Modulation of the alkaline transition in cytochrome *c* and cytochrome *c*-T by full or specific partial acetimidylation

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Acetimidylated horse cytochrome *c* and related derivatives exhibit more or less marked changes, both upscale and downscale, in apparent *pK* of the alkaline transition. This transition occurs when the normal methionine-80 residue is replaced at the sixth haem co-ordination position by another strong-field ligand. Analysis of the relationship between structural change and *pK* shift in these derivatives supports the view that the replacement ligand is a lysine residue, probably 72 or 79, and contradicts an alternative hypothesis. The results add further detail to a comprehensive view of the mechanism of this isomerization.

Ferricytochrome *c* undergoes a number of spectral changes with increasing pH. This alkaline transition, with an apparent *pK* in the horse protein of 8.8–9.4, depending on ionic strength, causes a shift in most of the major absorption bands of the cytochrome, and the elimination of a weak absorption at 695 nm, although the protein remains in the low-spin state. The 695 nm band, and an associated shoulder at 650 nm, has been attributed to the bond between the ferric iron atom and the sulphur atom of Met-80 (Schechter & Saludjian, 1967), and its loss to a replacement of the methionine ligand by another strong-field ligand (Blumberg & Peisach, 1971; Davis *et al.*, 1974). Most studies have concluded that the replacement ligand is a lysine residue, specifically Lys-79 (Dickerson *et al.*, 1972; Wilgus & Stellwagen, 1974; Harris, 1977); but Pettigrew *et al.* (1976) proposed the triggering ionization to be that of the imidazole imino nitrogen of the His-18 ligand and that a small change in iron-sulphur bond length leads to the loss of the 695 nm band. More recently, two groups, assuming the Lys-79 hypothesis to be correct, have proposed a mechanism of ligand exchange (Osheroff *et al.*, 1980; Falk *et al.*, 1981), whereas Bosshard (1981) has presented evidence against Lys-79, and Smith & Millett (1980) conclude that Lys-79, or more often Lys-72, depending on the specific cytochrome *c* under study, is the replacement ligand.

I now report studies of the alkaline transition in a range of cytochrome *c* derivatives. These contradict some of the results reported by Pettigrew *et al.*

(1976), and support the view that the replacement ligand is a lysine residue. The results also permit an elaboration of the proposed mechanism of ligand replacement.

Experimental

Materials

Horse heart cytochrome *c* (type III) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Methyl acetimidate hydrochloride was prepared by the method of Hunter & Ludwig (1962). Other reagents were obtained from Fluka, Buchs, Switzerland, or Merck, Darmstadt, West Germany, and were of analytical grade.

Acetimidylation of cytochrome *c*

This was performed by the method of Wallace & Harris (1984). Cytochrome *c*-T (the non-covalent complex between tryptic fragments 1–38 and 39–104 of the protein; Harris & Offord, 1977), acetimidylated cytochrome *c*-T and chimerae (covalent and non-covalent complexes formed from one protected and one unprotected component) of cytochrome *c* and cytochrome *c*-T were prepared as indicated by Wallace (1984).

Spectrophotometric titrations

These were performed at room temperature with either a Varian Superscan 3 or a Cary 210 spectrophotometer. Solutions of cytochromes *c* (about 0.5 mM) were made up in potassium phosphate buffer of the appropriate molarity, and titrations were performed in both directions by the addition of either KOH of the same molarity or 0.1 M-HCl.

Abbreviations used: Hse, homoserine; Acim, acetimidyl.

Cytochromes *c*-T were made up in 0.1 M-Tris/HCl buffer, pH 9.4, and titrated with 0.1 M-HCl. The pH was determined before each measurement with a Radiometer 84 pH-meter. Spectra were drawn on the Cary 210 instrument.

Results and discussion

Fig. 1 shows an example of the spectrophotometric titration of the 695 nm band in native horse cytochrome *c*. *pK* values of transition obtained for this protein and its derivatives are given in Table 1, and those for cytochromes *c*-T are in Table 2.

The value for native cytochrome *c* is in agreement with values reported by very many other authors. The values determined for 19-*N*- ϵ -acetimidylated cytochrome *c* are higher by 0.45–0.7 pH unit, depending on ionic strength, than the equivalent value for the native protein and are thus at variance with the result reported by Pettigrew *et al.* (1976). They show, as does the transition in horse cytochrome *c*, a dependence of *pK* on ionic strength and thus accord with the value determined by Harris (1977) at low ionic strength.

The reason for this discrepancy (of up to 0.8 pH unit) is not known, though it should be noted that the method of acetimidylation used by Pettigrew *et al.* (1976) can give rise to multiple molecular forms of the protein on ion-exchange chromatography, whereas that employed in the present study does not (Wallace & Harris, 1984). Of the chimerae, Acim-(1–65)-(66–104)-[Hse⁶⁵]cytochrome *c* has a *pK* similar to that of the native protein, whereas that of (1–65)-Acim-(66–104)-[Hse⁶⁵]cytochrome *c* is closer to that of the fully acetimidylated protein.

