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## Fluorescent Probe Study of Temperature-Induced Conformational Changes in Cytochrome Oxidase in Lecithin Vesicle and Solubilized Systems<sup>†</sup>

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**ABSTRACT:** A protein-bound label, *N*-(1-anilino-naphthyl-4)-maleimide (ANM), was used to investigate conformational changes in bovine heart cytochrome oxidase. The fluidity of cytochrome oxidase vesicles was monitored by a lipophilic probe, 1,6-diphenyl-1,3,5-hexatriene. The fluorescence intensity and emission anisotropy of these probes were examined between 4 and 60 °C in enzyme-dipalmitoyllecithin vesicles, in enzyme-dimyristoyllecithin vesicles, in enzyme-dioleoyllecithin vesicles, and in the soluble enzyme. The tempera-

ture-dependent changes in these quantities indicated that there were two types of conformational changes in oxidized cytochrome oxidase: one was attributed to an intrinsic enzyme conformational change which occurred around 20 °C, and the other was attributed to a conformational change induced by the lipid phase transition. Although ANM-reactive subunits of cytochrome oxidase in these four lecithin vesicle and solubilized systems were different from each other, subunit I always reacted with ANM in preference to other subunits.

**C**ytochrome oxidase is the terminal enzyme of the respiratory chain in the mitochondrial inner membrane (Boyer et al., 1977). Three-dimensional analysis of the structure of cytochrome oxidase in vesicle crystals (Henderson et al., 1977) has shown that the enzyme molecule sticks out on both side surfaces of the vesicle.

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An interesting question regarding membrane proteins is how their functions and conformations are influenced by the physical states of membrane lipids (Vanderkooi, 1974; Yu et al., 1975; Carroll & Racker, 1977; Jost et al., 1977; Longmuir et al., 1977). Sharp breaks have been observed in the temperature dependences of the activities of several membrane enzymes (Inesi et al., 1973; Houslay et al., 1975; Lee et al., 1974; Hesketh et al., 1976). These breaks were often attributed to lipid phase transitions or lateral lipid phase separations.

Raison et al. (1971) and Erecińska & Chance (1972) observed a break at around 20 °C in the Arrhenius plot of cytochrome oxidase activity in rat liver and pigeon heart mitochondria, respectively. They attributed the break to the lipid phase transition. However, the mitochondrial membranes of rat liver, pigeon heart, and bovine heart have been shown to be in the liquid-crystalline state above 10 °C (Blazyk & Steim, 1972; Vanderkooi, 1973; Cannon et al., 1975; Shinitzky

cytochrome oxidase activity. Since these systems have no phase transitions above 0 °C, the break has been attributed to an intrinsic conformational change of cytochrome oxidase.

It is well-known that fluorescence techniques are useful to monitor structural changes in proteins (Weber, 1952; Oh-yashiki et al., 1974). In the present paper, by using the fluorescence intensity and polarization of *N*-(1-anilino-naphthyl-4)maleimide (ANM)<sup>1</sup> and 1,6-diphenyl-1,3,5-hexatriene (DPH), we have examined the temperature-induced conformational changes in oxidized cytochrome oxidase in three kinds of lecithin vesicles whose transition temperatures are 40 °C (dipalmitoyllecithin), 23 °C (dimyristoyllecithin), and -20 °C (dioleoyllecithin) and in phosphate buffer containing Emasol.

### Experimental Procedures

**Materials.** Cytochrome oxidase (EC 1.9.3.1) was prepared from beef heart muscle by the method of Okunuki et al. (1958) with some modifications. The final ammonium sulfate precipitate was dissolved in 50 mM sodium phosphate buffer (pH 7.4) containing 0.25% (v/v) Emasol 1130 and 0.1% (w/v) sodium cholate (ECP) and dialyzed against a sufficient volume of the same ECP buffer in the cold. The dialysate was stored frozen in liquid nitrogen. In the present experiments, we used five different cytochrome oxidase preparations of slightly varying purity:  $A_{280\text{nm}}/A_{420\text{nm}}$  (oxidized) = 2.35–2.57 (10–11 nmol of heme *a* per mg of protein). The concentration of cytochrome oxidase was determined spectrophotometrically by using a millimolar extinction coefficient difference of 16.5 [ $\Delta\epsilon_{605-630\text{nm}}$ (reduced)].

