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Detection, Characterization, and Quenching of the Intrinsic Fluorescence of Bovine Heart Cytochrome *c* Oxidase†

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ABSTRACT: The intrinsic fluorescence of lauryl maltoside solubilized bovine heart cytochrome *c* oxidase has been determined to arise from tryptophan residues of the oxidase complex. The magnitude of the fluorescence is approximately 34% of that from *n*-acetyltryptophanamide (NATA). This level of fluorescence is consistent with an average heme to tryptophan distance of 30 Å. The majority of the fluorescent tryptophan residues are in a hydrophobic environment as indicated by (1) the fluorescence emission maximum at 328 nm and (2) the differing effectiveness of the quenching agents: Cs^+, I^-, and acrylamide. Cesium was ineffective up to a concentration of 0.7 M, whereas quenching by the other surface quenching agent, iodide, was compiex. Below 0.2 M, KI was ineffective whereas between 0.2 and 0.7 M 15% of the tryptophan fluorescence was found to be accessible to iodide. This pattern indicates that protein structural changes were induced by iodide and may be related to the chaotropic character of KI. Acrylamide was moderately effective as a quenching agent of the oxidase fluorescence with a Stern–Volmer constant of 2 M^{-1} compared with acrylamide quenching of NATA and the water-soluble enzyme aldolase having Stern–Volmer constants of 12 M^{-1} and 0.3 M^{-1}, respectively. There was no effect of cytochrome *c* on the tryptophan emission intensity from cytochrome *c* oxidase under conditions where the two proteins form a tight, 1:1 complex, implying that the tryptophan residues near the cytochrome *c* binding site are already quenched by energy transfer to the hemes of the oxidase. The lauryl maltoside concentration used to solubilize the enzyme did not affect the fluorescence of NATA. In contrast, the fluorescence spectral maximum of indole was shifted to a shorter wavelength near the critical micelle concentration of lauryl maltoside, indicating that indole had partitioned into the detergent micelle. The fluorescence spectrum of indole in a lauryl maltoside micelle resembles the tryptophan fluorescence of cytochrome *c* oxidase. These data support the idea that in a membrane protein there are two possible environments that may give rise to the fluorescence properties of tryptophan seen with cytochrome *c* oxidase: the interior of the protein and the protein/lipid or protein/detergent interface. The ability of Cs^+ and I^- to quench the fluorescence of indole in lauryl maltoside micelles is much greater than the ability of these agents as quenchers of the oxidase fluorescence. These quenching data suggest that in cytochrome *c* oxidase the fluorescent tryptophan residues are buried in the protein. Thus, quenching studies represent a method for distinguishing between the two possible types of hydrophobic environments in integral membrane proteins. It is concluded that the fluorescent tryptophans in cytochrome *c* oxidase are asymmetrically located in the complex, removed from the cytochrome *c* binding region, and buried in the interior of the protein rather than at the protein/lipid interface.

Fluorescence spectroscopy has been an important tool in the study of the dynamics of protein conformation. Intrinsic fluorescence of protein molecules most commonly arises from the aromatic amino acid tryptophan (Lakowicz, 1983). Tryptophan fluorescence is highly sensitive to environment and is thus a useful structural probe (Burstein et al., 1973).

Cytochrome *c* oxidase (EC 1.9.3.1; cytochrome *c*:oxygen oxidoreductase) is an integral protein of the inner mitochondrial membrane. This protein consists of 10–13 nonidentical subunits with a total molecular weight of 200 000 and contains

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two heme groups and two copper atoms noncovalently bound to the protein matrix [for reviews, see Wikström et al. (1981) and Freedman & Chan (1984)]. It has been suggested that conformational changes in the protein structure of the oxidase may be an important part of the electron transfer or energy-coupling mechanism employed by this protein (Wikström et al., 1981). Although there are some 50 tryptophan residues in the oxidase complex, intrinsic fluorescence from tryptophan has not been reported. Intrinsic tryptophan fluorescence offers a measure of the protein conformational state independent of the spectroscopic properties of the heme groups.

In this paper, we report that the intrinsic fluorescence of detergent-solubilized cytochrome *c* oxidase arises from tryptophan residues of the protein complex and is of a considerable magnitude. We have characterized this fluorescence and compared it to the simple model system of the tryptophan analogue indole in lauryl maltoside micelles. By this approach, we have concluded that the fluorescent tryptophans of cytochrome *c* oxidase are buried in a hydrophobic environment and located asymmetrically in the complex removed from the cytochrome *c* binding, cytoplasmic domain.

