Involvement of Lysines-72 and -79 in the Alkaline Isomerization of Horse Heart Ferricytochrome *c*†

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ABSTRACT: Spectrophotometric titrations of five singly modified horse heart ferricytochromes *c*, specifically (trifluoromethyl)phenylcarbamylated ($CF_3PhNHCO$–) or trifluoroacetylated (CF_3CO–) at lysines-13, -72, and -79, were carried out. The $CF_3PhNHCO$-Lys-13, Lys-79, and CF_3CO-Lys-79 derivatives all underwent alkaline isomerization with loss of the 695-nm band to low-spin species with an apparent p*K* of about 8.9, as did the unmodified cytochrome. However, modification of lysine-72 appeared to alter the reaction pathway since the $CF_3PhNHCO$-Lys-72 derivative isomerized to a high-spin form with an apparent p*K* of 9.3, while the CF_3CO-Lys-72 derivative isomerized to a low-spin species with an apparent p*K* of 9.6, indicating that lysine-72 may be the normal sixth iron ligand in the native protein alkaline isomer. These results, together with those of other workers, suggest a model for the alkaline transition in which replacement of the methionine iron ligand is dependent on a number of factors, including the local availability and relative affinities of possible ligands for the heme iron and the effects of ionic and hydrophobic interactions on the tertiary structure of the molecule.

Theorell & Åkesson (1941) first investigated the five discrete pH-dependent conformational states of horse heart cytochrome *c*. Since these early studies, these isomerizations and the mechanisms by which they take place have been much studied. In particular, the state III to state IV[1] transition, which occurs with a p*K* of 9.35, has been of considerable interest, and there have been suggestions that mitochondrial cytochrome *c* may be chemically similar to its alkaline aqueous form (Letellier & Schejter, 1973; Davis et al., 1974; Looze et al., 1978).

The III and IV transition occurs with the disappearance of the 695-nm band, indicating the replacement of methionine-80 as an iron ligand by another strong-field ligand, probably a nitrogenous base (Hettinger & Harbury, 1964; Redfield & Gupta, 1971; Gupta & Koenig, 1971; Blumberg & Peisach, 1971; Morton, 1973). The pH-linked ligand substitution parallels a lowering of the redox potential (Margalit & Schejter, 1973) and nonreducibility by ascorbate and ferrocyanide (Greenwood & Palmer, 1965; Brandt et al., 1966; Lambeth et al., 1973; Davis et al., 1974).

Modification of all of the lysine residues by guanidination or trifluoroacetylation prevents the normal III to IV transition (Hettinger & Harbury, 1964; Fanger & Harbury, 1965; Morton, 1973; Stellwagen et al., 1975), suggesting that a lysine ε-amino group replaces the methionine-80 ligand in the native protein under alkaline conditions. Selective guanidination of the eight lysine residues in peptide 66–104 of the horse heart cytochrome also abolished the normal alkaline isomerization, implicating one of those lysines as the alkaline iron ligand (Wilgus & Stellwagen, 1974). On the basis of the X-ray crystallographic model (Dickerson et al., 1971), workers have proposed that lysine-79 is the iron ligand in the native protein but not in the modified proteins (Redfield & Gupta, 1971; Lambeth et al., 1973; Davis et al., 1974; Wilgus & Stellwagen, 1974; Stellwagen et al., 1975; Looze et al., 1978). However, this assignment has been disputed (Pettigrew et al., 1976), although there is no direct evidence to support either claim.

We report here on the use of five horse cytochrome *c* derivatives, specifically modified at lysine residues 13, 72, and 79, to show that under certain conditions either lysine-72 or lysine-79 can become the sixth ligand in the state IV isomer.

Experimental Procedure

Materials. Horse heart cytochrome *c* (type VI) was obtained from Sigma Chemical Co. $CF_3PhNHCO$[2]-Lys-13, $CF_3PhNHCO$-Lys-72, $CF_3PhNHCO$-Lys-79, and native cytochromes *c* were prepared and purified as described by Smith et al. (1977). CF_3CO-Lys-72 and CF_3CO-Lys-79 cytochromes *c* were prepared and purified according to Staudenmayer et al. (1976, 1977) and M. B. Smith, J. Stonehuerner, A. J. Ahmed, N. Staudenmayer, and F. Millett (unpublished experiments).

