ON THE INTERPRETATION OF ENERGY LINKED 1-ANILINO-8-NAPHTHALENE SULFONIC ACID FLUORESCENCE CHANGES IN MITOCHONDRIAL FRAGMENTS

Angelo AZZI and Mario SANTATO

Institute of General Pathology and Center for the Study of Mitochondrial Physiology, University of Padova, Italy

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1. Introduction

The study of the energy transfer system of mitochondrial membranes has recently taken advantage of the use of fluorescent probes [1–4] to monitor structural changes associated with energy conservation [5–9].

A fluorescence increase of 1-anilinonaphthalene-8-sulfonic acid (ANS) occurs when mitochondrial fragments are "energized" either from respiratory substrates, ATP or by the use of a cationic gradient [10]. Such a fluorescence enhancement has been attributed to an increase in ANS binding as a consequence of an increased affinity of the membrane for the probe. In fact uncoupler and inhibitory sensitive binding changes can be measured by different techniques [7, 10] during cycles of "energization" and "de-energization" of the mitochondrial membrane.

An alternative explanation of the fluorescence changes of ANS, namely an increased quantum yield [11] of bound probe during energy conservation in mitochondria has been proposed (cf. however [12]).

Those ANS molecules having a higher quantum yield would decay to a low fluorescent state upon uncoupling, in a rapid first order reaction preceding in time other molecules slowly leading out of the membrane [13]. (Note however that the conclusion that the uncoupler induced fluorescence decay was the sum of two *consecutive* reactions was based on its analysis in terms of two *parallel* reactions with different rate constants [11]).

More recently, a mechanism was proposed [14] in which fast and slow fluorescence changes were considered the consequence of parallel first order reactions, in contrast with previous conclusions [13]. Moreover the state of "residual energization" [14] was described, in which ANS molecules bound to the membrane conserve a high quantum yield, despite the presence of an inhibitor such as KCN.

2. Methods and materials

Mitochondria and fragments were prepared according to previously published methods [15–17].

1, 8-ANS from K and K was recrystallized as Mg^{2+} salt. S_{13} (5-Cl, 3-t-butyl, 2′-Cl, 4′-NO_2-salicylanilide) was a gift of Dr. Metcalf of Monsanto Chemical Company. FCCP (carbonylcyanide p-trifluoromethoxyphenylhydrazone) was a gift of Dr. Heytler of DuPont Company. All other chemicals were reagent grade commercial products.

Fluorescence was measured in a Eppendorf filter fluorometer using a 366 nm interference filter for excitation, a Wratten 2 c for emission.

3. Results and discussion

3.1. *Comparison between the fluorescence quantum yield of ANS in mitochondrial fragments in the presence of ATP or succinate*

When the reciprocal of ANS fluorescence, measured in the presence of different amounts of membrane fragments is plotted as a function of the reciprocal of protein concentration a straight line is obtained. Similarly a straight line is obtained in the presence of succinate (fig. 1A) but the intercept with the ordinate is at a lower value. This finding has been interpreted as an increase in ANS quantum yield asso-

