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## Relation between ANS fluorescence and energy states of mitochondria

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# Relation between ANS fluorescence and energy states of mitochondria\*

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## Abstract

1. ANS fluorescence change at various energized stages of mitochondria was investigated.
2. Freshly prepared mitochondria manifest ANS fluorescence change during anaerobic-aerobic transition, but aged and inner mitochondrial membrane show remarkable changes.
3. These data suggest that freshly prepared mitochondria or those in energized state exhibit less hydrophobic environments or decrease the binding site of ANS.
4. Energy dependent light scattering changes indicating the configurational changes of mitochondria cannot be said to be identical with the pattern of ANS fluorescence changes indicating the conformational change of mitochondrial membrane.
5. Polarity of the membrane structure and binding site of ANS in submitochondrial particles and mitochondrial membranes have been discussed.

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## RELATION BETWEEN ANS FLUORESCENCE AND ENERGY STATES OF MITOCHONDRIA

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A number of investigators have reported that the metabolic states of mitochondria correlate with the conformational changes of the mitochondrial membrane structure (1-5). More recently WRIGGLESWORTH and PACKER (4) have shown changes in optical rotatory dispersion and circular dichroism signals which depend on ultrastructure and metabolic states or oscillatory states of mitochondria. GREEN *et al.* (5) also documented energized and non-energized states of mitochondria, associated with the characteristic conformational changes of the mitochondrial membrane. However, these findings were noted only after fixation of mitochondria, and showing no dynamic changes of the conformation of mitochondrial membrane. On the other hand, AZZI *et al.* (6) reported that a fluorescent dye ANS (8-anilino-1-naphthalene sulfonic acid) bound to the mitochondrial membrane indicates indirectly conformational changes of mitochondrial membrane. They observed that sonicated particles of beef heart mitochondria show an increase in fluorescence intensity by the energized state and decrease by the non-energized state. The increase in fluorescence could be induced not only by the respiratory substrates but also by ATP. Azzi (7) also reported that the direction of ANS fluorescence change bound to mitochondria was opposition to the one of submitochondrial particles. From these ANS fluorescence studies, Azzi (7) considered that the polarity of the inner membrane of intact mitochondria was reversed in the membrane of submitochondrial particles. We have confirmed these differences in the direction of ANS fluorescence change between mitochondria and submitochondrial particles. But no ANS fluorescence change was observed by the change of metabolic state of intact mitochondria, and if aged mitochondria were used, a big ANS fluorescence change was observed depending upon the changes of mitochondrial metabolic state. In this report we describe the conformational changes of mitochondrial membrane detected by the change in ANS fluorescence intensity under various energy states of mitochondria. The paper also reports that

the fluorescence change of ANS bound to mitochondrial membrane does not show a similar pattern of the light scattering change of mitochondria and that the fluorescence change seems to reflect the true conformational change of mitochondria.

#### MATERIALS AND METHODS

Rat liver mitochondria were isolated in 0.33 M sucrose containing 1 mM EDTA and 1 mM Tris-HCl at pH 7.4 as described previously (8). Aged mitochondria were prepared by the incubation of isolated mitochondria in 0.33 M sucrose at 0° or 25°C. Beef heart mitochondria and sonicated submitochondrial particles were obtained from Dr. O. HATASE. Inner mitochondrial membranes were prepared by the technique of LOTTOCASA *et al.* (9). Fluorescence was measured at 560 m $\mu$ , with excitation at 360 m $\mu$ . Oxygen consumption was measured polarographically using a rotating platinum electrode. Swelling-shrinkage of mitochondria were followed by recording the 90° light scattering change using 600 m $\mu$  light beam. All three parameters were recorded simultaneously in some cases (10). To achieve anaerobic condition for mitochondria, the incubation medium was flashed with nitrogen gas prior to the addition of mitochondria. Protein was determined according to LOWRY *et al.* (11).

