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Membrane Permeability Transition Promoted by Phosphate Enhances 1-Anilino-8-Naphthalene Sulfonate Fluorescence in Calcium-Loaded Liver Mitochondria

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Phosphate and a number of other compounds induce membrane permeability transition (MBT) in Ca^{2+}-loaded mitochondria. 1-Anilino-8-naphthalene sulfonate (ANS) was used as a fluorescent probe to investigate perturbations on the inner membrane during MBT. Induction of MBT caused ANS fluorescence enhancement with a biphasic rate that reached a plateau. The enhancement is analogous to that reported for de-energization of mitochondria. The fluorescence level was independent of whether ANS was added before or at different times after phosphate. In the absence of ANS, fluorescence was low and remained unchanged. The initial time course of MBT, as followed by large-amplitude swelling, was similar to that of fluorescence enhancement. Ruthenium red, EGTA, ADP, and cyclosporin A inhibited the enhancement. Only EGTA + ADP (or ATP) reversed the enhancement when added after phosphate. Efflux of matrix Ca^{2+} by sodium acetate or A23187 did not alter ANS fluorescence. The binding parameters (K_d and number of binding sites) were not significantly different, but the fluorescence maximum was more than doubled after MBT. Although the fluorescence of bound ANS showed a nonlinear relationship, it was always higher (73.0 ± 19.0%) after reaching the plateau. Since ANS binding to membranes is nonspecific, the exact mechanism of the enhanced fluorescence is not apparent. The dependence of the initial rate of fluorescence enhancement on Ca^{2+} concentration was nonlinear, with 45 μM at half-maximal rate. The dependence on phosphate was hyperbolic with 0.7 mM at half-maximal rate, which is close to the K_m value of phosphate carrier. The kinetics is compatible with Ca^{2+} binding to some membrane component(s) during MBT and cause ANS fluorescence enhancement. It is suggested that the bilayer–nonbilayer (hexagonal$_{II}$) transition consequent to Ca^{2+} binding to protein-phospholipid domains containing cardiolipin may play a role in fluorescence enhancement and MBT.

KEY WORDS: Membrane permeability transition; liver mitochondria; 1-anilino-8-naphthalene sulfonate; fluorescence; mitochondrial swelling; calcium efflux.

INTRODUCTION

Accumulation of Ca^{2+} by liver mitochondria in the presence of phosphate has been known for some time to decrease oxidative phosphorylation (Hunter and Ford, 1955) and result in ultrastructural changes of the organelle (Hackenbrock and Caplan, 1969). Detailed studies by Hunter and Haworth (1979*a,b*; Haworth and Hunter, 1979) further showed that Ca^{2+} triggered an increase of nonspecific permeability of the inner membrane which manifests as large-amplitude swelling and efflux of matrix Ca^{2+}. Since permeabilization response was transient and reversible, it was interpreted as a gating effect (or pore opening) that would allow movement of neutral and charged molecules (< 1500 molecular weight) across

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the inner membrane. Kinetically, swelling response to Ca^{2+} was sigmoidal and the process was described as Ca^{2+}-induced membrane transition. Since then, a number of findings have aroused interest in the mitochondrial membrane permeability transition (Gunter and Pfeiffer, 1990). A large diverse group of compounds (SH reagents, metabolites, pharmacological and toxicological agents) can replace phosphate to promote the permeability transition (Gunter and Pfeiffer, 1990). Cellular damage caused by many of the toxicological agents has been ascribed to the induction of the transition and the oxidation of GSH (Reed, 1991; Rapuano and Maddaiah, 1988). Recently, the immunosuppressant drug cyclosporin A is found to be a potent inhibitor of the permeability transition (Halestrap and Davidson, 1990; Gunter and Pfeiffer, 1990). The channel is found to be identical with 1.35 ns multi-conductance mitochondrial megachannel (Szabo and Zoratti, 1992).

