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Anilinonaphthalenesulfonate fluorescence changes induced by non-enzymatic generation of membrane potential in mitochondria and submitochondrial particles

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SUMMARY

Changes in the fluorescence of 1-anilino-8-naphthalenesulfonate (ANS^-) accompanying non-enzymatic generation of the membrane potential in mitochondria and sonicated submitochondrial particles have been demonstrated. Generation of the membrane potential was induced by addition of an ionophore (valinomycin for K^+, or tetrachlorotrifluoromethylbenzimidazole for H^+) under conditions where there existed K^+ (or H^+) gradients across the mitochondrial membrane. The ANS^- fluorescence decreased when the mitochondrial (or particle) interior became more negative, and increased when it became more positive. Collapse of the membrane potential reversed the ANS^- responses. A hypothesis is put forward to explain the energy-dependent ANS^- responses in mitochondria and particles by the membrane potential-induced redistribution of ANS^- between the membrane and water phases.

Recently it has been demonstrated[1-7] that energization of mitochondria and "inside-out" submitochondrial particles is accompanied by some changes in the fluorescence of 1-anilino-8-naphthalenesulfonate (ANS^-). The fluorescence intensity decreased in mitochondria and increased in submitochondrial particles. Azzi[8] noticed that the particles take up ANS^- and mitochondria extrude ANS^- in an energy-dependent fashion. The direction of responses of the fluorescent cation, auramine O, was opposite. Neither the fluorescence, nor the concentration of a non-ionized ANS derivative (ANS-amide) were influenced by changes in the energy level[8]. The effect of energization on the distribution of ANS^- between mitocondria (submitochondrial particles) and the external solution closely resembles that described in experiments with synthetic penetrating anions of tetraphenylboron, phenyldicarbaundecaborane and picrate. These ions are accumulated

Abbreviations: ANS^-, 1-anilino-8-naphthalenesulfonate anion; PS^-, pyrene-3-sulfonate anion; TTFB, tetrachlorotrifluoromethylbenzimidazole.

by submitochondrial particles[9] and extruded from mitochondria[10] upon transition to the energized state, the effect being explained in terms of "transmembrane electrophoresis". We examined whether or not this explanation also holds for the energy-dependent responses of the ANS^- anion. To this end we used non-enzymatic generation of a membrane potential, a method applied earlier by Jackson and Crofts[11] to the study of carotenoid responses in chromatophores.

Mitochondria or submitochondrial particles suspended in potassium-free solution containing ANS^- were treated with valinomycin to induce K^+ efflux, thus generating a membrane potential (negative inside). Then a high concentration of KCl was added which resulted in a change of direction of electric field in the membrane (positive inside). In other experiments the membrane potential was generated by a pH gradient in the presence of an uncoupler, tetrachlorotrifluorobenzimidazole (TTFB) as protonophore. Mitochondria stored at pH 7.5 were added to a solution of pH 5.5. Subsequent addition of TTFB resulted in H^+ influx, thus charging the membrane (positive inside). All samples contained cyanide.

Measurements of ANS^- fluorescence showed (Fig. 1) that it changed characteristically under these treatments. The fluorescence decreased when the inside of the mitochondria (Fig. 1A,B) or submitochondrial particles (Fig. 1D) became negative, and increased when the mitochondrial (Fig. 1A,H) or submitochondrial particles (Fig. 1D)

