Interaction of Cytochrome *c* Peroxidase with Cytochrome *c*†

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ABSTRACT: We have studied the binding of 8-anilino-1-naphthalenesulfonate to apocytochrome *c* peroxidase (apoCCP). We measured a rotational relaxation time of 48 nsec. The polarization ratio increases in the presence of ferro- or ferricytochrome *c*. The quenching of the 8-anilino-1-naphthalenesulfonate fluorescence by ferro- or ferricytochrome *c* indicates formation of a one to one complex. The quenching of porphyrin fluorescence from the protoporphyrin–apoCCP complex (P–CCP) by added ferro- or ferricytochrome *c* also indicates formation of a one to one complex. The affinity for ferricytochrome *c* is less than that for ferrocytochrome *c*.

We have recently used the magnesium salt of 8-anilino-1-naphthalenesulfonic acid and free base protoporphyrin IX as fluorescent probes to study the oxy to deoxy transition of hemoglobin in aqueous buffer (Leonard and Yonetani, 1973). The porphyrin is a particularly interesting probe since it combines with apoprotein and occupies the heme site. Thus, the apoprotein–porphyrin conjugate must be very similar to the holoheme protein.

Cytochrome *c* peroxidase (CCP)[1] which catalyzes the peroxidatic oxidation of ferrocytochrome *c* was discovered in and purified from bakers' yeast (Altschul *et al.*, 1940). An isolation and purification method has recently been reported (Yonetani and Ray, 1965). It has also been reported (Yonetani, 1967) that the apoenzyme may be prepared by the acid–butanone method of Teale (1959) and that the apoenzyme readily combines with free base protoporphyrin IX with the porphyrin occupying the heme-binding site (Asakura and Yonetani, 1969).

Extensive mechanistic studies on peroxidases have been carried out (Chance, 1943, 1949a–c, 1950, 1951a,b; Keilin and Mann, 1937; Keilin and Hartree, 1951). The postulated mechanisms involve two intermediate enzyme–peroxide compounds. The peroxide compound of CCP has been detected, isolated, and crystallized (Yonetani *et al.*, 1966; Yonetani and Schleyer, 1966). The complex with cytochrome *c* has been more elusive. Recently, nuclear magnetic resonance experiments which suggest formation of a one to one complex with ferricytochrome *c* have been reported (Gupta and Yonetani, 1973). We report here the results of our fluorescence studies on ANS-labeled apocytochrome *c* peroxidase (ANS–CCP) and protoporphyrin-labeled apocytochrome *c* peroxidase (P–CCP). The results suggest formation of a one to one complex with ferrocytochrome *c* (cyt *c*) and ferricytochrome *c* (cyt c^+). The cyt *c* complex is the more stable.

Experimental Section

Materials. CCP, apoCCP, P–CCP, cyt *c*, and cyt c^+ from bakers' yeast were prepared as described previously (Yonetani and Ray, 1965; Yonetani, 1967; Asakura and Yonetani, 1969). A purified sample of magnesium 8-anilino-1-naphthalenesulfonic acid was kindly donated by Dr. Daniel Quimby.

Methods. All fluorescence and phosphorescence spectra were recorded with a Perkin-Elmer MPF-2A fluorescence spectrophotometer using an R446 HTV photomultiplier tube for porphyrin fluorescence and an R106 tube for all other measurements. Phosphorescence experiments were done at 77°K in a deoxygenated 50% glycerol–100 mM potassium phosphate buffer (pH 7). Phosphorescence decay times were determined from the trace of phosphorescence decay recorded with a storage oscilloscope. Fluorescence decay times were determined at room temperature in buffer solution with an Ortec 7200 fluorescence lifetime spectrophotometer. Data were stored on magnetic tape and computer analyzed.

Results

Binding of ANS to ApoCCP. When 10 μM ANS is added to a 10 μM apoCCP solution in 100 mM potassium phosphate buffer (pH 7), the characteristic blue fluorescence of protein-bound ANS (Stryer, 1965) appears at 475 nm with excitation at 290 or 385 nm (Figure 1). There is a decrease in protein tryptophan fluorescence on binding indicating energy transfer to bound ANS. Titrations show that apoCCP binds one ANS molecule per heme, thus indicating that ANS occupies the heme site. Bound ANS may be displaced by added hemin. The rotational relaxation time of ANS–CCP was determined by the method of Perrin (1926). Experiments were done at 25 ± 0.2°; the temperature to viscosity ratio was varied by sequential additions of sucrose. The results are shown in Figure 2. The rotational relaxation time was calculated to be 48 nsec. The molecular weight of apoCCP is about 4×10^4 (Yonetani, 1967). The rotational relaxation time for a spherical protein of this molecular weight is 37 nsec. The discrepancy probably reflects a deviation from spherical symmetry. The observed polarization ratio increases on addition of cyt *c* or cyt c^+ thus indicating association between cyt *c* or cyt c^+ and ANS–CCP.

