BBA 45981

THE NATURE OF COMPLEX FORMATION BETWEEN CYTOCHROME *c* AND CYTOCHROME *c* PEROXIDASE

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(Received February 23rd, 1970)

SUMMARY

1. The nature of the forces involved in complex formation between cytochrome *c* and yeast cytochrome *c* peroxidase have been examined.

2. Polycations (*e.g.* polylysine of 3000 and 150000 mol. wt. and salmine) act as competitive inhibitors of cytochrome *c* peroxidation by cytochrome *c* peroxidase thereby supporting the previous ideas that electrostatic forces play a predominant role in complex formation between the two proteins.

3. Kinetic and physical studies employing acetylated and guanidinated cytochromes *c* provide additional support for the significance of electrostatic interactions in complex formation and stress the importance of the net positive charge on cytochrome *c* rather than the lysine residues *per se*.

4. Cytochrome *c* also forms a Sephadex-detectable complex with the dimethyl protoheme ester derivative of cytochrome *c* peroxidase. This observation, along with other kinetic studies, suggests that the cytochrome *c* binding site on cytochrome *c* peroxidase may be associated with the protein rather than the heme.

5. The similarities of cytochrome *c* peroxidase in its reaction with cytochrome *c* to that of mammalian cytochrome oxidase are discussed.

INTRODUCTION

Kinetic[1,2] and physical[3] studies have indicated that cytochrome *c* can form a reversible complex in a 1 to 1 molar ratio with yeast cytochrome *c* peroxidase. Such an interaction has also recently been indicated by DROTT[4] employing a spin-labeled cytochrome *c*. The inhibition of the cytochrome *c* peroxidase-catalyzed cytochrome *c* peroxidation reaction by high ionic strengths and polycations[2] supported the idea that electrostatic forces[5] play a predominant role in complex formation. This report contains a further analysis of the polycation inhibition kinetics along with a study of the essential groups on cytochrome *c* necessary for the formation of complexes with cytochrome *c* peroxidase.

Recently ASAKURA AND YONETANI[6], employing synthetic cytochrome *c* peroxidases, have shown the importance of the side chains at positions 6 and 7 of the

Abbreviations: PL-3, polylysine, mol. wt. 3000; PL-150, polylysine, mol. wt. 150000.

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porphyrin ring for the peroxidase activity of cytochrome *c* peroxidase. In addition, from their preliminary kinetic studies, they had suggested that modification of these positions may interfere with electron transfer between the hemes in the enzyme and cytochrome *c*. In view of these observations, the ability of cytochrome *c* to form complexes with the dimethyl protoheme ester derivative of cytochrome *c* peroxidase was also examined.

The results from the present study strengthen the idea that electrostatic forces associated with the protein moieties are predominant in cytochrome *c* complex formation with cytochrome *c* peroxidase and further stress the kinetic[7,8] and physical[9] similarities of this enzyme in its reaction with cytochrome *c* to that of mammalian cytochrome oxidase. A preliminary account of this work has appeared previously[10].

MATERIALS AND METHODS

Cytochrome *c* peroxidase was prepared from bakers yeast according to the method of YONETANI AND RAY[11]. The studies employed a twice-recrystallized fraction of the enzyme (purity index $A_{408\,m\mu}/A_{230\,m\mu} = 1.25$) or a highly purified form (*e.g.* homogeneous in the ultracentrifuge and gave one band on disc electrophoresis) with a purity index of 1.1. Both enzymes behaved similarly. The dimethyl protoheme derivative of cytochrome *c* peroxidase was a generous gift from Dr. T. Asakura and was prepared as described by ASAKURA AND YONETANI[6]. The concentrations of cytochrome *c* peroxidase and dimethyl protoheme cytochrome *c* peroxidase were determined employing extinction coefficients at 408 mμ of 93 $mM^{-1}\cdot cm^{-1}$ and 81 $mM^{-1}\cdot cm^{-1}$, respectively.

Horse heart cytochrome *c* (Type III), salmine sulfate (Grade I) and polylysine of molecular weights 3000 (Type II) (PL-3) and 150000 (Type I) (PL-150) were obtained from the Sigma Chemical Co. The reduced form of cytochrome *c* was prepared by passing a $Na_2S_2O_4$-reduced sample through a Sephadex G-25 column[12]. 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[13].

Guanidinated cytochrome *c* was essentially prepared as described by HETTINGER AND HARBURY[14]. Cytochrome *c* (200 mg) was added to 20 ml of 0.5 M *o*-methylisourea (obtained from Aldrich)at pH 8.5, and the pH was adjusted to 11.0 with 6 M NaOH. The reaction was stirred at 4° for approx. 120 h, dialyzed against distilled water and finally against 0.5 M potassium phosphate buffer (pH 7.0). Denatured material was removed from the dialysate by passing through a Sephadex G-25 column. The eluate was then adsorbed onto carboxymethylcellulose (previously equilibrated with 0.01 M potassium phosphate buffer (pH 6.0) and subjected to a stepwise elution fractionation. One major homogeneous protein peak, which was eluted with 0.2 M potassium phosphate buffer (pH 6.0) was observed.

The oxidized and reduced spectra of the derivative at pH 7.0 were identical with that of native cytochrome *c*. At alkaline pH (10.5), however, the spectrum of the oxidized form of this derivative differed from native cytochrome *c* in that the 528-mμ absorption was broadened and shifted toward the red, and two additional broad bands at 570 and 600 mμ appear (*cf.* Fig. 3 of ref. 14). This alkaline spectrum

corresponds to that for fully guanidinated cytochrome *c* previously reported by HETTINGER AND HARBURY[14].

Acetylated cytochrome *c* was essentially prepared as described by WADA AND OKUNUKI[16] by slowly adding a 200-fold (or 20-fold for less drastic acetylation) molar excess of acetic anhydride to 2 mM cytochrome *c* in 50% saturated sodium acetate solution. The pH of the resulting solution was adjusted to 7.2 with 1 M NaOH and stirred at 4° for various time intervals (15–60 min) and dialyzed against 0.02 M potassium phosphate buffer (pH 6.0). This procedure resulted in a number of small cytochrome *c* fractions of varying degrees of acetylation which could be purified to homogeneity by column chromatography. Small amounts of native cytochrome *c*, as well as partially acetylated cytochromes *c*, were obtained by stepwise elution on carboxymethylcellulose at 0.01 M potassium phosphate buffer (pH 6.0). Some of the acetylated cytochrome *c* was not adsorbed and passed through the column. These derivatives of acetylated cytochrome *c* could be further fractionated by stepwise elution on DEAE-cellulose equilibrated with the 0.01 M potassium phosphate buffer (pH 6.0). The properties of the derivatives employed in the present study are summarized in Table I. Cellulose acetate electrophoresis in a Tris–glycine buffer at pH 8.0 indicated a close correspondence between the ability of the various acetylated derivatives to migrate towards the cathode and their degree of adsorption to the exchange resins. The derivatives with the least affinity for carboxymethylcellulose had the smallest migration towards the cathode. On this basis, the degree of acetylation is No. 3 < No. 2 < No. 1.

