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KINETIC STUDIES ON REDOX REACTIONS OF HEMOPROTEINS

I. REDUCTION OF THERMORESISTANT CYTOCHROME *c*-552 AND HORSE HEART CYTOCHROME *c* BY FERROCYANIDE

HIROSHI KIHARA[a], HIROSHI NAKATANI[b], KEITARO HIROMI[b], KOYU HON-NAMI[c] and TAIRO OSHIMA[c]

[a]*Department of Physics, Jichi Medical School, Yakushiji, Tochigi,* [b]*Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, Kyoto, and* [c]*Mitsubishi-kasei Institute of Life Sciences, Machida, Tokyo (Japan)*

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SUMMARY

The oxidation-reduction reaction of horse heart cytochrome *c* and cytochrome *c* (552, *Thermus thermophilus*), which is highly thermoresistant, was studied by temperature-jump method. Ferrohexacyanide was used as reductant.

$$\text{cytochrome } c^{3+} + Fe(CN)_6^{4-} \underset{k_{ox}}{\overset{k_{red}}{\rightleftharpoons}} \text{cytochrome } c^{2+} + Fe(CN)_6^{3-}$$

Thermodynamic and activation parameters of the reaction obtained for both cytochromes were compared with each other. The results of this showed that (1) the redox potential of cytochrome *c*-552, +0.19 V, is markedly less than that of horse heart cytochrome *c*. (2) $\Delta H_{ox}^{\ddagger}$ of cytochrome *c*-552 is considerably lower than that of horse heart cytochrome *c*. (3) $\Delta S_{ox}^{\ddagger}$ and $\Delta S_{red}^{\ddagger}$ of cytochrome *c*-552 are more negative than those of horse heart cytochrome *c*. (4) k_{red} of cytochrome *c*-552 is much lower than that of horse heart cytochrome *c* at room temperature.

INTRODUCTION

Cytochrome *c*-552 was first extracted by Hon-nami and Oshima [1] from *Thermus thermophilus* HB8, which grew in very hot springs with an optimum temperature of 75 °C [2, 3]. Cytochrome *c*-552 itself is also highly thermoresistant. It has an absorption peak at 408 nm in the oxidized form, and peaks at 552, 522 and 417 nm in the reduced form, as shown in Fig. 1. Its molecular weight is about 15 000 and its isoelectric point is 10.8 [1]. It contains one heme per one protein. The 690 nm band of the oxidized form, which is believed to be characteristic of the sulfur ligation at the sixth coordinate site of the heme iron atom in C-type cytochromes [4], remains up to 80 °C (Hon-nami and Oshima, unpublished observation). The 690 nm band of the oxidized form also does not change between pH 5 and 12. The ligand exchange oc-

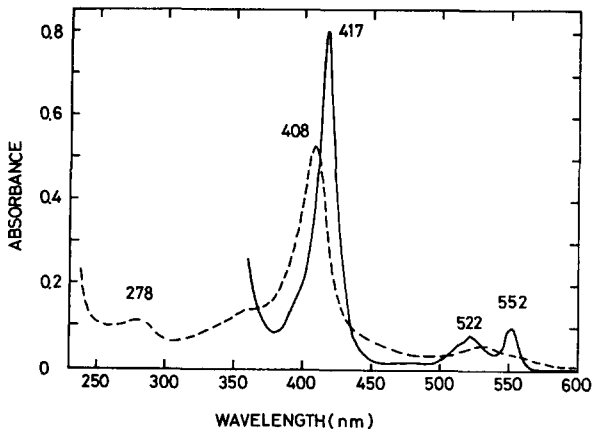


Fig. 1. Absorption spectra of cytochrome *c*-552 at pH 7 in the oxidized (– – –) and reduced (—) state.

curring in horse heart cytochrome *c* with p*K* 9.3 is not found below pH 12 (Kihara et al., unpublished observations).

The purpose of this work is to investigate the relationship between structure and function, by comparing the temperature dependence of the equilibrium and rate constants of the redox reaction of cytochrome *c*-552, the reduction by ferrocyanide. with that of horse heart cytochrome *c*.

