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KINETIC STUDY OF ISOMERIZATION OF FERRICYTOCHROME *c* AT ALKALINE pH

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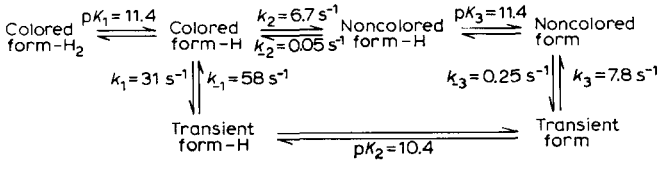
SUMMARY

Kinetic studies of the isomerization reaction of horse heart ferricytochrome *c* between pH 8.5 and pH 12.1 have been carried out by using stopped-flow and rapid scanning stopped-flow techniques. Below pH 10, our results were in good agreement with the scheme proposed earlier (Davis, L. A., Schejter, A. and Hess, G. P. (1974) J. Biol. Chem. 249, 2624–2632). Above pH 10, another faster first-order process was observed, which suggested the existence of a transient species in the isomerization reaction between the species with and without a 695 nm band. The probable scheme of the isomerization reaction is considered to be



where H denotes a proton, the colored forms are the species predominant at neutral pH with a 695 nm band and the noncolored forms are the species without a 695 nm band. The transient species has a small 695 nm absorbance which suggests that the sixth ligand is still Met-80, although the protein conformation might be different from that at neutral pH.

INTRODUCTION

Ferricytochrome *c* takes several different conformations, depending on pH [2, 3]. It is in low spin state at neutral pH, and has Met-80 as the sixth ligand [4].

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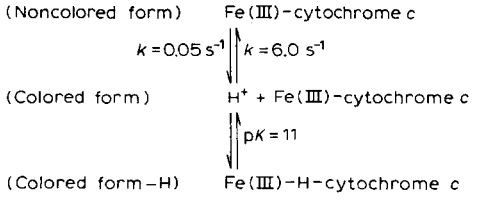
Its absorbance spectrum has a shoulder at 695 nm [2, 5]. This species will be termed the colored form. Above pH 8, another type of low-spin state appears, which has no shoulder at 695 nm [2, 5], which will be termed the noncolored form. The sixth ligand of the noncolored form is not Met-80, but speculated to be Lys-79 by several authors [6–9]. Davis et al. [1] studied the kinetic properties of the isomerization reaction of horse heart ferricytochrome *c* between pH 8.5 and pH 10.5 by a stopped-flow technique, and proposed the following scheme as the minimal necessary scheme for the conformational conversion.



The purpose of this report is to investigate the kinetics of the isomerization reaction between pH 8.5 and pH 12.1 in more detail by a pH jump method. A new transient species was found above pH 10, and its structure is discussed.

EXPERIMENTAL

Materials and Methods

Horse heart cytochrome *c* was purchased from Sigma (Type VI) and oxidized by

TABLE I

COMPOSITIONS OF SOLUTIONS USED FOR THE STOPPED-FLOW AND THE RAPID SCANNING STOPPED-FLOW EXPERIMENTS

For each measurement, Solution A was mixed with equal volume of solution B (buffer solution). Ionic strength was adjusted to 0.1 or 0.5 by adding NaCl.

Method	Solution A		Solution B	
	pH	Materials	pH	Materials
pH jump (up)	7 >	Ferricytochrome *c* NaCl	8 ~ 11	Glycine NaOH NaCl
			11	NaOH NaCl
pH jump (down)	> 11	Ferricytochrome *c* NaOH NaCl	9	Glycine NaOH NaCl
			7 ~ 8	NaH_2PO_4 Na_2HPO_4 NaCl
	9 ~ 11	Ferricytochrome *c* Glycine NaOH NaCl	7 ~ 8	NaH_2PO_4 Na_2HPO_4 NaCl

potassium ferricyanide. Ferricytochrome *c* was adsorbed onto a CM-cellulose column equilibrated with 0.01 M potassium phosphate buffer at pH 7.0. After washing the column with the same buffer, ferricytochrome *c* was eluted with 0.5 M phosphate buffer at pH 7.0. The main fraction of the elute was used in the present investigation.

Stopped-flow measurements were carried out using a stopped-flow apparatus (Union Giken SF-70, Japan). The optical path of the observation cell was 10 mm or 2 mm, depending on the reaction conditions. The dead time was 1.4 ms for the 10 mm cell and 1.0 ms for the 2 mm cell. The obtained absorbance changes were memorized in a digital memory, Quick Signal Converter (Kawasaki Electronica, Japan) and recorded on a pen recorder. Rapid scanning stopped-flow measurements were carried out by using an RA-1300 apparatus (Union Giken). The composition of the buffer solution used in the kinetic experiments is given in Table I. The difference spectrum was measured with a Shimadzu D-40 DFS spectrophotometer.

The buffer solution used in the difference spectrum experiments was Tris · HCl at neutral pH and glycine/NaOH at alkaline pH. All the experiments were performed under nitrogen atmosphere to prevent pH change due to carbon dioxide. Temperature was regulated at 25 °C throughout. Ionic strength was adjusted to 0.5 with NaCl unless otherwise stated.

RESULTS

pH jump (up) by the stopped-flow method

The kinetics of the isomerization reaction between colored and noncolored forms were studied by observing the approach to equilibrium just after the pH jump from neutral to alkaline. Ionic strength was adjusted to 0.1 or 0.5. The wavelength was fixed at 390 nm, where the absorbance change between the colored and the transient form newly found was the largest. When the final pH was below 10, the absorbance increased to the equilibrium state, following first-order kinetics (Fig. 1A). Above pH 10, however, the absorbance change involved two first-order processes (Fig. 1B). Above pH 11, the absorbance increased quickly just after mixing and then decreased slowly (Fig. 1C). The observed rate constants of the fast and the slow processes were plotted as a function of pH in Fig. 2. The ionic strength did not seriously influence on the pH dependence of the observed rate constants. The rate constants observed by Davis et al. [1] were also plotted in Fig. 2. Their data are in excellent agreement with ours as far as the slow process is concerned, in spite of the difference in wavelength and temperature employed.

