Biochimica et Biophysica Acta, 634 (1981) 93—104

BBA 47983

COMPARISON OF THE REDOX REACTIONS OF VARIOUS TYPES OF CYTOCHROME *c* WITH IRON HEXACYANIDES

HIROSHI KIHARA

Jichi Medical School, Department of Physics, Yakushiji 3311-1, Tochigi 329-04 (Japan)

(Received February 11th, 1980)
(Revised manuscript received September 12th, 1980)

Key words: Cytochrome c; Redox reaction; Iron hexacyanide; Temperature jump; Electron transfer

Summary

The dynamic behavior of various types of cytochromes *c* in the redox reaction with iron hexacyanides was studied using a temperature-jump method in order to elucidate the molecular mechanism of the redox reaction of cytochromes with their oxidoreductants.

Transmittance after the temperature jump changed through a single exponential decay for all cytochromes investigated.

Under a constant concentration of anion, the redox reaction of various types of cytochrome *c* with iron hexacyanides was analyzed according to the scheme:

$$\underset{}{\mathrm{C(III) + Fe(II)}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{step A}}{\mathrm{C(III)\cdot Fe(II)}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{step B}}{\mathrm{C(II)\cdot Fe(III)}} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{\text{step C}}{\mathrm{C(II) + Fe(III)}}$$

$K_i = k_i/k_{-i}$ $(i = 1, 2, 3)$

where C(III) and C(II) are ferric and ferrous cytochromes, respectively, Fe(III) and Fe(II) are ferri- and ferrocyanides, respectively, C(III) · Fe(II) is the ferricytochrome-ferrocyanide complex and C(II) · Fe(III) is the ferrocytochrome-ferricyanide complex. When step B is slower than the other two steps A and C, τ^{-1} can be represented approximately as

$$\tau^{-1} = \frac{k_2 K_1([\overline{\mathrm{Fe}}(\mathrm{II})] + [\overline{\mathrm{C}}(\mathrm{III})])}{1 + K_1([\overline{\mathrm{Fe}}(\mathrm{II})] + [\overline{\mathrm{C}}(\mathrm{III})])} + \frac{k_{-2}([\overline{\mathrm{Fe}}(\mathrm{III})] + [\overline{\mathrm{C}}(\mathrm{II})])}{K_3 + ([\overline{\mathrm{Fe}}(\mathrm{III})] + [\overline{\mathrm{C}}(\mathrm{II})])}$$

where the bar over the variables denotes the equilibrium value. In a large excess

of ferrocyanide against cytochrome, we can estimate k_2, k_{-2}, K_1 and K_3 independently. In the case of horse cytochrome *c* at 18°C in 0.1 M phosphate buffer at pH 7 with 0.3 M KNO_3, the estimated parameters are $k_2 = 100 \pm 50\ s^{-1}$, $k_{-2} = (3.5 \pm 1.0) \cdot 10^3\ s^{-1}$, $K_1 = 15 \pm 7\ M^{-1}$ and $K_3 = (8.5 \pm 1.5) \cdot 10^{-4}$ M. From the same experiments for seven cytochromes (cytochrome *c* from horse, tuna, *Candida krusei*, *Saccharomyces oviformis*, *Rhodospirillum rubrum* cytochrome c_2, *Spirulina platensis* cytochrome *c*-554 and *Thermus thermophilus* cytochrome *c*-552), the following results can be deduced. (1) Each parameter defined in the scheme above (k_2, k_{-2}, K_1, K_3) diverged beyond the error range. Above all, k_2 values of cytochromes *c*-554 and *c*-552 are as large as $1 \cdot 10^4\ s^{-1}$ and much larger than those for the other cytochromes (to 50 approx. $700\ S^{-1}$). (2) The variance of k_2K_1 and k_{-2}/K_3 are relatively less than the variances of individual parameters (k_2, k_{-2}, K_1 and K_3), which suggests that the values of k_2K_1 and k_{-2}/K_3 have been conserved during the course of evolution.

Introduction

In the present study, the redox reaction of various types of cytochrome *c* was studied using a temperature-jump method in order to elucidate the molecular mechanism of the redox reaction of cytochromes with their oxidoreductants. Iron hexacyanides were used as oxidoreductants, because (1) the reaction is reversible and (2) they are metal complexes that may be considered as the models of the physiological oxidoreductant (cytochrome oxidase and reductase). The reaction of cytochrome *c* with iron hexacyanides has been investigated by many workers [1—22]. However, no one has succeeded in obtaining the rate constants of the internal electron transfer directly. In the present article, a method for calculating the rate constants of the interconversion between two intermediate complexes (ferricytochrome-ferrocyanide and ferrocytochrome-ferricyanide complexes) is given, and according to this calculation method, the redox reaction of various types of cytochrome *c* with iron hexacyanides was studied.

