difficult. By contrast it should be easily possible to qualitatively distinguish between "lone-pair" type orbitals on O bonded to one Si and one H and those with O bonded to two Si, simply by inspecting the number of peaks in $\rho(q)$. Extended to solid surfaces this would provide a direct means of monitoring silanol, Si-O-H, and bridging oxygen, Si-O-Si, sites on the surface of a silica glass or silicate mineral.

Acknowledgment. The work was supported by the National Science Foundation, Grants CHE-88-08589 and EAR-86-03499.

Communications to the Editor

Semisynthesis of Axial-Ligand (Position 80) Mutants of Cytochrome *c*

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Received August 6, 1990

Axial ligands play important roles in the functions of heme proteins.[1-3] Although changes in axial ligation in cytochrome *c* can be made both by biological and chemical techniques,[4-10] we have found that chemical semisynthesis[7,11] is particularly useful in that it allows the production of reasonable quantities of mutants that vary widely in the electronic character of the axial group at position 80. Of special interest is our finding that substitution of methionine by cysteine (cyt *c*-Cys80)[12,13] results in a mutant protein with a reduction potential of −390 mV, thereby demonstrating that axial ligand replacement alone can shift the reduction potential well over 600 mV from native cytochrome *c* (262 mV).[14]

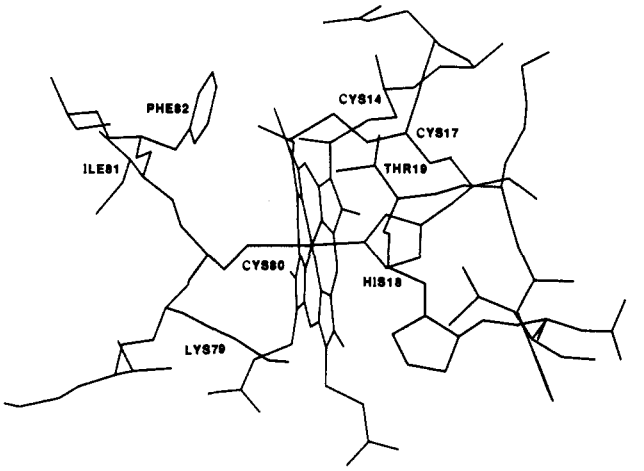

Figure 1. Heme area of cyt *c*-Cys80.

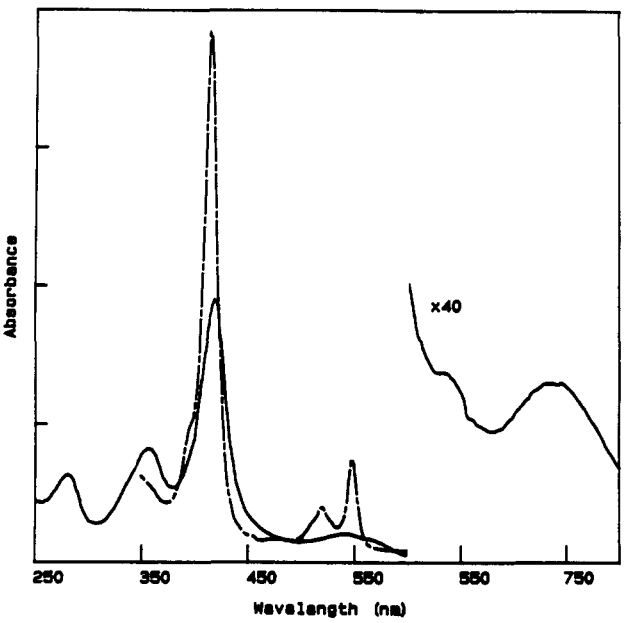

Figure 2. Absorption spectra of oxidized (—) and reduced (- - -) cyt *c*-Cys80 in 50 mM sodium phosphate buffer, pH 7.0.

Ferric cyt *c*-Cys80 also is strikingly similar spectroscopically to cytochrome P-450.

The heme area of cyt *c*-Cys80 is pictured in Figure 1.[15] Proteins with histidine (cyt *c*-His80)[7] and leucine (cyt *c*-Leu80)[16]