Materials and Methods

Sodium cholate, obtained from Sigma, was recrystallized from ethanol. Aldolase (type I), *n*-acetyltryptophanamide (NATA)[1] and cytochrome *c* (type III) were also supplied by Sigma. Acrylamide was the twice recrystallized variety obtained from Serva. Lauryl maltoside was from Calbiochem. Indole was from Fisher.

Cytochrome *c* oxidase was prepared by a modification of the method of Kuboyama et al. (1972), but cholate was used rather than Emasol-1130 in the final fractionation steps. The final pellet, following fractionation in cholate, was washed and then resuspended in 0.025 M sodium phosphate buffer, pH 7.4, containing 1 mg/mL lauryl maltoside. Measurements were also made on material that was prepared according to a modified version (Capaldi & Hayashi, 1972) of the method of Fowler et al. (1962). Both types of enzyme preparation were further purified on Triton X-100, glycerol gradients according to the method of Robinson et al. (1980). Since the available samples of Triton X-100 were found to be highly fluorescent, it was necessary to exchange the Triton X-100 in these preparations for lauryl maltoside as described by Robinson et al. (1984). These preparations have a heme to protein ratio of 9–10 nmol of heme A/mg of protein. The concentration of heme was determined by using an extinction coefficient ΔE(605–630 nm) (reduced–oxidized) = 13.5 (Nicholls, 1979), and protein concentration was determined by the modified biuret method (Gornall et al., 1949).

Absorbance data were obtained on either a Zeiss PM6K or a Cary 219 spectrophotometer. Steady-state fluorescence measurements were made on a Perkin-Elmer MPF 44A fluorometer operated in the ratio mode. All measurements were made at 25 °C.

Inner filter effects due to the protein and to acrylamide in quenching experiments were corrected by the formula (Lakowicz, 1983):

$$F_c = F \text{ antilog } [(A_{ex} + A_{em})/2] \quad (1)$$

where F_c is the corrected fluorescence intensity, F is the measured intensity, A_{ex} is the absorbance of the solution at the wavelength of fluorescence excitation, and A_{em} is the absorbance of the sample at the wavelength of fluorescence emission. The relative quantum yield for cytochrome *c* oxidase was calculated by comparing the area of the emission profile of the oxidase with that of NATA after correction for the inner filter effect and normalizing for their relative absorbances at the wavelength of excitation. Stern–Volmer quenching constants (K_{SV}) were determined for systems having a static quenching component by using a modified Stern–Volmer equation [eq 2 (Eftink & Ghiron, 1976b)]:

$$\frac{F_0}{F_Q \exp(V[Q])} = 1 + K_{SV}[Q] \quad (2)$$

where F_0 is the fluorescence intensity in the absence of quencher, F_Q is the fluorescence intensity in the presence of a concentration of quencher, Q, V is the parameter to account for static quenching, and K_{SV} is the Stern–Volmer quenching constant.

In Förster's theory of resonance energy transfer [see Stryer (1978)] the efficiency of energy transfer, E, is

$$E = 1 - Q_p/Q_a \quad (3)$$

where Q_p is the quantum yield in the presence of the acceptor and Q_a is the quantum yield in the absence of the acceptor. The value of Q_a for tryptophan is 0.2 (Weber & Teale, 1959). The efficiency of energy transfer is related to the distance between the donor and acceptor groups by

$$E = r^{-6}/(r^{-6} + R_0^{-6}) \quad (4)$$

where r is the distance between the donor and acceptor groups and R_0 is the distance at which the donor fluorescence is quenched by 50%. R_0 is defined by the equation:

$$R_0 = 9.79 \times 10^3 (K^2 n^{-4} Q_a J)^{1/6} \quad (5)$$

where K^2 is the relative orientation between the donor and acceptor transition dipole moments, n is the refractive index of the medium separating donor and acceptor, taken to be 1.4 (Stryer, 1978), Q_a is the quantum yield of the donor in the absence of acceptor and is equal to 0.2 (Weber & Teale, 1959), and J is the spectral overlap integral. The overlap integral, J, between the tryptophan emission and heme absorption is described by the equation:

$$J = \frac{\int F_\lambda \epsilon_\lambda \lambda^4 \, d\lambda}{\int F_\lambda \, d\lambda} \quad (6)$$

where F_λ is the donor emission intensity at wavelength λ (nm) and ϵ_λ is the acceptor extinction coefficient at λ (nm). The value of J was determined numerically over the spectral region from 300 to 400 nm as described by Campbell and Dwek (1984). The value of J for tryptophan emission and heme absorption in cytochrome *c* oxidase is 6.05×10^{-14} cm^3 M^{-1}.

