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AUTOCATALYTIC PEROXIDATION OF FERROCYTOCHROME *c*

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SUMMARY

1. Ferricytochrome *c* acts as a catalyst in the peroxidation of ferrocytochrome *c* thereby giving rise to an autocatalytic reaction.

2. The rate of the peroxidation reaction is proportional to the concentration of H_2O_2 and ferricytochrome *c* but is independent of the concentration of ferrocytochrome *c* in the concentration ranges studied.

3. Integration of the rate equation, $d[c^{3+}]/dt = k[c^{3+}][H_2O_2]$, gives a theoretical expression which fits the experimental time courses for the ferrocytochrome *c* peroxidation reaction.

4. No direct spectral evidence was found for the formation of a catalytically active ferricytochrome *c*–H_2O_2 derivative. Kinetic evidence is presented, however, which indicates the existence of such an intermediate.

5. Ferricytochrome *c* was more susceptible than ferrocytochrome *c* to an apparent degradation reaction caused by excess H_2O_2, thus supporting the idea that the cytochrome *c* heme iron is more accessible in the oxidized form.

INTRODUCTION

In addition to the common peroxidases many other hemoproteins, such as hemoglobin[1], myoglobin[2], and cytochrome *c*[3,4], catalyze peroxidation reactions. The present report demonstrates that ferricytochrome *c* can catalyze the peroxidation of ferrocytochrome *c* in a manner which may be analogous to the classical peroxidases. Because the catalyst, ferricytochrome *c*, is also the reaction product, the peroxidation is autocatalytic. The peroxidase activity of ferricytochrome *c* towards ferrocytochrome *c* is considerably lower than that of yeast cytochrome *c* peroxidase[5]. It is, however, large enough to complicate the time courses for the peroxidation of ferrocytochrome *c* catalyzed by both yeast[5–7] and horseradish peroxidases[8].

MATERIALS AND METHODS

Horse heart cytochrome *c* (Sigma Type III) was employed directly without further purification. No marked effects of CO on the present reaction kinetics were observed. This indicates a negligible contribution by possible trace amounts of modified cytochrome *c*. The reduced form (approx. 93%) was prepared by passing a dithionite-

reduced sample through a Sephadex G-25 column[9]. The oxidized form was prepared by the addition of a 10-fold excess of $K_3Fe(CN)_6$ followed by dialysis against 0.5 % NaCl. H_2O_2 (30 % Superoxol obtained from Merck) was calibrated using cytochrome *c* peroxidase[9]. Spectrophotometric measurements were carried out with a Cary (Model 15) recording spectrophotometer. The kinetics of ferrocytochrome *c* oxidation were measured by following the decrease in absorbance at 550 mμ. The temperature was 26°.

RESULTS

Fig. 1, Trace A illustrates the oxidation of ferrocytochrome *c* by H_2O_2. As can be seen, a low initial rate is followed by an acceleration. This suggested that the reaction was catalyzed by a reaction product. Accordingly, when the experiment was repeated with the addition of ferricytochrome *c*, it was found, that the peroxidation rate was increased (Trace B).

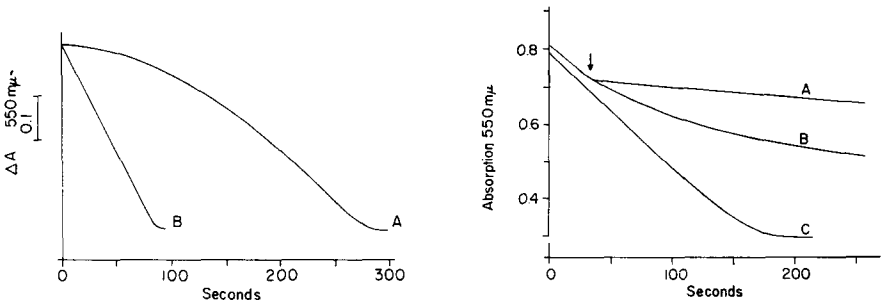

Fig. 1. Time course of cytochrome *c* oxidation by H_2O_2. Ferrocytochrome *c* oxidation was followed by measuring the decrease in absorbance at 550 mμ upon the addition of 176 μM H_2O_2 to solutions containing a mixture of ferro- and ferricytochrome *c* in 0.05 M sodium acetate buffer (pH 6.0, 26°). Trace A was obtained with 23.8 μM ferrocytochrome *c* and 1.8 μM ferricytochrome *c*, Trace B with 23.8 μM ferrocytochrome *c* and 15.8 μM ferricytochrome *c*.

