

Alkaline Isomerization of Ferricytochrome *c*: Identification of the Lysine Ligand

(heme protein/peptide reconstitution/guanidination)

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ABSTRACT Changes in the visible absorbance spectra of complexes of horse heart cytochrome *c* hemopeptide 1-65, peptide 66-104, and their guanidinated counterparts are compared with those characteristic of native and fully guanidinated ferricytochrome *c* over the pH range 7 to 11. Upon raising the pH, the methionine ligand in the guanidinated hemopeptide 1-65-peptide 66-104 complex is replaced by a strong field ligand. By contrast, the methionine ligand in the hemopeptide 1-65-guanidinated peptide 66-104 is replaced by a weak field ligand. These results demonstrate that lysine 13 does not ligate with the heme iron upon isomerization of ferricytochrome *c* and that the ligand in the horse heart protein is one of the eight lysine residues in the 66-104 segment of the polypeptide, most likely lysine 79.

The sulfur atom of methionine 80 is ligated with the heme iron of cytochrome *c* at neutral pH (1). As the pH of solutions of ferri-, but not ferro-, cytochrome *c* is raised, the sulfur ligand is replaced by a strong field ligand which forms a low spin complex having the electron paramagnetic resonance spectrum characteristic of a primary amine (2). This ligand substitution prevents reduction of the heme iron by ascorbate or ferrocyanide (3, 4). Replacement of the sulfur ligand by lysine after carboxymethylation of methionine 80 produces a modest conformational change (5) which also prevents reduction of the heme iron by ascorbate or ferrocyanide and which makes the protein incapable of transferring electrons when added to cytochrome *c* depleted mitochondria (6, 7). Inspection of the crystallographic model of horse heart ferricytochrome *c* has led several investigators (8-10) to assume that the primary amine ligand is the ϵ -amino group of lysine 79 because of its accessibility to the heme iron. However, the crystallographic model also suggests that the ϵ -amino group of lysine 13 is accessible to the heme iron. In the case of small hemopeptides of cytochrome *c* (11, 12), lysine 13 is known to ligate with the heme iron.

The pH dependent ligand exchange of ferricytochrome *c* is most easily described by observing the loss of a small absorption maximum at 695 nm characteristic of sulfur ligation (13). In the case of horse heart ferricytochrome *c*, the loss of $\epsilon_{695 \text{ nm}}$ with increasing pH describes a titration curve involving a single proton dissociation having an apparent pK ranging from 8.9 to 9.3 (10, 14). This transition is distinct from the gross alkaline denaturation of cytochrome *c* which has an apparent pK of 12.5 (15). The kinetics of the changes of absorbance at 695 nm with pH have been analyzed in terms of an ionization having an intrinsic pK of 10.9 coupled with a ligand replacement isomerization having an equilibrium con-

stant of 83 at 21° (14). The intrinsic pK of 10.9 very likely represents the pK of the ϵ -amino group of the lysine ligand. Since only the dissociated amino group can ligate, the coupling of dissociation with ligation results in $\text{pK}_{\text{app}} < \text{pK}_{\text{intrinsic}}$.

Conversion of all 19 lysine residues of ferricytochrome *c* to homoarginine residues by exhaustive reaction with *O*-methyl isourea does not alter the physical, chemical, or biological activity of the protein at neutral pH (16). Since the intrinsic pK of homoarginine is substantially higher than that of lysine, homoarginine cannot replace the methionine sulfur ligand of ferricytochrome *c* in the pH range from 7 to 11. Absorption and electron paramagnetic resonance spectral titrations of guanidinated ferricytochrome *c* (17) demonstrate that its methionine ligand is replaced by a solvent hydroxide ion which forms a high spin complex and that this replacement has an apparent pK of 9.4.

Corradin and Harbury (18) have recently demonstrated that hemopeptide 1-65 and nonheme peptide 66-104 can be combined noncovalently to form a complex having the physical and biological properties characteristic of native cytochrome *c*. This system then presents a method for distinguishing whether the ligand for the alkaline isomer of ferricytochrome *c* is lysine 13 or lysine 79. If lysine 79 is the ligand which displaces methionine 80, then a hybrid complex consisting of hemopeptide 1-65-guanidinated peptide 66-104 should produce an absorption spectrum characteristic of a high spin complex upon titration to pH 10. By contrast, formation of the opposite hybrid complex, consisting of guanidinated hemopeptide 1-65-nonguanidinated peptide 66-104 should produce a typical low spin absorbance spectrum at pH 10. The converse spectra should be observed if lysine 13 is the ligand which displaces methionine 80 in the alkaline isomer of ferricytochrome *c*.

