Structurally Engineered Cytochromes with Novel Ligand-Binding Sites: Oxy and Carbon Monoxy Derivatives of Semisynthetic Horse Heart Ala80 Cytochrome c

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We have shown that semisynthesis[1] can be employed to manipulate the structure of the heme pocket of horse heart cytochrome *c* (cyt *c*).[2,3] Replacement of the axial Met80 ligand yields cytochromes with novel spectroscopic, electrochemical, and ligand-binding properties. Substitution of His for Met80 results in a bis-histidine (b_5-like) heme (His80cyt *c*) with a reduction potential of 41 mV vs NHE, over 200 mV lower than that of native cyt *c*.[2] Dramatic changes in cyt *c* properties were achieved in the Met80 → Cys transformation, which gives a protein with a P-450-like absorption spectrum and a reduction potential of −390 mV (Cys80cyt *c*).[3] We have also demonstrated that cyt *c* refolding can occur in the absence of a position-80 ligand by semisynthesis of Leu80cyt *c*, a variant that oxidizes rapidly in the presence of dioxygen.[3] Of particular interest is the finding that a related mutant (semisynthetic Ala80cyt *c*)[4,5] forms a stable O_2 adduct with striking spectroscopic similarities to oxymyoglobin (O_2Mb).[5] The engineering of a cavity in the position-80 region of cyt *c* has allowed us to probe the ligand-binding properties of a heme that is buried in the interior of an electron-transfer protein.

Horse heart Ala80cyt *c* was prepared according to standard semisynthetic methods[2-7] and purified to homogeneity by using cation-exchange chromatography. The mutant migrates with native cyt *c* on an SDS/PAGE gel and is nearly as α-helical as the native as determined by circular dichroism spectroscopy.[8] The EPR spectrum of ferriAla80cyt *c* at pH 7[9] is characteristic of low-spin Fe(III) in a rhombic environment (g_x = 1.85, g_y = 2.18, g_z = 2.58) and is nearly identical with that of ferriMb at pH 10 (g_x = 1.84, g_y = 2.16, g_z = 2.59).[10] The corresponding ligand-field parameters indicate axial His–OH^- ligation.[11] The absorption spectrum of ferriAla80cyt *c* is pH dependent, with a p*K* of 6.2. The analogous transition in Mb (p*K* 8.95, sperm whale Mb)[12] involves deprotonation of the axial H_2O.[13]

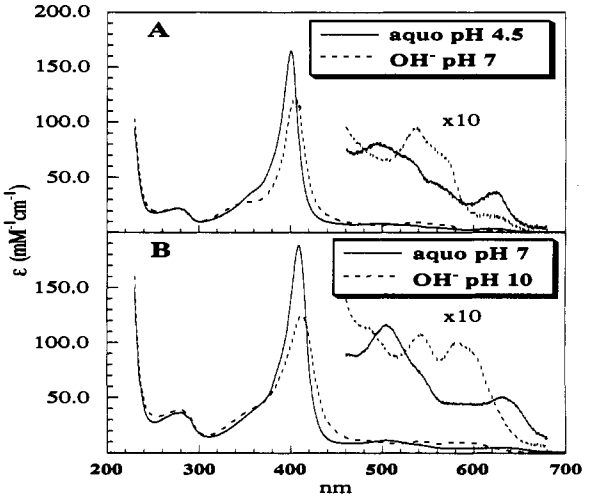

Figure 1. Absorption spectra of high- and low-pH forms of ferriAla80cyt *c* (A) and horse heart Mb (B) at room temperature. Spectra were obtained on a Cary 14 spectrophotometer in a 1-cm quartz cell. Samples were in μ = 50 mM NaP_i (pH 7), μ = 50 mM NaOAc (pH 4.5), or μ = 50 mM $NaHCO_3$ (pH 10.0). Absorption maxima for ferriAla80cyt *c* are as follows: λ (ε) 275 (22.5), 352 (28.1), 405 (121.7), 538 (9.5), 569 (6.8), 622 (1.6) at pH 7; 279 (22.2), 400 (164.4), 495 (8.1), 622 nm (3.7 mM^{-1} cm^{-1}) at pH 4.5. Extinction coefficients were determined by using the pyridine hemochrome method (Berry, E. A.; Trumpower, B. L. *Anal. Biochem.* **1987**, *161*, 1-15).