Several mechanisms have been proposed to explain the Ca^{2+}-triggered membrane permeability transition. One of the earliest is the gating effect to open up nonspecific hydrophilic pores (Hunter and Haworth, 1979*a,b*). Another is the induction of phase defects by lysophospholipids (Broekemeier *et al.*, 1991). Yet another is the opening of a nonspecific pore by an interaction of cyclophilin with ADP/ATP carrier (Halestrap and Davidson, 1990) or another component regulated by Ca^{2+} and adenine nucleotides (McGuinness *et al.*, 1990). In spite of many studies, the nature of membrane perturbation that may occur during the permeability transition and its relationship to Ca^{2+} is not known. Useful information about membrane perturbation that occurs during mitochondrial energization has been obtained by using fluorescent probes (Azzi *et al.*, 1969; Jassaitis *et al.*, 1971; Radda and Vanderkooi, 1972; Maddaiah *et al.*, 1981). Because of large fluorescence response to electrostatic and/or hydrophobic changes, 1-anilino-8-naphthalene sulfonate (ANS) is one of the widely used membrane probes (Slavik, 1982). It is now well known that energization of whole mitochondria results in quenching of ANS fluorescence. Although binding of the probe to mitochondria is of a complex nature, elaborate studies of Robertson and Rottenberg (1983) have suggested that quenching is more related to transmembrane potential generated by energization than to changes in membrane surface charge or potential. It is shown here that induction of permeability transition in Ca^{2+}-loaded mitochondria by phosphate resulted in an enhancement of ANS fluorescence with little or no change of binding parameters. Dependence of fluorescence enhancement on Ca^{2+} concentration was nonlinear whereas on phosphate it was rectangular hyperbolic. A similar fluorescence enhancement occurred when Ca^{2+} was added to vesicles of mitochondrial lipids.

MATERIALS AND METHODS

Liver mitochondria from male Sprague Dawley rats (250–300 g body weight) maintained on Purina laboratory chow were isolated as described (Maddaiah *et al.*, 1981; Rapuano and Maddaiah, 1988) in medium A that contained 0.22 M mannitol, 0.07 M sucrose, 0.2 mM EGTA, 0.5% bovine serum albumin, and 0.05 M Hepes (pH 7.4), washed and suspended in medium A without EGTA and albumin to obtain a protein concentration in the range of 90–100 mg/ml.

Lipids were extracted from freshly prepared mitochondria by the method of Folch *et al.* (1957) as previously described (Clejan *et al.*, 1980; Clejan and Maddaiah, 1986). Vesicles were prepared in 0.12 M KCl and 0.05 M Hepes (pH 7.4) by vortexing followed by sonication (Clejan *et al.*, 1981).

Fluorescence intensity measurements were made in a Hitachi F-2000 fluorescence spectrophotometer equipped with a thermostated cell holder, magnetic stirrer, and a window on top of the cell compartment suitable to add or take out aliquots of the reaction mixture without interrupting measurements. Excitation and emission wavelengths were 380 and 480 nm, respectively. Reactions were carried out in cuvettes at 25°C in a total volume of 3 ml that contained 0.12 M KCl, 0.05 M Hepes (pH 7.4), 4 μM rotenone, 5 mM potassium succinate, and other additions as required. Stock solutions of ANS, $CaCl_2$, potassium succinate, and potassium phosphate were adjusted to pH 7.4. Ammonium salt of ANS was recrystallized 2–3 times to obtain a molar extinction value of 6×10^3 (Harris, 1971) at 360 nm. Routinely, concentration of free ANS in reaction mixtures was estimated fluorometrically by the method of Robertson and Rottenberg (1983) as described below.

In studies of ANS binding to mitochondria before and after phosphate-promoted transition, 1 ml of the reaction mixture was taken out after completion of Ca^{2+} uptake but before phosphate was added and also after fluorescence intensity plateaued (Fig. 1). The samples were centrifuged for

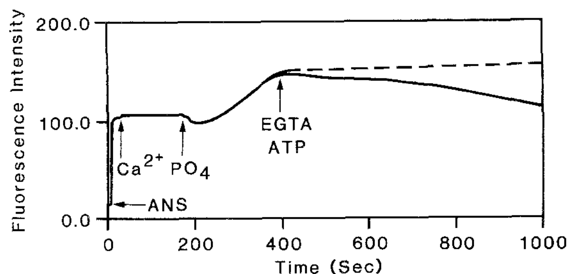

Fig. 1. Real-time measurement of fluorescence intensity (arbitrary units) after adding ANS (25 μM), Ca^{2+} (60 μM), and phosphate (2 mM) to mitochondria (1 mg/ml protein) in a medium containing 0.12 M KCl, 0.05 M Hepes (pH 7.4), 5 mM potassium succinate, and 4 μM rotenone. Additions were made at times indicated by arrows. Broken line—no further additions; solid line—2 mM EGTA followed by 1 mM ATP.

