Biochimica et Biophysica Acta, 548 (1979) 161—165

BBA Report

BBA 41317

INTRAMOLECULAR ELECTRON TRANSFER AND BINDING CONSTANTS IN IRON HEXACYANIDE-CYTOCHROME *c* COMPLEXES AS STUDIED BY PULSE RADIOLYSIS

YIGAL ILAN[a] and AVIGDOR SHAFFERMAN[b,*]

[a] *Department of Physical Chemistry, The Hebrew University and* [b] *Israel Institute for Biological Research, P.O.B. 19, Ness Ziona (Israel)*

(Received July 12th, 1979)

Key words: Cytochrome c; Iron hexacyanide; Electron transfer; Pulse radiolysis

Summary

Internal oxidation and reduction rates of horse cytochrome *c* in the complexes CII·Fe(III)$(CN)_6^{3-}$ and CIII·Fe(II)$(CN)_6^{4-}$, are $4.6 \cdot 10^4 s^{-1}$ and $3.3 \cdot 10^2 s^{-1}$, respectively. The binding site of the iron hexacyanide ions on either CII or CIII are kinetically almost indistinguishable; binding constants range from $0.87 \cdot 10^3$ to $2 \cdot 10^3 M^{-1}$. The present pulse radiolytic kinetic data is compared with that from NMR, T-jump and equilibrium dialysis studies.

The redox reactions between cytochrome *c* and iron hexacyanides:

$$\text{CII} + \text{Fe(III)}(\text{CN})_6^{3-} \underset{k_{red}}{\overset{k_{ox}}{\rightleftharpoons}} \text{CIII} + \text{Fe(II)}(\text{CN})_6^{4-} \quad (1)$$

served as a model system for the study of redox heme-proteins involved in one-electron transfer processes [1—13].

Stellwagen and Shulman [8] were first to propose a detailed reaction scheme for this system:

$$\text{CII} + \text{Fe(III)}(\text{CN})_6^{3-} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{CII}\cdot\text{Fe(III)}(\text{CN})_6^{3-} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{CIII}\cdot\text{Fe(II)}(\text{CN})_6^{4-} \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}}$$

$$\text{CIII} + \text{Fe(II)}(\text{CN})_6^{4-} \quad (2)$$

by monitoring changes in line widths of proton resonances of the protein

Abbreviations: CII, ferrocytochrome *c*, CIII, ferricytochrome *c*.

*To whom correspondence should be addressed.

under different concentrations of cytochrome-C and iron hexacyanide ions they [8] have determined the rate for the internal reduction (k_{-2}), calculated K_3, and estimated a value for k_2 (see Table I).

Using stopped flow [4,6,9] and T-jump techniques [2,3,7,10] the overall rate of oxidation k_{ox} ($6-16 \cdot 10^6 M^{-1} \cdot s^{-1}$) was determined, T-jump studies allowed also determination of k_{red} $2.5 \cdot 10^4 M^{-1} \cdot s^{-1}$. We have used the pulse radiolytic technique [14] to study this redox system [10,13,15]. This technique enabled us to measure directly redox processes on a time scale shorter than that available in flow methods and unlike T-jump in the pulse radiolytic method short lived transient not in equilibrium could be followed [10,14,17]. In the present communication, we extend the kinetic information previously presented [10] for this system. Direct evidence is presented for the internal oxidation of CII in the complex CII·Fe(III)$(CN)_6^{3-}$ allowing determination of k_2 and the binding constant K_1. Also on the basis of the results obtained we revaluated the previously estimated values for K_4, K_5 and corrected the value for k_{-2}.

$$\text{CII} + \text{Fe(II)}(CN)_6^{4-} \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} \text{CII} \cdot \text{Fe(II)}(CN)_6^{4-} \qquad (3)$$

$$\text{CIII} + \text{Fe(III)}(CN)_6^{3-} \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} \text{CIII} \cdot \text{Fe(III)}(CN)_6^{3-} \qquad (4)$$