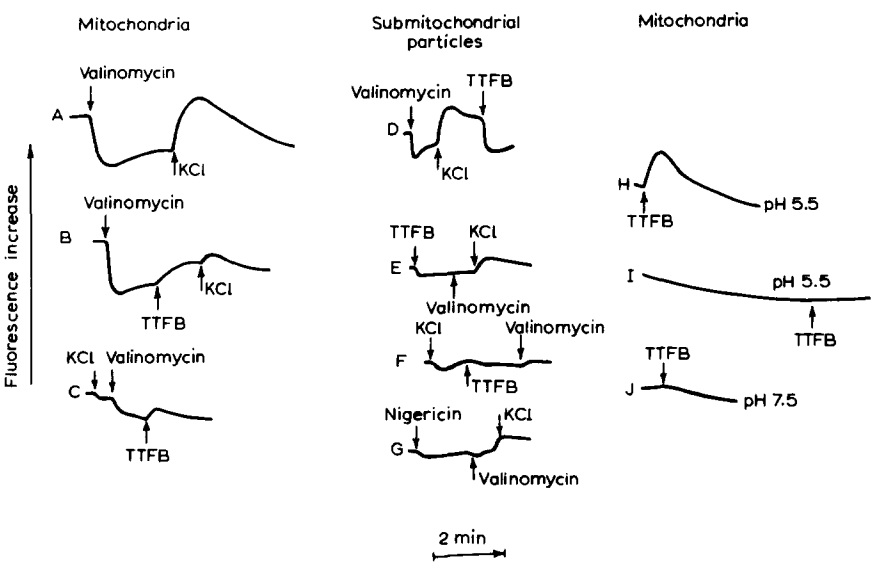

Fig. 1. ANS^- fluorescence responses on non-enzymatic generation of the membrane potential in mitochondria and submitochondrial particles. Reaction mixture: A, B, C, D, E, F, G: 0.2 M sucrose, 5 mM NaCN, 50 mM Tris–HCl (pH 7.5), 2 mg of mitochondrial protein and 1 mg of submitochondrial particle protein per mL; H, I, J: 0.1 M KCl, 5 mM NaCN, 50 mM potassium acetate, pH 5.5 (H, I), or 50 mM Tris–HCl, pH 7.5 (J), 2.3 mg of mitochondrial protein per ml. ANS^- concentration, $1 \cdot 10^{-5}$ M. Additions: $2 \cdot 10^{-8}$ M valinomycin. 0.1 M KCl, $2 \cdot 10^{-5}$ M TTFB, 1 μg/ml nigericin. Fluorescence excitation wavelength, 360 nm; fluorescence measured at 460 nm. Beef heart mitochondria and submitochondrial particles were prepared as described earlier[9,10], stored at 0° in a solution which contained 0.25 M sucrose for mitochondria, and 0.25 M sucrose, 10 mM Tris–HCl (pH 7.5), 5 mM $MgSO_4$, 5 mM $MnCl_2$, 1 mM succinate and 1 mM ATP for submitochondrial particles.

interior became more positive. Discharge of the potential by TTFB (Fig. 1B,D,E) or nigericin (Fig. 1G) greatly decreased the ANS^- response.

The ANS^- fluorescence changes accompanying non-enzymatic generation of the membrane potential were of the same magnitude as that induced by energization with respiratory substrate or ATP. However, the hypothesis that the ANS^- responses observed are due to changes in the level of energization of the enzymatic system in the mitochondrial membrane fails to explain all the data shown in Fig. 1. These data indicate that both submitochondrial particles and mitochondria (at pH 7.5) should be characterized as initially de-energized, since neither TTFB (Fig. 1C,E,F,J) nor a combination of nigericin and valinomycin (Fig. 1G) influences the ANS^- fluorescence under conditions where no membrane potential is generated non-enzymatically. This is not surprising, as the mitochondria and submitochondrial particles were incubated without energy sources. Respiration was arrested by 5 mM NaCN. ATP included in the submitochondrial particle suspension medium was diluted to a final concentration of $2 \cdot 10^{-5}$ M in the experimental sample. Apparently, this small amount of ATP was hydrolyzed during the few minutes of preincubation required for the equilibration of the ANS^- solution with the particles.

Since the submitochondrial particles were already de-energized, it is difficult to explain how valinomycin induced a decrease in ANS^- fluorescence by de-energization of the particles. Furthermore, the decay of the ANS^- response upon the addition of TTFB (Fig. 1H) proves to be inexplicable if the TTFB-induced fluorescence increase had been a result of de-energization. It is most probable that the decay is accounted for by dissipation of the pH gradient and the membrane potential due to progessive acidification of the mitochondrial interior. As is seen in Fig. 1I, TTFB did not affect the fluorescence if added after the decay was over. Thus the data suggest that the above ANS^- responses are primarily associated with formation of the membrane potential rather than with energization of the enzymatic system.

