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# NANOSECOND TIME-RESOLVED FLUORESCENCE INVESTIGATIONS OF TEMPERATURE-INDUCED CONFORMATIONAL CHANGES IN CYTOCHROME OXIDASE IN PHOSPHATIDYLCHOLINE VESICLES AND SOLUBILIZED SYSTEMS

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## Summary

Intrinsic and lipid phase transition-induced conformational changes in cytochrome oxidase in phosphatidylcholine vesicle and solubilized systems were examined by the fluorescence lifetime of *N*-(1-anilinonaphthyl-4)-maleimide conjugated with the enzyme. The time-dependent fluorescence intensity of *N*-(1-anilinonaphthyl-4)-maleimide attached to cytochrome oxidase was described as a triple exponential decay. Both the intrinsic and lipid phase transition-induced conformational changes were detectable in plots of the average lifetime against temperature. In most cases a peak occurred at the temperature of the conformational change.

The time-dependent emission anisotropy showed that *N*-(1-anilinonaphthyl-4)-maleimide embedded in cytochrome oxidase in phosphatidylcholine vesicles underwent a rapid restricted wobbling within a cone. The half-angle of the cone was around 30° for cytochrome oxidase in dimyristoyl phosphatidylcholine vesicles.

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Abbreviation: ANM, *N*-(1-anilinonaphthyl-4) maleimide.

## Introduction

Cytochrome oxidase is the terminal enzyme of the respiratory chain catalysing transfer of electrons from cytochrome *c* to molecular oxygen [1]. The enzyme is a multipeptide transmembrane protein embedded in the inner mitochondrial membrane [2—4].

How functions and conformations of membrane proteins are influenced by the physical states of membrane lipids is an interesting problem [5,6]. A sharp break around 20°C in the Arrhenius plot of cytochrome oxidase activity has been observed in several kinds of mitochondrial membrane [7,8] and reconstituted vesicle and in the soluble enzyme [9]. Taking into account subsequent study of the steady-state fluorescence intensity and polarization of protein-bound probe ANM in cytochrome oxidase vesicles and the solubilized cytochrome oxidase [10], this break is an intrinsic conformational change in the enzyme [11] which is independent of the lipid phase transitions. Furthermore, the steady-state fluorescence study has shown a conformational change in the enzyme which is induced by the lipid phase transition [10].

Here we investigate further the conformational changes in cytochrome oxidase in phosphatidylcholine vesicle and solubilized systems with the time-resolved nanosecond fluorescence spectroscopy of ANM reacted with the SH group of the enzyme.

## Experimental

### *Materials*

Cytochrome oxidase (EC 1.9.3.1) was prepared from beef heart muscle by the method of Okunuki et al. [12] with some modifications. Purified preparation 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 (the Emasol-cholate-phosphate buffer). Purity of cytochrome oxidase preparations were 10—11 nmol heme *a*/mg protein ($A_{280nm}/A_{420nm}$ (oxidized) = 2.35—2.57). The concentration of cytochrome oxidase was determined spectrophotometrically by using a millimolar extinction coefficient difference of 16.5 ($\Delta\epsilon_{605\text{-}630nm}$, reduced).

Dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine and dioleoyl phosphatidylcholine were purchased from Sigma and used without further purification. ANM was purchased from Teika Seiyaku.

### *Preparation of cytochrome oxidase vesicles*

Dipalmitoyl phosphatidylcholine, dimyristoyl phosphatidylcholine and dioleoyl phosphatidylcholine in chloroform were dried under a stream of $N_2$ gas. Each of these residues was dispersed in 50 mM sodium phosphate buffer (pH 7.4) containing 2 mM $MgSO_4$, and a 20% (w/v) solution of sodium cholate was added to a final concentration of 1%. The phosphatidylcholine-cholate mixture was vortexed vigorously with occasional heating to above 40°C. After a few minutes of this treatment, the phosphatidylcholine-cholate mixture became clear. The solution was cooled to 0°C and cytochrome oxidase in Emasol-cholate-phosphate buffer 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 $MgSO_4$ for 20 h at 4°C with four changes of the outer solution.

