NANOSECOND FLUORESCENCE DECAY STUDY OF MITOCHONDRIA AND MITOCHONDRIAL MEMBRANES

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SUMMARY

The nanosecond fluorescence decay of the tryptophan residues of rat liver mitochondria and mitochondrial membranes has been measured. Apparent fluroescence lifetimes of 3 to 11 nanoseconds have been observed. Authentic membrane proteins suspended in 50% DMSO exhibit fluorescence properties almost identical to membrane proteins in situ. It is found that the fluorescent membrane components appear to remain firmly bound to the membranes in fresh preparations, but can be, at least in part, released into the solvent phase as a consequence of freeze thaw procedures.

INTRODUCTION: A thorough understanding of the function of membrane bound transport systems will only be achieved when their in situ molecular motion has been described. Recent efforts in this direction have employed exogenously supplied spin label or fluorescent probes (1, 2), to aid in elucidating the movement of lipids or protein in membranes. The main disadvantage of these methods is the uncertain location of the probes under varying physiological conditions. A more attractive approach to this problem is to deliniate the movement of well localized naturally occuring membrane components (3, 4). One such component is the naturally fluorescent tryptophan residue of membrane proteins. In this communication, we report the results of a study in which the fluorescence lifetime of quiescent mitochondrial membrane proteins was determined. Fluorescent components, presumed to be tryptophan residues of membrane proteins, are shown to be present in intact mitochondria, mitochondrial ghosts, inner membranes plus matrix, water washed inner membranes and membrane proteins. Moreover, it is

shown that unlike exogenously supplied probes these fluorescent membrane components remain firmly bound to the membrane in fresh preparations, but can be at least in part, released into the solvent phase as a consequence of freeze thaw procedures.

MATERIALS AND METHODS: Rat liver mitochondria were prepared as previously described (5). Inner membranes + matrix (IM+M) were prepared by the digitonin procedure of Schnaitman, Irwin and Greenawalt (6). Digitonin concentration was adjusted for optimal solubility of outer membrane using monoamine oxidase as a measure of outer membrane content. Monoamine oxidase activity was determined as described earlier (7). The IM+M fraction exhibited ADP acceptor respiratory control under the conditions described by Schnaitman and Greenawalt (8). Respiratory activity was determined polarographically (9). Water washed inner membranes (IM ghosts) were prepared by applying the procedure of Caplan and Greenawalt (10). The IM+M fraction was washed three times using one milliliter distilled water per gram original liver tissue. Whole mitochondrial ghosts (inner and outer membranes) were prepared as described earlier except that the membrane suspensions were pelleted at 43,500 X g in a Sorvall RC-2B centrifuge.

In freeze-thaw studies, freshly prepared mitochondrial ghosts were suspended in distilled water (20 mg ghost protein per ml.), frozen at -15° for 4 hours, thawed at room temperature, and centrifuged at 165,000 X g for 30 min., in a Spinco L-350 to separate membranes from the solvent phase.

Mitochondrial membrane proteins were prepared by a modification (11) of the method of Fleischer and Fleischer (12).

For fluorescence spectroscopy, mitochondria and IM+M were suspended in 0.25 M sucrose. Freshly prepared whole ghosts, freeze thawed whole ghosts and IM ghosts were suspended in distilled water.

Digitonin was purchased from Sigma Chemical Co. and recrystallized from hot ethanol. Protein assays were as previously described (13).

The fluorescence experiments were carried out on a modified Ortec nano-

second fluorescence spectrometer with single-photon counting. The technique has been discussed by several authors (14-16).

An air flash lamp was used as a light source. An exitation wavelength of 295 nm was selected by a Schoffel monochromator. The primary protein fluorphor under these conditions is the tryptophan residue with an emission maximum at about 350 nm. The sample was contained in a 2.0 ml, quartz cuvette mounted in the sample compartment to permit observation of front surface fluorescence. All fluorescence measurements were performed at room temperature.

