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Rotational Diffusion of the ADP/ATP Translocator in the Inner Membrane of Mitochondria and in Proteoliposomes*

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The ADP/ATP translocator was selectively labeled with the triplet probe eosin-5-maleimide (EMA) after pretreatment with *N*-ethylmaleimide in beef heart mitochondria, as reported previously for submitochondrial particles (Müller, M., Krebs, J. J. R., Cherry, R. J., and Kawato, S. (1982) *J. Biol. Chem.* 257, 1117–1120). The EMA binding was completely inhibited by carboxyatractylate. 0.7–1.1 molecules of EMA conjugated with 1 molecule of the dimeric translocator with M_r ~ 65,000. The EMA binding decreased [^{14}C]ADP uptake by about ~25%.

The EMA-labeled translocator·bongkrekate complex was purified and reconstituted in liposomes by removing Triton X-100 with Amberlite XAD-2. The liposomes were composed of phosphatidylcholine/phosphatidylethanolamine/cardiolipin and the lipid to protein ratio by weight was (L/P) = 60.

Rotational diffusion of the ADP/ATP translocator around the membrane normal was measured in reconstituted proteoliposomes and in the mitochondrial inner membranes by observing the flash-induced absorption anisotropy, $r(t)$, of EMA. In proteoliposomes with L/P = 60, the translocator was rotating with an approximate average rotational relaxation time of $\phi \simeq 246$ μs and a normalized time-independent anisotropy $[r_3/r_r(0)]_{min} \simeq 0.55$. In intact mitochondria, values of $\phi \simeq 405$ μs and $r_3/r_r(0) \simeq 0.79$ were obtained. The higher value of $r_3/r_r(0)$ in mitochondria compared with proteoliposomes indicates the co-existence of rotating and immobile translocator ($\phi > 20$ ms) in the inner mitochondrial membrane. Based on the assumption that all the translocator is rotating in the lipid-rich proteoliposomes, the population of the mobile translocator at 20 °C was calculated to be ~47%. By removing the outer membrane, the mobile population was increased to ~70% in mitoplasts, while ~53% of the translocator was rotating in submitochondrial particles.

The above results indicate a significant difference in protein-protein interactions of the ADP/ATP translocator in the different types of inner membranes of mitochondria. The immobile population of the translocator could be due to nonspecific protein aggregates caused by the very high concentration of proteins in the inner membrane of mitochondria (L/P ~ 0.4).

The ADP/ATP translocator catalyzes the vectorial exchange between cytosolic ADP and the matrix ATP across the inner membrane of mitochondria, which is a key process in the cellular energy supply of aerobic organisms (for review see Refs. 1–3). In beef heart mitochondria, the translocator is the most abundant integral protein, being about 10% of the total mitochondrial protein (4). The M_r of the monomeric translocator is ~32,500.

The translocator has been isolated as the complex with CAT[1] or bongkrekate using extraction with Triton X-100 and it was found that the complex is a dimer (5, 6). A complete amino acid sequence of the translocator revealed that the monomeric protein contains 4 cysteines over 297 amino acids (7). Masking or chemical modification of sulfhydryl groups, which are essential for the nucleotide translocation, causes the inhibition of the translocation activity (2). At least one sulfhydryl group is masked when the translocator is liganded by CAT.

The translocator was successfully reconstituted in lipid vesicles (8, 9) and the nucleotide transport activity was shown to be dependent on the lipid composition (9, 10), the membrane potential (11), and the nucleotide distribution (12).

It has been postulated that the translocator is functionally and perhaps structurally linked to the proteins responsible for the ADP phosphorylation and transport, *i.e.* ATP-synthase, mitochondrial creatine phosphokinase, and phosphate carrier (2, 13, 14). However, to date, an inadequate amount of studies has been performed for direct measurements of the topological organization for these proteins. A promising approach to this problem is to investigate protein rotational diffusion, which is particularly sensitive to protein-protein interactions (15–17).

Recent investigations showed that integral proteins in the inner membrane of mitochondria are able to undergo rotational and lateral diffusion (16–20). However, a significant fraction of membrane proteins such as cytochrome oxidase was observed to be immobile within a millisecond time range in mitochondria (16, 17).

We have demonstrated selective labeling and rotational diffusion of the ADP/ATP translocator in SMP using the EMA probe (21). Co-existence of mobile and immobile translocator was observed.

Here we extend the study to quantitatively examine the mobility of the translocator in mitochondria, mitoplasts, and SMP by comparing its rotational diffusion with that observed in proteoliposomes.

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[1] The abbreviations used are: CAT, carboxyatractylate; D-mitoplasts, mitoplasts prepared by the digitonin procedure; EMA, eosin-5-maleimide; FP-mitoplasts, mitoplasts prepared by the French press procedure; L/P, lipid to protein weight ratio; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulfate; SMP, submitochondrial particles.