Ninhydrin analysis[15,16], employing native cytochrome *c* as a standard, indicated that Fraction No. 3 had 68% of its amino groups acetylated (on the basis of a dimer, *cf.* Fig. 7), whereas Fraction No. 1 had 26%. Insufficient quantities of Fraction No. 2 prevented a ninhydrin analysis.

TABLE I

SUMMARY OF PROPERTIES OF ACETYLATED AND GUANIDINATED CYTOCHROMES *c*

Fractions No. 1 and No. 2 were eluted from CM-cellulose as described in MATERIALS AND METHODS with 0.1 M and 0.01 M potassium phosphate buffer (pH 6.0), respectively, whereas Fraction No. 3 was eluted from DEAE-cellulose with 0.1 M of the same buffer. Spectra were recorded in phosphate buffer (pH 7.0).

Derivative	*Electrophoretic behavior**	*$-NH_2$ groups modified (%)***	*Spectrum (mμ)*		
			Oxidized	*Reduced*	*CO comple*
Native	100	0	409, 528	415, 521, 550	—
Guanidinated	105	—	409, 528 531***, 570, 600	415, 521, 550	—
Acetylated					
Fraction No. 1	50	26	409, 528	415, 521, 550	—
Fraction No. 2	25	—	409, 528	415, 521, 550	—
Fraction No. 3	0§	68	405, 526	415, 521, 550	+

* Relative movement compared to native ferricytochrome *c* towards cathode in Tris–citrate buffer (pH 8.0) on cellulose acetate electrophoresis.

** On the basis of ninhydrin analysis[15,16].

*** pH 10.5.

§ Migrates towards anode at this pH.

In agreement with the observations of WADA AND OKUNUKI[16], the spectrum of the oxidized and reduced forms of the partially acetylated derivatives (*i.e.* Fractions No. 1 and No. 2) were identical with that of native cytochrome *c*. In addition, the reduced forms of these derivatives did not combine with CO. On the other hand, Fraction No. 3 differed spectrally from oxidized native cytochrome *c* in that the absorption maxima were shifted, from 528 to 526 mμ and from 409 to 405 mμ. In addition, the reduced form of this derivative reacted with CO and had a net negative charge at pH 8.0.

Spectrophotometric measurements were carried out with a Cary Model-15 recording spectrophotometer. Estimates of the reactivities of the various cytochromes *c* were examined by following the rate of reduction and oxidation of cytochrome *c* at 550 mμ in 0.01 M potassium phosphate buffer (pH 6.0). Reduction rates were followed by adding a small excess of sodium ascorbate to a solution containing the cytochrome *c*. When the cytochrome *c* was approx. 70% reduced, cytochrome *c* peroxidase and H_2O_2 were added. The initial velocities were calculated from the product of the first-order rate constant (determined from log plots) and ferrocytochrome *c* concentration. For more detailed analysis of the reactivity of cytochrome *c* peroxidase towards the various modified cytochromes *c*, ferrocytochrome *c* concentration was varied, and kinetic parameters were obtained form Lineweaver–Burk and Dixon plots. The reduced form of the modified cytochrome *c*, in this case, was prepared by passing a $Na_2S_2O_4$-reduced sample through a Sephadex G-25 column as described for native cytochrome *c* above.

Column chromatography studies of complex formation between modified cytochrome *c* and cytochrome *c* peroxidase were performed on Sephadex G-75. Elution patterns were monitored by following the Soret absorbance of each fraction. The column was calibrated by the gel-filtration method of ANDREWS[17], employing horse heart ferricytochrome *c* (mol. wt. = 12363), catalase (mol. wt. = 240000), cytochrome *c* peroxidase (mol. wt. = 34400) and myoglobin (mol. wt. = 17000) as standards. The ratio of guanidinated cytochrome *c* to enzyme in each fraction, or after the addition of $Na_2S_2O_4$, was calculated from the following relationship which was based on the respective mM extinction coefficient values for reduced guanidinated cytochrome *c* and cytochrome *c* peroxidase at 550 mμ of 27.7 and 7.7, at 535 mμ of 12 and 8.0, and the mM extinction coefficient values for oxidized guanidinated cytochrome *c* and cytochrome *c* peroxidase at 408 mμ of 106 and 93, respectively:

$$\frac{[\text{cytochrome } c]}{[\text{cytochrome } c \text{ peroxidase}]} = \frac{0.061\,(A_{550\,m\mu} - A_{535\,m\mu}) - 0.003\,A_{408\,m\mu}}{0.014\,A_{408\,m\mu} - 0.07\,(A_{550\,m\mu} - A_{535\,m\mu})}$$

The ratio of acetylated cytochrome *c* to cytochrome *c* peroxidase in each fraction was obtained in a similar manner employing the respective mM extinction coefficient values for the reduced forms of acetylated cytochrome *c* and cytochrome *c* peroxidase at 550 mμ of 25 and 7.0, at 535 mμ of 12 and 8, and for the oxidized forms at 405 mμ of 130 and 90:

$$\frac{[\text{cytochrome } c]}{[\text{cytochrome } c \text{ peroxidase}]} = \frac{0.082\,(A_{550\,m\mu} - A_{535\,m\mu}) - 0.004\,A_{405\,m\mu}}{0.016\,A_{405\,m\mu} - 0.113\,(A_{550\,m\mu} - A_{535\,m\mu})}$$

Ratios of native cytochrome *c* to the protomethyl ester derivative of cytochrome *c* peroxidase in each fraction were obtained from the oxidized spectrum employing

mM extinction coefficients of 106 (408 mμ), 66 (398 mμ) and 31 (280 mμ) for ferricytochrome *c*, and 81 (408 mμ), 66 (398 mμ) and 76 (280 mμ) for the modified cytochrome *c* peroxidase according to the following:

$$\frac{[\text{cytochrome } c]}{[\text{cytochrome } c \text{ peroxidase}]} = \frac{0.014\,(A_{408\ m\mu} - A_{280\ m\mu}) - 0.001\,A_{398\ m\mu}}{0.016\,A_{398\ m\mu} - 0.014\,(A_{408\ m\mu} - A_{280\ m\mu})}$$

RESULTS

Inhibition of cytochrome c peroxidase by polycations

In another study[2] we extended the work of BETTLESTONE[5] on the inhibition of the cytochrome *c* peroxidase-catalyzed peroxidation of cytochrome *c* by the basic protein salmine and showed that other polycations, such as PL-3 and PL-150, were also effective inhibitors of this reaction. Fig. 1 illustrates a characteristic inhibition plot for the polycations. In this particular case, it can be seen that salmine is a competitive inhibitor with respect to ferrocytochrome *c* concentration of the peroxidation reaction. Both PL-3 and PL-150 also exhibited competive inhibition patterns, the K_i values for the various polycations are summarized in Table II. On the molar basis, the effectiveness of the inhibitors is PL-150 > salmine > PL-3. However, if the polycations are considered on an equivalent charge basis (*i.e.* per lysine or arginine residue), they are all of approximately the same effectiveness. This observation stresses the importance of the polycation charge in exerting its inhibitory action.

