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ARTICLE · JUNE 2008

DOI: 10.1016/0003-9861(73)90672-3

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Eur. J. Biochem. 60, 199–207 (1975)

Cytochrome *c* Interaction with Membranes

Absorption and Emission Spectra and Binding Characteristics of Iron-Free Cytochrome *c*

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(Received July 1 / September 9, 1975)

A cytochrome *c* derivative from which iron is removed has been prepared and characterized. Several lines of evidence indicate that native and porphyrin cytochrome *c* have similar conformations: they have similar elution characteristics on Sephadex gel chromatography; in both proteins the tryptophan fluorescence is quenched and the pK values of protonation of the porphyrin are identical. Porphyrin cytochrome *c* does not substitute for native cytochrome *c* in either the oxidase reaction or in restoring electron transport in cytochrome-*c*-depleted mitochondria. It does however competitively inhibit native cytochrome *c* in these reactions, the K_i for inhibition being larger than the K_m for reaction. The absorption and emission spectra, and the polarized excitation spectrum of the porphyrin cytochrome *c* are characteristic of free base porphyrin. The absence of fluorescence quenching of porphyrin cytochrome *c* when the protein is bound to cytochrome oxidase suggests that heme to heme distance between these proteins is larger than 0.5 to 0.9 nm depending upon orientation. Binding of the porphyrin cytochrome *c* to phospholipids or to mitochondria increases the fluorescence polarization of a positively polarized absorption band, which indicates that the bound form of the protein does not rotate freely within the time scale of relaxation from the excited state.

Cytochrome *c* is a hemeprotein whose physiological function is intimately concerned with rapid changes in valency of the iron. Although the iron moiety seems to be the most vital part of cytochrome *c*, both the porphyrin and the surrounding protein provide a structure of the enzyme which is most efficient for electron transfer reactions. The polypeptide chain supplies a three-dimensional framework in which the heme is conveniently "housed" [1, 2]. It can also be expected to account for the binding properties to the membranes and isolated enzymes, which ensures specificity and proper orientation of the interactions. The porphyrin macrocycle contributes to the electronic properties of cytochrome *c* by mixing its electron cloud with that of the iron, and determines the firmness and stability of the protein network by stabilizing it through extra heme-protein interactions.

In our previous work [3–7], we have investigated the protein part of native cytochrome *c*, its binding properties and effects of modification of the polypeptide chain on the electronic properties of the heme. Here we aim to characterize the properties of the porphyrin and polypeptide chain in the absence of iron and thus obtain insight into the contribution of the central metal ion to the properties of the enzyme.

EXPERIMENTAL PROCEDURE

Horse heart cytochrome *c* (type III and type VI) and phospholipids were obtained from Sigma Chemical Company (St. Louis, Missouri). Matheson (Patterson, N. J.) supplied anhydrous hydrogen fluoride. All other chemicals were of reagent grade. Water was glass distilled and deionized.

Pigeon heart mitochondria were prepared by the method of Chance and Hagihara [8] and rat liver mitochondria were isolated according to Schneider [9]. Cytochrome *c* was extracted from the mitochondria essentially as described by the method of Jacobs and Sanadi [10] as modified by Boveris *et al.* [11]. Phospholipid suspensions were prepared by sonication of the lipids for about 5 min using a Branson sonifier.

Preparation of Iron-Free Porphyrin Cytochrome c

Preparation of iron-free porphyrin cytochrome *c* was carried out with minor modifications of the methods of Flatmark and Robinson [12] and of Fisher *et al.* [13]. One hundred milligrams of cytochrome *c* (type III) were cooled in an open Teflon beaker suspended in a Dewar flask containing liquid nitrogen.

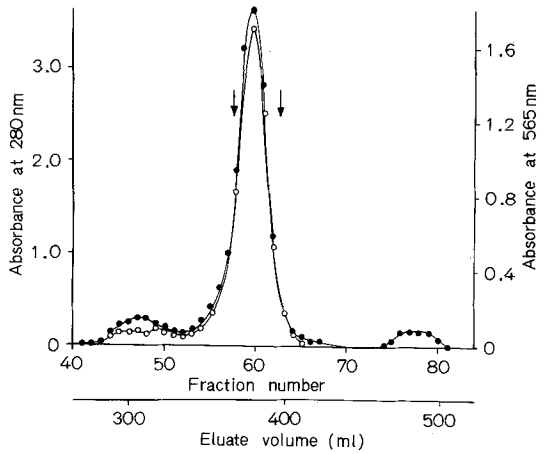

Fig. 1. *Elution of porphyrin cytochrome* c *from Sephadex G-50 column.* Gel filtration was carried out at room temperature as described in the Experimental Procedure. (●——●) Absorbance at 280 nm; (○——○) absorbance at 565 nm. Fractions between the arrows were pooled