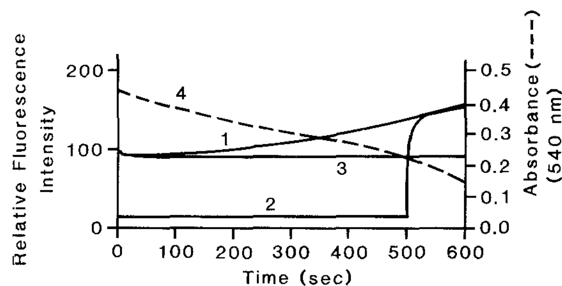

Fig. 2. Time course of the effect of adding phosphate (2 mM) to Ca^{2+}-loaded mitochondria on large-amplitude swelling (curve 4, absorbance at 540 nm) and fluorescence intensity (curves 1–3). Details are described under Fig. 1 Curve 1, phosphate was added at 0 time. Curve 2, ANS was added 500 sec after phosphate. Curve 3, reaction medium contained 3 μM ruthenium red or 2 mM EGTA or 50 nM cyclosporin A or 1 μM A23187 which was added instead of phosphate.

5 min in a Beckman microfuge. An aliquot (1–5 μl) of the supernatant was made up to 1 ml with 1% Triton X-100 + 10% NaCl and mixed by vortexing. Fluorescence intensity was measured with excitation and emission wavelengths set at 380 and 480 nm, respectively. ANS standards and appropriate blanks in 1% Triton X-100 + 1% NaCl were set up during each determination. The standard curve was linear from 0.1 to 5 μM ANS. The concentration of the samples was adjusted by dilution so that emission intensities were in the range of the standards.

Large-amplitude swelling of mitochondria was measured by the decrease in absorbance at 540 nm in an Aminco DW-2 recording spectrophotometer. Calcium uptake and release was measured using antipyralazo III as described (Rapuano and Maddaiah, 1988). All chemicals and biochemicals were from Sigma Chemical Co. (St. Louis, Missouri). Cyclosporin A was generously provided by Dr. D. L. Winter of Sandoz Pharmaceutical Co. (East Hanover, New Jersey).

RESULTS

Effect of Phosphate and Other Modifiers of Permeability Transition on ANS Fluorescence

Fluorescence intensity changes with time after adding ANS (25 μM) followed by Ca^{2+} (60 μM) and phosphate (2 mM) to succinate-energized mitochondria (1 mg protein per ml) are shown in Fig. 1. Phosphate or any other promoter of permeability transition was added 2 min after Ca^{2+} by which time uptake of added Ca^{2+} was complete (Rapuano and Maddaiah, 1988). Fluorescence of mitochondria was small but was increased upon the addition of ANS. Regardless of concentration, addition of Ca^{2+} elicited only a slight further increase in fluorescence. On the other hand, addition of phosphate increased the fluorescence with an initial slow rate followed by a faster raise to reach a plateau (broken line, Fig. 1). A similar increase of fluorescence was observed with other promoters of permeability transition (not shown) such as *t*-butylhydroperoxide (0.5 mM), acetoacetate (2 mM), or atractyloside (1 mM) + phosphate (0.1 mM). Intrinsic fluorescence of mitrochondria was small and remained unchanged when Ca^{2+} and phosphate were added in the absence of ANS (discussed later). Addition of EGTA or Ca^{2+} uptake inhibitor, ruthenium red, before Ca^{2+} did not elicit fluorescence enhancement. In separate experiments, the time course of uptake and release of Ca^{2+} was followed spectrophotometrically (Rapuano and Maddaiah, 1988). Release of 90% of the added Ca^{2+} was almost complete by the time fluorescence enhancement reached the plateau level (not shown).

Large-amplitude swelling is considered to be a quantitative measure of mitochondrial membrane permeability transition. The temporal relationship of ANS fluorescence increase (curve 1) with swelling (curve 4) after the addition of phosphate is shown in Fig. 2 (the sequence of addition is as shown in Fig. 1). Presence of ANS did not have any effect on the rate of swelling. After a short delay, the fluorescence increase closely followed the time course of swelling, which continued even after the fluorescence plateaued, probably because solute and water movements have not yet reached equilibrium. In order to further establish

the relationship between membrane permeability transition and fluorescence enhancement, known inhibitors of the transition were used. Addition of cyclosporin A (50 nM) or ADP (5 mM) or EGTA (3 mM) or ATP (1 mM) before phosphate inhibited fluorescence enhancement completely. Release of Ca^{2+} without swelling or permeability transition has also been observed. For example Na^{+}-dependent Ca^{2+} release has been demonstrated in liver mitochondria (Wingrove and Gunter, 1986). Addition of 20 mM sodium acetate instead of phosphate initiated slow Ca^{2+} release but did not alter ANS fluorescence. Similarly, replacement of phosphate by the ionophore A23187 also did not cause fluorescence increase.