Reactivity of modified cytochromes c with cytochrome c peroxidase

WADA AND OKUNUKI[16] and HETTINGER AND HARBURY[14], respectively, have shown that acetylation and guanidination of cytochrome *c*, carried out under the

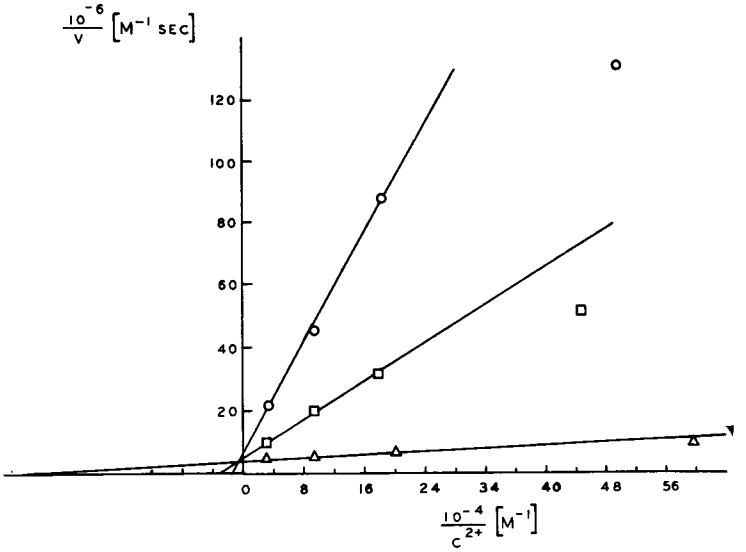

Fig. 1. Polycation inhibition of the cytochrome *c* peroxidase-catalyzed peroxidation of cytochrome *c*. Lineweaver–Burk plot of the ferrocytochrome *c* peroxidation obtained with 100 μM H_2O_2 and 0.5 mμM cytochrome *c* peroxidase at 0.01 M potassium phosphate buffer (pH 7.0) in the presence and absence of salmine: △, −salmine; ◇, +0.4 μM salmine; ○, +1.0 μM salmine.

TABLE II

K_i VALUES FOR POLYCATION INHIBITION OF CYTOCHROME *c* PEROXIDATION BY CYTOCHROME *c* PEROXIDASE

K_i values represent average values obtained from Dixon and Lineweaver–Burk plots with 0.5 mμM cytochrome *c* peroxidase, 100 μM H_2O_2, in 0.01 M potassium phosphate buffer (pH 7.0).

Inhibitor	K_i (*μM inhibitor*)*	K_i (*μM inhibitor*)**
PL-3	0.18	3.78
PL-150	0.004	4.12
Salmine***	0.05	1.15

* On molar basis.
** On equivalent charge basis.
*** Calculations based on mol. wt. 5000 and 80% arginine.

TABLE III

REACTIVITY OF MODIFIED CYTOCHROMES *c* WITH ASCORBATE AND CYTOCHROME *c* PEROXIDASE

Rates of reduction and oxidation of cytochrome *c* were followed at 550 mμ in 0.01 M potassium phosphate buffer (pH 6.0). Reduction was followed by adding sodium ascorbate (40 μM) to a solution containing 14 μM of the designated cytochrome *c*. When the cytochrome *c* was approx. 70% reduced, 4.2 mμM cytochrome *c* peroxidase and 117 μM H_2O_2 were added. Velocities are represented as μM cytochrome *c* oxidized (or reduced) per sec.

Cytochrome c derivative	$v_{reduced}$ (*μM/sec*)	*Activity remaining* (%)	$v_{oxidized}$ (*μM/sec*)	*Activity remaining* (%)
Native*	0.07	100	2.9	100
Native	0.10	100	2.9	100
Acetylated**				
Fraction No. 1	0.035	35	1.2	40
Fraction No. 2	0.015	21	0.57	20
Fraction No. 3	0.002	2	0	0
Guanidinated	0.013	19	2.6	90

* Conditions same as above except 20 μM ascorbate added.
** Where degree of acetylation is: Fraction No. 3 > No. 2 > No. 1; see Table I, MATERIALS AND METHODS.

conditions described in the present study, lead to a relatively specific modification of the free amino groups of lysine. Table III compares the reactivity of native, guanidinated and acetylated cytochromes *c* towards ascorbate and cytochrome *c* peroxidase. As can be seen, partial acetylation (Fractions No. 1 and No. 2) of cytochrome *c* lysine residues gives rise to derivatives which have a decreased reactivity towards both ascorbate and cytochrome *c* peroxidase. Whereupon further acetylation (Fraction No. 3) leads to a cytochrome *c* which is completely unreactive towards cytochrome *c* peroxidase and has only a low rate of reactivity with ascorbate. These observations illustrate the importance of the lysine residues in the reactivity of cytochrome *c* towards cytochrome *c* peroxidase.

In contrast to the effects of acetylation, guanidination of cytochrome *c* has much less effect on the reactivity towards cytochrome *c* peroxidase (Table III). Since guanidination of cytochrome *c* replaces the lysine residues with homoarginyl

residues[14], it appears that the positive charge of the lysine residue is the critical factor in the reactivity towards cytochrome *c* peroxidase. This was further illustrated in a more detailed analysis of the reactivity of the modified cytochromes *c*. Figs. 2A and 2B, show that the time-courses of cytochrome *c* peroxidase peroxidation for the acetylated and guanidinated derivatives of ferrocytochrome *c*, like the native form[2], follow first-order kinetics under the indicated conditions. The maximum turnover numbers (TN_{max}) and K_m values for native and modified cytochromes *c* obtained from Lineweaver–Burk plots are listed in Table IV. The effect of guanidination

TABLE IV

KINETIC PARAMETERS FOR THE PEROXIDATION OF GUANIDINATED AND ACETYLATED FERROCYTOCHROME *c* BY CYTOCHROME *c* PEROXIDASE

Reactions were run in 0.01 M potassium phosphate buffer (pH 7.0) with 58.6 μM H_2O_2 and 0.58 mμM cytochrome *c* peroxidase at varying ferrocytochrome *c* concentrations. Maximum turnover numbers (TN_{max} = moles cytochrome c^{2+} oxidized per mole of enzyme per sec) were obtained from Lineweaver–Burk plots, K_i values for cytochrome c^{3+} from Dixon plots.

