*A FLUORESCENCE PROBE OF ENERGY-DEPENDENT STRUCTURE CHANGES IN FRAGMENTED MEMBRANES**

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Abstract and Summary.—The reaction of the fluorochrome, 8-anilino-1-naphthalene-sulfonic acid (ANS), with fragmented membranes from beef heart mitochondria has been studied. ANS fluorescence is found to be enhanced 25-fold on binding to the membrane fragments in the absence of energy conservation, and this enhancement is increased to 35-fold in the membrane energized by substrate plus oxygen. The fluorescence of bound ANS depends upon the energy state of the membrane fragments, as indicated by the effects of ATP, substrates of the respiratory chain, oligomycin, and uncouplers. It is concluded that the changes of ANS fluorescence indicate structural changes of the mitochondrial membrane associated with energy conservation. The time course of energization is readily followed by ANS, and has a half-time of two seconds at 26°.

Introduction.—A number of changes of mitochondrial properties have been observed in transitions from high- to low-energy states: the oxidation of cytochrome *b*,[1] the "low-amplitude" light-scattering change,[2, 3] the diminution of the H^+ gradient indicated by bromthymol blue (BTB),[4, 5] and the disappearance of cytochrome b_{555} absorbancy in pigeon heart mitochondria.[6] Except in the last case, it is not possible to determine whether these changes are linked primarily to the energized state of the system or are secondary responses due, for example, either to the equilibration of cation concentration gradients or, in the case of the cytochromes, to alterations in the ratio of the oxidized to the reduced form. Thus, evidence for the high-energy state based upon cytochrome changes should be sought in membranes in which electron transport is blocked or across which minimal ionic concentration gradients exist.[6]

In addition, the energy state of the membrane may be probed by an indicator which responds only to the state of the dielectric in which it is bound and is insensitive both to the oxidation-reduction state of the electron transport system and to ionic concentration gradients across the membrane. Such an indicator would appear to afford ideal properties for interpreting or identifying changes in the mitochondrial membrane associated with its energized state.

8-Anilino-1-naphthalene-sulfonic acid (ANS) meets many of these requirements. It has been employed extensively in the study of changes of protein conformation, where it has provided a new insight into the structure, kinetics, and interactions of proteins.[7, 8] Its use in the study of changes of membrane conformation in mitochondria is reported here.

ANS is not fluorescent in water but becomes fluorescent in such hydrophobic environments as organic solvents and the nonpolar regions of proteins.[9, 10] Changes

in ANS fluorescence, excited in the region 360–390 nm, with emission measurements at 560 nm, are thus a measure of changes in the polarity of its environment as a consequence of spatial rearrangements of the protein molecules or of the protein-lipid relationships in the mitochondrial membranes.

Materials and Methods.—Three apparatuses have been used for the measurement of ANS fluorescence in fragments of mitochondrial membranes. First, ANS fluorescence and cytochrome absorbancy were measured simultaneously with a double-beam spectrophotometer and an attached fluorometer. Suitable guard filters were employed on the photomultipliers to avoid "cross-talk" between the two measurements. The apparatus has been described in detail elsewhere,[11] as has the regenerative flow apparatus used for rapid measurements.[12]

Secondly, changes in ANS fluorescence and pyridine nucleotide absorbancy and fluorescence were followed in a time-sharing fluorometer and double-beam spectrophotometer in which pyridine nucleotide absorbancy was measured at 347–380 nm. Fluorescence excitation was obtained from a water-cooled mercury arc through a band-pass filter with a half-width of 50 nm and a peak transmission at 366 nm. Fluorescence emission was measured through similar filters with peak transmission at 412 (reduced nicotinamide-adenine dinucleotide, NADH) and 570 (ANS) nm.