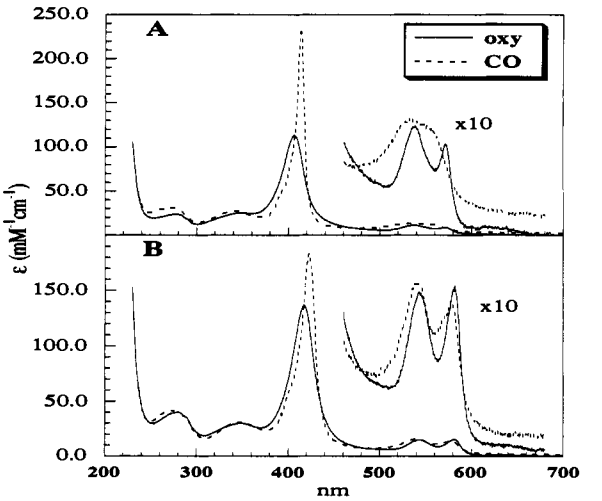

Figure 2. Absorption spectra of oxy and carbon monoxy derivatives of ferroAla80cyt *c* (A) and horse heart Mb (B) at room temperature. All derivatives were prepared by reduction of deoxygenated protein solutions by excess dithionite, followed by passage down a Sephadex G-25 column eluted with deoxygenated μ = 50 mM NaP_i, pH 7.0, under anaerobic conditions and equilibration of the sample under 1 atm of O_2 or CO. Absorption maxima are as follows: λ (ε) 278 (23.5), 348 (25.0), 406 (113.2), 538 (11.0), 571 (8.7) for O_2Ala80cyt *c* and 270 (31.3), 342 (27.3), 413 (233.6), 528-560 nm (broad, ca. 13 mM^{-1} cm^{-1}) for COAla80cyt *c*.

The absorption spectra of axially ligated derivatives of Ala80cyt *c* are very similar to those of analogous Mb species. The assignment of His–H_2O ligation at pH ≤6.2 is supported by the ferriAla80cyt *c* absorption spectrum at pH 4.5 (Figure 1). The position and intensity (ε = 164.4 mM^{-1} cm^{-1}) of the Soret band are indicative of His–H_2O coordination.[14] Proteins such as

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(7) Ala80cyt *c* was prepared by incubation of 0.3 M synthetic cyt *c*(66-104)(Ala80) peptide with 0.4 M cyt *c*(1-65) lactone[6a] in 50 mM NaP_i, pH 7.0, at room temperature. The solution was thoroughly degassed and reduced with 2 equiv of sodium dithionite under a nitrogen atmosphere. After the solution was stirred under nitrogen for 45 h, the product was purified by cation-exchange chromatography (Pharmacia FPLC, Mono S 10/10 column).
(8) CD data were obtained between 190 and 240 nm at ambient temperature on a Jasco-600 spectrometer. Sample was 12 μM in protein in 50 mM NaP_i, pH 7.0.
(9) EPR data were obtained at 33 K on a Bruker ESP 300 instrument with a liquid helium cryostat. Sample was 0.1 mM in protein in a 50% 100 mM HEPES, pH 7.0, 50% glycerol glass.
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Aplysia Mb that lack an axial water in the ferric state[15] have broad, blue-shifted Soret bands with $\epsilon \approx 100$ mM^{-1} cm^{-1}.[14] The spectrum of the ferrous-deoxy species of Ala80cyt *c* differs from Mb, with a Soret band at 411 nm and a shoulder at 434 nm, possibly due to a mixture of spin states. Addition of dioxygen or carbon monoxide to the deoxy protein gives a red or pink product, respectively, with spectral features similar to those of Mb, including the characteristic narrow, intense Soret band for the CO derivative (Figure 2).

The dioxygen adduct of ferroAla80cyt *c* (O_2Ala80cyt *c*) is remarkably stable. Ala80cyt *c* binds dioxygen with an affinity greater than that of Mb[16] and autooxidizes with a half-life of days (k_{ox} = 0.01 h^{-1}, 22 °C),[17] while Mb autooxidizes in hours (k_{ox} = 0.08–0.22 h^{-1}, 22 °C).[18] The high stability of O_2Ala80cyt *c* with respect to O_2Mb is especially surprising in light of recent reports that nearly all distal pocket mutants of Mb show low O_2 affinities[19-23] and high autooxidation rates.[19] A notable exception is Leu29 → PheMb, which is extremely stable in the oxy state, possibly due to favorable interactions of the iron-bound dioxygen with the positive edge of Phe29.[23] Molecular modeling suggests that Phe82 could play a similar role in Ala80cyt *c* (Figure 3). In addition, modeling shows that Tyr67 is positioned in the cytochrome pocket to donate a hydroxyl proton to dioxygen, playing a role analogous to that of the distal histidine in Mb.

