The Effect of Methylation on Cytochrome c Fragment Complementation*

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The heme propionates of heme peptide 1-25 from horse heart cytochrome c were methylated in acidified methanol. The methylated heme peptide combines with apocytochrome c in a single second order kinetic phase whose rate constant is within a factor of four of that measured for the unmethylated heme peptide. The dissociation constants for the two complexes are also within a factor of four. The methylated and unmethylated complexes exhibit similar fluorescence quenching, far ultraviolet dichroic spectra, and catalytic activities. The methylated complex has a T m about 10 °C lower than that of the unmethylated complex. The charge transfer band of the methylated complex occurs at 720 nm instead of 695 nm as observed for the unmethylated complex. It is proposed that methylation of the outer heme propionates causes a small steric effect which alters the geometry of the methionine 80 ligand with the heme iron.

The heme moiety of cytochrome c is critical to the acquisition and stabilization of its native globular conformation. Since apocytochrome c (1-104) has the properties of a random coil in solution (1, 2). In contrast to most heme proteins, the heme propionyl side chains are inaccessible to solvent (3). Since the propionyl carboxyl groups are both ionized in the native conformation (4), it would be expected that they would be buried with identifiable positive charges forming salt linkages. However, the crystallographic structure suggests that the formal negative charges of each propionate is only diminished by a network of hydrogen bonds (5).

In this report we attempt to assess the contribution of this unusual bonding situation to the acquisition and stabilization of the native conformation. We have chosen to eliminate the propionate formal charge by esterification with methanol so as to minimize any steric consequences of modification. Since free heme cannot be combined with apocytochrome c, it is necessary to esterify the heme while conviently attached to the polypeptide. In order to minimize coincident esterification of aspartate and glutamate side chain carboxyl groups, we have esterified the smallest heme peptide known to form a functional complex with apoprotein, namely heme peptide 1-25 termed (1-25)H. This peptide has two glutamic acids and a single aspartic acid all of which are known to be exposed to the solvent in the native structure (5). Consequently, esterification of the amino acid carboxyl groups would be expected to have little effect.

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EXPERIMENTAL PROCEDURES

Materials—Horse heart cytochrome c, type VI, was purchased from Sigma and served as the source for all fragments used in this study. Apocytochrome c and heme peptide (1-38)H were prepared using the procedures of Fisher et al. (2) and Parr et al. (6). Heme peptide (1-25)H was obtained by tryptic digestion of the (1-38)Hapocytochrome c complex as described by Parr et al. (6). Each fragment was eluted from a calibrated Sephadex G-50 column (7) as a single symmetrical component in a volume appropriate to its size and had an amino acid composition following hydrolysis for 24 h at 110 °C in 6 N HCl consistent with its assignment.

Esterification—Heme peptide (1-25)H was esterified in methanol as described by Stellwagen et al. (8). The extent of esterification of the heme propionates in (1-25)H was measured by thin layer chromatography following cleavage of the heme from the esterified peptide using Ag2SO4 (1). The cleaved heme was extracted with ether containing 25% (v/v) acetic acid and the ether extract washed with 5% (w/v) NaCl. An aliquot of the washed ether extract was applied to cellulose and chromatographed using 52% kerosene, 45% chloroform, and 5% 1-propanol (v/v/v). In contrast to heme cleaved from unesterified (1-25)H which remains at the origin, all the heme cleaved from (1-25)H-OMe migrated as a single component with an R f of 0.8. No heme could be detected remaining at the origin. By varying the amount of peptide applied, it was deduced that no less than 97% of the heme in (1-25)H-OMe is dimethylated.

Spectral Measurements—All spectral measurements were made at 25 °C using 100 mM phosphate buffer, pH 7.0, as the solvent unless noted otherwise. Absorbance spectra were obtained using a Cary model 17 recording spectrophotometer. The concentration of solutions of ferriheme c and peptide complexes, ferriheme peptides (1-25)H and (1-25)H-OMe, and apocytochrome c were determined using absorptivities of 106 mm -1 cm -1 at 409 nm, 133 mm -1 cm -1 at 406 nm, and 115 mm -1 cm -1 at 276 nm, respectively. Circular dichroic spectra in the far ultraviolet were obtained using a Cary model 60 spectropolarimeter having a circular dichroic attachment. Equilibrium fluorescence measurements were obtained with a Hitachi model MPF-2A fluorometer. Bandwidths were 6 nm for the 280 nm excitation beam and 12 nm for the observed emission at 340 nm. The relative fluorescence intensity of peptide complexes is defined as the measured emission intensity in arbitrary units per µM apocytochrome c. Correction was made for the absorbance of heme fragments by comparing the fluorescence intensity of apocytochrome c and heme peptide-apocytochrome c mixtures in 6 M guanidine hydrochloride. The kinetics of fluorescence changes associated with peptide complexation was measured using a Durrum-Gibson stopped flow spectrophotometer having a fluorescence attachment. Measurements were made using a 280 nm excitation beam and a Corning type 0-54 filter to minimize scattering below 300 nm.