Fig. 3 illustrates the absorbance change of the total process from the initial to the final states, together with those of the individual fast and slow processes above pH 8.5 observed with the stopped-flow method. The pK value of the total process was found to be 9.26. This value coincided with that obtained in equilibrium at 695 nm, which has been reported earlier [2, 3, 6].

pH jump (up) by rapid scanning stopped-flow experiments

The kinetics of the isomerization reaction between colored and noncolored forms were also studied by rapid scanning stopped-flow technique. The time at which the scanning started after the flow stopped was (1) 0 ms, (2) 45 ms, (3) 300 ms and (4) 20 s at pH 10.5 and (1) 0 ms, (2) 20 ms, (3) 95 ms and (4) 800 ms between pH 10.5

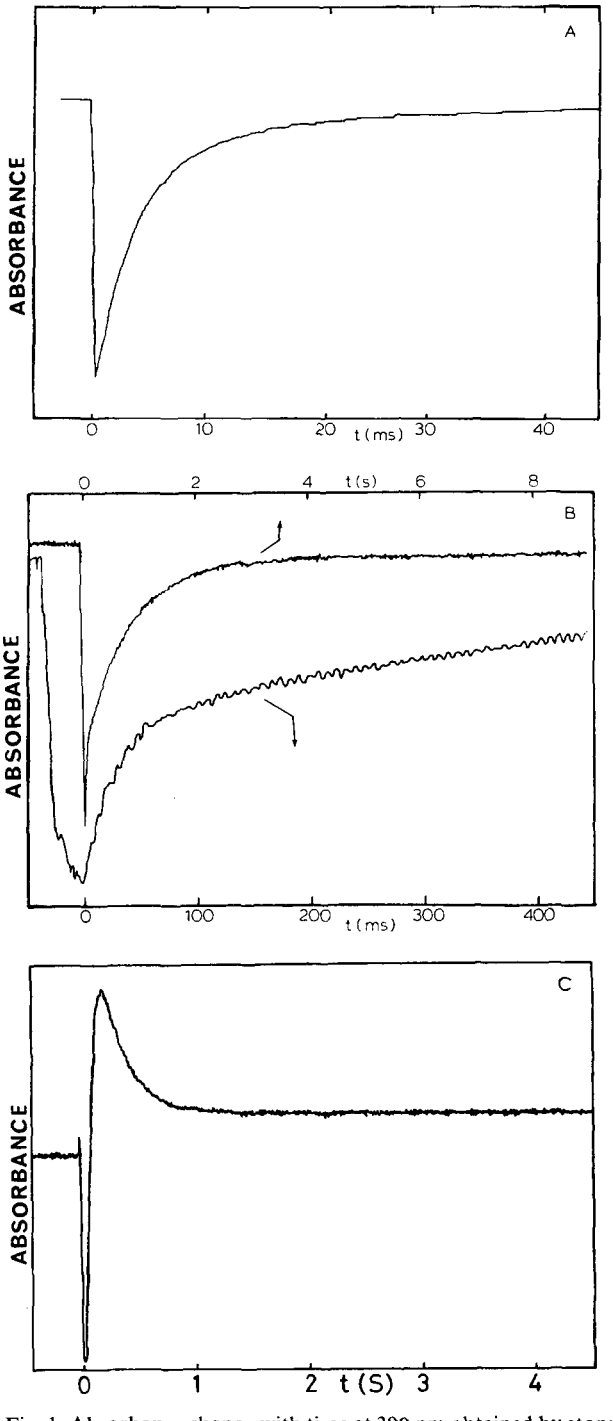


Fig. 1. Absorbance change with time at 390 nm obtained by stopped-flow experiments. Equal volumes of Solutions A and B (see Table I) were mixed in a stopped-flow apparatus. Ferricytochrome *c*, 7.6 μM; starting pH, 6.7; final pH (A) 9.8, (B) 10.4, (C) 12.1.

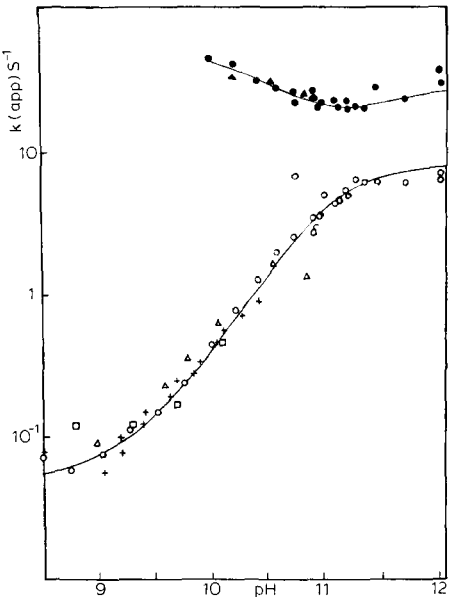


Fig. 2. pH dependence of the observed rate constants by pH jump(up) method. Details of the composition of the buffer solution are summarized in Table 1. 390 nm; ferricytochrome *c*, 7.6 μM; starting pH 6.7. The value of pH indicated is the pH after mixing. Each point represents the average of at least three determinations: (▲) fast process, ionic strength 0.1; (△) slow process, ionic strength 0.1; (●) fast process, ionic strength 0.5; (○) slow process, ionic strength 0.5. The solid lines are the theoretical curves calculated with Eqns. 1 and 2, using the constants listed in Table II. (□), rate constants obtained by pH jump (down) method, starting pH 12.0. For the purpose of comparison, the data of pH jump (up) (+) obtained by Davis et al. [1] at 21 °C, 695 nm are also shown.

and pH 12.1. Two typical examples of the spectra obtained at pH 10.2 and pH 12.0 are shown in Fig. 4. At pH 10.2, no transient spectrum appeared, judging from the isosbestic points. At pH 12.0, however, the absence of the isosbestic points clearly shows the existence of a transient species other than the colored and the noncolored forms. To obtain further information about the isomerization processes between the colored and the noncolored forms, the isosbestic points between (1) and (2), (3) and (4), and (1) and (4) were plotted as a function of pH in Fig. 5, since they are considered to represent the isosbestic points of the fast, the slow and the total processes, respectively. As clearly shown in the figure, the isosbestic points of the fast and the total processes are independent of pH, whereas the isosbestic points of the slow process changed appreciably between pH 10 and pH 11. The result suggests that the slow processes consist of two components, one of which is dominant below pH 10, the other dominant above pH 11, and the both components co-exist between pH 10 and pH 11.