Results

Uncorrected fluorescence emission and excitation spectra for cytochrome *c* oxidase are shown in Figure 1. When excited at either 280 or 295 nm, the protein emits with a maximum at 328 nm and a bandwidth of 51 nm (Figure 1a). When the emission spectra obtained by excitation at either 295 or 280 nm are normalized at 365 nm, they are identical. In addition, the excitation spectrum has a peak at 280 nm with a shoulder at 292 nm (Figure 1b). These features suggest that the observed fluorescence arises nearly entirely from tryptophan residues and that if there is any contribution from tyrosine, it must be very small.

As fluorescence from heme proteins is expected to be weak, it is important to rule out the possibility that such fluorescence results from any contaminating protein present in the purified

[1] Abbreviations: NATA, *n*-acetyltryptophanamide; Tris, tris(hydroxymethyl)aminomethane; cmc, critical micelle concentration.

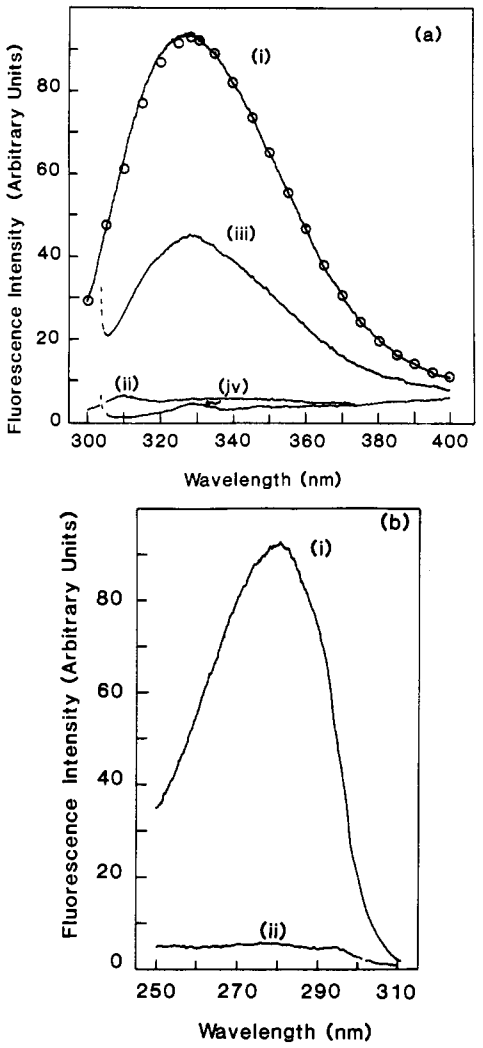

FIGURE 1: Intrinsic fluorescence of cytochrome *c* oxidase. (a) Emission spectra of cytochrome *c* oxidase (0.051 mg/mL, i.e., 0.025 μM) in 20 mM Tris, pH 7.8, with 0.1 mg/mL lauryl maltoside at 25 °C and a bandwidth of emission and excitation monochromators of 6 nm. (i) Cytochrome *c* oxidase emission with excitation wavelength of 280 nm. (ii) Buffer background fluorescence with excitation wavelength of 280 nm. (iii) Cytochrome *c* oxidase emission with an excitation wavelength of 295 nm. (iv) Background fluorescence of the buffer with an excitation wavelength of 295 nm. The open circles (O) represent points along the emission spectrum of cytochrome *c* oxidase at an excitation wavelength of 295 nm multiplied by the ratio of the fluorescence intensities at 365 nm produced by excitation at 280 and 295 nm. (b) Excitation spectrum of cytochrome *c* oxidase. The conditions are identical with those described above. The excitation spectrum was scanned with the emission monochromator set at 328 nm. (i) Cytochrome *c* oxidase excitation spectrum. (ii) Background excitation from the buffer.