Spectrophotometric Titrations. Samples of native and modified cytochromes *c* equilibrated in 50 mM KCl–50 mM borate (pH 6.5) or in 50 mM KCl (pH 6.5) and oxidized with a small amount of 0.1 M $K_3Fe(CN)_6$ were passed through a Bio-Gel P-60 column equilibrated with the same buffer to remove the ferricyanide and any polymeric material. Titrations of 30–50 μM solutions of cytochromes *c* in KCl–borate (3-mL volume) were carried out in 1-cm path length cuvettes by addition of 4 M NaOH and 4 M HCl with rapid stirring, and titrations of 7–12 μM solutions of cytochromes in KCl were carried out in the same manner by addition of 0.5 M NaOH and 0.5 M HCl. After spectra were recorded at the lowest pH, the samples were titrated to the highest pH, spectra were immediately recorded, and back-titration was begun. The pH was determined with a Radiometer 27 pH meter, and absorption spectra were recorded on a Cary 14 or Aminco DW-2a spectrophotometer.

Results

The absorbance changes resulting from the alkaline titration of native and modified cytochromes *c* were followed from 450 to 750 nm in the pH range 6.4–11.4. As shown in Figures 1A and 2, the 695-nm band for the native protein disappeared with an apparent p*K* of about 8.9. This is in agreement with earlier studies of Theorell & Åkesson (1941), Greenwood &

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[1] The numbering of pH-dependent conformation states is in the terminology of Theorell & Åkesson (1941).

[2] Abbreviations used: $CF_3PhNHCO$, (trifluoromethyl)phenylcarbamyl; CF_3CO, trifluoroacetyl; EPR, electron paramagnetic resonance; ^1H NMR, proton magnetic resonance.

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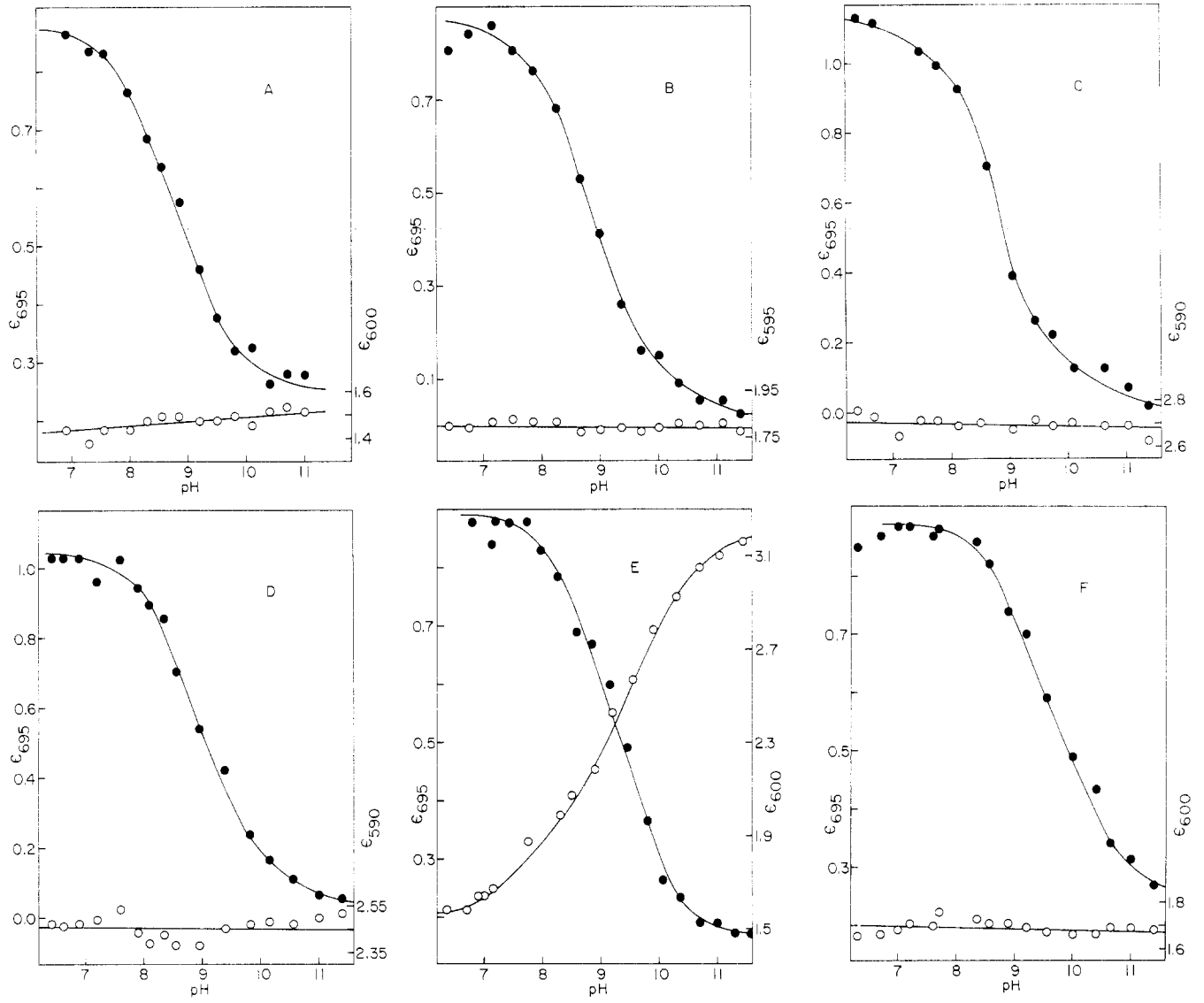

