THE ROLE OF THE LYSINES IN THE ALKALINE HEME-LINKED IONIZATION OF FERRIC CYTOCHROME c

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SUMMARY

Fully amidinated cytochrome c has a 695 nm band which is lost with a pK of 9.2 in a transition to a low-spin state. Fully maleylated cytochrome c can also exist in a low-spin state without the 695 nm band although the pH of the transition is very dependent on the salt concentration. Neither of these derivatives have free lysine groups. It is concluded that the heme-linked alkaline ionization of native cytochrome c is not triggered by deprotonation of a lysine residue, and that lysine does not necessarily coordinate the iron in the low-spin alkaline species.

Ferric cytochrome c undergoes a heme-linked ionization with a pK of 9.3 in which the iron remains in the low-spin state while the 695 nm band disappears (1). It was suggested that the process involves a change of iron ligand, the methionine being replaced by a lysyl ϵ-amino nitrogen (2). The pK of the triggering ionization (3) is compatible with that of a lysine side chain. Indeed, rotation of the lysyl-79 side-chain in the 3-dimensional model brings its free nitrogen close to the iron atom (4). Furthermore, complete guanidination of the protein yields a product that undergoes a spectral transition with pK of about 10, but to a high spin form (2). In a recent study on trifluoroacetylated and guanidinated cytochrome c it was proposed that lysine-79 is the iron ligand in the active protein, but not in the modified cytochromes (5). Evidence that is incompatible with the lysine hypothesis is presented here, based on spectrophotometric titrations of two new derivatives - fully amidinated and maleylated horse cytochrome c. Other properties of these preparations will be reported separately.

MATERIALS AND METHODS

Maleylation was based on the method of Butler et al. (6). To 8 ml of 0.75% solution of horse cytochrome c (Sigma, type III) in 0.1 M $NaHCO_3$, pH 9.0 at 0° C, 60 µl portions of 1 M maleic anhydride in dioxane were added every 5 min

Abbreviations:

TNBS, 2,4,6 trinitrobenzenesulfonic acid;

SDS, sodium dodecyl sulfate.

for 1 hour. This constitutes an 8-fold molar excess over free amino groups. The pH was maintained at 9.0 by addition of 1 M NaOH. Excess of reagent was removed on a Sephadex G-25 column equilibrated with 0.025 M phosphate pH 7.0. The product was adsorbed on a DEAE cellulose column (Whatman DE52, 1.5 x 5 cm) equilibrated in the same buffer and chromatography was carried out in 0.025 M phosphate buffer containing 0.4 NaCl. The material was eluted as a single band which, after pooling, bound 0.1-0.2 mol TNBS/mol. In routine preparations the chromatography step was ommitted.

Amidination was carried out according to Reynolds (7). To 40 mg of cytochrome c in 5 ml 0.1 M borax-NaOH pH 10.0 three additions of 325 mg of methyl-acetamidinate (8) were made at 20 min intervals. pH was corrected with 6N NaOH. The product was desalted on a Sephadex G-25 column, assayed with TNBS and lyophilysed. It contained no free amino groups.

Estimation of free amino groups was performed with the TNBS method (9). Approximately 30 nmol of a given preparation were incubated with 0.2 ml of 1% TNBS in 2% $NaHCO_3$, pH 8.5 at 40° C for 2 hours. The reagent was then removed by gel-filtration on Sephadex G-25 in 0.05 M NaCl. Equal portions of the eluate were taken for absorption measurement at 340 nm (in 0.15 M HCl, 2% SDS) and for estimation of cytochrome concentration by the pyridine hemochrome method (10). The contribution of heme absorption at 340 nm was subtracted (ε_{340nm}= 27.5 $mM^{-1}cm^{-1}$ for unmodified ferricytochrome in 0.15 M HCl, 2% SDS) and the number of free amino groups of the assayed material estimated by dividing by 14 $mM^{-1}cm^{-1}$ (the extinction coefficient of a trinitrophenyl group).

Spectra were recorded on a Cary 118 Spectrophotometer. pH titrations were performed directly in a cuvette with a Radiometer pH meter 26.

RESULTS

The uv spectrum of fully maleylated cytochrome c showed the expected increase in absorption at 250 nm due to maleyl groups (3). The visible spectrum was very similar to the spectrum of native protein except for an increase in the extinction of the oxidized Soret peak to 127 $mM^{-1}cm^{-1}$.

