OSCILLATIONS OF 8-ANILINONAPHTHALENE-1-SULFONIC ACID FLUORESCENCE IN MITOCHONDRIA

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SUMMARY

Simultaneous measurements of ANS fluorescence and 90° light scattering have been made during energized oscillations of mitochondrial volume. The time course of the measurements show that membrane conformational changes, as indicated by ANS fluorescence, precede the utilization of intermediates of energy conservation as indicated by configurational changes associated with ion transport. Where functional changes do not involve energy coupling both fluorescence and configurational changes occur together within a period of less than one second.

8-Anilinonaphthalene-1-sulfonic acid (ANS) is a dye which exhibits strong fluorescence in a hydrophobic environment but loses its fluorescence intensity as the solvent polarity is increased (Weber and Lawrence, 1954; Stryer, 1965; McClure and Edelman, 1966). This property makes the compound extremely sensitive to conformational* changes when bound to non-polar regions of proteins (Alexander and Edelman, 1965; McClure and Edelman, 1967; Deluca, 1969) thus providing a valuable technique for studies on protein-protein and protein-lipid interactions in biological membranes (Tasaki, Watanabe, Sandlin and Carnay, 1968; Azzi, Chance, Radda and Lee, 1969). Azzi et al. (1969) have demonstrated that changes in the fluorescence of bound ANS in fragmented mitochondrial membranes precede reverse electron flow but lag behind changes

* The term "conformation" is restricted to secondary tertiary and quarternary protein structure. The term "configuration" is used to describe ultrastructural appearance as demonstrated by electron microscopy.

in oxidation-reduction state of the electron transport chain. In the present work, we have investigated the fluorescence of ANS bound to "intact" mitochondria capable of oxidative phosphorylation and ion transport. By following the phase relationships between structural and functional parameters in the oscillatory state (Utsumi and Packer, 1967) it is shown that conformational changes as indicated by ANS fluorescence precede the energy-linked functions of ion transport. The technique thus provides a useful probe for investigating the sequence and consequences of energy metabolism in mitochondria.

METHODS

Rat liver mitochondria were isolated in 0.3 M sucrose, 1.0 mM Tris-EDTA at pH 7.4 and oscillations induced by adding permeant ions as previously described (Utsumi and Packer, 1967). Front-face fluorescence between 455 nm and 578 nm was detected at an angle of approximately 20° to the incident exciting beam (290 nm to 404 nm). A simultaneous monitor of 90° light scattering was made using 640 nm to 700 nm light. There was no significant cross interference between the fluorescence and light scattering systems. The reaction medium was mixed by use of a Teflon-coated stirring bar operated by a magnetic stirrer located below the cuvette.

RESULTS

The number of ANS binding sites ($\bar{N}$) expressed as nmoles per mg mitochondrial protein obtained from titration curves by the method of Daniel and Weber (1966) is shown in Table 1. Osmotic expansion of the inner membrane does not cause any significant increase in binding site number but after disruption in distilled water, $\bar{N}$ approximately doubles and reaches a value similar to that of membrane fragments prepared by sonication. These results would indicate a barrier to ANS penetration in mitochondria making approximately 50 % of the mitochondrial protein inaccessible to binding when the inner membrane is intact. A similar conclusion is suggested by the work of Azzi and Chance (1969).

TABLE 1

EFFECT OF MITOCHONDRIAL SWELLING ON THE NUMBER OF ANS BINDING SITES ($\bar{N}$)

Samples were suspended in the indicated medium plus 1.0 mM Tris-EDTA, pH 7.4, and 2.0 μgm/ml rotenone. $\bar{N}$ was calculated according to Daniel and Weber (1966).

Samples	$\bar{N}$ nmoles ANS/mg protein
Mitochondria in 300 mM sucrose	21 ± 5
Mitochondria in 100 mM sucrose	17 ± 2
Mitochondria in distilled water	35 ± 5
Mitochondria membrane fragments	37 ± 5

Figure 1 shows a simultaneous monitor of ANS fluorescence and 90° light scattering of mitochondria in the oscillatory state. After addition of an energy source and permeant ions, the volume of the inner membrane compartment as followed by light scattering (lower trace) exhibits a damped sinusoidal oscillation (Deamer, Utsumi and Packer, 1967). When the reaction mixture be-

