

## Kinetic Studies of the Interaction of Ferricytochrome c with Potassium Ferrocyanide by a Chemical Relaxation Technique

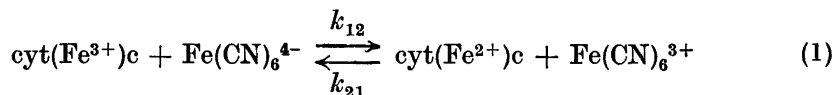
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A chemical relaxation technique, the temperature-jump method, is applied to the study of the kinetics of the interaction of ferricytochrome c with potassium ferrocyanide. The rate constants for both directions of the electron transfer were evaluated. The equilibrium constant derived from these rate constants agreed well with data obtained from spectrophotometric titration and measurement of potentials.

The pH-dependence of the rate constant in the forward direction was determined. In general, this curve followed the protolytic titration curve (and  $E^\circ$ ) but specific effects of considerable magnitude were found in the vicinity (within  $\pm 1/4$  pH-unit) of pH 7.1, 9.0, (rate increase) and 4.8 (rate decline). The narrowness and symmetry of these extrema indicate that the origin is interaction between conjugate pairs of ionizing groups in the sidechains, possibly carboxyl groups and heme-bound and non-heme-bound imidazole groups.

The »temperature-jump» method has been used to elucidate the kinetics of a great variety of chemical reactions,<sup>1</sup> but so far its application to biological pigments has been confined to a gross characterization of complicated systems.<sup>2</sup> However, the method can readily be used to evaluate the rate constants of the electron transfer reaction between a respiratory protein as cytochrome c\* and a redox couple as ferricyanide/ferrocyanide and possibly also for a study of conformational changes in the protein which appear to accompany the electron transfer. The reaction:



has previously been studied by Sutin and Christman,<sup>3</sup> who measured  $k_{21}$

\* Horse heart ferricytochrome c, prepared according to Keilin and Hartree, saltfree, twice crystallized, purchased from Boehringer & Soehne, Mannheim. Affinity for carbon monoxide found to be minimal (Tso's method).<sup>7</sup>

with a stopped-flow apparatus. The other rate constant,  $k_{12}$ , was not measurable by that technique. The expression for the relaxation rate,  $1/\tau$  ( $\tau$  = relaxation time), for the equilibrium above (1), is:

$$1/\tau = k_{21}(K(c_1 + c_0 - 2x) + 2x) \quad (2)$$

where  $c_1$  = initial concentration of  $K_4Fe(CN)_6$

$c_0$  = " " " ferricytochrome c

$x$  = equilibrium " " ferrocyclochrome c

$x$  = " " " ferricyanide,

$x = (-c_1 + \sqrt{c_1^2 + 4c_1c_0/K}) (K/2)$ , since  $c_0 \ll c_1$  and  $K \ll 1$ ,

$$K = k_{12}/k_{21} \quad (3)$$

The initial concentrations of ferrocyclochrome c and ferricyanide are zero. A plot of  $1/\tau$  versus  $(K(c_0 + c_1 - 2x) + 2x)$  yields  $k_{21}$  (Figs. 1 and (2)). The equilibrium constant,  $K$ , was determined by spectrophotometric titration (this value was used in Fig. 1) and by measurement of potentials. The other rate constant,  $k_{12}$ , was calculated from  $K$  and  $k_{21}$  (3). The parameters  $k_{12}$  and  $k_{21}$  can also be evaluated from a plot of  $1/\tau$  versus  $(c_0 + c_1 - 2x)$  using data in the range where  $x$  is independent of  $c_1$  ( $x \cong c_0$ ). The concentration  $c_0$  was kept constant in that series. The agreement between the values of  $K$ ,  $k_{12}$ ,

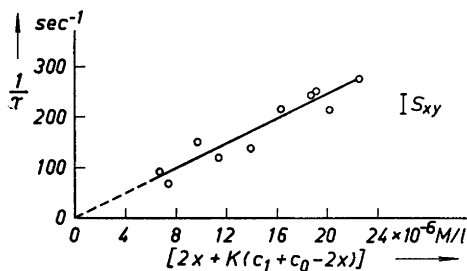


Fig. 1. Graph for evaluation of the rate constant of the reaction of ferricytochrome c with potassium ferrocyanide at pH 7.00 and 12°C in 0.14 M tris buffer. Ionic strength: 0.17 (tris,  $KNO_3$ ). Ferricytochrome c ( $c_0$ ):  $7.0 \times 10^{-6}$  –  $12.0 \times 10^{-6}$  moles/l; potassium ferrocyanide ( $c_1$ ):  $1.0 \times 10^{-3}$  –  $12.4 \times 10^{-3}$  moles/l. The line is calculated by linear regression. Slope =  $k_{21} \text{ sec}^{-1} \text{ M}^{-1}$ . The other symbols: See text. Standard error of the regression:  $s_{xy} = \pm 22 \text{ sec}^{-1}$ .