Anhydrous HF (about 6 ml) was passed into the beaker and the cytochrome *c* turned purple, indicating that a reaction had occurred. The solution was stirred manually with a teflon stirring rod and anhydrous HF (about 6 ml) was again added to the beaker. This procedure took about 3 min. The beaker was transferred from the liquid nitrogen container to room temperature and HF was removed under a stream of nitrogen. The protein was dissolved in 3 ml 0.05 M ammonium acetate, pH 5.0, and elution was carried out with the same medium. A typical elution pattern is shown in Fig. 1. The porphyrin cytochrome *c* eluted in the same fraction as normal ferricytochrome *c*, which indicated that their molecular sizes are similar. The fractions shown in the figure were pooled, dialyzed against 0.01 M phosphate buffer, pH 7.4, and concentrated by applying the solution to an Amberlite CGH-50 column (0.5 × 0.5 cm) equilibrated with 0.01 M phosphate, followed by elution with 0.5 M PO_4 buffer, pH 7.4. A small amount which remained on the column was discarded. Care was taken during all of the above manipulations to avoid exposure of the porphyrin derivative to light, although we have no evidence that porphyrin cytochrome *c* is extremely light-sensitive. The absorption coefficient at 404 nm was taken to be 160 mM^{-1} cm^{-1} [12].

Binding

Binding of cytochrome *c* (or porphyrin cytochrome *c*) was determined from the amount found in the mitochondrial pellet after centrifuging down cytochrome-*c*-depleted mitochondria (10 min at 8000 × *g*) from the suspending medium (0.2 M sucrose/0.05 M morpholinopropane sulfonate buffer, pH 7.0). The pellet was suspended in 0.1 M phosphate buffer, pH 7.2 containing 1% Triton X-100. The concentration of native cytochrome *c* was estimated from the difference in absorbance between total oxidized (+ 5 mM ferricyanide) – total reduced (+ dithionite) measured at 550–540 nm in a Johnson Foundation dual wavelength spectrophotometer. The absorption coefficient was taken to be 19.7 mM^{-1} cm^{-1}. The concentration of porphyrin cytochrome *c* was estimated from the absorbance measurements at 404 nm which were carried out against a blank containing the same amount of cytochrome-*c*-depleted mitochondria (or supernatant), but no added porphyrin cytochrome *c*. It was found that this eliminated the interference from the absorbance of cytochromes *b* and c_1 at the measuring wavelength of porphyrin cytochrome *c*.

Oxygen Uptake

Oxygen uptake was measured at 24 °C with a Clark oxygen electrode in 0.20 M sucrose/0.05 M morpholinopropane sulfonate/0.01 M phosphate, pH 7.0 buffer with succinate as substrate. Cytochrome oxidase activity was measured in 0.1 M phosphate buffer, pH 6.5 using reduced cytochrome *c* as substrate. The purified oxidase was isolated from pigeon breast mitochondria by the methods of Kuboyama *et al.* [14]. Cytochrome *a* was determined from absorbance measurements at 605–630 nm using an absorption coefficient of 24 mM^{-1} cm^{-1} (reduced-oxidized). The protein was determined by the biuret method [15].

Fluorescence Spectra

Fluorescence spectra were obtained using an Hitachi MPF-2A spectrofluorometer equipped with an HTV R446 photomultiplier. Fluorescence polarization was measured by insertion of a 105PB Polacoat filter between the exciting beam and the cuvette, and between the cuvette and the detector. Polarization, *p*, is defined as:

$$p = \frac{I_{\parallel} - cI_{\perp}}{I_{\parallel} + cI_{\perp}} \tag{1}$$

where $I_{\parallel}$ and $I_{\perp}$ are light intensities parallel and perpendicular to an exciting beam polarized parallel to the laboratory axis and *c* is a factor used to correct for the unequal detection of differently polarized light. A phase-modulating lifetime instrument was utilized to determine fluorescent lifetime, τ [16]. The rotational correlation time, ϱ, was calculated according to the Perrin equation:

$$\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\varrho}\right) \tag{2}$$

where P_0 is the fluorescence polarization in the absence of molecular motion.

Spectral overlap exists between the α absorption band of cytochrome oxidase and the emission bands of porphyrin cytochrome *c* which, in theory, should result in distance-dependent quenching. The distance, R_c, at which porphyrin is 50% quenched can be calculated according to Förster's theory by:

$$R_c^6 = \frac{9000\,(\ln 10)\,K}{128\,\pi\,n^4 N}\,\phi_f \int_0^\infty \frac{F_D\,\varepsilon_A\,d\bar{\nu}}{\bar{\nu}^4} \qquad (3)$$

where N is Avogadro's number, n is the refractive index, taken to be 1.4, F_D is the normalized fluorescence emission spectrum of the donor (porphyrin cytochrome *c*, ε_A is the absorption spectrum of the acceptor (cytochrome oxidase), and $\bar{\nu}$ is the wavelength. R_c depends critically on the orientation factor, K, which is determined from:

$$K^2 = (\cos\alpha - 3\cos\beta\cos\gamma)^2 \qquad (4)$$

where α is the angle between donor and acceptor transition moments, β is the angle between the donor transition moment and the vector joining the centers of the donor and acceptor, and γ is the angle between the acceptor moment and the vector joining the centers of the donor and acceptor.