Theoretical

The analyses were done on the basis of Scheme I defined by Stellwagen and Shulman [7];

$$\underset{}{\mathrm{C(III) + Fe(II)}} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{step A}}{\mathrm{C(III) \cdot Fe(II)}} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \underset{\text{step B}}{\mathrm{C(II) \cdot Fe(III)}} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} \underset{\text{step C}}{\mathrm{C(II) + Fe(III)}} \qquad \text{(I)}$$

where C(III), C(II), Fe(III) and Fe(II) designate ferric and ferrous cytochromes, ferricyanide and ferrocyanide, respectively, and C(III) · Fe(II) and C(II) · Fe(III) represent the intermediates of ferricytochrome-ferrocyanide and ferrocytochrome-ferricyanide complexes, respectively. τ^{-1} can be obtained for two extreme cases as follows; (i) k_2, $k_{-2} >> k_1$ ([C(III)] + [Fe(II)]), k_{-1}, k_3, k_{-3} ([C(II)] + [Fe(III)]).

The reciprocal relaxation time, τ^{-1}, can be calculated according to a simpler

scheme (Scheme II)

$$C(III) + Fe(II) \underset{k_{ox}}{\overset{k_{red}}{\rightleftharpoons}} C(II) + Fe(III) \qquad (II)$$

When a large excess of ferrocyanide is mixed with cytochrome, Eqn. 1 holds [23].

$$\frac{\tau^{-1}}{[Fe(II)]_0} = k_{red} + k_{ox} \cdot \frac{([Fe(III)]_0 + 2[\overline{C}(II)] - [C(II)]_0)}{[Fe(II)]_0} \qquad (1)$$

where subscript zero denotes the initial value before reaction. Hence, the values of k_{red} and k_{ox} can be determined from the intercept and slope of the plots of the experimental data, respectively, by changing the concentration of ferrocyanide.

The parameters k_{red} and k_{ox} are related to those in Scheme I as,

$$k_{red} = k_1/(1 + K_4) \qquad (2)$$

$$k_{ox} = K_4 k_{-3}/(1 + K_4) \qquad (3)$$

where

$$K_4 = k_{-2} \cdot k_{-1}/k_2 \cdot k_3 \qquad (4)$$

(ii) $k_2, k_{-2} << k_1$ ([C(III)] + [Fe(II)]), k_{-1}, k_3, k_{-3} ([Fe(III)] + [C(II)]).

In this case, the reciprocal relaxation time, τ^{-1}, can be calculated as

$$\tau^{-1} = \frac{k_2 K_1([\overline{Fe}(II)] + [\overline{C}(III)])}{1 + K_1([\overline{Fe}(II)] + [\overline{C}(III)])} + \frac{k_{-2}([\overline{Fe}(III)] + [\overline{C}(II)])}{K_3 + ([\overline{Fe}(III)] + [\overline{C}(II)])} \qquad (5)$$

where a bar over the parameter indicates the value at equilibrium [23]. The parameters k_2, k_{-2}, K_1 and K_3 can be estimated by performing the following two series of experiments.

(a) $[Fe(II)]_0$ constant and $[Fe(III)]_0$ variable. When $[Fe(II)]_0$ is constant and in large excess, the sum of [Fe(II)] and [C(III)] can be considered as nearly equal to $[Fe(II)]_0$, and the first term of Eqn. 5 can be regarded as constant. Eqn. 5, then, can be represented as

$$\frac{1}{\tau^{-1} - A} = \frac{1}{k_{-2}} + \frac{K_3}{k_{-2}([\overline{Fe}(III)] + [\overline{C}(II)])} \qquad (6)$$

where

$$A = \frac{k_2 K_1 [Fe(II)]_0}{1 + K_1 [Fe(II)]_0} \text{ (constant).} \qquad (7)$$

$[\overline{Fe}(III)]$ and $[\overline{C}(II)]$ in the second term of the right side of Eqn. 6 are obtained from Eqns. 8–10:

$$[\overline{Fe}(III)] = [Fe(III)]_0 + ([\overline{C}(II)] - [C(II)]_0) \qquad (8)$$

$$[\overline{C}(II)]_s = [\overline{C}(II)] + [\overline{C(II) \cdot Fe(III)}] \qquad (9)$$

$$k_3[\overline{C(II) \cdot Fe(III)}] = k_{-3}[\overline{C}(II)] \cdot [\overline{Fe}(III)] \qquad (10)$$

where $[\overline{C}(II)]_s$ denotes the concentration of the reduced cytochromes calculated from the optical absorption of the α-band at equilibrium. Hence, the parameters K_3 and k_{-2} were estimated from the experimental values of τ^{-1}, $[C(II)]_0$, $[Fe(III)]_0$ and $[C(II)]_s$ and Eqns. 8—10 by the least-squares methods, A being a parameter.