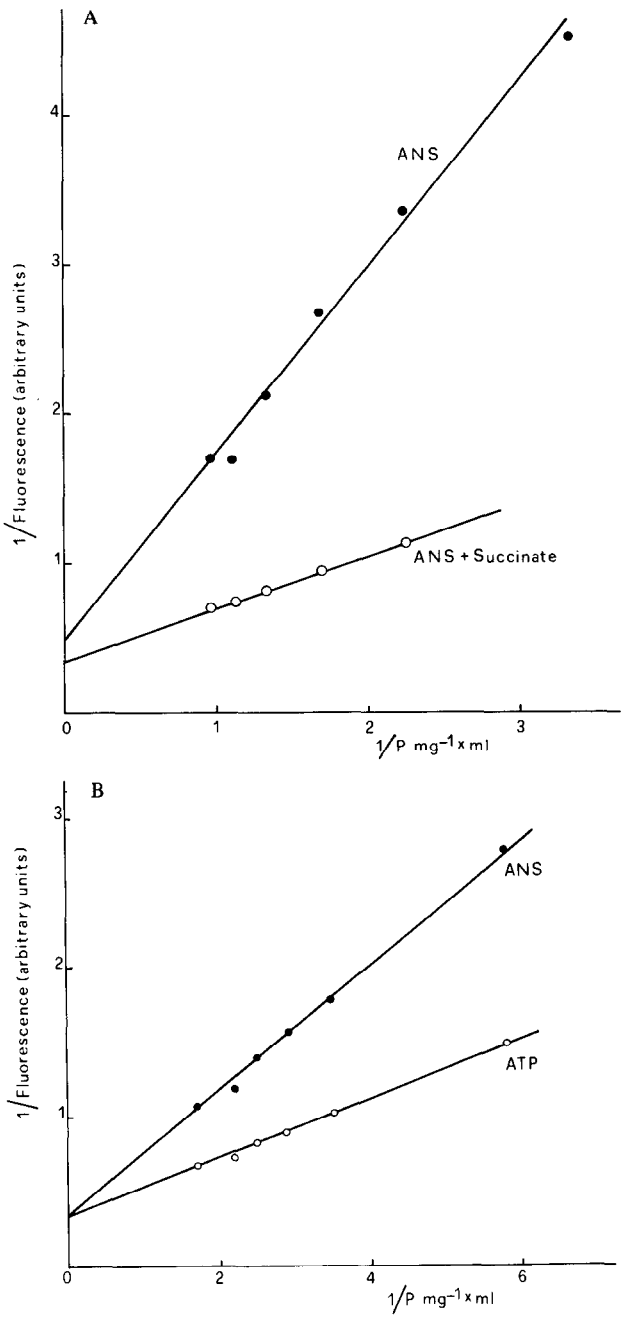

Fig. 1. ANS fluorescence changes in mitochondrial fragments as a function of protein concentration. The incubation medium contained: 250 mM sucrose, 5 mM Tris-HCl pH 7.5, 2 mM $MgCl_2$, 12.5 μM ANS. In A, (lower curve) 5 mM succinate (Tris salt) and in B, 1 mM ATP (Tris-salt) were also added. Protein concentration was varied according to the figure where data are in form of a double reciprocal plot.

ciated with energy conservation [11]. In fig. 1B is shown that the presence of ATP does not produce an increase in ANS quantum yield. Energy conservation cannot therefore be considered the cause of the quantum yield increase, in agreement with previous [10, 12], and also more recent data [14].

3.2. *Relationship between increase in ANS fluorescence yield and fast fluorescence changes induced by uncoupling*

Addition of uncouplers such as FCCP or S_{13} to membrane fragments in the presence of succinate has been shown [13] to result in a biphasic decay (fig. 2). The two first order processes have half-times of 2.5 and 12 sec.

When ATP is added instead of succinate, to energize the membrane fragments, the uncoupler also produces a biphasic decay and the kinetics, resolved as two parallel first order processes, which do not differ significantly from those produced when succinate is the substrate.

It appears from the comparison between fig. 1A

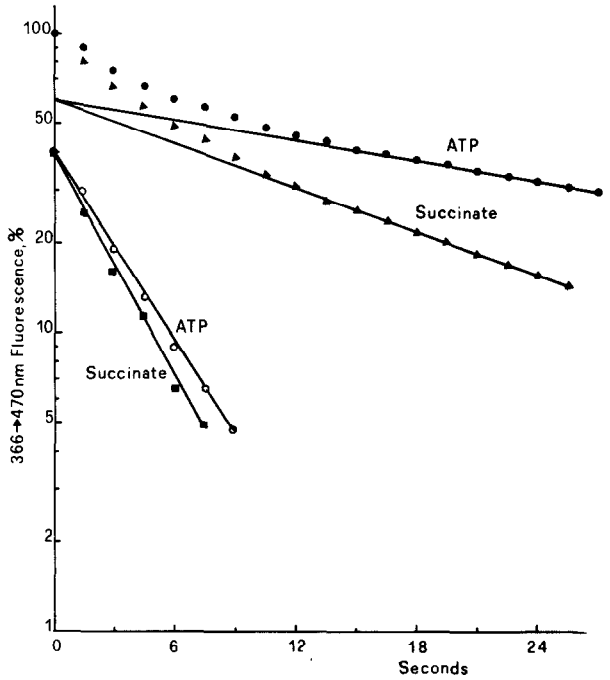

Fig. 2. Kinetics of S_{13} induced ANS fluorescence decay. Under the conditions of fig. 1 after the addition of 5 mM ATP or 1 mM succinate 2×10^{-7}M S_{13} was added. Protein concentration was 0.3 mg/ml. Data are presented in a semilog plot of % fluorescence *vs.* time.

and B with this figure that the fast fluorescence process is not related to the decay of ANS molecules with a high quantum yield. The fast fluorescence decrease is present when either ATP or succinate are used to energize the membrane, the high quantum yield with succinate only.

3.3. *Relationship between residual energization and the fast ANS fluorescence decay*

The relative magnitudes of the fluorescence components, decaying with different rate constants can be calculated by extrapolation of the slow process to zero ordinate in a semilog plot of the percent of fluorescence versus time (cf. [8]). In fig. 2 the fast component has a fluorescence emission corresponding to about 40% of the total. If, after addition of succinate, anaerobiosis is reached or 2 mM KCN is added, the extrapolated fluorescence quantum yield does not disappear [14]. Under these conditions 0.6 μM FCCP or S_{13} promote a fluorescence decrease of approx. 5% of the total fluorescence decay produced by KCN. This figure is 8 times smaller than that obtained by extrapolation of the kinetics of the slow component. Thus the two processes, "residual energization" and rapidly decaying fluorescent component do not seem to be quantitatively related.