*L*- $\alpha$ -Dipalmitoyllecithin (DPL), *L*- $\alpha$ -dimyristoyllecithin (DML), and *L*- $\alpha$ -dioleoyllecithin (DOL) were purchased from Sigma Chemical Co. and used without further purification. *N*-(1-Anilino-naphthyl-4)maleimide (ANM), 1,6-diphenyl-1,3,5-hexatriene (DPH), and *p*-(chloromercuri)benzoate (pCMB) were purchased from Teika Seiyaku, Aldrich, and Sigma, respectively.

**Preparation of Cytochrome Oxidase Vesicles.** DPL, DML, and DOL dissolved in chloroform were dried under a stream of N<sub>2</sub> gas. Each of these residues was dispersed in 50 mM sodium phosphate buffer (pH 7.4) containing 2 mM MgSO<sub>4</sub>, and a 20% (w/v) solution of sodium cholate was added to a final concentration of 1%. The lecithin–cholate mixture was vortexed vigorously with occasional heating to above 40 °C. After a few minutes of this treatment, the lecithin–cholate mixture became clear. The solution was cooled to 0 °C and cytochrome oxidase in ECP was added. This mixture in a dialysis tube was dialyzed against 1000 volumes of 50 mM sodium phosphate buffer (pH 7.4) containing 2 mM MgSO<sub>4</sub> for 20 h at 4 °C with four changes of the outer solution. Incorporation of the enzyme into lecithin vesicles was analyzed by sucrose density gradient ultracentrifugation, and no pure lecithin vesicles without cytochrome oxidase were detected in the present condition (Yoshida et al., 1979).

Sonicated pure lecithin vesicles were prepared essentially as described by Kawato et al. (1977).

**Fluorescence Labeling.** To 6 mL of the suspension of cytochrome oxidase vesicles (0.5 mg/mL in lecithin and 4.2  $\mu$ M in the enzyme), 3–12.5  $\mu$ L of 1 mM ANM in acetone was

added and incubated at 18–19 °C for 2–3 h. To 3 mL of the solution of cytochrome oxidase in ECP containing 12 and 29  $\mu$ M enzyme, 18 and 9  $\mu$ L of 1 mM ANM in acetone were added, respectively, and the samples were incubated at ~18 °C for 2 h. The molar ratio of added ANM to the enzyme was in the range 1:10 to 1:2 in the present experiments.

To 6 mL of the suspension of the vesicles (0.4 mg/mL in lecithin and 3.4  $\mu$ M in the enzyme), 15.8  $\mu$ L of 190  $\mu$ M DPH in tetrahydrofuran was added, and the sample was incubated at 0 °C for 2 days.

The reaction of ANM with cytochrome oxidase and the incorporation of DPH into the vesicles were followed as an increase in the fluorescence intensity. After the incubation, when the fluorescence intensity reached a plateau, the fluorescence measurements were performed.

The activity of ANM-labeled cytochrome oxidase in ECP was measured spectrophotometrically at 25 °C by following the oxidation of reduced cytochrome *c* with a Cary spectrophotometer, Model 15, at 550 nm. The reaction mixture in a cuvette contained 15  $\mu$ M reduced cytochrome *c* in 3.0 mL of 75 mM sodium phosphate buffer, pH 6.0.

**Fluorescence Measurements.** The steady-state fluorescence intensity and anisotropy were measured with a self-constructed instrument which was described previously (Kinoshita et al., 1976). The exciting light was polarized vertically and the fluorescence from the sample was automatically analyzed into vertically and horizontally polarized components  $I_V$  and  $I_H$  by an analyzer. These intensities were measured as numbers of photons per unit of time interval. From these quantities the total fluorescence intensity  $I_T$  and the emission anisotropy  $r$  were obtained as

$$I_T = I_V + 2I_H \quad (1)$$

$$r = (I_V - I_H)/(I_V + 2I_H) \quad (2)$$

The excitation wavelength was selected at 360 nm for ANM and DPH by a monochromator. All fluorescence was collected through cutoff filters above 390 nm for ANM (Hoya L-39 filter) and above 420 nm for DPH (Hoya L-39 and L-42 filters). All experiments were performed as the sample was agitated by a magnetic stirrer. Error in the emission anisotropy values was estimated to be within 1% in all experiments. The depolarization due to the scattering of exciting and emitted lights by vesicles was negligible in the present vesicle suspensions of low lipid concentration (0.4 and 0.5 mg/mL). Temperature dependence of the fluorescence intensity and the emission anisotropy of probes was usually recorded as temperature was raised unless stated otherwise.