(b) $[Fe(III)]_0$ constant, $[Fe(II)]_0$ variable and in large excess. In this case, the second term of Eqn. 5 can be calculated using the values of K_3 and k_{-2} obtained in case (a). Eqn. 5 can be rewritten as

$$\frac{1}{k_{app} - B} = \frac{1}{k_2} + \frac{1}{k_2 K_1 \cdot [\overline{Fe}(II)] + [\overline{C}(III)]} \tag{11}$$

where

$$B = \frac{k_{-2}}{K_3 + ([\overline{Fe}(III) + [\overline{C}(II)])} \tag{12}$$

The sum of $[\overline{Fe}(II)]$ and $[\overline{C}(III)]$ may be regarded as $[Fe(II)]_0$, since $[Fe(II)]_0$ is in large excess. As $[\overline{Fe}(III)]$ and $[\overline{C}(II)]$ can be calculated from the experimental values of $[\overline{C}(II)]_s$, $[C(II)]_0$ and $[Fe(III)]_0$ according to Eqns. 8—10, the value of B in Eqn. 12 can be calculated. Therefore, the parameters k_2 and K_1 can be estimated from Eqns. 11 and 12 by the least-squares method.

Thus, four rate constants (k_2, k_{-2}, k_{ox} and k_{red}) and two dissociation constants (K_1 and K_3) can be estimated from two series of experiments. Once the parameters k_2, k_{-2}, K_1 and K_3 are obtained, the concentration of ferrocytochrome at equilibrium can be estimated. The details of the calculation are described elsewhere [23].

Experimental

Methods

In the present experiment, a Union-Giken T-jump spectrophotometer, model RA-1200, was used. The details are described elsewhere [23]. Temperature change after heating is 3°C. The initial temperature of the solution was maintained at 15°C ± 0.05°C throughout.

The absorbance in the ultraviolet and visible regions was monitored by a Shimadzu UV-200 spectrophotometer or a Cary-17 spectrophotometer.

The pH value was monitored by a Beckman pH meter, model 76 with an electrode of number 39013. The pH value of the solution was adjusted to pH 7.00 ± 0.01 throughout by adding HCl or NaOH. The temperature of the solution for optical absorbance and pH measurements was kept at 18 ± 0.1°C throughout the experiment. The solvent used was 0.1 M phosphate buffer with 0.3 M of KNO_3* for cytochromes and iron hexacyanides in the experiments.

---

* The parameters in Scheme I can be estimated independently of the concentrations of protein and oxidoreductants in the constant concentrations of buffer and anions [23].

Materials

Cytochromes *c* of horse heart, tuna heart and *Candida krusei* were purchased from Sigma Chemical Co. (type VI, XI and VII, respectively). Cytochrome *c* of *Saccharomyces oviformis* was kindly supplied by Sankyo Co., Ltd. (Tokyo, Japan). They were further purified on a carboxy-methyl-cellulose (CM-52) column.

Cytochrome c_2 of *Rhodospirillum rubrum* was a gift from Professor Horio. It was used without further purification.

Cytochrome *c*-554 (*Spirulina platensis*) was purified from the precipitate of *S. platensis* by 70% saturated ammonium sulfate according to the procedure of Yamanaka et al. [24], and was a gift from Professor T. Yamanaka.

Cytochrome *c*-552 (*Thermus thermophilus*) was extracted from the thermophilic bacteria HB8, and purified according to the procedure of Hon-nami and Oshima [25].

Results

The temperature-jump of the solution of cytochomes with iron hexacyanides gave rise to a decrease in the transmittance at the wavelength of the α-band with a single exponential relaxation process, when both oxidized and reduced states were in co-existence.

According to the procedure described in the theoretical section, two rate constants, k_2 and k_{-2}, and two dissociation constants, K_1 and K_3, specified in