EXPERIMENTAL PROCEDURES

Membranes and Protein Preparations—Beef heart mitochondria were prepared according to Hatefy and Lester (22) as modified by Bock and Fleischer (23) and stored frozen at −80 °C. D-mitoplasts were prepared by the digitonin procedure as described by Krebs *et al.* (24). FP-mitoplasts were prepared by the French press procedure as described by Decker and Greenawalt (25). After the preparation, all the membranes were resuspended in a medium containing 220 mM mannitol, 70 mM sucrose, 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, and KOH to adjust the pH to 7.4 (buffer A), at a protein concentration of 10–15 mg/ml. EMA-labeled translocator·bongkrekate complex was purified from mitochondria according to Klingenberg *et al.* (26). For reconstitution in liposomes, the pass-through fraction of the hydroxylapatite column chromatography was used. Triton X-100 was removed from the lipid/protein/detergent mixture using Amberlite XAD-2 beads in batch procedure at 2 °C. After 3 h of incubation, the beads were replaced by fresh ones. After further incubation for 3 h, the turbid solution was centrifuged 6 h at 160,000 × *g*. The pellets were resuspended in glycerol solution for rotational diffusion measurements.

Selective Labeling of the Membranes—Mitochondria were labeled with EMA (Molecular Probes Inc., Junction City, OR) as follows: 10 μg of NEM/mg of protein were added to the membranes and incubated for 5 min at 0 °C, then 15 μg of EMA/mg of protein were added to the membranes and incubated for 30 min in the dark at 0 °C. The reaction was stopped by adding 0.5 mg of dithiothreitol/mg of protein. After a 30-min incubation at 0 °C, the samples were washed several times with buffer A to remove any free EMA. Addition of bovine serum albumin to buffer A increased the efficency of the removal of free EMA, especially for large scale labeling experiments. The amount of bound EMA was determined spectrophotometrically as described by Cherry (27). FP- and D-mitoplasts were prepared from EMA-labeled mitochondria. FP-SMP were prepared from EMA-labeled mitochondria by the French press procedure as described previously (21). SMP were also prepared from EMA-labeled D-mitoplasts by sonication for 4 min using a Branson sonifier with pulsed mode (50%) at 50-watt output at 0 °C under a N_2 stream.

pH Treatment of Mitochondria and Mitoplasts—The pH treatment was performed as follows: the EMA-labeled membranes were adjusted to pH 6 with HCl and to pH 10 with KOH at 10–15 mg of protein/ml and incubated for 1 h at room temperature. Subsequently, the samples were transferred into glycerol solution and the mobility of the translocator was measured.

[^{14}C]ADP Uptake Measurements—Freshly isolated rat heart mitochondria were diluted in buffer A to 200 μg of protein/ml. Labeling of mitochondria with EMA was performed as described in the above section. The reaction at 20 °C was started by the addition of [^{14}C] ADP (500 cpm/μg of protein). Aliquots of 100 μl were filtered on Millipore filters (0.45 μm pore size) and washed with buffer A containing CAT (10 μg/ml). The Millipore filters were transferred into scintillation liquid and analyzed for radioactivity.

SDS-Polyacrylamide Gel Electrophoresis—A slightly modified method of Laemmli (28) was used as previously described (21). Fluorographs were obtained by illuminating the slab gel with UV light and photographing the fluorescence through cut-off filters. Subsequently, the gel was stained conventionally with Coomassie blue R-250.

Rotational Diffusion Measurements and Analysis of Absorption—For transient dichroism experiments, EMA-labeled mitochondrial inner membranes were suspended in 80% (w/w) glycerol, in order to eliminate vesicle tumbling and to reduce light scattering. The final concentration of EMA was 8–10 μM. Prior to measurements, oxygen was displaced from the samples by a stream of argon as described previously (16). The flash photolysis apparatus is described in detail elsewhere (27). Briefly, the sample was excited at 532 nm by a vertically polarized flash of 15-ns duration from a Nd/YAG laser (JK Laser, second harmonic). Absorbance changes due to ground state depletion of EMA were measured at 520 nm. The signals were analyzed by calculating the absorption anisotropy $r_r(t)$ and the total absorbance change $A(t)$, given by

$$r_r(t) = [A_V(t) - A_H(t)]/[A_V(t) + 2A_H(t)] \quad (1)$$

$$A(t) = A_V(t) + 2A_H(t) \quad (2)$$

where $A_V(t)$ and $A_H(t)$ are, respectively, the absorbance changes for vertical and horizontal polarization at time t after the flash. In each experiment, 1024 signals were averaged using a Datalab DL 102 A signal averager. A further improvement of the signal to noise ratio was achieved by averaging data from several experiments. The ADP/ATP translocator maintains a fixed orientation with respect to the membrane plane (1, 3, 8), implying that rotation occurs about the normal to the plane of the membrane. Therefore, decays in absorption anisotropy, $r_r(t)$, were analyzed based on a "rotation-about-membrane normal" model (15, 29). When there is a single rotating species of the translocator with the rotational relaxation time, $\phi_\parallel$, $r_r(t)$ is given by

$$r_r(t)/r_r(0) = 3\sin^2\theta_N \cos^2\theta_N \exp(-t/\phi_\parallel) + \tfrac{3}{4}\sin^4\theta_N \exp(-4t/\phi_\parallel) + \tfrac{1}{4}(3\cos^2\theta_N - 1)^2 \quad (3)$$

where θ_N is the angle between the absorption moment of EMA and the membrane normal. However, because EMA is very rapidly wobbling with a subnanosecond time constant (21), the above θ_N should be considered as a time-averaged angle over about 10 μs (a dead time of the transient dichroism measurement). In the present study, multiple rotating species of the ADP/ATP translocator with different $\phi_\parallel$ are considered by analyzing the data by the following equation:

$$r_r(t) = r_1\exp(-t/\phi) + r_2\exp(-4t/\phi) + r_3 \quad (4)$$

where ϕ is the approximate average rotational relaxation time. Here, it should be noted that $r_3 = \tfrac{1}{4}(3\cos^2\theta_N - 1)^2$ when all the translocator is rotating, even in the presence of multiple rotating species. Curve-fitting of the data by Equation 4 was accomplished by a Hewlett-Packard HP 9825 A desk top computer.

