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SHORT COMMUNICATION

Axial Ligand Replacement in Horse Heart Cytochrome *c* by Semisynthesis

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ABSTRACT **Semisynthesis has been employed to replace the axial methionine in horse heart cytochrome *c* with histidine. The reduction potential of the His-80 protein (cyt *c*-His-80) is 41 mV vs NHE (0.1 M phosphate; pH 7.0; 25°C). The absorption spectra of oxidized and reduced cyt *c*-His-80 are very similar to those of the native protein in the porphyrin region, but the 695 nm band is absent in the oxidized His-80 protein.**

Key words: cytochrome *c*, axial ligand, semisynthesis

INTRODUCTION

Changes in axial ligation affect the electron-transfer (ET) properties of cytochromes.[1–5] In the case of cytochrome *c*, stabilization of Fe(II) relative to Fe(III) by the axial methionine is thought to be a key factor in the relatively high reduction potential of the protein.[3–5] The development of reliable methods to vary the axial ligands in cytochrome *c* will lead to the generation of novel proteins for investigations of ET mechanisms.

Work in our laboratory has been concerned with the rates and mechanisms of long-range ET in proteins.[6,7] Modification of cytochrome *c* (both Zn-substituted and native Fe) at histidine 33 with $[a_4RuL]^{3+}$ (a = NH_3; L = NH_3, pyridine, isonicotinamide) has provided a versatile system for ET rate/driving force analyses.[8] We are extending our investigation of this well-characterized system to include an examination of a wider range of driving forces as well as the effect of reorganization energy on ET rate. Variation of the axial Fe ligand in cytochrome *c* will perturb the reduction potential of the iron; it also will induce changes in the polarity of the heme environment, which in turn will affect the outer-sphere ET reorganization energy.[9]

Semisynthesis is a promising methodology for making axial ligand changes in cytochrome *c*.[10] Indeed, we have successfully employed this technique to replace the axial methionine in horse heart cytochrome *c* with histidine. The resulting protein contains a bis-histidine ("b_5-like") heme (pictured in Fig. 1).[11]

MATERIALS AND METHODS

Horse heart cytochrome *c* was obtained from Sigma (Type VI) and was purified by cation-exchange chromatography before use. Mono S and Superose 12 FPLC columns were purchased from Pharmacia.

Cytochrome *c* was cleaved with cyanogen bromide at methionines 65 and 80,[10,12] and peptide 1–65 containing the covalently bound heme was isolated and purified. This cleavage method produces a homoserine (Hse) as the carboxyl terminal residue which then cyclizes to the Hse lactone, the residue which allows reformation of the peptide bond in protein reconstitution. A 15 mg/ml solution of horse heart cytochrome *c* in 70% formic acid was reacted with a 50-fold excess of cyanogen bromide for 24 hours. The solution, in 7% formic acid, was run on both Sephadex G-50 and Superose 12 gel filtration columns to separate 1–65 from other peptide fragments (1–80, 66–104, 81–104) that result from cleavage. A 10/10 Mono S cation-exchange column was then used to separate the 1–65 Hse carboxylate from the Hse lactone terminus.

Peptide 66–104 with histidine at position 80 was synthesized using the stepwise solid phase method performed on an ABI 43A synthesizer. The amide peptides were desalted on Dowex AG.1-X2 resin and purified by reverse-phase HPLC. The quality of the peptides was determined by amino acid analysis and peptide microsequencing.[13,14]

Protein refolding was carried out in 0.1 M sodium phosphate buffer, pH 7.0, using a ~0.3 mM solution of each peptide, for 24 hours. The solution containing 1–65 and 66–104 peptides was treated with ~1 mM dithionite at the start of the reaction. Refolding was monitored using cation-exchange chromatography (10/10 Mono S column, eluted with a linear gra-

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Dedicated to the memory of Irving S. Sigal.