*Fluorescence labeling*

Fluorescence labeling of cytochrome oxidase with ANM was achieved by addition of several $\mu$l of 1 mM ANM in acetone to several ml of the suspension of cytochrome oxidase vesicles or the solution of cytochrome oxidase in Emasol-cholate-phosphate buffer. The concentration of added ANM and cytochrome oxidase were 1—1.2 $\mu$M and 2—2.5 $\mu$M, respectively. The reaction of ANM with cytochrome oxidase was followed as an increase in the fluorescence intensity. After the incubation at approx. 20°C for 2—3 h, when the fluorescence intensity reached a plateau, the fluorescence decay measurements were performed.

The depolarization due to the scattering of exciting and emitted lights by vesicles was negligible in the present vesicle suspensions of low lipid concentration (0.25—0.3 mg/ml in phosphatidylcholine, 2—2.5 $\mu$M in the enzyme).

*Fluorescence decay measurements*

The nanosecond fluorometer used for fluorescence decay measurements is detailed elsewhere [13,14]. Briefly, the sample was excited by a vertically polarized light of 1—2 ns duration and wavelength 360 nm. All emission above 390 nm was collected through cutoff filters and detected by a Hamamatsu TV R943-02 photomultiplier. The signals were analyzed by calculation of the total fluorescence intensity, $I_T(t)$, and the emission anisotropy, $r(t)$, given by:

$$I_T(t) = I_V(t) + 2I_H(t) \tag{1}$$

$$r(t) = (I_V(t) - I_H(t))/I_T(t) \tag{2}$$

where $I_V(t)$ and $I_H(t)$ are the fluorescence intensities for vertical and horizontal polarization, respectively, at time $t$ after the flash. These quantities, $I_T(t)$ and $r(t)$, are related to $I_T^\delta(t)$ and $r^\delta(t)$, responses to a impulsive excitation expressed as $\delta(t)$, by the following equations:

$$I_T(t) = \int_0^t g(t')\, I_T^\delta(t - t')\, dt' \tag{3}$$

$$r(t)\, I_T(t) = \int_0^t g(t')\, r^\delta(t - t')\, I_T^\delta(t - t')\, dt' \tag{4}$$

where $g(t)$ is the response function of the apparatus [14]. The fluorescence lifetime $\tau_i$ and the rotational relaxation time $\phi_j$ were determined by a curve-fitting procedure, by assuming that $I_T^\delta(t)$ and $r^\delta(t)$ were expressed as sums of exponential functions:

$$I_T^\delta(t) = \sum_{i=1}^{N} I_i \exp(-t/\tau_i) \tag{5}$$

$$r^{\delta}(t) = \sum_{j=1}^{M} r_j \exp(-t/\phi_j) \tag{6}$$

The average lifetime is defined as

$$\langle \tau \rangle = \sum_{i=1}^{N} \alpha_i \tau_i \tag{7}$$

where

$$\alpha_i = I_i / \sum_{k=1}^{N} I_k$$

($i = 1, ..., N$) is an exponential fraction.

## Results and Discussion

Fig. 1 shows representative decay curves of $I_T(t)$ and $r(t)$ for ANM conjugated with cytochrome oxidase in dimyristoyl phosphatidylcholine vesicles at 25°C. Time courses of $r(t)$ curves in cytochrome oxidase vesicles were biphasic, including a fast phase corresponding to the rapid restricted rotation of ANM embedded in cytochrome oxidase, followed by an almost constant phase. All $I_T(t)$ curves of ANM were described as a triple exponential decay in the present solubilized and vesicle systems. These complex decays of $I_T(t)$ is not necessarily due to the multiple binding sites of ANM in the enzyme, since even ANM which specifically reacted with subunit I stoichiometrically 1 : 1 per heme *a* in the soluble enzyme has three lifetime components [10].

Fluorescence lifetime of ANM reacted with β-mercaptoethanol, bovine serum albumin and cytochrome oxidase are summarized in Table I. ANM reacted with a small compound had essentially a single lifetime, while ANM bound to protein had three lifetime components. The average lifetime $\langle \tau \rangle$, which is proportional to the quantum yield of the fluorescent probe, is a proper index for

TABLE I

FLUORESCENCE LIFETIME (ns) OF ANM CONJUGATED WITH A COMPOUND AND PROTEINS AT APPROX. 20°C

Oxidase in dipalmitoyl phosphatidylcholine and in dimyristoyl phosphatidylcholine were measured at 18.6°C and 21.0°C, respectively. Other samples were measured at 20.0°C. Concentrations were: ANM, 10 μM and mercaptoethanol, 12 μM; ANM, 6 μM and albumin, 12 μM; ANM, 1—1.2 μM and oxidase 2—2.5 μM in all ANM-cytochrome oxidase systems.