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(12) Syntheses involved reaction of 1-65 lactone (CNBr cleavage product of cytochrome c^2) and peptide 66-104.[7,13] Cyt *c*-Cys80 was synthesized by using 0.35 mM 1-65 lactone and peptide 66-104(Cys80) in 5 mM sodium phosphate buffer, pH 7.0, which was degassed and reduced with 4 equiv of sodium dithionite. After reaction under Ar for 36 h at ambient temperature with addition of 0.5 equiv of dithionite every 12 h, the products were purified by using cation-exchange chromatography (Pharmacia FPLC 10/10 Mono S column) with a 0.15-0.40 M NaCl gradient in 0.05 M sodium phosphate buffer, pH 7.0. Isoelectric focusing showed that cyt *c*-Cys80 was one species, with an isoelectric point equal to that of cytochrome *c*. Amino acid analyses agreed well with calculated values (in most cases within 2%).
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Table I. Reduction Potentials

heme protein	iron ligands	heme type	$E^{\circ\prime}$ (mV vs NHE)	ref
cytochrome *c*	Met/His	*c*	262	14
cyt *c*-His80	His/His	*c*	41 (10)	7
cytochrome b_5	His/His	*b*	5	25
cyt *c*-Leu80	His/H_2O[a]	*c*	−45 (10)	this work
myoglobin	His/H_2O	*b*	58.8 (2)	29
horseradish peroxidase	His/H_2O	*b*	−260	30
cyt *c*-Cys80	Cys/His	*c*	−390 (10)	this work
cytochrome P-450	Cys/H_2O	*b*	−360 to −170	31a

[a] Probable axial ligation; however, see ref 21.

substituted for methionine also have been synthesized.[17,18] Electronic spectra of cyt *c*-His80 and cyt *c*-Leu80 are virtually identical with the spectrum of native cytochrome *c* in the porphyrin absorption region: Soret band at 408 nm (1.1×10^5 M^{-1} cm^{-1}); α,β band at 528 nm (1.0×10^4 M^{-1} cm^{-1}).[7,19] Cyt *c*-Cys80, however, exhibits red-shifted Soret and α,β bands at 416 (6.7×10^4) and 540 nm (8.3×10^3 M^{-1} cm^{-1}) (Figure 2) that are similar to absorptions in cytochrome P-450 [417 nm (1.15×10^5 M^{-1} cm^{-1}); 535 and 569 nm (1.16×10^4 and 1.19×10^4 M^{-1} cm^{-1})], which has thiolate–Fe(III) ligation.[20] The close analogy of cyt *c*-Cys80 to P-450 is further displayed by sulfur to Fe(III) charge-transfer bands that are resolved at lower energy [635 and 734 nm (1.1×10^3 and 1.07×10^3 M^{-1} cm^{-1})].[21] Upon reduction of cyt *c*-Cys80, the spectrum [416 nm (1.4×10^5 M^{-1} cm^{-1}); 520 nm (1.6×10^4 M^{-1} cm^{-1}); 550 nm (2.8×10^4 M^{-1} cm^{-1})] (Figure 2) appears very similar to that of native cytochrome *c*, implying that the thiolate–iron bond is no longer intact.

Reduction potentials were determined by using differential pulse polarography (DPP) (cyt *c*-His80)[22] and spectroelectrochemistry [cyt *c*-Leu80 and cyt *c*-Cys80 (Figure 3)].[23] Results are given in Table I with comparisons to native proteins.[24] Replacement of Met80 by His results in a −229-mV change in the potential, which is comparable to variations (−148 to −180 mV) that are documented for the substitution of a thioether by imidazole.[3] Interestingly, the potential of cytochrome b_5, 5 mV,[25] is in good agreement with the potential of cyt *c*-His80 (41 mV).[26] Other

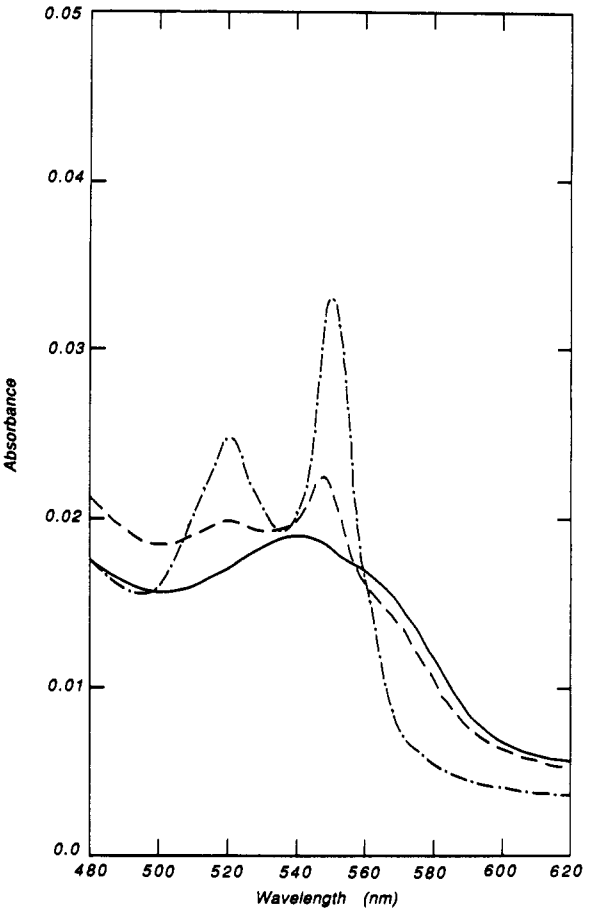

Figure 3. Absorption spectra of cyt *c*-Cys80 (82 μM) in the presence of the redox mediator anthraquinone-2-sulfonate (0.71 mM) at applied potentials of 146 (—), −254 (– –), and −564 mV vs NHE (– · –).