#### RESULTS

*Energy dependent changes of ANS fluorescence in mitochondria:* We observed about 20% increase in ANS fluorescence intensity by energized sonicated particles of beef heart mitochondria with respiratory substrates, such as succinate,  $\alpha$ -ketoglutarate, and with ATP. In comparison with the large increase, intact mitochondria show only a small changes in ANS fluorescence intensity by ATP or by substrate oxidation at different energy states such as state 3, 4 and 5. But the big ANS fluorescence change was observed in aged mitochondria. Namely, the rate and degree of ANS fluorescence changes by anaerobic-aerobic or aerobic-anaerobic transitions were increased with the time of incubation at 25°C, as shown in Fig. 1. Similar observations were made by incubating mitochondria overnight at 0°C and by washing several times with sucrose solution (Fig. 2). In this case the fluorescence change was observed only in the inner membrane system. Of course, the aged or well-washed mitochondria decreased or lost the activity of respiratory control by ADP.

*Effects of various inhibitors on ANS fluorescence:* The above data suggest that the uncoupled state of mitochondria manifest a greater ANS fluorescence change. Therefore, we tested the effect of various uncouplers on ANS fluorescence change of mitochondria. As shown in Fig. 3,

ANS Fluorescence and Mitochondria Energy States

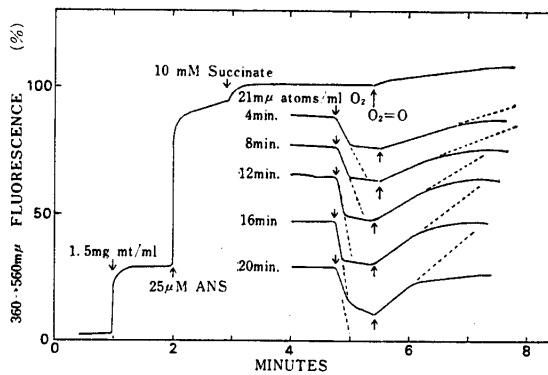


Fig. 1 ANS fluorescence change by anaerobic-aerobic or aerobic-anaerobic transition of mitochondria under various anaerobic incubation stages.

Rat liver mitochondria (1.5mg protein/ml) were incubated in 150mM choline chloride, pH 7.1, at 25°C. Total incubation mixture was 2.5ml. Oxygen pulse was applied by adding incubation mixture. ANS fluorescence was measured at 560 mμ with excitation at 360 mμ as described in text. The concentration of added substances are indicated in the figure. Dotted lines show the initial velocity of fluorescence change by anaerobic-aerobic and aerobic-anaerobic transitions. This figure shows an increased ANS fluorescence change depending on the change of energy state by anaerobic incubation.

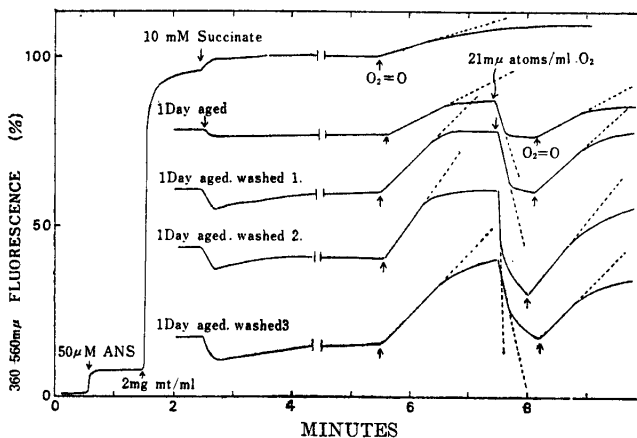


Fig. 2 Effect of washing and aging on ANS fluorescence change by anaerobic-aerobic or aerobic-anaerobic transitions.

Conditions as in Fig. 1, except mitochondria were aged one day at 0°C in 0.25 M sucrose and washed with 0.25 M sucrose. Mitochondrial protein was 2 mg/ml. Aging or washing of mitochondria increases the velocity and extent of ANS fluorescence change by aerobic-anaerobic or anaerobic-aerobic transitions,