	TN_{max} (sec^{-1})	K_m (μM cytochrome c^{2+})	K_i (μM cytochrome c^{3+})
Native cytochrome *c*	630	3.4	2.6
Guanidinated cytochrome *c*	580	5.4	4.5
Acetylated cytochrome *c* (Fraction No. 1)	390	6.5	5.8

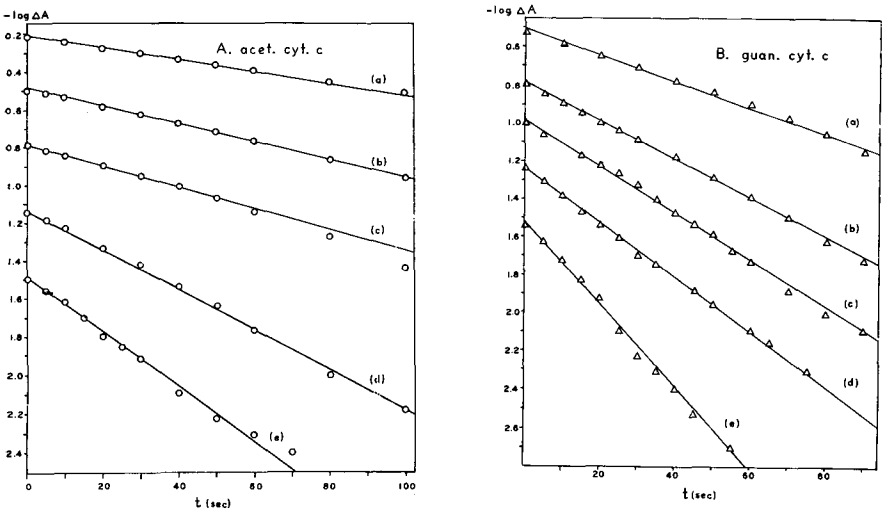

Fig. 2. Time-courses for peroxidation of modified cytochromes *c* by cytochrome *c* peroxidase. The peroxidation of acetylated (A) cytochrome *c* (Fraction No. 1) and guanidinated (B) cytochrome *c* was followed by observing the decrease in absorbance at 550 mμ upon the addition of 0.58 mμM cytochrome *c* peroxidase to a solution containing the indicated concentration of ferrocytochrome *c* and 58.6 μM H_2O_2 in 0.01 M potassium phosphate buffer (pH 7.0), 25°. No appreciable initial oxidation occurred in the absence of cytochrome *c* peroxidase. Log ΔA corresponds to the logarithm of the change in absorbance of cytochrome *c* at time *t minus* the absorbance of completely oxidized cytochrome *c*. The initial concentrations (μM) of acetylated ferrocytochrome *c* were: a, 30.7; b, 16.1; c, 8.3; d, 3.7; e, 1.6. The initial concentrations (μM) of guanidinated ferrocytochrome *c* were: a, 15.3; b, 8.1; c, 5.1; d, 2.9; e, 1.5.

is to cause a slight increase in the K_m and a small decrease in maximum turnover number, whereas partial acetylation leads to an increased K_m and a considerably larger decrease in maximum turnover number.

Inhibition of cytochrome c peroxidase activity by modified oxidized cytochromes c

Since ferricytochrome *c* can act as a competitive inhibitor of the peroxidation of ferrocytochrome *c* by cytochrome *c* peroxidase[2], it was of added interest to examine the effectiveness of the modified cytochromes *c* as inhibitors of ferrocytochrome *c* peroxidation. From the time-courses in Fig. 2, it can be seen that, as with native cytochrome *c*, the value of the apparent first-order rate constant for the oxidation of the cytochrome *c* derivatives is inversely proportional to the concentration of the reduced form of the derivative, thereby suggesting that the oxidized form of the cytochrome *c* derivative is inhibiting the reaction[18]. The inhibitory effects of the oxidized partially acetylated and guanidinated cytochromes *c* are illustrated in the form of Dixon plots in Figs. 3A and 3B, respectively. Both forms of ferricytochrome *c* act as competive inhibitors of their reduced forms. The K_i value for acetylated ferricytochrome *c* was 5.8 μM, while the K_i for guanidinated ferricytochrome *c* was 4.5 μM. The K_i values for the oxidized derivatives are approximately equal to the K_m values for the reduced forms (Table IV). This finding is consistent with the first-order kinetics[8,18].

Fig. 4 shows the results of a similar experiment where the inhibition of cytochrome *c* peroxidase activity by these derivatives towards native ferrocytochrome *c* was examined directly. In this case the guanidinated and most drastically acetylated (*i.e.* Fraction No. 3) oxidized derivatives were compared to native ferricytochrome. It is clear that guanidinated ferricytochrome *c* is an inhibitor of approximately the same effectiveness as native ferricytochrome *c*, whereas this acetylated cytochrome *c* derivative, which is inactive towards cytochrome *c* peroxidase, also has lost its capacity to inhibit the cytochrome *c* peroxidase–native ferrocytochrome *c* reaction.

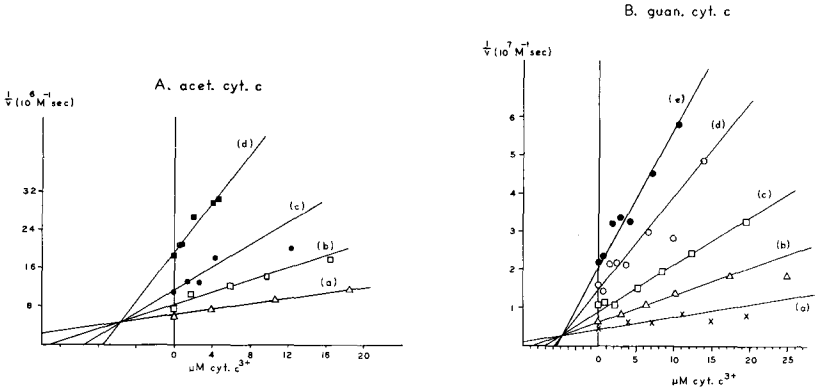

Fig. 3. Inhibitory effects of modified ferricytochromes *c*. Initial velocities of ferrocytochrome *c* peroxidation in the presence of the indicated concentrations of ferricytochrome *c* were determined indirectly from the time-course curves listed in Fig. 2 and represented here in the form of Dixon plots. A. Initial concentrations (μM) of acetylated ferrocytochrome *c* (Fraction No. 1) were: a, 12.1; b, 6.3; c, 3.6; d, 1.6. B. Initial concentrations (μM) of guanidinated ferrocytochrome *c* were: a, 11.4; b, 5.1; c, 2.9; d, 1.5; e, 1.0.