FIGURE 1: Spectrophotometric titrations of horse heart ferricytochromes *c* in 50 mM KCl. (A) native: mM extinction at 695 nm (●); mM extinction at 600 nm (○). (B) $CF_3PhNHCO$-Lys-13: mM extinction at 695 nm (●); mM extinction at 595 nm (○). (C) $CF_3PhNHCO$-Lys-79: mM extinction at 695 nm (●); mM extinction at 590 nm (○). (D) CF_3CO-Lys-79: mM extinction at 695 nm (●); mM extinction at 590 nm (○). (E) $CF_3PhNHCO$-Lys-72: mM extinction at 695 nm (●); mM extinction at 600 nm (○). (F) CF_3CO-Lys-72: mM extinction at 695 nm (●); mM extinction at 600 nm (○).

Wilson (1971), Davis et al. (1974), and Stellwagen & Cass (1974), in which it was shown that the pH-dependent transition involving loss of the 695-nm band and dissociation of a single proton occurs with an apparent p*K* of 8.9–9.3, depending on the ionic strength. No concomitant change was observed in the spectral region around 600 nm. Spectrophotometric titrations of $CF_3PhNHCO$-Lys-13, $CF_3PhNHCO$-Lys-79, and CF_3CO-Lys-79 cytochromes *c* under the same conditions also resulted in the disappearance of the 695-nm band with an apparent p*K* of around 8.9 in each case (Figures 1B–D and 2). As with the unmodified cytochrome, no significant changes were observed in the region near 600 nm over the same pH range. Parts E and F of Figure 1 and Figure 2 show that spectral titration of the $CF_3PhNHCO$-Lys-72 and CF_3CO-Lys-72 derivatives again resulted in the disappearance of the 695-nm band with increasing pH, although in both cases with a somewhat higher apparent p*K* (9.3 in the former; 9.6 in the latter). However, while little change was seen in the spectral region around 600 nm for the CF_3CO-Lys-72 derivative, disappearance of the 695-nm band of the $CF_3PhNHCO$-Lys-72 derivative was accompanied by the appearance of a maximum at 600 nm, characteristic of a complex in which the strong-field methionine-80 sulfur ligand is replaced by a weak-field solvent hydroxyl ligand (Morton, 1973). The pH-induced changes were not affected by choice of buffer (KCl–borate or KCl) and were reversible in every instance. All of the high pH solutions were clear and red in color.

Discussion

Since Theorell & Åkesson (1941) first reported the five distinct pH-dependent conformational states of ferricytochrome *c*, it has been of interest to determine on a molecular level the conformational changes which take place with the disappearance of the weak 695-nm absorption band in moderately alkaline aqueous solutions. The decrease in absorbance at 695 nm occurs concomitantly with the loss of a single proton with an apparent p*K* of 8.9–9.3 as mentioned earlier. While it is generally accepted that the 695-nm band is due to ligation of the heme iron by the sulfur atom of methionine-80, it is not as clear what the replacement ligand is if methionine-80 is replaced nor is the nature of the triggering ionization known.

The EPR studies of Blumberg & Peisach (1971) and Morton (1973) are consistent with the displacement of the sulfur ligand by a strong-field nitrogenous base in alkaline

