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The reduction mechanism of ferricytochrome *c*

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SUMMARY

The second-order rate constant of the reaction between the hydrated electron and ferrinitrocytochrome *c* exhibits a marked pH dependence that could not be fully ascribed to changes in geometrical parameters and in net charge of the protein molecule.

The correlation between the pH dependence of the rate constant, the 695-nm absorbance and the ionization state of the nitrated tyrosyl-67 residue indicates that tyrosine-67 is of importance in maintaining the specific structure for the electron transfer mechanism in ferricytochrome *c* upon reduction.

Previously we have shown[1], that for the reaction of metmyoglobin and methaemoglobin with hydrated electrons (e^-_{aq}) the second-order rate constant (k_{obsd}) is pH dependent. This behaviour was ascribed to the pH dependence of the net charge on the protein and the resulting pH dependence of the electrostatic interaction energy of the reactants. For ferricytochrome *c*, however, between pH 8 and 10 a larger change in k_{obsd} was found than could be expected from the change in net charge on the protein and it was suggested that this is due to structural changes. The conformational change of ferricytochrome *c* between pH 8 and 10 (refs 2–13), characterized by a decrease in 695-nm absorbance ($A_{695\ nm}$)[4–7], affects mainly the structure in the vicinity of the haem[8–13] and seems to have little effect on the geometrical parameters of the molecule[3,6,7,13,14]. That the change in reactivity is caused by a change in radius (see also Braams and Ebert[15,16]) is unlikely, since for the observed decrease in reactivity to about 10% of the value at neutral pH the radius of ferricytochrome *c* at alkaline pH must at least be halved. We therefore suggest that changes in the structure around the haem affect the reactivity of ferricyto-

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chrome *c* towards e^-_{aq}. To check the validity of this hypothesis we investigated the effect of modification of an amino acid residue in the vicinity of the haem.

Sokolovsky *et al.*[5] and Skov *et al.*[17] reported that nitration of Tyr-67 shifts both the pK_a of its hydroxyl group and the pH value for maximal change in $A_{695\ nm}$ from 9 to 6. This indicates that the ionization state of Tyr-67 affects the ligand field around the haem iron. Therefore, we have studied the effect of pH on the reactivity of ferrinitrocytochrome *c*.

Fig. 1 shows the pH dependence of the second-order rate constant (k_{obsd}) for the reaction of e^-_{aq} with ferricytochrome *c* (x——x) and ferrinitrocytochrome *c* (○——○), respectively. In agreement with earlier observations[1], the k_{obsd} decreases sharply between pH 6 and 7 between 9 and 10 and is nearly constant from pH 7 → 9. At pH 6 → 7 the k_{obsd} for ferrinitrocytochrome *c* decreases to a larger extent than for the native protein. No gross changes in reactivity are observed at alkaline pH.

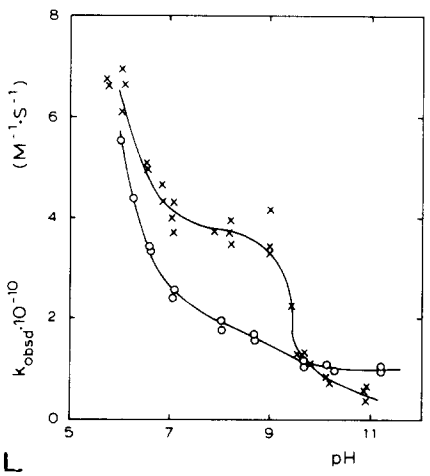

Fig. 1. pH dependence of the second-order rate constant for the reaction of ferricytochrome *c* (x——x) and ferrinitrocytochrome *c* (○——○) with e^-_{aq}. The rate constants are corrected for the reactivity of the matrix solution[1]. Monomeric ferricytochrome *c* ($A_{280\ nm}/A_{550\ nm}$ = 1.30) was isolated from horse hearts according to the method of Margoliash and Walasek[18]; ferrinitrocytochrome *c* (horse heart) was prepared according to the method of Sokolovsky *et al.*[5]. The concentration of the protein solutions (4–5 μM) was determined spectroscopically using a $\Delta A_{550\ nm}$ (reduced *minus* oxidised) = 21.1 $mM^{-1}\cdot cm$ for both ferricytochrome *c* and ferrinitrocytochrome *c*. Other conditions as described previously[1].