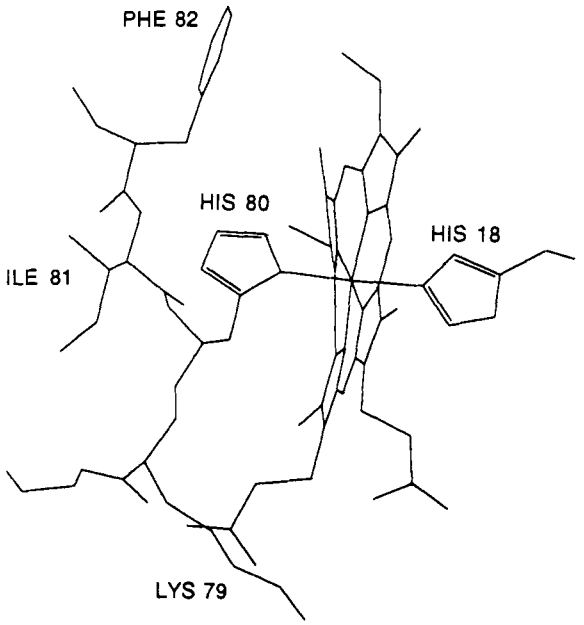


Fig. 1. Structure of the heme region of cytochrome *c* showing amino acids 79–82 with histidine substituted for methionine at position 80.

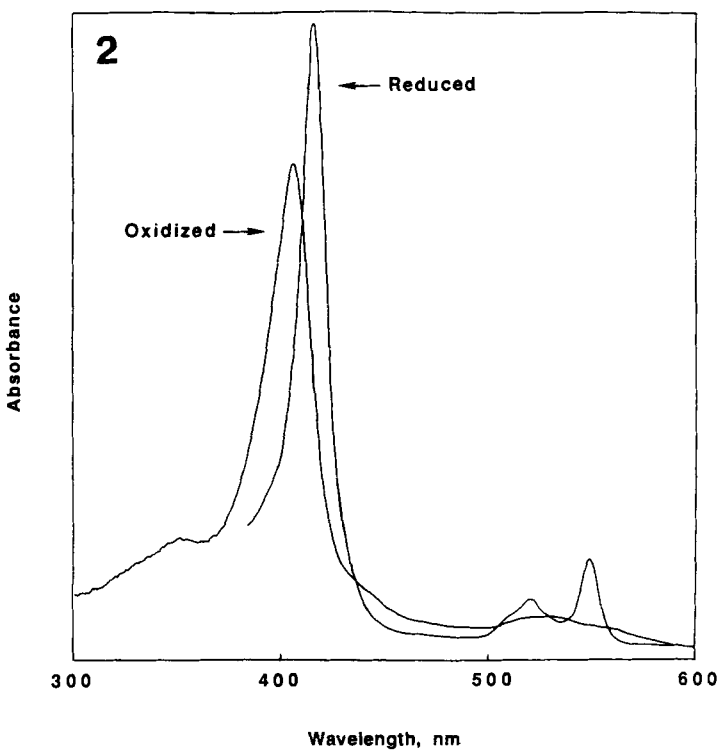
dient from 0.21 to 0.25 M NaCl, in 50 mM sodium phosphate buffer, pH 7.0).[10,14] The complete protein and the 1–65 peptide have different retention times on cation-exchange chromatography columns; it is assumed that all of the isolated protein contains the reformed peptide bond between residues Hse-65 and Glu-66.

Reduction potentials were determined using differential pulse polarography.[15] These measurements employed a gold disk electrode; the solution was 0.4 mM cyt *c*-His-80 with 0.01 M 4,4′-bipyridine in 0.1 M sodium phosphate buffer, pH 7.0 (25°C).

RESULTS AND DISCUSSION

Protein refolding proceeded in 70% yield after making modifications of the method outlined by Corradin and Harbury.[10] Two conformers of the protein containing His-80 (cyt *c*-His-80) were formed initially with reduction potentials of −100 and ~0 mV vs NHE; these subsequently converted to a single species with a potential of 41(5) mV vs NHE.