RESULTS: As shown in Fig. 1, digitonin solubilizes outer mitochondrial membranes with the concommitant loss of the outer membrane marker enzyme monoamine oxidase. In our hands complete solubility of monoamine oxidase is achieved at 0.21 mg

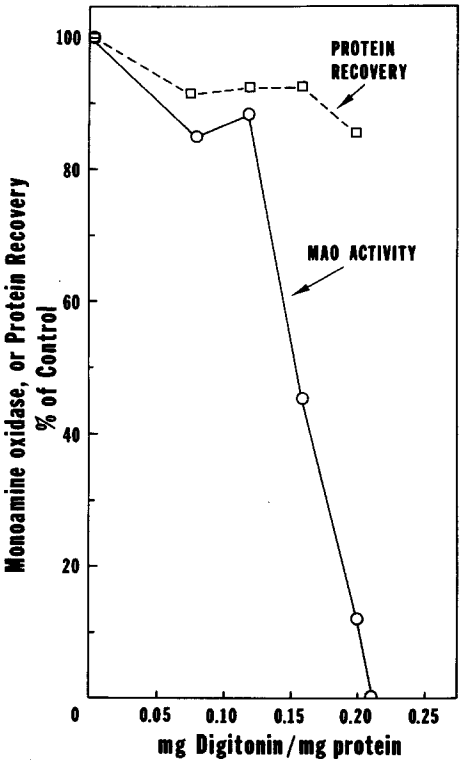

Fig. 1 - Digitonin solubalization of outer mitochondria membrane: Protein recovery is the percentage of a control without digitonin; MAO is monoamine oxidase activity measured in the pelleted fraction relative to that found in a control without digitonin.

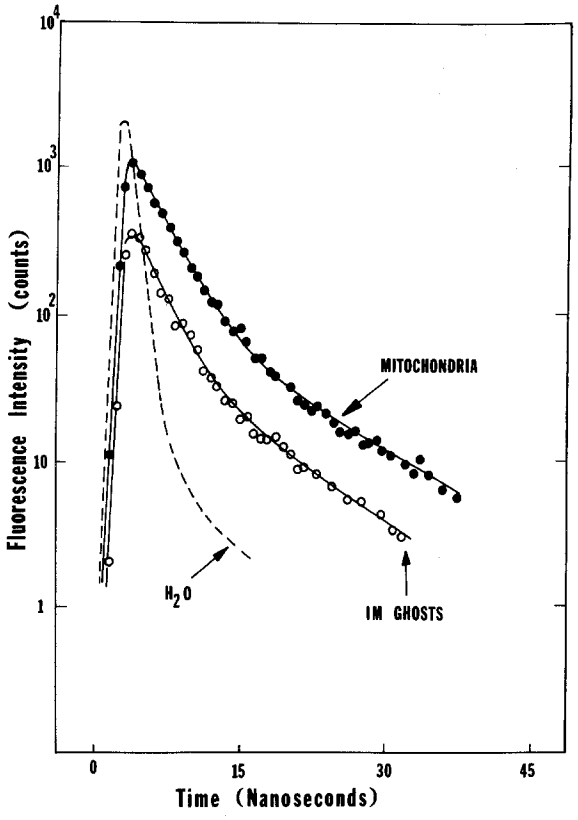

Fig. 2 - Fluorescence decay of intact rat liver mitochondria (1.0 mg/ml) IM ghosts (1.0 mg/ml) and water blank. Exitation wavelength, 295 nm.

digitonin per mg mitochondrial protein.

Figure 2 contains representative decay curves obtained from intact mitochondria, IM ghosts and water. The area under the decay curve is proportional to the sample fluorescence intensity while the apparent fluorescence lifetime is obtained from the slope(s) of the decay curve. The decay curves seem to indicate that only two main components are present with apparent lifetimes of 3-4 ns and 7.5-10.5 ns respectively. Like the foregoing membrane preparations, IM+M also exhibits fluorescence properties characterized by a lifetime similar to that of intact mitochondria.

Since it is of interest to determine the location of the fluorescing species, whole ghosts suspended in distilled water were centrifuged at 165,000 X g

for 30 min. and the supernatant solution examined for its fluorescent properties. The supernatant solution exhibited a fluorescence decay curve similar to that of whole ghosts but with the intensity decreased 40 fold (see Table 1). The membrane fluorophors thus appear to be rather firmly retained by fresh membrane

Table 1

PREPARATION	FLUORESCENCE INTENSITY
INTACT MITOCHONDRIA	100
WHOLE GHOSTS	41
FRESHLY PREPARED WHOLE GHOST WASH, SUPERNATANT FLUID	1
FROZEN WHOLE GHOST WASH, SUPERNATANT FLUID	6
INNER MEMBRANE + MATRIX	97
INNER MEMBRANE GHOSTS	28

Table 1: Relative fluorescence intensity of mitochondria and mitochondrial membranes. Supernatant fluids were obtained as described in the text, from 10 mg/ml suspensions of the various membrane preparations. Protein concentrations of membrane preparations are 1.0 mg/ml.

preparations. However, when whole ghosts suspended in distilled water are freeze-thawed and centrifuged at 164,000 X g for 30 min., the supernatant solution contains about 6 times more fluorescent material than the corresponding supernatant solution from fresh ghosts (Table 1). It is proposed that the phase transitions induced by the freeze-thaw procedure lead to the release of relatively loosely bound membrane proteins.