To evaluate if addition of the probe after initiation of permeability transition had any effect on the time course of fluorescence enhancement or plateau level, ANS was added at different times after phosphate. When added at the time the fluorescence intensity would have plateaued, as determined in a separate experiment, the same fluorescence level was attained without any time lag, as shown in Fig. 2 (curve 2). Similarly, if ANS was added at different times after phosphate, the fluorescence reached the same level without a time lag and followed the rest of the time course similar to that of ANS addition before Ca^{2+} and phosphate (not shown). The data in Fig. 2 (curve 2) also shows that the fluorescence intensity of mitochondria without ANS is small and remained constant (< 10%) throughout the permeabilization process. Therefore, light scattering consequent to large-amplitude swelling during the permeabilization process does not contribute to the observed fluorescence intensity changes of ANS.

In the inhibitor studies, it was interesting to find that when cyclosporin A or EGTA was added at different times after phosphate only further enhancement of fluorescence was inhibited but was not reversed. However, addition of ADP or ATP either in the middle or at the end of the process caused the fluorescence enhancement to reverse. Addition of EGTA (2 mM) followed by ATP (1 mM) was most effective in reversing the fluorescence increase (solid line, Fig. 1). The kinetics of reversal of fluorescence increase was biphasic with an initial fast drop followed by a slow decrease.

Binding of ANS to Mitochondria before and after Fluorescence Enhancement

Whether fluorescence enhancement resulted from increased affinity and/or number of ANS binding sites on mitochondria was evaluated by determining the parameters of binding equilibria before and after phosphate was added to Ca^{2+}-loaded mitochondria. As discussed by Robertson and Rottenberg (1983), in earlier binding studies, a direct relationship between added ANS and fluorescence intensity was assumed while deriving binding parameters. The assumption may not be entirely correct since not all added ANS will be bound to mitochondria and not all bound ANS contributes to fluorescence equally. Therefore, in the present studies, free ANS was quantitatively determined as described under Methods and Materials. Bound ANS was obtained as the difference between added and free ANS in the supernatant after centrifugation of the mitochondrial suspension. In order to evaluate the effects of membrane permeability transition on binding to mitochondria, free ANS was determined in aliquots of the reaction mixture taken out before adding phosphate and after fluorescence intensity had plateaued following the sequence of reagent additions as shown in Fig. 1. In these experiments, the concentration of ANS was varied keeping mitochondrial protein (1 mg/ml), Ca^{2+} (60 μM), and phosphate (2 mM) constant.

Increasing the concentration of ANS did not change the time course but only increased the fluor-

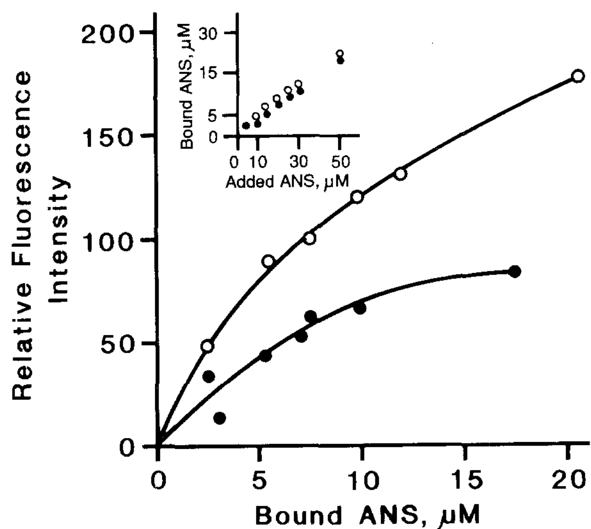

Fig. 3. Fluorescence and binding of ANS to Ca^{2+}-loaded mitochondria (60 μM) before adding phosphate (2 mM) (●) and after phosphate-induced fluorescence had plateaued as shown in Fig. 1 (○). Varying concentrations of ANS (5–50 μM) were added at constant 1 mg/ml of mitochondrial protein. The medium is as described under Fig. 1. Bound ANS was calculated from free ANS and total ANS (added) as described under Materials and Methods. The inset is a plot of bound ANS versus added ANS.

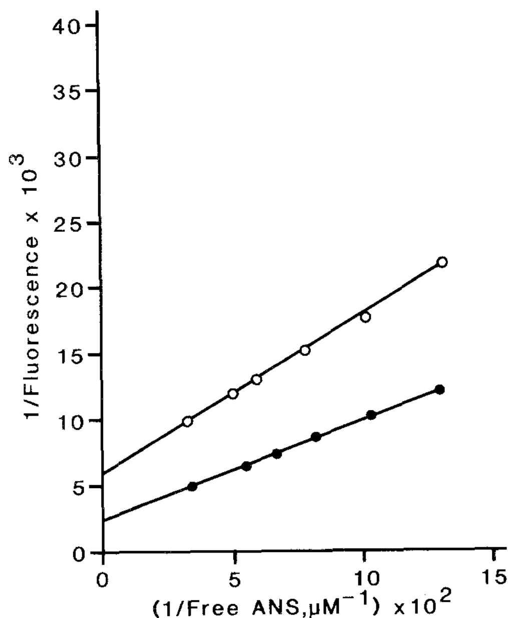

Fig. 4. Fluorescence intensity as a function of free ANS concentration before adding phosphate (2 mM) to Ca^{2+}-loaded mitochondria (1 mg protein/ml) (○) and after phosphate-induced fluorescence had plateaued as shown in Fig. 1 (●). Details are as described under Fig. 3.