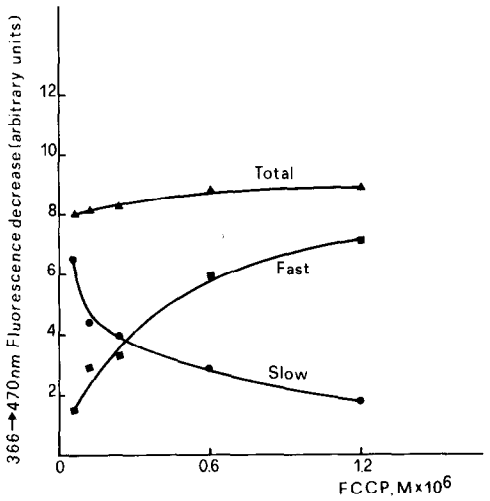

Fig. 3. Extent of fast and slow ANS fluorescence decrease as a function of FCCP concentration. Experimental conditions as in fig. 1; succinate was added as substrate and the fluorescence decrease induced by the different concentrations of FCCP indicated was measured. Fast and slow components were separated as in fig. 2.

3.4. *The effect of uncouplers concentration on the extent of the fast decaying fluorescent component*

In fig. 3 a plot of the extent of fluorescence decrease on addition of different concentrations of FCCP and the relative extents of fast and slow components is plotted.

The total fluorescence change remains approximately constant on adding 10^{-7}M FCCP or more (in the presence of 0.2 mg protein/ml). Instead the relative extents of fast and slow components are significantly different. At 1.2 μM FCCP the fast component accounts for more than 80% of the total while at 0.12 μM the fast is only 19% of the total. It appears therefore that fast and slow components do not pre-exist in definite relative amounts in the "energized state" (extrapolation at zero time after addition of uncoupler) but their relative extents depend upon the amount of uncoupler interacting with the membrane. Similarly the extent of the fast phase induced by addition of 0.5 μM S_{13} can be evaluated in the presence of different concentrations of ANS (fig. 4).

If the fast component were due to a number of

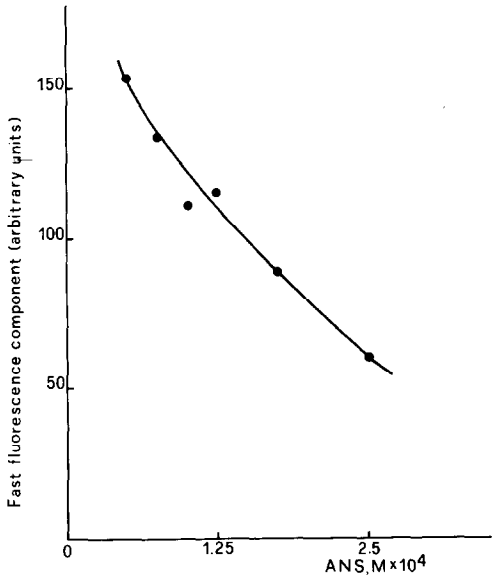

Fig. 4. Extent of fast fluorescence decrease as a function of ANS concentration. Experimental conditions as in fig. 1. ANS concentration was varied according to the figure. Protein concentration of the mitochondrial fragments was 0.3 mg/ml. Succinate (3 mM) produced a fluorescence increase which was inverted by 5×10^{-7}M S_{13}. The fast component was calculated as in fig. 2 for each ANS concentration.

bound ANS molecules decaying to a low fluorescence yield on addition of an uncoupler, one would expect that by increasing ANS concentration this component would either increase or remain constant depending on the degree of saturation of ANS binding sites responsible for this effect. On the other hand, the fast decaying component is smaller at high than at low ANS concentrations. This and the previous experiment suggest that the relative concentrations of ANS and uncoupler are of importance in the onset of the fast fluorescence decay process, this being larger at high uncoupler and low ANS concentrations. A direct interaction with the same binding sites on the membrane of ANS and uncouplers would result in an uncoupler induced detachment of ANS molecules larger at high uncoupler and low ANS concentrations. Viceversa a small release of ANS would be expected at low uncoupler, high ANS concentrations.

4. Conclusions

The fluorescence changes of ANS have been flexible and important indicators of the onset of energization in mitochondrial fragments [5, 19, 20]. The changes of ANS binding in fragments, opposite to those in intact mitochondria, have suggested that membrane fragments acquire an electrical charge more negative and mitochondria more positive on energy conservation [7].

Changes in fluorescence quantum yield extrapolated at low ANS/protein ratios [11] are not consequence of energization, since ATP does not produce them.

A pool of fluorescent molecules undergoing fast fluorescence quenching upon addition of FCCP or S_{13} does not exist prior to the addition of the uncoupler. The extent of the rapid fluorescence changes depend upon the relative concentrations of ANS and uncouplers and may be due to interaction of uncoupler and ANS with the same binding site. Moreover the fast reacting ANS molecules are in large excess with respect to those involved in the process of "residual energization". Finally they are not related to the changes in the apparent quantum yield observable under some conditions in mitochondrial fragments.

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