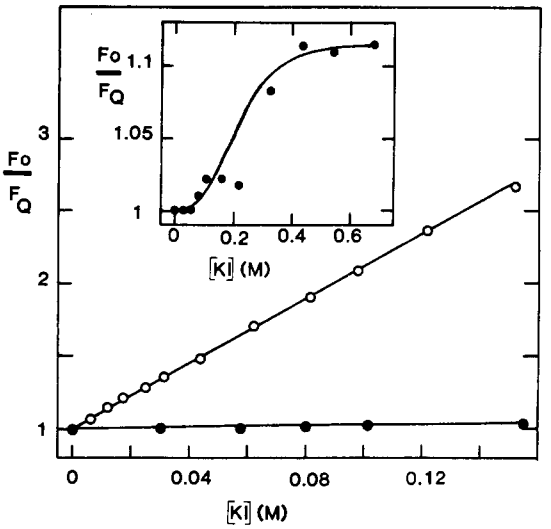

FIGURE 2: Iodide quenching of cytochrome *c* oxidase fluorescence. A Stern–Volmer plot of iodide quenching of NATA (O) and cytochrome *c* oxidase (0.038 mg/mL) (●). The buffer for the oxidase was 0.02 M Tris, pH 7.8, with 0.1 mM EDTA and 1 mg/mL lauryl maltoside. In the case of NATA the buffer was 0.02 M Tris, pH 7.8, with 0.1 M NaCl. The buffer was supplemented with 1.1 mM $Na_2S_2O_3$ to prevent the formation of I_2. Iodide was added from a freshly prepared stock of 5.1 M KI in 0.02 M Tris, pH 7.8, with 0.1 M NaCl and 1.1 mM $Na_2S_2O_3$. The ionic strength was maintained constant with NaCl added from a 5.1 M stock in the same buffer. The emission intensity was corrected for dilution and inner filter effect at the excitation and emission wavelengths for each concentration of iodide. F_0 is the initial fluorescence intensity, and F_Q is the intensity in the presence of the quencher. The excitation wavelength was 280 nm for both the oxidase and NATA while the intensity of emission was monitored at 328 nm for the oxidase and 352 nm for NATA. The temperature was 25 °C, and the slits of both the excitation and emission monochromators were set at 6 nm. The inset shows the Stern–Volmer plot for I^- quenching of cytochrome *c* oxidase up to 0.7 M KI on an expanded ordinate scale.

preparation. Therefore, the enzyme preparation was purified further with Triton X-100–glycerol gradient centrifugation. This procedure, which is known to remove lipid and a majority of heavy molecular weight protein contaminants that are often seen in preparations of cytochrome *c* oxidase (Robinson et al., 1980), did not affect the fluorescence spectrum. In addition, fluorescence from preparations made by either of the procedures described under Materials and Methods gave the same results as those described in Figure 1, which are from a sample prepared by the method of Kuboyama et al. (1972).

The fluorescence quantum yield of the sample shown in Figure 1 is 0.325 that of NATA in the same buffer. The absolute quantum yield (i.e., the ratio of the number of photons emitted to the number of photons absorbed) of NATA in water is about 0.20 (Teale, 1960) and is not changed in the lauryl maltoside buffer used here. Therefore, the absolute quantum yield for cytochrome *c* oxidase is estimated to be 0.065 as compared with a value of 0.002 for hemoglobin (Weber & Teale, 1959).

The ionic quenchers Cs^+ and I^- and the polar nonionic quencher acrylamide were each examined for their ability to quench the intrinsic fluorescence of cytochrome *c* oxidase. Cs^+, up to a concentration of 0.7 M, did not quench the intrinsic fluorescence of the oxidase. I^- and acrylamide were both effective as quenching agents. In Figure 2 a Stern–Volmer plot of I^- quenching of cytochrome *c* oxidase and the tryptophan analogue NATA shows that I^- quenches the protein fluorescence very weakly compared with its quenching of NATA. Above 0.2 M the oxidase becomes more sensitive to I^- and is quenched to a maximum of 15% at 0.7 M KI (see inset to Figure 2).

In Figure 3 a Stern–Volmer plot of the quenching by acrylamide of the intrinsic tryptophan fluorescence of cytochrome *c* oxidase is compared with the acrylamide quenching of NATA and the soluble enzyme aldolase. NATA and aldolase represent two extremes in their sensitivity to acrylamide quenching. The NATA plot curves upward, indicating a static as well as a dynamic component to the quenching process. Static quenching may indicate there is an association between the quencher and fluorophore such that the collisional process is instantaneous. Static quenching by acrylamide is commonly seen with a freely exposed fluorophore. In such cases the Stern–Volmer constant (K_{SV}), which is a measure of the dynamic quenching, may be derived from a modified Stern–Volmer plot [Eftink & Ghiron, 1976b; see Materials and

