Biochimica et Biophysica Acta, 636 (1981) 129–135
Elsevier/North-Holland Biomedical Press

BBA 48074

ELECTRON TRANSFER BETWEEN HORSE HEART AND *CANDIDA KRUSEI* CYTOCHROMES *c* IN THE FREE AND BOUND STATES

TETSURO YOSHIMURA, TAKAYUKI SOGABE and KENJI AKI

Institute for Enzyme Research, School of Medicine, University of Tokushima, Tokushima 770 (Japan)

(Received November 25th, 1980)

Key words: Cytochrome c; Phosvitin; Electron transfer; Collision frequency; (Stopped-flow kinetics)

Electron transfer between horse heart and *Candida krusei* cytochromes *c* in the free and phosvitin-bound states was examined by difference spectrum and stopped-flow methods. The difference spectra in the wavelength range of 540–560 nm demonstrated that electrons are exchangeable between the cytochromes *c* of the two species. The equilibrium constants of the electron transfer reaction for the free and phosvitin-bound forms, estimated from these difference spectra, were close to unity at 20°C in 20 mM Tris-HCl buffer (pH 7.4). The electron transfer rate for free cytochrome *c* was $(2–3) \cdot 10^4$ $M^{-1} \cdot s^{-1}$ under the same conditions. The transfer rate for the bound form increased with increase in the binding ratio at ratios below half the maximum, and was almost constant at higher ratios up to the maximum. The maximum electron exchange rate was about $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$, which is 60–70 times that for the free form at a given concentration of cytochrome *c*. The activation energy of the reaction for the bound cytochrome *c* was equal to that for the free form, being about 10 kcal/mol. The dependence of the exchange rate on temperature, cytochrome *c* concentration and solvent viscosity suggests that enhancement of the electron transfer rate between cytochromes *c* on binding to phosvitin is due to increase in the collision frequency between cytochromes *c* concentrated on the phosvitin molecule.

Introduction

In mitochondrial electron transfer, cytochrome *c* plays crucial roles and acts as a mediator between its reductase and oxidase. However, the mechanism of electron transfer between these proteins is still obscure. Studies on protein-protein and protein-small molecule electron transfer in solution can provide clues to this problem. There have been several studies on protein-protein electron transfer in which *c*-type cytochromes are involved; namely studies on electron transfer between cytochromes *c* [1–4], cytochrome *c* and c_{551} [5,6], cytochromes c_{551} [7], cytochrome *c* and c_{553} [8], cytochrome c_1 and *c* [9], cobalt and iron cytochromes *c* [10,11], and cytochrome *c*, c_{551} or c_{553} and azurin [6,8,12–14] or plastocyanine [8,15]. Some mechanistic models have been proposed to explain these electron transfer processes [8,10,11].

Previously, we reported that *Candida krusei* cytochrome *c* forms a molecular complex with the phosphoprotein phosvitin. In these complexes the maximum binding ratio of cytochrome *c* to phosvitin is 22 : 1, the affinity for phosvitin is much higher with binding ratios below half the maximum [16], and the electron transfer rate is repressed in the tightly bound state, but is restored as the binding becomes weaker [17]. Since phosvitin-bound cytochrome *c* seems to be a useful model system for the membrane-bound form [18], studies on electron transfer between cytochromes *c* bound to phosvitin might be available for understanding of the mechanism of electron transfer in the membrane-bound state. This paper reports

Abbreviations: CII_h and $CIII_h$, horse heart ferrous and ferric cytochrome *c*; CII_c and $CIII_c$, *Candida krusei* ferrous and ferric cytochrome *c*.

investigations showing that electrons are exchangeable between horse heart and *C. krusei* cytochromes *c*, and that the electron exchange rate is greatly enhanced by binding of the cytochromes to phosvitin.

Materials and Methods

Cytochrome *c* of *C. krusei* was obtained from Sankyo Co., and horse heart cytochrome *c* (Type VI) and phosvitin were purchased from Sigma Chemical Co. Other chemicals were commercial products of reagent grade. The ferric and ferrous forms of cytochrome *c* were prepared by gel filtration on Sephadex G-25 of samples oxidized with potassium ferricyanide and reduced with sodium ascorbate, respectively. Concentrations of cytochrome *c* were determined spectrophotometrically assuming millimolar extinction coefficients for CII_c and CII_h of 32.7 $mM^{-1} \cdot cm^{-1}$ at 549 nm and 29.8 $mM^{-1} \cdot cm^{-1}$ at 550 nm [19], respectively. Concentration of phosvitin was determined with a Hitachi protein refractometer using bovine serum albumin as a standard. All solutions were in 20 mM Tris-HCl/0.1 mM EDTA (pH 7.4).