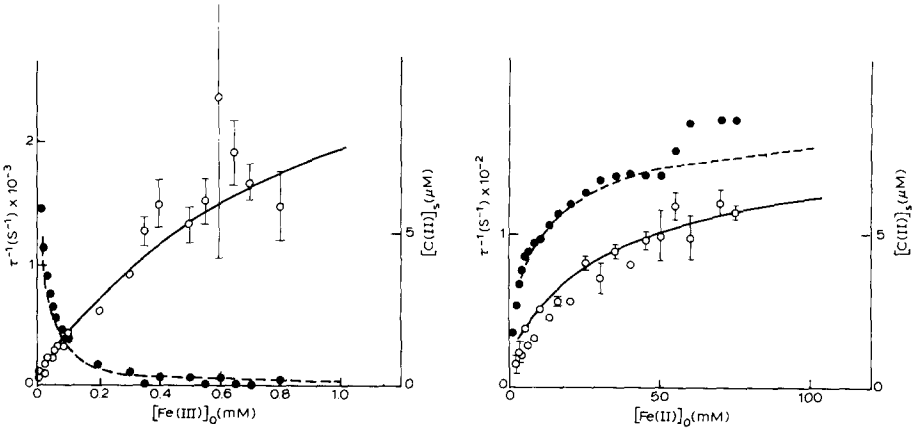

Fig. 1. Reciprocal relaxation time, τ^{-1}, and the concentration of ferrocytochrome at equilibrium of horse heart cytochrome *c* against the concentration of ferricyanide. $[Fe(III)]_0$, initial concentration of ferricyanide (variable). Initial concentration of ferrocyanide, 50 mM. Initial concentrations of ferric and ferrous cytochromes were 12.4 μM and 0 μM, respectively. 0.1 M of phosphate buffer (pH 7) with 0.3 M KNO_3. Initial and final temperatures are 15°C and 18°C, respectively. Pressure 5 atm. Each reciprocal relaxation time was estimated from four kinetic runs. ○, reciprocal relaxation time; ●, concentration of ferrocytochrome at equilibrium. The solid and broken lines in the figure are calculated according to Eqn. 5 and Section 2.2.2 of Ref. 23 with the parameters in Table I.

Fig. 2. Reciprocal relaxation time and the concentration of ferrocytochrome at equilibrium against ferrocyanide, horse cytochrome *c*. Ferrocyanide variable and no ferricyanide. For the other descriptions, see Fig. 1.

Scheme I were separately estimated from the reciprocal relaxation times and the concentration of ferrocytochrome measured for both the ferrocyanide- and the ferricyanide-variable systems.

(1) The ferricyanide-variable system

The experiments were performed with concentration of ferrocyanide constant at 50 mM, and a variable concentration of ferricyanide. Cytochromes in the oxidized state were used in the experiment except for cytochrome *c*-554. In the case of cytochrome *c*-554, the reduced cytochrome was used, since it was more stable [24]. The values of τ^{-1} obtained for horse cytochrome *c* were plotted against the concentration of ferricyanide in Fig. 1. Similar profiles were obtained for all cytochromes investigated. From the figure, we can obtain the values of K_3 and k_{-2} according to Eqns. 8—10.

(2) The ferrocyanide-variable system

The temperature-jump experiments were performed with variable concentrations of ferrocyanide and no ferricyanide, except in the case of cytochrome *c*-554. In that case, ferricyanide of 0.1 mM was mixed with variable concentrations of ferrocyanide. Cytochromes in the oxidized state were used except cytochrome *c*-554. For the last cytochrome, the reduced cytochrome was used. The obtained reciprocal relaxation times, τ^{-1}, of horse cytochrome *c* were plotted against the concentrations of ferrocyanide in Fig. 2. Similar profiles were obtained for all cytochromes investigated. The parameters K_1 and k_2 can be obtained from the data under conditions in which the concentration of ferrocyanide is relatively high, according to Eqns. 11 and 12.

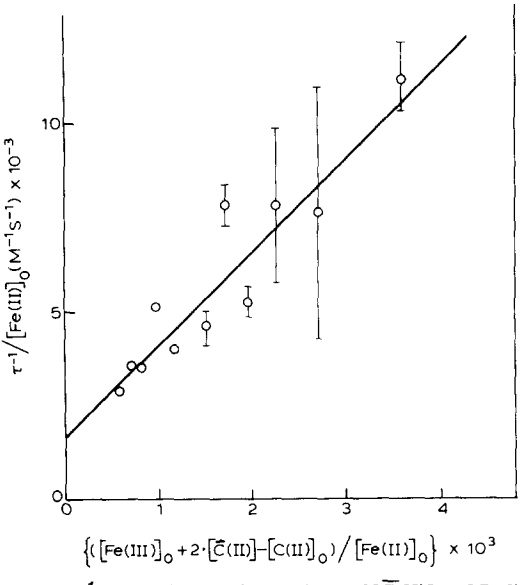

Fig. 3. $\tau^{-1}/[Fe(II)]_0$ **vs.** $([Fe(III)]_0 + 2[\bar{C}(II)] - [C(II)]_0)/[Fe(II)]_0$. **Ferrocyanide variable and no ferricyanide. Other descriptions are the same as for Fig. 1.**