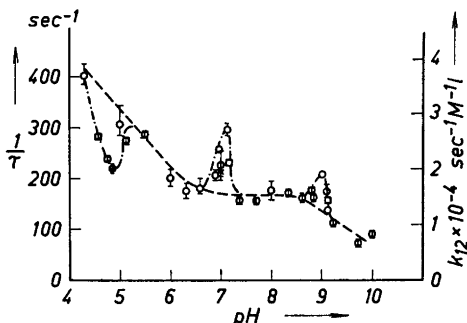


Fig. 2. The pH-dependence of the relaxation rate and  $k_{12}$  for the cytochrome c-potassium ferricyanide/potassium ferrocyanide system at 12°C. Tris, acetate and phosphate buffers, 0.01–0.15 M. Symbols: O, 0.26 M ionic strength;  $\times$ , 0.17 M ionic strength. Cytochrome c:  $2.5 \times 10^{-5} \pm 0.3 \times 10^{-5}$  M assuming a molecular weight of 12 000.  $K_4Fe(CN)_6$ :  $1.09 \times 10^{-2} \pm 0.03 \times 10^{-2}$  M. The estimated error is indicated by a vertical line through the point. Wavelength:  $540 \pm 5 \text{ m}\mu$  ( $\alpha$ -band). Each point represents the average of about 6 observations. In general: One solution for each point. Single observation:  $\square$ . Extrapolated to time of mixing:  $\bullet$ . — — — This curve is parallel to the titration curve. - - - Specific effects.

pH was measured at 23°C instead of at 12°C. The pH-values above 7 should therefore be raised about 0.05 pH-unit.

Table 1. Kinetic and static parameters characterizing the reaction (1).

Origin of data	$k_{21} \times 10^{-7}$ sec <sup>-1</sup> M <sup>-1</sup> l	$k_{12} \times 10^{-4}$ sec <sup>-1</sup> M <sup>-1</sup> l	$K \times 10^3$	$-E^\circ_{\text{FCN}}$ mV <sup>a</sup>	$-E^\circ_{\text{cyt}}$ mV
Ref. 3, 25°, pH 6.0 phos.buf., $\mu = 0.1$	1.6 ± 1				
This work, 12°, pH 7 tris, <sup>b</sup> $\mu = 0.17$	1.23 ± 0.10 <sup>c</sup>				
Spectrophot., 12.9° Measurement of potentials, 13.9°			0.91 ± 0.01 <sup>c</sup>		
From $k_{12}/k_{21}$ , 12°			1.33 ± 0.05 <sup>c</sup>		
This work, 14°, pH 7			1.27 ± 0.28		297 ± 2 <sup>c,d</sup>
Ref. 4, 14°, pH 7					260
This work, 19.9°				443 ± 2 <sup>c, d</sup>	
This work, 14.0°				461 ± 2 <sup>c, d</sup>	
Ref. 5, 25°				460	
From $1/\tau$ vs. ( $c_0 + c_1 - 2x$ )	1.20 ± 0.30 <sup>c</sup>	1.56 ± 0.32 <sup>c</sup>			
From $K \times k_{21}$		1.12 ± 0.27			

<sup>a</sup> Standard potential of the potassium ferricyanide/potassium ferrocyanide couple referred to the H<sub>2</sub>/2H<sup>+</sup> half cell.

<sup>b</sup> Tris(hydroxymethyl)amino methane.

<sup>c</sup> Standard error.

<sup>d</sup> Measured with Radiometer pH-meter and Radiometer Pt-electrode. Temperature control with water jacket around sample cell.

and  $k_{21}$  determined by different methods and between the experimental and the literature values of  $k_{21}$  and the standard potentials,  $E^\circ$ , is good (Table 1).