Based on well-determined rates for the reactions of the water redox species (e_{aq}^-, H, OH), generated by the radiolytic pulse, with CII, CIII, Fe(II)$(CN)_6^{4-}$ and Fe(III)$(CN)_6^{3-}$ [10,13], and a judicious choice of concentration of these molecules and scavengers (N_2O or *tert*-butanol [14]) it is possible to follow oxidation of CII by Fe(III)$(CN)_6^{3-}$ in two alternative ways. In one, the starting solution, contains CII and excess of Fe(II)$(CN)_6^{4-}$ over CII, Fe(II)$(CN)_6^{4-}$ is then oxidized by OH radicals, and the reaction between CII and the radiolytically produced Fe(III)$(CN)_6^{3-}$ is followed. Using this approach, we determined k_{ox} and the internal rate (k_{-2}) of reduction of CIII [10] within the newly formed complex CIII·Fe(II)$(CN)_6^{3-}$. The second alternative is to start with a solution containing CIII and Fe(III)$(CN)_6^{3-}$ pulse reduce CIII with e_{aq}^- and H atoms and follow the reaction between Fe(III)$(CN)_6^{3-}$ and the CII produced in situ. In order to follow the internal oxidation of CII in the complex CII·Fe(III)$(CN)_6^{3-}$ it is required that the starting solution will contain a substantial amount of the complex CIII·Fe(III)$(CN)_6^{3-}$. To achieve this we used a large excess of Fe(III)$(CN)_6^{3-}$ over CIII (see Fig. 1). Under such conditions, we generate after the pulse Fe(II)$(CN)_6^{4-}$ besides CII and CII·Fe(III)$(CN)_6^{3-}$. However, the presence of Fe(II)$(CN)_6^{4-}$ in the solution does not complicate the interpretation of the kinetic data for CII oxidation, since reduction of CIII by Fe(II)$(CN)_6^{4-}$ is well time resolved ($\tau_{1/2} \geq 1$ sec; $k_{red} = 1.3-2.5 \cdot 10^4 M^{-1} \cdot s^{-1}$ and [CIII] = $2 \cdot 10^{-5}$M) from the oxidation process of CII. On the other hand due to the competition between Fe(III)$(CN)_6^{3-}$ and CIII for the reducing radicals (e_{aq}^-, H) there exists an upper limit to the concentration of Fe(III)$(CN)_6^{3-}$ (about 2 mM) beyond which the amount of CII produced in situ would be too small to allow accurate kinetic analysis. In

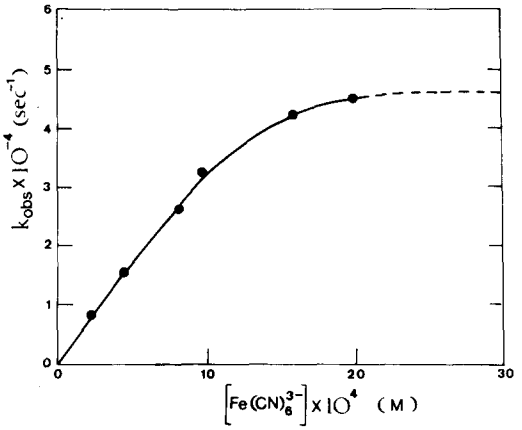

Fig. 1. Dependence of the observed rate constants (k_{obs}) on the concentration of $Fe(III)(CN)_6^{3-}$ in the oxidation process of CII produced in situ. pH = 7.1, $[CIII]_0 = 2 \cdot 10^{-5}$M, the monitored wavelength 550 nm; $I = 0.02$. Each point is an average of 5 independent experiments.