Difference spectra were measured at 20°C in a Hitachi 323 automatic recording spectrophotometer. Paired tandem cells were used.

Stopped-flow experiments were performed at 20°C with a Union RA-401 stopped-flow spectrophotometer, equipped with a Sord M223 microcomputer. The slit-width was set at 1.4 nm. The delay-time and dead-time of the apparatus were about 20 and 0.5 ms, respectively.

Results

Evidence for electron transfer between cytochromes c. In a characteristic absorption spectrum of the ferrous form of cytochrome *c*, the positions of the α bands of CII_c and CII_h are 549 and 550 nm, respectively. This difference of about 1 nm should make it possible to detect electron transfer between the two. To test this possibility, we measured difference spectra as follows: One side of a paired cell was filled with a solution of $CIII_h$ of known concentration, and the other side with a solution of CII_c of the same concentration. The two sides of another paired cell were filled with solutions of CII_h and $CIII_c$ of the same concentrations. Then the difference spectrum, $(A_{CIII_h} + A_{CII_c}) - (A_{CII_h} + A_{CIII_c})$, was recorded. The results are shown in Fig. 1A. Positive and negative bands were observed at about 548 and 553 nm, respectively. The difference between the millimolar extinction differences ($\Delta\epsilon$) at the wavelengths for the maximum and minimum difference, $\Delta\epsilon_{max-min}$, was estimated

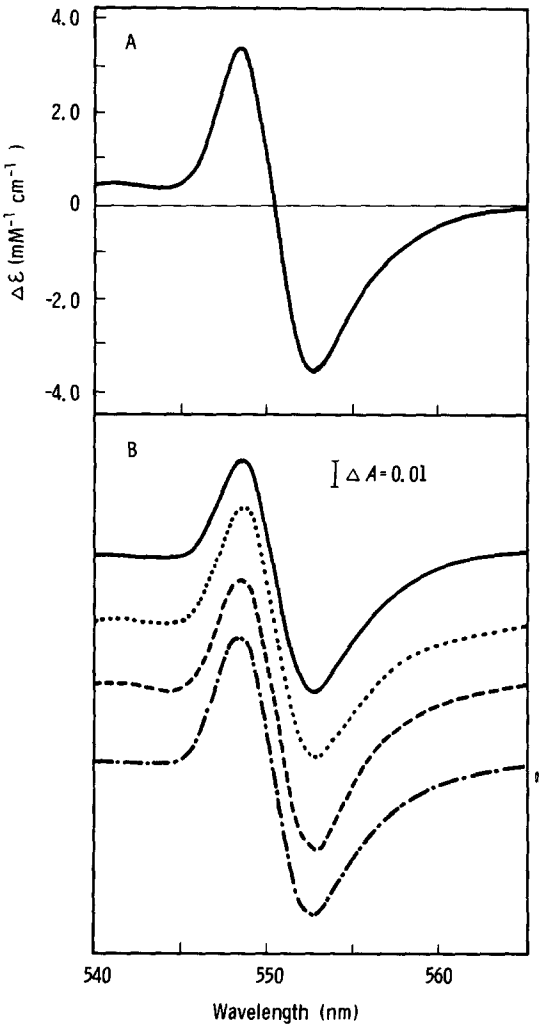

Fig. 1. Difference spectra between CII_c and $CIII_h$ in a paired cell and $CIII_c$ and CII_h in another paired cell, $(\epsilon_{CII_c} + \epsilon_{CIII_h}) - (\epsilon_{CIII_c} + \epsilon_{CII_h})$ (A), and between samples of CII_h and $CIII_c$, both in one side of a paired cell and each in each of two sides of another paired cell (B). The spectra for free (———) and phosvitin-bound cytochrome *c* at ratios of 21.3 (· · · · ·), 10.6 (- - - - -) and 2.1 (— · — · —) were measured at 20°C in 20 mM Tris-HCl buffer (pH 7.4). The concentrations of horse and *Candida* cytochrome *c* were all 51.7 μM (A), and 33.4 and 30.3 μM, respectively (B).

to be 7.13 $mM^{-1} \cdot cm^{-1}$. Fig. 1B shows the difference spectra between mixed and separated samples of CII_h and $CIII_c$ in the presence and absence of phosvitin. The spectra also had positive and negative bands at about 548 and 553 nm, respectively, indicating that electrons are exchangeable between the cytochromes *c* of the two species both in the free and bound states.

