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Polymethionyl, -valyl and -glycyl Derivatives of Equine Heart Cytochrome *c* Heme Octapeptide

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Abstract

Polymethionyl ($(Met)_{5.3}$-H8PT†), polyvalyl ($(Val)_{4.4}$-H8PT) and polyglycyl ($(Gly)_{3.2}$-H8PT) derivatives of the heme octapeptide of equine heart cytochrome c were prepared with the aid of the N-carboxyl anhydrides of their respective amino acids. Only for the $(Met)_{5.3}$-H8PT did the γ-peak in the absorption spectrum of the reduced form shift from 412.5 nm, the value for the unsubstituted octapeptide, to 414 nm, the value close to the γ-peak of intact ferrocytochrome c. The γ-peak of the oxidized form of the octapeptide did not shift significantly from 397 nm. Amino acids attached to the N-terminal cysteine of the octapeptide, with methionine excepted, have little or no effect on the spectrum, and apparently cannot serve as either an intramolecular or intermolecular ligand for the iron. This result is in marked contrast to those previously obtained with the substituted cytochrome c heme undecapeptide (ref. 8) where all three derivatives had altered spectra. For the octapeptide, the ratio, absorbance of the γ-peak of the oxidized form to absorbance of the γ-peak of the reduced form, did not change appreciably for any of the derivatives, although when the reaction products from excess methionine anhydride were present (before centrifugation and dialysis) the ratio was lower and approached the ratio for cytochrome c.

Introduction

Hydrolysis of equine heart cytochrome c with pepsin results in a heme undecapeptide [1, 2] which when treated with trypsin to remove the

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†H8PT, heme octapeptide prepared with pepsin and trypsin.

N-terminal sequence, Val-Gln-Lys, yields the following heme octapeptide [2]:

Cys-Ala-Gln-Cys-His-Thr-Val-Glu
└——Heme——┘

Harbury and Loach [3, 4] studied the oxidation-reduction proton functions in the octa- and undecapeptides, as well as the interaction of some nitrogenous ligands with them. Harbury *et al.* [5] added N-acetyl-DL-methionine, N-acetyl-DL-methionine methyl ester, diethyl sulfide, β-methylmercaptopropionic acid, 2,2′-thiodiglycol or imidazole to the heme octapeptide and found that the thioether compounds transformed a broad flat maximum in the visible absorption spectrum into a typical α- and β-peaked hemochrome spectrum which persisted down to pH 2.34. Imidazole also produced a two-peaked spectrum, but no evidence of it remained at pH 4. Kowalsky [6] found by NMR spectroscopy that there was a specific interaction between the S-methyl protons of N-acetylmethionine and the heme octapeptide. Nanzyo and Sano [7] added N-acetyl-L-methionine, L-tryptophane, Pro-Gly-Thr-Lys-Met-Ile-Phe-Ala, N-acetyl-Thr-Gly-Lys-Gly or N-acetyl-Thr-Trp-Lys-Gly to the octapeptide and concluded that since tryptophane, alone or in a synthetic tetrapeptide, could coordinate with the iron, the possibility existed that a mixed hemochrome, such as Trp-Fe-His, might also be formed in cytochrome c.

Having earlier synthesized polyamino acid derivatives of the heme undecapeptide [8], we were interested in producing the same derivatives of the heme octapeptide in order to study the effect on the absorption spectrum, even though there is no longer any question but that His-18 and Met-80 are two of the six ligands for the iron in cytochrome c [9].

Methods

Cytochrome c heme octapeptide was prepared by hydrolysis of Sigma Chemical Co., Type IV, equine heart cytochrome c with pepsin [1, 2] and trypsin [2, 3]. N-Carboxy-glycine anhydride [10] was prepared from N-carbobenzoxy-glycine, while N-carboxy-L-methionine anhydride and N-carboxy-L-valine anhydride [11] were prepared from their respective amino acids with phosgene. The octapeptide was reacted at room temperature in 0.196 M phosphate buffer, pH 7.45, for one h each after three successive additions of ten molar excess of the appropriate amino acid anhydride. The reaction mixtures were finally centrifuged to remove precipitates and were dialyzed for 12 h against water at 4° and then freeze-dried. Amino acid analyses were performed with a Beckman Model 120B analyzer. The octapeptide had the expected composition. The average polyglycylated H8PT contained 3.2 residues of glycine

($(Gly)_{3.2}$-H8PT), while the polyvalylated H8PT contained 4.4 added residues of valine ($(Val)_{4.4}$-H8PT), and the polymethionylated H8PT contained 5.3 residues of methionine ($(Met)_{5.3}$-H8PT). Spectra were recorded with a Cary Model 11 spectrophotometer after dissolving the polyamino acid derivatives in 0.1 M phosphate buffer of the designated pH. Reduction was accomplished when necessary with $Na_2S_2O_4$. The heme concentration was determined by analyzing for iron with a Perkin-Elmer, Model 303, atomic absorption spectrophotometer.