RESULTS

Optical Spectra of Porphyrin Cytochrome c

The absorption spectra of porphyrin cytochrome *c* are given in Fig. 2. At neutral pH the visible spectrum shows the characteristic four bands observed for all free base porphyrins [17] with absorption maxima at 506, 540, 568, and 620 nm. Acidification results in collapse of the visible spectra into two bands with major absorption maximum at 554 and a minor band at 595 nm. The Soret peak of the base form absorbs at 412 nm; protonation results in sharpening of the peak and a shift in absorbance maximum to 410 nm. Protonation of the ring occurs with a pK at pH 2.5 (Fig. 3) which is identical to that in the native cytochrome *c*.

Both the base and acidic forms of porphyrin cytochrome *c* exhibit red fluorescence. In Fig. 4, the fluorescence excitation and emission spectra are presented. The excitation spectra are identical to the absorption spectra (Fig. 2), the differences being accounted for by the different instruments. Moreover, the excitation and emission spectra are independent of the wavelengths used for emission or excitation, respectively which indicates the presence of one emitting species. The fluorescent lifetime of the base form is 6.5 ns (Tatsuro Yoshida, personal communication), a value similar to that calculated for free base porphyrin in organic solvent [18]. The fluorescent yield was identical in H_2O and ^2H_2O buffer; the absence of enhancement in the ^2H_2O medium suggests that the porphyrin in cytochrome *c* is shielded from the surrounding water. On the other hand, in 6 M guanidine-HCl, the fluorescence yield decreases about 20% from that observed in the H_2O medium. Presumably in this case, the unfolding

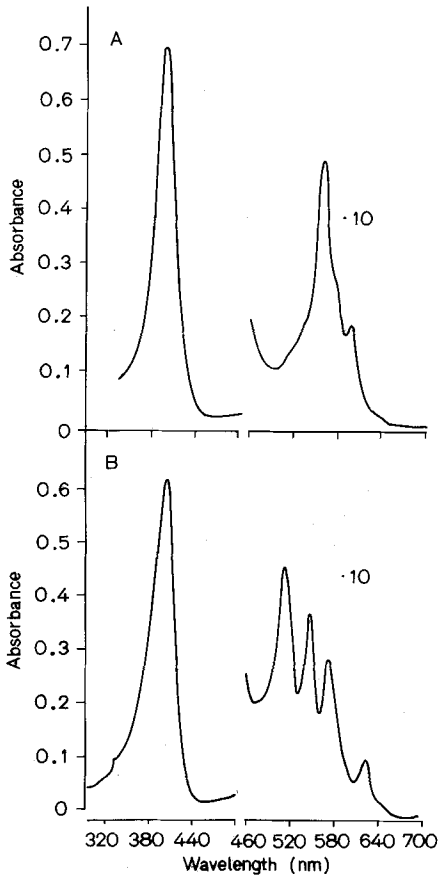

Fig. 2. *Absorption spectra of porphyrin cytochrome* c. Sample consisted of 3.8 μM porphyrin cytochrome *c* in 10 mM PO_4 at pH 1.3 (A) or 7.0 (B)

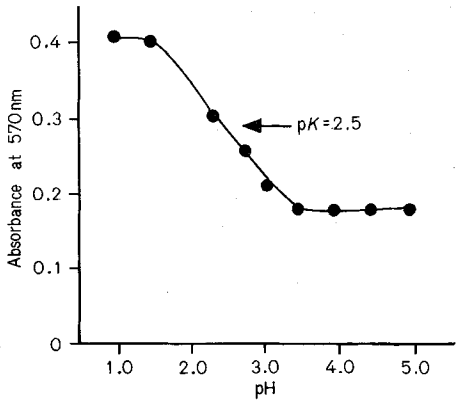

Fig. 3. *The pH dependence of protonation of porphyrin.* Absorbance at 570 nm was measured as a function of pH. The medium contained 10 mM PO_4 and 40 μM porphyrin cytochrome *c*

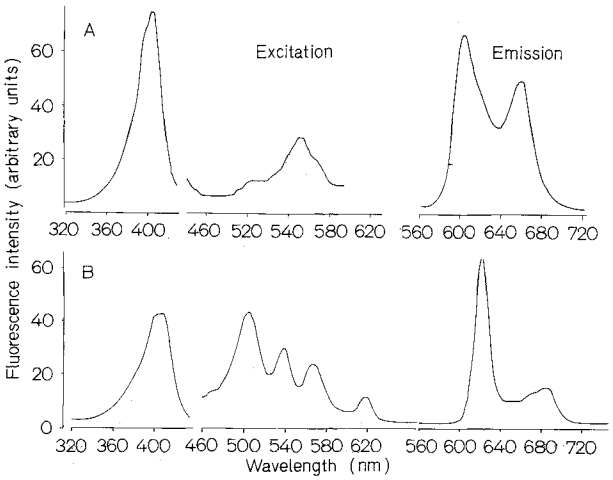

Fig. 4. *Fluorescence excitation and emission spectra of porphyrin cytochrome* c. Sample contained 3 μM porphyrin cytochrome *c* and 10 mM PO_4 at pH 1.3 (A) and 7.0 (B). (A) Excitation at 550 nm; emission, 605; (B) excitation at 500 nm; emission at 620 nm. Slit widths: 10 nm. Spectrum carried out at ambient temperature (24 °C)