EXPERIMENTAL

Materials

Preparation of cytochrome c-552. Cytochrome *c*-552 was extracted from *T. thermophilus* HB8 (ATCC 27634). The cells of the bacteria were grown at 75 °C with vigorous aeration and mechanical agitation. They were collected at late log phase [2].

The collected cells (500 g wet wt.) were ground using a mechanical motor with aluminum oxide, and mixed with 1 l of 0.05 M Tris · HCl buffer, pH 7.8, containing 1 mM EDTA, 10 mM $MgCl_2$, 135 mM KCl and 7 mM β-mercaptoethanol. The resultant slurry was centrifuged to separate the extract from alumina and cell debris. The supernatant solution was centrifuged at 22 300 $\times$ *g* for 15 h to yield a clear brown supernatant solution. The supernatant was dialyzed against 0.01 M phosphate buffer, pH 7.0, containing 1 mM EDTA and 7 mM β-mercaptoethanol.

The dialysate was adsorbed on Amberlite CG-50 resin (Rohm and Haas, Type II). The cytochrome adsorbed was eluted with the phosphate buffer with 1 M NaCl. The colored eluate was dialyzed against the phosphate buffer and centrifuged to remove the precipitates.

The supernatant solution was adsorbed onto a column (3.5 $\times$ 25 cm) of Amberlite CG-50 resin. The chromatogram was developed with a linear gradient between 1 l of the phosphate buffer and 1 l of the phosphate buffer with 1 M NaCl.

The fractions containing the cytochrome were pooled, concentrated, and then passed on a column of Sephadex G-75. Protein was eluted with the phosphate buffer and the hemocolored zone was pooled. The purified cytochrome gave a single band

on disc-gel electrophoresis under acidic condition. The absorbance ratio (408/280 nm) was 4.7.

Preparation of horse cytochrome c. Horse heart cytochrome *c* was purchased from Sigma (Type VI), and purified by the same method as mentioned in a previous paper [5].

Physicochemical measurements. The buffer solutions of pH 7.0, used for spectrophotometric and temperature-jump measurements, contained 0.02 M potassium phosphate, 0.001 M EDTA and potassium nitrate, the concentration of which was 0.05 M for spectrophotometric measurements and changed to make ionic strength constant at 0.18 for temperature-jump measurements. The concentration of horse heart cytochrome *c* and cytochrome *c*-552 in the reaction mixture were $2 \cdot 10^{-5}$ M and $2.3 \cdot 10^{-5}$ M, respectively, whereas that of ferrocyanide was varied. Ferrocyanide was dissolved just before the experiment under a nitrogen atmosphere and was kept in the dark throughout the experiment.

Temperature-jump experiments were performed with a RA-1200 T-jump spectrophotometer (Union Giken) with a cell specially designed for high temperature anaerobic measurements. The cell was maintained at a constant temperature within 0.01 °C. Spectrophotometric measurements were carried out with Thunberg cells on a recording spectrophotometer, Cary Model 17.

Methods

Redox reactions in equilibrium. The reaction for the electron transfer between ferricytochrome and ferrohexacyanide may be written as

$$C^{III} + Fe^{II} \underset{k_{ox}}{\overset{k_{red}}{\rightleftharpoons}} C^{II} + Fe^{III} \qquad \text{Scheme 1}$$

where C^{III} and C^{II} represent ferric and ferrous cytochrome *c* respectively, and Fe^{III} and Fe^{II} represent ferri- and ferrohexacyanide.

For the system, the over-all equilibrium constant may be defined by:

$$K_{eq} = \frac{\overline{C^{III}} \cdot \overline{Fe^{II}}}{\overline{C^{II}} \cdot \overline{Fe^{III}}} \qquad (1)$$