This electron exchange reaction is written as

$$CII_h + CIII_c \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CIII_h + CII_c \qquad (1)$$

The equilibrium constant for this reaction was estimated from the concentration of products, which was calculated by the equation

$$[CIII_h](=[CII_c]) = \Delta A_{max-min}/\Delta\epsilon_{max-min} \qquad (2)$$

where $[CIII_h]$ and $[CII_c]$ are the millimolar concentration of product, and $\Delta A_{max-min}$ and $\Delta\epsilon_{max-min}$ are the differences between absorption and millimolar extinction differences at the wavelengths for the maximum and minimum difference, respectively, the latter being 7.13. As shown in Table I, the equilibrium constants for the free and bound forms were close to unity, which indicates that the rate constant for the forward reaction (k_1) is almost equal to that for the backward reaction (k_{-1}).

Electron transfer rate between cytochromes c. The rate of electron transfer between CII_h and $CIII_c$ was measured at 548 and 553 nm, using a stopped-flow spectrophotometer. If the equilibrium constant is close to unity, this electron transfer reaction described in Eqn. 1 should obey first-order kinetics:

$$A_\infty - A_t = (A_\infty - A_0)\exp(-k't) \qquad (3)$$

where A_∞, A_0 and A_t represent the absorbance at equilibrium, time $t = 0$ and time t, respectively, and $k' = k[C]_0$, $k = k_1 = k_{-1}$, and $[C]_0 = [CII_h] + [CIII_h] + [CII_c] + [CIII_c]$ (total concentration of cytochrome *c*). The data could be analyzed in terms of the first-order expression. The first-order rate constant for free cytochrome *c* was broadly proportional to the total concentration of cytochrome *c* (Fig. 2A), and a second-order rate constant of $(2–3) \cdot 10^4$ $M^{-1} \cdot s^{-1}$ was obtained. In Fig. 2A, the plot of the data deviates somewhat from linearity, possibly because the maximum change in absorbance was only about one-tenth of the total absorbance.

TABLE I

EQUILIBRIUM CONSTANTS OF THE ELECTRON TRANSFER REACTION FOR FREE AND BOUND CYTOCHROMES *c*

Experimental conditions were the same as described in Fig. 1. The concentration of the products was calculated by the equation: $[CIII_h]$ $(=[CII_c]) = \Delta A_{max-min}/7.13$; where $\Delta A_{max-min}$ was obtained from the spectra shown in Fig. 1. The equilibrium constants were estimated using the equation: $K = [CIII_h][CII_c]/([CII_h]_0 - [CIII_h])$ $([CIII_c]_0 - [CII_c])$ where $[CII_h]_0$ and $[CIII_c]_0$ are the initial concentrations of CII_h and $CIII_c$, which are 33.4 and 30.3 μM, respectively.

Cytochrome *c*	Added cytochrome *c*/phosvitin	$\Delta A_{max-min}$	*K*
Free	–	0.106	0.8
Bound	2.1	0.129	1.8
	10.6	0.127	1.6
	21.3	0.117	1.1

On addition of phosvitin, three kinds of experiment were performed: in the stopped-flow cell, (i) CII_h in the presence of phosvitin was mixed with $CIII_c$ in its presence, (ii) CII_h in the presence of phosvitin was mixed with $CIII_c$ in its absence, and (iii) CII_h in the absence of phosvitin was mixed with $CIII_c$ in its presence, where the ratio of total cytochrome *c* to phosvitin was kept constant. As shown in Fig. 3, similar results were obtained in the three experiments. The second-order rate constant increased with increase in the binding ratio at ratios below half the maximum, was almost constant at higher ratios up to the maximum, and decreased when ratios exceed the maximum. * The maximum rate constant was about $2 \cdot 10^6$ $M^{-1} \cdot s^{-1}$ at 20°C and pH 7.4, which is 60–70 times that for the free form at a cytochrome *c* concentration of 45 μM. The data could, of course, be analyzed on the basis of the first-order expression. In

* Since all the added cytochrome *c* is bound to phosvitin up to the maximum binding ratio [16], the amount of added cytochrome *c* can be considered to be equal to that of bound cytochrome *c*.