bis-histidine heme proteins have potentials that range down to −340 mV,[27] and heme-area side-chain packing, polarity, and solvent exposure are thought to account for these large differences.[28]

Methionine substitution by leucine at position 80 (cyt *c*-Leu80) shifts the reduction potential into the range observed for proteins with His/H_2O heme ligation, e.g., myoglobin (58 mV)[29] and peroxidases (−190 to −260 mV).[30] It is particularly striking that the cyt *c*-Cys80 reduction potential (−390 mV) is similar to the potentials of cytochrome P-450 and chloroperoxidase (Cys/H_2O ligation): the range for the native thiolate-ligated proteins is −360 to −140 mV.[31]

The ferrous cytochrome *c* derivatives react with carbon monoxide at pH 7.0, forming low-spin species with Soret absorption maxima centered at 411 ± 1 nm (the α,β bands are shifted to 530 nm).[32] The Soret absorptions of the cyt *c*-His80 and cyt *c*-Cys80

(16) For cyt *c*-Leu80, 0.07 mM 1–65 lactone and 66–104(Leu80) were combined in a two-compartment cell with medium-porosity frit in degassed 5 mM sodium phosphate solution; the counter-electrode solution contained 0.04 M NaCl and 0.56 mM $[Ru(NH_3)_6]Cl_3$ in the same buffer. A potential of −450 mV vs SCE was applied for 16 h at ambient temperatures with stirring, using a Princeton Applied Research (PAR) Model 175 universal programmer in conjunction with a PAR Model 173 potentiostat/galvanostat. Platinum baskets were used as the working and counter electrodes. Products were purified as above (ref 12).

(17) The overall reaction yields for the Cys, Leu, and His mutants were 37, 12, and 60%. Greater than 20 mg of the Cys and His derivatives can be synthesized conveniently.

(18) Aspartate substitution was attempted by using a variety of synthetic conditions (0–0.2 M NaCl, pH 5–8, 22–50 °C, 24–48 h) in a procedure similar to that for cyt *c*-Cys80, but yields were less than 5%.

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(22) DPP measurements were conducted by using a PAR Model 174A polarographic analyzer and gold disk electrode on 0.2 mM protein solutions containing 0.01 M 4,4'-bipyridine, 0.2 M NaCl in 0.05 M sodium phosphate pH 7.0 (25 °C). Electrode potentials were measured with a Keithley 177 microvoltmeter, relative to SCE reference. Results were recorded on a Houston Instruments Omnigraphics 2000 recorder.

(23) Spectroelectrochemical determination of reduction potentials was carried out at 22 °C in 0.2 M NaCl and 0.05 M sodium phosphate buffer, pH 6.8, under anaerobic conditions. For procedures, see: Ellis, W. R., Jr.; Wang, H.; Blair, D. F.; Gray, H. B.; Chan, S. I. *Biochemistry* **1986**, *25*, 161–167. $[Ru(NH_3)_6]Cl_3$ (cyt *c*-Leu80) and anthraquinone-2-sulfonate (cyt *c*-Cys80) were used as mediators.

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(32) Carbon monoxide derivatives of the proteins were produced by degassing protein solutions (~50 mM) in 50 mM sodium phosphate buffer, pH 7.0, reducing with sodium dithionite (~5 equiv), and flushing with CO for 30 min.

CO derivatives are similar to that of cyt *c*-CO at pH 13.7 (414 nm, 2.54×10^5 M^{-1} cm^{-1}).[33] In contrast, cytochrome P-450-CO, with thiolate-Fe(II)-CO ligation, has an absorption maximum at 446 nm.[34] These results indicate that residue 80, not His18, is displaced from Fe(II) upon reaction with CO.

We have shown that replacement of the axial methionine in cytochrome *c* can lead to dramatic changes in the heme reduction potential; that cytochrome *c* refolding can be achieved in the absence of a position-80 ligand; and that the ligand-binding properties of cytochrome *c* can be significantly altered. The position-80 cytochrome *c* derivatives should be useful in studies of long-range donor-acceptor electronic couplings in proteins, and work in this area is underway.

Acknowledgment. We thank S. Horvath for peptide preparation. A.L.R. acknowledges postdoctoral fellowship support from the NIH. This research was supported by National Science Foundation Grant CHE88-22988.