The difference spectrum

The difference spectrum between alkaline and neutral ferricytochrome *c* was measured between pH 7.0 and pH 11.5 to see whether or not some species other than the noncolored form exists in equilibrium (Fig. 6). The figure clearly shows isosbestic

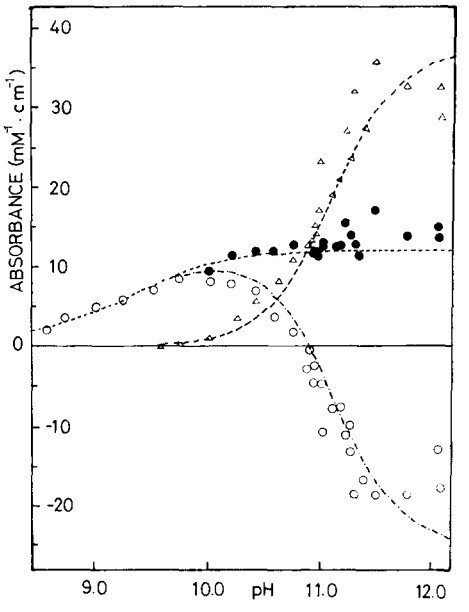


Fig. 3. pH dependence of the absorbance change at 390 nm obtained by pH jump (up) method. The colored form was employed as the reference. (△) Fast process; (○) slow process; (●) total process. The curves (———, —·— and - - -) are the theoretical curves calculated with Eqns. a10, a11 and a19–21, using the constants listed in Tables II. (See appendix).

points between the spectra of the species existing at alkaline pH and neutral pH. This leads to the conclusion that the transient species is absent at equilibrium.

pH jump (down) by stopped-flow method

The kinetics of the isomerization reaction were also studied by observing the approach to equilibrium just after the pH jump from the alkaline to the neutral region. The decrease in absorbance accompanied by pH jump (down) from pH 11.8 to pH 7.3

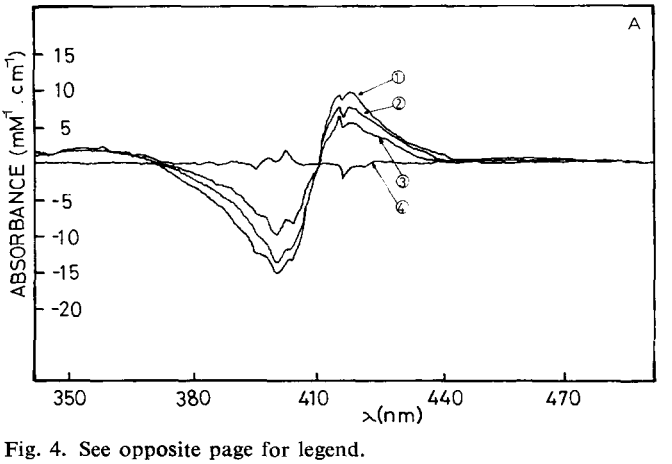


Fig. 4. See opposite page for legend.

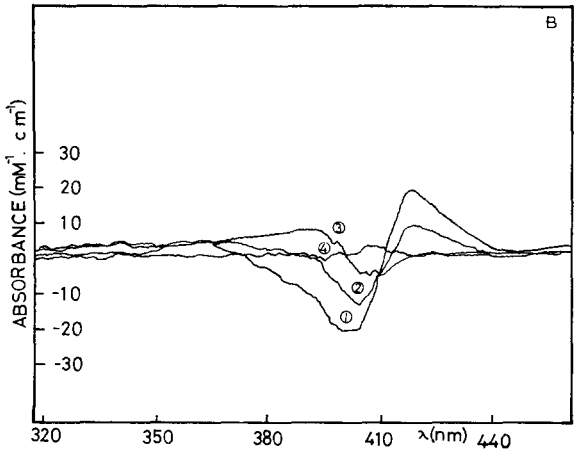


Fig. 4. Variation with time of the difference spectrum obtained by the rapid scanning stopped-flow technique. Scanning speed was 30 nm/ms. Starting pH 7.0; final pH (A) 10.2, (B) 12.0. The reference was the spectrum 50 s after mixing. The scanning of spectra (1), (2), (3) and (4) were started at (A) 0 ms, 45 ms, 300 ms and 20 s, and (B) 0 ms, 20 ms, 95 ms and 800 ms, after the flow stopped, respectively.

is shown in Fig. 7. The figure shows the absence of an observable faster process, and the relaxation process is proved to be a single first-order process. It is also shown that the absorbance change observed with the stopped-flow method was equal to that observed statically before and after the pH jump. Some of the observed rate constants

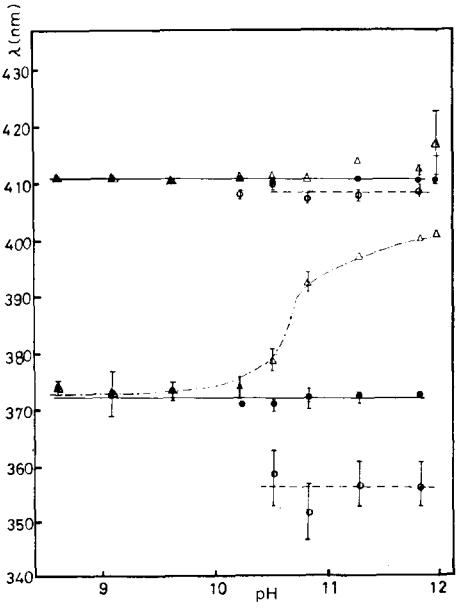


Fig. 5. Variation with pH of the isosbestic points of the fast (○), slow (△) and total (●) processes.