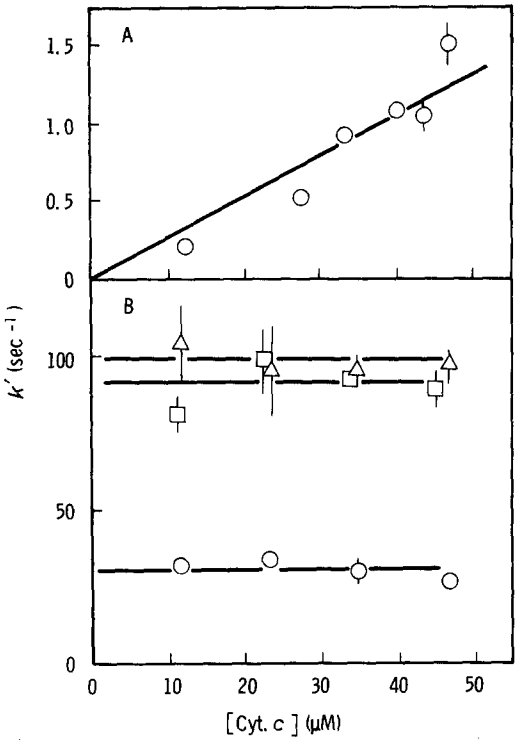

Fig. 2. Dependence of the observed first-order rate constant (k') on the total concentration of free (A) and phosvitin-bound (B) cytochromes *c* (cyt. *c*). CII_h and $CIII_c$ (A), and CII_h in the absence of phosvitin and $CIII_c$ in its presence (B) were mixed in the stopped-flow cell, and absorbance changes at 548 and 553 nm were measured at 20°C in 20 mM Tris-HCl buffer (pH 7.4). The final ratios of added cytochrome *c* to phosvitin were 2.3 (○), 9.6 (□) and 17.6 (△). The ratio of CII_h to $CIII_c$ was fixed at 1.0.

0.5 M NaCl (pH 7.4) where cytochrome *c* does not form a complex with phosvitin, the rate was equal to that for free cytochrome *c*, being $4 \cdot 10^3$ $M^{-1} \cdot s^{-1}$. Since both $CIII_h$ and CII_h also form a complex with phosvitin at a maximum ratio of 20 : 1 [20], these findings indicate that the electron transfer rate is much greater between bound cytochromes *c*.

The electron transfer reactions between bound cytochromes *c* are of two types: electron transfer between cytochromes *c* on the same phosvitin molecule and that between cytochromes *c* on separate phosvitin molecules, that is, 'intramolecular and intermolecular' electron transfer. To distinguish the former from the latter, the electron exchange rate between CII_h and $CIII_c$ was examined in the presence of excess phosvitin. Fig. 4 shows that the second-order rate

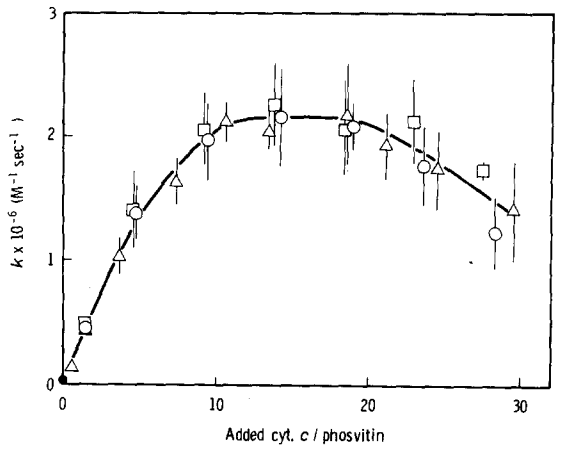

Fig. 3. Plot of the second-order rate constant as a function of the ratio of added cytochrome *c* (cyt. *c*) to phosvitin. CII_h in the presence of phosvitin was mixed with $CIII_c$ in its presence (○), CII_h in the absence of phosvitin was mixed with $CIII_c$ in its presence (□), and CII_h in the presence of phosvitin was mixed with $CIII_c$ in its absence (△) in the stopped-flow cell, where the ratio of total cytochrome *c* to phosvitin was kept constant. The total concentration of cytochrome *c* and the ratio of CII_h to $CIII_c$ were fixed at 45 μM and 1.0, respectively. Other experimental conditions were the same as described in Fig. 2. The value in the absence of phosvitin is shown on the ordinate.