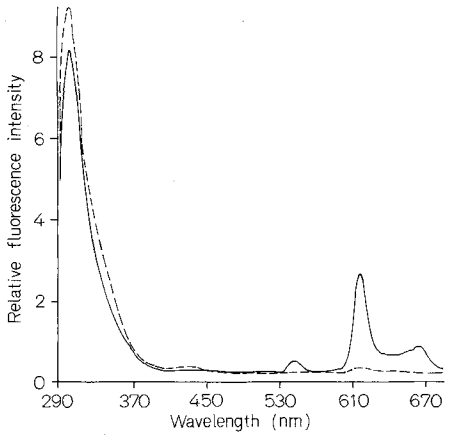

Fig. 5. *Tryptophan to porphyrin energy transfer.* Sample contained 1 μM porphyrin cytochrome, 10 mM phosphate, pH 7.2 (——) or 6 M guanidine-HCl (– – –). Gain for guanidine-HCl sample was reduced by 3. Excitation: 285 nm

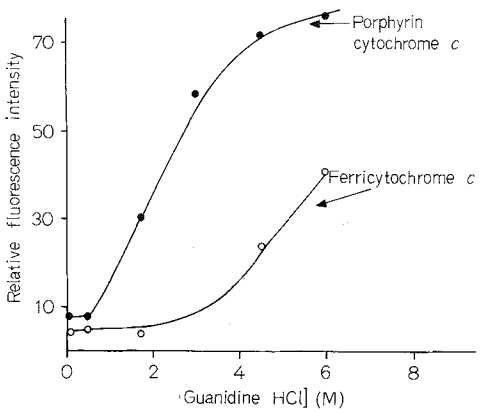

Fig. 6. *Effect of guanidine-HCl on tryptophan fluorescence in native and porphyrin cytochrome* c. Sample contained 1 μM porphyrin cytochrome *c* (●) or 1 μM ferricytochrome *c* (○), 10 mM PO_4, pH 7.2 and guanidine-HCl in concentration indicated. Excitation, 285 nm; emission, 340; slits, 5 nm

of the polypeptide chain leads to exposure of the porphyrin to the medium which results in quenching of the fluorescence.

In porphyrin cytochrome *c* the fluorescence of tryptophan is quenched [19] (Fig. 5) but, as shown in Fig. 6, the excitation of tryptophan at 280 nm results in the fluorescence emission spectrum of the porphyrin. This sensitized porphyrin emission provides unequivocal evidence that Förster-type energy transfer [20] from the tryptophan to the heme is responsible for the quenching of tryptophan fluorescence in the porphyrin cytochrome *c*. It indicates, moreover, that even in the absence of the central metal the tryptophan residue is in close proximity to the porphyrin ring. The energy transfer is abolished in the presence of 6 M guanidine-HCl which unfolds the protein and leads to an increased distance between the tryptophan and the porphyrin. In the latter condition the fluorescence of tryptophan is enhanced and a single-banded emission spectrum with maximum at 340 nm and with no evidence of heterogeneity, can be observed. The concentration profile for denaturation of porphyrin-cytochrome *c* by guanidine-HCl indicates that the iron-free derivative is less "firm" than the native cytochrome *c* because its sensitivity to denaturation (Fig. 6) is higher. A similar finding was made by Cohen *et al.* [19] who observed that porphyrin cytochrome *c* is more sensitive to heat denaturation than ferricytochrome *c*.

Binding of Porphyrin Cytochrome c *to Cytochrome-*c*-Depleted Pigeon Heart Mitochondria*

Porphyrin cytochrome *c*, like its parent molecule, binds to cytochrome-*c*-depleted mitochondria. Fig. 7 presents the binding of native cytochrome *c* and porphyrin cytochrome *c* to cytochrome-*c*-depleted pigeon heart mitochondria in the form of Scatchard plots. The amounts of the bound proteins are expressed relative to cytochrome *a* concentration (rather than protein content) because this affords a more reliable basis for comparison of the results and, in addition, provides us with a better insight into the stoichiometry of binding.

In agreement with our previous results [4, 6] Scatchard plots for the binding of native cytochrome *c* were non-linear, which indicates heterogeneity in the binding sites. With native cytochrome *c*, the high affinity binding sites with a K_d of $\approx$ 0.02 μM were present in the amount of 2 per cytochrome *a* (obtained from the extrapolation of the steeper part of the binding curve). The Scatchard plot for the binding of porphyrin cytochrome *c* was also non-linear; the initial steeper part of the binding curve extrapolated to the value of 1 – 2 porphyrin cytochrome *c* bound per cytochrome *a*, but the binding constant was approximately 5 – 10 times higher, *i.e.* K_d = 0.1 μM. Low affinity binding sites were present in considerably larger numbers and