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Helix Formation of Melittin on Poly(L-glutamic acid) and Poly(D-glutamic acid)

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Received October 1, 1990

Melittin consists of 26 amino acid residues with six positive and no negative charges: H_3N^+Gly-Ile-Gly-Ala-Val-Leu-Lys^+-Val-Leu-Thr_{10}-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile_{20}-Lys^+-Arg^+-Lys^+-Arg^+-Gln-Gln-NH_2.[1] Many investigators have studied conformational changes of melittin induced by high pH,[2-4] high salt,[4,5] and high melittin concentration,[2,4-6] as well as by the addition of surfactants,[3,7] lipid/membrane,[8] methanol,[9] and calmodulin.[10] However, we now report unprecedented conformational changes of melittin lying on anionic polymers.

Melittin (Sigma) was decolored by gel permeation chromatography with 10% acetic acid as the eluent.[3] The protein concentration was determined with $\epsilon_{280} = 5600$.[2,3] Poly(L-glutamic acid) (PLGA) and poly(D-glutamic acid) (PDGA) were used as the anionic polymers (both from Sigma). The circular dichroism (CD) spectra of melittin-PLGA (A) and melittin-PDGA (B) systems, measured on a JASCO J-600 instrument, are shown in Figure 1. When we subtracted the spectrum of polymer from that of the melittin-polymer mixture, we obtained the spectrum of melittin alone, which was indicative of α-helical structure in both cases. If such a spectrum of melittin was observed only in the melittin-PLGA mixture, there remained a possibility that some parts of the polymer also adopted helical structures induced by the protein. However, the spectrum of melittin alone with the same intensity was observed also in the melittin-PDGA mixture (if the helical structure of PDGA is induced by melittin, the negative intensity of the spectrum should decrease more or less). Therefore, the present results clearly indicate that the helices of melittin are formed on these polymers irrespectively of their optical activity.

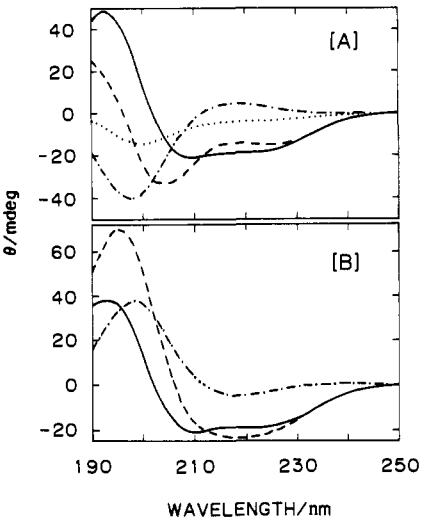

Figure 1. CD spectra of melittin-PLGA (A) and melittin-PDGA (B) systems at neutral pH and 25 °C. Each shows CD spectra of a melittin-polymer mixture (---), polymer along (-·-), and the difference spectrum (—) between them which corresponds to melittin alone in the presence of PLGA (A) and PDGA (B). The dotted curve in part A indicates the spectrum of melittin in the absence of PLGA. Polymerization degree of polymer: 88 (PLGA) and 90 (PDGA). Polymer concentration: 0.52 mM (residue). Melittin concentration: 20 μM (0.52 mM in residue concentration). Light path of cell: 2 mm.

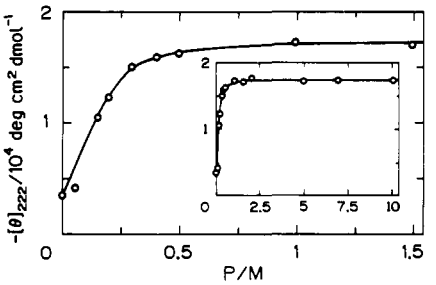

Figure 2. The dependence of $[\theta]_{222}$ for melittin alone on P/M (see text) in melittin-PLGA (polymerization degree: 88) system. The inset shows the data over a wide range of P/M.

The changes of CD spectra of melittin depended on the mixing ratio, P/M (P, PLGA or PDGA; M, melittin, both in residue concentration). Figure 2 shows the dependence of $[\theta]_{222}$ for melittin alone on P/M. The value of $[\theta]_{222}$ abruptly increased until $P/M = 0.4$ (the solution was slightly turbid below $P/M = 0.3$), attaining an approximately constant magnitude of $-18\,000$ to $-17\,000$ deg cm^2 dmol^{-1} at P/M higher than 1. The saturated residue ellipticity is appreciably larger than that at pH 12 and is smaller than that in sodium dodecyl sulfate.[3] We used PLGA with polymerization degrees of 88 and 380 and PDGA with polymerization degrees of 90 and 380. However, the dependence

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