Fluorescence spectra were obtained with a Shimadzu spectrofluorometer, Model RF-502.

Decays of the fluorescence polarization and the fluorescence intensity were measured by a method similar to that described by Kawato et al. (1977). The excitation wavelength and the filters for fluorescence were the same as those of the steady-state measurements. The fluorescence lifetime  $\tau_i$  and the rotational relaxation time  $\phi_j$  were determined by a curve-fitting procedure. We assumed that the fluorescence intensity  $I_T^\delta(t)$  and the emission anisotropy  $r^\delta(t)$ , responses to a truly impulsive excitation expressed as  $\delta(t)$ , Dirac's delta function, were expressed as sums of exponential functions:

$$I_T^\delta(t) = \sum_{i=1}^N I_i \exp(-t/\tau_i) \quad (3)$$

$$r^\delta(t) = \sum_{j=1}^M r_j \exp(-t/\phi_j) \quad (4)$$

<sup>1</sup> Abbreviations used: ANM, *N*-(1-anilino-naphthyl-4)maleimide; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPL, *L*- $\alpha$ -dipalmitoyllecithin; DML, *L*- $\alpha$ -dimyristoyllecithin; DOL, *L*- $\alpha$ -dioleoyllecithin; ECP, 50 mM sodium phosphate buffer (pH 7.4) containing 0.25% (v/v) Emasol 1130 and 0.1% (w/v) sodium cholate; pCMB, *p*-(chloromercuri)benzoate; NaDodSO<sub>4</sub>, sodium dodecyl sulfate.

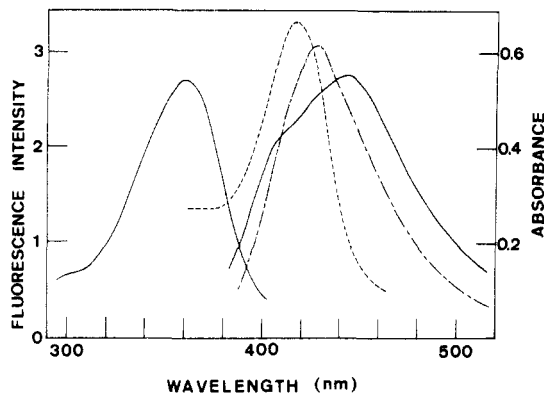


FIGURE 1: Uncorrected excitation and emission spectra of ANM conjugated with cytochrome oxidase in ECP [(—) 5  $\mu$ M ANM; 10  $\mu$ M enzyme] and the absorption spectrum of oxidized cytochrome oxidase in ECP around 420 nm [(---) 10  $\mu$ M enzyme]. The emission spectrum of ANM conjugated with noncolored protein bovine serum albumin is shown [(--·) 6  $\mu$ M ANM; 12  $\mu$ M protein].

*Tracings of NaDodSO<sub>4</sub>-Polyacrylamide Gel Electrophoresis Samples.* Cytochrome oxidase in ECP, DPL vesicles, DOL vesicles, and DML vesicles (195  $\mu$ M in the enzyme and 23 mg/mL in lecithin) was reacted with equimolar ANM at 25 °C (enzyme-ECP, enzyme-DPL and enzyme-DOL), 20 °C (enzyme-DML), or 28 °C (enzyme-DML) for 3 h. Any remaining free ANM was conjugated with a 50-fold molar excess of *N*-acetyl-L-cysteine to ANM. NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis was performed essentially according to Yamamoto & Orii (1974), using a 10% gel with 2-mm thickness (130  $\times$  135 mm). The fluorometric tracing was performed on a Shimadzu CS-910 TLC scanner with an excitation wavelength of 365 nm and the cutoff filters Hoya L-39 and L-42 as emission filters. After the gel was stained with Coomassie blue and destained with 7% acetic acid in 30% aqueous methanol, the densitometric tracing was also performed on the Shimadzu CS-910 scanner at 640 nm.