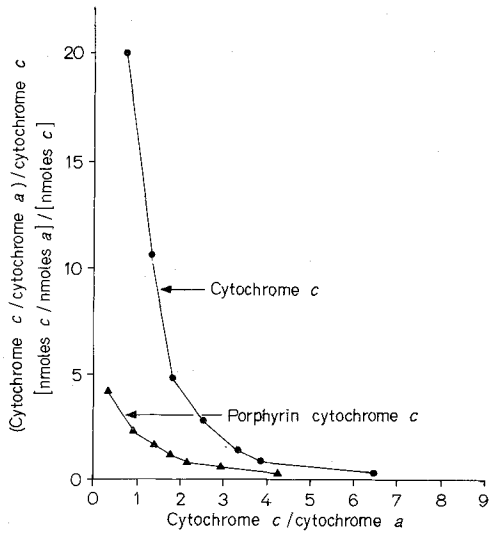

Fig. 7. *Binding of native cytochrome* c *and porphyrin cytochrome* c *to cytochrome-*c*-depleted pigeon heart mitochondria.* Cytochrome-*c*-depleted pigeon heart mitochondria (2.9 nmol cytochrome *a*) were incubated in 0.20 M sucrose/0.050 M morpholinopropane sulfonate pH 7.0 buffer with various amounts of cytochrome *c* (or porphyrin cytochrome *c*) for 2 min at room temperature. The samples were aerated during the incubation time. The mitochondria were removed by centrifugation at $8000 \times g$ for 10 min at 4 °C. The pellet was suspended in 0.1 M phosphate buffer pH 7.2 containing 1% Triton X-100. The concentration of cytochrome *c* in the pellet and in the supernate was determined as described in the Methods section. (●) Native cytochrome *c*; (▲) porphyrin cytochrome *c*

the K_d value was approximately 0.1 – 0.2 μM for both the native and porphyrin cytochrome *c*.

Reaction of Porphyrin Cytochrome c *with Cytochrome-*c*-Depleted Pigeon Heart Mitochondria*

The addition of cytochrome *c* to cytochrome-*c*-depleted mitochondria elicits an increase in oxygen uptake. The extent of stimulation depends on the concentration of cytochrome *c* added; the rate is essentially zero in the absence of cytochrome and it increases until saturation is reached at a concentration of approximately 0.2 nmol cytochrome *c* per 2 mg protein used in the experimental system presented in Fig. 8. Porphyrin cytochrome *c* by itself does not stimulate oxygen uptake of the depleted mitochondria but, when added to the incubation mixture prior to native cytochrome *c*, it prevents the enhancement of respiration. In Fig. 8 two different concentrations of porphyrin cytochrome *c* were added and the respiratory activity was determined as a function of increasing amounts of native cytochrome *c*. It is seen that, in the presence of porphyrin cytochrome *c*, higher concentrations of the native enzyme are required to attain the same respiratory activity. However, the final respiratory rate was the same in the absence and presence of porphyrin cytochrome *c*. This behavior pattern may be suggestive

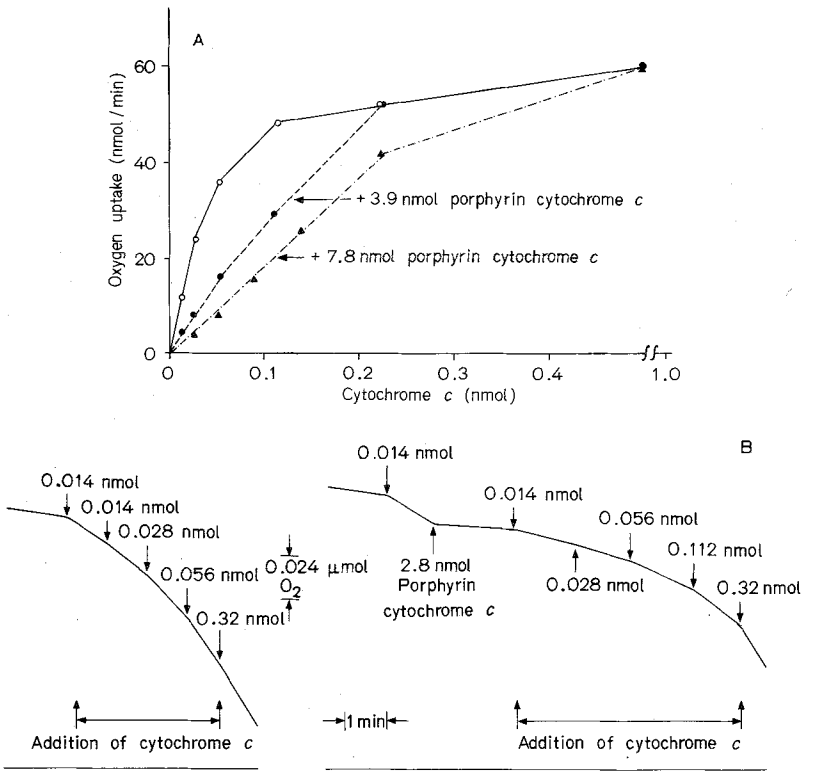

Fig. 8. *The effect of porphyrin cytochrome* c *on the restoration of oxygen uptake by native cytochrome* c *in cytochrome-*c*-depleted rat liver mitochondria.* Rat liver mitochondria (2.1 mg protein) were suspended in 2.5 ml of 0.2 M sucrose/0.050 M morpholinopropane sulfonate/0.010 M phosphate pH 7.0 buffer. The respiratory substrate was 10 mM succinate. All concentrations shown are additions of cytochrome *c*, except the addition of 2.8 nmol porphyrin cytochrome *c* in (B)