Tryptophan Phosphorescence. Phosphorescence spectra at 77°K of tryptophan in glycerol buffer, apoCCP, and P–CCP are shown in Figure 3. The spectrum of tryptophan in water agrees with that reported previously (Freed and Salmre, 1958). There is a slight red shift in the phosphorescence maxima in apoCCP. There is a striking change in the phosphorescence spectrum from P–CCP; all vibronic structure is lost. Phosphorescence lifetimes are presented in Table I.

Fluorescence Decay Times. The ANS–CCP and P–CCP fluorescence decay times of ANS and porphyrin, respectively,

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[1] Abbreviations used are: CCP, cytochrome *c* peroxidase; ANS, 8-anilino-1-naphthalenesulfonic acid.

TABLE I: Emission Data

Sample	Emission	Lifetime	$(X^2/R^6) \times 10^{-40}$	R (Å)	
				A	B
ApoCCP	Tryptophan phosphorescence	4.50 sec			
P-CCP	Tryptophan phosphorescence	4.00 sec			
ANS-CCP	ANS fluorescence	11.5 nsec			
P-CCP	Porphyrin fluorescence	16.6 nsec			
ApoCCP-cyt c	Tryptophan phosphorescence	3.84 sec	1.100	18.7	19.9
ApoCCP-cyt c^+	Tryptophan phosphorescence	3.80 sec	1.100	19.0	20.3
P-CCP-cyt	Tryptophan phosphorescence	3.19 sec	1.500	17.3	18.9
ANS-CCP-cyt c	ANS fluorescence	1.5 nsec	0.888	19.4	20.7
ANS-CCP-cyt c^+	ANS fluorescence	1.6 nsec	0.880	19.5	20.8
P-CCP-cyt c	Porphyrin fluorescence	5.3 nsec	5.682	14.3	15.2
P-CCP-cyt c^+	Porphyrin fluorescence	3.2 nsec	5.180	14.5	15.4

are presented in Table I. In the absence of cyt c or cyt c^+, the fluorescence decay from either sample is a simple, single exponential. As cyt c or cyt c^+ is added however, the decay exhibits two exponentials (Figure 4). One has the same lifetime as in the absence of cytochrome while the other is much faster. The contribution of this fast phase to the total decay increases as the concentration of cyt c or cyt c^+ increases. This indicates formation of a complex and the resulting quenching of fluorescence by the heme in cyt c or cyt c^+. One may plot the fractional contribution of the fast phase (which is readily obtained by the computer deconvolution of the observed fluorescence decay into the sum of two exponentials) *vs.* the concentration of cyt c or cyt c^+ to obtain the stoichiometry of the complex (Figure 5). For ANS-CCP or P-CCP with cyt c or cyt c^+, the stoichiometry was found to be one to one.

Fluorescence Titrations and Binding Constants. Since addition of cyt c or cyt c^+ to solutions of ANS-CCP or P-CCP resulted in complex formation and fluorescence quenching, one should be able to carry out steady-state fluorescence titrations. One must select exciting wavelengths that minimize effects due to absorption by the added cyt c or cyt c^+ yet give a sufficient fluorescence intensity. The results of such experiments are shown in Figures 6 and 7. Each experiment showed an inflection point corresponding to one to one stoichiometry. From the quantitative knowledge of fluorescence quenching obtained from the lifetime measurements, one may estimate binding or dissociation constants from the steady-state fluorescence titrations (see Appendix). The binding constants for ANS-CCP with cyt c or cyt c^+ and

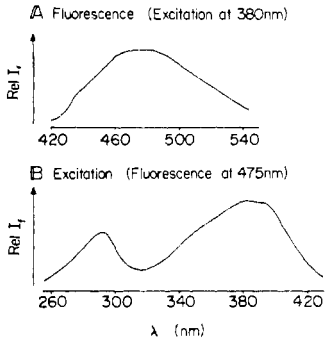

FIGURE 1: Fluorescence and excitation spectra of ANS-apocytochrome c peroxidase (ANS-CCP) in 100 mM potassium phosphate buffer (pH 7).

