THE KINETICS OF OXIDATION OF A TRIFLUOROACETYLATED DERIVATIVE OF CYTOCHROME C BY FERRICYANIDE

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Summary: The kinetics of oxidation of a trifluoroacetylated derivative of ferrocytochrome c, which does not bind the oxidizing agent, by ferricyanide were measured. A rate constant of 3.0 x 10^5 $M^{-1}s^{-1}$, an enthalpy of activation of 0.4 kcal/mole and an entropy of activation of -33 e.u. were obtained. Because of the low enthalpy of activation we suggest that the mechanism involves a preequilibrium of the cytochrome c derivative with a conformation which is more easily oxidizable and that the actual electron transfer takes place through this conformation.

A number of recent studies have focused on the oxidation of ferrocytochrome c by inorganic oxidizing agents (1-4). One of the most useful of these is ferricyanide. However it has been observed that the activation energy is near zero for the oxidation of ferrocytochrome c by ferricyanide (5) and by ferricyanide derivatives (6). Based on this observation we proposed two possible mechanisms (6). The first involved a rapid preequilibrium consisting of binding of the ferricyanide to the protein followed by electron transfer. The second involved a preequilibrium of cytochrome c with another conformation which was more easily oxidizable by the oxidizing agent. The first mechanism was strengthened by the demonstration of binding between cytochrome c and ferricyanide by Stellwagen and Cass (7). That the binding was determined by electrostatic interactions between the negatively charged ferricyanide and positively charged lysine residues on the protein was substantiated by the observation that a trifluoroacetylated derivative of cytochrome c, in which all of

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the lysine residues were modified, did not bind ferricyanide. Thus, in order to elucidate the specific role of binding of ferricyanide in the overall mechanism of the oxidation of ferrocytochrome c, we have determined the kinetic parameters of the oxidation of the trifluoroacetylated derivative of cytochrome c by ferricyanide.

Materials and Methods: Trifluoroacetylated cytochrome c was prepared by reacting horse heart cytochrome c (Type III from Sigma) with ethylthioltrifluoroacetate (Aldrich) according to the method of Fanger and Harbury (8). The reduced derivative was prepared by incubating the oxidized species with potassium ascorbate and removing the ascorbate on a Sephadex G-25 column. These procedures were carried out in solutions which had previously been flushed with nitrogen to prevent slow autoxidation. The procedures below were carried out under anaerobic conditions by flushing solutions with nitrogen in flasks equipped with serum caps near the bottom. Transfers were carried out with Hamilton gas tight syringes. E_o values were determined by incubating the derivative with mixtures of $FeCDTA^-$ and $FeCDTA^{2-}$ ($CDTA^{4-}$ is trans-1,2-diaminocyclohexane-N,N,N'N'-tetraacetate) and measuring the ratio of the oxidized to reduced derivative spectrophotometrically on an Acta CV spectrophotometer as had previously been done for myoglobin (9). Based on five points we obtain a value of 0.20 ± 0.01 volts for the reduction potential of the derivative. The kinetic experiments were carried out on an Aminco-Morrow stopped-flow apparatus. The temperature was controlled to $\pm 0.1^o$ with a circulating water bath. In addition, all solutions were brought to the temperature of the experiment by preequilibrium in a water bath.

Results: The kinetic data were determined under pseudo-first order conditions. The ionic strength was maintained at 0.1 M with added KCl; the pH was maintained at 7 with 0.01 M phosphate buffer. The observed rate constant, k_{obs}, for the oxidation of the trifluoroacetylated derivative of ferrocytochrome c was determined as a function of ferricyanide concentration. The results are shown in Figure 1. From the slope of the line, the rate constant is 3.0×10^5 $M^{-1}s^{-1}$ compared to 8.0×10^6 $M^{-1}s^{-1}$ for the oxidation of ferrocytochrome c. Activation parameters were determined by measuring the rate constant as a function of temperature. The results are shown in Figure 2. As in the case of the oxidation of ferrocytochrome c, the oxidation

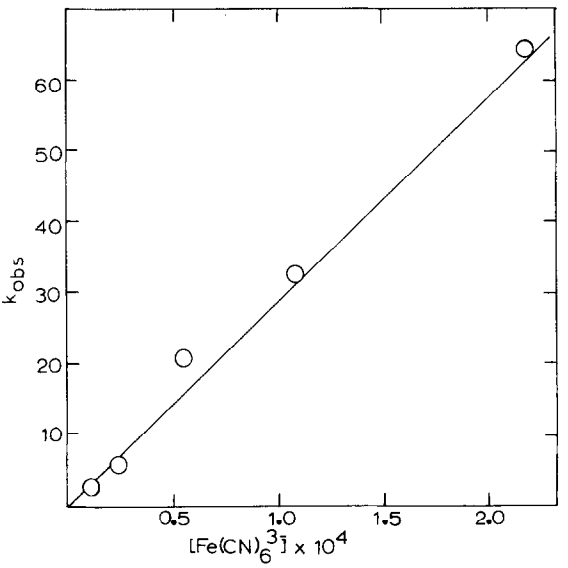

Figure 1. The observed rate constant, k_{obs}, (s^{-1}) as a function of $[Fe(CN)_6^{3-}] \times 10^4$ for the oxidation of the trifluoroacetylated derivative of horse heart ferrocytochrome c by ferricyanide. The ionic strength was 0.1 M and temperature was 25°.

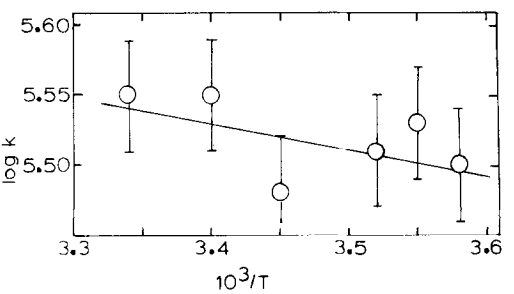

Figure 2. Arrhenius plot for the oxidation of the trifluoroacetylated derivative of horse heart ferrocytochrome c by ferricyanide.

of the derivative is characterized by a near zero enthalpy of activation. A value of 0.4 kcal/mole was obtained compared to that of 1.1 kcal/mole obtained for ferrocytochrome c (6). The difference observed in the rate constants lies in a difference in the entropy of activation with values of -33 e.u. and -20 e.u.

being observed for the derivative and native cytochrome c (6), respectively.

Discussion: Based on the observation that the activation energy for the oxidation of ferrocytochrome c by ferricyanide was near zero, we proposed the two possible mechanisms discussed above as explanations for this phenomenon. The trifluoroacetylated derivative, while structurally similar to the native protein was shown not to bind ferricyanide as a result of the removal of the positive charges on the lysine residues responsible for the binding (7). As a result of this modification, we observe a twenty-fold reduction in the rate constant but, more significantly, the same low activation enthalpy as observed for the oxidation of ferrocytochrome c. The reduction in the rate constant is attended by a reduction in the entropy of activation and can be explained by a reduction in the electrostatic interactions involved in the formation of the activated complex. This is consistent with the decrease in the entropy of activation since in the case of the oxidation of the derivative, there is less charge neutralization and hence more solvent ordering in the formation of the activated complex. In the case of the derivative, the low activation energy cannot be explained by formation of a complex between the protein and the ferricyanide. Thus we are left with the explanation that the protein is in equilibrium with a conformation which is more readily oxidized than the predominant conformation. We would suggest that if true, this conformation resembles the one which exists naturally for oxidized cytochrome c.

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