The effect of membrane potential on ANS^- fluorescence can be easily explained if we take into account the properties of this compound. ANS^- displays a high fluorescence yield only in a hydrophobic medium[14,15]. Therefore, the fluorescence of ANS^- in a suspension of mitochondria or of submitochondrial particles is due to the membrane-bound portion of the ANS^- pool. The concentration of ANS^- in the mitochondrial membrane must be a function of its concentration in the incubation mixture and in the intramitochondrial solution. However, if we are dealing with a phenomenon related to a redistribution of ANS^- between the extra- and intramitochondrial spaces, the amount of ANS^- in the membrane is determined entirely by the ANS^- concentration in the solution inside the mitochondria. For instance, the efflux of the whole portion of ANS^-, which was initially in the intramitochondrial water, cannot increase the ANS^- concentration in the mitochondrial exterior significantly. Extruded ANS^- is diluted in the "ocean" of extramitochondrial water which occupies by far the greater portion of the experimental sample than the mitochondria. As a result, the ANS^- concentration will decrease more inside mitochondria than it will increase outside. Therefore, the concentration of ANS^- in the membrane of the mitochondria and, hence, the intensity of the fluorescence, should decrease as the ANS^- concentration inside the mitochondria decreases. Similarly, accumulation of ANS^- inside the mitochondria should increase both the membrane-bound ANS^- and the fluorescence. When the interior of the mitochondrial or submitochondrial

particles becomes more negative (*e.g.* under K^+ efflux) ANS^- leaves electrophoretically, resulting in a decrease in the ANS^- concentration in the internal water and, hence, in the membrane, the effect revealing itself as a lowering of the fluorescence. The opposite changes must take place when the mitochondria and particle interiors become more positive. The latter response was observed with both K^+ influx in the presence of valinomycin, and influx of H^+ in the presence of TTFB.

Since transition to the energized state results in a decrease of ANS^- fluorescence in the experiments with mitochondria[5,6], one can conclude that the mitochondrial interior is negatively charged under energization. The opposite direction of the ANS^- response in submitochondrial particles[1-4,7] could be due to the reverse polarity of the particle membrane whose energization results in the positive charging of the particle interior.

It is noteworthy that some factors other than membrane potential could be involved in the energy-dependent ANS^- responses. So, the ANS^- fluorescence extrapolated to infinite concentration of particles suggests an energy-dependent change in the quantum yield[7], which can hardly be a consequence of ANS^- redistribution. It is more probable that the quantum yield changes are associated with some conformational transitions of the ANS^- binding proteins[7].

However, at any realistic particle concentration, the contribution due to the increase in ANS^- quantum yield (the fast component of the energy-dependent fluorescence enhancement) is much smaller than that of the slower component resulting from ANS^- influx[7]. Apparently, in these cases, the role of the membrane potential-induced redistribution of ANS^-, but not that of changes in protein conformation, is predominant. In this way one could explain why energization of mitochondria and "inside-out" particles containing the same enzymatic system for energy coupling induce opposite changes in fluorescence of ANS^-.

The membrane potential concept allows one to explain also the energy-dependent shift of the fluorescence excitation spectrum of pyrene-3-sulfonate (PS^-) in submitochondrial particles, the effect described recently by Brocklehurst *et al.*[7]. The shift observed indicates that PS^- is greatly concentrated in particles under energization. It should be stressed that PS^- fluorescence does not depend on medium polarity changes which are usually believed to account for the ANS^- fluorescence responses.

Thus, the results of the fluorescent probe experiments give further impetus to the conclusion of the existence and orientation of an electric field in the energized mitochondrial membrane, arrived at when studying fluxes of natural and synthetic ions[16,17]

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