Fig. 1, we present the data obtained in the above described experiments. Fig. 1 clearly demonstrates that the rate of oxidation becomes independent of $Fe(III)(CN)_6^{3-}$ at high concentrations of the latter. At a concentration of 2 mM $Fe(III)(CN)_6^{3-}$ the oxidation observed is therefore mainly that which occurs within the complex $CII \cdot Fe(III)(CN)_6^{3-}$. Extrapolation to higher concentrations of $Fe(III)(CN)_6^{3-}$ yields a value of $4.6 \cdot 10^4 s^{-1}$ for the rate constant of the intramolecular electron transfer. A value which is in good agreement with the estimated rate constant given by Stellwagen and Shulman (see Table I). At low concentrations of $Fe(III)(CN)_6^{3-}$ we would have mainly non-bound CII reacting with $Fe(III)(CN)_6^{3-}$, thus the initial slope in Fig. 1, gives the value of k_{ox} (at $I = 0.02$) which is $4 \cdot 10^7 M^{-1} \cdot s^{-1}$, in good agreement with previously determined rate constants at this ionic strength [10,13,15]. When the amount of complexed CII is negligible, and using the experimental observation that $k_2 \gg k_{-2}$, the derivation of the kinetic law for the oxidation shows that $k_{ox} = k_1 k_2/(k_{-1}+k_2)$. If we assume $k_{-1} > k_2$ we obtain $k_{ox} = k_1 k_2/k_{-1}$ i.e. $K_1 = k_1/k_{-1} = k_{ox}/k_2 = 4 \cdot 10^7/4.6 \cdot 10^4 = 0.87 \cdot 10^3 M^{-1}$. The data in Fig. 1 allow us also to give a more accurate estimate for K_5, which we previously estimated [10] to be approx. $10^4 M^{-1}$. According to Fig. 1 at a concentration of 2 mM $Fe(III)(CN)_6^{3-}$ most of CIII is bound to $Fe(III)(CN)_6^{3-}$ it may be calculated therefore, that $K_5 \simeq 2 \cdot 10^3 M^{-1}$. Note that only binding sites affecting electron transfer can be revealed in our studies. Previously [10] we also estimated a value of approx. $10^4 M^{-1}$ for K_4 based on the fact that only one phase of oxidation was observed in solutions containing prior to the pulse 1—4 mM of $Fe(II)(CN)_6^{4-}$ and 10—50 μM of CII. Thus by calculating K_4 on the assumption that no more than 1% of CII is present in the non-bound state at 1 mM $Fe(II)(CN)_6^{4-}$ we obtained $K_4 = 10^4 M^{-1}$. However, a closer examination of the data indicate that even if 20% of CII was present in a non-bound state (i.e. $K_4 = 2 \cdot 10^3 M^{-1}$) it would have been difficult to observe two phases of oxidation (one with bound CII and the other with non bound CII). Indeed Fig. 1 in Ref. 10 shows that consistent lower rates for oxidation were obtained as the concentration of $Fe(II)(CN)_6^{4-}$ was increased from 1 mM to 4 mM. We

would like therefore to suggest that a more correct estimate for K_4 is $\geqslant 2 \cdot 10^3\ M^{-1}$. This conclusion suggests also that the extrapolated value for k_{-2} (Fig. 1 in Ref. 10) should be derived from rates determined in the presence of 4 mM $Fe(II)(CN)_6^{4-}$. The value thus obtained for k_{-2} is 330 s^{-1} instead of 400 s^{-1}.

Table I summarizes values obtained for the system of study by us and others. The agreement of kinetic data for reactions in Equation 2 obtained by the pulse radiolytic study and NMR studies is remarkable. As shown in Table I

TABLE I

Eqn. 1 $K=k_{ox}/k_{red}$	Eqn. 2				Eqn. 3 K_4	Eqn. 4 K_5
	k_2	k_{-2}	K_1	K_3^{-1}		
220—340 (a,b,c)	$4.6 \cdot 10^4\ s^{-1}$ (d)	$330\ s^{-1}$ (d,a)	$0.87 \cdot 10^3\ M^{-1}$ (d)	—	$2 \cdot 10^3\ M^{-1}$ (d,a)	$2 \cdot 10^3\ M^{-1}$ (d)
	$2 \cdot 10^4\ s^{-1}$ (e)	$208\ s^{-1}$ (e)	—	$0.4 \cdot 10^3\ M^{-1}$ (e)	$0.4—1.0 \cdot 10^3\ M^{-1}$ (f)	$0.4—1.0 \cdot 10^3\ M^{-1}$

[a,b,c] Refs. 10, 7, 3: T-jump studies.
[d] Present study.
[e] Ref. 8.
[f] Ref. 18. Equilibrium dialysis study.

