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ON THE ELECTRON-TRANSFER-COUPLED PROTON RELEASE OF CYTOCHROME *c*

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SUMMARY

Horse heart ferrocytochrome *c* was oxidized by injection of ferrihexacyanide and the amount of protons released was measured quantitatively over the pH range 7–11, leading to the two electron-transfer-linked pK_H's of 9.1 and 10.4. The kinetic result that the proton changes took place more rapidly than the changes in the 695-mμ absorption band for both types of rapid pH changes on oxidized cytochrome *c* (pH 7 → 10 and pH 10 → 7) led to the proposal of a cyclic reaction scheme. Involved proton-releasing amino acid residues are discussed.

During the *Symposium on Energy Coupling in Electron Transport* at the Federation Meeting, 1967, the involvement of protons in the electron-transfer-process was profoundly stressed by both the defendants[1,2] and the opponents[3] of the MITCHELL[4] theory. It was emphasized[3], that no proton change is associated with the electron transfer in cytochrome *c* at physiological pH. This fact was shown already by PAUL[5] over twenty years ago: proton coupling to electron transfer occurs only at alkaline pH. However, it is highly probable that suitable biochemical effectors shift the proton coupling to the electron transfer into the physiological pH range. A deeper mechanistic understanding of any such shifts requires first a clear picture of the proton coupling without effectors present.

Earlier experiments indicated[6,7] that oxidized cytochrome *c* is linked to protonic dissociations through a slow monomolecular interconversion (causing the rate constants in the electron transfer process to be independent of pH). No proton coupling to the electron-transfer process is detectable on the side of reduced cytochrome *c* up to at least pH 12. The pH dependence of the redox potential of cytochrome *c* is thus solely due to protonic dissociations of the oxidized form.

The oxidized form shows two electron-transfer-coupled dissociation constants between pH 7 and 11 (at pH 9.1 and 10.4). We did not investigate an additional $pK_H \approx 11.6$ any further, as complications arose from very slow structural changes, probably due to deamination. Of particular interest in this investigation was the precise determination of the two electron-transfer-linked apparent dissociation constants by oxidizing reduced cytochrome *c* and measuring the proton release quantitatively.

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Kinetic investigations were to follow to link the protonic dissociations to changes in the absorption band at 695 mμ. THEORELL AND AKESSON[8] had already shown that this band is associated with ferricytochrome *c* at pH 7, while it disappeared at high pH. GREENWOOD AND PALMER[9] discussed the pH-dependence of this band further.

The protonic dissociation constants were determined with a Radiometer pH-stat TTT-1 in a thermostatted reaction vessel, containing a magnetically driven stirring bar. A rubber stopper with various inlets (NaOH from pH-stat, N_2, micropipets) and outlets (combination glass electrode, N_2) sealed the vessel. N_2 flow over the solution provides for the exclusion of CO_2 as the solutions are only weakly buffered (by 1–5 mM sodium phosphate in Na_2SO_4 to give ionic strength 0.1, 25°). Horse heart cytochrome *c* was prepared according to MARGOLIASH AND FROHWIRT[10]. 3.2 ml of 0.2 mM ferrocytochrome *c* solution are vigorously stirred at a present pH and the electron transfer is initiated by the addition of 25 μl sodium ferrihexacyanide solution of the same pH and a concentration, which assures complete oxidation (about 10 % above equivalence). Released protons were neutralized by NaOH from the syringe of the pH-stat.

Some of our kinetic experiments were conducted in the same reaction vessel. The outlet for N_2 was then used for a coaxial insertion mixer employing the same infinite-jet principle, discussed previously[11]. Unbuffered cytochrome *c* solution at one pH was mixed with weak buffer at another pH to result in a desired terminal pH. Any slow proton change was followed with a glass electrode, connected to a Radiometer pH-meter and recorded on Tektronix 564 storage oscilloscope. Prior to each experiment, the vessel was dried and the electrode wetted. The experiments resulted in proton changes which were slower than the overall response of the detection system, by a

