IS LYSINE 79 A LIGAND FOR IRON HEXACYANIDES BOUND TO CYTOCHROME C?

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Summary. Levels of iron hexacyanides sufficient to saturate 90 percent of the available binding sites are found to exert no significant change on the ferricytochrome c heme linked ionization with a pK of approximately 9. If lysine 79, postulated to be involved in a ligand interchange with that pK, were crucial for one hexacyanide binding site, a change of greater than 1 pK unit would have been expected.

Introduction

One approach to the elucidation of the path of the electron flow through cytochrome c has been to attempt to identify the residue(s) involved in the formation of the precursor complex. Recently, Stellwagen and Cass (1) have measured the association constants for complex formation between ferricytochrome c and the iron hexacyanides. Their equilibrium dialysis studies defined two separate sites for each hexacyanide. In the case of ferrohexacyanide, one site could be made to disappear upon carboxymethylation of methionines 65 and 80, whereas no effect could be observed when histidine 33 and methionine 65 were specifically carboxymethylated. Likewise, addition of 1 M imidazole at pH 8 to displace the methionine from the heme did not alter the number of sites. The two ferrohexacyanide binding sites were found to disappear with a pK of approximately 9, similar to that ascribed to the alkaline isomerization reaction of cytochrome c. From this data, the authors concluded that lysine 79 played a crucial role in one of the hexacyanide binding sites.

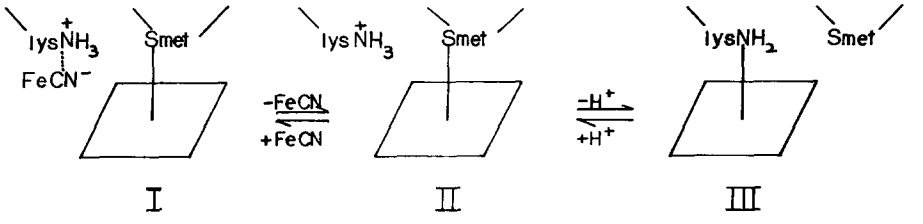
We would like to point out that their conclusion is inconsistent with the postulated role of lysine 79 in the alkaline isomerization reaction; in this communication we show that this residue cannot simultaneously perform both functions.

Methods and Materials

Type VI cytochrome c, purchased from Sigma Chemical Company in the ferric form, was titrated from neutral to alkaline pH in the manner of Stellwagen and Cass (1). An 0.1 mM solution of the protein initially at pH 7.2 in 50 mM Tris-HCl or Tris-acetate buffer was titrated to pH 11 by the incremental addition of 0.5 N NaOH. Similar titrations were carried out in the presence of either 40 mM ferrohexacyanide or ferrihexacyanide. Based on the equilibrium dialysis data, better than 90 percent of the available binding sites should have been occupied. The titrations, carried out at 25 C, were monitored from 750 nm to 480 nm in a Cary 17 recording spectrophotometer. The data were recorded either as an absolute spectrum with the blank containing everything except cytochrome c, or as a difference spectrum with the blank containing all of the components at the original pH, 7.2.

Results and Discussion

Our results are interpreted on the basis of the following thermodynamic considerations. If the ligand interchange and the hexacyanide binding site are common to lysine 79, one must propose the following scheme:



where FeCN stands for the appropriate hexacyanide.