A line over the symbol denotes equilibrium concentration. In our experiments, a large excess of ferrohexacyanide was initially mixed with ferricytochrome, so that the relationship $\overline{Fe^{III}} = \overline{C^{II}}$ always holds. Thus Eqn. 1 may be rewritten to a good approximation, as:

$$K_{eq} = \frac{(C_0^{III} - \overline{C^{II}})Fe_0^{II}}{\overline{C^{II}}^2} \qquad (2)$$

where the subscript zero denotes initial concentration. $\overline{C^{II}}$ is given by

$$\overline{C^{II}} = \frac{\Delta A_\lambda}{\Delta \varepsilon_\lambda} \qquad (3)$$

where λ is the wavelength (550 nm for horse heart cytochrome *c* and 552 nm for cytochrome *c* 552), ΔA_λ is the absorbance difference between the oxidized and reduced

state at λ, and $\Delta\varepsilon_\lambda$ is the millimolar absorptivity difference between the oxidized and reduced cytochromes at λ. A value of 19.0 and 13.0 for $\Delta\varepsilon_\lambda$ ($mM^{-1} \cdot cm^{-1}$) of horse heart cytochrome *c* and cytochrome *c*-552, were adopted, respectively.

Redox reactions in kinetics. Under the conditions of the experiments with $Fe_0^{II} \gg C_0^{III}$, the expression for the relaxation time τ for the reaction $C^{III} + Fe^{II} \leftrightarrows C^{II} + Fe^{III}$ becomes:

$$\tau^{-1} = k_{app} = k_{red}Fe_0^{II} + 2k_{ox}\overline{C^{II}}$$
$$= k_{red}Fe_0^{II}\{1 + 2K_{eq}\overline{C^{II}}(Fe_0^{II})^{-1}\} \quad (4)$$

since

$$K_{eq} = k_{ox}/k_{red} \quad (5)$$

k_{red} can be expressed as

$$k_{red} = \kappa \frac{kT}{h} \exp(-\Delta G_{red}^{\ddagger}/RT) \quad (6)$$

according to the theory of the rate processes [6] where κ is the transmission coefficient and assumed to be equal to unity hereafter in the present analysis, R is the gas constant, k is Boltzmann's constant and h is Planck's constant. Taking the logarithm of k_{red}/T and expressing $\Delta G_{red}^{\ddagger}$ by the activation enthalpy $\Delta H_{red}^{\ddagger}$ and the activation entropy $\Delta S_{red}^{\ddagger}$, Eqn, 7 holds:

$$\log_{10}(k_{red}/T) = -\frac{\log_{10} e}{R}\left(\frac{\Delta H_{red}^{\ddagger}}{T} - \Delta S_{red}^{\ddagger}\right) + \log_{10}\frac{k}{h} \quad (7)$$

where e is the base of natural logarithms. k_{ox} can be derived from Eqn. 5 and k_{red}. The same equation as Eqn. 7 can be derived for k_{ox}.

$$\log_{10}(k_{ox}/T) = -\frac{\log_{10} e}{R}\left(\frac{\Delta H_{ox}^{\ddagger}}{T} - \Delta S_{ox}^{\ddagger}\right) + \log_{10}\frac{k}{h} \quad (8)$$

RESULTS

Spectrophotometric titrations

K_{eq} was measured in the temperature range between 19 and 33 °C for horse heart cytochrome *c* and between 25 and 75 °C for cytochrome *c*-552, their van't Hoff plots being shown in Fig. 2. This indicates that, in both cases, log K_{eq} is linear to $1/T$ (T, absolute temperature) over the temperature range investigated.

The enthalpy change, ΔH_{eq}, and the standard entropy change, ΔS_{eq} at 25 °C for the reduction of C^{III} were calculated from the straight lines obtained by the least squares method in Fig. 2, and are shown in Table I. The enthalpy change of cytochrome *c*-552 was found to be rather larger than that of horse heart cytochrome *c*. The standard free energy change, ΔG_{eq}, for the two cytochromes is plotted in Fig. 3, from which the ΔG_{eq} of cytochrome *c*-552 is seen to be much more negative than that of horse heart cytochrome *c* in the temperature range investigated. The result suggests that,