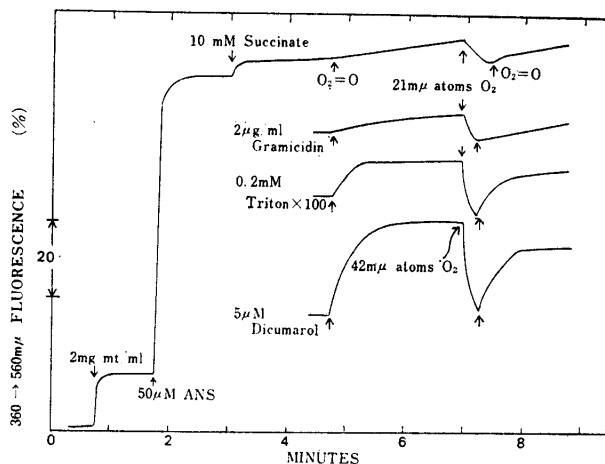


Fig. 3 Effect of uncouplers on ANS fluorescence change during anaerobic-aerobic transitions.

Conditions as in Fig. 1, except mitochondrial protein was 2 mg/ml and ANS concentration 50  $\mu$ M. ANS fluorescence change depending on the change of energy state was increased by the treatment with various uncouplers without aging or washing of mitochondria.

gramicidin, triton and dicumarol, at concentrations known to uncouple oxidative phosphorylation, vary in their effects. Dicumarol induces a large fluorescence change, while an intermediate change was produced by triton and a much smaller change by gnamacidin. From these data it cannot be concluded directly that the increase in ANS fluorescence intensity is due to the membrane of uncoupled mitochondria.

*Effect of ATP level on ANS fluorescence:* It is well known that mitochondrial ATP level decreases with aging or repeated washing. We attempted to study the effect of external ATP or intra-mitochondrially formed ATP on ANS fluorescence. As described above, anaerobic-aerobic transition induces a large fluorescence change in aged mitochondria. This large fluorescence change is abolished on addition of ATP and the ATP effect is inhibited by adding oligomycin. Similar inhibition of fluorescence change was observed under the conditions of ATP formation from ADP and Pi in mitochondria (Fig. 4). These results suggest that the energy level of mitochondria and membrane intactness are the determinant of the intensity of ANS fluorescence. Namely, the high energy level or unimpaired membrane such as intact mitochondria or the mitochondria in the medium containing ATP or ATP generating system show little change on ANS fluorescence. Even in anaerobiosis, an energized state or

## ANS Fluorescence and Mitochondria Energy States

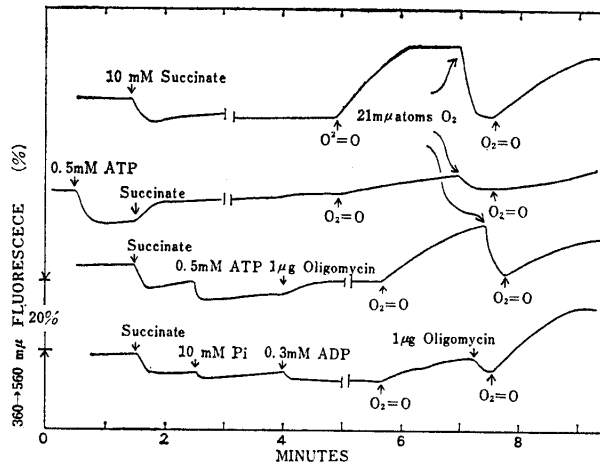


Fig. 4 Effect of ATP, ATP generation system and oligomycin on anaerobic-aerobic transition dependent changes of ANS fluorescence.

One-day aged mitochondria (2 mg protein/ml) were incubated in a medium of 150 mM choline chloride. Succinate concentration was always 10 mM. Big change of ANS fluorescence in aged mitochondria depending on the change of energy state was reduced by addition of ATP or ATP generating system. This reduced ANS fluorescence change was reversed to a big extent on addition of oligomycin.

high energy level of mitochondria may exist provided it is freshly prepared because the endogenous ATP is present without hydrolysis by latent ATPase. But aged mitochondria have impaired membranes and cannot keep an energized state or high energy level under the anaerobic condition because of latent ATPase activity and of increased membrane permeability. This explains why the change of fluorescence intensity by anaerobic-aerobic transition is so remarkable in aged mitochondria (Figs. 1, 2 and 4).