Complex formation between cytochrome c peroxidase and modified cytochrome c

The above kinetic studies of the reactivity of the modified cytochromes *c* might be interpreted as indicating that guanidinated cytochrome *c* is forming complexes with cytochrome *c* peroxidase whereas acetylated cytochrome *c* is not. As a supplement to these studies, physical studies of complex formation on Sephadex were performed. A mixture containing guadininated ferricytochrome *c* and cytochrome *c* peroxidase was placed on a Sephadex G-75 column. The elution profile of the fractions monitored at 408 mμ is illustrated in Fig. 5 and clearly indicates the separation of two distinct hemoprotein peaks. The ratio of guanidinated cytochrome *c* to enzyme in each fraction was estimated as described in MATERIALS AND METHODS. The formation of a complex between the enzyme and guanidinated cytochrome *c* corresponding to the first peak is indicated by the constancy of the ratio in the major portion of the peak. This is followed by a rise in the ratio corresponding to the second peak which contains exclusively guanidinated cytochrome *c*. The presence of free enzyme is indicated by the low ratio in the leading edge of the first peak. The systems appears to be indicative of a typical associating–dissociating equilibrium in which free enzyme, enzyme–guanidinated cytochrome *c* complex and free cytochrome *c* are present, and thus resembles the reaction of native cytochrome *c* with cytochrome *c* peroxidase[3]. The lower ratio of guanidinated cytochrome *c* to cytochrome *c* peroxidase (0.65) compared with that of native cytochrome *c* (0.9–1.0, see ref. 3) may indicate a lower affinity of the enzyme for the guanidinated derivative which would be consistent with the higher K_m and K_i values observed above for

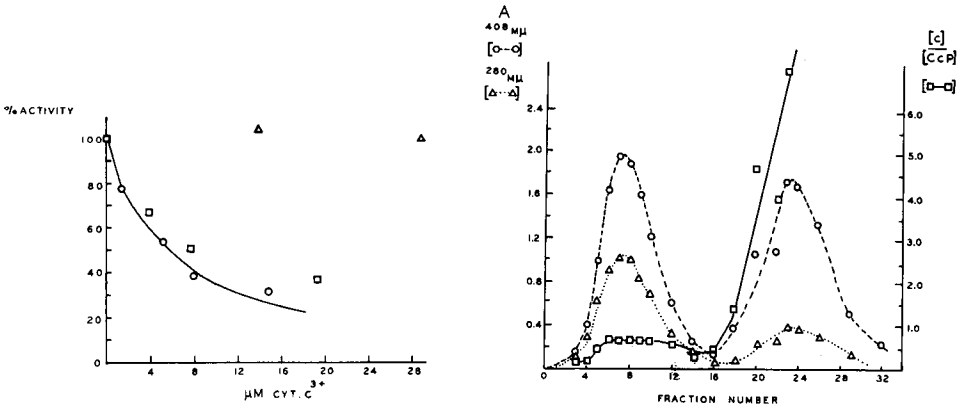

Fig. 4. Inhibition of cytochrome *c* peroxidase-catalyzed ferrocytochrome *c* peroxidation by native and modified cytochromes *c*. The peroxidation rate of approx. 5 μM ferrocytochrome *c*, in the presence of the indicated quantity of the designated form of ferricytochrome *c*, was obtained with 0.58 mμM cytochrome *c* peroxidase and 58.6 μM H_2O_2 in 0.01 M potassium phosphate buffer (pH 7.0): □, guanidinated; ○, native; △, acetylated. These rates were divided by the rate determined in the absence of the inhibitor to obtain the percent activity remaining.

Fig. 5. Elution profile of cytochrome *c* peroxidase and guanidinated cytochrome *c* on Sephadex. A 0.3-ml mixture containing an excess of guanidinated ferricytochrome *c* to cytochrome *c* peroxidase (610:167 μM) in 5 mM potassium phosphate buffer (pH 7.0), 4°, was placed on a 1 cm × 31 cm Sephadex G-75 column which had been previously equilibrated with the same buffer. The flow rate of the column was approx. 3 ml/h and 0.5-ml fractions were collected. The absorbance at 408 mμ (○---○) and 280 mμ (△···△) was recorded for each fraction. The ratio of guanidinated cytochrome *c* to enzyme in the fractions ([C]/[CcP], □—□) was determined as described in MATERIALS AND METHODS.

this derivative (*cf.* Table IV), if the K_m and K_i values do approximate dissociation constants under these conditions[2,3].

Fig. 6 contains a similar elution profile for acetylated ferricytochrome *c* (Fraction No. 3) and cytochrome *c* peroxidase. In this case, the results are more complicated in that there is no clear separation of the acetylated cytochrome *c* and cytochrome *c* peroxidase. The explanation for this observation becomes clear, however, when acetylated cytochrome *c* was run in the absence of cytochrome *c* peroxidase (Fig. 7). Here it is seen that the acetylated cytochrome *c* is apparently a dimer, since it has a molecular weight of approximately 24000. The single peak in Fig. 6, then, represents a mixture of cytochrome *c* peroxidase and acetylated cytochrome *c*. Determination of the ratio of acetylated cytochrome *c* to cytochrome *c* peroxidase in the various fractions, as described in MATERIALS AND METHODS, indicated that since only a very small fraction of cytochrome *c* appeared in the leading fractions, no appreciable complex formation occurred between the acetylated cytochrome *c* and cytochrome *c* peroxidase. (The very small degree of complex formation observed is either due to a lack of sensitivity in estimating the ratios of cytochrome *c* to cytochrome *c* peroxidase at low concentrations or to the presence of trace amounts of native cytochrome *c* in the acetylated cytochrome *c* fraction employed.) This conclusion was supported by the rise in the ratio concomitant with the appearance of the acetylated cytochrome *c* peak. (Since cytochrome *c* peroxidase has a much greater 280-mμ absorbance than cytochrome *c*, the absorption at 280 mμ can be used to identify the cytochrome *c* peroxidase peak which is shown to be at the leading edge of the Soret peak.) It is apparent from this experiment that since a cytochrome *c* dimer