Fig. 2. Inhibition of the peroxidation of cytochrome *c*. Effect of inhibitors on the oxidation of 20.0 μM ferrocytochrome *c* by 176 μM H_2O_2 observed in the presence of 9.0 μM ferricytochrome *c* in 0.2 M potassium phosphate buffer (pH 6.0): A, + 3.3 mM KCN; B, + 3.3 mM NaN_3; C, + 3.3 mM NaF and without inhibitor.

Further evidence for the participation of ferricytochrome *c* in the peroxidation of ferrocytochrome *c* comes from the inhibition studies shown in Fig. 2. Cyanide and azide, ligands that form complexes with ferricytochrome *c*, are inhibitors of the oxidation reaction[10]. On the other hand, fluoride, another classical heme binding ligand which does not, however, form a complex with ferricytochrome *c* at this pH, has no effect on the rate of peroxidation of ferrocytochrome *c*. From the degree of inhibition by cyanide and azide, the respective approximate values for the dissociation constants, K_d, are estimated to be 0.7 mM and 12 mM. These values are in the range of reported K_d values for similar conditions of 1 mM for cyanide[11], and 150 mM for azide[12].

A slight pH optimum in the range 3.6–6.0 for the peroxidation reaction was localized around 4.9. All the following experiments were performed at that pH. The dependency of the initial rate of the reaction on ferricytochrome *c* concentration is presented in Fig. 3, and can be seen to be linear. The initial reaction rate also increases

linearly with the concentration of H_2O_2 (Fig. 4). The data from Figs. 3 and 4 give values of 84 and 79 $M^{-1} \cdot sec^{-1}$, respectively, for the bimolecular rate constant of the ferricytochrome c–H_2O_2 reaction. In contrast to the effects of H_2O_2 and ferricytochrome c, the initial reaction rate does not depend on the concentration of ferrocytochrome c (Fig. 5), at least down to a concentration of 2 μM of this substance.

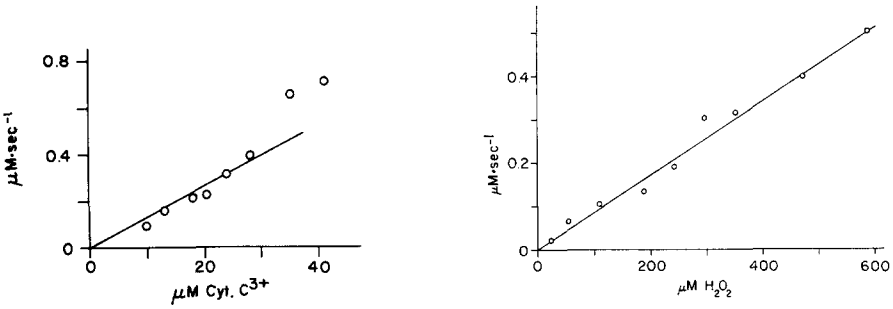

Fig. 3. Effects of ferricytochrome c on the initial rate of ferrocytochrome c peroxidation. The initial velocities for the oxidation of 29 μM ferrocytochrome c by 176 μM H_2O_2 in 0.05 M sodium acetate buffer (pH 4.9, 26°) were measured with varying quantities of ferricytochrome c.

Fig. 4. Effect of H_2O_2 on the initial velocity of ferrocytochrome c oxidation. The initial oxidation rate of 32 μM ferrocytochrome c in the presence of 10.6 μM ferricytochrome c was measured with varying amounts of H_2O_2. The reactions were run in 0.05 M sodium acetate buffer (pH 4.9, 26°).

Since the above studies suggested that ferricytochrome c participated in the peroxidation of ferrocytochrome c it was of interest to determine whether a peroxide derivative of ferricytochrome c could be detected. The addition of a stoichiometric amount of H_2O_2 to 7 μM ferricytochrome c had no effect on the spectrum of ferricytochrome c in the visible or ultraviolet regions. However, in agreement with the observations of others[13] the presence of a 10-fold excess H_2O_2 caused a slow decrease in absorbance in the Soret region. Measuring the absorption spectrum in the Soret region at various time intervals revealed that the absorption decreased equally in all regions of the spectrum. This indicated that excess H_2O_2 caused a degradation of ferricyto-