The effects of salts and pH on the 695 nm band of maleylated cytochrome c were, however, markedly different. At pH 7.0 in water a low-spin spectrum was observed lacking the 695 nm band (Fig. 1A). On addition of salt the absorbance at 695 nm increased and reached the normal extinction of 0.8 mM^{-1} cm^{-1} above 0.3 M NaCl (Fig. 1B). Like native cytochrome c the 695 nm band of maleylated cytochrome in 0.3 M NaCl disappeared above pH 9.0 (Fig. 2A, Fig. 1C) although the Hill plot did not show a slope of 1. The product was a low-spin species.

At ionic strengths lower than 0.3 M the 695 nm band disappeared at lower pH values (Fig. 2). Thus at 0.05 M NaCl the maximal extinction of the band was at pH 6.0 while at pH 7.3 it was completely abolished. Below pH 6.0

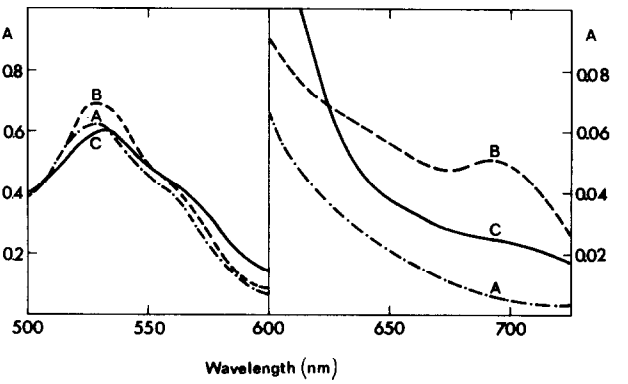

Fig 1. Spectrum of 6.2×10^{-5} M fully maleylated cytochrome c (A) pH 7.0, 0.025M phosphate buffer (B) pH 7.0, 0.33M NaCl (C) pH 10.9, 0.33M NaCl.

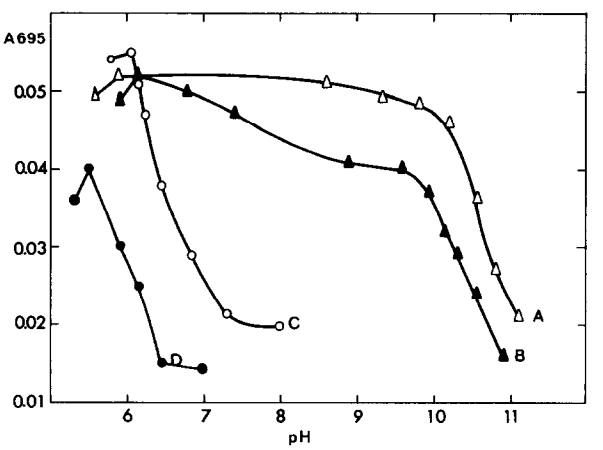

Fig. 2. Titration of the 695 band in maleylated cytochrome c at different ionic strengths: (A) 0.3M (B) 0.15M (C) 0.05M (D) 0.005M. Cytochrome concentration was 7.10^{-5}M.

the 630 nm and 490 nm absorbance increased indicating the formation of high-spin material and this was more pronounced the lower the salt concentration. At salt concentrations below 0.05 M the full 695 nm absorption was not observed (Fig. 2D) as the band was lost in a very sharp transition above pH 6.0 and below this pH value high-spin material was formed.

Fully amidinated cytochrome c, on the other hand, was very similar to

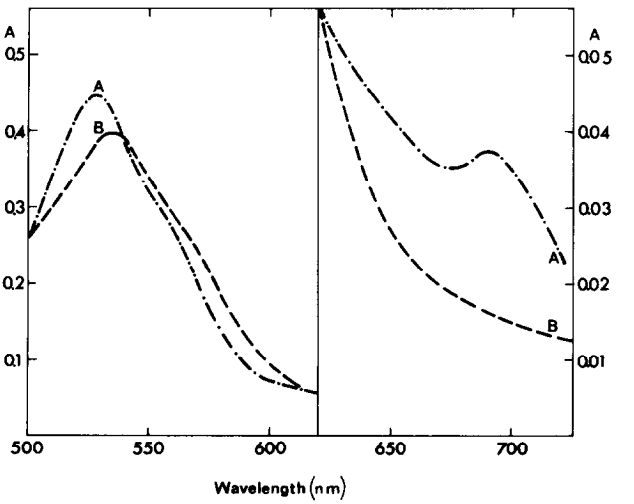

Fig. 3. Visible spectrum of 3.9×10^{-5} M amidinated cytochrome c: (A) pH 7.0 (B) pH 10.8.