Thirdly, excitation and emission difference spectra, as well as polarization spectra, were obtained in a differential fluorometer in which two cuvettes were mechanically oscillated through the excitation beam; the photocurrents due to fluorescence emission were subtracted and plotted as a function of time. This technique has been previously employed for the measurement of flavoprotein[11] and pyridine nucleotide[13] spectra and polarization effects.[11]

Membrane fragments prepared from beef heart mitochondria following sonication in the presence of EDTA (E-SMP[14] fragments) or of magnesium ion and ATP (M-ASP[15] fragments) were used. Fragments prepared by treatment with ammonia and urea (ASU[16] fragments) were kindly donated by Dr. E. Racker.

Results.—Binding of ANS to mitochondrial membrane fragments: Table 1 illustrates the increase of ANS fluorescence caused by the addition of fragmented membranes from a suspension of 30 mg E-SMP protein per ml to a cuvette containing a solution of 54 μM ANS in a buffered medium. After 2 mg protein had been added, giving an increase of 38 fluorescence units, separation of the membranes from the supernatant by centrifugation showed that more than 53 μM of the 54 μM originally present was bound to the fragmented membranes. Similar experiments in which 54 μM ANS was dissolved in ethanol gave a fluorescence increase of 150 units.

Based upon Stryer's value of 0.37 for the quantum efficiency of ANS in ethanol,[9] the value for ANS bound to the membrane fragments is 0.1; this may be compared with the value of 0.004 for ANS in water. A fluorescence efficiency equal to that of the membrane-bound ANS is obtained by dissolving ANS in a solution of 80 per cent ethanol in water, which corresponds to a decrease of dielectric constant of water to a value of 35.[17]

The reverse titration of membrane fragments with ANS indicates the number of ANS binding sites in E-SMP membranes to be 200 nmoles per mg protein, with a dissociation constant of 5.7×10^{-5} M as calculated from the linear portion of a Scatchard plot (A. Azzi and G. K. Radda, unpublished observations). Thus, the content of ANS binding sites is 300-fold greater than the cytochrome a content.

TABLE 1. *The effect of protein or ethanol on ANS fluorescence.*

E-SMP (mg protein/ml)	Ethanol in water (%)	Increase in fluorescence, 390 → 560 nm arbitrary units
0.4	—	12.5
0.8	—	23.0
1.2	—	30.0
1.6	—	36.0
2.0	—	38.0
2.4	—	41.0
3.0	—	40.0
—	100	150.0
—	80	40.0

The experiment was carried out in a differential fluorometer by adding aliquots of E-SMP to both cuvettes, one of which contained 54 μM ANS. The suspending medium contained 0.3 *M* mannitol-sucrose, 20 mM Tris-HCl, pH 7.4. In the case of ethanol, the deflection obtained by adding 54 μM ANS was recorded. The excitation wavelength was 390 nm, and the emission was 560 nm.

The polarization of ANS fluorescence is 0.194 when 3 μmoles of ANS are bound to 1 gm of fragmented membranes and decreases to 0.159 when 15 μmoles of ANS per gm of protein are bound. This decrease of polarization is characteristic of energy transfer between ANS molecules, a phenomenon which occurs at a distance of less than 20 Å.[18] Since the density of the fragmented membranes is 1.19,[19] the volume per gm of fragmented membrane is 0.84 cc or 8.4×10^{23} Å^3; thus, each of the 9×10^{18}-ANS molecules occupies a volume of approximately 10^5 Å^3. At the maximum distance of 20 Å, the ANS molecules must be bound in the same plane, each occupying an area of 400 Å^2. The ANS molecules cover only approximately 25 per cent of the 50 to 70 Å thick membrane observed electron micrographically for the membranes of intact mitochondria[20] and membrane fragments obtained by sonication (D. F. Parsons, personal communication). Presumably, ANS binding occurs at specific chemical sites (possibly phospholipids) clustered in discrete areas on the inner surfaces of vesicles. 150–200 μmoles of ANS per gm of protein may be bound to the fragmented membranes before saturation occurs, suggesting that additional binding sites not only are located in the clusters on the inner surfaces but are also distributed throughout the thickness of the membrane.