The normalized time-independent anisotropy $r_3/r_r(0)$ is related to the fraction of mobile ADP/ATP translocator (29). When all the translocator is mobile, $r_3/r_r(0)$ has a minimal value $[r_3/r_r(0)]_{min}$. The fraction of mobile population, f_m, can be calculated by

$$r_3/r_r(0) = f_m \cdot [r_3/r_r(0)]_{min} + (1 - f_m) \quad (5)$$

Other Methods—The mitochondrial heme *a* content was determined at room temperature using an extinction coefficient of 12 mM^{-1} cm^{-1} ($\Delta\epsilon_{605-630\,nm}$) (30). Dithionite-reduced minus air-oxidized difference spectra were obtained with an Aminco DW-2a spectrophotometer. The protein concentration was determined following the biuret procedure (31) unless otherwise stated. When required, we used the procedure of Bradford (32) using the concentrate reagent of Bio-Rad or the method of Lowry *et al.* (33). The electron transfer activity from succinate to oxygen was determined polarographically by measuring the oxygen consumption with a Clark electrode at 25 °C in 0.8-ml volume in the presence of 3 μM rotenone. Stimulation of the respiration was induced by the addition of ADP aliquots.

RESULTS

Selective Labeling of the Translocator with EMA

Selective labeling of the translocator with EMA in intact mitochondria or mitoplasts was accomplished by preincubating the membranes with NEM (10 μg/mg of protein) and subsequently incubating with EMA (15 μg/mg of protein).

A typical example is shown on sodium dodecyl sulfate polyacrylamide gel in Fig. 1 (*left, lane 2*). When prior to the above labeling procedure, the membranes were preincubated with CAT, a specific inhibitor of the translocator, no EMA-labeled band is observed on the gel (*left, lane 3*). Therefore, it is clearly indicated that the observed EMA-labeled protein is the ADP/ATP translocator and that the EMA binding site is overlapping with the CAT binding site which is located on the cytoplasmic side of the inner membrane of mitochondria. Preincubation of the membranes with ADP or ATP didn't affect the selective labeling.

The ratio of bound EMA to the dimeric translocator molecule (M_r ~65,000) was determined spectrophotometrically to be 0.7–1.1 (mol/mol) in mitochondria,[2] based on a translocator

[2] In Ref. 21, we published a wrong value of EMA/monomeric translocator of ~1 (mol/mol) in SMP. The correct value is ~0.5 EMA/monomeric translocator (mol/mol).

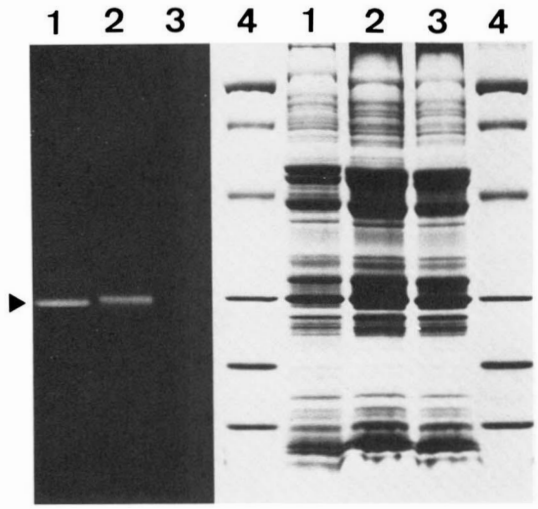

FIG. 1. **Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of mitochondria after labeling with EMA.** *Left*, fluorograph of the gel before staining with Coomassie blue. Labeling of mitochondria was performed with 15 μg of EMA/mg of protein for 30 min at 0 °C, resulting in a multiple labeling (*lane 1*). By treatment of the mitochondria with 10 μg of NEM/mg of protein before EMA labeling, selective labeling of the ADP/ATP translocator (*arrow*) was achieved (*lane 2*). Preincubation with CAT (3.6 μg/mg of protein), 10 min, 20 °C, prior to the NEM and EMA labeling resulted in an almost complete disappearance of the EMA fluorescence (*lane 3*). Mitochondria were labeled at a protein concentration of 10 mg/ml. *Right*, Coomassie blue stain of the same gel (*lanes 1–3*). Marker proteins are phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme whose apparent M_r are, respectively, 92,500, 66,200, 45,000, 31,000, 21,500, and 14,400 (*lane 4*).

content of 1.3–2.0 nmol of dimer/mg of protein (1). From this, the molar ratio of EMA/heme *a* was calculated to be 1.6–1.9, based on the measured heme a content. The presence of bongkrekate didn't change the above characteristics of EMA binding to the translocator in mitochondria.