The kinetics of CO recombination to ferroAla80cyt *c* after photolysis have been investigated by using transient absorption spectroscopy.[24] Carbon monoxide recombines anomalously slowly ($k' = (8.7 \pm 0.8) \times 10^3$ M^{-1} s^{-1}) relative to its reaction with sperm whale Mb ($k' = 5.0 \times 10^5$ M^{-1} s^{-1}).[13] The quantum yields of CO and O_2 dissociation from ferroAla80cyt *c* (Φ_{CO} = 0.02, $\Phi_{O_2} \approx$ 0.001) are exceedingly low;[25] geminate recombination[26] was not observed on a nanosecond time scale for O_2- or COAla80cyt *c*.

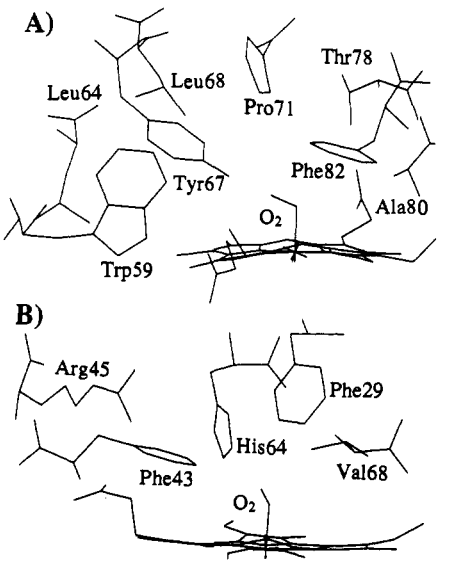

Figure 3. Molecular models of the Ala80cyt *c* (A) and Phe29Mb (B) distal pockets. Modeling was performed by using BIOGRAF version 2.2 (BioDesign, Inc.) on a VaxStation 3500. The Leu29 → Phe mutant of sperm whale Mb was modeled on the 1.6-Å sperm whale O_2Mb structure (Phillips, S. E. V. *J. Mol. Biol.* **1980**, *142*, 531–554) by replacement of Leu29 with Phe and local energy minimization. Ala80cyt *c* was built on the 1.94-Å horse heart cyt *c* structure (Bushnell, G. W.; Louie, G. V.; Brayer, G. D. *J. Mol. Biol.* **1990**, *214*, 585–595) by substitution of Ala for Met80, placement of dioxygen at the heme iron, and local energy minimization. Phe82 was rotated from its position in the native structure before local energy minimization to show the potential Phe–O_2 interaction. The Phe Cζ → O_2(2) distance in this model is 3.4 Å, compared with 3.2 Å reported for Leu29PheMb.[20] Tyr67, if protonated, may make a hydrogen bond with bound O_2; the Tyr67 Oη → O_2(2) distance is 2.65 Å, compared with the His64 Nϵ_2 → O_2(2) distance of 2.77 Å in SWMb.

The low yields with which O_2 and CO escape from the heme pocket of Ala80cyt *c* in addition to the slow CO recombination demonstrate that the iron site is much less accessible in this protein than in Mb.[27] Dynamics calculations suggest that particular amino acids in Mb facilitate diffusion between the solvent and the heme iron.[28] Such a ligand channel apparently is not available in Ala80cyt *c*. The striking stability of O_2Ala80cyt *c* can be partially attributed to the highly protected ligand-binding site.

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(16) Ala80cyt *c* was found to bind O_2 with $K = 2.6 \pm 1.4$ μM^{-1}, in comparison with K = 0.4–1.2 μM^{-1} for Mb (various species).[13] The Ala80cyt *c* binding constant was estimated by adding small amounts of dioxygen to a fully deoxygenated sample while observing changes in the absorption spectrum; the error is large because there is some autooxidation at low oxygen pressures during the time (≈1 h) required to reach equilibrium in this system.

(17) Rate for freshly synthesized O_2Ala80cyt *c*. The rate for oxyprotein prepared from ferriAla80cyt *c* is higher (k_{ox} = 0.04 h^{-1}).

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(24) The excitation source for transient absorption experiments was a Lambda Physik FL3002 dye laser (coumarin 480 dye) pumped by a Lambda Physik LPX210i eximer laser with a pulse width of 25 ns (480 or 500 nm) or a frequency-doubled Q-switched Quanta-Ray Nd:YAG laser with a pulse width of 20 ns (532 nm). A xenon arc lamp provided the probe light. Ligand recombination was monitored both near 430 (all samples) and near 414 (CO) or 406 nm (O_2). Transient absorption experiments were performed at room temperature on 3–10 μM protein in 50 mM NaP_i, pH 7.0. The protein was fully deoxygenated and reduced before being equilibrated under 1 atm of CO or O_2.

(25) Quantum yield refers to production of fully dissociated product capable of rebinding in bimolecular reactions. Quantum yields were calculated with respect to COMb, for which $\Phi = 1$.[13] The low O_2 dissociation yield precluded an accurate determination of k'_{O_2}.

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