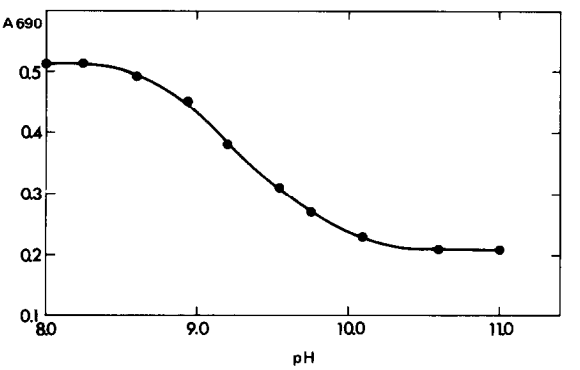

Fig. 4. Spectrophotometric titration of 5.8×10^{-4} M amidinated cytochrome c.

the native protein (Fig. 3), both in the spectra and its dependence on pH. The 695 nm band of this derivative disappeared at alkaline pH with a pK of 9.2 (Fig. 4) resulting in a low-spin spectral form.

DISCUSSION

Neither maleylated nor amidinated horse cytochrome c have lysyl residues that can ionize or coordinate iron. However, both modified proteins undergo heme-linked ionizations with spectroscopic changes identical to those

observed in the native enzyme. It is obvious, therefore, that the ionization of a lysine side chain is not a necessary requirement for such a reaction to occur.

Although free amidino-groups have a pK of 12.5, that is, 2 pH units higher than the pK of ε-amino groups, the heme-linked pK of amidinated cytochrome c is 9.2, almost the same as in the native molecule. The main difference between guanidinated and amidinated cytochrome c is that in the alkaline form the former is in the high-spin state (2,5,11), while the latter remains low-spin. Since substitution of a $-CH_3$ group for an $-NH_2$ group in guanidine decreases the denaturing power of the molecule(12); it is possible that the large number of guanido-groups present at the molecular surface of the guanidinated protein affects its non-covalent interactions, especially at high pH, while a much less pronounced effect is caused by the amidino-groups.

The observation (13) that guanidination of the lysines of the 66-104 peptide yields a high-spin alkaline species, while the same modification of the 1-65 peptide yields a low-spin alkaline species, can be a reflection of the differences in hydrogen bonding and hydrophobic packing of the two sides of the cytochrome c molecule (14).

Maleylated cytochrome c presents another problem: the existence of a low spin, no-695 species depends very markedly on the concentration of salt as well as pH (Fig. 2). This behaviour can be explained by electrostatic considerations. In native and amidinated cytochrome c, positive and negative charges are distributed all over the protein surface; the maleylated derivative is highly anionic, so that the repulsion forces acting at the protein surface become very important, and the native tertiary structure is already affected at pH 6. In the presence of salts, the screening effect of the counterions on the charged groups (15) increases the pH at which the change is observed.

Regardless of the nature of the ionization process, the striking fact remains that two cytochrome c derivatives in which no lysines are available for iron coordination, are in the low-spin state at pH values where the

695 nm band is absent. The only direct evidence for the displacement of methionine is the disappearance with pK 9, of its methyl proton resonances from the upfield region of the nmr spectrum (16). However, the shift of the proton resonances is so sensitive to the paramagnetic and ring current interactions with the ferric heme, that even a small change in the length or geometry of the sulfur-iron bond may cause a large effect on the position of the methionine methyl peak. Such a change should also affect the energy of the iron d_{z^2} orbital, shifting the 695 nm band towards spectral regions where it is masked or undetectable (17).

A possible cause for a small change in the sulfur-iron bond is the ionization of the iron-bound imidazole. It has been shown that the imino proton of imidazole in the imidazole-myoglobin complex dissociates with pK 10 (18). This led to the assignment of the alkaline ionization of cytochrome c to an iron-linked histidine (19). Recently, it was reported that the downfield-shifted, heme-methyl nmr signals of imidazole-myglobin, shift about 4 ppm upfield upon ionization of the imino group of imidazole with pK close to 10 (20). The upfield shift of the heme-methyl peaks observed for cytochrome c at pH 9 (16) is strikingly similar to that of imidazole-myoglobin (20), and supports the hypothesis that the imino group of histidine-18 is the ionizing species, while methionine-80 remains iron-linked in the alkaline form.

Finally, if methionine is not the iron ligand, a plausible alternative remaining is an OH^- ion. For methionine alkylated cytochrome c, the low-spin, no-695 band neutral species was postulated to have an iron coordinated water (21). Similar arguments should apply in the present case to OH^-, which has a stronger ligand field than water. Of course this would necessitate a reinterpretation of the kinetic experiments and a different formulation of the mechanism (3).

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