escence intensity. The relationship between bound ANS and fluorescence intensity before phosphate was added and after fluorescence plateaued is shown in Fig. 3. As can be seen, the fluorescence intensity did not bear a linear relationship with bound ANS but showed a saturation trend. A similar relationship has been reported for ANS binding to unenergized mitochondria (Robertson and Rottenberg, 1983). More importantly, the fluorescence intensity of bound ANS after reaching the plateau level was always higher (73.0 ± 19.0%) than that before the addition of phosphate at the concentrations of ANS used. In contrast, bound ANS showed a fairly linear relationship to added (total) ANS, and the increase of bound probe after reaching the plateau level was small (13.0 ± 9.0%).

Double reciprocal plots of fluorescence intensity versus free ANS concentration measured at varying concentrations of added ANS is shown in Fig. 4.

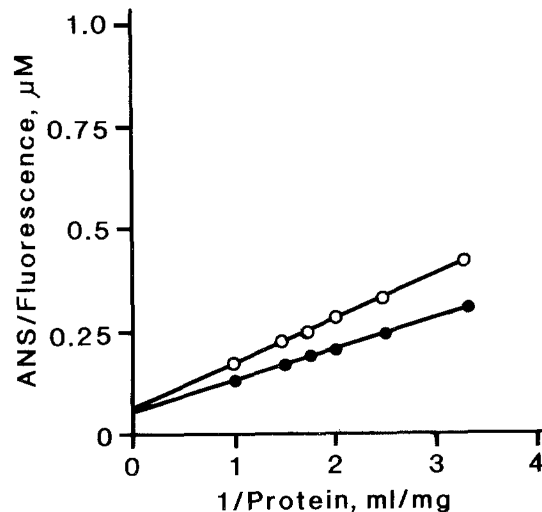

Fig. 5. Fluorescence intensity as a function of mitochondrial protein concentration (mg/ml) at a fixed concentration of ANS (10 μM) before adding phosphate (2 mM) (○) and after phosphate-induced fluorescence had plateaued (●) as shown in Fig. 1. Details are as described under Fig. 3.

Numerical values for the dissociation constant of ANS binding to mitochondria (K_d) and maximum fluorescence intensity (F_m) before and after adding phosphate were derived from the equation

$$\frac{1}{F} = \frac{1}{F_m} + \frac{K_d}{F_m} \times \frac{1}{[\mathrm{ANS}]} \tag{1}$$

and are shown in Table I. Here F is the fluorescence intensity, F_m is the maximum fluorescence intensity, K_d is the dissociation constant, and [ANS] is the free ANS concentration.

The number of ANS binding sites n on mitochondria was determined by following the formalism developed by Wang and Edelman (1971) according to the relationship

$$\frac{[\mathrm{ANS}]}{F} = \frac{1}{Q} + \frac{K_d}{Q_n} \times \frac{1}{P} \tag{2}$$

Here Q is the constant related to quantum efficiency, n is the number of binding sites, and P is the mitochondrial protein, mg/ml. In these experiments, mitochondrial protein (mg/ml) was varied but ANS (10 μM), Ca^{2+} (60 μM), and phosphate (2.0 mM) were held constant.

Table I. Parameters of ANS Binding to Energized Mitochondria Loaded with 60 μM Calcium

	Before addition of phosphate (2 mM)	After fluorescence plateaued
Dissociation constant K_d (μM)	10.0	11.6
Fluorescence maximum F_m at 1 mg/ml of protein	170	365
Number of binding sites n (nmol/mg protein)	11	15

Plots of [ANS]/F versus the reciprocal of mitochondrial protein concentration are shown in Fig. 5. The value of Q was obtained from the intercept, and the value of n was calculated from the slope using the corresponding numerical value of K_d generated from Fig. 4. The numerical values of binding parameters (Table I) are smaller than those reported by Williams *et al.* (1977) but closer to the estimates of Roberston and Rottenberg (1983). The latter investigators have attributed the earlier high estimates partly to contamination of the probe with bis-ANS. In the present study, ANS was recrystallized at least twice in water and free ANS was quantitatively determined. It is important to note that K_d and n increased only slightly after phospate was added and fluorescence intensity plateaued.