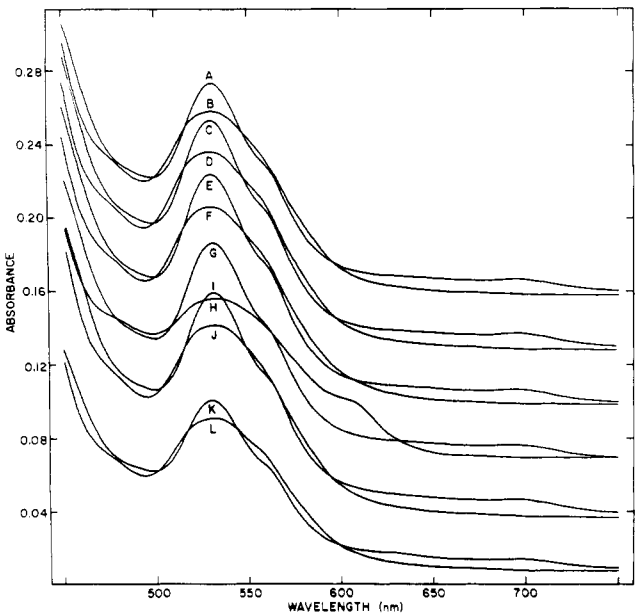

FIGURE 2: Visible absorption spectra of horse heart ferricytochromes *c* in 50 mM KCl. 10.4 μM native: (A) pH 6.5; (B) 11.4. 11.2 μM $CF_3PhNHCO$-Lys-13: (C) pH 6.4; (D) pH 11.4. 11.3 μM CF_3CO-Lys-72: (E) pH 6.4; (F) pH 11.4. 10.6 μM $CF_3PhNHCO$-Lys-72: (G) pH 6.4; (H) pH 11.4. 10.9 μM CF_3CO-Lys-79: (I) pH 6.4; (J) pH 11.4. 8.4 μM $CF_3PhNHCO$-Lys-79: (K) pH 6.4; (L) pH 11.4. The curve pairs A–B, C–D, E–F, G–H, I–J, and K–L are sequentially offset by 0.03 absorbance unit.

ferricytochrome *c*. Further, the ^1H NMR studies of Redfield & Gupta (1971) and Gupta & Koenig (1971) support the notion that at pH values above about 9 the methionine-80 ligand is displaced and that it is replaced by a strong-field ligand, probably a lysine ϵ-amino nitrogen. However, as Pettigrew et al. (1976) pointed out, pH-induced conformational changes affecting the histidine-18–iron bond could also account for the changes in the ^1H NMR spectra.

Studies with cytochrome *c* derivatives in which all of the lysines were modified by guanidination (Hettinger & Harbury, 1964; Morton, 1973), by trifluoroacetylation (Morton, 1973; Stellwagen et al., 1975), and by amidination and maleylation (Pettigrew et al., 1976) suggest that deprotonation of a lysine is not the triggering ionization in the isomerization reaction since the transition occurs even when all lysines are blocked. EPR and spectral studies of the modified cytochromes *c* (Morton, 1973; Hettinger & Harbury, 1964; Wilgus & Stellwagen, 1974; Stellwagen et al., 1975) demonstrate the appearance, with increasing pH, of EPR signals characteristic of a high-spin iron coordination complex and of an absorption maximum around 600 nm similar to the absorption bands in high-spin, type II ferricytochrome *c* (Theorell & Åkesson, 1941). Furthermore, Wilgus & Stellwagen (1974) showed that a hybrid molecule in which only the segment from residues 66 to 104 was guanidinated underwent alkaline isomerization to a high-spin form, while a hybrid in which only the segment from residues 1 to 65 was guanidinated showed the normal transition to a low-spin form, suggesting that one of the eight lysine residues in the peptide 66–104 is the sixth iron ligand in state IV ferricytochrome *c*.

We have found that specific (trifluoromethyl)phenylcarbamylation and trifluoroacetylation of lysine ϵ-amino groups of horse heart cytochrome *c* produce derivatives in which the positive charge on an unmodified lysine is replaced by an uncharged $CF_3PhNHCO$ or CF_3CO group on the modified residue (Smith et al., 1977; Staudenmayer et al., 1976, 1977; M. B. Smith, J. Stonehuerner, A. J. Ahmed, N. Staudenmayer, and F. Millett, unpublished experiments). Spectrophotometric titrations of five of these derivatives, $CF_3PhNHCO$-Lys-13, $CF_3PhNHCO$-Lys-72, $CF_3PhNHCO$-Lys-79, CF_3CO-Lys-72, and CF_3CO-Lys-79 cytochromes *c*, revealed the disappearance of the 695-nm band with increasing pH in the same manner as in the case of the unmodified protein. Furthermore, while changes in the spectral region around 600 nm paralleled those of native cytochrome for the $CF_3PhNHCO$-Lys-13, $CF_3PhNHCO$-Lys-79, CF_3CO-Lys-72, and CF_3CO-Lys-79 derivatives, specific (trifluoromethyl)phenylcarbamylation of lysine-72 resulted in the appearance of an absorption maximum at about 600 nm with an apparent p*K* of about 9.3. These observations are consistent with the earlier lysine modification studies discussed above but suggest, in addition, that under certain conditions either lysine-72 or lysine-79 can become the sixth ligand of the heme iron in the alkaline isomer of ferricytochrome *c*.