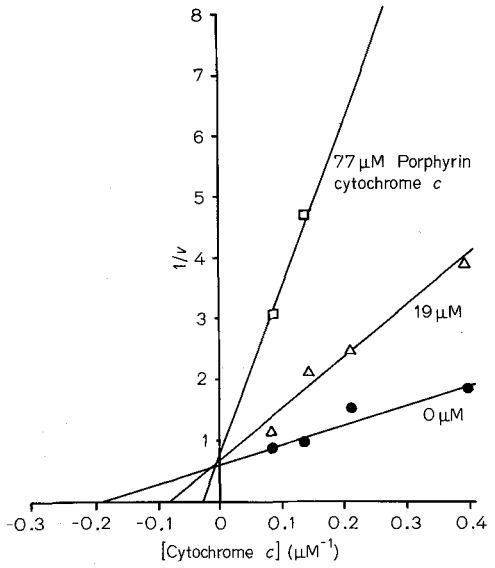

Fig. 9. *Oxidation of ferrocytochrome* c *by cytochrome oxidase.* The reaction medium contained 0.02 μM cytochrome oxidase, 20 mM PO_4 buffer, pH 7.2 and initial concentrations of type VI Sigma horse heart cytochrome *c* indicated in the figure. Oxidation of cytochrome *c* was monitored by the disappearance of absorption at 550 nm as described by Yonetani and Ray [22]. The temperature was ≈ 23 °C. *v* is expressed in μmol/min

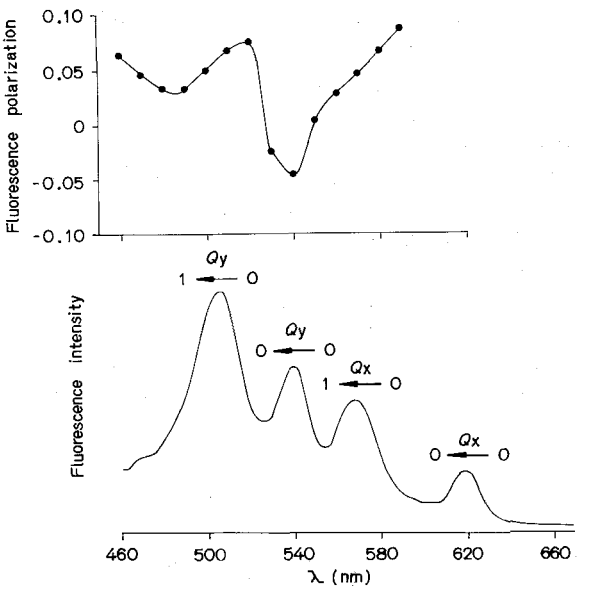

Fig. 10. *Polarized excitation spectrum for porphyrin cytochrome* c. Sample contained 0.3 μM porphyrin cytochrome *c* in 90% glycerol. Temperature: 20 °C. Emission, 620 nm. Excitation slits: 8 nm; emission slits, 10 nm

of competition between the native cytochrome *c* and the porphyrin cytochrome *c* for a common site.

The inhibitory effect of porphyrin cytochrome *c* is also seen when the experiment is carried out in yet another way. Oxygen uptake is stimulated by the addition of a small concentration of cytochrome *c* (Fig. 8) and then inhibited by subsequent introduction of porphyrin cytochrome *c*. The inhibition can be reversed by the addition of a higher concentration of native cytochrome *c* and the maximal respiratory rate, equal to that in the absence of porphyrin cytochrome *c*, is reached.

A more direct test of the competition between native and porphyrin cytochrome *c* is a spectrophotometric assay of the oxidation of ferrocytochrome *c* by purified cytochrome oxidase [21,22] carried out in the absence and presence of porphyrin cytochrome *c*. The double reciprocal plots presented in Fig. 9 show that the value for the *y* intercept is the same in the presence and absence of porphyrin cytochrome *c*. This confirms our earlier suggestion that the porphyrin derivative competes with the native cytochrome *c* for the same site. The K_m for the oxidation of ferrocytochrome *c* is 5 μM under conditions described in the legend. The inhibitory constant, K_i, of porphyrin cytochrome *c* is somewhat higher, about 10 μM.

Fluorescence Characteristics of Bound Porphyrin Cytochrome c

The competitive binding of porphyrin cytochrome *c* with the native cytochrome suggests that the binding sites are identical. If so, we are afforded an excellent opportunity to characterize the cytochrome binding site through use of porphyrin cytochrome *c* fluorescence characteristics. The results are as follows:

The Fluorescence Yield is not changed upon binding to mitochondria or oxidase which indicates that the heme environment is not measurably altered upon binding, and that the heme is sufficiently far from paramagnetic and colored centers in the membrane so that quenching does not occur. The relationship between the distance and orientation required for quenching is presented in the Discussion.