Effect of EMA on the ADP Translocation Activity

The translocation of [^{14}C]ADP in freshly isolated rat heart mitochondria was investigated under different conditions as illustrated in Fig. 2. The translocation activity was decreased by ~25% after the selective labeling of the translocator by EMA (30-min labeling of the membranes with EMA at 0 °C). Addition of CAT to the labeled mitochondria completely inhibited the translocation activity (Fig. 2, *curve b*). After a 2-h labeling of the membranes with EMA at 0 °C, a ~60% decrease in [^{14}C]ADP translocation activity was observed. Also, in this case, the addition of CAT blocked the translocation activity completely. The measured EMA/dimeric translocator ratio was 1.3–2.0 after 2 h of incubation. Thus, although CAT prevents completely the EMA binding to the translocator, even the presence of more than 1 EMA/dimeric translocator does not prevent the CAT binding to the translocator as suggested by the observed results. This proposes that the EMA and CAT binding sites are overlapping but not identical.

Rotational Diffusion of the ADP/ATP Translocator

Rotational diffusion of the translocator was measured in beef heart mitochondria, mitoplasts, SMP, and reconstituted vesicles. Data were analyzed by Equation 4 and decay parameters are summarized in Table I.

Proteoliposomes—To perform calculations of the mobile

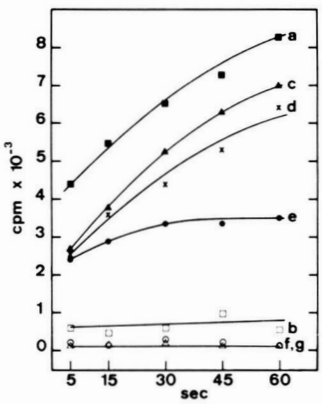

FIG. 2. **Effect of NEM, EMA, and CAT on the [^{14}C]ADP uptake in freshly isolated rat heart mitochondria.** Conditions are described under "Experimental Procedures." *Curve a*, control mitochondria (■); *curve b*, control inhibition by CAT (4 μg/mg of protein, 10-min preincubation) (□); *curves c*, *d*, and *e*, mitochondria were preincubated for 5 min with NEM (10 μg/mg of protein) and further incubated with EMA (15 μg/mg of protein) for, respectively, 5 (▲), 30 (×), 120 (●) min; *curves f* and *g*, mitochondria of the *curves d* and *e* were further incubated with CAT (4 μg/mg of protein) for 10 min (○, △). All incubations were performed at 10 mg of mitochondrial protein/ml at 0 °C.

and immobile populations of the translocator, it is necessary to determine the minimal value of the normalized time-independent anisotropy $[r_3/r_r(0)]_{min}$ which corresponds to $r_3/r_r(0)$ in Equation 4, when all the translocator is rotating (29). For this, the purified translocator, stabilized by bongkrekate and labeled with EMA, was reconstituted in vesicles composed of phosphatidylcholine/phosphatidylethanolamine/cardiolipin = 4:2:1 by weight, at L/P = 60. As shown in Fig. 3, $r_3/r_r(0) = 0.55 \pm 0.05$ and $\phi = 246 \pm 5$ μs have been obtained. Assuming that all the translocator is rotating in these proteoliposomes (*i.e.* $[r_3/r_r(0)]_{min} = 0.55$), we can calculate the mobile population by Equation 5 in the inner membrane of mitochondria.

Inner Mitochondrial Membranes at 20 °C (Fig. 3)—In all inner mitochondrial membranes examined (*i.e.* mitochondria, mitoplasts, and SMP), $r_3/r_r(0)$ was larger than 0.55, indicating the existence of immobile translocator within the experimental time range of 1 ms. Judging from the approximate average rotational relaxation time ϕ, it was also evident that the mobile translocator was rotating more slowly in the inner membrane of mitochondria ($\phi \simeq 350$–550 μs) than in proteoliposomes ($\phi \simeq 246$ μs). The mobile population of the translocator, calculated from $r_3/r_r(0)$, was the smallest in mitochondria ($f_m \simeq 47\%$), the middle in SMP ($f_m \simeq 47$–58%), and the largest in mitoplasts ($f_m \simeq 70\%$). No significant difference was observed between D-mitoplasts and FP-mitoplasts in both f_m and ϕ ($\phi \simeq 400$ μs). Differences in decay parameters were not negligible between SMP prepared from D-mitoplasts by sonication ($f_m \simeq 47\%$ and $\phi \simeq 358$ μs) and SMP prepared from mitochondria by the French press procedure ($f_m \simeq 58\%$ and $\phi \simeq 547$ μs). In our previous study on SMP (21), the accuracy of the data did not justify use of the full Equation 4. Therefore, the data were analyzed with the simplified equation $r_r(t) = A_1\exp(-t/\phi_r) + A_2$ where ϕ_r is the rotational time constant. ϕ_r does not have a simple theoretical relationship with ϕ in Equation 4. Therefore, all the experiments of SMP were repeated with an improved signal to noise ratio in order to compare different types of inner mitochondrial membrane as quantitatively as possible with the full Equation 4.