TABLE I

A LIST OF KINETIC PARAMETERS

	horse *c* KNO_3	tuna *c*	*C. krusei c*	*S. oviformis c*	*R. rubrum* c_2	*S. platensis* c-554	*T. thermophilus* c-552
$k_2(s^{-1})$	$(1.0 \pm 0.5) \cdot 10^2$	$(4.9 \pm 0.5) \cdot 10$	$(3.5 \pm 0.5) \cdot 10^2$	$(7 \pm 2) \cdot 10^2$	$(4.9 \pm 0.5) \cdot 10^2$	$(1.2 \pm 0.2) \cdot 10^4$	$\sim 10^5$ $>10^4$
$k_{-2}(s^{-1})$	$(3.5 \pm 1.0) \cdot 10^3$	$(3 \pm 1) \cdot 10^3$	$(1.5 \pm 0.5) \cdot 10^4$	$(4 \pm 0.5) \cdot 10^3$	$\sim 4 \cdot 10^4$ $>2 \cdot 10^4$	$\sim 10^5$ $>5 \cdot 10^4$	$\sim 10^4$ $>10^4$
$K_1(M^{-1})$	15 ± 7	17 ± 3	10 ± 2	5 ± 1	6.1 ± 1	1.0 ± 0.5	$>5 \cdot 10^{-2}$ $\sim 5 \cdot 10^{-3}$
$K_3(M)$	$(8.5 \pm 1.5) \cdot 10^{-4}$	$(7 \pm 2) \cdot 10^{-4}$	$(2.1 \pm 0.4) \cdot 10^{-3}$	$(7 \pm 2) \cdot 10^{-4}$	$\sim 4.6 \cdot 10^{-2}$	$\gtrsim 0.02$	$\gtrsim 7 \cdot 10^{-4}$
k_{-2}/K_3 $(M^{-1} \cdot s^{-1})$	$(4.1 \pm 1.0) \cdot 10^6$	$(4.3 \pm 0.7) \cdot 10^6$	$(7.1 \pm 0.7) \cdot 10^6$	$(5.7 \pm 1.6) \cdot 10^6$	$(8.8 \pm 0.6) \cdot 10^5$	$(4.5 \pm 0.3) \cdot 10^6$	$(1.4 \pm 0.2) \cdot 10^7$
$k_2 K_1$ $(M^{-1} \cdot s^{-1})$	$(1.5 \pm 0.7) \cdot 10^3$	$(8.3 \pm 0.3) \cdot 10^2$	$(3.5 \pm 0.5) \cdot 10^3$	$(3.5 \pm 1) \cdot 10^3$	$(3.0 \pm 0.5) \cdot 10^3$	$(1.1 \pm 0.4) \cdot 10^4$	$(4.7 \pm 0.3) \cdot 10^2$
k_{red} $(M^{-1} \cdot s^{-1})$	$(1.6 \pm 1.8) \cdot 10^3$	$(2.0 \pm 1.5) \cdot 10^3$	$(5.4 \pm 2.0) \cdot 10^3$	$(3.7 \pm 2.8) \cdot 10^3$	$(2.6 \pm 1.7) \cdot 10^3$	$(2.3 \pm 3) \cdot 10^4$	$(1.0 \pm 2.5) \cdot 10^3$
k_{ox} $(M^{-1} \cdot s^{-1})$	$(2.5 \pm 1.5) \cdot 10^6$	$(2.4 \pm 0.9) \cdot 10^6$	$(5.4 \pm 0.6) \cdot 10^6$	$(5.2 \pm 0.8) \cdot 10^6$	$(8.5 \pm 0.9) \cdot 10^5$	$(4.4 \pm 0.5) \cdot 10^6$	$(4.1 \pm 0.2) \cdot 10^6$
K	$3.6 \cdot 10^{-4}$	$1.9 \cdot 10^{-4}$	$4.9 \cdot 10^{-4}$	$6.1 \cdot 10^{-4}$	$1.75 \cdot 10^{-3}$	$5.6 \cdot 10^{-3}$	$3.2 \cdot 10^{-5}$
$E(V)$ (18° C)	0.23	0.22	0.24	0.24	0.27	0.30	0.17

Estimate of k_{ox} and k_{red}

At a low concentration of ferrocyanide, Scheme I can be reduced to a simpler scheme, Scheme II and the parameters k_{ox} and k_{red} can be estimated according to Eqn. 1. For six cytochromes except cytochrome *c*-554, ferrocyanide was mixed with ferricytochrome, whereas for cytochrome *c*-554, ferrocyanide, 0.1 mM of ferricyanide and ferrocytochrome were mixed. Dependence of τ^{-1} of horse cytochrome *c* on the concentration of ferrocyanide was plotted in Fig. 3.

The estimated kinetic parameters are summarized in Table I.

Discussion

1. Examination of the validity of the kinetic parameters

Kinetic parameters estimated for cytochrome *c* were checked by several critical points. (i) The equilibrium constant of horse cytochrome *c* with iron hexacyanide was calculated to be $3.6 \cdot 10^{-4}$, from which the redox potential was estimated to be 0.23 V. It is in fairly good agreement with the value observed in equilibrium (0.24 V [23]). (ii) The reciprocal relaxation times and the concentrations of ferrocytochromes were calculated according to Eqns. 1 and 5, and Section 2.2.2 of Ref. 23 with the parameters in Table I. The simulated curves thus obtained for horse cytochrome *c* are shown in Figs. 1—3. The agreement with the experimental data is good for all the cytochromes investigated (not shown here). (iii) The reciprocal relaxation times and the concentrations of ferrocytochrome were also calculated for horse cytochrome *c* with respect to the system with variable concentrations of ferrocyanide and 0.1 mM of ferricyanide, according to Eqn. 5 and Section 2.2.2 of Ref. 23 with the parameters listed in Table I. The simulated curves of the reciprocal relaxation times and the concentration of ferrocytochrome were compared with the corresponding experimental data. Their agreement is quantitatively good [23].