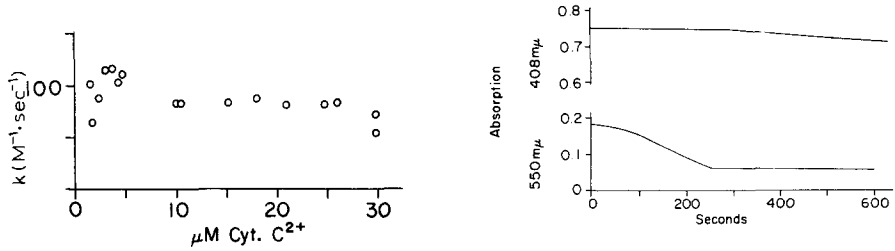

Fig. 5. Effects of varying ferrocytochrome c on the initial rate of ferrocytochrome c peroxidation. The initial rates of ferrocytochrome c peroxidation by 176 μM H_2O_2 at varying initial concentrations of ferrocytochrome c were measured in 0.05 M sodium acetate buffer (pH 4.9, 26°). The initial velocities thus obtained were divided by the peroxide concentration and the appropriate concentration of ferricytochrome c to give the rate constant k.

Fig. 6. Comparison of ferrocytochrome c peroxidation with ferricytochrome c degradation. The time course for the peroxidation of 7.2 μM ferrocytochrome c by 176 μM H_2O_2 and 2 μM ferricytochrome c in 0.05 M sodium acetate buffer (pH 4.9) was monitored at 550 mμ and 409 mμ (an isosbestic point for the ferrocytochrome c–ferricytochrome c couple).

chrome *c*. The finding that only 75 % of the ferricytochrome *c* was dithionite reducible after treatment with excess H_2O_2 supported this view. The observation of this degradation raises the question as to whether the degraded form of ferricytochrome *c*, rather than the native protein, is the catalyst in the peroxidation of ferrocytochrome *c*. However, this possibility is ruled out by the result of an experiment where the degradation of ferricytochrome *c* was examined during the peroxidation of ferrocytochrome *c* (Fig. 6). Here the peroxidation of ferrocytochrome *c* at 550 mμ is compared to that followed at 409 mμ (an isosbestic point of the ferrocytochrome–ferricytochrome *c* couple). It is clear that the major degradation reaction does not begin until all the ferrocytochrome *c* has been oxidized. It was also noted that, like ferrocytochrome *c* peroxidation, the degradation reaction of ferricytochrome *c* could be inhibited by cyanide.

DISCUSSION

The experiments discussed above demonstrated rather conclusively that ferricytochrome *c* can catalyze the peroxidation of ferrocytochrome *c*. The initial reaction rate varies linearly with the concentrations of ferricytochrome *c* and H_2O_2, and is independent of ferrocytochrome *c* at concentrations greater than 2 μM. For high concentrations of ferrocytochrome *c*, then, the rate law for the overall reaction is

$$-\frac{d[c^{2+}]}{dt} = k[c^{3+}]\,[H_2O_2] \qquad (1)$$

The conservation of cytochrome *c* gives the equation:

$$[c^{2+}] + [c^{3+}] = [c^{2+}]_0 + [c^{3+}]_0 \qquad (2)$$

and the assumption that one molecule of H_2O_2 oxidizes two molecules of ferrocytochrome *c* yields:

$$[H_2O_2] = [H_2O_2]_0 - \tfrac{1}{2}([c^{2+}]_0 - [c^{2+}]) \qquad (3)$$

where the suffix 0 indicates initial concentration. Integration of Eqn. 1, using the condition $[c^{2+}] = [c^{2+}]_0$ for $t = 0$, and the mass conservation Eqns. 2 and 3 yields:

$$t = \frac{2}{k([c^{3+}]_0 + 2[H_2O_2]_0)}\left(\log_e \frac{2[H_2O_2]_0}{[c^{3+}]_0} - \log_e \frac{2[H_2O_2]_0 - [c^{2+}]_0 + [c^{2+}]}{[c^{3+}]_0 + [c^{2+}]_0 - [c^{2+}]}\right) \qquad (4)$$

Fig. 7 compares the theoretical points calculated from Eqn. 4 with the experimental time courses at different initial ferricytochrome *c* concentrations. It can be seen that the fit is perfect except for the lower portions of the curves where ferrocytochrome *c* may become limiting. In the calculation of the theoretical curves in Fig. 7, the rate constant which gives the best fit was determined in each case. The values of the rate constant determined in this way were found to vary between 70 and 80 $M^{-1} \cdot sec^{-1}$.