## Results

*Characterization of ANM-Labeled Cytochrome Oxidase.* ANM reacts specifically with SH groups in preference to other nucleophilic residues in proteins. The free ANM is non-fluorescent while the reaction product of ANM with SH groups is strongly fluorescent (Kanaoka et al., 1973).

Cytochrome oxidase has seven SH groups per heme *a* (Orii et al., 1965). The reaction of ANM with cytochrome oxidase was complete within  $\sim$ 2 h at 18–19 °C in ECP and lecithin vesicles. The fluorescence intensity of ANM reached a plateau within  $\sim$ 2 h in the above conditions and remained almost constant for more than 5 h. ANM in lecithin vesicles without cytochrome oxidase was nonfluorescent.

The excitation and emission spectra of ANM conjugated with oxidized cytochrome oxidase in ECP are shown in Figure 1. Since the emission spectrum of ANM attached to cytochrome oxidase partially overlaps the Soret absorption band of oxidized heme *a*, the observed emission spectrum had a depression around 420 nm. Similar spectra were obtained in cytochrome oxidase-lecithin vesicles.

The specific activity of nonlabeled cytochrome oxidase in ECP at 25 °C, pH 6.0, was 10.6 s<sup>-1</sup> per mg of protein per cuvette. The specific activity of ANM-labeled cytochrome oxidase decreased as the molar ratio of ANM(added)/enzyme was raised up to 1, beyond which the activity remained unchanged. The activity of ANM-labeled cytochrome oxidase at the molar ratio of ANM(added)/enzyme = 1 was about three-fifths of that of the nonlabeled cytochrome oxidase.

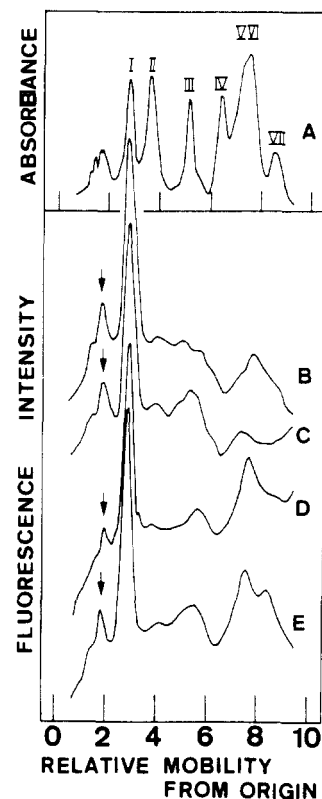


FIGURE 2: Fluorometric and densitometric tracing patterns in NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis of cytochrome oxidase labeled by ANM. (A and B) Enzyme-ECP; (C) enzyme-DPL vesicles; (D) enzyme-DOL vesicles; (E) enzyme-DML vesicles. After the fluorometric tracings (B–E), the gels were stained and the absorbance difference between 640 and 750 nm was measured (A). Labeling was performed at 25 °C (B–D) and 20 °C (E). Arrows indicate the fluorescence of ANM conjugated with contaminant.

*ANM-Labeled Subunits of Cytochrome Oxidase.* The reactive subunits of cytochrome oxidase with ANM were determined by comparing the fluorometric tracing with the densitometric tracing of the NaDodSO<sub>4</sub>-polyacrylamide gel electrophoresis sample (Figure 2). In Figure 2, a subunit was regarded as being labeled by ANM when its fluorescence intensity was stronger than that of the contaminant (indicated by arrows). In the present preparation of soluble bovine heart cytochrome oxidase, subunit I specifically reacted with ANM. Furthermore, the back-titration of SH groups by pCMB which has been shown to react with three SH groups of cytochrome oxidase per heme *a* (S. Yoshida and Y. Orii, unpublished experiments) showed that ANM reacted with soluble cytochrome oxidase stoichiometrically 1:1 per heme *a*.