MATERIALS AND METHODS

Horse heart cytochrome *c* was obtained from Sigma Chemical Co. (Type III). The protein was guanidinated by reaction with 0.5 M *O*-methylisourea for 11 days at pH 11.0. Amino acid analysis of the product after acid hydrolysis showed the presence of 0.2 residues of lysine and 18.8 residues of homoarginine per molecule of protein. The apparent persistence of 0.2 lysine residues can be ascribed to the 1% conversion of homoarginine to homoornithine during acid hydrolysis (24 hr at 110°) and the coelution of lysine and homoornithine with a pH 5.28 elution buffer during routine amino acid analysis at 50° (19).

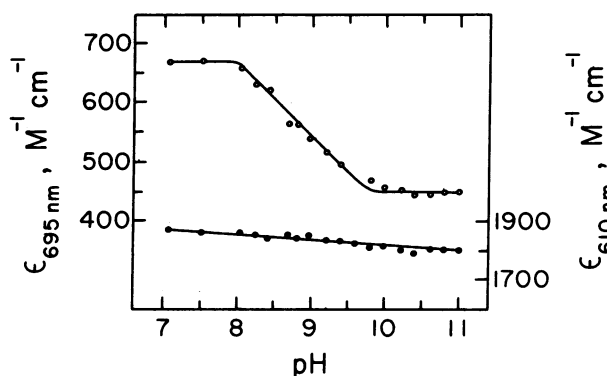


FIG. 1. Spectrophotometric titration of the ferrihemopeptide 1-65-peptide 66-104 complex. O, molar extinction at 695 nm; ●, molar extinction at 610 nm.

Both native and guanidinated cytochrome *c* were partially digested with a 3-fold molar excess of cyanogen bromide for 24 hr in 70% formic acid. The peptides in the digest were purified by repeated exclusion chromatography on Sephadex G-50F. The purified peptides 1-65 and 66-104 had amino acid compositions within $\pm 3\%$ of the expected values. Equimolar amounts of the desired peptides were recombined at 4° *in vacuo* for 24 hr in 0.1 M phosphate buffer, pH 6.0, containing excess dithionite. The complexes were then dialyzed against 0.1 M phosphate pH 7; excess ferricyanide was added and dialysis continued against the phosphate buffer and then against 50 mM KCl. All complexes at neutral pH exhibited absorption spectra, circular dichroic spectra, and biological activity characteristic of native ferricytochrome *c*.

Spectrophotometric titrations were performed at room temperature in 50 mM KCl by addition of 1 M NaOH. The concentrations of aliquots of the hemopeptides 1-65 and the complexes were determined by formation of their pyridine hemochromes (18). The concentrations of peptides 66-104 were calculated from their near ultraviolet absorption using the molar extinctions for the constitution aromatic residues. Absorption spectra were obtained with a Cary model 14 spectrophotometer.

RESULTS AND DISCUSSION

The complex consisting of hemopeptide 1-65 and peptide 66-104 at neutral pH exhibits a maximum at 695 nm indicating the presence of a sulfur ligand. As the pH of solutions of this

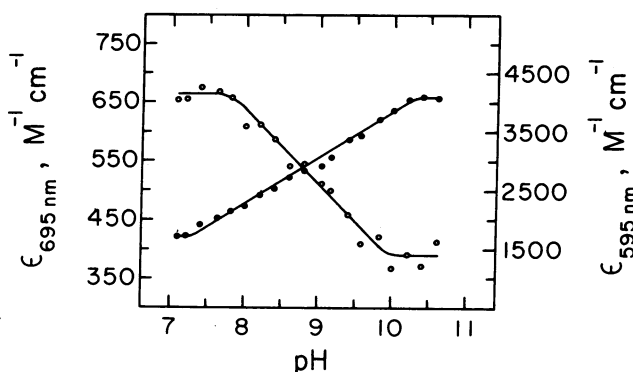


FIG. 2. Spectrophotometric titration of fully guanidinated horse heart ferricytochrome *c*. O, molar extinction at 695 nm; ●, molar extinction at 595 nm.