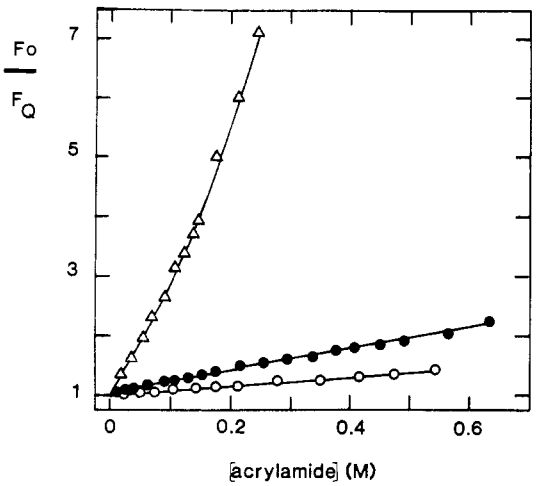

FIGURE 3: Stern–Volmer plots of quenching by acrylamide of the fluorescence of cytochrome *c* oxidase, NATA, and aldolase. Cytochrome *c* oxidase was at a concentration of 0.05 mg/mL in 20 mM Tris, pH 7.4, buffer with 0.1 mg/mL lauryl maltoside. NATA and aldolase were dissolved in the same buffer without detergent. The excitation wavelength was 295 nm, and the emission intensity was monitored at 328 nm for the oxidase, 352 nm for NATA, and 322 nm for aldolase. Acrylamide was added from a concentrated stock solution to give the final concentration shown in the figure. The fluorescence intensity was corrected for dilution and the inner filter effect. The symbols designate cytochrome *c* oxidase (●), aldolase (○), and NATA (△).

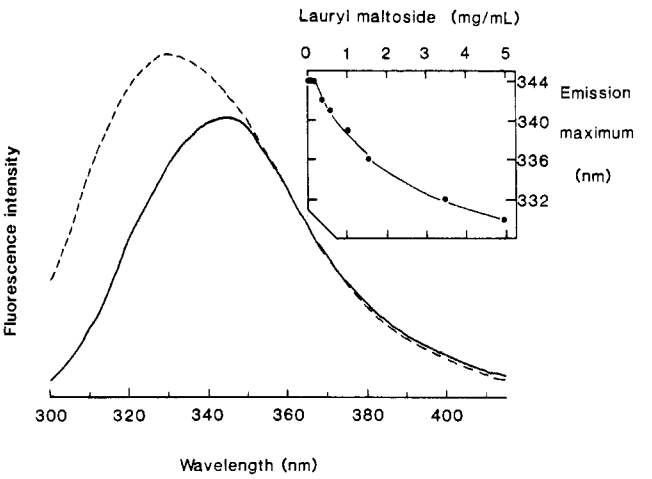

FIGURE 4: Effect of lauryl maltoside on the fluorescence emission spectrum of indole. The buffer was 20 mM Tris, pH 7.8, with 0.1 M NaCl. Lauryl maltoside was added from a concentrated stock solution. The concentration of indole was 3.5 μM. The concentration of detergent in the lauryl maltoside spectrum was 5 mg/mL. The temperature was 25 °C, and the bandwidths of the emission and excitation monochromators were set at 6 nm. The solid line spectrum is in the absence of lauryl maltoside.

Methods). The K_{SV} for acrylamide quenching of NATA is 12 M^{-1}, and this may be compared with the values obtained for the quenching of cytochrome *c* oxidase and aldolase which are 2 M^{-1} and 0.3 M^{-1}, respectively.

Cytochrome *c* forms a stable 1:1 complex with cytochrome *c* oxidase at low ionic strength, and it would be expected to quench any fluorescent tryptophans near its binding site on the oxidase. When cytochrome *c* is added to solutions of the oxidase at low ionic strength, no effect is seen on the oxidase fluorescence due to energy transfer to the heme of cytochrome *c*.