Table 1. *Fluorescent polarization of porphyrin cytochrome* c. Excitation 500 nm (8 nm slit); emission, 620 nm (10 nm slit). Medium was 10 mM PO_4 buffer, pH 7.2. Temperature was 25 °C. Limiting polarization was taken to be 0.13

Conditions	*P*	*ϱ*
		ns^{-1}
1 μM porphyrin cytochrome *c*	0.044	9.42
1 μM porphyrin cytochrome *c* + 0.5 mg phosphatidic acid and 1.0 mg dimyristoyl lecithin/ml	0.120	216.0
0.3 μM porphyrin cytochrome *c* and 3 μM cytochrome oxidase	0.125	immobilized
0.5 μM porphyrin cytochrome *c* + 3 mg cytochrome-*c*-depleted mitochondria/ml	0.13	immobilized

Polarization measurements allow estimation of rotational mobility. Fig. 10 presents the polarized excitation spectrum of the visible bands of porphyrin cytochrome *c*. The spectrum which shows both positive and negative bands is characteristic of free base porphyrins [23]. The fluorescence polarization increases when porphyrin cytochrome *c* binds to mitochondria or to artificial membranes (Table 1). The rotational correlation time can be obtained from the polarization according to Eqn (2) (Materials and Methods). The polarization of the bound porphyrin cytochrome *c* approaches the limiting value, which indicates that the rotational motion of the bound cytochrome is slower than the relaxation time of the excited state porphyrin.

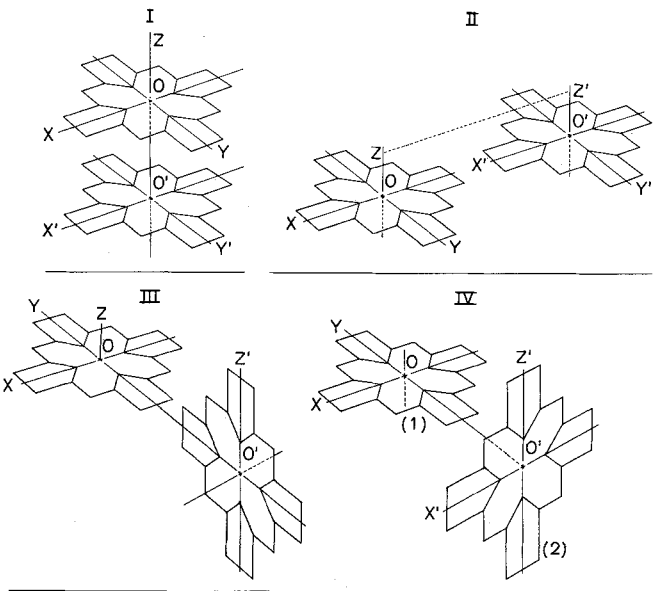

Fig. 11. *Orientation factors for energy transfer between porphyrins*

DISCUSSION

The heme environments in native cytochrome *c* and in its iron-free derivative appear similar in several aspects. First, the identical pH dependences of protonation of the porphyrin and ferri-forms of the cytochromes suggest either that the presence of iron in the porphyrin does not shift the pK significantly or that any contribution of the polypeptide chain in altering the pK is the same in the native and iron-free cytochrome. Secondly, the fluorescence of tryptophan 59 is quenched in both native and iron-free cytochrome *c*. As demonstrated in the Results section, Förster-type energy transfer is responsible for the quenching of tryptophan fluorescence in the porphyrin derivative. The distance from the tryptophan to the heme edge in crystalline cytochrome *c* is under 1 nm, a value which would be predicted to favor energy transfer according to Förster's theory. It can be conjectured that the same mechanism is responsible for the quenching of tryptophan fluorescence in native cytochrome *c* [24, 25]. And, finally, the absence of enhancement of porphyrin fluorescence in ^2H_2O suggests that the porphyrin ring is located in an environment from which water is excluded. The higher sensitivity of porphyrin cytochrome *c* to denaturation both by guanidine-HCl (Fig. 7) and heat [19] and lower affinity of binding to cytochrome *c* oxidase (Fig. 10) and cytochrome-*c*-depleted membranes (Fig. 9) indicate that the polypeptide chain is somewhat altered. However, it is difficult to tell whether this is due to overall conformational change caused by the loss of the ligands between the metal and the protein, or whether the preparative procedure itself leads to destabilization of the protein conformation.