Variation of the pH changes the net charge of the cytochrome *c* molecules, which may cause the observed dependence of the reactivity on the pH. We therefore calculated $k = k_{obsd}/f_c$ in which f_c (the Debye factor[1]) describes the effect of the electrostatic interaction between the spherically charged protein and e^-_{aq} on the second-order rate constant at high ionic strength. The net charge of the protein at different pH values was determined from the titration curve of ferricytochrome *c*[19]. The charge of ferrinitrocytochrome *c* was obtained from that of ferricytochrome *c* taking into account the shift of the pK_a of Tyr-67 from 9 to 6 (refs 5, 17).

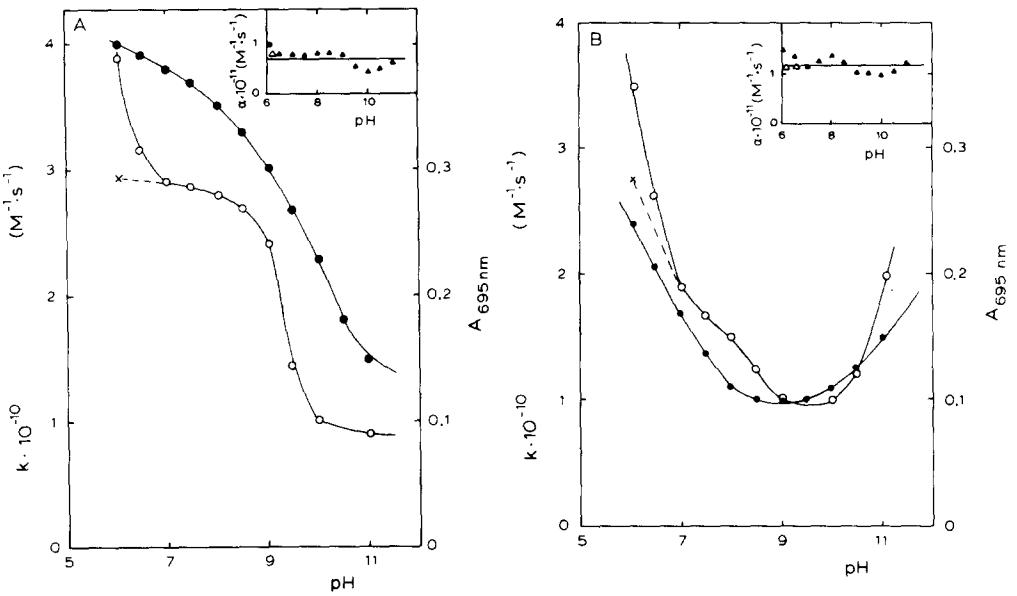

Fig. 2. The pH dependence of the ratio $k = k_{obsd}/f_c$ (○——○) and the 695-nm absorbance (●——●). (A) Ferricytochrome *c*. (B) Ferrinitrocytochrome *c*.
The values for $A_{695\ nm}$ are from Skov *et al.*[17]. The dashed line (x- - - -x) between pH 6 and 7 represents the estimate of the reactivity after subtraction for the reactivity of protonated histidyls. In the insets is plotted $\alpha = k/A_{695\ nm}$ (▲——▲) against the pH (see text). △, represents α, taking into account the histidyl reactivity.

Fig. 2A shows that even after application of the Debye factor for charge effects the relationship between the reactivity of ferricytochrome *c* and the pH is still biphasic. As suggested by Braams[20,21] the decrease in reactivity around pH 6.5 is most likely due to deprotonation of histidyls. The dashed line represents an estimate of the pH dependence of *k* after subtraction of the histidyl reactivity.

The reason for the sharp decrease in reactivity of ferricytochrome *c* around pH 9 is less clear, but the observation that the 695-nm absorbance of ferricytochrome *c* also decreases in this pH region suggested to us that these two parameters are related and thus we plotted $A_{695\ nm}$ against the pH in Fig. 2A. To show the similarity in behaviour of the reactivity and the 695-nm absorbance in more detail we have plotted the ratio $k/A_{695\ nm}$ against the pH (inset Fig. 2A). This ratio is nearly constant.