| | $\alpha_1$ | $\tau_1$ | $\alpha_2$ | $\tau_2$ | $\alpha_3$ | $\tau_3$ | $\langle \tau \rangle$ |
|---|---|---|---|---|---|---|---|
| β-Mercaptoethanol | 0.998 | 1.2 | 0.002 | 7.9 | | | 1.2 |
| Bovine serum albumin | 0.342 | 0.7 | 0.472 | 5.1 | 0.186 | 9.9 | 4.5 |
| Oxidase | | | | | | | |
| in Emasol-cholate-phosphate buffer | 0.572 | 0.7 | 0.295 | 3.7 | 0.133 | 8.4 | 2.6 |
| in dipalmitoyl phosphatidylcholine | 0.768 | 0.3 | 0.203 | 2.0 | 0.029 | 7.5 | 0.9 |
| in dimyristoyl phosphatidylcholine | 0.625 | 0.8 | 0.308 | 3.5 | 0.067 | 8.7 | 2.2 |
| in dioleoyl phosphatidylcholine | 0.692 | 0.4 | 0.263 | 2.7 | 0.045 | 8.1 | 1.4 |

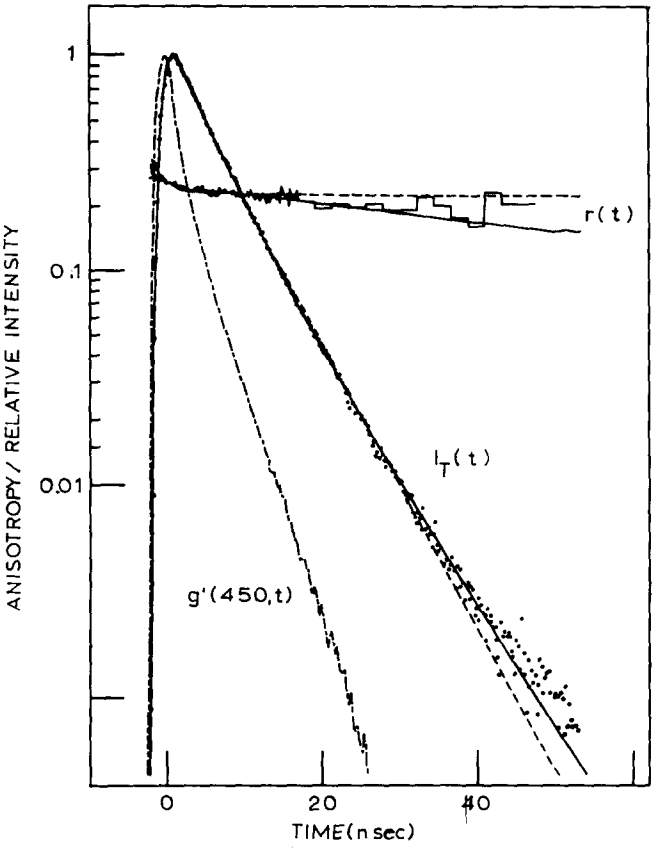


**Fig. 1.** Time-dependent fluorescence intensity $I_T(t)$ (dots) and emission anisotropy $r(t)$ (zig-zag curve) of ANM conjugated with cytochrome oxidase in dimyristoyl phosphatidylcholine vesicles at 25°C. The $I_T(t)$ curve: the solid line is the best fit curve by a triple exponential approximation ($\alpha_1 = 0.584$, $\tau_1 = 0.3$ ns, $\alpha_2 = 0.342$, $\tau_2 = 2.5$ ns, $\alpha_3 = 0.074$, $\tau_3 = 7.6$ ns), and the dashed line is the best fit curve by a double exponential approximation. The $r(t)$ curve: the solid line is the best fit curve by a double exponential approximation ($r_1 = 0.039$, $\phi_1 = 1.1$ ns, $r_2 = 0.240$, $\phi_2 = 99.8$ ns), and the dashed line is the best-fit curve according to Eqn. 8 ($\phi_1 = 0.2$ ns, $r_\infty = 0.227$). The chain line is the apparatus response function $g'$ (450 nm, $t$).