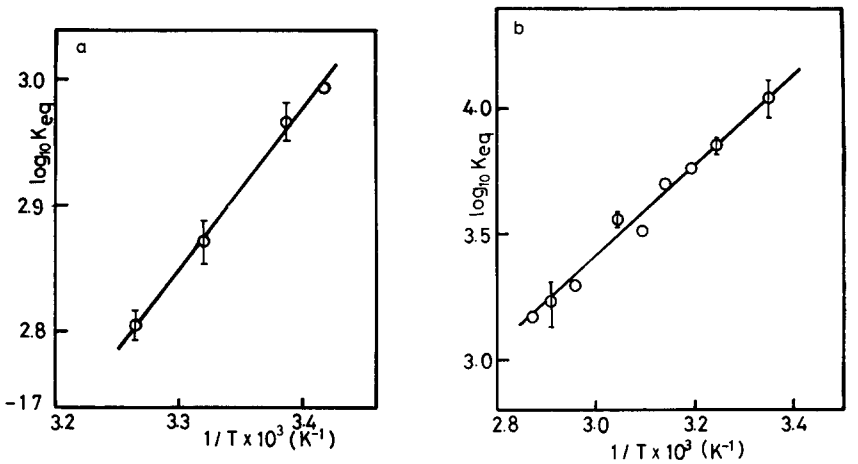


Fig. 2. The equilibrium constants of the redox reaction between C^{III} and ferrocyanide vs. the reciprocal absolute temperature, $1/T$, at pH 7. The concentration of ferrocyanide was changed from 8 to 16 mM. (a) C^{III}, horse heart cytochrome *c*; (b) C^{III}, cytochrome *c*-552. The straight lines were obtained by least squares method.

TABLE I

THE THERMODYNAMIC PARAMETERS OBTAINED FROM THE REDOX REACTION BETWEEN FERRICYTOCHROMES AND FERROCYANIDE

Parameter values are expressed in kJ/mol for ΔH and J/mol · K for ΔS.

	Horse heart cytochrome *c*	Cytochrome *c* 552
ΔH_{eq}	−24.8	− 34.4
$\Delta H_{ox}^{\ddagger}$	20.8	0.65
$\Delta H_{red}^{\ddagger}$	45.6	35.0
ΔS_{eq} *	−27.4	− 37.9
$\Delta S_{ox}^{\ddagger}$ *	−42.6	−108.8
$\Delta S_{red}^{\ddagger}$ *	−15.2	− 70.9

* Calculated at 300 K.

compared with horse heart cytochrome *c*, the oxidized state of cytochrome *c*-552 is thermodynamically much more stable than the reduced state. The redox potential of cytochrome *c*-552 was calculated to be 0.19 V at 25 °C.

Temperature jump experiments

The experiments were performed within about the same temperature range as those used to measure the equilibrium constants. In Fig. 4, a typical relaxation curve, obtained from the temperature-jump experiments was found to be a single exponential decay. In Fig. 5, the dependence of the apparent rate constants on the concentration of ferrocyanide is shown. The value of k_{app}/Fe_0^{II} (the ordinate) is expected to be linear to the value of $\overline{C^{II}}/Fe_0^{II}$ (the abscissa) according to Eqn. 4, which would

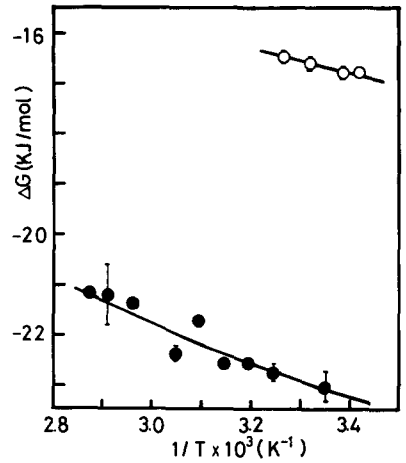
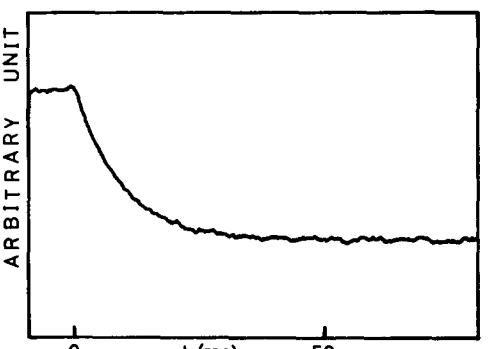


Fig. 3. Standard free energy change for the reduction of C^{III} vs. the reciprocal absolute temperature, $1/T$. (pH 7). Horse heart cytochrome *c*, ○; cytochrome *c*-552, ●. The solid lines were obtained using the thermodynamic parameters ΔH_{eq} and ΔS_{eq} shown in Table I.