2. Comparison of the kinetic parameters with values in the other literature

(i) The values of k_{ox} and k_{red} obtained for horse cytochrome *c* in early reports have been summarized in our previous paper [16]. The value of k_{ox} is between $6.5 \cdot 10^6$ and $1.6 \cdot 10^7\ M^{-1} \cdot s^{-1}$, and the value of k_{red} lies between $1.1 \cdot 10^4$ and $3.4 \cdot 10^4\ M^{-1} \cdot s^{-1}$. The values obtained in the present work are smaller than the values mentioned above. These slight discrepancies might be due to the difference in experimental conditions, since the measurements in the literature were almost all done in a relatively low concentration of salts.

(ii) Six rate constants in Scheme I were estimated by Miller and Cusanovich [8] with the aid of the values estimated by Stellwagen and Shulman [7]. Stellwagen and Shulman obtained k_2 from the saturated value of a plot of $\Delta\nu$ (the broadening of the line width) vs. [Fe(II)], and k_{-2} from the assumption of $K_1 = K_3^{-1}$ and a plot of $\Delta\nu$ vs. [Fe(III)]. Miller and Cusanovich analyzed the reaction according to the scheme,

$$C(III) + Fe(II) \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} C \cdot Fe \underset{k'_{-2}}{\overset{k'_2}{\rightleftharpoons}} C(II) + Fe(III) \qquad (III)$$

assuming one intermediate complex. τ^{-1} is then derived as

$$\tau^{-1} = \frac{k_2' K' [\text{Fe(II)}]}{1 + K' [\text{Fe(II)}]} \tag{13}$$

where $K' = k_1'/(k_{-1}' + k_2')$, neglecting the backward process of k_{-2}'[C(II)][Fe(III)]. They derived k_1' from the initial slope of a plot of τ^{-1} vs. [Fe(II)], and k_2' and K' from Eqn. 13. The value of k_2' was ascribed to the value of k_3 in Scheme I, and $k_1' = k_1$ and $k_{-1}' = k_{-1}$. The value of k_2 is calculated from the other rate constants and overall equilibrium constant, with the use of k_{-2} estimated by Stellwagen and Shulman [7] and k_{ox} obtained by Brandt et al. [2] as k_{-3}. The values are shown in Table II. However, as already pointed out in the theoretical section and the results above, (1) the term k_{-2}' [C(II)][Fe(III)] cannot be neglected, (2) k_1' and k_{-3} cannot be estimated only from the procedure described above because the reaction is reversible. Moreover, (3) the assumption $K_1 = K_3^{-1}$ made by Stellwagen and Shulman is unreasonable and has no evidence to support it, and (4) k_2' should be ascribed to k_2 rather than k_3, because the absorbance change at Soret peak was used as monitor of the production of ferrocytochrome in the analysis of Miller and Cusanovich and that chance occurred through process B in scheme I. The value of 1500 s^{-1} (k_2 of Miller and Cusanovich) should be ascribed to k_3 according to their analysis.

Considering these facts, the agreement of k_2 and k_{-2} between the present results and Miller and Cusanovich's is good, though there is a large difference for K_1. Since the dissociation constants K_1 and K_3 are extremely salt concentration dependent, there are nothing to say about the discrepancy of the dissociation constants between two experiments.

3. Divergence of the kinetic parameters and its physiological meaning

From the values in Table I, it can be seen that each kinetic parameter diverged beyond the error range among the species examined. To estimate the divergence of the parameters, the logarithms of the parameters were plotted in Fig. 4. The variances of the logarithms of the parameters were estimated among seven species and also among four species of eukaryotes. They are also shown in Fig. 4. From the figure, the variances in eukaryotes were smaller and similar to each other, whereas among seven species those of k_2, k_{-2}, K_3, K_1 were larger, and the variances of the complex values k_{-2}/K_3, k_2K_1, k_{red} and k_{ox} are not so large. These results suggest that the values of k_2K_1 and k_{-2}/K_3 (and k_{red} and k_{ox}) have been conserved during the course of the evolution, notwithstanding the divergence of each parameter (k_2, k_{-2}, K_1 and K_3).

k_2 and k_{-2} (Step B in Scheme I). As specified in Scheme I, the parameters k_2 and k_{-2} are considered to be the rates of the electron transfer from ferrocyanide to ferricytochrome and from ferrocytochrome to ferricyanide in the

TABLE II

RATE CONSTANTS ESTIMATED IN EARLIER WORK

k_1	$1.7 \cdot 10^4$ $M^{-1} \cdot s^{-1}$	[8]	k_{-1}	7 s^{-1}	[8]
k_2	$1.5 \cdot 10^3$ s^{-1}	[8]	k_{-2}	$2.1 \cdot 10^4$ s^{-1}	[7]
	208 s^{-1}	[7]			
k_3	132 s^{-1}	[8]	k_{-3}	$8.7 \cdot 10^6$ $M^{-1} \cdot s^{-1}$	[2]