Electron-transport-induced changes of ANS fluorescence in membrane fragments: Electron transport is most rapidly activated when oxygen is added to the anaerobic membranes. The flow apparatus traces of Figure 1 show that the kinetics of ANS fluorescence change parallel those of the oxidation and reduction of cytochrome *a* in a suspension supplemented with succinate to bring the system to anaerobiosis and with oligomycin for maximal energy coupling[14] of the membranes. The addition of 15 μM oxygen induces a rapid oxidation of cytochrome *a* (upper trace), which has an independently measured half-time of 500 μsec.[12] The ANS response is slower by a factor of 4000; the half-time is approximately two seconds. On expenditure of oxygen, both the cytochrome *a* and ANS changes are reversed, and again the reduction of cytochrome *a* (half-time, 1 sec) is considerably faster than the decrease of ANS fluorescence (half-time, 7 sec).

Electron transport may also be activated by addition of a substrate to aerobic membranes. As shown in Figure 2, each addition of NADH induces a cycle of

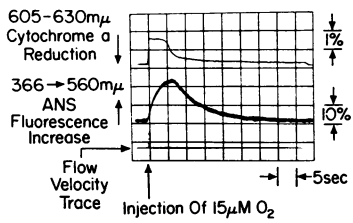

FIG. 1.—Flow apparatus traces of the response of ANS and cytochrome *a* to an oxygen pulse. 2 mg E-SMP protein per ml in 0.3 *M* mannitol-sucrose, 20 mM Tris-HCl, pH 7.4, supplemented with 0.5 μg per ml of oligomycin and 100 μM ANS, in the presence of 10 mM succinate as substrate.

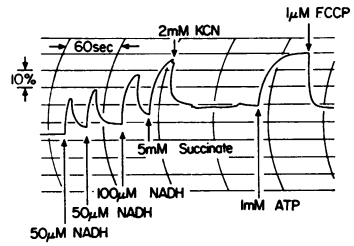

FIG. 2.—The response of ANS fluorescence to respiratory substrates and ATP. 1 mg M-ASP protein per ml in 0.3 *M* mannitol sucrose, 20 mM Tris-HCl, pH 7.4, supplemented with 4 mM $MgCl_2$ and 100 μM ANS. As in the other figures, an increase of ANS fluorescence is indicated by an upward deflection.

ANS fluorescence similar to that shown in Figure 1; under these conditions, the addition of succinate causes similar changes. Addition of cyanide to block the terminal oxidase decreases the ANS fluorescence to the value prior to succinate addition. Independent measurements indicate that the oxidation-reduction changes of cytochromes (cf. Fig. 1) and pyridine nucleotide (cf. Fig. 4) are more rapid than the changes of ANS fluorescence. From these experiments, it is apparent that the increases of ANS fluorescence are related to, but not synchronous with, the electron-transport process and the oxidation-reduction changes of the respiratory carriers.

Energy-induced changes of ANS fluorescence and BTB absorbancy in fragmented membranes: Effect of energization by ATP and uncoupling by FCCP: In previous studies of the energy-dependent responses of fragmented membranes,[4] the membrane-bound pH indicator, bromthymol blue, showed that the energized state is associated with increased acidity in the fragmented membrane. This phenomenon, in which energization of the membrane by the addition of 140 μM NADH in the absence of oligomycin causes only small deflections of the ANS and BTB traces, which subside when the added NADH is oxidized, is shown in Figure 3. Addition of oligomycin blocks energy-dissipating reactions in the membranes and increases the degree of respiratory control, the rate and extent of reversed electron transport (cf. Fig. 4), and the transhydrogenase activity.[14] Thus, following the addition of 2 μg oligomycin per ml, a second addition of NADH causes large and closely synchronized changes of BTB and ANS as the NADH is oxidized. Titrations with oligomycin under these conditions give similar end points: 0.2 μg oligomycin per mg protein for ANS, and 0.4 μg per mg protein for BTB.