In lecithin vesicles the main subunit which reacted with ANM was subunit I. Besides subunit I, subunits V and VI reacted with ANM in DOL vesicles, and subunits V–VII reacted with ANM in DML vesicles. A small amount of ANM reacted with subunit III in these three lecithin vesicles. Although the subunits of cytochrome oxidase in DML vesicles labeled by ANM were essentially the same at 20 and 28 °C (i.e., below and above the phase transition temperature of DML), the ratio of the fluorescence intensity of subunits V and VI to that of subunit I was greater above than below the phase transition.

*Temperature Dependence of the Fluorescence Intensity and the Emission Anisotropy.* The steady-state fluorescence intensity,  $I_T$ , and the emission anisotropy,  $r$ , of ANM bound to cytochrome oxidase and of DPH incorporated in the hydrocarbon region of vesicles (Kawato et al., 1978) are plotted against temperature in Figure 3.

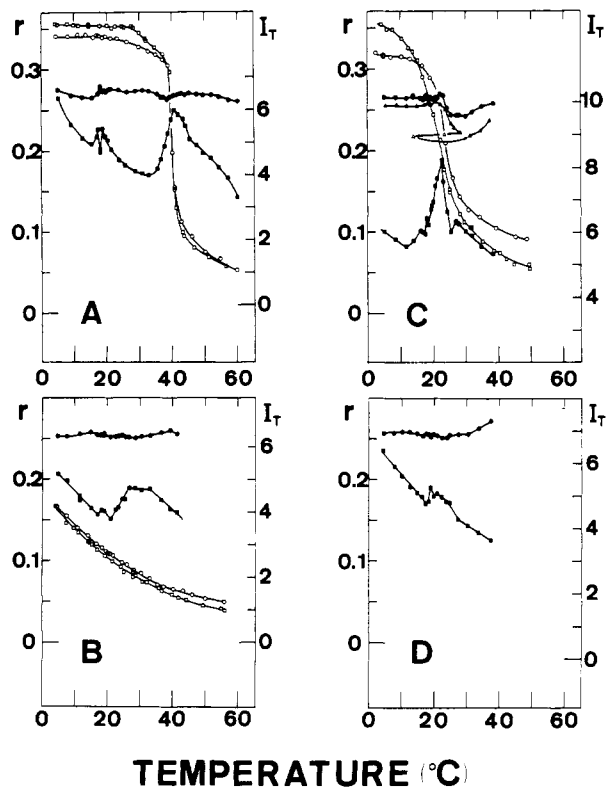


FIGURE 3: Temperature dependence of the fluorescence intensity  $I_T$  (■) and the emission anisotropy  $r$  [(●) ANM; (○) DPH] in cytochrome oxidase vesicles and ECP. (A) Enzyme-DPL; (B) enzyme-DOL; (C) enzyme-DML [(●)  $r_1$  curve of ANM; (▲ and △)  $r_2$  curve of ANM]; (D) enzyme-ECP. Temperature dependence of DPH emission anisotropy (□) in each pure lecithin vesicle is shown. The concentrations are 1.4  $\mu$ M ANM, 4.2  $\mu$ M enzyme, and 0.5 mg/mL lecithin in cytochrome oxidase vesicles. In ECP the concentrations are 6  $\mu$ M ANM and 12  $\mu$ M enzyme. The concentrations are 0.5  $\mu$ M DPH, 3.4  $\mu$ M enzyme, and 0.4 mg/mL lecithin in cytochrome oxidase vesicles. In pure lecithin vesicles the concentrations are 0.3  $\mu$ M DPH and 0.3 mg/mL lecithin.

(1) *Cytochrome Oxidase-DPL Vesicles (Figure 3A)*. In the  $I_T$  curve of ANM, two sharp peaks were observed in the range between 15 and 25 °C, and a large broad peak was observed in the range between 33 and 60 °C. A small sharp peak at 18 °C and a depression around 38 °C were observed in the  $r$  curve of ANM. The value of  $r$  of DPH decreased sharply at ~40 °C and decreased monotonously at ~20 °C. The  $r$  curve of DPH in pure DPL vesicles prepared by sonication is shown in Figure 3. The presence of cytochrome oxidase did not affect the value of  $r$  in the liquid-crystalline phase.