The reaction was followed by measurement of the spectral changes in the  $\alpha$ -band (540 m $\mu$ ), which is characteristic of the reduction of cytochrome c. The degree of reduction was determined before and after each rate experiment since autoxidation, if excessive, affects the measurement. The protolytic equilibria do not influence  $\tau$  under the present conditions.

The temperature dependence of the  $1/\tau$ -value, which here equals that of  $k_{12}$  ( $1/\tau = k_{12}c_1$ ,  $c_1 = \text{const.}$ ,  $c_1 \gg c_0$ ,  $x$ ), was found to be only slight (energy of activation for reaction  $1 \rightarrow 2 = \text{ca. } 2 \text{ kcal/equiv.}$ ) at pH 7.0 and ionic strength 0.17 (range 12–25°C). Usually, the value for electron transfer falls in the range: 7–14 kcal/equiv.

An attempt was made to gain some insight in the function of the protein moiety. For this purpose, the following systems, which both lack protein, were tested in the presence of Fe(CN)<sub>6</sub><sup>4-</sup>/Fe(CN)<sub>6</sub><sup>3-</sup>:

protoporphyrin IX + Fe<sup>3+</sup>/Fe<sup>2+</sup> + 2 histidine and  
Fe<sup>3+</sup>/Fe<sup>2+</sup> + 2 histidine.\*

In both cases, a relaxation effect similar to that found for complete cytochrome c (but faster) appeared. Thus, the protein is not essential to the appearance of the effect studied. The latter must be associated with the iron atom.

\* A suspension of a blue complex was soon formed, but the relaxation effect persisted.

Table 2. Interaction of ferricytochrome c with a number of redox couples.

System	Reaction	Reduction of cyt(Fe <sup>3+</sup> ) c	Relaxation effect	Comment
1	Cyt(Fe <sup>3+</sup> ) c × K <sub>4</sub> Fe(CN) <sub>6</sub>	+	+	See Table 1
2	» + dichlorophenol- indophenol	+	+	τ < 10 μsec
3	» + <i>p</i> -phenylene- diamine	+	+	τ < 10 μsec
4	» + Na <sub>2</sub> S <sub>2</sub> O <sub>4</sub>	+	+	τ < 10 μsec
5	» + K <sub>3</sub> Fe(CN) <sub>6</sub>	—	—	No reaction

A number of redox couples were tested in an attempt to detect a possible specificity in the interaction with cytochrome c (Table 2). The systems 1–4 appear to act in accordance with the potential, *i.e.* the change in free energy. No indication of specificity was found. Case 5 shows that the relaxation effect only appears when ferricytochrome is partially reduced. This confirms the view that an electron transfer is observed.

The pH-dependence of the relaxation rate,  $1/\tau$ , which within experimental error is proportional to that of  $k_{12}$  ( $1/\tau = k_{12}c_1$ ,  $c_1 = \text{const.}$ ), was measured because it might reveal the identity of groups essential to the observed reaction. The general rise in relaxation rate (and in  $k_{12}$ ) toward low pH-values is probably mainly due to changes in the net charge of the molecule which unfolds the protein by electrostatic repulsion. This may make the heme group more accessible to the other redox couple. The narrow peak near pH 7.1 may be interpreted as due to catalysis of electron transfer by 2 groups, presumably non-heme-bound imidazole sidechains, in a co-operative fashion. Only one of the groups of  $pK$  7.1 should be protonated for maximal effect. Similarly, the peak at pH 9.0 points toward the occurrence of conjugate catalysis by 2 groups of  $pK$  9.0, possibly the 2 histidine groups which are covalently bonded to Fe.<sup>6,8</sup> The rate minimum near pH 4.8 appears to implicate the carboxyl groups. Again, a pairwise interaction seems to operate. The pH-dependence of  $k_{12}$  is probably sooner due to variations in the protein stability than in the resonance energy since the sensitivity of the  $\alpha$ -band to pH near neutrality is minor. Moreover, the increase in  $k_{12}$  is paralleled by an increase in the rate of autoxidation which is known to be accompanied by protein denaturation and inactivation with respect to coupling with the cytochrome oxidase system. A blue precipitate is formed when a solution of cytochrome c and ferrocyanide is left for 10 h at pH 4.3–4.5 and room temperature. Iron must have been removed from cytochrome to form a ferrocyanide complex after major protein denaturation.

This example shows the ease with which kinetic data for mechanistic studies of biological reactions such as those involving pigments can be collected by use of a chemical relaxation technique and the variety of such data.

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