Relationship between ANS Fluorescence and Ca^{2+} and Phosphate Concentrations

All of the known promoters of membrane permeability transition require accumulation of Ca^{2+} in mitochondria (Gunter and Pfeiffer, 1990) and the relationship has already been shown to be sigmoidal (Haworth and Hunter, 1979). The only exception is the bifunctional SH reagent phenylarsine oxide which can induce permeability transition in the presence of EGTA (Bernardi *et al.*, 1992). To elucidate the relationship between fluorescence enhancement and Ca^{2+} and phosphate, the effects of varying concentrations of these agents on the kinetics of fluorescence enhancement were investigated. When the concentration of Ca^{2+} was increased, keeping the concentrations of ANS, mitochondria, and phosphate constant, neither the initial nor the plateau levels of fluorescence were affected, but the time course to reach the plateau level was shortened. Similarly, increasing the concentration of only phosphate shortened the time course without affecting fluorescence intensity. Therefore, fluorescence data were analyzed (1) by obtaining the initial rate of fluorescence increase (increase per minute) during the initial 5 min after the addition of phosphate and (2) by deriving apparent first-order rate constants from the slopes of plots of log fluorescence intensity versus time. Semilog plots of the fluorescence versus time were linear with a correlation coefficient of at least 0.98. A plot of the initial rate versus Ca^{2+} concentration while holding mitochondria (1 mg/ml), ANS (25 μM), and phosphate (2 mM) constant is shown in Fig. 6. The relationship was nonlinear and the Ca^{2+} concentration at half-maximal rate was 45 μM. A significant cooperative effect is apparent after about 10 μM Ca^{2+}. A similar nonlinear relationship was also found in the plot of apparent first-order rate constant versus Ca^{2+} (not shown). The plot of phosphate concentration versus initial rate, while keeping the concentration of mitochondria (1 mg/ml), ANS

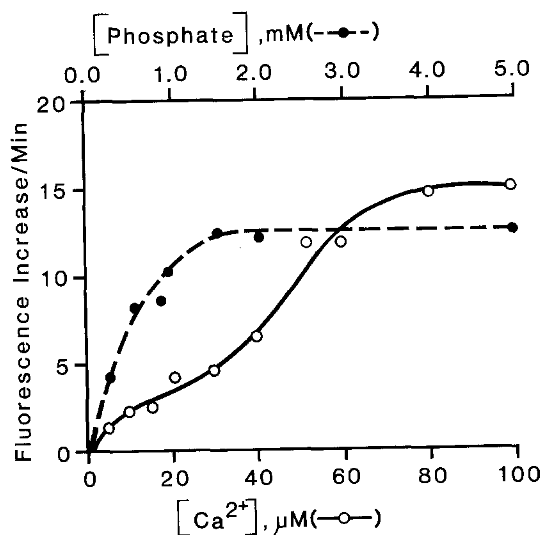

Fig. 6. Effect of varying concentrations of Ca^{2+} and phosphate on the initial rate of fluorescence increase during the initial 5 min after adding phosphate at zero time. The medium is as described under Fig. 1 (25 μM ANS) ○, varying Ca^{2+} concentration (5–100 μM) at fixed 2 mM phosphate; ●, varying phosphate concentration (0.5–5.0 mM) at fixed 60 μM Ca^{2+}.

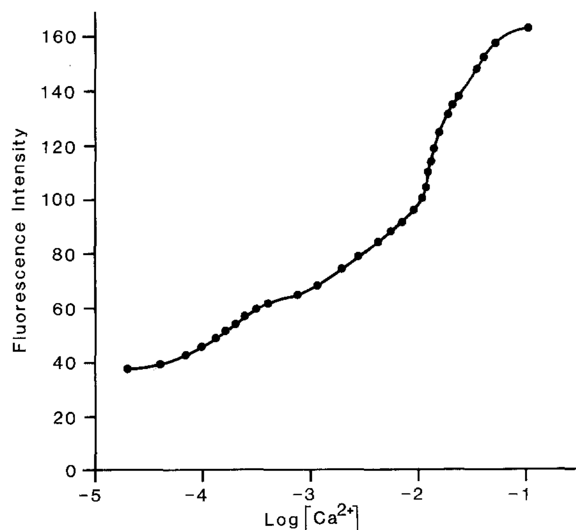

Fig. 7. Effect of varying concentrations of Ca^{2+} on fluorescence intensity of ANS (25 μM) in vesicles of lipids extracted from liver mitochondria. The lipid concentration is equivalent to 1 mg mitochondrial protein per ml.