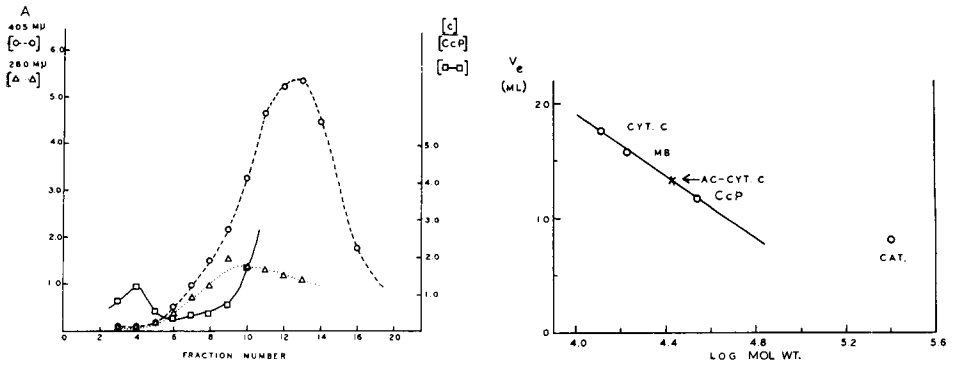

Fig. 6. Elution profile of cytochrome-*c* peroxidase and acetylated cytochrome *c* on Sephadex. A 0.3-ml mixture containing an excess of acetylated ferricytochrome *c* to cytochrome *c* peroxidase (454:113 μM) in 5 mM potassium phosphate buffer (pH 7.0), 4°, was placed on a 1 cm × 31 cm Sephadex G-75 column which had been previously equilibrated with the same buffer. The flow rate of the column was approx. 3 ml/h and 0.5-ml fractions were collected. The absorbance at 405 mμ (○---○) and 280 mμ (△ · · · △) was recorded for each fraction. The ratio of acetylated cytochrome *c* to enzyme in the fractions ([C]/[CcP], □—□) was determined as described in MATERIALS AND METHODS.

Fig. 7. Molecular weight estimation of acetylated cytochrome *c* (Fraction No. 3). Elution volumes (V_e) plotted as a function of logarithm of molecular weight. Approx. 1-mg samples of native cytochrome *c*, myoglobin (MB), cytochrome *c* peroxidase (CcP), and catalase (CAT.) were employed as standards, and placed on a 1 cm × 31 cm Sephadex G-75 column (previously equilibrated with 5 mM potassium phosphate buffer (pH 7.0)). The proteins were eluted with the same buffer and identified by their characteristic absorbance peaks. The elution volume for each of the proteins corresponded to the maximum concentration as determined from the elution profile.

is evidently formed, the possibility that a masking of the cytochrome *c* 'active site', rather than acetylation of lysine residues, has lead to the inability of this cytochrome *c* derivative to form a complex with cytochrome *c* peroxidase cannot be eliminated. Table V summarizes the complex formation studies on Sephadex.

TABLE V

SUMMARY OF COMPLEX FORMATION BETWEEN CYTOCHROME *c* AND CYTOCHROME *c* PEROXIDASE ON SEPHADEX

Indicated quantities of cytochrome *c* and cytochrome *c* peroxidase were placed on a 1 cm × 31 cm Sephadex G-75 column equilibrated with 5 mM potassium phosphate buffer (pH 7.0) at 4°. Ratios of cytochrome *c*: cytochrome *c* peroxidase in the leading peaks of the elution profile were determined as described in MATERIALS AND METHODS.

Experimental conditions	*Ratio of cytochrome c: cytochrome c peroxidase in leading peak*
610 μM guanidinated cytochrome c^{3+} + 167 μM cytochrome *c* peroxidase	0.65
454 μM acetylated cytochrome c^{3+} + 113 μM cytochrome *c* peroxidase	—
550 μM cytochrome c^{3+} + 150 μM cytochrome *c* peroxidase	0.95
630 μM cytochrome c^{3+} + 190 μM modified cytochrome *c* peroxidase	0.9

Reactivity of cytochrome c with modified cytochrome c peroxidase

ASAKURA AND YONETANI[6] have shown that apocytochrome *c* peroxidase can readily combine with protoheme alkyl esters, which contain modified side chains at positions 6 and 7 of the porphyrin ring, to give synthetic enzymes which reacted with H_2O_2 and other ligands in a manner analogous to native cytochrome *c* peroxidase. On the other hand, the synthetic enzymes had a much lower activity than the native enzyme towards cytochrome *c*. Fig. 8 illustrates the time-course for ferrocytochrome *c* peroxidation catalyzed by the dimethylprotoheme-modified enzyme. As with native cytochrome *c* peroxidase in phosphate[2], the time-courses follow apparent first-order kinetics, the apparent first-order rate constant decreasing with increasing concentrations of cytochrome *c*. The TN_{max} and K_m values for both native and modified cytochrome *c* peroxidase are listed in Table VI. The results are essentially in agreement with those previously reported by ASAKURA AND YONETANI[6] under somewhat different conditions and confirm the conclusion of these workers that esterification of heme at positions 6 and 7 of the heme phorphyrin ring causes a marked decrease in TN_{max} (approx. 98%) while only slightly increasing the K_m value.

Fig. 9 compares the effects of varying H_2O_2 on the reaction of cytochrome *c* with both native and modified cytochrome *c* peroxidase. With modified cytochrome *c* peroxidase, like native cytochrome *c* peroxidase[1,2], H_2O_2 becomes saturating at high concentrations, the concentration of H_2O_2 required for 50% saturation being in the range of 5–10 μM for both enzymes. Thus it would appear that the differences between the two enzymes in their kinetic parameters for cytochrome *c* peroxidation (*cf.* Table VI) are not due to any kinetically significant alteration in the reactivity

of the modified enzyme towards H_2O_2 (*i.e.* formation of the peroxide intermediate is not rate limiting). This suggests that the decrease in reactivity towards cytochrome *c* observed with the modified cytochrome *c* peroxidase can be attributed primarily to the reaction with cytochrome *c*. The observation[6] that the reactivity towards ferrocyanide for the modified enzyme is not appreciably altered supports this conclusion.

As can be seen from Fig. 8, the apparent first-order rate constants for ferro-

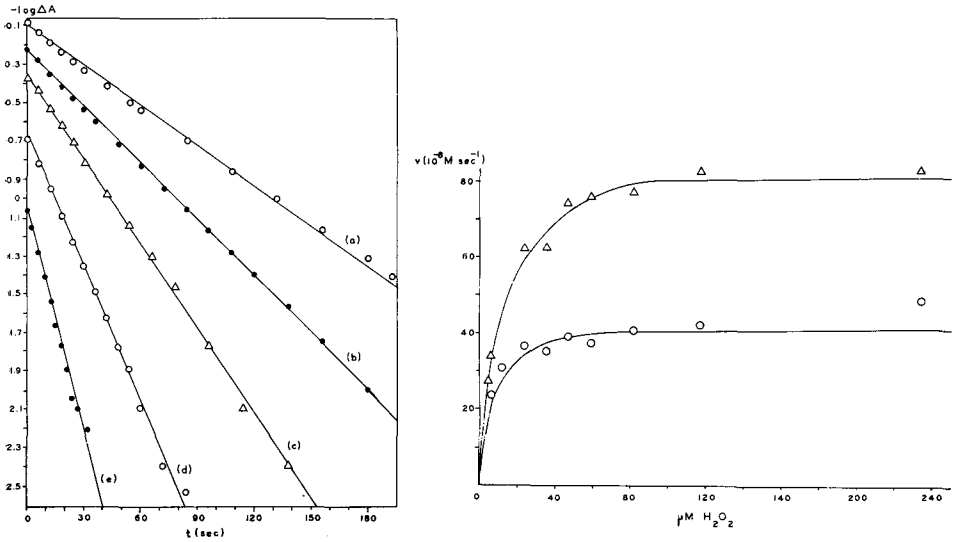

Fig. 8. Time-course for peroxidation of cytochrome *c* by the protomethyl ester derivative of cytochrome *c* peroxidase. The peroxidation of the indicated quantities of native cytochrome *c* by 69 mμM modified cytochrome *c* peroxidase in the presence of 58.6 μM H_2O_2 in 0.01 M potassium phosphate buffer (pH 7.0), 25°, was followed as described in Fig. 2. The initial concentrations (μM) of ferrocytochrome *c* were: a, 41.7; b, 31.0; c, 21.6; d, 10.4; e, 4.5.