K_1 and K_3^{-1} are similar (within a factor of two) thus the overall equilibrium (Equation 1) may be given by $k_2/k_{-2} = 140$ a value consistent (within a factor of two) with values obtained by T-jump measurements (Table I). Note that while k_2 and k_{-2} are independent of ionic strength all the binding K_1, K_3^{-1}, K_4 and K_5 are ionic strength dependent. One important feature which emerges from Table I is the similarity in the binding of $Fe(II)(CN)_6^{4-}$ or $Fe(III)(CN)_6^{3-}$ to either CII or CIII. While it might be expected that both iron hexacyanides may have similar binding constants with a cytochrome molecule at the same oxidation state, it is not expected that binding to both oxidation states (CII and CIII) would be similar, unless it is assumed that the binding sites are insensitive to the conformational differences between CII and CIII. This conclusion well fits the assumed location of the binding site · · lysines 87, 13, 8 [10] (see also Ref. 19), a location which is hardly affected by the redox state of cytochrome [20].

Acknowledgment

We acknowledge the support of the U.S. ERDA under contract E(11-1)3009, and E(11-1)3221.

References

1 Sutin, N. and Christman, D.R. (1961) J. Am. Chem. Soc. 83, 1773—1774
2 Havsteen, B.H. (1965) Acta Chem. Scand. 19, 1227—1231
3 Brandt, K.G., Parks, P.C., Czerlinski, G.H. and Hess, G.P. (1966) J. Biol. Chem. 241, 4185
4 Morton, R.A., Overnell, J. and Harbury, H.A. (1970) J. Biol. Chem. 245, 4653—4657
5 Lambeth, D.O., Campbell, K.L., Zand, R. and Palmer, G. (1973) J. Biol. Chem. 248, 8130—8136
6 Creutz, C. and Sutin, N. (1974) J. Biol. Chem. 249, 6788—6795
7 Zabinski, R.M., Tatti, K. and Czerlinski, G.H. (1974) J. Biol. Chem. 249, 6125—6129
8 Stellwagen, E. and Shulman, R.G. (1973) J. Mol. Biol. 80, 559—573
9 Cassat, J.C. and Marini, C.P. (1974) Biochemistry 13, 5323—5328

10 Ilan, Y., Shafferman, A. and Stein, G. (1976) J. Biol. Chem. 251, 4336—4345
11 Wherland, S. and Gray, H.B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 2950—2954
12 Peterman, B.F. and Morton, R.A. (1977) Can. J. Biochem. 55, 796—803
13 Ilan, Y. and Shafferman, A. (1978) Biochim. Biophys. Acta 501, 127—135
14 Shafferman, A. and Stein, G. (1975) Biochim. Biophys. Acta 416, 287—317
15 Ilan, Y., Shafferman, A., Feinberg, B.A. and Lau, K.Y. (1979) Biochim. Biophys. Acta 548, in the press
16 Shafferman, A. and Stein, G. (1974) Science 183, 428—430
17 Shafferman, A. and Stein, G. (1977) Biochim. Biophys. Acta 462, 161—170
18 Stellwagen, E. and Cass, R.D. (1975) J. Biol. Chem. 250, 2095—2098
19 Ferguson-Miller, S., Brantigan, D.L. and Margliash, E. (1978) J. Biol. Chem. 253, 149—159
20 Dickerson, R.E. and Timkovich, R. (1975) in The Enzymes (Beyer, P., ed.), 3rd edn. Vol. 11, pp. 397—547, Academic Press, New York