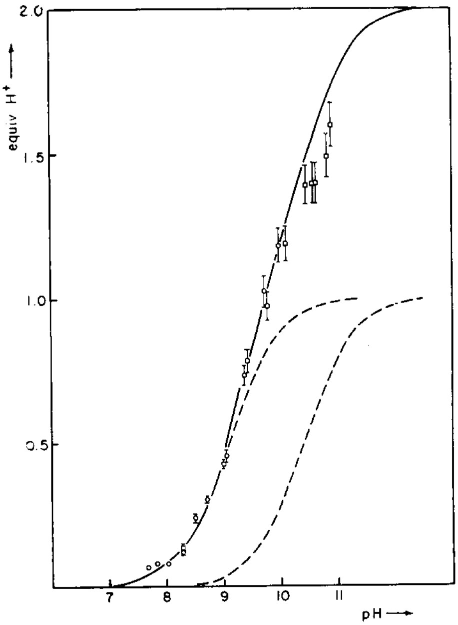

Fig. 1. Results from injecting ferrihexacyanide into reduced cytochrome *c* solutions in thermostatted vessel, normalized with H_2SO_4 injections (25 μl of acid of a concentration which corresponds after dilution to exactly one proton per cytochrome *c* molecule). The dashed curves represent titration curves for pK_H 9.1 and 10.4, added together in the full curve.

factor of 5 in the worst case. All proton changes were calibrated by addition of a known amount of H_2SO_4 (equivalent to about half the amount of cytochrome *c*). To correlate proton changes with changes in the 695-mμ band we used the Durram-Gibson stopped-flow apparatus. Phosphate was used for buffering near pH 7, glycine for buffering near pH 10; the buffer with the cytochrome *c* solution was quite weak, buffer alone in the second syringe was stronger (determining the final pH).

The results of the equilibrium experiments at the various pH values are shown in Fig. 1. The error-widths comprise the overall variations in the experiments. The curves fitted to the data are associated with the mentioned two pK_H values. The initially mentioned slow changes at high pH cause the deviations near pH 11; pK 10.4 is therefore less precise than pK 9.1.

A summary of our kinetic experiments is presented in Fig. 2. When the pH is quickly changed from basic to neutral, the changes at 695 mμ are slowest with an apparent monomolecular rate constant of 0.025 sec^{-1}. For the inverse process, the apparent monomolecular rate constant for the change at 695 mμ is 0.078 sec^{-1}. However, for either pH change the proton changes are much faster than the changes at

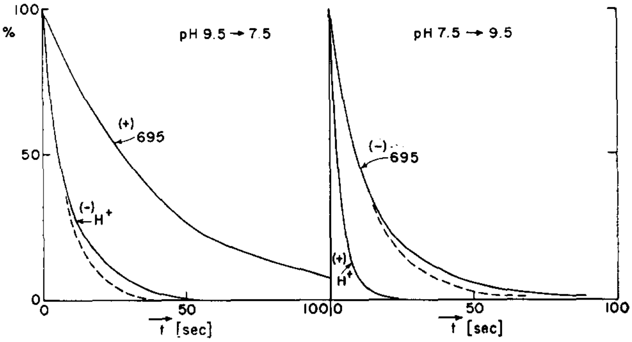

Fig. 2. Representative normalized curves from pH-"switching" experiments. The dashed sections are theoretical for an ideal exponential decay. (+) means appearance of the indicated observable, (−) its disappearance.

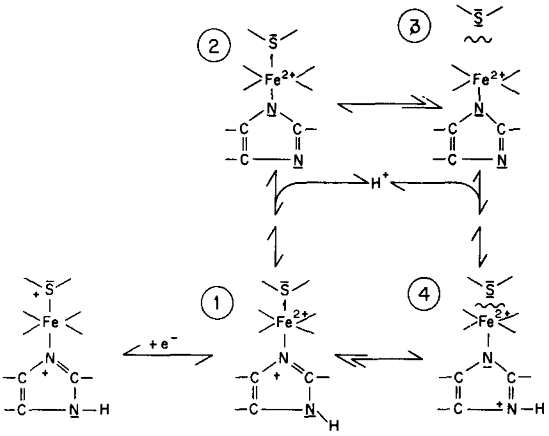

Fig. 3. Reaction diagram for the fifth and sixth coordinate position of iron in cytochrome *c* for 7.0 < pH < 10.0, incorporating all information available to date. Only the most probable limiting electromeric forms are indicated.

695 mμ, the difference being close to factor 5. This result requires that the proton change must precede the change at 695 mμ for any direction of the pH change. One is thus forced to assume a reaction cycle as shown in Fig. 3.