The competitive binding of the native cytochrome *c* and of the iron-free derivative (Fig. 9) and the identical number of "high affinity" sites (Fig. 8) suggest that those binding sites for the two molecules are identical; thus, porphyrin cytochrome *c* can be used instead of the native enzyme to characterize interactions with either the oxidase or the reductase. It was found that the visible absorption of cytochrome oxidase overlaps with the emission spectrum of porphyrin cytochrome *c* and therefore one might expect quenching which results from energy transfer of the Förster-type between the oxidase and porphyrin cytochrome *c*. The extent of porphyrin quenching depends critically on three factors Eqn (2): distance between the oxidase and the porphyrin cytochrome *c*, the orientation of the two hemes (or a heme and a porphyrin) toward each other, and the spectral overlap between the absorption of the oxidase and the emission of porphyrin cytochrome *c*. Since the spectral overlap is greater with the reduced oxidase one would expect more pronounced quenching in this state of the enzyme. Experimentally we find no quenching of porphyrin cytochrome *c* fluorescence either by reduced or oxidized cytochrome oxidase which means either that the porphyrin-heme distance is too large and/or the orientation factor for energy transfer is unfavorable. It should be pointed out that in the metal porphyrin system the symmetry is D_{4h}; that is, there are two equivalent dipole transitions in the x and y directions [17]. Because of this, a special situation arises in which the orientation factor assumes a minimum value which is always greater than zero. Diagrams of extreme cases for the possible orientation of the heme and the porphyrin rings are depicted in Fig. 11 and described in Table 2. The orientation most favorable for energy transfer occurs when the two rings lie on the same plane; the least favorable orientation is when the planes are perpendicular and one of the center atoms does not lie in the plane of the other. The distance, R_c, for 50 % quenching thus varies greatly

Table 2. *Orientation factors,* K^2, *for the porphyrin to heme system, and distances,* R_c, *for 50% energy transfer in the porphyrin cytochrome* c *to cytochrome oxidase system*
K^2 is the orientation factor for energy transfer from porphyrin (two transition moments—in plane) to heme (assumed degenerate transitions in the plane). R_c is the distance for 50% quenching calculated according to Eqn (3). The spectrum of dithionite reduced cytochrome oxidase was used to calculate the overlap integral

Case	Requirements	K^2	R_c
			nm
I	Porphyrin rings lie superimposed in two parallel planes	1	0.44
II	Porphyrin rings lie in the same plane	2	0.88
III	Porphyrin rings lie in two perpendicular planes such that the plane of one bisects the ring of the other (note that the central atom of one porphyrin is in the plane of the other)	2	0.88
IV	Porphyrins lie in planes which are perpendicular to each other, but while the central atom of (2) lies in the plane of (1), the central atom of (1) does not lie in the plane (2)	1	0.44

according to orientation and ranges from 0.44 to 0.9 nm. Of course the orientations most favorable for the energy transfer may not necessarily be the same as those for electron transfer.

The difficulties in determing the distances between the chromophores is well demonstrated by the experiments carried out on cytochrome *c*—cytochrome *c* peroxidase complexes. Gupta and Yonetani [26] using the nuclear magnetic resonance technique estimated the distance between the respective hemes to be greater than 2.5 nm; on the other hand, Leonard and Yonetani [24], on the basis of their fluorescence studies, obtained a value of approximately 1.9 nm for the same gap. In spite of these inadequacies, our own data and those of Yonetani and coworkers [26,27] suggest that the electron can pass through rather large distance, and these need to be taken into account when mechanism for electron transfer are considered.

It is of further interest to note that the molecular rotation of cytochrome *c* bound to phospholipids or mitochondria is slower than the times scale provided by the relaxation of excited state porphyrin (Table 1). The rotational correlation time of bound cytochrome *c*, estimated from the polarization data for porphyrin cytochrome *c*, was found to be less than 200 ns. This value provides another upper limit on the rotation correlation time, which previously was estimated to be greater than 6 ns based on tryptophan fluorescence polarization of tuna cytochrome *c* [28]. Since cytochrome *c* is a polycationic molecule, it is likely to be attached to the membrane through several ionic linkages and therefore it can be expected that the rotational diffusion of the bound cytochrome is slow.

It is generally accepted that iron is involved in the equilibrium redox state of the cytochrome *c* molecule, but it has also been proposed [29] that the porphyrin ring itself participates in electron transfer with the generation of a porphyrin radical. We find that porphyrin cytochrome *c* does not stimulate oxygen uptake in cytochrome-*c*-depleted mitochondria supplemented with an oxidizable substrate and its absorption and fluorescence spectra remain unaltered under these conditions. This finding does not unequivocally eliminate the participation of a porphyrin radical in the detailed reaction mechanism; however, it may be interpreted to indicate that the midpoint redox potential of the porphyrin radical is sufficiently displaced from those of cytochrome *c*, and cytochrome *a* so that electron transfer does not occur.

The authors wish to thank Drs J. Callis and F. Adar for stimulating conversations, and Dr P. George for use of laboratory equipment. This work was supported by USPHS GM-12202 and GM-21699-01. One of us (J. V.) is supported by Career Development award USPHS GM-53-01.

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Note Added in Proof (November 17, 1975). It has recently come to our attention that Schwartz *et al.* have discussed singlet transfer rates by dipole-dipole coupling [Schwarz, F. F., Gouterman, M., Muljiani, Z. & Dolphin, D. H. (1972) *Bioinorg. Chem. 2*, 1–32]. They observed that in covalently linked cobalt and zinc porphyrins no singlet energy transfer occurred over a distance of 0.5 to 1.0 nm.