*Relation between swelling and ANS fluorescence intensity in mitochondria:* According to Azzì *et al.* (6), the change of ANS fluorescence indicates structural change of the mitochondrial membrane associated with energy conservation. In the previous paper (8), we established that changes in light scattering reflect the configurational changes of membrane such as swelling-contraction which depends on ion translocation. Therefore, we attempted to test the relationship between configurational changes of mitochondrial membrane, mitochondrial swelling induced by ion translocation and ANS fluorescence changes. As shown in Fig. 5, the ANS fluorescence change does not completely parallel with the light scattering change. Anaerobic-aerobic transitions by oxygen pulse, in the presence of dicu-

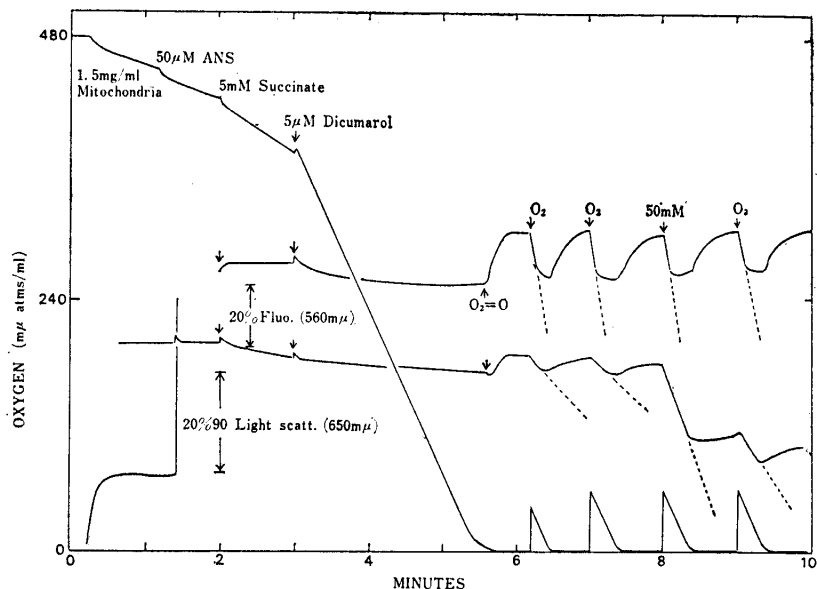


Fig. 5 Simultaneous measurements of oxygen consumption, light scattering and ANS fluorescence changes of mitochondria.

Conditions as in Fig. 1. No close correlation was observed between the changes in ANS fluorescence and in light scattering intensity.

marol, induce a large ANS fluorescence change and also a small light scattering change, but the addition of a large amount of Pi results in mitochondrial swelling without concomitant change of ANS fluorescence: Always the maximum level of light scattering changes lagged behind that of ANS fluorescence changes and no close similarity was observed the extent of changes between light scattering and fluorescence changes. We also examined the relationship between light scattering and fluorescence changes, at various aging states. At 25°C, ANS fluorescence change increases with the lapse of incubation time within 40-minute, then gradually decreases, in accordance with the decrease in velocity. (Fig. 6). On the other hand, light scattering change does not show the pattern similar to ANS fluorescence change. As shown in Fig. 7, both velocity and the extent of decrease in light scattering by anaerobic-aerobic transition were raised with the lapse of incubation, 100-minute aging. From these data we conclude that the conformational change expressed in terms of ANS fluorescence change does not correlate directly with the degree of membrane configurational change expressed by light scattering change.



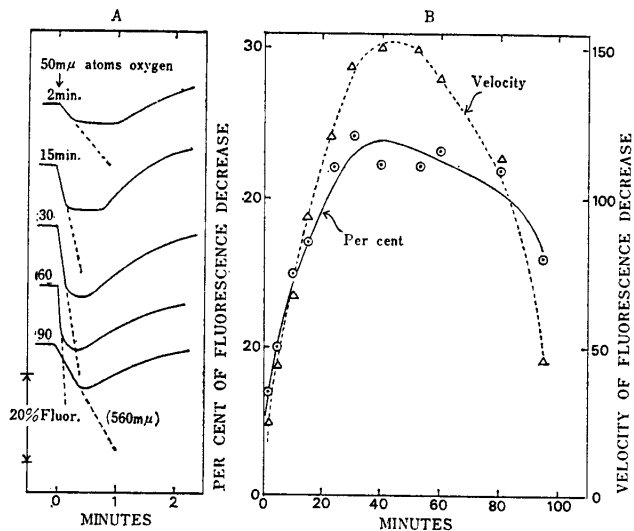


Fig. 6 Effect of anaerobic preincubation on the degree of ANS fluorescence change during anaerobic-aerobic transition.