Since isolated, detergent-solubilized cytochrome *c* oxidase is known to bind about 200 molecules of detergent (Suarez et al., 1984), we have examined the possibility that the characteristics of the tryptophan fluorescence of the oxidase may be due to their presence in a hydrophobic, detergent binding domain of the protein. Neither the fluorescence of NATA nor its behavior toward quenching agents was affected by the detergent lauryl maltoside at concentrations used to solubilize the enzyme. However, the fluorescence of indole was perturbed by the addition of the detergent lauryl maltoside. Figure 4 shows the effect of lauryl maltoside on the emission spectrum of indole. In buffer, indole fluoresces with an emission maximum at 343 nm, whereas after the addition of 5 mg/mL lauryl maltoside the emission maximum is shifted to 330 nm, and the intensity is slightly increased. Additions of lauryl maltoside above this level have no effect on the fluorescence of indole. The inset to Figure 4 shows the dependence of the emission maximum on lauryl maltoside concentration. At levels below 0.2 mg/mL of the detergent, the emission is unaffected. At a concentration of about 0.2 mg/mL lauryl maltoside, the fluorescence spectrum begins to shift and then proceeds to shift monotonically to a low wavelength limit of 330 nm as the detergent concentration is increased to 5 mg/mL. Since there are about 150 lauryl maltoside molecules per micelle (Suarez et al., 1984), the concentration of micelles at 5 mg/mL lauryl maltoside is about 65 μM. Therefore, under these conditions the concentration of micelles is well in excess of that of indole, and on average there is less than one molecule of indole per micelle. The concentration of lauryl maltoside at which the initial spectral shift is observed is near the critical micelle concentration [cmc values of 0.08 and 0.3 mg/mL for lauryl maltoside have been reported by De Grip & Bovee-Geurts (1979) and Knudson & Hubbell (1978), respectively] for this detergent, and our initial conclusion is that indole is partitioning into the hydrophobic environment of the lauryl maltoside micelle. Indirect evidence for this view comes from studies with another nonionic detergent, octyl glucoside. In this system the fluorescence of indole is unaffected until a much higher concentration of detergent, at a level which is near the much higher cmc, about 25 mM (De Grip & Bovee-Geurts, 1979), for octyl glucoside (data not shown).

In Figure 5, Stern–Volmer plots for the quenching of indole by Cs^+, I^-, and acrylamide in the presence and absence of lauryl maltoside are shown. In each, the effectiveness of the quenching agent is greatly reduced in the presence of lauryl maltoside micelles. The forms exhibited in the Stern–Volmer plots are also informative. The plot for I^- quenching (Figure 5A) of indole in water is linear, indicating a simple collisional mechanism for the quenching process. The Cs^+ plot (Figure 5B) is curved downward, indicating that this quencher is sensing a heterogeneity in the indole population, which is probably a result of the low solubility of indole in water. The plot for acrylamide quenching of indole in water is curved upward as a result of a static quenching component. The Stern–Volmer plots for the quenching of indole in lauryl maltoside micelles show a different pattern. In the Stern–Volmer plot for acrylamide quenching (Figure 5C) of indole in lauryl maltoside, the static component seen in water is absent, which is consistent with the idea that indole has partitioned into the lauryl maltoside micelle. Both of the charged agents, Cs^+ and I^-, are still effective as quenchers of indole fluorescence in the presence of detergent, and the Stern–Volmer plots curve downward. This is consistent with either a considerable mobility of indole in the micelle such that it spends a considerable time at the micelle surface exposed to solvent or that there is a heterogeneity in the distribution of indole in the micelle structure. The Stern–Volmer quenching constants under each condition are reported in Table