Although no spectral evidence was found for the existence of a ferricytochrome *c*–H_2O_2 complex, the inhibition of ferrocytochrome *c* peroxidation by cyanide and azide suggests that the formation of such a derivative is proceeding in a manner analogous to the common peroxidases (*i.e.* reaction of the heme prosthetic group with peroxide). The cyanide inhibition of the H_2O_2 degradative reaction of ferricytochrome

c provides additional support for this view. The above kinetic results, then, might be interpreted in terms of the classical peroxidatic mechanism[14] where the experimental rate law (*cf.* Eqn. 1) could arise because the formation of a reactive ferricytochrome *c*–H_2O_2 intermediate is rate limiting. The lack of spectrally detectable quantities of the intermediate when ferricytochrome *c* is treated with H_2O_2 could be explained by a high dissociation rate for this compound which results in a low equilibrium concentration.

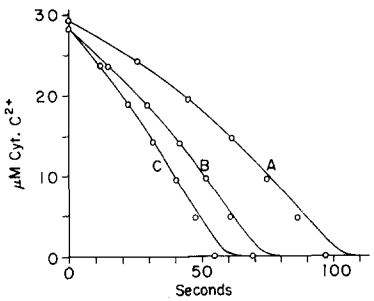

Fig. 7. Theoretical fit to the experimental time courses for ferrocytochrome *c* peroxidation. The theoretical points calculated from Eqn. 4 are compared with experimental time courses obtained in 0.05 M sodium acetate buffer (pH 4.9) with approx. 28 μM ferrocytochrome *c*, 176 μM H_2O_2 and the following ferricytochrome *c* concentrations: A, 13.1 μM; B, 18.0 μM; C, 24.3 μM.

The finding that ferricytochrome *c* is only degraded by H_2O_2 in the absence of ferrocytochrome *c* indicates that the postulated ferricytochrome *c*–H_2O_2 derivative may also be an intermediate in the degradation reaction. As long as ferrocytochrome *c* is present the steady state concentration of the intermediate is low. However, when ferrocytochrome *c* is no longer available to reduce the intermediate, its steady state concentration increases so that now the degradative reaction with H_2O_2 will be more rapid.

It is of additional interest to note that the results in Fig. 6 illustrate that only the oxidized form of cytochrome *c* is significantly degraded by H_2O_2. Thus the reaction with H_2O_2 can be added to the long list of physicochemical and reactivity differences between ferro- and ferricytochrome *c* (see review by MARGOLIASH AND SCHEJTER[15]). All of these observations suggest that the heme iron is more accessible in the oxidized form.

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REFERENCES

1 D. KEILIN AND E. F. HARTREE, *Biochem. J.*, 49 (1951) 88.
2 P. GEORGE AND D. H. IRVINE, *Nature*, 168 (1951) 164.
3 Y. NAKAMURA, T. SAMEJIMA, K. KURIHARA, M. TOKJO AND K. SHIBATA, *J. Biochem. Tokyo*, 48 (1960) 862.

4 T. FLATMARK, *Acta Chem. Scand.*, 20 (1966) 1470.
5 T. YONETANI AND G. S. RAY, *J. Biol. Chem.*, 241 (1966) 700.
6 J. BEETLESTONE, *Arch. Biochem. Biophys.*, 89 (1960) 35.
7 P. NICHOLLS AND E. MOCHAN, in preparation.
8 P. NICHOLLS, *Arch. Biochem. Biophys.*, 106 (1964) 25.
9 T. YONETANI, *J. Biol. Chem.*, 240 (1965) 4509.
10 W. D. BUTT AND D. KEILIN, *Proc. Roy. Soc. London, Ser. B*, 156 (1962) 429.
11 P. GEORGE AND C. L. TSOU, *Biochem. J.*, 50 (1952) 440.
12 B. L. HORECKER AND J. N. STANNARD, *J. Biol. Chem.*, 172 (1948) 589.
13 P. J. O'BRIEN, *Biochem. J.*, 100 (1966) 68P.
14 B. CHANCE, *Advan. Enzymol.*, 12 (1951) 153.
15 E. MARGOLIASH AND A. SCHEJTER, *Advan. Protein Chem.*, 21 (1966) 113.