The decrease in the reduction potential of the native protein (260 mV vs NHE) to 41 mV in cyt *c*-His-80 confirms that Fe(III) is relatively stabilized, as in cytochrome b_5($E^{\circ\prime}$ ~5 mV vs NHE).[16] The isoelectric point of cyt *c*-His-80 is approximately 8, as compared to 10 for the native cytochrome.[17] The



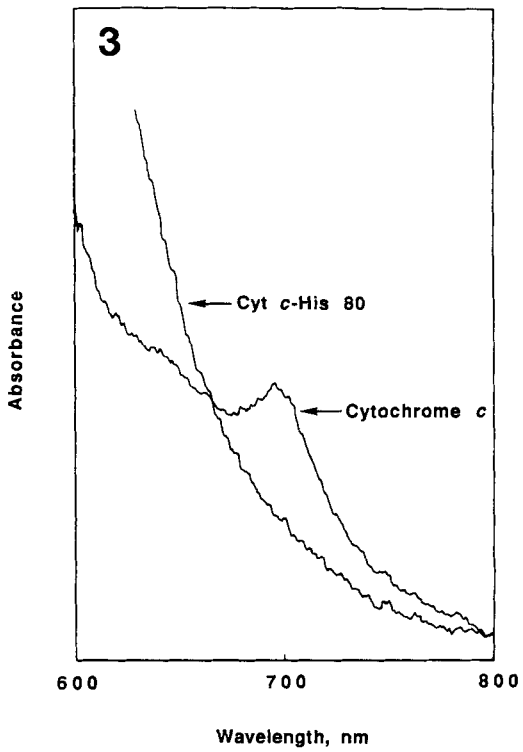


Fig. 2. UV-vis spectra of ~60 μM oxidized and reduced cyt *c*-His-80 in 50 mM sodium phosphate buffer, pH 7.0. The full absorbance scale is 0.75.

Fig. 3. Absorption spectra (600–800 nm) of 0.1 mM cyt *c*-His-80 and 0.1 mM cytochrome *c* in 50 mM sodium phosphate buffer, pH 7.0. The full absorbance scale is 0.2.

overall charges of the proteins should be nearly the same, so polarity differences in regions of the protein surface may be induced by the substitution. The UV-vis absorption spectra of both oxidized and reduced forms of cyt *c*-His-80 are very similar to those of the native protein in the porphyrin region [Fig. 2: $\epsilon_{524} = 1.2 \times 10^4\ M^{-1}\ cm^{-1}$ (oxidized), $\epsilon_{415} = 1.5 \times 10^5$, $\epsilon_{520} = 1.6 \times 10^4$, and $\epsilon_{550} = 2.5 \times 10^4$ (reduced)]. For native cytochrome *c*, $\epsilon_{528} = 1.1 \times 10^4\ M^{-1}\ cm^{-1}$ (oxidized), $\epsilon_{416} = 1.3 \times 10^5$, $\epsilon_{520} = 1.6 \times 10^4$, and $\epsilon_{550} = 2.8 \times 10^4$ (reduced).[18] Significantly, the 695 nm absorption band, which is believed to be due to sulfur (methionine) charge transfer to Fe(III),[5] is absent in oxidized cyt *c*-His-80 (Fig. 3).

Using random mutagenesis, Sherman and co-workers have replaced the axial His-18 of yeast iso-1-cytochrome *c* with Tyr and Asn, and Met-80 with Arg and Ile.[1] Of these, Arg and Ile-80 proteins were produced, but the proteins were largely nonfunctional. In addition, using site-directed mutagenesis in yeast iso-2-cytochrome *c*, Sorrell and co-workers have substituted His-18 with Arg, and isolated an active cytochrome *c* with a reduction potential similar to that of the native protein, but with a reduced ET self-exchange rate.[2]

We have demonstrated that an axial ligand substitution can be made efficiently in cytochrome *c* by semisynthesis. This method should be useful in further investigations of the role of the axial ligand in cytochrome *c* reactions; it also should allow incorporation of unnatural amino acids into the protein.

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