Fig. 9. Effects of H_2O_2 on cytochrome *c* oxidation by native and the protomethyl derivative of cytochrome *c* peroxidase. Initial peroxidation rates of 30 μM ferrocytochrome *c* (followed as described in Fig. 2) were observed at various H_2O_2 concentrations, 0.01 M potassium phosphate buffer (pH 7.0), 25°. The concentration of native cytochrome *c* peroxidase (○—○) was 0.58 mμM and the concentration of modified cytochrome *c* peroxidase (△—△) was 69 mμM.

TABLE VI

KINETIC PARAMETERS FOR THE PEROXIDATION OF CYTOCHROME *c* BY THE PROTOMETHYL ESTER DERIVATIVE OF CYTOCHROME *c* PEROXIDASE

Reactions were run in 0.01 M potassium phosphate buffer (pH 7.0) with 58.6 μM H_2O_2, 69 mμM modified cytochrome *c* peroxidase and 0.58 mμM native cytochrome *c* peroxidase at varying ferrocytochrome *c* concentrations. Maximum turnover numbers (T_{max} = moles cytochrome c^{2+} oxidized per mole of enzyme per sec) were obtained from Lineweaver–Burk plots, K_i values for cytochrome c^{3+} from Dixon plots.

	TN_{max} (sec^{-1})	K_m (μM cytochrome c^{2+})	K_i (μM cytochrome c^{3+})
Native cytochrome *c* peroxidase	700	3.1	2.2
Modified cytochrome *c* peroxidase	13	5.5	4.5

cytochrome *c* peroxidation by the modified cytochrome *c* peroxidase, as with the native enzyme, are inversely proportional to the cytochrome *c* concentration. As pointed out for the native enzyme above, this phenomenon most likely results from the ability of the product, ferricytochrome *c*, to competitively inhibit the peroxidation reaction. This also seems to be the case for the modified enzyme (Fig. 10). As with the native enzyme, the K_i for ferricytochrome *c* was approximately equal to the K_m for ferrocytochrome *c* (Table VI).

Fig. 11 presents the results of a column chromatography study where the ability of the modified cytochrome *c* peroxidase to form a complex with ferricyto-

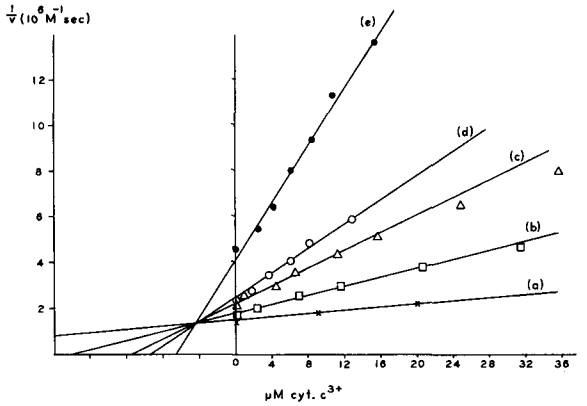

Fig. 10. Inhibitory effects of native ferricytochrome *c* on modified cytochrome *c* peroxidase. The initial velocities obtained from the time-courses in Fig. 8 were treated as described in Fig. 3. Initial concentrations (μM) of ferrocytochrome *c* were: a, 21.8; b, 10.4; c, 6.1; d, 4.5; e, 1.9.

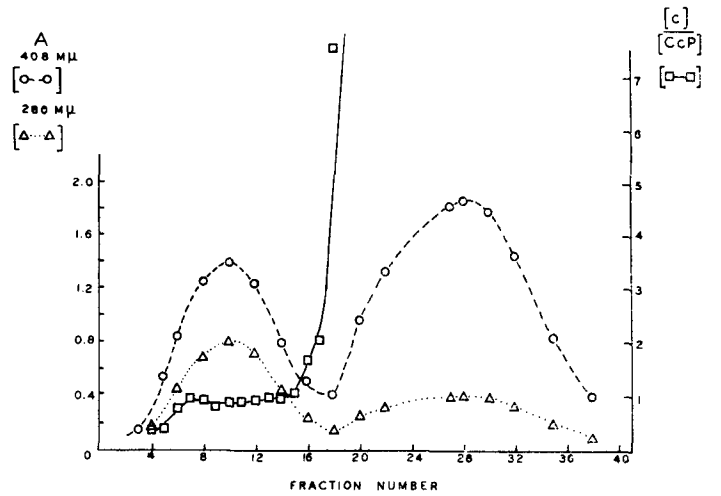

Fig. 11. Elution profile of cytochrome *c*–modified cytochrome *c* peroxidase mixture on Sephadex. A 0.3-ml mixture containing an excess of native ferricytochrome *c* to the protomethyl ester derivative of cytochrome *c* peroxidase (630:190 μM) in 5 mM potassium phosphate buffer (pH 7.0), 4°, was placed on a 1 cm × 31 cm Sephadex G-75 column. The absorbance at 408 mμ (○---○) and at 280 mμ (△···△) was recorded for each of the 0.5-ml fractions. The ratio of ferricytochrome *c* to modified cytochrome *c* peroxidase ([c]/[CcP], □—□) was determined as described in MATERIALS AND METHODS.

chrome *c* was examined. The elution profile is essentially identical with that observed with native cytochrome *c* peroxidase, the formation of a stoichiometric complex between modified cytochrome *c* peroxidase and cytochrome *c* being indicated by the constancy of the ratio of cytochrome *c* to enzyme in each fraction of approximately 1.0 in the major portion of the leading peak. From a comparison of the spectra of the free components with that of the ferricytochrome *c*–modified cytochrome *c* peroxidase complex, there appeared to be no marked changes in the spectra of cytochrome *c* or the modified cytochrome *c* peroxidase when they are in a complex. A similar situation also seems to be true for the cytochrome *c*–native cytochrome *c* peroxidase complex[3] and the guanidinated cytochrome *c*–native cytochrome *c* peroxidase complex.