Acetimidylation affects only lysine residues and produces only minor changes in the structural and functional properties of the protein (Wallace, 1984). The observed change in *pK* of transition supports the notion that a lysine residue provides the substitute ligand. The correspondences be-

tween certain chimerae and the native protein and others and the acetimidyl-protein, for both cytochrome *c* and cytochrome *c*-T, demonstrate that the residue(s) involved occurs in the 66–104 tract; the same conclusion was reached by Wilgus & Stellwagen (1974). Furthermore, it is the (1–65)-Acim-

Fig. 1. Spectrophotometric titration of the 695 nm band of horse cytochrome *c*

For experimental details see the text. Lower left, the variation in the 600–800 nm spectrum with pH from 7 to 11. Upper right, titration curve of ΔA_{695} .

Table 1. *pK*_(app.) values of alkaline haem-linked transitions of cytochrome *c* and some derivatives at various ionic strengths determined in the present and other studies

For experimental details used in the present work see the text.

Cytochrome derivative	<i>pK</i> _(app.)			
	<i>I</i> 0.01	<i>I</i> 0.06	<i>I</i> 0.15	<i>I</i> 0.25
Cytochrome <i>c</i>	8.9*†	9.25‡	9.32 ± 0.05	9.4‡
Acim-(1–65)-(66–104)-[Hse ⁶⁵]cytochrome <i>c</i>			9.18	
Acetimidyl-cytochrome <i>c</i>	9.5†	9.70	9.98 ± 0.08	9.92; 9.2§
(1–65)-Acim-(66–104)-[Hse ⁶⁵]cytochrome <i>c</i>			9.67	9.75

* Greenwood & Wilson (1971).

† Harris (1977).

‡ Osheroff *et al.* (1980).

§ Pettigrew *et al.* (1976) (ionic strength was not given, but their value is placed in this column because it was compared with the value for cytochrome *c* obtained at high ionic strength).

Table 2. $pK_{(app.)}$ values of alkaline haem-linked isomerizations of cytochromes *c*-T

All titrations were performed in 0.1M-Tris/HCl buffer, pH9.4, by addition of 0.1M-HCl, at room temperature. For full experimental details see the text.

Cytochrome <i>c</i> derivative	$pK_{(app.)}$
Cytochrome <i>c</i> -T	7.10
Acim-(1-38)-(39-104)-cytochrome <i>c</i> -T	7.00
Acetimidyl-cytochrome <i>c</i> -T	8.15
(1-38)-Acim-(39-104)-cytochrome <i>c</i> -T	8.30

(66-104)-[Hse⁶⁵]cytochrome *c* chimera, displaying biological and n.m.r.-spectroscopic properties identical with those of native cytochrome *c* (Boswell *et al.*, 1983; Wallace, 1984), that shows the alkaline-transition characteristics of acetimidyl-cytochrome *c*.

N.m.r. observations of cytochrome *c* on varying the pH over the range of the alkaline transition show no evidence of major structural reorganization (Boswell *et al.*, 1983; Robinson *et al.*, 1983), so that the substitute lysine ligand must lie close to the haem group. The obvious candidates are Lys-79 or, at a slightly greater distance, Lys-72.

However, although the values for the Acim-(1-65)-(66-104)-[Hse⁶⁵]cytochrome *c* chimera and cytochrome *c* are very close to one another, there is a greater discrepancy between those of the other pair (Table 1), which suggests that the modification of a specific amino acid may not be the only contributory factor to the raised pK .

A possible explanation lies in the observation that changes in the pattern of surface charge and thus of salt bridges stabilizing the haem crevice can alter the apparent pK (Osheroff *et al.*, 1980). A small general effect of the raised pK of the acetimidylated lysine side chains on this pattern might thus exist, but would only be detectable as the apparent pK of transition approached the value of the pK values of the lysine side chains involved in those salt bridges.

Thus, when the apparent pK of transition is raised to a higher value by the acetimidylation of a specific residue in the 66-104 region, this analogue becomes sensitive to a general effect of further acetimidylation in the 1-65 region; but, at the lower apparent pK of the native molecule, acetimidylation of these same residues in the Acim-(1-65)-(66-104)-[Hse⁶⁵]cytochrome *c* chimera have no such general effect.

In their study on amidinated derivatives of cytochrome *c*, Wilgus & Stellwagen (1974) showed that the equivalent transition resulted in a high-spin ferricytochrome *c* at alkaline pH; but the alkaline form of acetimidyl-cytochrome *c* remains completely in the low-spin state. Hence the acetimidino

group (pK 12.7; G. R. Moore & C. J. A. Wallace, unpublished work) is capable of substituting for the amino group of Lys-79 as a replacement for the Met-80 thioether, albeit at a higher pH, whereas the guanidino group (pK 12.6) is not.