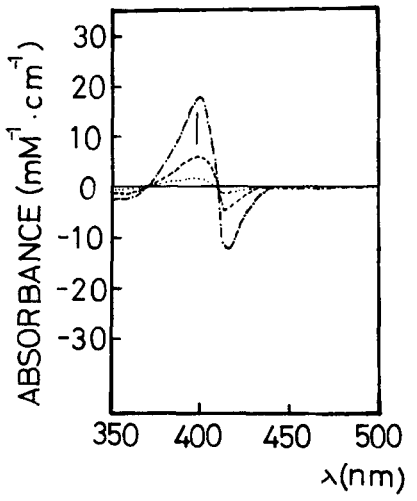


Fig. 6. Difference spectra of ferricytochrome *c* between alkaline and neutral pH. The reference solution was adjusted at pH 7.0. Ferricytochrome *c*, 3.3 μM, 23 °C. The pH values of the sample solutions are (———) 7.0, (. . .) 8.5, (– – –) 9.5 and (–·–) 11.5.

were plotted as a function of the final pH in Fig. 2, the initial pH being fixed at 12. It shows that pH dependence of the rate constants obtained in pH jump (down) are similar to that of the slow process in pH jump (up), indicating that the isomerization from the noncolored to the colored form (direct path or via transient form) occurs with the same rate constant k_{app} at the same pH.

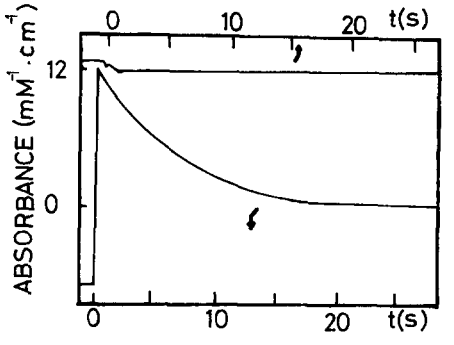
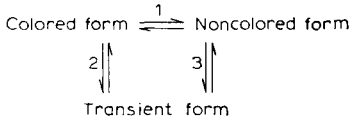


Fig. 7. Absorbance changes of ferricytochrome *c* at 390 nm with time by pH jump (down) method. Compositions of the buffer solutions are listed in Table I. The initial and final pH values were 11.8 and 7.3, respectively. Ferricytochrome *c*, 15.7 μM. Optical path, 10 mm. The upper line shows the absorbance change at 390 nm with time with both initial and final pH 11.8, shown for comparison.

ANALYSIS AND DISCUSSION

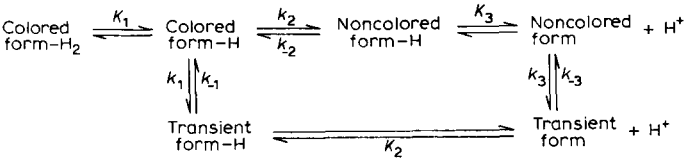
The above results lead to the following scheme for the isomerization reaction:



(Scheme 1)

where processes 1 and 3 are slow and process 2 is fast. Process 1 is dominant below pH 10 and processes 2 and 3 become dominant above pH 11. The transient form newly found may have a conformation different from those of the colored and the noncolored forms.

To determine the minimal probable scheme, computer simulation of the rate constants shown in Fig. 2 was made by the least square method, assuming that the proton uptake (or release) is much faster than the conformational change of the protein*. The scheme must satisfy at least the following two requirements. (1) A correct scheme must include two processes, one of which represents the isomerization between the colored and the noncolored form directly and the other represents the process from the colored to the noncolored forms via transient species. (2) No detectable amount of the transient species exists in equilibrium. The scheme shown below was found to be a minimal probable one among several schemes examined. K_1, K_2 and K_3 are dissociation constants and k_1, k_{-1}, k_2, k_{-2}, k_3 and k_{-3} are rate constants specified in the scheme. The details of the calculations are shown in the appendix.



(Scheme 2)

The rate constant of the fast process, k_f, and the slow process, k_s, are represented as follows, respectively (see Eqns a5 and a16 in the appendix);

$$k_f = \frac{k_1}{\alpha_1} + \frac{k_{-1}\dfrac{[H^+]}{K_2}}{\alpha_2} \tag{1}$$

$$k_s = \frac{1}{\alpha_1 \alpha_2 k_f} \cdot \left(k_2 k_{-1} \frac{[H^+]}{K_2} + k_1 k_{-3}\right) + \frac{1}{\alpha_3}\left(k_{-2}\frac{[H^+]}{K_3} + k_3\right) \tag{2}$$

where

---

* Ilgenfritz and Schuster [10] reported that the relaxation times of the acid-alkaline transitions of methemoglobin and metmyoglobin are less than 100 μs, which are considered to be due to protonation (or, otherwise, association and dissociation of OH^-). Davis et al. [1] reported that the rate constants observed for the isomerization of ferricytochrome *c* by pH jump were independent of the method of measurement (the change in 695 nm absorbance and proton uptake or release). In view of these results, the assumption mentioned above would not be far from the truth and will be employed in the analysis of the present results.

TABLE II

DISSOCIATION AND RATE CONSTANTS OBTAINED FOR SCHEME 2 BY A LEAST SQUARES METHOD

pK_1	11.4	k_1	30.6 s^{-1}	k_{-1}	58.4 s^{-1}
pK_2	10.4	k_2	6.68 s^{-1}	k_{-2}	0.05 s^{-1}
pK_3	11.4	k_3	0.25 s^{-1}	k_{-3}	7.75 s^{-1}

$$\alpha_1 = 1 + \frac{[H^+]}{K_1} \tag{3}$$

$$\alpha_2 = 1 + \frac{[H^+]}{K_2} \tag{4}$$

$$\alpha_3 = 1 + \frac{[H^+]}{K_3} \tag{5}$$

The constants specified in the scheme were obtained by computer simulation and summarized in Table II. The theoretical simulation curves calculated according to Eqns. 1 and 2 with the constants in Table II are shown in Fig. 2. They are in good agreement with the experimental values. The simulation of the relative absorbance change of the transient species and the noncolored form were also obtained by least square method, using the colored form as reference. The simulated curves of the absorbance change for the fast, the slow and the total processes are shown by the curves in Fig. 3, which are also in good agreement with the experimental data. The details of the calculation of the absorbance change should be referred to in the appendix.