At this point, only the numbers in Fig. 3 should be considered. Form 1 is stable at pH 7 and is the form which participates in the electron transfer. Form 3 is stable at pH 10 and inactive in electron transfer. If the pH is quickly changed from 7 to 10, a proton is quickly released to give Compound 2, which slowly converts to 3. If the pH is quickly changed from 10 to 7, a proton is rapidly picked up by Form 3, which thereby becomes Form 4. And Compound 4 then rearranges slowly to Compound 1. Various constants may be defined in the reaction scheme of Fig. 3. Their definitions are given in Table I together with values for the constants. The previously mentioned apparent pK_H may then be written in the two forms $pK_H = pK'_H - pK_{3,4}$ and $pK_H = pK''_H - pK_{8,7}$ (with $K_{i,j} = k_i/k_j$).

TABLE I

DEFINITION OF CONSTANTS AND THEIR VALUES

c_i indicates the equilibrium concentration of the i-th component (or of H+) in Fig. 3.

Constant	K'_H	K''_H	k_3	k_8	$K_{3,4}$	$K_{8,7}$
Definition	$\frac{c_2c_H}{c_1}$	$\frac{c_3c_H}{c_4}$	rate constant $2 \rightarrow 3$	rate constant $4 \rightarrow 1$	$\frac{c_3}{c_2}$	$\frac{c_1}{c_4}$
Value	$10^{-9.6}$	$10^{-7.8}$	0.078	0.025	3	20
Units	M	M	sec^{-1}	sec^{-1}	None	None

At pH 7, the ratio $k_8/k_7 = K_{8,7}$ can reasonably well be estimated from the experiments of SCHEJTER AND GEORGE[12]. Their value of 20 gives for pK''_H the number 7.8. If the two slow interconversions have equal unsymmetry, one could obtain $pK'_H = 10.4$ requiring the proton release to follow mainly the time-course of the 695-mμ change for rapid pH changes from 7 to 10. As such a result was not obtained experimentally, pK'_H has to be at least 9.6. This value results in $K_{3,4} = k_3/k_4 = 3$. Rapid pH changes to different pH values would quantitatively establish the magnitude of pK'_H.

If the proton-dissociation steps between Components 1 and 2 or 4 and 3 would be diffusion limited, one would expect the measurement of the proton changes to coincide with the overall time constant of the glass-electrode detection system. However, proton changes were slower than this time constant in both instances (by factor 5 in the worst case). The proton steps in Fig. 3 were therefore split into two parts, a rapid bimolecular step and a slower monomolecular step next to Compound 1 (or 4). This result is in agreement with the fact that both rate constants in the electron transfer reaction of ferrocytochrome *c* with ferrihexacyanide were found to be independent of pH (ref. 6).

The details of Fig. 3 are based in part on suggestions which THEORELL[13] made in conjunction with work on the magnetic susceptibility of cytochrome *c*. He found that reduced cytochrome *c* has no unpaired electrons, leading thus to the structure shown in the lower left corner. Compound 1 has about 1.6 unpaired electrons and a weakened bond between sulfur and iron. This apparently leads to the very strong effect, iron-III can exert upon imidazole to shift its second pK_H down by several units. Regarding imidazole being responsible for pK 9.1 we follow the original suggestion of THEORELL AND AKESSON[14]. Our pK 10.4 is presumably associated with tyrosine, as derived from the spectrophotometric titrations of STELLWAGEN[15], excluding the consideration of this residue for the first protonic dissociation.

Recent results of DICKERSON *et al.*[16] from X-ray studies on ferricytochrome *c* crystals confirm the structural details, but make the proton dissociation of His-18 somewhat unlikely: the imidazole residue in the fifth ligand position of the heme-iron is located in a rather hydrophobic environment and its proton is hydrogen-bonded to a peptide CO. The other two imidazole residues are about 10 and 11 Å away from the sulfur atom of Met-80, the sixth ligand of the heme-iron. Another choice for protonic dissociation is available in Tyr-67, only 5 Å away from Met-80 and 5 Å above one of the pyrrol rings of the porphyrin system. The phenolic OH of Tyr-67 is also hydrogen-bonded, but might dissociate without showing up spectrophotometrically in the titrations of STELLWAGEN[15]. No spectrophotometric discrimination of the protonic dissociation may in part result from the hydrophobic environment of Tyr-67, in part upon an interaction between Tyr-67 and Met-80, causing the disappearance of the band at 695 mμ. In either case one would expect a comparatively slow release of the proton, as found experimentally. These authors prefer the involvement of the imidazole residue, as a tyrosine residue seems clearly to be coupled in after the dissociation of the first proton (Fig. 1), and pK 10.4 is therefore tentatively attributed to Tyr-67.

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