Biological Activity—The initial rate of reduction of ferriheme c, ferriheme peptides, and ferriheme peptide complexes by yeast lactate dehydrogenase was observed spectrophotometrically at 417 nm. Reduction was initiated by addition of 0.04 unit of dehydrogenase to a cuvette containing 3 mM l-lactate, 3 mM ferriheme, and 0.1 M potassium phosphate buffer, pH 7.0, at 25 °C, in a total volume of 1.0 ml.

RESULTS

The fluorescence of the single tryptophan residue in horse heart cytochrome c located at position 59 is largely quenched by its proximity to the heme moiety in the native protein (2). Denaturation of cytochrome c and/or removal of the heme to
form apocytochrome c, a random coil (1, 2), results in at least an order of magnitude increase in the fluorescence intensity of tryptophan 59. This change in fluorescence intensity has been used to monitor the kinetics and extent of folding of cytochrome c (9) and the association of heme peptides with apocytochrome c (10). Addition of incremental portions of ferriheme peptide (1-25)H to a fixed concentration of apocytochrome c results in a systematic quenching of tryptophan fluorescence reaching a maximal change at a hemepeptide/apoprotein molar ratio of one as shown in Fig. 1A. Using the procedure of Richards and Vithayathil (11) and Hantgan and Taniuchi (10), the stepwise diminution in fluorescence intensity shown in Fig. 1A indicates formation of a (1-25)H-(1-104) complex having a dissociation constant of 40 nM, a value in agreement with that reported previously (12). Addition of methylated ferriheme peptide (1-25)H-OMe to apocytochrome produces a similar stepwise quenching of tryptophan fluorescence as shown in Fig. 1A, indicative of formation of a stoichiometric (1-25)H-OMe-(1-104) complex having a dissociation constant of 140 nM. However, the peptide complex containing (1-25)H-OMe is somewhat less thermostable than the complex containing the unmethylated heme peptide (1-25)H as shown in Fig. 1B. The increase in fluorescence intensity of the (1-25)H-OMe-(1-104) complex occurs in a rather broad single transition centered at about 40 °C while that for the complex (1-25)H-(1-104) occurs in a sharper transition centered at 50 °C. It should be noted that the fluorescence for each sample attains that of a comparable concentration of free tryptophan at the completion of the transition.

The kinetics of association of the heme peptides with apoprotein were measured by stopped flow spectrofluorimetry at 25 °C using a 1.5-fold molar excess of heme peptide to minimize the contribution of complex dissociation to the observed process. Fluorescence changes were analyzed using a second order kinetic expression for unequal concentrations of reactants (12). Typical results are shown in Fig. 2. Ferriheme peptide (1-25)H complexes with apoprotein with a rate constant of 4.2 μM⁻¹ s⁻¹ in agreement with previous measurements (12) while ferriheme peptide (1-25)H-OMe associates with apoprotein with a rate constant of 1.2 μM⁻¹ s⁻¹.

The far ultraviolet circular dichroic spectra of ferriheme peptide complexes (1-25)H-(1-104) and (1-25)H-OMe-(1-104) are similar as shown in Fig. 3A. The dichroic spectra of these complexes are characteristic for native ferriheme peptide and distinct from the dichroic spectra of the unstructured individual components as seen in Fig. 3A.

The visible absorbance spectra of peptide complexes (1-25)H-(1-104) and (1-25)H-OMe-(1-104) are characteristic for a low spin coordination complex having two strong field protein ligands in the axial positions such as that found in native cytochrome c. Both complexes exhibit absorbance maxima at 415, 520, and 550 nm in the ferro form having A₄₁₅/A₅₅₀ and A₄₁₅/A₅₂₀ ratios of 1.81 ± 0.06 and 0.19 ± 0.02, respectively. The ferri forms of both complexes exhibit maxima at 407 and 530 nm having a A₄₀₇/A₅₃₀ ratio of 17.9 ± 0.4. Ferri complex (1-25)H-(1-104) exhibits a weak charge transfer absorbance band centered at 695 nm as shown in Fig. 3B. This band is found in the native ferriheme c and is diagnostic for ligation of methionine 80 in an axial coordination position (13). While ferricomplex (1-25)H-OMe-(1-104) also exhibits a weak charge transfer band it is centered at 720 nm as shown in Fig. 3B.

Acidification of ferriheme c in high concentrations of NaCl results in changes in the Soret absorbance at 395 nm indicative of the stepwise dissociation of the two protein ligands in the axial coordination positions (14). The dissociation of the axial ligand with a pK of 3.7 in 3 M NaCl has been assigned to methionine 80 (8). As shown in Fig. 4A, the increase in the 395 nm absorbance of the two ferriheme complexes describe a common transition having an apparent pK of 3.5 suggesting that each complex possesses a methionine ligand at neutral pH.

As shown in Fig. 4B, incremental addition of ferriheme
The major difference observed in the properties of complexes (1-25)H-OMe is in their acid pK values for both methionine 80 and 5.2 A from the sulfur-iron ligation. Since charge transfer bands are particularly sensitive to geometry, the maximum for the 695 nm band could be readily shifted upfield to 720 nm by the steric perturbation resulting from methylation of the buried outer propionate carboxylate. In this context we note that the charge transfer band does not have a constant absorbance maximum in all native cytochromes c, e.g. for Euglena cytochrome c is centered at 700 nm (15). However, it is likely that amidination of the heme propionate carboxylate further decreases the absorbance of these bands.

REFERENCES