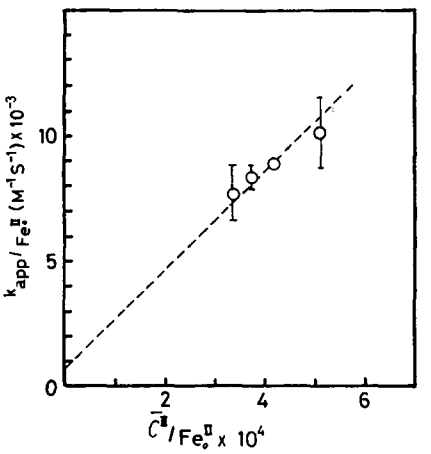


Fig. 4. Typical temperature-jump trace of transmittance at 552.5 nm with respect to time. Temperature (final), 30 °C. Ferric cytochrome *c*-552, $2.37 \cdot 10^{-5}$ M; ionic strength, 0.18.

Fig. 5. Dependence of the apparent rate constant on the concentration of the ferrocyanide in the case of cytochrome *c*-552 at 20 °C. Cytochrome *c*-552, $2.3 \cdot 10^{-5}$M; ferrocyanide, 5 to 13 mM; wavelength, 552.5 nm; pH 7; ionic strength, 0.18, k_{obs}/Fe_0^{II} of the ordinate is linear to the C^{II}/Fe_0^{II} of the abscissa, according to Eqn. 4. The explanation of the dashed line is given in the text.

be the case in the present experiment with less than 13 mM of ferrocyanide within the experimental error. Thus the analysis was performed on the basis of Scheme 1. The observed rate constants are summarized in Fig. 6, the concentration of ferrocyanide being variable. $\mathrm{Log}_{10}\ (k_{red}/T)$ and $\log_{10}\ (k_{ox}/T)$ calculated from these data and Eqns. 4 and 5 are plotted in Fig. 7, showing them to be linear to $1/T$. The calcu-

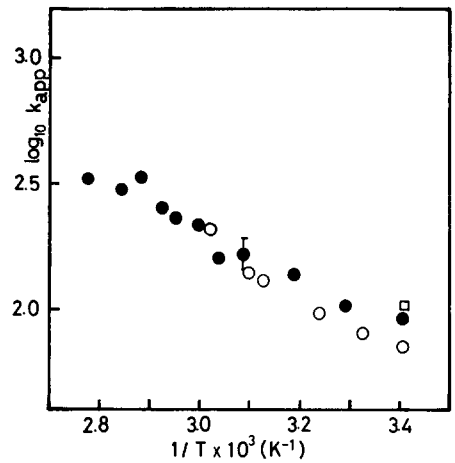


Fig. 6. The apparent rate constants obtained from temperature-jump of the redox reaction between ferricytochrome *c*-552 and ferrocyanide vs. the reciprocal absolute temperature, $1/T$. Wavelength, 552.5 nm; ionic strength, 0.18; pH 7; cytochrome *c* 552, $2.3 \cdot 10^{-5}$ M; ferrocyanide: ○, 0.008 M; ●, 0.01 M; □, 0.012 M.