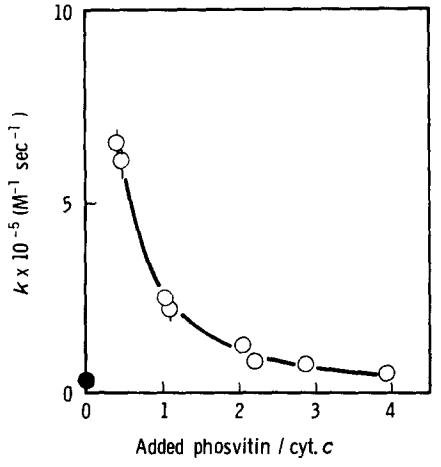

Fig. 4. Plot of the second-order rate constant as a function of added phosvitin to cytochrome *c* (cyt. *c*). CII_h in the presence of phosvitin was mixed with $CIII_c$ in its presence in the stopped-flow cell. The total concentration of cytochrome *c* and the ratio of CII_h to $CIII_c$ were fixed at 40 μM and 1.0, respectively. Other experimental conditions were the same as described in Fig. 2. The value in the absence of phosvitin is shown on the ordinate.

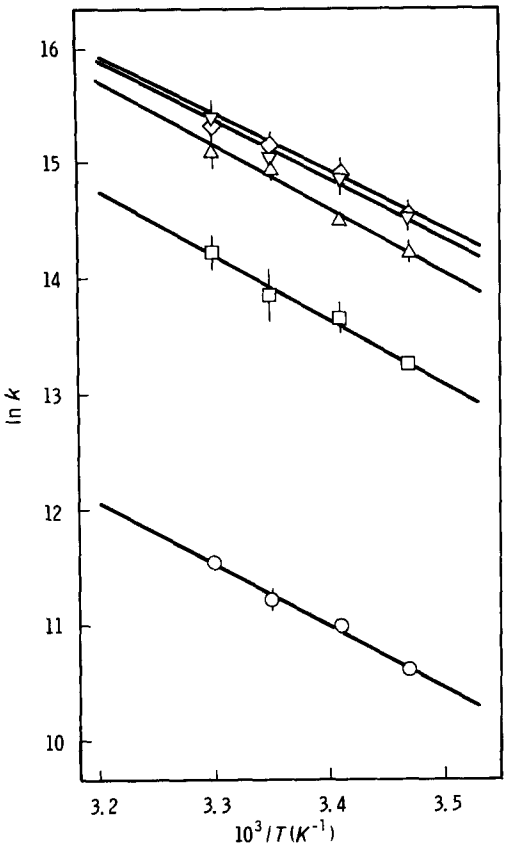

Fig. 5. Temperature dependence of the second-order rate constants, plotted as ln k vs $1/T$. CII_h and $CIII_c$ (○), and CII_h and $CIII_c$ both in the presence of phosvitin at ratios of 2.3 (□), 7.5 (△), 11.7 (▽) and 17.5 (◇), were mixed in the stopped-flow cell. The total concentration of cytochrome c and the ratio of CII_h to $CIII_c$ were fixed at 35 μM and 1.0, respectively. Other experimental conditions were the same as described in Fig. 2.

constant decreased with increase in the ratio of phosvitin to cytochrome c to close to that for free cytochrome c. This result indicates that the intermolecular electron transfer rate is as low as that for the free form.

Effects of temperature, cytochrome c concentration and solvent viscosity on the electron transfer rate. As shown in Fig. 5, the electron exchange rate in both the free and bound state was dependent on temperature, and the activation energies of the reaction, obtained from the Arrhenius plots, were 10.4 kcal/mol for free cytochrome c and 10.9, 10.8, 10.1 and 9.8 kcal/mol for the bound forms at respective ratios

TABLE II

EFFECT OF SUCROSE CONCENTRATION ON THE RATE OF ELECTRON TRANSFER FOR FREE AND BOUND CYTOCHROMES c

In the stopped-flow cell, CII_h was mixed with $CIII_c$ or $CIII_c$ in the presence of phosvitin. The total concentration of cytochrome c and the ratio of CII_h to $CIII_c$ were fixed at 28 μM and 1.0, respectively. Other experimental conditions were the same as described in Fig. 2. In each case, the electron transfer rate in the sucrose solution was expressed as the value relative to that in buffer only.