The studies made by Davis *et al.* (1974) show that isomerization is a two-step reaction, involving an ionization followed by a conformational rearrangement. The apparent pK of the isomerization thus depends on the intrinsic pK of the ionizing group and the conformational equilibrium constant. Hence the increased pK of the acetimidino group leads to a higher pK of transition for the acetimidylated protein. Substitution of these values, however, into the expression derived by Davis *et al.* (1974) for the relationship:

$$pK_{(app.)} = p(K_{(intrinsic)}/K_c)$$

shows that acetimidylation also leads to a change in the value for K_c , the conformational equilibrium constant, to 1500, from 125 when lysine is the replacement ligand. The incapacity of the homo-arginine residue, with intrinsic pK identical with that of acetimidyl-lysine, of amidinated cytochrome *c* to act as a substitute ligand indicates a much lower K_c for this protein. As the conformational equilibrium constant is a measure of the affinity of the deprotonated base for the hydrophobic interior of the cytochrome haem crevice, these observations seem entirely consistent with the relative polarities of the acetimidino, amino and guanidino groups.

However, relative polarity of the substitute ligand is not the only determining factor for K_c . Cleavage of the 38-39 peptide bond, as in cytochrome *c*-T, causes a shift in the apparent pK of about 2 pH units, representing an increase in K_c to about 8000 (35000 in acetimidyl-cytochrome *c*-T). In cytochrome *c*-T, the stability of the interior of the haem crevice is decreased, as shown by changes in redox potential and biological activity (Wallace, 1984) and in the n.m.r. spectrum (G. R. Moore & D. E. Harris, unpublished work). A looser, more solvent-accessible, internal structure would favour entry of the substitute ligand.

Osheroff *et al.* (1980) have shown that differences in certain of the forces stabilizing the exterior of the haem crevice that exist between different mammalian species, or can be produced by chemical modification, lead to considerable variation in $pK_{(app.)}$, which, as the substitute ligand does not vary, must be due to changes in K_c . A general effect on these forces due to acetimidylation of the lysine residues involved may also contribute to the changes in K_c for the modified protein discussed above.

Pettigrew *et al.* (1976) also studied the alkaline transition in fully maleylated cytochrome *c*. The

apparent pK was very much more dependent on ionic strength than in any other derivative, but at high ionic strength was similar to that of the native protein and resulted in a low-spin alkaline form. This observation was adduced as further evidence that the transition could not be due to ligand substitution by a lysine residue (being, in this case, blocked by a dicarboxylic acid). The observation is certainly at variance with the mechanism described above, but the conflict may be resolved by assuming that the cytochrome when maleylated undergoes denaturation on rising pH, allowing the replacement of Met-80 as a ligand by a strong-field histidine imidazole group, such as is the case in the isolated 1-65 fragment of cytochrome *c* (Babul *et al.*, 1972) or the urea-denatured form of the native protein (Babul & Stellwagen, 1971). The data of Schejter *et al.* (1979) on the fluorescence-emission and c.d. changes accompanying the loss of the 695 nm absorption in the maleyl-protein are indicative of severe denaturation.

One important question remains yet unanswered. Is the alkaline transition a purely fortuitous event, or does the universality of the phenomenon, the strict conservation throughout eukaryotic evolution of lysine residues at positions requiring minimal conformational change to achieve substitution, the surprising ease with which Met-80 is displaced compared with an alternative ligand, and the sensitivity to modulation by a wide range of structural and environmental factors, indicate a physiological role for the transition?

Conclusions

(1) Acetimidylation of a lysine residue (or residues) in horse cytochrome *c* shifts the apparent pK of the alkaline transition upward by 0.45–0.7 pH unit. In one partially acetimidylated analogue this change occurs without accompanying alteration of any other of the biological or spectroscopic properties of the protein. The discrepancy between this result and the report by Pettigrew *et al.* (1976) that acetimidylation did not alter the apparent pK may result, at least in part, from their use of an unsatisfactory method of acetimidylation.

(2) The altered tertiary structure at the bottom left of the haem crevice in cytochrome *c*-T causes a decrease in the apparent pK of about 2 pH units.

(3) The apparent pK values of the chimeric forms locate the affected residue(s) in the 66–104 fragment, confirming previous observations. N.m.r. spectroscopy shows only slight conformational change in the protein on raising the pH, so that it seems likely that the alkaline transition in cytochrome *c* is caused by displacement of the methionine ligand by the deprotonated amino

group of residue 72 or residue 79, both of which lie fairly close to the haem iron. This process is subject to modulation by the pK of the amine (or substituted amine) group on the side chain of this lysine residue, by the polarity of this group, by internal changes in the haem crevice, by changes in the surface conformation on either side of the haem crevice as a result of interspecific variation or chemical modification, and by the ionic strength and composition of the medium. Modulation by these or other factors *in vivo* could provide sensitive control of electron-transfer rates.

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