Spectrum of the transient species

The spectrum of the transient species was reproduced from the data of the rapid scanning stopped-flow method, and the results are shown in Fig. 8. The procedure is as follows. First, the absorbance change at 95 ms in Fig. 4B is multiplied by a factor $1/(1-e^{-k_s t})$, since the fast process has been finished at this time and, as a result, any colored form does not exist. Next the absorbance change thus obtained was added to the spectrum of the noncolored species (the final state). It can be seen that the transient species has evidently an absorbance between 680 and 710 nm, though it is less than that of the colored form.

Nature of the transient species

The 695 nm band is reported to be characteristic of the sulfur ligation of Met-80 at the sixth coordination site [11]. The sixth ligand of the transient species would then be Met-80, although the coordination nature is different from that of the colored form. Ikeda et al. [12] reported that cytochrome b_5 exists in a distorted structure above pH 12, at which no exchange of ligand could occur, but the coordination nature was distinctly different from that of the species at neutral pH. The conformational change following the proton release by the path Colored form-H_2 $\rightleftharpoons$ Colored form-H

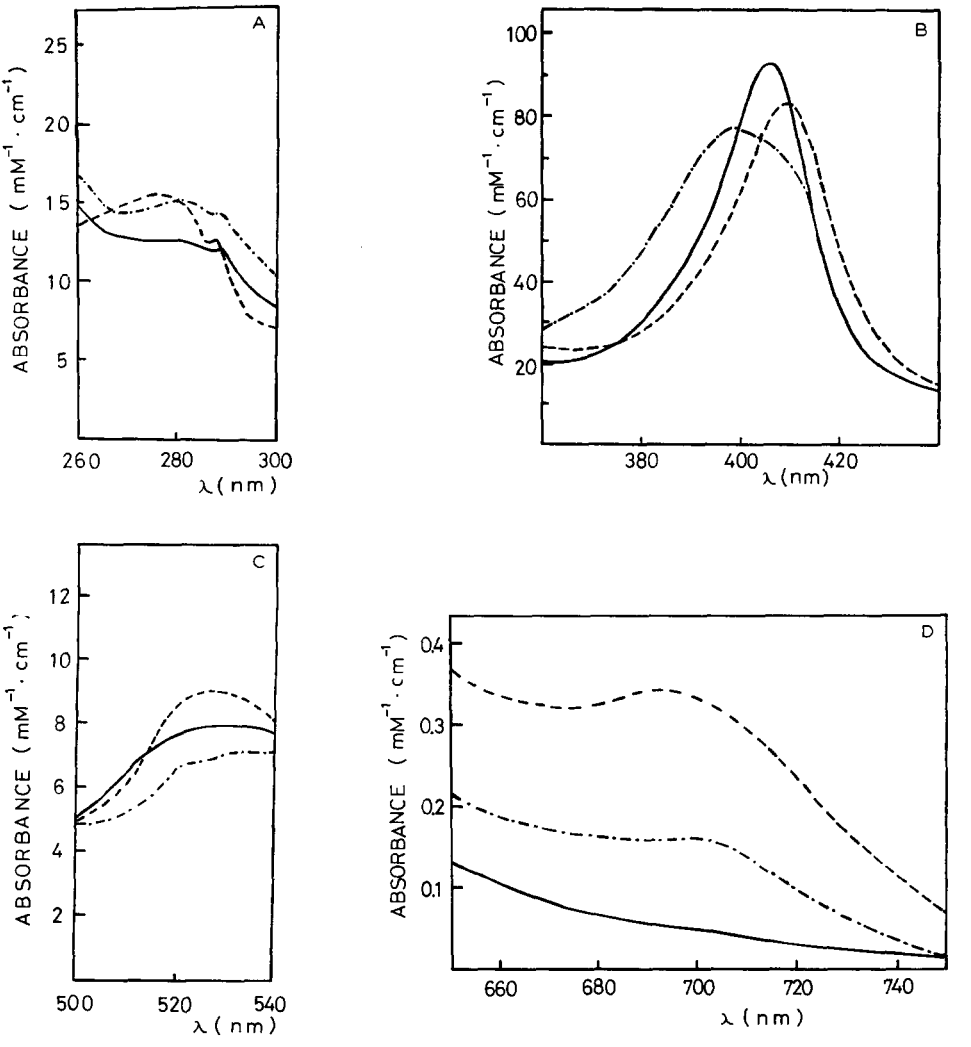


Fig. 8. Spectra of the colored (---), the transient (-·-) and the noncolored (———) forms reproduced from data obtained by the rapid scanning stopped-flow experiments. The ranges of the wavelength are (A) 260–300 nm, (B) 360–440 nm, (C) 500–540 nm and (D) 650–750 nm.

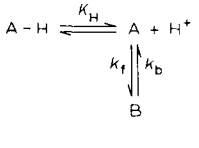
$+H^+ \rightleftharpoons$ Noncolored form-H$+H^+$ would correspond to the ligand exchange from Met-80 to Lys-79 [1], since the sixth ligand at alkaline pH would probably be Lys-79, judging from the results obtained by several authors [6–9]. The dissociation Colored form-$H_2 \rightleftharpoons$ Colored form-H$+H^+$ would then involve a proton of ε-amino residue of Lys-79. The process Colored form-H $\rightleftharpoons$ Transient form-H may be due to some local conformational change including the main chain, since the rate constants k_1 and k_{-1} are much slower than the acid-alkaline transition observed for metmyoglobin [10]. The conformational change by the fast process is accompanied by an absorbance change around 280 nm (Fig. 8A), which did not cause the disappearance of the 290 nm band. The result suggests that the dissociation of tyrosine hydroxyl group may

accompany the conformational change of the fast process. The value of pK_2, which is responsible for the dissociation equilibrium Transient form-H $\rightleftharpoons$ Transient form $+ H^+$ is found to be 10.4, which is in conformity with the pK of a normal tyrosine hydroxyl group (10.1). Dickerson et al. [7] reported that the polypeptide chains of residues 79–83 of cytochrome *c* must move upon ligand exchange from Met-80 to Lys-79, which might influence the environment of the residue 70–82 loop. The conformational change on the fast process would then accompany the rearrangement of the conformation of the main chain residues including a part of the residues 70–82, which will induce the environmental change of Tyr-67 and/or Tyr-74. The residue responsible for the dissociation equilibrium Noncolored form-H $\rightleftharpoons$ Noncolored form $+ H^+$ still remains to be investigated.