(2) *Cytochrome Oxidase-DOL Vesicles (Figure 3B)*. Both small and large peaks were observed in the  $I_T$  curve of ANM between 17 and 40 °C. The temperature dependence of  $r$  of DPH in the enzyme-DOL vesicles was smooth and was similar to that in sonicated pure DOL vesicles.

(3) *Cytochrome Oxidase-DML Vesicles (Figure 3C)*. Four peaks at 16, 18, 22.5, and 27 °C were observed in the  $I_T$  curve of ANM. The  $r$  curves of ANM showed a large decrease at ~24 °C and exhibited irreversibility. The  $r_1$  curve was obtained with steadily increasing temperature, and the  $r_2$  curve was obtained with increasing and decreasing temperature. In the  $r$  curve of ANM, the greatest difference of the value before and after the large decrease at ~24 °C was 0.07 (not shown in the figure) in six experiments. At temperatures above the large decrease, the value of  $r$  of ANM did not reverse and remained at a similar value ( $r_2$  curve) when the temperature was decreased from 28.5 to 14.3 °C. On the other hand, the

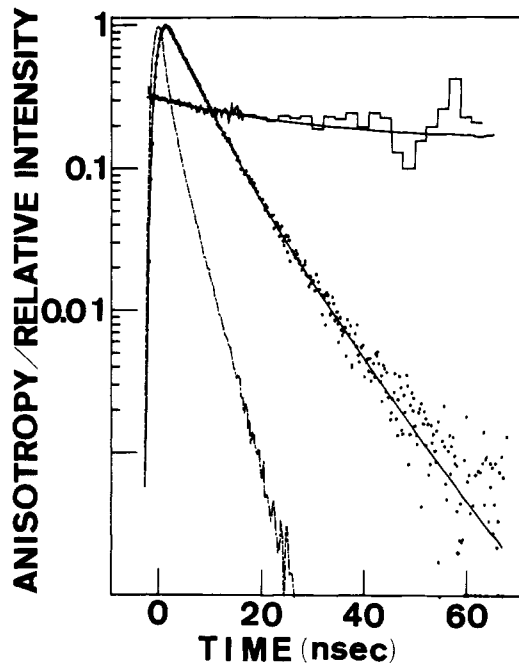


FIGURE 4: Decay curves of the total fluorescence intensity  $I_T(t)$  (dots) and the emission anisotropy  $r(t)$  (zigzag curve) of ANM attached to cytochrome oxidase in ECP at 20 °C. The total fluorescence  $I_T(t)$  was described as a triple exponential decay with three lifetimes (solid line),  $\tau_1 = 0.7$  ns (57%),  $\tau_2 = 3.7$  ns (30%), and  $\tau_3 = 8.4$  ns (13%), where the percentage represents the amplitude of each lifetime. The emission anisotropy  $r(t)$  was described as a double exponential decay with two rotational relaxation times (solid line),  $\phi_1 = 4.9$  ns (20%) and  $\phi_2 = 128.2$  ns (80%), where the percentage represents the amplitude of each relaxation time. The chain line is an apparatus response function  $g'(455 \text{ nm}, t)$ . The concentrations of ANM and the enzyme are 1 and 2.7  $\mu$ M, respectively.

value of  $r$  of ANM reversed almost perfectly when the temperature was decreased from 10–20 °C to lower temperature. In the  $r_1$  curve, a zigzag part was observed between 17 and 20 °C. As compared with the  $r$  curve of DPH in sonicated pure DML vesicles, the presence of cytochrome oxidase increased the emission anisotropy of DPH in the liquid-crystalline phase.

(4) *Cytochrome Oxidase in ECP (Figure 3D)*. A peak in the  $I_T$  curve of ANM was observed between 17 and 28 °C. The increase of the emission anisotropy above 30 °C seems to be due to denaturation of cytochrome oxidase (Orii & Miki, 1979).

*Time-Dependent Emission Anisotropy and Fluorescence Lifetime of ANM.* Decays of the emission anisotropy and the fluorescence intensity of ANM-labeled cytochrome oxidase in ECP were measured at several temperatures. Time courses of the emission anisotropy were biphasic, including a fast phase which corresponded to the rapid restricted rotation of ANM, followed by a slower phase which corresponded to the rotation of cytochrome oxidase (Figure 4). The rotational relaxation time of cytochrome oxidase was longer than 100 ns. According to a triple exponential approximation in the total fluorescence decay curve, the longest component of the fluorescence lifetime was about 8–9 ns in all experiments.

