*A*) and analogous HRP and cytochrome $P450_{cam}$ (cyt $P\text{-}450_{cam}$) species (*B*). All proteins were in 50 mM sodium phosphate buffer (pH 7.0), except for the alkaline form of HRP, which was prepared in 100 mM $NaHCO_3$ (pH 11.4), and the imidazole (Im) derivative of cytochrome $P450_{cam}$, which was kept in 100 mM sodium phosphate/88 mM imidazole/22 μM camphor, pH 7.0.

system (2 mg/liter) is comparable to that of other expression systems used to generate functional iso-1-cytochrome *c* variants (7, 8, 23). The M80A mutant protein constitutes 25–30% (0.5–0.6 mg/liter) of the total cytochromes *c* purified from this system; to our knowledge it represents the first recombinant, nonfunctional axial-ligand mutant of cytochrome *c* produced in quantities that permit extensive biophysical characterization.

The yeast M80A exhibits nearly all the properties of a heme protein with an available sixth ligation site. In the reduced form, the mutant can either have a vacant coordination site (deoxy form) or bind small exogenous ligands such as O_2 and CO (Fig. 2*A*). The spectroscopic properties of the yeast protein are similar to those of the horse heart M80A variant (16, 17). In a survey of derivatives of naturally occurring heme proteins, the closest resemblance can be drawn to HRP (Fig. 2*B* and Table 1). The observation that the electronic absorption bands of the various M80A species are slightly blue-shifted relative to those of their HRP analogs can be accounted for by the difference in π conjugation between *c* and *b* hemes (heme *b* having two extra vinyl substituents) (25).

At neutral pH, the UV/visible spectrum of the oxidized M80A mutant is analogous to that of the alkaline form of ferri-HRP (Fig. 3). It is likely that the sixth ligand is OH^- rather than H_2O, since EPR and other spectroscopic measurements are consistent with His-OH^- coordination in the M80A horse heart protein at pH 7 (17). Acid titration of the mutant produces spectral changes that indicate direct protonation of the hydroxyl ligand to form the aquoferric derivative. Interestingly, the pK_a ($\approx$6.5) is much lower than that of horse Mb (8.9) (28). Addition of thiomethoxide (CH_3S^-) to the oxidized M80A protein results in an absorption spectrum similar to that of the imidazole derivative of cytochrome $P450_{cam}$, with slight blue shifts attributable to the different heme types (*c* vs. *b*).

Our approach based on the combined use of a *cyc1*$^+$/*cyc1*$^-$ expression vector and metal-affinity separation expands the possibilities of incorporating structural changes in regions of cytochrome *c* that are not accessible by either semisynthetic (e.g., region 1–64) (15–17) or previous recombinant DNA strategies. Using this methodology, we have successfully expressed and purified a cytochrome *c* derivative that possesses unusual ligand-binding properties. Our findings represent an important step toward the long-term goal of developing a deeper understanding of heme-mediated processes; with such an understanding, it should be possible to engineer useful catalytic functions into cytochrome *c* and related heme-containing molecules.

We thank Michael Smith for the yeast iso-1-cytochrome *c* expression system, Frances Arnold for the metal-chelating cytochrome *c* gene, Torbjörn Pascher for providing purified cytochrome $P450_{cam}$, and Lynn Williams for the sequence analyses. K.L.B. acknowledges a Kodak fellowship. This work was supported by the National Science Foundation, the National Institutes of Health, and the Arnold and Mabel Beckman Foundation.

1. Pettigrew, G. W. & Moore, G. R. (1987) *Cytochromes c: Biological Aspects* (Springer, New York).
2. Moore, G. R. & Pettigrew, G. W. (1990) *Cytochromes c: Evolutionary, Structural and Physicochemical Aspects* (Springer, New York).
3. Sherman, F. (1990) *Genetics* **125**, 9–12.
4. Elöve, G. A. & Roder, H. (1991) in *Protein Refolding*, American Chemical Society Symposium, eds. Georgiou, G. & Bernardez-Clark, E. (Am. Chem. Soc., Washington, DC), Series 470, pp. 50–63.
5. Mauk, A. G. (1991) *Struct. Bond. (Berlin)* **75**, 131–157.
6. Winkler, J. R. & Gray, H. B. (1992) *Chem. Rev.* **92**, 369–379.
7. Pielak, G. J., Mauk, A. G. & Smith, M. (1985) *Nature (London)* **313**, 152–154.
8. Holzschu, D., Principio, L., Conklin, K. T., Hickey, D. R., Short, J., Rao, R., McLendon, G. & Sherman, F. (1987) *J. Biol. Chem.* **262**, 7125–7131.
9. Hilgen, S. E. & Pielak, G. J. (1991) *Protein Eng.* **4**, 575–578.
10. Garcia, L. L., Fredericks, Z., Sorrell, T. N. & Pielak, G. J. (1992) *New J. Chem.* **16**, 629–632.
11. Hampsey, D. M., Das, G. & Sherman, F. (1988) *FEBS Lett.* **231**, 275–283.
12. Arnold, F. H. & Haymore, B. L. (1991) *Science* **252**, 1796–1797.
13. Umana, P., Kellis, J. T. & Arnold, F. H. (1993) in *Biocatalyst Design for Stability and Specificity*, American Chemical Society Symposium, eds. Himmel, M. E. & Georgiou, G. (Am. Chem. Soc., Washington, DC), Series 516, pp. 102–108.
14. Dumont, M. E., Ernst, J. F. & Sherman, F. (1988) *J. Biol. Chem.* **263**, 15928–15937.
15. Raphael, A. L. & Gray, H. B. (1991) *J. Am. Chem. Soc.* **113**, 1038–1040.
16. Wallace, C. J. A. & Clark-Lewis, I. (1992) *J. Biol. Chem.* **267**, 3852–3861.
17. Bren, K. L. & Gray, H. B. (1993) *J. Inorg. Biochem.* **51**, 111.
18. Wuttke, D. S., Bjerrum, M. J., Winkler, J. R. & Gray, H. B. (1992) *Science* **256**, 1007–1009.
19. Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 488–492.
20. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
21. Faye, G., Leung, D. W., Tatchell, K., Hall, B. D. & Smith, M. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 2258–2262.
22. Becker, D. M. & Guarente, L. (1991) *Methods Enzymol.* **194**, 182–187.
23. Cutler, R. L., Pielak, G. J., Mauk, A. G. & Smith, M. (1987) *Protein Eng.* **1**, 95–99.
24. Keilin, D. & Hartree, E. F. (1951) *Biochem. J.* **49**, 88–104.
25. Rux, J. J. & Dawson, J. H. (1991) *FEBS Lett.* **290**, 49–51.
26. Berry, E. A. & Trumpower, B. L. (1987) *Anal. Biochem.* **161**, 1–15.
27. Louie, G. V. & Brayer, G. D. (1990) *J. Mol. Biol.* **214**, 527–555.
28. Antonini, E. & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands* (North-Holland, Amsterdam), pp. 47–48.