Of further interest in this study is a determination of the polar nature of biological membranes. Figure 3 indicates that purified membrane proteins suspended in 50% DMSO exhibit fluorescent properties virtually identical to natural membranes. The presence of two distinct lifetimes in 50% DMSO suggests the location of tryptophan residues in the interior of the proteins, in a hydro-

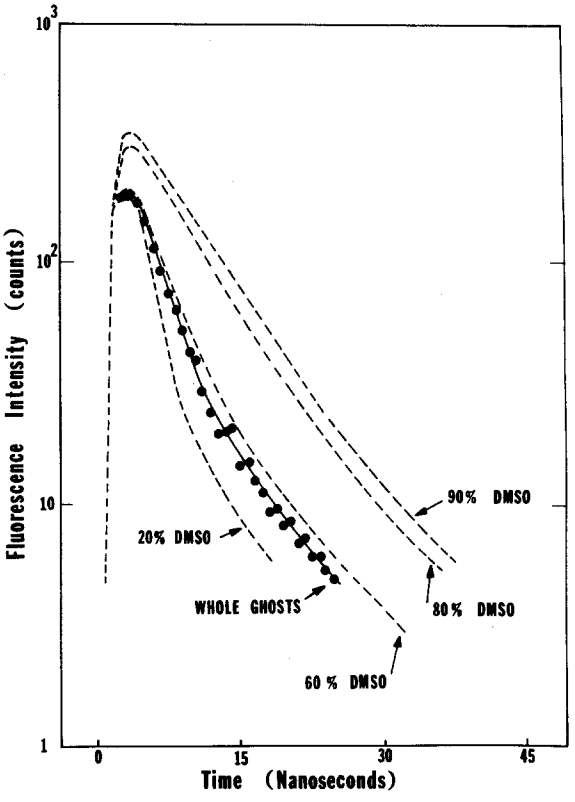

Fig. 3 - Fluorescence decay of isolated membrane proteins in DMSO - H_2O solutions (---) and whole ghosts in distilled water (-●-●-) Protein 1.0 mg/ml. DMSO is dimethylsufoxide.

phobic environment, and on the exterior exposed to the more polar 50% DMSO solution.

DISCUSSION: The emphasis of this investigation has been to demonstrate that tryptophan residues of proteins bound to mitochondrial membranes exhibit fluorescence decay in the nanosecond range and that the use of this naturally occurring fluorophor permits experimental evaluation of the physical state of biological membranes.

Since little information is available on the tryptophan content of membrane proteins, the identity of the tryptophan bearing membrane proteins remain obscure. However, of more importance in this study is the fact that the membrane fluorophors

observed here exhibit lifetimes of fluorescence decay similar to that of authentic membrane proteins in 50% DMSO solutions. From this observation it might be concluded that 50% DMSO approximates the environment of tryptophane residues in situ. However, 50% DMSO possesses a calculated (17) dielectric constant of 59 which is much greater than the value of 5.5 obtained from direct capacitance measurements of biological membranes (18). Azzi, et al (19) have shown that the flurophore Analino-Naphthalene-Sulfonic Acid (ANS) exhibits a fluorescence enhancement when bound to membranes similar to that of ANS in 80% ethanol. They have thus concluded that the environment of membrane bound ANS was equivalent to that of an 80% Ethanol solution. The dielectric constant of 80% Ethanol is 31, again far larger than that of biological membranes.

Since fluorescence intensity is dependent on a variety of factors including the dielectric constant of the environment and the availability of quenching species in that environment it may be concluded that simple fluorescence enhancement measurements in solutions such as in 50% DMSO or 80% Ethanol yield little information on the intramembrane environment of a probe such as ANS, or the tryptophane residue of membrane proteins. Conversly, in situ and in vitro determinations of fluorescence lifetimes and the relative intensity of species fluorescing with different lifetimes can allow an estimate of the proportion of probe in one or another environment.

From the results presented here we can infer that the lifetimes of 3-4 ns and 7.5-10.5 ns represent the localization of tryptophan residues in two major environments. These environments may be tentatively identified as possessing a high and low dielectric constant respectively and may in fact reflect the existance of tryptophan residues in the aqueous environment outside the membrane (highly polar, highly quenching)and within the membrane, (low polarity, small quenching). An extension of these measurements will ultimately permit us to estimate the porportions of membrane bound proteins buried within the lipid phase of the membrane and accessible to the aqueous environment.

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