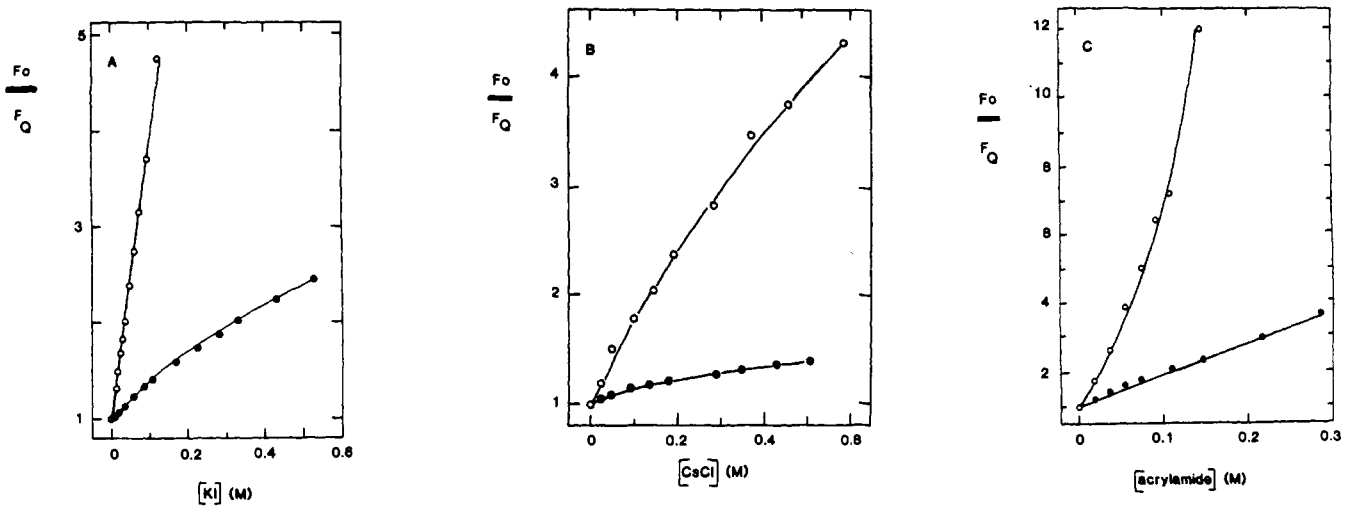

FIGURE 5: Quenching of indole fluorescence in the presence (●) and absence (○) of 5 mg/mL lauryl maltoside. (A) Quenching by I^-. The indole concentration was 4.45 μM in 20 mM Tris, pH 7.8, with 0.1 M NaCl. The buffer was supplemented with 1.1 mM $Na_2S_2O_3$, and iodide was added from a stock of 5.1 M KI in the same buffer. (B) Quenching by Cs^+. The concentration of indole was 3.35 μM in 20 mM Tris, pH 7.8, with 0.1 M NaCl. CsCl was added from a stock solution of 5 M in the same buffer. (C) Quenching by acrylamide. The concentration of indole was 2.22 μM in 20 mM Tris, pH 7.8, with 0.1 M NaCl.

Table I: Stern–Volmer Quenching Constants for the Quenching of Indole in Buffer and in Lauryl Maltoside Micelles by Cesium, Iodide, and Acrylamide

	K_{SV} (M^{-1}) when 5 mg/mL lauryl maltoside is	
quenching agent	absent	present
I^-	28	2
Cs^+	8	1
acrylamide	40	10

I. These values show the relative influence of the micellar environment on the dynamic quenching ability of these agents. The effectiveness of iodide to quench indole fluorescence is reduced 14-fold and cesium 8-fold by the incorporation of indole into the lauryl maltoside micelle. Quenching by the neutral agent acrylamide is the least affected, being reduced by a factor of 4.

Discussion

The characteristics of the intrinsic fluorescence of cytochrome *c* oxidase allow us to assign this signal to some of the tryptophan residues in the protein complex. Fluorescence from tryptophan residues in heme proteins is usually largely quenched by efficient energy transfer from tryptophan to heme (Lakowicz, 1983). Hemoglobin has an absolute quantum yield of about 0.002 (Weber & Teale, 1959) compared with the value obtained here of 0.065 for bovine heart cytochrome *c* oxidase. When these values are normalized for the number of fluorescence donors (i.e., tryptophan residues), energy transfer acceptors (i.e., heme groups) per protein molecule, and the relative size of the two proteins, then the oxidase is roughly 5.5 times as fluorescent as hemoglobin. Similar levels of fluorescence, as reported here for the oxidase, have also been reported for two other hemeproteins: horseradish peroxidase (Weber & Teale, 1959) and cytochrome b_5 (Dufourq et al., 1975).

Förster's theory of resonance energy transfer defines the parameters that relate the efficiency of energy transfer to the distance and orientation between the donor and acceptor groups (see Materials and Methods). To determine the distance between a donor–acceptor pair, their relative orientation must be known. For a protein such as cytochrome *c* oxidase, to calculate a distance for each donor–acceptor pair is not feasible. However, it is possible to estimate a distance over which energy transfer will occur. The value for the orientation factor has a high probability of being within certain limits for a donor–acceptor pair with multiple emission and absorption moments, as is known to be the case for tryptophan and heme. This places a limit on the error of a calculated distance (Haas et al., 1978). In addition, if the tryptophans have a random orientation relative to the heme groups, irrespective of their mobility, and a value of 0.67 is used for the orientation factor (the value for K^2 when the donor or acceptors are freely mobile), the calculated distance will be within 10% of the actual distance (Haas et al., 1978). When an orientation factor of 0.67 was used, the R_0 value for the oxidase was calculated to be 34 Å, giving a value for r of 30 Å. The value of 30 Å represents an "average" distance between the hemes and tryptophans within cytochrome *c* oxidase and is meant to give a qualitative estimate for the distance over which energy transfer might occur in this protein.