(25 μM), and Ca^{2+} (60 μM) constant, is shown in Fig. 6. In contrast to the nonlinear effect of Ca^{2+}, the initial rate (Fig. 6) or apparent first-order rate constant (not shown) showed a rectangular hyperbolic relationship with phosphate concentration. Phosphate concentration at half-maximal rate was 0.7 mM.

Ca^{2+} and other bivalent metal ions are known to increase ANS binding particularly to anionic phospholipids (Haynes, 1974). Since ANS binding sites on mitochondria constitute protein as well as phospholipid domains, effects of Ca^{2+} on ANS fluorescence with phospholipids extracted from mitochondria were investigated. As can be seen in Fig. 7, Ca^{2+} increased ANS fluorescence intensity and the dependence was nonlinear, as has been reported for phospholipid mixtures (Haynes, 1974).

DISCUSSION

ANS is a permeable anion and its fluorescence when added to mitochondria is considered to be mostly due to binding to lipids and/or proteins of the inner membrane. It is generally thought that a large part of the aromatic ring of ANS lies in the hydrophobic domain, probably between phospholipid head groups, while the anionic sulfonate is exposed to the aqueous membrane interface (Slavik, 1982; Kumbar and Maddaiah, 1977). Binding of ANS to mitochondria is considered to be heterogeneous with at least two different affinity sites (Robertson and Rottenberg, 1983). Because of the nonspecific nature of the probe, interpretation of changes in binding parameters is equivocal. However, in the present studies, no significant changes in binding parameters were found. Binding to mitochondria may depend to a large extent on surface charge. To eliminate the surface-charge effect as a contributing factor, the present experiments were carried out in 0.12 M KCl. However, similar fluorescence enhancement was also observed in sucrose-mannitol medium (our unpublished results). Since ANS, at concentrations larger than 100 μM, was found to inhibit mitochondrial respiration (Williams *et al.*, 1977), the probe concentration was kept no greater than 50 μM.

The membrane permeability transition induced by phosphate and other promoters in Ca^{2+}-loaded mitochondria is associated with a drop in membrane potential, oxidation of matrix NAD(P)H and GSH, pyridine nucleotide hydrolysis, and efflux of Ca^{2+} and other solutes (Gunter and Pfeiffer, 1990). The permeability transition and the membrane potential decline are strongly indicative of changes of inner membrane function. Depletion of matrix NAD(P)H and GSH can affect membrane structure and function consequent to oxidation and cross linking of SH groups of membrane proteins (Fajian *et al.*, 1990). Although a cause and effect relationship of these changes is not known, significant membrane structural perturbations are indicated.

The important observation in this study is that enhancement of ANS fluorescence is induced by phosphate and other promoters of the transition and is also inhibited by cyclosporin A and ADP (or ATP) that are specific to permeability transition. Therefore, fluorescence enhancement should be closely associated with permeability transition of the inner membrane. As presented earlier, ANS was once widely used as a fluorescent probe of mitochondrial energization (Azzi *et al.*, 1969; Jassaitis *et al.*, 1971; Radda and Vanderkooi, 1972; Maddaiah *et al.*, 1981). In whole mitochondria, energization quenches fluorescence while in sonicated mitochondrial particles it enhances the fluorescence. Explanations such as generation of membrane potential (Jassaitis *et al.*, 1971), increase of net negative charge on the membrane surface (Azzi *et al.*, 1969), and lowering of the quantum yield in the new membrane state or conformation (Radda and Vanderkooi, 1972) have been offered as causes of fluorescence quenching in whole mitochondria. Recent elegant studies of Robertson and Rottenberg (1983) have suggested that changes in surface charge are a less likely cause of quenching because salt response of the unquenched fluorescence in energized mitochondria was identical to that of nonenergized system. Energization was further shown to decrease ANS binding affinity without affecting the number of binding sites on mitochondria.

Therefore energization-dependent generation of membrane potential inducing an extrusion of inner membrane-bound ANS is considered more likely. The above discussion makes it apparent that the association of membrane potential drop and fluorescence increase with deenergization of mitochondria is analogous to the changes presently observed with membrane permeability transition. However, there are two important differences. Firstly, during the Ca^{2+}-triggered process, the membrane potential drop is largely due to the increase of nonspecific membrane permeability, whereas in deenergization it occurs

because of specific proton leak by uncouplers or protonophores.