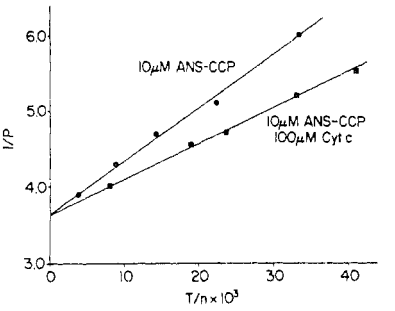

FIGURE 2: Perrin plots for (1) 10 μM ANS-CCP and (2) 10 μM ANS-CCP with 100 μM cyt c. Measurements done in 100 mM potassium phosphate buffer (pH 7).

P-CCP with cyt c or cyt c^+ along with dissociation constants of the resulting complexes are presented in Table II. These are only estimates of the equilibrium constants since inner filter effects still partially interfere with the titration. Calculations based on the lifetime titrations would be even less precise due to the numerical uncertainty in the computer-fitted contributions.

Discussion

All the data suggest the formation of a one to one complex between cytochrome c peroxidase and cyt c or cyt c^+. The dissociation constant for cyt c^+ complex is larger than that for cyt c complex. The difference is largest for P-CCP complexes which should be much more similar to the holoenzyme complex. One would expect the affinity for cyt c to be larger than for cyt c^+ in view of the catalytic nature of the enzyme.

From the phosphorescence and fluorescence quenching, the distance between emitter and heme of cyt c or cyt c^+ may be calculated. The calculation is based on the assumption that the emitter and heme are so far apart that there can be no

TABLE II: Binding Constants

Reaction	K_b
ANS-CCP + cyt c	8.55×10^4
ANS-CCP + cyt c^+	7.56×10^4
P-CCP + cyt c	1.22×10^5
P-CCP + cyt c^+	7.56×10^4

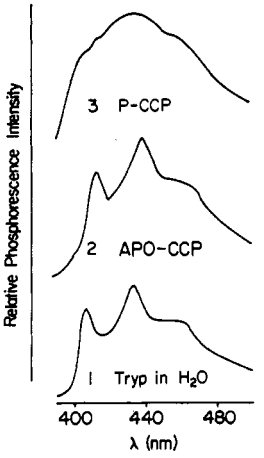

FIGURE 3: Tryptophan phosphorescence spectra at 77°K in a 50% glycerol–50% 100 mM potassium phosphate buffer (pH 7). Excitation was at 290 nm.

direct electronic interaction between them, thus, triplet–triplet transfer can be approximated by a dipole–dipole mechanism. This assumption is supported by the work of Gupta and Yonetani and by our observation that the absorption spectrum of P–CCP in the presence of cyt *c* (recorded by a difference technique) is identical to that of P–CCP in the absence of cyt *c*. If there is no direct electronic interaction, then the quenching must result from longer range dipole–dipole or Förster-type energy transfer to heme.

This type of energy transfer has been studied by Förster (1959). Its efficiency is known to depend on the distance between donor and acceptor molecules, the relative orientation of their transition dipoles, and the spectral overlap of the donor fluorescence spectrum with the acceptor's absorption spectrum. Förster gives the following expression for the "rate constant" for energy transfer

$$k_t = (8.940 \times 10^{-26})\left(\frac{X^2}{\tau_0 R^6}\right)\int_0^\infty f_s(\nu)\epsilon_a(\nu)\frac{d\nu}{\nu 4} \qquad (1)$$

$_s(\nu)$ is the normalized and corrected donor fluorescence spectrum and $\epsilon_a(\nu)$ is the acceptor absorption spectrum in molar absorptivity both measured on a wave-number scale. R is the distance between donor and acceptor. τ_0 is the natural radiative lifetime of the donor, and X^2 is an orientation factor.

From this expression, and the value of k_t which is calculated from the lifetime in the presence and absence of cytochrome, one may calculate values of X^2/R^6. In the absence of other data, one must assume a value for X^2. It is equal to 0.4761 for random rigidly oriented dipoles and to 0.6667 for

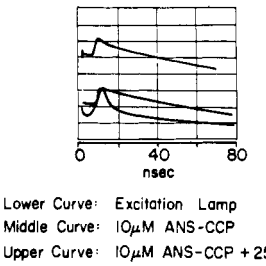

FIGURE 4: ANS fluorescence decay curves: lower curve, excitation lamp; middle curve, 10 μM ANS–CCP; upper curve, 10 μM ANS–CCP and 25 μM cyt *c*; all in 100 mM potassium phosphate buffer (pH 7).