Effect of Temperature—In all inner mitochondrial membranes examined, an increase of temperature from 20 °C to

TABLE I

Decay parameters of the time-dependent absorption anisotropy of the ADP/ATP translocator in mitochondrial inner membranes and proteoliposomes analyzed by Equation 4

Experimental conditions are described in Fig. 3 but with varying pH and temperature.

Membrane	pH	ϕ	$r_3/r_r(0)$	Mobile translocator[a]	r_1/r_2	Temperature
		μs		%		°C
Mitochondria	7.4	405 (36)[b]	0.79 (0.03)	47 (7)	2.2 (0.9)	20
	7.4	241 (18)	0.78 (0.001)	49 (0.2)	3.9 (2.5)	37
	6[c]	217 (35)	0.92 (0.02)	18 (4)		20
	10	433 (37)	0.81 (0.01)	42 (2)	3.3 (1.6)	20
D-mitoplasts	7.4	402 (79)	0.68 (0.01)	71 (2)	2.4 (1.3)	20
	7.4	184 (72)	0.68 (0.01)	71 (2)	5.4 (4.0)	37
	6[c]	790 (221)	0.77 (0.02)	51 (4)		20
	10[c]	342 (7)	0.81 (0.01)	42 (2)		20
FP-mitoplasts	7.4	412 (73)	0.69 (0.01)	69 (2)	1.9 (0.2)	20
	6[c]	191 (9)	0.89 (0.01)	24 (2)		20
	10[c]	171 (4)	0.91 (0.005)	20 (1)		20
SMP from D-mitoplasts[d]	7.4	358 (15)	0.79 (0.02)	47 (4)	2.0 (0.1)	20
FP-SMP	7.4	547 (4)	0.74 (0.01)	58 (2)	2.1 (0.1)	20
	7.4	286 (42)	0.73 (0.01)	60 (2)	1.5 (0.5)	37
Proteoliposomes	7.4	246 (5)	0.55 (0.05)	100 (11)	0.6 (0.1)	20

[a] The percentage of mobile population of the translocator was calculated from Equation 5 using $[r_3/r_r(0)]_{min} = 0.55$.

[b] Numbers in brackets are standard deviation.

[c] Since the accuracy of the data in this case does not justify use of the full Equation 4 because of the shallow decay in $r_r(t)$, we employed the simplified equation $r_r(t) = [r_r(0) - r_3]\exp(-t/\phi) + r_3$.

[d] SMP were prepared by sonication from D-mitoplasts.

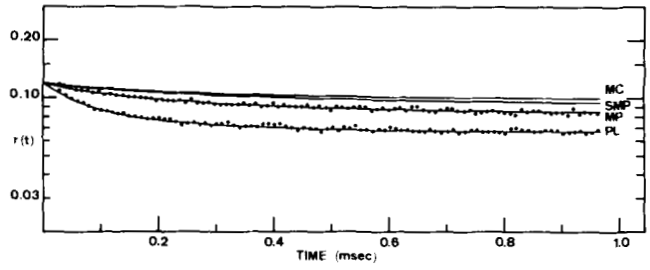

FIG. 3. **Time-dependent absorption anisotropy of the ADP/ATP translocator in mitochondria, mitoplasts, SMP, and proteoliposomes at 20 °C, pH 7.4.** Samples (8–10 μM in EMA, pH 7.4) were photolyzed by a vertically polarized laser flash at 532 nm and $r_r(t)$ was recorded at 520 nm. All measurements were performed in 80% (w/w) glycerol (~0.6 poise). Curves are: *MC*, mitochondria; *SMP*, FP-SMP; *MP*, D-mitoplasts; *PL*, proteoliposomes of L/P = 60. *Solid lines* were obtained by fitting the data to Equation 4. Data points of mitochondria and SMP are omitted for clarity. Curves other than SMP are slightly normalized to the same $r_r(0) = 0.12$ of SMP in order to facilitate a comparison. This is justified because although $r_r(0)$ depends on the intensity of the photoselecting flash, $r_r(t)/r_r(0)$ is independent of the flash intensity (29).

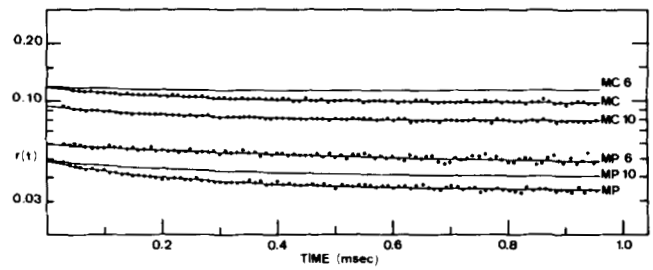

FIG. 4. **Effect of pH on the time-dependent absorption anisotropy of the ADP/ATP translocator in mitochondria and mitoplasts at 20 °C.** Experimental conditions are described in Fig. 3 but with varying pH. Curves are: *MC6*, mitochondria at pH 6; *MC*, control mitochondria at pH 7.4; *MC10*, mitochondria at pH 10; *MP6*, D-mitoplasts at pH 6; *MP10*, D-mitoplasts at pH 10; *MP*, control D-mitoplasts at pH 7.4. *Solid lines* were obtained by fitting the data to either Equation 4 (MC, MC10, and MP) or the simplified equation $r_r(t) = [r_r(0) - r_3]\exp(-t/\phi) + r_3$ (MC6, MP6, and MP10). This simplified equation is used, since the accuracy of the data of MC6, MP6, and MP10 does not justify use of the full Equation 4 because of the small decay in $r_r(t)$. CUrves other than MC6 and MC10 are vertically displaced for clarity.