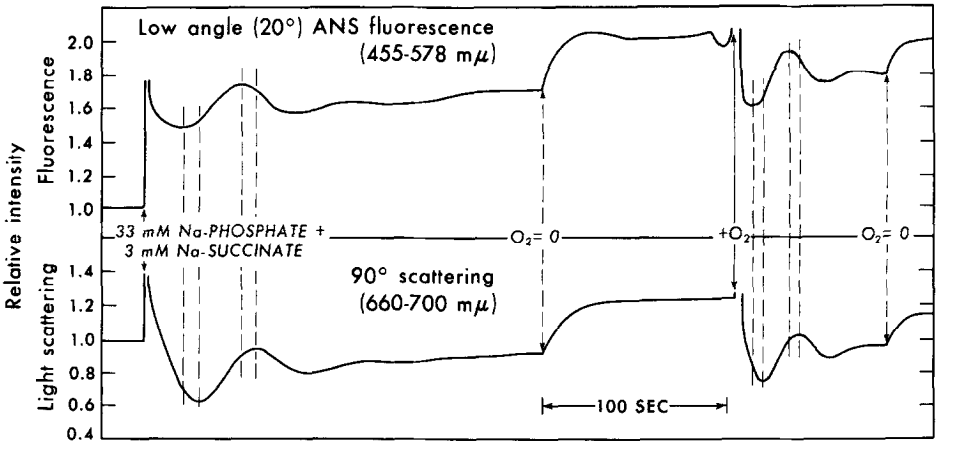

Fig. 1 - Simultaneous measurement of oscillations of ANS fluorescence and light scattering in mitochondria. Conditions of oscillation were: sucrose, 100 mM; Tris-EDTA, 1.0 mM, pH 7.8; rotenone, 6.0 μgm/ml; oligomycin, 6.0 μgm/ml; mitochondrial protein, 1.3 mg/ml; ANS, 30 μM. Oscillations were initially induced by the addition of sodium phosphate, 30 mM, pH 7.8, and sodium succinate, 3.0 mM, in a total volume of 3.3 ml.

comes anaerobic, the mitochondria contract to a steady state level. Oscillations can be re-initiated by a short period of oxygen perfusion. Qualitatively similar changes occur in ANS fluorescence (upper trace) but several important quantitative differences can be seen. The absolute level of fluorescence increases after the initial addition of phosphate and succinate, and remains higher than the initial level throughout the two subsequent oscillations. Experiments where substrate and permeant ion are added separately show that this initial fluorescence increase occurs following the addition of phosphate. A second feature apparent in Fig. 1 is that oscillations of ANS fluorescence precede those of light scattering by a constant phase difference of approximately 7 sec, but at anaerobis both fluorescence and light scattering change together within a time period of less than 1 sec. These phase relationships are maintained whether the oscillations are induced by permeant ion or by oxygen perfusion.

Several controls (Fig. 2) eliminated the possibility of artifacts arising from cross interference of fluorescence and light scattering in the sytem. This would also be expected from the non-constant phase relationship between the two during the oscillation experiment. Varying the level of light

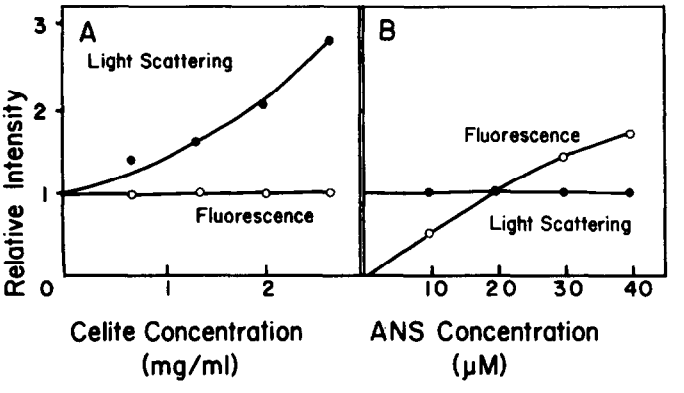

Fig. 2 - Independence of ANS fluorescence and 90° light scattering simultaneously measured in the same system.
A contained a constant level of 52 μM ANS in isopropanol, to which Celite was added.
B contained a constant level of 1.0 mg/ml Celite in isopropanol, to which ANS was added.

scattering by the addition of an inert compound, Celite, did not affect ANS fluorescence (Fig. 2a). Similarly, changes in the absolute fluorescence intensity did not alter the light scattering level (Fig. 2b). The presence of ANS in the concentrations used was not found to affect either the amplitude or period of the light scattering trace.

DISCUSSION

An increase in ANS fluorescence can arise both from a change in the hydrophobic environment of the molecule and from an increase in the number of binding sites. Since there is no significant change in ANS binding with osmotic expansion, alterations in ANS fluorescence with mitochondrial volume change would seem to reflect environmental changes of the ANS molecules. The specific site of ANS binding is as yet unknown but the work of Martonosi (1969) and Gitler (1969) has shown that both phospholipid and protein may be involved. The apparent increase in ANS binding when mitochondria are suspended in distilled water probably arises from the availability of matrix protein for reaction with ANS after disruption and not from a true increase in the number of binding sites per protein molecule. Thus, in intact mitochondria, a change in ANS fluorescence would be a measure of a conformational change in membrane rather than matrix protein.

Previous studies (Utsumi and Packer, 1967) have established that oscillations of several functional parameters accompany the oscillations of mitochondrial volume. These include respiration, pyridine nucleotide oxidation-reduction (inhibited by rotenone in the present experiments) and proton production. It was found that the time for peak amplitude of the volume changes lagged behind that of the electron transfer parameters. The present work shows that membrane conformation changes, as indicated by ANS fluorescence, also precede the energy-dependent configurational changes. Since these configurational changes are driven by ion transport and are uncoupler sensitive (Packer and Wrigglesworth, 1968), the results clearly show that membrane conformation changes precede the utilization of intermediates of energy conservation.

Where the functional changes do not involve energy coupling, as in the aerobic-anaerobic transition, both conformational and configurational changes occur together within a period of less than one sec. The precise nature of the conformational changes in membrane protein is as yet obscure but studies using optical rotary dispersion and circular dicroism (Wrigglesworth and Packer, 1968) suggest that they are confined to quarternary structural changes.

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