ATP addition causes similar ANS and BTB changes when added to the cyanide-blocked

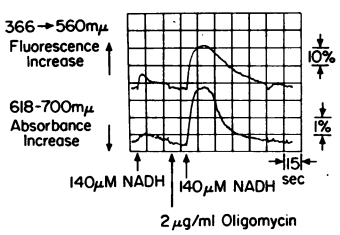

FIG. 3.—The energy dependence of the ANS response. 1 mg ASU protein per ml in 0.3 *M* mannitol-sucrose, 20 mM Tris-HCl, pH 7.4, supplemented with 10 μM BTB and 120 μM ANS.

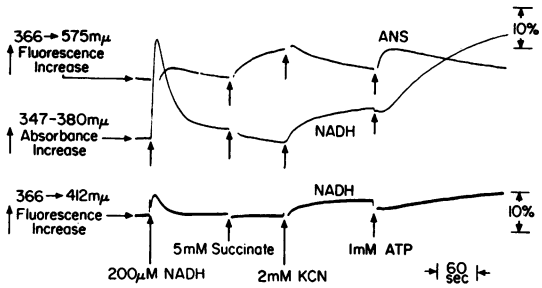

FIG. 4.—The relationship between the ATP-induced reversal of electron transport and the ANS response. Experimental conditions as in Fig. 2.

membrane fragments (Fig. 2). An uncoupling agent, carbonyl scyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), abolishes the response to NADH, succinate, or ATP.

Effect of pH changes of ANS fluorescence and BTB absorbancy: Glass electrode measurements[4, 21] of pH changes external to the mitochondrial membranes clearly show that hydrogen-ion movements accompany the activation of electron transport and energy coupling, and such changes are believed to be indicated by BTB as well.[4] The possibility that ANS measures these pH changes can be excluded on the basis of several observations. First, a pH change of almost three units (from 7.4 to 10.15) does not affect the ANS response in the membrane fragments by more than 20 per cent (A. Azzi, unpublished observations). Secondly, ANS in 80 per cent ethanol is not affected by variations in pH from 3 to 11. Thirdly, titrations of ANS with bovine serum albumin, reported by Chen,[22] show that no fluorescence response is obtained by varying the pH between 5 and 9.

The similarity of the ANS and BTB responses suggests that the hydrogen ions to which BTB responds may arise from a conformation-induced change in the pK of the membrane constituents, or from a sensitivity of BTB to the membrane dielectric constant. Responses to changes in the ratio of dye-bound to dye-free are minimized by the use of BTB and ANS in large excess. Thus, it appears that ANS indicates conformation changes and is insensitive to alterations of pH, while BTB may be capable of indicating changes in both properties.

The relationship between changes of ANS fluorescence and energy utilization in NAD reduction: The relationship between the membrane conformation indicated by ANS fluorescence and energy utilization in reversed electron transport is shown in Figure 4, in which the three traces show the response of ANS to an addition of NADH followed by succinate, as well as absorbancy and fluorescence measurements of the rate of NADH oxidation. As in Figure 2, the addition of cyanide reverses the increase of ANS fluorescence following succinate addition and stimulates the reduction of pyridine nucleotide as well. This effect can be attributed to reversed electron transport from succinate to NAD,[23, 24] since the energy requirement is met by the oxidation of succinate during the period of partial oxidase inhibition by cyanide. Addition of ATP at this point induces an increase in ANS fluorescence, as would be expected from all agents that increase the energy level of the system, and a further reduction of NAD.

The most interesting feature of Figure 4 is the interval of several seconds observed between the plateau in the ANS response and the initiation of NAD reduction, as shown both in the absorbancy and fluorescence traces. The response of ANS is 70 per cent complete before the NAD reduction starts; the function indicated by ANS clearly precedes the energy-linked reversal of electron transport.

Discussion.—The ANS binding site: ANS is bound by membrane fragments, as indicated by centrifugation experiments and by the enhancement of ANS fluorescence when membrane fragments are added. The binding site for ANS has, therefore, a more hydrophobic environment than that of the water phase surrounding the fragments.[25] The degree of rotational motion of the ANS molecules at the binding site is fairly high, as suggested by the relatively low values of polarization (P = 0.19) when the excitation wavelength maximum is 390 nm and the ANS concentration is 3 μmoles per gm of protein.