comparing the character of the fluorescent probe in different surroundings. The order of magnitude of $\langle\tau\rangle$ for ANM bound to the above compound and proteins around 20°C was as follows: $\langle\tau\rangle$ (albumin) > $\langle\tau\rangle$ (soluble oxidase) > $\langle\tau\rangle$ (oxidase-dimyristoyl phosphatidylcholine) > $\langle\tau\rangle$ (oxidase-dioleoyl phosphatidylcholine) ≃ $\langle\tau\rangle$ (mercaptoethanol) > $\langle\tau\rangle$ (oxidase-dipalmitoyl phosphatidylcholine. The quantum yield of the fluorescence of ANM is sensitive to the polarity around ANM [15] and the energy transfer between ANM and heme *a* [10], so that the above differences in $\langle\tau\rangle$ are probably due to these two factors.

Fig. 2 shows that the temperature dependence of all components of lifetime and exponential fraction of ANM conjugated with cytochrome oxidase in dipalmitoyl phosphatidylcholine vesicles has a peak or a depression around 45°C. While there are no observable peaks or depressions around 18°C. The changes in lifetimes and exponential fractions around 45°C can be assigned to the conformational change in cytochrome oxidase induced by the dipalmitoyl phos-

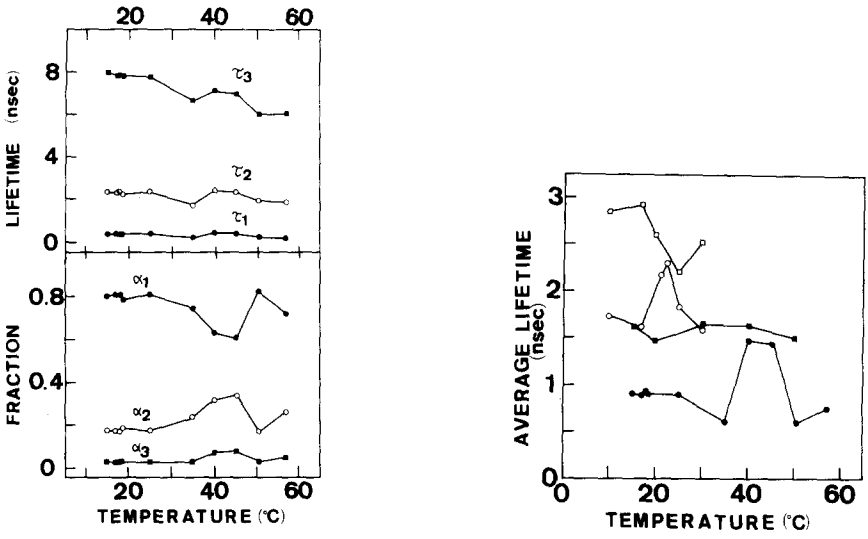


Fig. 2. Temperature dependence of fluorescence lifetimes and exponential fractions of ANM conjugated with cytochrome oxidase in dipalmitoyl phosphatidylcholine vesicles. These parameters were calculated by a triple exponential approximation.

Fig. 3. Temperature dependence of the average lifetime of ANM conjufated with cytochrome oxidase in dipalmitoyl phosphatidylcholine (●), dimyristoyl phosphatidylcholine (○), dioleoyl phosphatidylcholine (■) and Emasol-cholate-phosphate buffer (□). The average lifetime was calculated by Eqn. 7 with $N = 3$.

phatidylcholine phase transition around 40°C, which has been observed as the temperature-induced peak around 40°C in the steady-state fluorescence intensity curve ($I_T^s$ curve) of ANM [10]. On the other hand, the intrinsic conformational change, which has been characterized by peaks around 18°C in the $I_T^s$ curve [10], was not detectable as changes in the fluorescence lifetime and the exponential fraction around 18°C.

The comparison of $\langle\tau\rangle$ curves of ANM bound to cytochrome oxidase in various phosphatidylcholine vesicles and Emasol-cholate-phosphate buffer is shown in Fig. 3. In comparing these $\langle\tau\rangle$ curves to $I_T^s$ curves in Fig. 3 in Ref. 10 in the same sample, a peak around 45°C in the enzyme-dipalmitoyl phosphatidylcholine and a peak around 22°C in the enzyme-dimyristoyl phosphatidylcholine in the $\langle\tau\rangle$ curves would reflect the conformational change in the enzyme induced by the lipid phase transition, and a broad peak over 20—50°C in the $\langle\tau\rangle$ curve of the enzyme-dioleoyl phosphatidylcholine would reflect the intrinsic conformational change. In contrast, no peak around 18—20°C was observed in the $\langle\tau\rangle$ curve of the enzyme-dipalmitoyl phosphatidylcholine. The decrease around 20°C in the $\langle\tau\rangle$ curve of the enzyme in Emasol-cholate-phosphate buffer may reflect the intrinsic conformational change.