Cytochrome c	Added cytochrome c/phosvitin	Relative electron transfer rate [Sucrose] (%, w/v)		
		0	12	24
Free	–	1.00	1.02	1.01
Bound	1.9	1.00	0.72	0.58
	8.5	1.00	0.75	0.55
	19.3	1.00	0.85	0.70

of cytochrome c to phosvitin of 2.3, 7.5, 11.7 and 17.5. The observed first-order rate constant for free cytochrome c was almost proportional to the concentration of cytochrome c (Fig. 2A), while that for the bound form was independent of its concentration (Fig. 2B), the reaction being apparently zero-order. On the contrary, the electron exchange rate for the free form was independent of the concentration of sucrose, whereas that for bound cytochrome c decreased with increase in its concentration (Table II). These differences can be explained by supposing that the electron exchange rate for bound cytochrome c depends on the collision frequency between cytochromes c concentrated on the phosvitin molecule.

Discussion

In the present work, the second-order rate constant for the electron exchange reaction was $(2–3) \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 20°C in 20 mM Tris-HCl buffer (pH 7.4), which is the same order as the values for the exchange reaction between cytochrome c and c_{551} ($1.6 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 4.5°C, pH 7.0 and an ionic strength of 0.2 [5] and $8 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 25°C, pH 7.0 and an ionic strength of 0.1 [6]) and between cytochrome c and c_{553} ($4.0 \cdot 10^4$ $M^{-1} \cdot s^{-1}$ at 25°C in

0.05 M phosphate buffer, pH 7 [8]). The reaction was dependent on temperature with an activation energy of 10 kcal/mol, which is also similar to reported values of 12 and 13 kcal/mol for the reactions between cytochrome *c* and c_{551} [5,6] and between cytochromes *c* [3], respectively.

The electron exchange reaction also occurred in the presence of phosvitin. In our experimental system, it is unclear whether electron transfers occurs between phosvitin-bound cytochromes *c* or between free and bound cytochromes *c*. However, similar results were obtained, whether bound cytochrome *c* was mixed with free or bound cytochrome *c*, indicating that this observed reaction can be regarded as a reaction between bound cytochromes *c*. The possibility of electron transfer between bound cytochromes *c* is also supported by the facts that the electron exchange rate decreased when the ratio of cytochrome *c* to phosvitin exceeds the maximum binding ratio (Fig. 3), and that the observed rate constant was independent of the cytochrome concentration.

The electron transfer rate between phosvitin-bound cytochromes *c* was much greater than that between free cytochromes *c*. Since this reaction between bound cytochromes *c* is of two types, 'intramolecular and intermolecular' electron transfer, it is uncertain which reaction rate was high. The intermolecular electron transfer rate was low when the binding ratio of cytochrome *c* to phosvitin was extremely low, but at higher ratios, there is no evidence that this intermolecular rate is low. However, the diffusion coefficient of phosvitin is smaller than that of cytochrome *c*, being $5.8 \cdot 10^{-7}$ cm$^2 \cdot$ s^{-1} [21], and the diffusion coefficient of the cytochrome *c*-phosvitin complex must be smaller at higher binding ratios because of increase of its molecular weight, which results in a lower intermolecular electron transfer rate. Independence of the rate constant on the cytochrome concentration also implies that the intermolecular rate is low. Thus, most of the exchange reaction is due to an intramolecular reaction, indicating that the intramolecular electron transfer rate is high on binding to phosvitin.

In considering the reason for this high rate, increase in the collision frequency between cytochromes *c*, the proximity of cytochrome molecules on phosvitin, and the charge effect of phosvitin must be taken into account.

When cytochromes *c* were bound to phosvitin, the apparent first-order rate constant was independent of the cytochrome concentration and was affected by the solvent viscosity, unlike the rate constant for free cytochromes *c*. The rate for the bound form increased with increase in the binding ratio at ratios below half the maximum (Fig. 3). These results suggest that the enhanced electron transfer rate is attributable to increase in the collision frequency between cytochromes *c* concentrated on the phosvitin molecule. This is likely, since phosvitin is a flexible polymer at neutral pH [22–24] and the rotational relaxation time for the segmental motion of a flexible polymer is estimated to be in the order of 1 ns [25,26], two or three orders of magnitude lower than the available time for electron transfer, and since a linear diffusion of bound cytochromes *c* should occur along the phosvitin chain.