The possibility that ANS movements on and off the binding sites may be responsible for the observed changes of fluorescence in such complex systems as the fragmented membranes studied here is opposed by the observation that more than 98 per cent of the ANS molecules are initially bound to the membrane fragments under our experimental conditions. Furthermore, increasing the saturation of the binding sites increases rather than decreases the ANS response to NADH addition, thus the migration of ANS from a binding site having a largely hydrophobic environment to one having a more hydrophilic environment, and vice versa, is probably much less responsible for the changes in ANS fluorescence. Thus, in membrane fragments derived from beef heart mitochondria, as in simpler proteins, ANS indicates changes in the structure of the membrane which are reflected in the environments of the ANS binding site.

The speed of the membrane-conformation change: Many probes for membrane structure, such as electron spin and nuclear magnetic resonance, do not permit the correlation of the time course of the observed changes with the functional state of the membrane. ANS, however, allows such correlations in fragments of the mitochondrial membrane.

In Figure 1, the regenerative flow apparatus provided an accurate comparison of the kinetics of cytochrome oxidation and those of the ANS fluorescence increase. It is apparent from this trace that there is no change in ANS fluorescence which may be directly linked to the oxidation-reduction state of this electron carrier. Other studies of rat liver mitochondria have shown that none of the electron carriers in the succinate-rotenone-blocked system respond as slowly as does ANS.[12] Thus, the energized state of the membrane is not activated simultaneously with the changes in oxidation-reduction states of the electron carriers, a conclusion in accord with that previously reached from rapid-flow studies with bromthymol blue as an indicator of the energized state of the membrane.[26]

Instead, it appears that the structural alteration of the membrane requires the turnover of the cytochrome components in order to build up a steady-state concentration of an intermediate or intermediates of energy conservation such as $X \sim I$.[27] Thus, an intermediate step is required between electron transport and alterations in the membrane structure, as indicated by the ANS changes

and possibly by the BTB changes as well, and in the pH of the environment, as indicated by the BTB changes. In addition, the relationship between the energy-induced conformation change of the membrane and an energy-utilizing process, such as reversed electron transport, indicates clearly that the membrane energization precedes the NAD reduction. This correlation may at present be taken to indicate that membrane-conformation changes are essential to, rather than secondary indications of, energy conservation.

The relationship to energy storage in mitochondrial membranes: Changes of gross mitochondrial morphology have been observed electron-microscopically by Hackenbrock[28] in the state 4 → 3 transition and by Green[29] in other transitions; in the latter case, correlations with the energy state of the membrane are stressed. No similar changes are observed in submitochondrial particles, and they are apparently not required for energy conservation or utilization in the fragmented membranes. It is possible, however, that the rearrangements of gross mitochondrial morphology bear the same relationship to the molecular rearrangements reported by ANS in the fragmented membranes as do the quaternary structure changes of hemoglobin to smaller secondary and tertiary structure rearrangements caused by ligand binding; the latter were suggested by two-dimensional[30] and three-dimensional[31, 32] studies of ligand binding in myoglobin. The possibility that such structural changes play a role in energy conservation has been presented elsewhere.[26, 33]

Relationship to electron transport: The changes of ANS fluorescence are too slow to be correlated with electron-transport changes in the respiratory chain. 5-Dimethylamino-1-naphthalene sulfonyl chloride (DNS) however, does show a fluorescence change as rapid as cytochrome *a* oxidation in fragmented membranes.[34] Such a rapid change in the membrane properties is required by computer-evaluated kinetics of the components of the respiratory chain in response to oxygen pulses.[35]

Abbreviations used: ANS, 8-anilino-1-naphthalene-sulfonic acid; NADH, reduced nicotinamide-adenine dinucleotide; BTB, bromthymol blue, 3,3′-dibromothymolsulfonphthalein; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; DNS, 5-dimethylamino-1-naphthalene sulfonyl chloride.

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