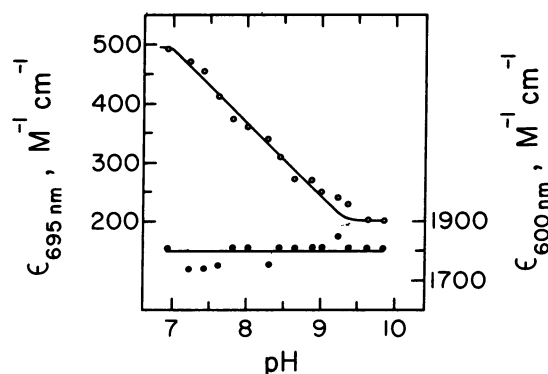


FIG. 3. Spectrophotometric titration of the ferri-guanidinated hemopeptide 1-65-peptide 66-104 complex. O, molar extinction at 695 nm; ●, molar extinction at 600 nm.

complex is raised, the 695-nm band disappears with an apparent pK of 8.9, as shown in Fig. 1, indicating the displacement of the sulfur ligand. No concomitant change in the spectral region about 600 nm is observed indicating that a strong field ligand, most probably a lysine residue, has replaced the sulfur ligand. This behavior is characteristic of the intact native protein. By contrast, spectra obtained upon increasing the pH of intact totally guanidinated ferricytochrome *c* exhibit a concomitant loss of the 695 nm absorption band and appearance of a maximum at 595 nm, characteristic of a high spin complex having a weak field hydroxide ligand. As shown in Fig. 2, both transitions have an apparent pK of 8.8.

As shown in Fig. 3, the spectral titrations observed with the hybrid complex, guanidinated hemopeptide 1-65-nonguanidinated peptide 66-104, are similar to those observed with complex formed from both nonguanidated peptides. This demonstrates that the lysine ligand is not one of the 11 lysine residues in hemopeptide 1-65. By contrast, the spectral titrations of the other hybrid complex, nonguanidated hemopeptide 1-65-guanidinated peptide 66-104 shown in Fig. 4, are characteristic of those of the totally guanidinated protein. The displacement of the sulfur ligand indicated by the loss of the 695 nm absorbance maximum is mirrored by the appearance of a maximum at 600 nm characteristic of the ligation of a weak field hydroxide anion.

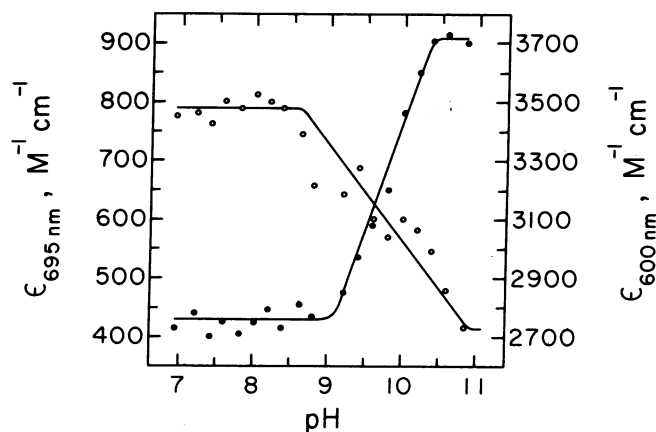


FIG. 4. Spectrophotometric titration of the ferrihemopeptide 1-65-guanidinated peptide 66-104 complex. O, molar extinction at 695 nm; ●, molar extinction at 600 nm.

These results clearly demonstrate that selective guanidination of the eight lysine residues in peptide 66-104 prevents ligation of a lysine residue in the alkaline isomer of ferricytochrome *c*. Since the alkaline isomerization of all native ferricytochromes *c* produces a low spin complex, the lysine ligand is likely to occupy an invariant position in the sequence or an equivalent position in the native conformation. Of the eight lysine residues in peptide 66-104, only the three lysine residues located at positions 72, 73, and 79 are reported to be invariant (1, 20). Lysine 72 is replaced by a serine residue in the sequence of *Tetrahymena pyriformis* cytochrome *c* (G. Tarr and W. M. Fitch, unpublished). Lysine 73 is replaced by an alanine residue in the equivalent conformational position of *Rhodospirillum rubrum* cytochrome *c*₂ (21). In view of the unique invariance of lysine 79 (or its conformational analog) in all sequences of cytochromes *c* and *c*₂, the easy access of lysine 79 to the heme iron in the crystallographic model of the pH 7 isomer of horse heart ferricytochrome *c*, and the small conformational perturbation characteristic of replacement of the methionine 80 ligand with a lysine (5), we propose that lysine 79 occupies the sixth coordination position of the heme ion in the alkaline isomer of ferricytochrome *c*.

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