Obviously, the distance between cytochromes *c* bound to phosvitin is small. The distance is in the range of 5–10 Å at the maximum binding, assuming that the length of the fully extended phosvitin chain is about 780 Å [20] and cytochrome *c* is a compact spheroid with a diameter of about 30 Å [27]. Since the separation between linking sites for electron transfer by tunneling was estimated to be 8–10 Å by Hopfield [28] and 12–13 Å by Jortner [29], it is postulated that electron tunneling also occurs in the cytochrome *c*-phosvitin complex. However, electron tunneling cannot be a important factor for the enhancement, because the activation energy of the reaction for the bound form was not so small as the value for tunneling [30,31]. A charge of phosvitin also seems ineffective, because the enhanced electron transfer rate decreased with decrease in the binding ratio, that is, increase in the net negative charge.

References

1 Kowarsky, A. (1965) Biochemistry 4, 2382–2388
2 Gupta, R.K., Koenig, S.H. and Redfield, A.G. (1972) J. Magn. Reson. 7, 66–73
3 Gupta, R.K. (1973) Biochim. Biophys. Acta 292, 291–295
4 Oldfield, E. and Allerhand, A. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3531–3535
5 Morton, R.A., Overnell, J. and Harbury, H.A. (1970) J. Biol. Chem. 245, 4653–4657
6 Greenwood, C., Finazzi Agrò, A., Guerrieri, P., Avigliano,

L., Mondovì, B. and Antonini, E. (1971) Eur. J. Biochem. 23, 321–327
7 Keller, R.M., Wütrich, K. and Pecht, I. (1976) FEBS Lett. 70, 180–184
8 Wherland, S. and Pecht, I. (1978) Biochemistry 17, 2585–2591
9 Yu, C.A., Yu, L. and King, T.E. (1973) J. Biol. Chem. 248, 528–533
10 Chien, J.C.W., Gibson, H.L. and Dickinson, L.C. (1978) Biochemistry 17, 2579–2584
11 Chien, J.C.W. (1978) J. Phys. Chem. 82, 2158–2171
12 Antonini, E., Finazzi Agrò, A., Avigliano, L., Guerrieri, P., Rotilio, G. and Mondovì, B. (1970) J. Biol. Chem. 245, 4847–4849
13 Wilson, M.T., Greenwood, C., Brunori, M. and Antonini, E. (1975) Biochem. J. 145, 449–457
14 Rosen, P. and Pecht, I. (1976) Biochemistry 15, 775–786
15 Wood, P.M. (1974) Biochim. Biophys. Acta 357, 370–379
16 Yoshimura, T., Matsushima, A. and Aki, K. (1979) Biochim. Biophys. Acta 581, 316–324
17 Yoshimura, T., Matsushima, A. and Aki, K. (1980) Biochim. Biophys. Acta 625, 100–108
18 Nicholls, P. (1974) Biochim. Biophys. Acta 346, 261–310
19 Margalit, R. and Schejter, A. (1970) FEBS Lett. 6, 278–280
20 Taborsky, G. (1970) Biochemistry 9, 3768–3773
21 Taborsky, G. and Mok, C.-C. (1967) J. Biol. Chem. 242, 1495–1501
22 Jirgensons, B. (1966) J. Biol. Chem. 241, 147–152
23 Perlmann, G.E. and Allerton, S.E. (1966) Nature 211, 1089–1090
24 Timasheff, S.N., Townend, R. and Perlmann, G.E. (1967) J. Biol. Chem. 242, 2290–2292
25 Nishijima, Y., Teramoto, A., Yamamoto, M. and Hiratsuka, S. (1967) J. Polym. Sci. A-2, 5, 23–35
26 Teramoto, A., Hiratsuka, S. and Nishijima, Y. (1967) J. Polym. Sci. A-2, 5, 37–45
27 Dickerson, R.E. and Timkovich, R. (1976) in The Enzymes (Boyer, P.D., ed.), Vol. 11, pp. 397–547, Academic Press, New York
28 Hopfield, J.J. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 3640–3644
29 Jortner, J. (1976) J. Chem. Phys. 64, 4860–4867
30 DeVault, D. and Chance, B. (1966) Biophys. J. 6, 825–847
31 Chance, B. (1974) Ann. N.Y. Acad. Sci. 227, 613–626