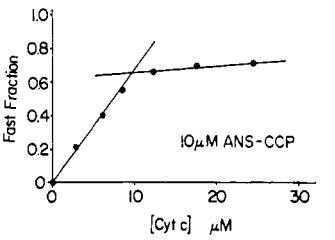

FIGURE 5: Fluorescence lifetime titration. 10 μM ANS–CCP in 100 mM potassium phosphate buffer (pH 7). The ordinate is the fast phase's contribution to the total decay.

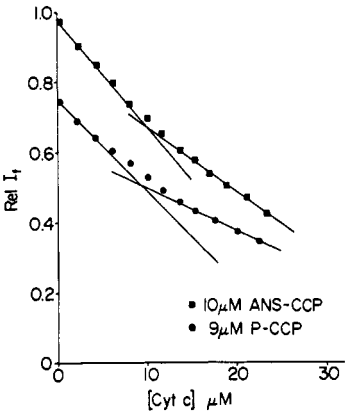

FIGURE 6: Steady-state fluorescence titrations of 10 μM ANS–CCP with cyt *c* (excitation at 340 nm, emission at 375) and 9 μM P–CCP with cyt *c* (excitation at 370 nm, emission at 620 nm). Experiments were done in 100 mM potassium phosphate buffer (pH 7).

random freely rotating dipoles. R values for each case are shown in Table I. There is about a 10% difference in R for each model, but for each model, the trend in the distances is quite reasonable. The tryptophan in the protein must be intimately associated with the heme-binding site as evidenced by the change in phosphorescence spectra in going from apo-CCP to P–CCP. The tryptophan–heme distance is about 19 Å. The ANS–heme distance is about the same. ANS is a small molecule compared to protoporphyrin and probably penetrates much further into the protein cavity. The much larger porphyrin cannot penetrate nearly so far into the cavity and thus gives a smaller heme distance, about 14 Å. It is significant that although quenching and overlap differ, calculation gives essentially the same porphyrin–heme distance in P–CCP–cyt *c*

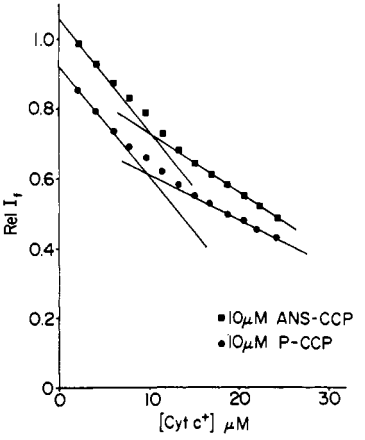

FIGURE 7: Steady-state fluorescence titrations of 10 μM ANS–CCP and 10 μM P–CCP with cyt c^+. Same conditions as in Figure 6.

and P–CCP–cyt c^+. This should correspond to the heme–heme distance in the holoenzyme complex.

Appendix

In the presence of cytochrome, the observed fluorescence intensity, $I(\text{cyt}) = c(X_1\phi_1 + X_2\phi_2)I_E$, where c is the total concentration of A–CCP conjugate, X_1 and X_2 are the mole fractions of uncomplexed and complexed material, ϕ_1 and ϕ_2 are the respective quantum yields (obtained from lifetime data), and I_E is the intensity of exciting light. Fluorescence intensity is a linear function of concentration in the concentration ranges used in this study. From the observed fluorescence intensity in the absence of cytochrome, $I(0)$, one may calculate X_1 (and thus also X_2) from the following

$$X_1 = \frac{I(\text{cyt}) - (\phi_2/\phi_1)I(0)}{I(0) - (\phi_2/\phi_1)I(0)} \quad (2)$$

Consideration of the expression for the cytochrome–CCP binding constant yields the following linear equation

$$\log (X_2/X_1) = \log K_b + \log ([\text{cyt}] - cX_2) \quad (3)$$

K_b may be calculated by plotting $\log (X_2/X_1)$ *vs.* $\log ([\text{cyt}] - cX_2)$ where [cyt] is the total concentration of added cytochrome. This analysis assumes I_E is constant during the titration so inner filter effects of added cytochrome must be minimized by a proper selection of exciting wavelengths. The plots were fairly linear, the worst correlation coefficient was 0.9844 and the best was 0.9995.

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