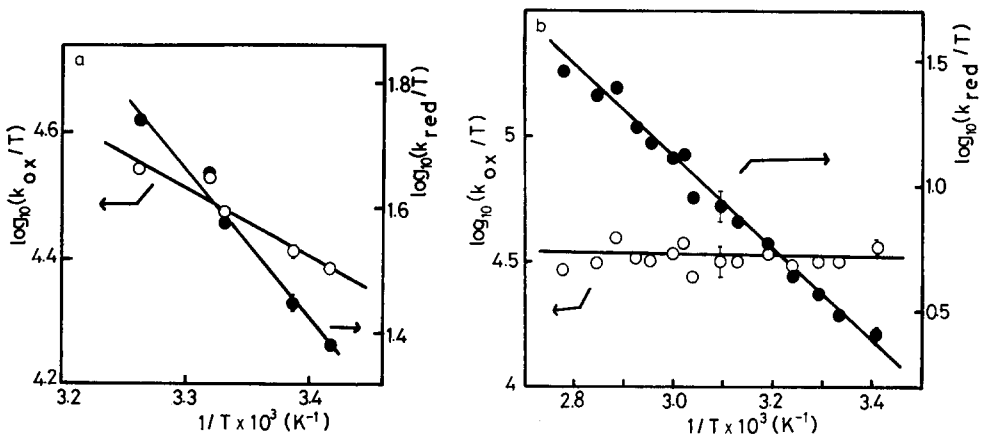
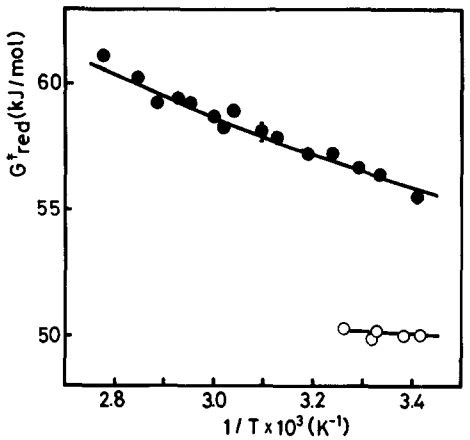


Fig. 7. log (k_{ox}/T), (○–○) and log (k_{red}/T), (●–●) vs. the reciprocal of the absolute temperature, calculated by Eqns. 4 and 5. T, absolute temperature. The straight lines were obtained by least squares method. (a), horse heart cytochrome *c*; (b), cytochrome *c*-552.

lated values of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are shown in Table I. This shows: (1) $\Delta H_{ox}^{\ddagger}$ of cytochrome *c* 552 is much less than that of horse heart cytochrome *c*. (2) Both $\Delta S_{ox}^{\ddagger}$ and $\Delta S_{red}^{\ddagger}$ of cytochrome *c*-552 are considerably more negative than those of horse heart cytochrome *c*. The activation free energy $\Delta G_{ox}^{\ddagger}$ and $\Delta G_{red}^{\ddagger}$ are shown in Figs. 8 and 9. $\Delta G_{red}^{\ddagger}$ of cytochrome *c*-552 is found to be much larger than that of horse heart cytochrome *c*, whereas $\Delta G_{ox}^{\ddagger}$ of both cytochromes at the same temperature are of the same order.



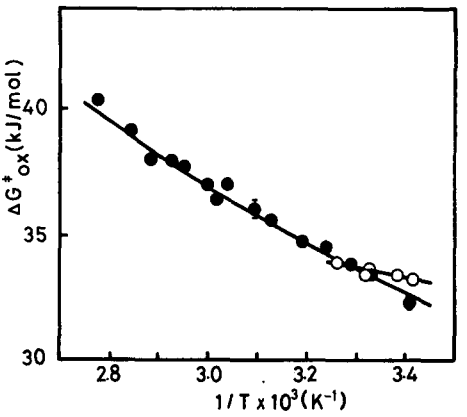


Fig. 8. Activation free energy for the reduction of ferricytochromes vs. the reciprocal of the absolute temperature. The solid lines were calculated using the activation parameters $\Delta H_{red}^{\ddagger}$ and $\Delta S_{red}^{\ddagger}$ in Table I. ○, horse heart cytochrome *c*; ●, cytochrome *c*-552.

Fig. 9. Activation free energy for the oxidation of ferrocytochromes vs. the reciprocal of the absolute temperature. The solid lines were calculated using the activation parameters $\Delta H_{ox}^{\ddagger}$ and $\Delta S_{ox}^{\ddagger}$ in Table I. ○, horse heart cytochrome *c*; ●, cytochrome *c*-552.