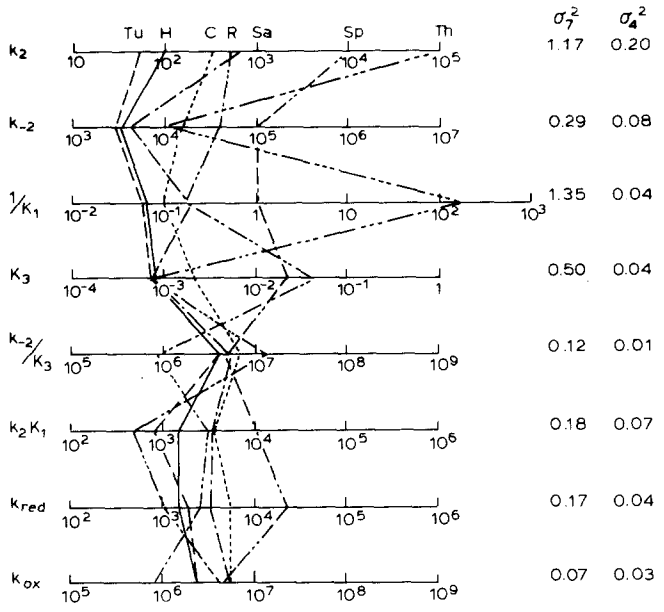

Fig. 4. Comparison of the divergence of the kinetic parameters. The column next to the right-hand side is the variances of logarithm of each parameter for seven species and the column at the right hand side represents the variances of logarithm of each parameter for four cytochrome *c*'s of eukaryotes. H, horse; Tu, tuna; C, *C. krusei*; Sa, *S. oviformis*; R, *R. rubrum*; Sp, *S. platensis*; Th, *T. thermophilus*.

cytochrome-iron hexacyanide complex. The divergence of k_2 and k_{-2} suggests that the environment of the electron pathway is not necessarily conserved throughout the molecular evolution.

4. Grouping to c_2- and c_6-types and its relation with kinetic parameters

The most characteristic feature of the kinetic parameters is that the k_2 values of cytochrome *c*-554 and *c*-552 are larger than the k_2 values of other cytochromes. From the spectral features these two cytochromes are considered to belong to the same subgroup, c_6-type. The conincidence of these spectral and kinetic classifications might be meaningful.

Wood and Cusanovich investigated the reaction of another c_6-type cytochrome, *c*-552, *E. gracilis*), with the iron hexacyanide, and reported the values of k_{red} and k_{ox} as $7.5 \cdot 10^3\ M^{-1} \cdot s^{-1}$ and $3.9 \cdot 10^4\ M^{-1} \cdot s^{-1}$, respectively, at 0.1 M buffer [26]. They also reported the values of k_{red} and k_{ox} for horse cytochrome *c* and *R. rubrum* c_2. A marked difference was seen among the values for three cytochromes, and they ascribed these differences to the difference in charged state at the reacting site, since the site of cytochrome *c*-552 (*E. gracilis*) is negatively charged in contrast to the positively charged reacting sites of horse cytochrome *c* and *R. rubrum* c_2.

On the other hand, the values of k_{red} and k_{ox} of cytochrome *c*-554 obtained in our experiments are $2 \cdot 10^4\ M^{-1} \cdot s^{-1}$ and $4 \cdot 10^6\ M^{-1} \cdot s^{-1}$ and are larger than the values obtained by Wood and Cusanovich for cytochrome *c*-552 of *E. gracilis*. The values are even larger than for horse cytochrome *c*. From these results, we can conclude that k_{ox} and k_{red} would not be adequate indices for classifi-

cation of cytochromes into c_2- and c_6-types, because k_{red} and k_{ox} depend strongly on the ionic atmosphere of the reacting sites which differs according to the difference of the distribution of the dissociation residues at the site.

5. The role of tyrosine or phenylalanine at position 46

Based on the reactivity of *c*-type cytochromes with *T. novellus* oxidase, Yamanaka [27] proposed a criterion of classification that *c*-type cytochromes can be subdivided into two classes due to whether the 46th residue is tyrosine or phenylalanine. However, there was no evidence that the cytochromes *c* of tuna, *C. krusei* and *S. oviformis* whose 46th residues were tyrosine were in the same group against horse cytochrome *c* (Phe-46). Therefore, this classification would be based on the specific recognition of the reacting molecule (oxidase). The difference of the 46th residue is indifferent to the reactivity with non-specific inorganic reagents such as iron hexacyanide.