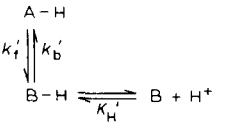
Biological meaning of the transient form newly found, e.g. the relationship between the transient form and the redox system, remains to be studied further.

APPENDIX

The scheme of the reaction of a proton release or uptake with conformational change between A and B can be represented as either one of the following schemes, A1 and A2.



(Scheme A1)



(Scheme A2)

where AH and BH are the protonated and A and B the deprotonated species, respectively. K_H and K'_H are the proton dissociation equilibrium constants, and k_f, k_b, k'_f and k'_b are the rate constants, as specified in the schemes.

The assumption that a proton uptake (or release) was much faster than the conformational change of the protein was employed throughout the following calculations. The discussion of this point was expressed in the footnote of the text.

The observed rate constants are expressed as follows:

$$k = k_b + \frac{k_f}{1 + \dfrac{[H^+]}{K_H}} \tag{a1}$$

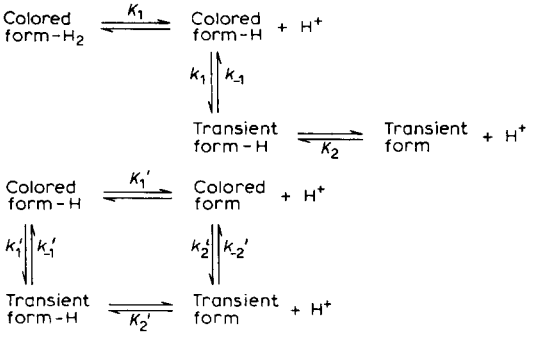
$$k' = k'_f + \frac{k'_b}{1 + \dfrac{K'_H}{[H^+]}} \tag{a2}$$

Eqn. a1 predicts that k will decrease with increasing $[H^+]$, whereas Eqn. a2 predicts that k' will increase with increasing $[H^+]$.

The fast process

The observed rate constants of the fast process increased with $[H^+]$ below pH 11 and decreased above pH 11, as shown in Fig. 2. The fast process, therefore, must bear the features expected from both schemes, A1 and A2. The simplest schemes

consistent with the results are the following two schemes obtained by combining Schemes A1 and A2:



(Scheme F1)

(Scheme F2)

where the abbrevations are expressed in the text.

(1) Scheme F1. The rate equation of the fast process of the isomerization reaction derived from Scheme F1 is;

$$\frac{\mathrm{d}([\text{Transient form-H}]+[\text{Transient form}])}{\mathrm{d}t} = k_1 \cdot [\text{Colored form-H}] - k_{-1} \cdot [\text{Transient form-H}] \tag{a3}$$

Upon integration

$$\alpha_2[\text{Transient form}] \equiv [\text{Transient form-H}]+[\text{Transient form}] = \frac{k_1 c_{\text{tot}}}{k_f \alpha_1} \cdot (1-\mathrm{e}^{-k_f t}) \tag{a4}$$

where k_f represents the rate constant of the fast process, and c_{tot} is the sum of the concentration of the colored form and the transient species at time t,

$$k_f = \frac{k_1}{\alpha_1} + \frac{k_{-1}}{\alpha_2} \cdot \frac{[\mathrm{H}^+]}{K_2} \tag{a5}$$

$$\alpha_1 = 1 + \frac{[\mathrm{H}^+]}{K_1} \tag{a6}$$

$$\alpha_2 = 1 + \frac{[\mathrm{H}^+]}{K_2} \tag{a7}$$

$$c_{\text{tot}} = \alpha_1 [\text{Colored form}] + \alpha_2 [\text{Transient form}] \tag{a8}$$

An index for error, S_1 is defined as follows:

$$S_1 = \sum (k_{\text{app (fast)}} - k_f)^2 \tag{a9}$$

where $k_{\text{app(fast)}}$ designates the rate constant obtained for the fast process in the experiment. When the time t is long enough to attain the pre-equilibrium after the fast process, the concentration of the transient species C(T) = ([Transient form-H]+[Transient form]) is given from Eqn. a4 by

$$C(T) = \frac{k_1}{\alpha_1 k_f} \cdot c_{tot} \tag{a10}$$

The concentration of the colored form, C(C) (=[Colored form-H_2]+[Colored form-H]), then becomes

$$C(C) = \frac{k_{-1}[H^+]}{\alpha_2 \cdot K_2 \cdot K_f} \cdot c_{tot} \tag{a11}$$

The rate constants k_1 and k_{-1}, and dissociation constants K_1 and K_2 were determined by the least square method, so as to minimize S_1, where the summation in Eqn. a9 was performed on all the experimental values through the pH range from 10 to 12.1. The obtained constants are summarized in Table II.

(*2*) *Scheme F2.* Considering that the free energy changes should be the same for the two paths

Colored form-H $\rightleftharpoons$ Transient form-H $\rightleftharpoons$ Transient form$+H^+$

and

Colored form-H $\rightleftharpoons$ Colored form$+H^+$ $\rightleftharpoons$ Transient form$+H^+$

we employed the least square method to minimize the sum of the squares of the difference between observed rate constants and theoretical rate constants calculated from Scheme F2. This procedure led to the conclusion that

$$k'_1 = k'_2 = 0 \tag{a12}$$

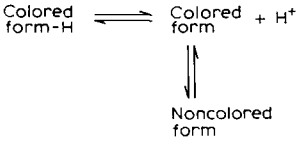
or

$$k'_1 = k'_{-1} = 0 \tag{a13}$$

When $k'_1 = k'_2 = 0$, no transient form can appear, which leads to the conclusion that no fast process can be observed in pH jump(up) experiments. This is obviously inconsistent with the experimental data shown in Fig. 2. Moreover, when $k'_1 = k'_{-1} = 0$, the observed rate constants must always decrease with increasing $[H^+]$. This is also inconsistent with the experimental data shown Fig. 2. Thus, Scheme F2 should be rejected.