The finding that both of the CF_3CO derivatives isomerize to low-spin species is in agreement with the notion that if either lysine-72 or lysine-79 is trifluoroacetylated, the unmodified residue of the pair can become the sixth iron ligand. On the other hand, while alkaline titration of the $CF_3PhNHCO$-Lys-79 derivative also produced a low-spin species, titration of the $CF_3PhNHCO$-Lys-72 derivative resulted in the appearance of a maximum around 600 nm characteristic of a high-spin complex (Morton, 1973). The simplest explanation of these combined observations is that lysine-72 can ligand if lysine-79 is modified and that (trifluoromethyl)phenylcarbamylation (but not trifluoroacetylation) of lysine-72 prevents the conformational change which permits ligation of lysine-79. However, the $CF_3PhNHCO$-Lys-72 and CF_3CO-Lys-72 derivatives isomerized at somewhat higher apparent p*K* values (9.3 and 9.6, respectively) than did the $CF_3PhNHCO$-Lys-13, $CF_3PhNHCO$-Lys-79, CF_3CO-Lys-79, and native cytochromes (which all isomerized with an apparent p*K* of about 8.9). Thus, modification of lysine-72 perturbs the reaction pathway, suggesting that lysine-72 is the normal sixth iron ligand in the native protein alkaline isomer. Looze et al. (1978) similarly argued that lysine-79 is the alkaline ligand on the basis of the finding that unmethylated iso-1 and methylated iso-1 (residue 72 is a trimethylated lysine) cytochrome *c* from *Saccharomyces cervisiae* both isomerized with the same apparent p*K*.

The results we have reported here, together with those of other workers, can be interpreted in terms of the following model, similar to that proposed by Stellwagen et al. (1975), for the alkaline isomerization of ferricytochrome *c*

$$\text{S–Fe–N} + \text{L} \rightleftharpoons \text{L–Fe–N}$$

where S–Fe–N represents the normal neutral state III ferricytochrome *c*, L represents a potential methionine sulfur replacement ligand, and L–Fe–N represents the alkaline state IV species. We propose that the nature of L and the position of the equilibrium, that is, the apparent p*K* of the transition, are dependent on a number of factors including the relative affinities for heme iron of possible ligands, local orientation or concentration of the possible ligands, and overall effects on the tertiary structure due to ionic and hydrophobic interactions. Thus, a lysine nitrogen is the strongest replacement ligand for methionine-80 sulfur if it becomes available by deprotonation. The alkaline three-dimensional structure appears to properly orient either lysine-72 or lysine-79 in the horse protein while only lysine-79 is available to ligand in the sequentially different trimethylated yeast cytochrome. If no lysine residue is available, either as a result of structural restrictions or as a result of chemical modification, then higher relative concen-

trations of hydroxyl ion in the vicinity of the heme iron can preferentially displace the methionine sulfur. This appears to be the case with the $CF_3PhNHCO$-Lys-72, totally guanidinated (Hettinger & Harbury, 1964; Morton, 1973; Wilgus & Stellwagen, 1974), totally trifluoroacetylated (Morton, 1973; Stellwagen et al., 1975), and partially guanidinated (Wilgus & Stellwagen, 1974) derivatives and with *Euglena gracilis* cytochrome *c* (Stellwagen & Cass, 1974). Pettigrew et al. (1976) prepared derivatives in which the lysines were modified by amidination, replacing positive charge with positive charge, or by maleylation, replacing positive charge with negative charge. The 695-nm band of the maleylated derivative disappeared at higher pH values with increasing ionic strength and with an apparent pK of 10.5 or more in 0.3 M NaCl. Below pH 6 at low salt concentrations, high-spin material was formed, while at higher pH and increasing salt, the product was reported to be low spin. These observations may be interpreted in terms of our proposal by assuming that in the highly anionic maleylated protein, repulsive ionic interactions are acting to disrupt the tertiary structure of the molecule. Therefore, state III isomerizes to state II at a pH of around 6 compared with the normal transition at around pH 2.5, and state III isomerizes directly to low-spin state V (Theorell & Åkesson, 1941). The observed effects of ionic strength reinforce this explanation. Finally, the totally amidinated derivative, although all of the lysines were blocked, isomerized to a low-spin form at a fairly normal apparent pK of 9.2. This could reflect ligand replacement by an acetamidino group with a pK somewhat reduced from the normal pK of >11 (Means & Feeney, 1971).

If our proposed mechanism is operative in aqueous solution, it could follow that similar considerations may explain the electron transfer events mediated by cytochrome *c* between its physiological reductant and its physiological oxidant in vivo.

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