The *k* for ferrinitrocytochrome *c* decreases rapidly between pH 6 and 8 and increases above pH 10 (Fig. 2B). The decrease around pH 6 is not a sole effect of deprotonation of histidyls since after subtraction of their contribution (x- - - -x) the rate constant still decreases sharply. The similarity of the 695-nm absorbance and the reactivity (inset Fig. 2B) indicates that the pH region for decrease of reactivity observed for ferricytochrome *c* around pH 9 is shifted to about pH 6 upon nitration. The increase in reactivity at pH 10 is not yet understood. It is interesting to note that even at these extreme pH values the reactivity and the 695-nm absorbance behave similarly.

Our observations that the changes in reactivity of ferricytochrome *c* and ferrinitrocytochrome *c* towards e^-_{aq} show a similar pH dependence as the 695-nm absorbance suggest that reactivity and the conformation around the haem are closely related. This is in line with the suggestion of Wilson and Greenwood[22] who showed that only the conformation of ferricytochrome *c* with 695-nm absorbance can be reduced by ascorbate.

Since Skov *et al.*[17] and Sokolovsky *et al.*[5] have observed that on nitration of Tyr-67 of ferricytochrome *c* both the pK_a of its hydroxyl group and the pH region for maximal change in 695-nm absorbance shift from about pH 9 to about 6 and since we have observed that nitration of Tyr-67 shifts the pH region for maximal change in reactivity also from about 9 to about 6 we conclude that the ionization state of Tyr-67 is of importance for the reactivity of ferricytochrome *c* towards e^-_{aq}.

For a better insight of the parameters determining the reactivity of cytochrome *c*, we have calculated the sum of the rate constants of all its individual amino acid residues (from Braams[20,21]). We found that this sum is too low to account for the observed rate constant ($k_{calcd} = \Sigma k_{amino\ acid}$, is approx. $3 \cdot 10^9\ M^{-1} \cdot s^{-1}$, electrostatic interactions neglected). The haem on the other hand is very reactive ($k_{obsd} = 3.0 \pm 0.3 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ at pH 7, in 0.1 M sodium formate, unpublished observations). Under these conditions the k_{obsd} for ferricytochrome *c* is nearly equal to that of the haem alone: $2.9 \cdot 10^{10}\ M^{-1} \cdot s^{-1}$ The haem, however, is buried rather deep inside the molecule[23] and therefore not readily accessible for e^-_{aq}. The exposed edge of the haem represents only a small part of the molecular surface and thus the collision frequency of the hydrated electron with the exposed part of the haem will be considerably smaller than the collision frequency for the cytochrome *c* molecule. It was found (refs 24, 25 and unpublished observations) that at neutral pH e^-_{aq} reduces the haem iron nearly stoichiometrically. These considerations suggest the occurrence of effective electron transfer from the surface of the molecule to the haem between pH 6 and 8.

Only the 695-nm conformation has a high reactivity and it is likely that this structure is essential for this electron transfer (see also ref. 22). A change in conformation around the haem induced by changes in pH or modification of amino acid side chains, prevents this effective transfer, causing a lower reactivity of cytochrome *c* towards e^-_{aq}. The remaining reactivity observed above pH 10 may be due to non-specific reactions, *e.g.* a direct accessibility of the haem group or *via* radical mechanisms (radicals may be generated from a reaction of e^-_{aq} with amino acid residues on the surface).

The high reactivity of ferricytochrome *c* ($k >> k_{calcd}$) is only observed at pH values where Tyr-67 is protonated, *e.g.* for ferricytochrome *c* at pH < 10 and for ferrinitrocytochrome *c* at pH < 7. This indicates that the protonated form of Tyr-67 is essential for the specific electron transfer mechanism.

In 1965 Winfield[26] proposed a general mechanism for electron transfer in haemoproteins, based on the presence of an aromatic residue near the haem iron. Dickerson *et al.*[27], using their three-dimensional model of ferricytochrome *c*[23], suggested that in this enzyme Tyr-67 is the aromatic residue. This suggestion is supported by our observation that the ionization state of Tyr-67 is of great importance for the specific electron transport in cytochrome *c*.

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