The slow process

Below pH 10, no fast process appeared, and pH dependence of the observed rate constants was consistent with those of Davis et al. [1]. Scheme C was proposed to explain the experimental data as minimum requirement by Davis et al. [1].

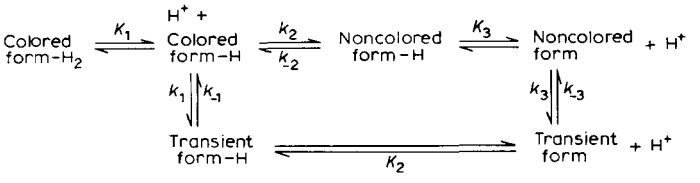


(Scheme C)

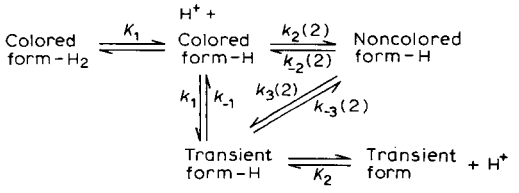
This scheme, however, cannot explain the data above pH 10, where at least the following two requirements must be satisfied, as already mentioned in the text. (1) A correct

scheme must include two processes, one of which represents the isomerization between the colored and the noncolored forms directly and the other which represents the process from the colored to the noncolored forms via transient species. (2) No detectable amounts of the transient species exists in the equilibrium.

Employing Scheme F1 as the fast process, the following two schemes (Schemes S1 and S2) are possible minimal ones that satisfy the above requirements.



(Scheme S1)



(Scheme S2)

(1) *Scheme S1.* The rate equations for the slow process are derived according to Scheme S1 as follows:

$$\frac{\mathrm{d}([\text{Noncolored form-H}]+[\text{Noncolored form}])}{\mathrm{d}t}$$

$$= k_2[\text{Colored form-H}]+k_{-3}[\text{Transient form}]$$

$$-(k_{-2}[\text{Noncolored form-H}]+k_3[\text{Noncolored form}]) \tag{a14}$$

$$\alpha_3\,[\text{Noncolored form}] \equiv [\text{Noncolored form-H}]+[\text{Noncolored form}]$$

$$= Ac^{\circ}(1-\mathrm{e}^{-k_s t}) \tag{a15}$$

$$k_s = \frac{1}{\alpha_1\,\alpha_2\,k_f}\cdot\left(k_2\,k_{-1}\,\frac{[\mathrm{H}^+]}{K_2}+k_1\,k_{-3}\right)+\frac{1}{\alpha_3}\left(k_{-2}\,\frac{[\mathrm{H}^+]}{K_3}+k_3\right) \tag{a16}$$

where k_s represents the rate constant of the slow process and c° represents the total concentration of cytochrome c, and

$$\alpha_3 = 1+\frac{[\mathrm{H}^+]}{K_3} \tag{a17}$$

$$A = \frac{1}{k_s\,k_f\,\alpha_1\,\alpha_2}\cdot\left(k_2\,k_{-1}\,\frac{[\mathrm{H}^+]}{K_2}+k_1\,k_{-3}\right) \tag{a18}$$

The concentration of the colored, the transient and the noncolored forms ($\overline{\mathrm{C}}$(C), $\overline{\mathrm{C}}$(T) and $\overline{\mathrm{C}}$(NC), respectively) in equilibrium are derived from Eqns. a10, a11 and a15 assuming t to be infinity in Eqn. a15, as follows:

$$\overline{\mathrm{C}}(\mathrm{C}) = \frac{k_{-1}[\mathrm{H}^+]c^0}{k_s\,k_f\,\alpha_2\,\alpha_3\,K_2}\cdot\left(k_{-2}\,\frac{[\mathrm{H}^+]}{K_3}+k_3\right) \tag{a19}$$

$$\overline{C}(T) = \frac{k_1 c^0}{k_s k_f \alpha_1 \alpha_3} \cdot \left(k_{-2} \frac{[H^+]}{K_3} + k_3 \right) \tag{a20}$$

$$\overline{C}(NC) = \frac{c^0}{k_s k_f \alpha_1 \alpha_2} \cdot \left(k_2 k_{-1} \frac{[H^+]}{K_2} + k_1 k_{-3} \right) \tag{a21}$$

The rate constants and the dissociation constants in the scheme must satisfy a requirement that the free energy changes should be equal for the paths

Colored form-H $\rightleftharpoons$ Noncolored form-H $\rightleftharpoons$ Noncolored form$+H^+$

and

Colored form-H $\rightleftharpoons$ Transient form-H $\rightleftharpoons$ Transient form$+H^+$ $\rightleftharpoons$ Noncolored form $+H^+$

which can be represented as:

$$\frac{k_2}{k_{-2}} \cdot K_3 = \frac{k_1}{k_{-1}} \cdot K_2 \cdot \frac{k_{-3}}{k_3} \tag{a22}$$

The constants were evaluated by a least square method with Eqn. a22, minimizing S_2;

$$S_2 = \sum (\log k_{\text{app (slow)}} - \log k_s)^2 \tag{a23}$$

where $k_{\text{app(slow)}}$ designates the rate constant of the slow process observed in the experiment. Summation in Eqn. a23 was performed for all the experimental values through the pH range from 8.5 to 11.4. The calculation was performed in the following procedure:

(a) expanding k_s in a power series with respect to $1/[H^+]$,

$$k_s = k_{-2} + \{K_1 k_2 + K_3 (k_3 - k_{-2})\}/[H^+] + \ldots \tag{a24}$$

The observed rate constants by the slow process were plotted as a function of $1/[H^+]$ in Fig. 9. The value of k_{-2} can be obtained from the vertical intercept of the straight line in the figure, to be

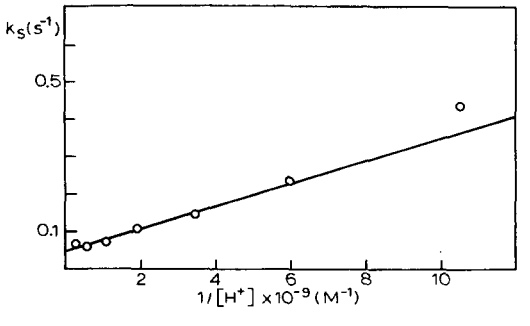


Fig. 9. Plot of the observed rate constant k_s for the slow process upon pH jump (up) vs. $1/[H^+]$, at ionic strength = 0.5. The vertical intercept, k_{-2} (Eqn. a24), was determined by a least squares method, using the data between pH 8.5 and pH 10.

