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On the nature of the energy-linked quantum yield change in anilinonaphthalene sulphonate fluorescence in submitochondrial particles

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SUMMARY

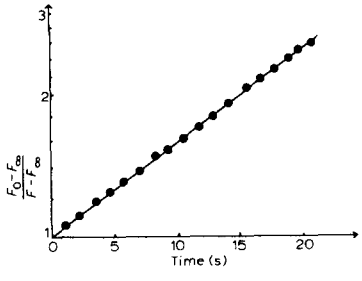
It is shown that the energy-linked uncoupler-sensitive increase in the fluorescence of anilinonaphthalene sulphonate probes in submitochondrial particles has two clearly resolvable components: an energy-dependent change in probe binding, and a quantum yield change. The latter is elicited by addition of substrates (succinate or NADH) to coupled submitochondrial particles and is reversed by the addition of uncouplers. Addition of electron transport inhibitors to energized particles results in a decrease in the amount of probe bound but not in the quantum yield, while energization without electron transport enhances binding but not the intrinsic quantum yield of the probe. These observations are explained in terms of two membrane states.

It has been demonstrated previously that energization of submitochondrial particles by succinate results in a fluorescence enhancement of the negatively charged probes 1-anilinonaphthalene 8-sulphonate (ANS) and 2-(*N*-methylanilino)naphthalene 6-sulphonate (MNS) which is reversed by uncouplers[1]. Two factors contribute to this enhancement: the amount of probe bound in the energized state is higher under most conditions, and the quantum yield of the bound probe is also doubled on energization[2,3]. These two effects can be most clearly separated when the kinetics of fluorescence decrease is followed after rapid addition of an uncoupler to energized particles[2-4]. The fluorescence decrease can be analysed in terms of two first-order rate processes. The fast change (with a $t_{1/2}$ of 2–3 s) is independent of the nature of the probe used and represents the change in quantum yield and not that in binding; the slow

Abbreviations: ANS, 1-anilinonaphthalene 8-sulphonate; MNS, 2-(*N*-methylanilino)naphthalene 6-sulphonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

decrease depends on the exclusion of the dye (and hence on the nature of the dye) from the deenergized membrane. (It should be noted that Azzi *et al.*[5] argued that the fast change also depends on the nature of the probe used. Their interpretation, however, was based on erroneous analysis of two first-order rates in a system where the rate constants for the two processes were not very different.) Such a biphasic decrease in fluorescence may arise as a result of one of two possible mechanisms. In the first the fast phase provides an obligatory intermediate for the release of the probe (the sequential mechanism), and in the second the two rates represent independent processes (the parallel mechanism). A hypothesis for the energy-linked ANS and MNS fluorescence changes proposed by one of us[3,4] assumed that the mechanism was sequential. The purpose of the present communication is to show that the parallel mechanism is the appropriate one and that the quantum yield changes in probe fluorescence reveal a new state of the mitochondrial membrane which is associated with energy coupling but not with energization *per se*.

The essential features of our observations are summarized in Figs 1–3. These show that anaerobiosis of energized submitochondrial particles, in contrast to the effect of uncouplers, did not result in a biphasic decrease in ANS fluorescence, in a decrease in the ANS quantum yield or in a decrease in the lifetime of MNS fluorescence. (For technical reasons fluorescence lifetime measurements are more accurate when MNS is used as the probe, however, no difference between the behaviour of ANS and MNS as energy-linked probes has been found[2].) The rate of ANS fluorescence decrease on anaerobiosis (Fig. 1) is identical to that measured for the release of ANS (as described before[2,4]) after the addition of uncoupler. Fig. 2 shows that the quantum



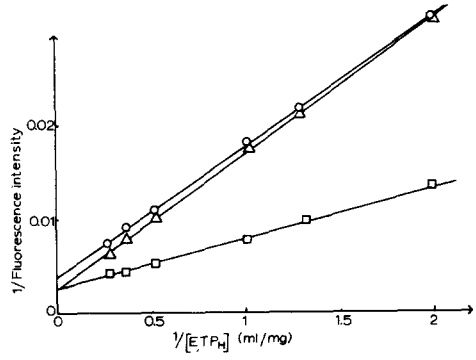
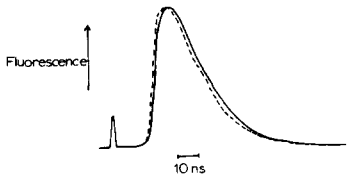


Fig. 1. Kinetics of the ANS fluorescence decrease on anaerobiosis of energized particles. The concentration of submitochondrial particles (ETP_H) was 1 mg/ml and of ANS 10 μM in 225 mM mannitol, 75 mM sucrose, 20 mM Tris buffer, pH 7.4; energization was by 5 mM succinate, excitation at 380 nm, emission at 480 nm.

Fig. 2. Double-reciprocal plots for titrations of ANS with submitochondrial particles (ETP_H). □—□, energized; ○—○, unenergized; △—△, anaerobic. Conditions as for Fig. 1.