## Discussion

*Rotation of Cytochrome Oxidase.* Cytochrome oxidase does not rotate appreciably in ECP within an ANM fluorescence lifetime shorter than 10 ns. In lecithin vesicles, the rotational relaxation time of cytochrome oxidase would be greater than 1  $\mu$ s considering recent results of protein rotation in membranes (Cone, 1972; Cherry et al., 1976, 1977). Since the longest

component of the ANM fluorescence lifetime bound to cytochrome oxidase in lecithin vesicles was also shorter than 10 ns (S. Kawato et al., unpublished experiments), within the ANM fluorescence lifetime cytochrome oxidase will be completely immobilized in lecithin vesicles.

Since the steady-state emission anisotropy,  $r$ , reflects the rotation of the probe averaged within its fluorescence lifetime, the temperature-dependent changes in  $r$  of ANM could not be due to the changes in the rotation of cytochrome oxidase.

**Temperature-Dependent Peaks in the Fluorescence Intensity of ANM.** The fluorescence intensity is very sensitive to the changes in the surroundings of a fluorescent probe. An important factor of the quenching in the present experiments is the thermal quenching which is caused by the collision of the probe with solvent molecules or surrounding amino acid residues. Since the collision is more frequent at higher temperatures, the fluorescence intensity will decrease monotonously with increasing temperature when there are no other quenching factors.

The present peaks in the  $I_T$  curves of ANM indicate that there are mechanisms of increasing or decreasing the ANM fluorescence intensity at a particular temperature. One probable mechanism of such temperature-dependent peaks is the change in the efficiency of resonance energy transfer between ANM and heme  $a$  which is expected from partial overlaps between the emission spectra of ANM bound to cytochrome oxidase and the Soret band of oxidized heme  $a$  (Dockter et al., 1978; Förster, 1965; Dale & Eisinger, 1976). ANM was fairly immobilized in cytochrome oxidase judging from the slight decay of the time-dependent emission anisotropy in the fast decreasing phase (Figure 4) or from high values of the steady-state emission anisotropy of ANM (above 0.25 except for the enzyme-DML vesicles in the liquid-crystalline phase) as compared with its limiting anisotropy of 0.365 conjugated with 2-mercaptoethanol in glycerin at  $-20^\circ\text{C}$  (S. Kawato, unpublished experiments). The orientation between the emission moment of ANM and the absorption moment of heme  $a$  will thus be restricted. Therefore, if a conformational change in cytochrome oxidase involving a change in the relative arrangement between ANM and heme  $a$  occurs, one might expect a change in the fluorescence intensity induced by the change in the efficiency of resonance energy transfer.

Since ANM fluorescence intensity appears to be sensitive to the solvent polarity (Kanaoka et al., 1973), it is also possible that a conformational change in cytochrome oxidase leading to a change in the polarity around ANM could induce the peak in the  $I_T$  curve.

The temperature-dependent changes in the absorption at 420 nm of soluble cytochrome oxidase and cytochrome oxidase in DOL vesicles were little and below 3% over a temperature range  $4$ – $35^\circ\text{C}$  (Y. Orii and T. Miki, unpublished experiments; S. Kawato et al., unpublished experiments). Therefore, we could eliminate the possibility that the observed peaks in the  $I_T$  curves were caused by a change in reabsorption of ANM fluorescence by heme  $a$ .

**Intrinsic Conformational Change in Cytochrome Oxidase.** All present samples containing cytochrome oxidase showed peaks in the  $I_T$  curves of ANM at  $\sim 17$ – $20^\circ\text{C}$ . However, in the enzyme-DOL vesicles, the peaks were observed over a wide temperature range,  $17$ – $40^\circ\text{C}$ . DPH emission anisotropy showed that DPL and DML are in the gel phase and DOL is in the liquid-crystalline phase in the above temperature regions. Therefore, these peaks in the  $I_T$  curves of ANM are not caused by the lipid phase transition, but such peaks would

be due to an intrinsic conformational change in cytochrome oxidase (Orii et al., 1977).