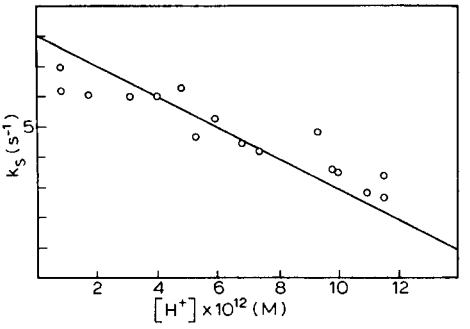


Fig. 10. Plot of the observed rate constant for the slow process upon pH jump (up) vs. $[H^+]$, at ionic strength = 0.5. The vertical intercept, which corresponds to first term of Eqn. a26, was obtained by a least squares method using the data between pH 11 and pH 11.5.

$$k_{-2} = 0.05\ s^{-1} \tag{a25}$$

(b) Expanding k_s in a power series with respect to $[H^+]$, we have

$$k_s = (k_3+k_{-3}) + \left[\frac{1}{K_2}\left\{\frac{k_{-1}}{k_1}(k_2-k_{-3})-k_{-3}\right\} + \frac{1}{K_3}(k_{-2}-k_3)\right]\cdot[H^+] + \ldots \tag{a26}$$

The observed rate constants were plotted as a function of $[H^+]$ in Fig. 10, from which the sum of the rate constants k_3+k_{-3} was estimated to be

$$k_3+k_{-3} = 8.0\ s^{-1} \tag{a27}$$

(c) The apparent equilibrium constant, K_{app}, between the colored and the noncolored form can be defined by

$$K_{app} = \frac{[\text{Noncolored form}][H^+]}{[\text{Colored form}]} \tag{a28}$$

Using Eqns. a19 and a21, we have:

$$K_{app} = \frac{\alpha_3(k_{-1}k_2+k_1k_{-3}K_2/[H^+])\cdot[H^+]}{\alpha_1 k_{-1}(k_3+k_{-2}\cdot[H^+]/K_3)} \tag{a29}$$

The value of pK_{app} was estimated to be 9.26 from the difference spectra in equilibrium (Fig. 6).

The rate and the dissociation constants were obtained by using least square method with Eqns. a22, a25, a27 and a29 are summarized in Table II.

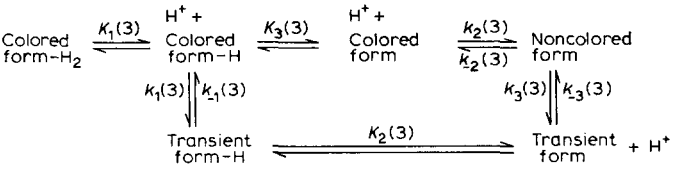
(2) Scheme S2. The rate constant k_s derived from scheme S2 becomes;

$$k_s(2) = (k_{-2}(2)+k_3(2))+(k_2(2)k_{-1}+k_{-3}(2)k_1)\cdot[H^+]/(K_2\alpha_1\alpha_2k_f) \tag{a30}$$

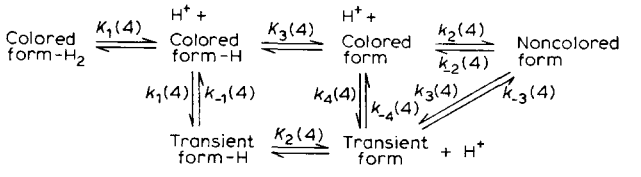
where α_1, α_2, k_f have been already defined in Eqns. A5–A7. This equation predicts that the value of $k_{s(2)}$ should converge to the same value $(k_{-2}(2)+k_3(2))$, as $[H^+]$ approaches infinity (acidic pH) and zero (alkaline pH). This is obviously inconsistent with the experimental results (see Fig. 2). Scheme S2 can then be rejected.

Next, as a little more complicated scheme for the fast process, two possible

schemes were examined (Schemes S3 and S4 shown below). However, both of them can be rejected for the following reasons.



(Scheme S3)



(Scheme S4)

(*3*) *Scheme S3.* This would require that the fast rate constant converge to zero when $[H^+]$ approaches zero. This is inconsistent with the experimental data (Fig. 2).

(*4*) *Scheme S4.* This would lead to the following equation for k_s:

$$k_s(4) = k_{-2}(4)+k_3(4)+\{k_2(4)\cdot(k_{-1}(4)\cdot[H^+]/K_2(4)+k_{-4}(4))+k_{-3}(4)\cdot(k_1(4)[H^+]/K_3(4)+k_4(4))\}/(k_f(4)\cdot\alpha_1(4)\cdot\alpha_2(4)) \quad (a31)$$

where

$$k_f(4) = (k_1(4)\cdot[H^+]/K_3(4)+k_4(4))/\alpha_1(4)+(k_{-1}(4)\cdot[H^+]/K_2(4)+k_{-4}(4))/\alpha_2(4) \quad (a32)$$

$$\alpha_1(4) = 1+\frac{[H^+]}{K_3(4)}\cdot\left(1+\frac{[H^+]}{K_1(4)}\right) \quad (a33)$$

$$\alpha_2(4) = 1+\frac{[H^+]}{K_2(4)} \quad (a34)$$

Expanding $k_s(4)$ in a power series with respect to $1/[H^+]$, the coefficient of $1/[H^+]$ becomes zero, which indicates that the tangent of $k_s(4)$ vs. $1/[H^+]$ converges to zero as $[H^+]$ increases. This contradicts the experimental result shown in Fig. 8.

In conclusion, only Scheme S1 is acceptable as a minimal probable scheme among several schemes examined above.

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