37 °C decreased ϕ by about 50%, while the mobile fraction of the translocator remained unchanged.

Effect of pH (Fig. 4)—Incubation of membranes at pH 6 for 1 h at room temperature caused a significant immobilization of the translocator. For all membranes examined (*i.e.* mitochondria, D- and FP-mitoplasts), about 20–45% of the total translocator was immobilized by the pH 6 incubation. The alkaline treatment (pH 10, 1-h incubation at room temperature) immobilized 30–50% of the translocator in mitoplasts, without affecting the $r_r(t)$ curve in mitochondria. pH 6 and pH 10 incubations for 20 min at 0 °C were also carried out. These milder incubations had qualitatively the same immobilizing effect on the translocator, however, to a smaller extent.

Triplet Lifetimes of the Eosin Probe

The triplet lifetime of EMA bound to the translocator exhibited a double exponential decay (see Table II). Lifetimes were $\tau_1 \simeq 120$ μs (≃30%) and $\tau_2 \simeq 1300$ μs (≃70%) at 20 °C, pH 7.4, for all membranes examined (*i.e.* mitochondria, mitoplasts, SMP, and proteoliposomes), the numbers in brackets representing the exponential amplitude. Although protein-protein interactions and aggregated states of the translocator are largely different among these inner mitochondrial membranes and proteoliposomes, no significant differences were observed in lifetimes.

Although pH 6 and pH 10 treatment of inner mitochondrial membranes decreased significantly the rotational mobility of the translocator, again little effect was observed on the lifetime of EMA. On the other hand, significantly shorter life-

TABLE II

Triplet lifetimes of EMA bound to the ADP/ATP translocator in mitochondrial inner membranes and proteoliposomes

The total absorbance change $A(t)$ of Equation 2 was fitted to a double exponential decay having time constants τ_1 and τ_2. $\alpha_1(\%)$ and $\alpha_2(\%)$ represent the exponential amplitude of τ_1 and τ_2 components, respectively. Experimental conditions are described in Fig. 3 but with varying pH and temperature.

Membrane	pH	τ_1	α_1	τ_2	α_2	Temperature
		μs	%	μs	%	°C
Mitochondria	7.4	116 (4)[a]	32 (0.2)	1285 (21)	68 (0.2)	20
	7.4	96 (1)	47 (2)	810 (16)	53 (2)	37
	6	122 (2)	31 (0.4)	1368 (34)	69 (0.4)	20
	10	113 (6)	33 (1)	1171 (26)	67 (1)	20
D-mitoplasts	7.4	122 (6)	28 (1)	1280 (19)	72 (1)	20
	7.4	107 (5)	43 (1)	741 (15)	57 (1)	37
	6	120 (4)	30 (0.1)	1328 (4)	70 (0.1)	20
	10	119 (5)	30 (0.1)	1116 (7)	70 (0.1)	20
FP-mitoplasts	7.4	119 (5)	28 (1)	1332 (12)	72 (1)	20
	6	112 (1)	30 (0.2)	1356 (8)	70 (0.2)	20
	10	120 (1)	30 (0.4)	1407 (3)	70 (0.4)	20
SMP from D-mitoplasts[b]	7.4	120 (4)	31 (1)	1290 (19)	69 (1)	20
FP-SMP	7.4	103 (2)	31 (1)	1290 (11)	69 (1)	20
	7.4	103 (7)	44 (0.1)	872 (10)	56 (0.1)	37
Proteoliposomes	7.4	126 (5)	32 (0.1)	1338 (34)	68 (0.1)	20

[a] Numbers in brackets are standard deviation.
[b] SMP were prepared by sonication from D-mitoplasts.

times of $\tau_1 \simeq 100$ μs (≃45%) and $\tau_2 \simeq 740$–870 μs (≃55%) were observed at 37 °C, pH 7.4, for mitochondria, mitoplasts, and SMP. Thus, the triplet lifetimes of EMA bound to the translocator are dependent on temperature, however, independent of pH and differences of membranes where the translocator exists. Generally, the lifetime of an optical probe is a sensitive indicator of changes in environment of the probe (*e.g.* polarity, interactions with amino acid residues, etc.) (34). Therefore, the above results suggest that the immediate surroundings of EMA in the translocator are very similar to each other in mitochondria, mitoplasts, SMP, and proteoliposomes, although the mobility, protein-protein interactions, and aggregated states of the ADP/ATP translocator are largely different.