Thus, the conformational change induced by the lipid phase transition is observed as a similar peak qualitatively in both the $\langle\tau\rangle$ curve and the $I_T^s$ curve. In the case of the enzyme intrinsic conformational change, however, the relationship between the $\langle\tau\rangle$ curve and the $I_T^s$ curve is not simple. Although the $I_T^s$ curve has the peak(s) around the enzyme intrinsic conformational change in all present systems, the $\langle\tau\rangle$ curve shows a peak (enzyme-dioleoyl phosphatidyl-

choline), a decrease (enzyme in Emasol-cholate-phosphate buffer) and no observable change (enzyme-dipalmitoyl phosphatidylcholine).

The fast decreasing phase, faster than 1 ns, in $r(t)$ curves of ANM bound to cytochrome oxidase shows the rapid independent motion of ANM [16]. Since cytochrome oxidase has been shown to rotate with a relaxation time of several hundred microseconds in both mitochondrial membranes and lipid vesicles [17] and to be completely immobilized within the experimental time range of approx. 100 ns, we analyzed $r(t)$ curves assuming $\phi_2 = \infty$ by a double-exponential approximation as follows:

$$r^{\delta}(t) = (r_0 - r_\infty) \exp(-t/\phi_1) + r_\infty \tag{8}$$

where the limiting anisotropy $r_0$ of ANM is taken as 0.365 [10] and $r_\infty$ is the residual time-independent anisotropy (see Fig. 1). One possible interpretation of Eqn. 8 is based upon a model which assumes random wobbling of ANM within a certain cone of half-angle $\theta_c$ which is formed by surrounding amino acid residues [18,19], since ANM may conjugate with cytochrome oxidase by a flexible linkage. In this 'wobbling-in-cone' model, $r^{\delta}(t)$ is closely approximated by the expression

$$r^{\delta}(t) = (r_0 - r_\infty) \exp(-D_w t/\langle\sigma\rangle) + r_\infty \tag{9}$$

where $\langle\sigma\rangle$ is a constant that depends only on $\theta_c$. The cone angle $\theta_c$ and the wobbling diffusion constant $D_w$ can be calculated by

$$r_\infty/r_0 = [\tfrac{1}{2} \cos\theta_c(1 + \cos\theta_c)]^2 \tag{10}$$

$$D_w = \langle\sigma\rangle/\phi_1 \tag{11}$$

Calculated values of $\theta_c$ and $D_w$ in the enzyme-dimyristoyl phosphatidylcholine vesicles are summarized in Table II. Thus, the motion of ANM embedded in cytochrome oxidase in dimyristoyl phosphatidylcholine can be described by the rapid wobbling ($\phi_1 \approx 0.5$ ns) within the cone of half-angle around 30°.

Consistent with the large decrease in the steady-state emission anisotropy of ANM [10], $\theta_c$ increased around 24°C, implying the conformational change in the enzyme induced by the dimyristoyl phosphatidylcholine phase transition.

TABLE II

ANALYSIS OF $r(t)$ CURVES IN THE CYTOCHROME OXIDASE-DIMYRISTOYL PHOSPHATIDYLCHOLINE SYSTEM BY THE 'WOBBLING-IN-CONE' MODEL

| Temperature (°C) | $r_\infty$ | $\theta_c$ (degree) | $\phi_1$ (ns) | $D_w$ ($ns^{-1}$) |
|---|---|---|---|---|
| 10 | 0.244 | 29.2 | 0.3 | 0.2 |
| 17 | 0.239 | 29.9 | 0.4 | 0.2 |
| 21 | 0.247 | 28.8 | 0.6 | 0.1 |
| 22.5 | 0.238 | 30.0 | 0.6 | 0.1 |
| 25 | 0.234 | 30.6 | 0.3 | 0.2 |
| 30 | 0.228 | 31.5 | 0.4 | 0.2 |

The variables were determined by Eqns. 7—10.

Similar restricted wobbling of ANM was observed in the soluble cytochrome oxidase [10] and other cytochrome oxidase vesicles.

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