Secondly, higher fluorescence in the de-energized state is largely due to higher ANS binding affinity, whereas in permeability transition neither binding affinity nor number of sites significantly change (Table I). Absence of changes of binding parameters is also supported by the observation that the time course as well as the plateau level of fluorescence when ANS was added at different times after induction of the permeabilization process was equivalent to that which would have been reached if ANS was added before Ca^{2+} and phosphate. If affinity and/or number of ANS binding sites were altered by the permeability transition process, such equivalence would have been highly unlikely. Since fluorescence of bound ANS was always higher after the Ca^{2+}-triggered permeabilization, fluorescence enhancement may be a reflection of higher quantum yield due to increased hydrophobicity of pre-existing ANS binding sites. Fluorescence enhancement by energy transfer from protein tryptophan and tyrosine residues should also be considered. Bivalent cations are known to neutralize surface charges and increase fluorescence intensity of ANS in phospholipids and membranes (Slavik, 1982). Addition of Ca^{2+} (up to $100\,\mu M$) to either energized or unenergized mitochondria caused a negligible fluorescence increase, suggesting that either the cation concentration is too low or membrane surface charges are inaccessible. Fluorescence enhancement occurred only with the induction of large-amplitude swelling and Ca^{2+} release. Efflux of Ca^{2+} by either A23187 or sodium acetate (Na^+-dependent Ca^{2+} release) which is not associated with swelling did not result in fluorescence enhancement.

Therefore, fluorescence enhancement is specifically associated with the process of membrane permeability transition rather than Ca^{2+} efflux per se. Its dependence on added Ca^{2+} concentration was nonlinear. Although the kinetics of Ca^{2+} uptake into mitochondrial matrix is itself a sigmoidal process (Affolter and Carafoli, 1981; Rapuano and Maddaiah, unpublished results), under the present experimental conditions the uptake is completed without any change in fluorescence before phosphate is added to initiate the permeabilization process. Therefore, the kinetics describes the relationship between accumulated matrix Ca^{2+} and fluorescence. It is suggestive of a cooperative binding of Ca^{2+} to some membrane component(s) during permeability transition. In fact, analysis of the data of Fig. 6 and similar kinetic data at different ANS concentrations by Hill plots gave an interaction factor of 1.4–1.5. An interaction factor of 1.85 has been reported for the dependence of swelling measured by light scattering on added Ca^{2+}, and the concentration at half-maximal rate was similar to the present value (Haworth and Hunter, 1979). The kinetic similarities suggest that the sites of ANS binding and permeability pore may exist in close proximity. On the other hand, the kinetic relationship between phosphate and fluorescence is rectangular hyperbolic. Phosphate may bind to its carrier because the concentration of 0.7 mM at half-maximal rate is close to the K_m value of the phosphate carrier (Coty and Predersen, 1974). The kinetics of Ca^{2+} is compatible with Ca^{2+} binding to activate an enzyme such as phospholipase A_2 to increase the lysophospholipid level (Broekemeier *et al.*, 1991). The binding component of the membrane could also be ADP/ATP translocator (Halestrap and Davidson, 1990) or other cyclophilin-interacting component (McGuinness *et al.*, 1990).

Requirement of adenine nucleotides for the reversal of fluorescence enhancement would favor the involvement of the nucleotide translocator. The kinetics is also compatible with Ca^{2+} binding to anionic sites of membrane phospholipids, as can be seen in Fig. 7. The possibility of Ca^{2+} binding to cardiolipin-rich domains of the inner membrane is of special significance. First, protein–cardiolipin–phosphatidylethanolamine is considered to be the active ADP/ATP carrier (Hoch, 1992). Second, charge neutralization of cardiolipin by Ca^{2+} or H^+ or cytochrome *C* is known to induce a bilayer–nonbilayer (hexagonal$_{11}$) transition in phosphatidylethanolamine–phosphatidylcholine–cardiolipin mixtures. Such changes in lipid structural morphology have been suggested to promote transmembrane proton flow and aqueous channel formation (Hoch, 1992). Occurrence of lipid phase transitions in mitochondrial processes have yet to be demonstrated, and exploration with nuclear magnetic resonance techniques should be worthwhile. It is, however, tempting to suggest that Ca^{2+} binding to cardiolipin-rich domains of the inner membrane such as ADP/ATP translocator may play a role in membrane permeability transition. The ensuing lipid phase transition would be able to explain, in a general way, the increase of nonspecific membrane permeability as well as the increase of quantum yield of bound

ANS. The findings of low swelling response (Rapuano and Maddaiah, 1988) and low fluorescence enhancement (Maddaiah, unpublished results), and 26% lower cardiolipin content of phospholipids (Clejan and Maddaiah, 1986) in hypophysectomized rat liver mitochondria compared to normal controls offer some support to the hypothesis.

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