DISCUSSION

EMA Labeling of Mitochondrial Membranes—The observed selective binding of EMA to the ADP/ATP translocator in intact mitochondria could be explained by the fact that eosin is amphiphilic and has a high affinity to nucleotide binding sites (35), *e.g.* the nucleotide binding site of the translocator on the cytoplasmic surface of the inner mitochondrial membrane (1, 3). However, in addition to the ADP/ATP translocator, there are several other proteins (including the phosphate-carrier) labeled by EMA in the absence of NEM (Fig. 1). A complete inhibition of the ADP-stimulated respiration was achieved in rat heart mitochondria within 2 min of labeling at 15 μg of EMA/mg of protein (data not shown). It seems very probable that the inhibition is mainly due to blocking the phosphate transport by EMA, because a sulfhydryl group of the phosphate-carrier is reported to be highly reactive (36). Furthermore, only an ~15% decrease in [^{14}C] ADP uptake was observed at 15 μg of EMA/mg of protein without NEM pretreatment supporting that the observed complete inhibition of the ADP-stimulated respiration is mainly due to inactivation of the phosphate-carrier (data not shown).

The molar ratio of EMA/dimeric translocator is calculated to be less than or equal to 1. A good evidence for this may be given by steady state fluorescence anisotropy r^s measurements. r^s of EMA is particularly sensitive to the energy transfer between EMA molecules (37). If there are 2 EMA molecules bound to the same dimeric translocator, a significant decrease in r^s may be expected due to the energy transfer between the two EMAs.[3] However, no significant decrease in r^s was observed for the samples with EMA/dimeric translocator = 0.1–1.0.[4] This supports that only 1 EMA molecule binds to the dimeric translocator under the present experimental conditions.

Nucleotide Binding Site—Overlapping between the EMA and the CAT binding site on the cytoplasmic side of the translocator is observed. Although CAT completely inhibits both EMA and ADP binding to the translocator, EMA binding does not prevent CAT binding to the translocator. Nucleotide transport is decreased by EMA to some extent. Based on the above results, Fig. 5 illustrates a possible arrangement of binding sites for these 3 molecules on the translocator.

It has been shown that among the 8 sulfhydryl groups in the dimeric translocator only two are accessible to different fluorescent sulfhydryl reagents.[5] The distance between donor and acceptor probes bound to these two sulfhydryl groups were measured to be ~3 nm.[5] This value of ~3 nm suggests the size of the nucleotide binding site of the translocator, assuming that one of the fluorescently labeled sulfhydryl group is the same sulfhydryl group which is reactive with EMA in our study. However, it should be noted that after the first sulfhydryl group was occupied by a probe, several hours were needed for another probe to react with the second sulfhydryl group. This may be due to steric hindrance, supporting our result that only 1 sulfhydryl group per dimeric translocator reacts with EMA within a short incubation of 30 min at 0 °C.

EMA undergoes a very rapid wobbling motion around the binding site of the translocator with a subnanosecond time constant (21). Using time-resolved fluorescence polarization

[3] R_0 is calculated to be 50–60 Å for eosin-eosin energy transfer, where R_0 is the distance between the donor and an acceptor eosin at which the probability for resonance energy transfer is 50%.

[4] S. Kawato and M. Müller, unpublished results.

[5] W. Riezler and M. Klingenberg, personal communication.

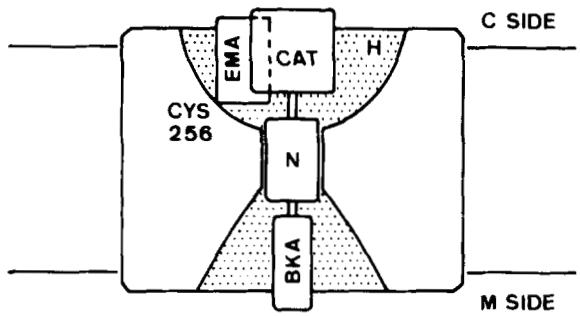

FIG. 5. **Schematic model of the ADP/ATP translocator.** The model illustrates a possible arrangement of the nucleotide binding site (*N*), bongkrekate, CAT, and EMA in a cross-section of the dimeric translocator. CAT and EMA bind to the translocator from the cytoplasmic side (*C*), whereas bongkrekate binds from the matrix side (*M*). After CNBr fragmentation of the translocator, cysteine 256 was identified as the preferred EMA-labeled sulfhydryl group.[6] This model is based on the results obtained from labeling experiments on intact mitochondria. (*H*, hydrophilic pocket of the translocator).

measurements, the time-dependent anisotropy $r_p(t)$ decays from the limiting value $r_0 = 0.38$ (38) to a time-independent anisotropy $r_p(\infty) \simeq 0.18$ within the time range of several tens of nanoseconds. Based on the "wobbling-in-cone" model (29, 39, 40), we can calculate the cone angle θ_c by

$$r_p(\infty)/0.38 = [\tfrac{1}{2} \cos \theta_c (1 + \cos \theta_c)]^2 \quad (6)$$

The value for $\theta_c \sim 40°$ indicates that the EMA wobbles relatively freely within a cone of half-angle of ~40°, suggesting that the nucleotide binding pocket is larger than the size of EMA.

