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MASTER

IRON HEXACYANIDE/CYTOCHROME-C - INTRAMOLECULAR
ELECTRON TRANSFER AND BINDING CONSTANTS -
(PULSE RADIOLYTIC STUDY).

PROGRESS REPORT

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Work carried out during the period:
December 1st 1979 to November 30, 1980

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S U M M A R Y

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

The redox reactions between cytochrome-C and iron hexacyanides:

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:

By monitoring changes in line widths of proton resonances of the protein 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 1).

Using stopped flow [4, 6, 9] and T-jump techniques [2, 3, 7, 10] the overall rate of oxidation k_{ox} ($6-16 \cdot 10^6 \text{ M}^{-1} \text{ S}^{-1}$) was determined, T-jump studies allowed also determination of k_{red} $2.5 \cdot 10^4 \text{ M}^{-1} \text{ S}^{-1}$. 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 $\text{CII} \cdot \text{Fe}^{\text{III}}(\text{CN})_6^{-3}$ allowing determination of k_2 and the binding constant K_1 . Also on the basis of the results obtained we reevaluated the previously estimated values for K_4, K_5 and corrected the value for k_{-2} .

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 *t*-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 $CIII \cdot Fe^{II}(CN)_6^{-3}$. 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 $CII \cdot Fe^{III}(CN)_6^{-3}$ it is required that the starting solution will contain a substantial amount of the complex $CIII \cdot Fe^{III}(CN)_6^{-3}$. 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 $CII \cdot Fe^{III}(CN)_6^{-3}$. 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 ($\tau_{1/2} \gg 1$ sec; $k_{red} = 1.3-2.5 \cdot 10^4 M^{-1} s^{-1}$ and $[CIII] = 2 \cdot 10^{-5}$) from the oxidation process of CII. On the other hand due to the competition between $Fe^{III}(CN)_6^{-3}$ and CIII on the reducing radicals (e_{aq}^- , H) there exists an upper limit to the concentration of $Fe^{III}(CN)_6^{-3}$ (~ 2 mM) beyond which the amount of CII produced in-situ would be too small to allow accurate kinetic analysis. In Fig. 1., we present the data obtained in the above described experiments. This Fig. 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 & Shulman (see Table 1). At low concentrations of

$\text{Fe}^{\text{III}}(\text{CN})_6^{-3}$ we would have mainly non-bound CII reacting with $\text{Fe}^{\text{III}}(\text{CN})_6^{-3}$, thus the initial slope in Fig 1., gives the value of k_{ox} (at $\mu=0.02$) which is $4.10^7 \text{M}^{-1}\text{s}^{-1}$, 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 $k_{\text{ox}} = k_1 k_2 / (k_{-1} + k_2)$. If we assume $k_{-1} > k_2$ we obtain $k_{\text{ox}} = k_1 k_2$ or $K_1 = 4.10^7 / 4.6 \cdot 10^4 = 0.87 \cdot 10^3 \text{M}^{-1}$. The data obtained in Fig. 1 allow us also to give a more accurate estimate for K_5 , which we previously estimated [10] to be $\sim 10^4 \text{M}^{-1}$. According to Fig. 1 at a concentration of 2mM $\text{Fe}^{\text{III}}(\text{CN})_6^{-3}$ most of CIII is bound to $\text{Fe}^{\text{III}}(\text{CN})_6^{-3}$ it may be calculated therefore, that $K_5 = 2.10^3 \text{M}^{-1}$. Note that only binding sites affecting electron transfer can be revealed in our studies. Previously [10] we also estimated a value of $\sim 10^4 \text{M}^{-1}$ for K_4 based on the fact that only one phase of oxidation was observed in solutions containing prior to the pulse 1-4mM of $\text{Fe}^{\text{II}}(\text{CN})_6^{-4}$ 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 1mM $\text{Fe}^{\text{II}}(\text{CN})_6^{-4}$ we obtained $K_4 = 10^4 \text{M}^{-1}$. However, a closer examination of the data indicate that even if 20% of CII was present in a non-bound state (i.e. $K_4 = 2.10^3 \text{M}^{-1}$) 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 $\text{Fe}^{\text{II}}(\text{CN})_6^{-4}$ was increased from 1 mM to 4 mM. We would like therefore to suggest that a more correct estimate for K_4 is $\geq 2.10^3 \text{M}^{-1}$. This conclusion suggest also that the extrapolated value for k_{-2} (Fig. 1. ref. 10) should be derived from rates determined in the presence of 4mM $\text{Fe}^{\text{II}}(\text{CN})_6^{-4}$. The value thus obtained for k_{-2} is 330s^{-1} instead of 400s^{-1} .

Table 1 summarizes values obtained for the system of study by us and others. The agreement of kinetic data for reactions in equation B obtained by the pulse radiolytic study and N.M.R studies is remarkable. As shown in this Table K_1 and K_3^{-3} are similar (within a factor of two) thus the overall equilibrium (equation A)

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 1). 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 1 is the similarities in the binding of $\text{Fe}^{\text{II}}(\text{CN})_6^{-4}$ or $\text{Fe}^{\text{III}}(\text{CN})_6^{-3}$ 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 19], a location which is hardly affected by the redox state of cytochrome [20].

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TABLE 1.

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

a,b,c Refs. [10], [7], [3] -T-jump studies

d - Present study

e - [8]

f - [18] Equilibrium dialysis study.

Legend to Figures

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