DISCUSSION

The redox reaction between horse heart ferricytochrome *c* and ferrohexacyanide have already been studied by several workers [7–14]. The rate constants reported so far are summarized in Table II, which includes the data obtained in the present study. The agreement of the data for horse heart cytochrome *c* is satisfactory, considering the difference in the experimental conditions. In contrast, k_{red} of cytochrome *c*-552 is much smaller than the corresponding values of horse heart cytochrome *c*.

TABLE II

RATE CONSTANT VALUES GIVEN IN THE LITERATURE

	Temperature (°C)	pH	Ionic strength	k_{ox} ($M^{-1} \cdot s^{-1}$)	k_{red} ($M^{-1} \cdot s^{-1}$)	Reference
Horse heart cytochrome *c*	22	7	0.18	$8.7 \cdot 10^6$	$2.4 \cdot 10^4$	7
	12	7	0.17	$1.23 \cdot 10^7$	$1.56 \cdot 10^4$	8
	25	6	0.1	$1.6 \cdot 10^7$		9
	24	7		$8.3 \cdot 10^6$		10
	20	7	0.4		$1.7 \cdot 10^4$	11
	17	7	0.18	$6.7 \cdot 10^6$		12
	25	7	0.1	$8.0 \cdot 10^6$	$3.4 \cdot 10^4$	13
	25	7	0.0894	$6.52 \cdot 10^6$	$2.39 \cdot 10^4$	14
	27	7	0.18	$8.84 \cdot 10^6$	$1.14 \cdot 10^4$	this work
cytochrome *c*-552	27	7	0.18	$9.53 \cdot 10^6$	$9.30 \cdot 10^2$	this work

TABLE III

RATE CONSTANTS CALCULATED ACCORDING TO SCHEME 2

k_1	$1.7 \times 10^4\ M^{-1}s^{-1}$ [a]	k_{-1}	$7\ s^{-1}$ [a]
k_2	$1.5 \times 10^3\ s^{-1}$ [a] $208\ s^{-1}$ [b]	k_{-2}	$2.1 \times 10^4\ s^{-1}$ [b]
k_3	$132\ s^{-1}$ [a]	k_{-3}	$8.7 \times 10^6\ M^{-1}s^{-1}$ [c]

[a] Miller and Cusanovich [11].
[b] Stellwagen and Shuhman [10].
[c] Brandt et al. [7].

Stellwagen and Shulman [10] investigated the rates of the same redox reaction system of horse heart cytochrome *c* by nuclear magnetic resonance, and proposed a reaction scheme, involving two intermediate complexes: $C^{III} \cdot Fe^{II}$ and $C^{II} \cdot Fe^{III}$, follows:

$$C^{III} + Fe^{II} \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} C^{III} \cdot Fe^{II} \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} C^{II} \cdot Fe^{III} \underset{k_{-3}}{\overset{k_3}{\rightleftarrows}} C^{II} + Fe^{III} \qquad \text{Scheme 2}$$

Miller and Cusanovich [11] also investigated the reaction by stopped-flow method, and estimated rate constants on the basis of the above scheme proposed by Stellwagen and Shulman. The values of rate constants obtained by these authors are summarized in Table III. They suggest that the interconversion between $C^{III} \cdot Fe^{II}$ and $C^{II} \cdot Fe^{III}$ will be rate-limiting at a high concentration of ferrocyanide. On the other hand, when the concentration of ferrocyanide is not very high, as in the present case, the binding of ferrocyanide to ferricytochrome and ferricyanide to ferrocytochrome will be rate-limiting and Scheme 2 reduces to Scheme 1. Hence the parameter k_{red} and k_{ox} in Scheme 1 correspond to k_1 and $(k_{-1})\,(k_{-2}/k_2)\,(k_{-3}/k_3)$ in Scheme 2, respectively. Based on Scheme 2, our result may be restated more emphatically, i.e. that k_1 of cytochrome *c*-552 differs appreciably from that of cytochrome *c*, whereas the values of $(k_{-1})\,(k_{-2}/k_2)\,(k_{-3}/k_3)$ are of the same order as each other, indicating that each of these rate constants is of the same order. Since the value of k_1 represents the rate constant for the binding of the reductant with the binding site of the protein, the difference in k_1 values may reflect the difference in the structure of the binding site for the reductant.

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