In order to obtain good signal-to-noise ratio in protein rotation measurements, we used a flash excitation of sufficient intensity, which decreases $r_r(0)$ (29). An accurate initial anisotropy $r_r(0) = 0.16$ was therefore determined with a weak flash excitation (21). It is shown that a significant loss of anisotropy from the limiting anisotropy for EMA of $r_0 = 0.38$ to $r_r(0) = 0.16$ is mainly due to a very rapid probe wobbling of EMA around the binding site of the translocator. Since there is only a slight gap between $r_p(\infty) \simeq 0.18$ and $r_r(0) = 0.16$, a possible large segmental motion of the translocator can be excluded. Direct measurements using time-resolved phosphorescence anisotropy $r_s(t)$ with a 20-μs time range have shown that the decay of $r_s(t)$ is similar to $r_r(t)$ of the present study, also suggesting the absence of a microsecond segmental motion of the translocator around the EMA binding site.[7]

Protein-Protein Interactions of the Translocator—The mobile population of the translocator in the inner mitochondrial membrane is calculated on the basis of $[r_3/r_r(0)]_{min} = 0.55$ obtained for the translocator reconstituted in liposomes at L/P = 60, assuming that all the translocator molecules are rotating freely within the experimental time range. Since no immobile fraction is observed for cytochrome oxidase and cytochrome P-450 vesicles at L/P = 30 and for bacteriorhodopsin vesicles at L/P = 4 (16, 41, 42), it is reasonable to assume that all the translocator molecules are rotating at an even higher lipid to protein ratio (L/P = 60).

Stable preparations of the purified translocator are obtained by the addition of bongkrekate prior to the solubilization of mitochondria with Triton X-100, since the presence of bongkrekate does not inhibit NEM binding to the translocator (43). In analogy to this finding, it was possible to label with EMA the bongkrekate stabilized translocator. The presence of bongkrekate in intact labeled mitochondria does not affect significantly the $r_r(t)$ curve of the translocator within the experimental error, ruling out the possibility of a conformational change and/or protein-protein interactions of the translocator induced by bongkrekate. When the translocator was purified without any inhibitor, more than 70% of the translocator was immobile even in Triton X-100 solution in the presence of 60% (w/w) sucrose, probably because of microaggregation of the translocator during purification procedures. Furthermore, the translocator was completely immobile after reconstitution of this preparation into liposomes. This finding may be correlated with the observation that only less than 10% of the so purified translocator actively translocates [^{14}C]ADP in liposomes (8, 9).

Co-existence of mobile and immobile populations of the translocator is demonstrated in the inner membrane of mitochondria. Only ~47% of the translocator is rotating in intact mitochondria. Although ~70% of the translocator becomes mobile by removing the outer membrane, the mobile population is reduced to only ~47–58% in SMP. The mobilization of the translocator in mitoplasts is probably due to reducing the steric hindrance of the translocator rotation by removing the outer membrane which would be in tight contact with the inner membrane. The decrease in the rotational mobility of the translocator by going from mitoplasts to SMP may indicate a rigidification of the membrane by decreasing the vesicle size.

The existence of a significant amount of immobile molecules of the translocator within the time range of 1 ms is probably due to a very high concentration of membrane proteins in the inner membrane of mitochondria (L/P ≃ 0.4) (24). Kawato and co-workers (16, 17, 42) have demonstrated that the mobility of integral membrane proteins such as cytochrome oxidase and cytochrome P-450 strongly depends on L/P. The presence of immobile cytochrome oxidase in the mitochondrial inner membrane is shown to be due to nonspecific protein aggregates caused by a low L/P ≃ 0.4. These nonspecific protein aggregates have a low affinity and can be easily dissociated by increasing L/P with enriching exogenous phospholipids into the inner membrane, resulting in mobilization of cytochrome oxidase (17). When anti-cytochrome c_1 antibodies were added to mitoplasts, the immobile population of the translocator was progressively increased, depending on the amount of antibodies added.[8] This proposes the presence of mobile nonspecific microaggregates including both cytochrome bc_1 and the translocator, because it is unlikely that the translocator forms a specific complex with cytochrome bc_1.

Effect of acidic and alkaline treatments on protein-protein interactions of the translocator was also examined. Both pH 6 and pH 10 treatments immobilize the translocator. Since a significant deviation from the neutral pH may be induced locally on the inner membrane of mitochondria during oxidative phosphorylation caused by proton translocation, a concomitant local immobilization of the translocator and other proteins, which should be reversible, might be expected.

CONCLUSIONS

Successful selective labeling and rotational diffusion of the ADP/ATP translocator are demonstrated in the inner membrane of mitochondria and proteoliposomes.

The EMA binding to the translocator does not efficiently inhibit the ADP translocation activity. CAT blocks com-

[6] M. Müller and S. Kawato, unpublished results.

[7] S. Kawato and E. Matayoshi, unpublished results.

[8] S. Kawato, A. Ohashi, and M. Müller, unpublished results.

pletely the ADP translocation even in the presence of EMA.

Co-existence of mobile and immobile populations of the translocator is demonstrated in the inner mitochondrial membrane. Calculated mobile population of the translocator strongly depends on the type of the membrane.

This approach of the rotation measurements can be applied to examine functional protein-protein interactions of the ADP/ATP translocator with other protein components of the oxidative phosphorylation (*e.g.* ATP-synthase, creatine phosphokinase, and phosphate-carrier).

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