It is necessary to consider whether the conformational change which is independent of the lecithin phase transition is caused by the phase transition of remaining phospholipids originating from mitochondria. The phospholipid content of the purified preparation of cytochrome oxidase was determined as 1.5 mol of phospholipid per mol of heme  $a$  (Y. Orii and Iba, unpublished experiments). It is unlikely that such a slight amount of phospholipid attached to cytochrome oxidase could cause the conformational change in this bulky enzyme [molecular weight of about  $2 \times 10^5$  in ECP (Orii et al., 1973)].

Since the intact mitochondrial membranes appear to be in the liquid-crystalline state above  $10^\circ\text{C}$  (Blazyk & Steim, 1972; Vanderkooi, 1973; Shinitzky & Inbar, 1976), the reported break at  $\sim 20^\circ\text{C}$  in the Arrhenius plot of cytochrome oxidase activity in mitochondria (Raison et al., 1971; Erecińska & Chance, 1972) would be due to the intrinsic conformational change in cytochrome oxidase and not to a lipid phase transition.

**Conformational Change Induced by Lipid Phase Transition.** The peak in the  $I_T$  curve of ANM between  $33$  and  $60^\circ\text{C}$  and the depression in the  $r$  curve of ANM around  $38^\circ\text{C}$  in the enzyme-DPL vesicles and the peaks in the  $I_T$  curve of ANM around  $24^\circ\text{C}$  and the large decrease of  $r$  of ANM around  $24^\circ\text{C}$  in the enzyme-DML vesicles may be assigned to a conformational change induced by the DPL or DML phase transition which was observed in the  $r$  curve of DPH embedded in these vesicles.

The peaks in  $I_T$  curves around the lipid phase transition may be alternatively interpreted as due to a change in the polarity around ANM by the displacement of cytochrome oxidase in lecithin bilayers, without any intramolecular structural change in the enzyme. In cytochrome oxidase vesicles, the lipid phase transition observed in the emission anisotropy of DPH was almost perfectly reversible. The change in  $r$  of ANM for such vertical and/or lateral displacements (Borochoy & Shinitzky, 1976) caused by the lipid phase transition alone should also be reversible. However, the emission anisotropy of ANM did not reverse at all through the phase transition (see the  $r_2$  curve in Figure 3C). Therefore, this possibility can be excluded.

**Reactivity of Subunits of Cytochrome Oxidase with SH Reagents.** Although subunit I of cytochrome oxidase was shown to be highly hydrophobic and buried deep in both the isolated enzyme and the mitochondrial membrane (Eytan et al., 1975), ANM reacted with subunit I in both ECP and lecithin vesicles in preference to other subunits. The fluorometric tracings showed that two other fluorescent SH reagents,  $N$ -(3-pyrene)maleimide and eosin-5-maleimide, also reacted specifically with subunit I in soluble cytochrome oxidase (S. Kawato et al., unpublished experiments). However, Kornblatt et al. (1973, 1975) showed that  $N$ -ethylmaleimide and 3-maleimido-2,2,5,5-tetramethylpyrrolidinyloxy reacted preferentially with subunit II of soluble and vesicular bovine heart cytochrome oxidase. Subunit II of soluble yeast cytochrome oxidase was shown to react specifically with  $N$ -[(iodoacetamido)ethyl]-1-aminonaphthalene-5-sulfonic acid (Dockter et al., 1978). The above inconsistency in the reactive subunits with SH reagents might be due to the structural differences in these SH reagents and/or in cytochrome oxidase preparations.

In contrast to the considerable motional freedom of the fluorescent SH reagents bound to subunit II of yeast cytochrome oxidase (Dockter et al., 1978), ANM bound to bovine heart cytochrome oxidase in the present systems exhibited

fairly restricted rotation (Kinosita et al., 1977; S. Kawato et al., unpublished experiments), suggesting that ANM is embedded in the enzyme.

Different lecithins and buffer changed the reactivity of subunits V-VII with ANM (Figure 2), and these changes suggest that the topography of these subunits depends on surrounding lipids and buffer. Our results complement the observation by Eytan & Broza (1978) that the surface-labeled subunits in cytochrome oxidase were different in buffer solution, acidic liposomes, and basic liposomes.

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