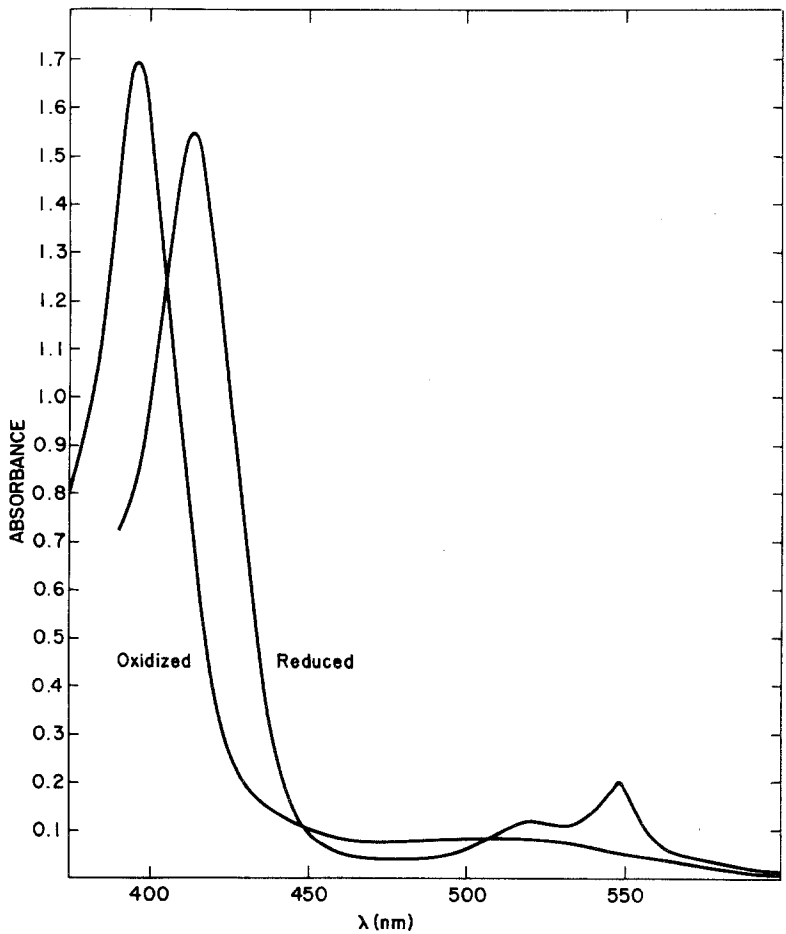

Figure 1. Spectra of oxidized and reduced heme octapeptide of equine heart cytochrome c at a concentration of $\sim 2.2 \times 10^{-5}$ M.

TABLE I. Absorption spectra maxima and absorbance ratios at pH 7.45 for cytochrome c heme octapeptide and three of its polyamino acid derivatives

Compounds or mixtures	Maxima				Ratios
	Oxidized	Reduced			Oxidized γ-peak:
	γ-peak	γ-peak	β-peak	α-peak	Reduced γ-peak
	nm	nm	nm	nm	
H8PT	397	412.5	521	548	1.14
H8PT + Met anhydride[a]	397.5	415	522	550	0.93
$(Met)_{5.3}$-H8PT[b]	397	414	521.5	549	1.07
H8PT + Val anhydride[a]	397	415	522	548	1.11
$(Val)_{4.4}$-H8PT[b]	396.5	412.5	521	548	1.15
H8PT + Gly anhydride[a]	397	413	520	547	1.07
$(Gly)_{3.2}$-H8PT[b]	397	413	520	548	1.06
Cytochrome c	408	415	520	550	0.82

[a] 30 molar excess; before centrifugation and dialysis.
[b] After centrifugation and dialysis.

Results

The spectra of the oxidized and reduced heme octapeptide at pH 7.45 are shown in Fig. 1. The curves are like those presented by Nanzyo and Sano [7] except that the γ-peak in the reduced curve in Fig. 1 is at 412.5 nm rather than at 414 nm. The ratio oxidized γ-peak : reduced γ-peak is > 1 in both instances.

The step-wise addition of any one of the three Leuch's anhydrides did not significantly shift the position of the γ-peak of the oxidized form to higher wavelengths from 397 nm (Table I). The position of the γ-peak of the reduced form was significantly nearer that for ferrocytochrome c only with the polymethionylated heme octapeptide, whereas the β-peak and the α-peak were not significantly affected. The ratio of the oxidized γ-peak : reduced γ-peak fell to < 1 only when N-carboxy-L-methionine anhydride was added. The ratio returned to > 1 when the precipitate in the reaction mixture was removed by centrifugation and the supernatant fluid was dialyzed. It would seem as though free methionine or polymethionine was responsible for the ratio < 1, which is also < 1 for cytochrome c. In one experiment with methionine anhydride, a prominent shoulder centered at 408 nm was found on the high wavelength side of the γ-peak of the oxidized octapeptide before centrifugation and dialysis. Since the γ-peak in the spectrum of oxidized cytochrome c is at 408 nm, this again suggested that polymethionine was a better ligand than L-methionine or N-acetyl-L-methionine, neither of which shifted the γ-peak of the oxidized form of the octapeptide to higher wavelengths from 397 nm. These data show that an amino acid not serving as a ligand for the iron, even though in the vicinity of the heme, has little effect on the spectrum. Furthermore, an amino acid added to the cysteine at the N-terminal, with methionine excepted, apparently cannot serve as either an intramolecular or intermolecular ligand to the iron. These results are in marked contrast to those previously obtained with the substituted cytochrome c heme undecapeptide [8] where all three derivatives had altered spectra.

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