6. Thermostability and kinetics of cytochrome c-552 (T. thermophilus)

A characteristic feature of *T. thermophilus* *c*-552 is that its redox potential is very low. The present study showed that its k_{red} and k_2K_1 are the lowest among seven species, whereas its k_{-2}/K_3 is the largest and its k_{ox} is comparatively large. Since the value of k_2 is the largest among seven species, the low values of k_2K_1 and k_{red} depend mainly on the low value of K_1. Similarly, the values of k_{-2}/K_3 and k_{ox} are large because the value of K_3 is low. Hence, the low value of the redox potential would be ascribed to the low value of K_1 and K_3. As pointed out in our previous paper [16], the characteristics of the kinetics of cytochrome *c*-552 with the iron hexacyanide as well as those described above, are (1) $\Delta H_{ox}^{\ddagger}$ is considerably lower than that of horse cytochrome *c*, (2) τ^{-1} at room temperature is slower than that of horse cytochrome *c*. The small value of τ^{-1} would be due to the low values of K_1 and K_3, owing to the reason described above. Although the details are unknown, the values of K_1 and K_3 would have been modified to make the cytochrome molecule fit the environment in spite of the divergence of the values of k_2 and k_{-2}.

Acknowledgements

The author would like to express his sincere thanks to Professor T. Iizuka of Keio University and Professor T. Yamanaka of Osaka University for their fruitful discussions. He wishes to give his thanks to Professor T. Horio of Osaka University, Professor T. Yamanaka, Mr. K. Hon-nami and Sankyo Co. Ltd. for their kind gifts of cytochrome c_2 of *R. rubrum*, *c*-554 (*S. platensis*), *c*-552 (*T. thermophilus*) and cytochrome *c* of *S. oviformis*, respectively. Thanks must also be given to Miss E. Takahashi for her skilful technical assistance. A part of this work was supported by a grant in aid from the Ministry of Education, Japan.

References

1 Havsteen, B.H. (1965) Acta Chem. Scand. 19, 1227—1231
2 Brandt, K.G., Parks, P.C., Czerlinski, G.H. and Hess, G.P. (1966) J. Biol. Chem. 241, 4180—4185
3 Watt, G.D. and Sturtevant, J.M. (1969) Biochemistry 8, 4567—4571
4 Morton, R.A., Overnell, J. and Harbury, H.A. (1970) J. Biol. Chem. 245, 4653—4657

5 Margalit, R. and Schejter, A. (1970) FEBS Lett. 6, 278—280
6 Czerlinski, G.H. and Dar, K. (1971) Biochim. Biophys. Acta 234, 57—61
7 Stellwagen, E. and Shulman, R.G. (1973) J. Mol. Biol. 80, 599—573
8 Miller, W.G. and Cusanovich, M.A. (1975) Biophyss. Struct. Mech. 1, 97—111
9 Cassatt, J.C. and Marini, C.P. (1974) Biochemistry 13, 5323—5328
10 Zabinski, R.M., Tatti, K. and Czerlinski, G.H. (1974) J. Biol. Chem. 249, 6125—6129
11 Stellwagen, E. and Cass, R.D. (1975) J. Biol. Chem. 250, 2095—2098
12 Ilan, Y., Shafferman, A. and Stein, G. (1976) J. Biol. Chem. 251, 4336—4345
13 Peterman, B.F. (1976) Doctoral Thesis, McMaster University, Hamilton, Canada
14 Peterman, B.F. and Morton, R.A. (176) Biophys. J. 16, 85a
15 Wood, F.E., Post, C.B. and Cusanovich, M.A. (1977) Arch. Biochem. Biophys. 184, 586—595
16 Kihara, H., Nakatani, H., Hiromi, K., Hon-nami, K. and Oshima, T. (1977) Biochim. Biophys. Acta 460, 480—489
17 Ilan, Y. and Shafferman, A. (1978) Biochim. Biophys. Acta 501, 127—135
18 Larroque, C., Marue, P. and Douzou, P. (1978) Biochim. Biophys. Acta 501, 20—32
19 Mizrahi, I.A., Wood, F.E. and Cusanovich, M.A. (1976) Biochemistry 15, 343—348
20 Sutin, N. and Christman, D.R. (1961) J. Am. Chem. Soc. 83, 1773—1774
21 Wherland, S. and Gray, H.B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2950—2954
22 Creutz, C. and Sutin, N. (1974) J. Biol. Chem. 249, 6788—6795
23 Kihara, H. (1979) Doctoral Thesis, University of Tokyo, Tokyo
24 Yamanaka, T., Fukumori, Y. and Wada, K. (1978) Plant Cell Physiol. 19, 117—126
25 Hon-nami, K. and Oshima, T. (1977) J. Biochem. (Tokyo) 82, 769—776
26 Wood, F.E. and Cusanovich, M.A. (1975) Arch. Biochem. Biophys. 168, 333—342
27 Yamanaka, T. and Fukumori, Y. (1977) FEBS Lett. 77, 155—158