DISCUSSION

Cytochrome *c* is a highly basic protein[19] whereas cytochrome *c* peroxidase is acidic[20]. Therefore, it is not unreasonable to expect that a predominant force in complex formation between the two proteins would be electrostatic in nature. Kinetic studies[1,2,5] showing that the cytochrome *c* peroxidase-catalyzed peroxidation of cytochrome *c* could be inhibited at high ionic strengths and by the basic protein salmine[2,5] supported this view.

From a more thorough examination of the polycation inhibition kinetics in the present study, it was observed that, in addition to salmine, PL-150 and PL-3 were effective inhibitors of the peroxidation of cytochrome *c* by cytochrome *c* peroxidase. All three inhibitors exhibited competitive kinetics with respect to ferrocytochrome *c*. Competitive inhibition of cytochrome *c* peroxidase by these substrate analogs, then, provides additional support for the electrostatic nature of cytochrome *c*–cytochrome *c* peroxidase complexes as well as for their participation in the catalytic activity of the enzyme[2]. As pointed out in the results, when the polycations are considered on an equal charge basis they are essentially equivalent in their inhibitor effectiveness. This observation, in addition to once again stressing the importance of the net positive charge on the polycations, might also be interpreted to suggest that low-molecular-weight polylysine has the same 'steric inhibitor effectiveness' as the higher-molecular-weight form. Thus a more detailed kinetic analysis of polycation inhibition of the cytochrome *c* peroxidase–cytochrome *c* interaction, as has recently been reported for the cytochrome oxidase–cytochrome *c* reaction[21–23], could conceivably yield information concerning the size of the cytochrome *c* 'positive binding site'.

Studies on the reactivity of modified cytochrome *c* towards cytochrome *c* peroxidase also signify the importance of electrostatic interactions between the two proteins. For neutralization of the positively charged lysine residues of cytochrome *c* by acetylation eventually leads to a form of cytochrome *c* which is inactive towards cytochrome *c* peroxidase, does not inhibit ferrocytochrome *c* peroxidation and does not form any appreciable complex with cyrochrome *c* peroxidase on Sephadex. Guanidination of the cytochrome *c* lysine residues, on the other hand, does not appreciably alter any of these properties. These observations emphasize the necessity of the net positive charge on the cytochrome *c* molecule, rather than the lysine residues *per se*, for maximum activity towards cytochrome *c* peroxidase. Although the present study has not been carried out in sufficient detail, it might

be anticipated from other evidence that not all of the positive residues on cytochrome *c* will be found to be necessary for maximum activity towards cytochrome *c* peroxidase. The results of OKUNUKI and co-workers[16,24] on the reaction of modified cytochromes *c* with mammalian cytochrome oxidase have implicated only six lysine residues (of a total of 18) on the beef cytochrome *c* molecule as necessary for reactivity towards the oxidase. Along similar lines not all of the positive residues on cytochrome *c* have remained invariant[19].

At present, much less is known about the cytochrome *c* peroxidase 'binding site'. The lack of any appreciable spectral changes of cytochrome *c* and/or cytochrome *c* peroxidase while in complex with each other has been previously[3] interpreted to indicate that the apparent negative binding site on cytochrome *c* peroxidase is most probably associated with the protein rather than heme moiety of cytochrome *c* peroxidase. This idea receives support in the present study from the observation that the dimethyl protoheme ester derivative of cytochrome *c* peroxidase, although only maintaining 2 % of native activity, can still form a Sephadex-detectable complex with cytochrome *c* with an apparent affinity similar to native cytochrome *c* peroxidase. This finding also suggests that the K_m value of cytochrome *c* peroxidase for cytochrome *c* may correspond to a dissociation constant[2], since there was little change in the observed K_m values for both forms of cytochrome *c* peroxidase. As suggested by ASAKURA AND YONETANI[6] and the results of this study, it would appear that the modified cytochrome *c* peroxidase may be altered in its electron transport reaction with cytochrome *c*, rather than its binding reaction. The ability to be competitively inhibited by ferricytochrome *c* further supports this view. It is also of interest to note that protomethyl ester cytochrome *c* peroxidase, in kinetic respects (*i.e.* low TN_{max} for cytochrome *c* oxidation), is very similar to the enzyme originally prepared by ALTSCHUL *et al.*[25] and others[2,5]. With these forms of cytochrome *c* peroxidase, it is possible that the heme of cytochrome *c* peroxidase is distorted in such a manner as not to alter appreciably its reaction with ligands and ferrocyanide[6] while significantly affecting the electron transfer reaction with the heme of cytochrome *c*.

As pointed out above, cytochrome *c* peroxidase and cytochrome oxidase show many kinetic and physical similarities in their reactions with cytochrome *c*. Both enzymes oxidize cytochrome *c* in a first-order process and follow Michaelis kinetics[1,2,8], and are inhibited by high ionic strengths and polycations[2,26] and competitively by the product, ferricytochrome *c*[2,8]. The kinetic observations for both enzymes can be explained in a mechanism that preserves a central role for reversible complexes between enzyme and both reduced and oxidized cytochrome *c*[2,7,8,18]. Physical detection of such complexes by column chromatography for both cytochrome *c* peroxidase[3] and cytochrome oxidase[9] supports this view. Employing chemically modified cytochromes *c*, the Japanese workers[16,24] have previously concluded that electrostatic forces play a predominant role in complex formation between cytochrome *c* and cytochrome oxidase. As discussed above, a similar conclusion has been reached in the case of cytochrome *c*–cytochrome *c* peroxidase complexes. A further similarity between the two enzymes is revealed in the present study from the observation that salmine and PL-150, as with cytochrome oxidase[10,22], are competitive inhibitors of cytochrome *c* peroxidase. Furthermore, the oxidation of cytochrome *c* by both enzymes remains first order (*i.e.* K_m cytochrome $c^{2+} \approx K_i$ cytochrome c^{3+}) in the presence of the polycations, suggesting that the polycations

are inhibiting both ferro- and ferricytochrome *c* complexes equally well. Along these lines it is also of interest to note that first-order kinetics were observed with modified cytochrome *c* peroxidase and modified cytochromes *c*. All these observations suggest a similar affinity, and possibly a common site, for ferro- and ferricytochrome *c*.

Certain differences in the two enzymes have also been indicated by the present study. Low-molecular-weight polylysine (*i.e.* PL-3), a competitive inhibitor of cytochrome *c* peroxidase, acts as a noncompetitive inhibitor of cytochrome oxidase[10]. Differences in the polycation K_i values for the two enzymes are also observed[10]. These differences, in part, may be a reflection of the particulate nature of the oxidase—a distinguishing feature between the two enzymes of possible functional relevance[27].

ACKNOWLEDGEMENTS

I would like to thank Dr. T. Asakura for his generous gift of modified cytochrome *c* peroxidase and for his helpful comments. I also thank Dr. T. Yonetani for his criticisms. This investigation was supported by a National Institutes of Health Fellowship 1-FO2-HE42,733-01 from the National Heart Institute.

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