Mitochondria (1 mg protein/ml) were incubated in 150 mM choline chloride and 50 μM atoms oxygen were injected to reaction vessels to produce oxygen pulse. A shows the actual traces of the change in ANS fluorescence intensity by anaerobic-aerobic transition at various aging states. B shows the changes in velocity and intensity of ANS fluorescence by anaerobic-aerobic transition at various aging states.

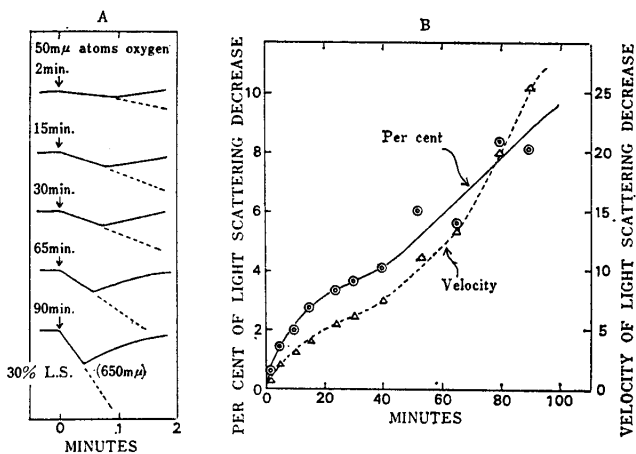


Fig. 7 Velocity and extent of light scattering changes in mitochondria by anaerobic-aerobic transition at various anaerobic preincubation stages.

Data in this figure were obtained simultaneously with those shown in Fig. 6.

DISCUSSION

ANS increases its fluorescence intensity when there is an increased number of binding sites to protein and an increase of hydrophobic environments. The specific site of ANS binding is not yet known but the most effective substances involved are possibly proteins and phospholipids of the mitochondrial membrane. We observed the ANS fluorescence change by anaerobic-aerobic transition only in aged or washed mitochondria. When mitochondria were tested immediately after isolation without repeated washing, no ANS fluorescence change was observed. Therefore, we may conclude that freshly prepared mitochondria and those at energized state or at the state of high energy level show a low extent of ANS fluorescence intensity, whereas aged, non-energized and or low energy state of membrane shows a high extent of fluorescence intensity. Further, the ANS fluorescence change is controlled by ATP level and energized state of mitochondria. According to AZZI (7), the polarity of the mitochondrial inner membrane in intact mitochondria differed from that of the membrane in submitochondrial particles, suggesting that the membrane of submitochondrial particles was inside out. Although there is no available data on specific binding site of ANS to the mitochondrial membrane, it is clear that, if ANS binds to both the outside and inside of inner membrane, we should see no difference in the direction of ANS fluorescence change between membranes of mitochondria and submitochondrial particles. Therefore, it seems that ANS binds outside the membranes of both mitochondrial and submitochondrial particles. Previous study (12) has shown that the pattern of light scattering change follows respiration, pyridine nucleotide oxidation-reduction and pH changes in a state of oscillation. The present study shows that the conformational changes in the membrane detected with the changes of ANS fluorescence also precede the changes of light scattering which is thought to be a functional change of mitochondria such as energy dependent ion translocation as indicated by PACKER *et al.* (13). The precise nature of membrane configurational changes in membrane protein indicated by light scattering change is still obscure but the conformational change of membrane structure expressed by ANS fluorescence was not similar to the light scattering change.

SUMMARY

1. ANS fluorescence change at various energized stages of mito-

chondria was investigated.

2. Freshly prepared mitochondria manifest ANS fluorescence change during anaerobic-aerobic transition, but aged and inner mitochondrial membrane show remarkable changes.

3. These data suggest that freshly prepared mitochondria or those in energized state exhibit less hydrophobic environments or decrease the binding site of ANS.

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