In the absence of the hexacyanide, the pK can be determined from titration using the usual assumption that the concentrations of the methionine ligated and the lysine ligated species can be determined accurately from the absorption at 695 nm.

$$pH = pK + \log (III/II)$$

$$= pK + \log ((A_{695}^{max} - A_{695})/(A_{695} - A_{695}^{min}))$$

However, when hexacyanide is added, the observed absorbance at 695 nm reflects not only the free but also the hexacyanide-bound methionine coordinated form. Under these conditions, the equation must be revised as follows:

$$pH = pK + \log ((K_{Fe})[FeCN] + 1) + \log (III/(I + II))$$

$$= pK_{apparent} + \log ((A_{695}^{max} - A_{695})/(A_{695} - A_{695}^{min}))$$

where $K_{Fe} = I/((II)[FeCN])$. In the conventional semi-logarithmic plot of the data, the pK is thus modified by $\log((K_{Fe})[FeCN] + 1)$. Here the electrostatic complex with the anionic hexacyanide stabilizes the positively charged form of lysine, thus requiring higher pH to accomplish deprotonation, a presumed condition for ligation. For example, using the association constants determined by Stellwagen and Cass (1) for the ferrohexacyanide binding, 100 M^{-1} and 300 M^{-1}, one would expect, in the presence of ferrohexacyanide, to observe an increase in the apparent pK of from 1.1 to 1.6 units.

The data from the three titrations are shown in figure one; it is clear that there are no significant differences between any of the sets of data, and thus there is no evidence for correlation between the ligand interchange and the hexacyanide binding. It follows that lysine 79 cannot simultaneously be involved in both processes. The conclusion that the loss of a binding site upon carboxymethylation of the axial methionine was due to a removal of lysine 79 now becomes untenable (1). Indeed the effect might be explained in terms of the introduction of a carboxyl group in the vicinity of the binding site rather than removal of the binding ligand.

The equilibrium dialysis data support the existence of two binding sites on cytochrome c for the hexacyanides, whose pK's reflect the possible role of lysines in the binding. However, the titration data of figure one show that lysine 79 cannot be both the alkaline heme ligand

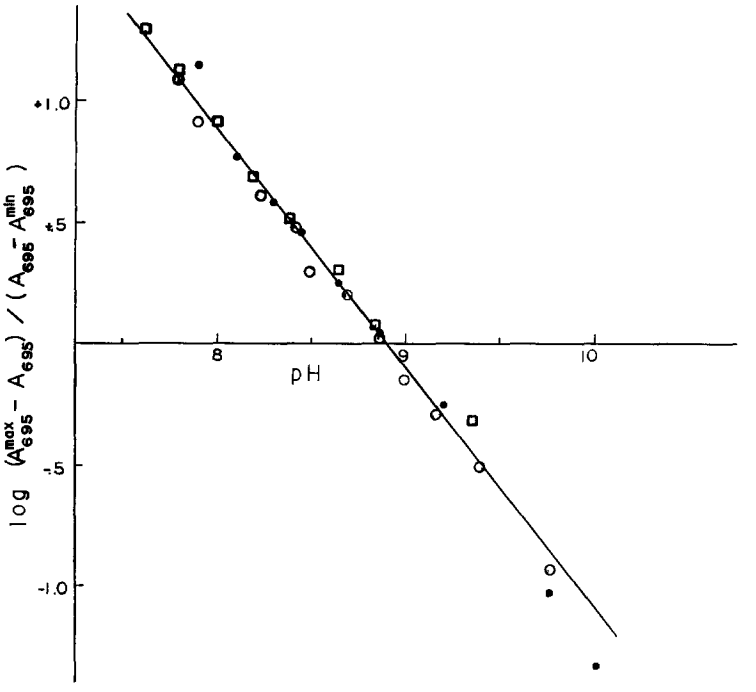


Figure 1. Effect of the addition of either 40 mM ferrihexacyanide or ferrohexacyanide on the alkaline pK of ferricytochrome c. (◘ ◘) ferricytochrome c only; (o o) with ferrohexacyanide; (· ·) with ferrihexacyanide. A theoretical line for a pK of 8.9 is drawn for comparison.

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and the crucial ligand of one iron hexacyanide binding site. While the data (2-8) for the role of lysine 79 in the ligand interchange is circumstantial, it is persuasive, and it therefore seems proper to conclude that binding of iron hexacyanides involves other residues whose identities remain to be elucidated.

Acknowledgement

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