This general picture for the relative position of the heme and tryptophan residues is further defined by the experiments with cytochrome *c*. Cytochrome *c* forms a 1:1 complex of high stability [K_d = 20 nM (Ferguson-Miller et al., 1976)] with cytochrome *c* oxidase at low ionic strength. In the formation of a similar type of complex between cytochrome *c* and adrenodoxin, the fluorescence of a tyrosine residue of adrenodoxin was quenched by resonance energy transfer to the heme of cytochrome *c* (Green & Millet, 1983). Cytochrome *c* is also able to quench the fluorescence of antibodies in antibody–cytochrome *c* complexes (Noble et al., 1969). Docktor et al. (1978) have shown by resonance energy transfer that the heme of cytochrome *c* is about 25 Å from the hemes of the oxidase. This distance would place any tryptophan residues in this region on the oxidase well within the range of energy transfer to the oxidase heme groups. Since the fluorescence of cytochrome *c* oxidase is unaffected by the binding of cytochrome *c*, it appears that there are no fluorescent tryptophans located near the cytochrome *c* binding site on subunit II in the cytoplasmic domain of cytochrome *c* oxidase.

Quenching studies with the hydrophilic, ionic quenchers I^- and Cs^+ suggest that very little of the intrinsic tryptophan fluorescence of cytochrome *c* oxidase arises from water-accessible, surface residues. This finding is consistent with the blue-shifted emission maximum of the fluorescence spectrum

of the oxidase, which indicates that the fluorescent tryptophans are in a hydrophobic environment (Burstein et al., 1973). The small degree of quenching by I^- above 0.2 M indicates that the accessibility of some tryptophan residues of the oxidase increases as the I^- concentration is raised. Evidence of iodide-induced protein structural changes has been observed previously and is related to the chaotropic action of KI (Ide & Engelborghs, 1981). The uncharged, polar quencher acrylamide is apparently able to penetrate into proteins and the degree of quenching with this probe is an indication of the accessibility of the fluorescent residues to solvent (Eftink & Ghiron, 1976a). The finding that acrylamide is weakly effective as a quenching agent of the intrinsic fluorescence of cytochrome *c* oxidase is also consistent with the previous conclusion that the fluorescent tryptophans are buried in a hydrophobic environment. In the case of a water-soluble protein this would imply that the residues were in the hydrophobic interior of the protein, but for an integral membrane protein there is a second hydrophobic environment, the lipid or detergent/protein interface.

It has been shown that some tryptophan analogues will partition into detergent micelles (Lakowicz & Keating, 1983; Turro et al., 1980; Eftink & Ghiron, 1976b). In this report we have demonstrated that the fluorescence emission of indole is shifted to a shorter wavelength in the presence of the detergent lauryl maltoside, the detergent used to solubilize the oxidase. This shift in fluorescence emission from indole begins at a concentration of lauryl maltoside near its cmc and, therefore, would suggest that indole is partitioning into the lauryl maltoside micelle. The other tryptophan analogue used here, NATA, whose fluorescence spectrum is also highly sensitive to environment, is not affected by lauryl maltoside. This difference between indole and NATA probably reflects their relative water solubilities. Therefore, we conclude that indole in detergent micelles may have some of the attributes of an appropriate model system for tryptophan residues at the hydrophobic surface of detergent-solubilized membrane proteins. However, the ionic quenchers Cs^+ and I^- are still effective when indole is incorporated into the lauryl maltoside micelles. This is in contrast to their ineffectiveness as quenchers of the tryptophan fluorescence of cytochrome *c* oxidase. Although some of the inaccessibility of tryptophan residues in cytochrome *c* oxidase to quenchers could be due to a screening effect of the hydrophobic detergent bound to the hydrophobic protein surface, the results are more consistent with the fluorescent tryptophans in cytochrome *c* oxidase being buried in the protein.

Our results indicate that the fluorescent tryptophans of cytochrome *c* oxidase are in a hydrophobic environment, located an average distance of 30 Å from the heme groups, and are located asymmetrically in the protein complex so as to be removed from the cytoplasmic, cytochrome *c* binding domain. At this time it is difficult to distinguish between the two possible types of hydrophobic sites in an intrinsic membrane protein: those buried in the protein and those at the protein/lipid interface. Model studies of indole in lauryl maltoside micelles support the idea that tryptophan at the protein/lipid interface would exhibit the fluorescence spectrum of a residue buried in the hydrophobic interior of the protein. However, the quenching studies indicate that in cytochrome *c* oxidase the fluorescent tryptophans are predominantly in the interior of the protein and not at the protein/lipid interface. These fluorescent tryptophan residues may be valuable probes of protein conformational changes induced by redox changes at the heme groups or in the putative proton pumping capacity of the enzyme (Wikström et al., 1981).

Registry No. NATA, 2382-79-8; cytochrome *c* oxidase, 9001-16-5; indole, 120-72-9; lauryl maltoside, 100839-36-9; aldolase, 9024-52-6; L-tryptophan, 73-22-3.

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