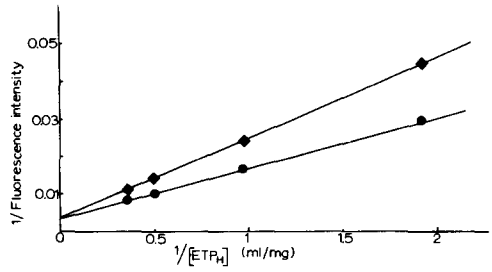


Fig. 3. Fluorescence decay curves for MNS-ETP$_H$. Excitation through Corning 7-60 filter and emission through a Corning 3-72 filter. MNS, 10 μM; otherwise conditions as Fig. 1. . . . . ., unenergized; ——, energized and anaerobic traces normalised to equal intensity.

Fig. 4. Double-reciprocal plots for titrations of ANS with submitochondrial particles (ETP$_H$). Conditions as for Fig. 2. Energisation by 0.5 M KCl and $5 \cdot 10^{-2}$ μg/ml valinomycin. ♦——♦, unenergized; ●——●, energized.

yield of bound ANS (obtained by extrapolation of the double-reciprocal plots to infinite protein concentration) is between 1.5- and 2- fold higher when membrane is energized by succinate than prior to the addition of substrate. The quantum yield, however, remains at this high level after anaerobiosis. The fluorescence lifetime measurements also support these observations (Fig. 3) in that the energized and anaerobic systems gave the same lifetimes for MNS but differed from those of the resting or uncoupled systems.

The increase in quantum yield is also elicited when NADH is used as substrate, and the biphasic fluorescence decrease is produced by a variety of uncouplers, including carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanalide (S-13) and a combination of KCl, valinomycin and nigericin (in concentrations of 33 mM, 1 μg/ml and 1μg/ml, respectively). When electron transport is inhibited by antimycin or KCN (using succinate for energization) or by rotenone (with NADH as substrate) the ANS fluorescence decrease is monophasic and the quantum yield for the probe fluorescence in the inhibited state remains the same as in the energized state. In contrast, when particles are energized in the absence of electron transport by the addition of KCl and valinomycin[6] the fluorescence enhancement does not produce a change in the quantum yield of the bound probe (Fig. 4). Energization by ATP in (Mg^{2+}) particles (J.A. Berden, G.K. Radda and E.C. Slater, unpublished observations) again gives rise to increase in ANS fluorescence without a quantum yield change.

These results suggest that the increase in quantum yield and in fluorescence lifetime of the anilinonaphthalene sulphonate probes requires both electron flow and a "coupled" membrane. We refer to the state of the membrane where the probe quantum yield is high as the symplectic or S-state (from the Greek, *symplectos*, referring to a state where two processes are linked) and the low quantum yield state as the aplectic or A-state (where no linking of the two processess of electron flow and energy conservation occurs). Thus we may have both energized and non-energized S-states and A-states. It is

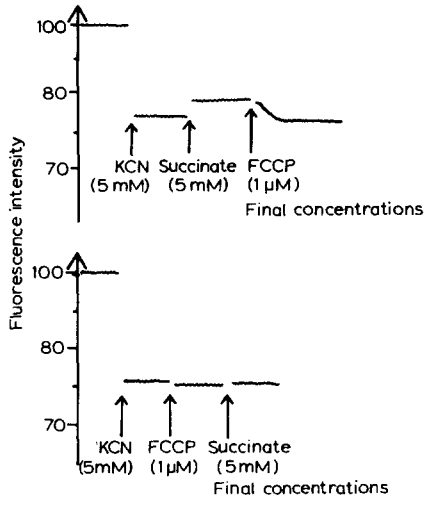


Fig. 5. ANS fluorescence changes in KCN-inhibited submitochondrial particles (ETP_H). Conditions as for Fig. 1. Fluorescence values were corrected for scattered light.

possible that the A–S transition requires several turnovers of the chain or that a single turnover could bring about this change. That the latter is the case can be shown by the following experiments. When succinate is added to submitochondrial particles after incubation with KCN in the absence of uncoupler, a small ANS fluorescence increase is observed which is shown to be uncoupler sensitive (it can be abolished by additon of uncoupler either prior to or after the introduction of the substrate) and is not a result of changes in the light-scattering properties of the solution (Fig. 5).

The explanation that our observations are a result of "residual" energization must now be considered. It is unlikely that residual energization is maintained by oxygen diffusion in the well sealed, strictly anaerobic system used by us. It is possible that leakage through the inhibitor sites again maintains a small extent of energization. However, the major change in ANS fluorescence after energization under conditions of high ANS to particle ratio is one in probe binding. It would be difficult to explain why, when the ANS binding appears to follow the degree of energization, the fluorescence lifetime and quantum yield of the probe in the fully energized state are identical to values observed for the state with "residual energy". The consistency in the observations that lack of biphasicity after inhibition is invariably accompanied by a lack of decrease in fluorescence lifetime and quantum yield indicates that our explanation is more likely to be correct.

It is worthwhile to point out that when uncoupler is added to particles energized by succinate there is a biphasic change in the cytochrome *b* spectrum (as observed at 565–558 nm), the slow phase of which has a $t_{1/2}$ of 2.5 ± 0.4 s, which is identical to the half-life for the S–A transition as observed in the fast phase of the probe fluorescence decrease. This observation was first made on a KCN-inhibited system by

Slater[7], and we have similar observations in the absence of inhibitor. It appears, therefore, that the A- and S-states are detectable not only by extrinsic but also by intrinsic probes in submitochondrial particles.

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