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FLUORESCENT LABELLING OF THE MITOCHONDRIAL INNER MEMBRANE

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SUMMARY

Submitochondrial particles from beef heart have been labeled covalently with three fluorescent dyes.

1. 1-Dimethylaminonaphthalene-5-sulfonyl chloride (DNS chloride) and 2-toluidinonaphthalene-6-sulfonyl chloride (TNS chloride) caused marked inhibition of succinate oxidase (75%) and lesser inhibition of NADH oxidase (50 and 25%) at levels of 67 and 50 nmoles/mg protein, respectively. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole caused similar inhibition but at a level of 3 nmoles/mg protein.

2. All three labels affected the primary succinate dehydrogenase, but not the primary NADH dehydrogenase.

3. It is suggested that the major sites of action of these labels on the respiratory chain are to the oxygen side of the flavoproteins.

4. The fluorescence properties of DNS and TNS bound covalently suggest that the binding sites are similar to the non-covalent binding sites, and are in a hydrophobic environment and close to protein.

5. Covalently bound TNS did not display an energy-dependent response (unlike the noncovalently bound probe). The significance of this for the mechanism of energy dependent fluorescent probe responses is discussed.

INTRODUCTION

The widespread use of fluorescent probes for studying the structure and function of biological macromolecules has been the subject of a number of excellent reviews[1–3]. In principle fluorescent probe techniques offer a number of advantages (*e.g.* high sensitivity, several measurable parameters of fluorescence) over other spectroscopic techniques. However, they are limited by the difficulty of interpreting the data

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonate; DNS chloride, 1-dimethylaminonaphthalene-5-sulfonyl chloride; TNS chloride, 2-toluidinonaphthalene-6-sulfonyl chloride; 7-chloro-NBD, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; PMS, phenazine methosulfate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; NBD-S, conjugated form of NBD and an –SH group; NBD-N, conjugated form of NBD and an $-NH_2$ group.

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obtained. This has been particularly the case with studies of the inner mitochondrial membrane.

1-Anilinonaphthalene-8-sulfonate (ANS) fluorescence is markedly enhanced and blue shifted by fragments of the inner mitochondrial membrane. The fluorescence is further enhanced when the membrane is "energized" by addition of ATP or oxidizable substrate; this further enhancement is reversed by uncouplers of oxidative phosphorylation[4]. The "energy-dependent" ANS fluorescence enhancement has been variously interpreted as resulting from a change in ANS quantum yield (as a result of a membrane conformational change) together with a change in binding parameters[5,6], a change in binding parameters only[7,8], or ANS migration along a membrane gradient[9].

In addition to the above differences of opinion, there is considerable contention over the location of the ANS binding sites in the membrane. Different workers prefer the protein[10], the lipids[11], or aquous/non-aquous interfaces[5,12] as the major areas of binding.

In an attempt to overcome these difficulties we have covalently labeled submitochondrial particles from beef heart with three fluorescent labels. 1-Dimethylaminonaphthalene-5-sulfonyl chloride (DNS chloride) — a commonly used fluorescent label — was used to determine optimum conditions for labeling. 2-Toluidinonaphthalene-6-sulfonate chloride (TNS chloride), which was expected to react like DNS chloride, would produce a conjugate with fluorescence properties much more like those of ANS than DNS. This label should tell whether or not movement is involved in the ANS response. 7-Chloro-4-nitrobenzo-2-oxo-1,3-diazole (7-chloro-NBD) has the novel property of being fluorescent only when it is conjugated to $-NH_2$ or $-SH$ groups[13]. Its fluorescence and absorption spectra vary with the type of group to which it is attached — which should give us some information on the nature of the covalent binding sites.

A preliminary report of this work has been published[14].

MATERIALS AND METHODS

Submitochondrial particles[15] derived from heavy beef heart mitochondria by sonication in the presence of EDTA were used throughout the studies presented here.

Labeled particles were prepared by several methods (see Results and Discussion). The extent of labeling was estimated spectrophotometrically. The absorbance of labeled samples (clarified by addition of 0.2% deoxycholate) were measured at 295 nm (corresponding to a trough in the DNS and TNS absorption spectra) and at the absorption maxima of the labels (DNS, 330 nm; TNS, 318 nm; conjugated form of NBD and an $-SH$ group (NBD-S), 420 nm; conjugated form of NBD and an $-NH_2$ group (NBD-N), 480 nm). The ratios of extinction coefficients for EDTA submitochondrial particles and the three labels, at 295 nm to the extinction coefficients at the absorption maxima of the labels were derived from absorption spectra of unlabeled EDTA submitochondrial particles, and the free labels in 50% aqueous ethanol. These ratios were used to solve simultaneous equations from which the amount of incorporated label (nmoles/mg protein) could be derived. This method proved to be very inaccurate for NBD due to interference by the Soret

absorption bands of the particles. NBD content was estimated by running parallel experiments and measuring the increase in absorbance at 420–550 or 480–550 nm with a Johnson Foundation dual wavelength spectrophotometer during a 15-min incubation.

Fluorescence data were obtained with a Perkin–Elmer Hitachi spectrofluorimeter (Model MPF-2A). Succinate (succinate:O_2) and NADH (NADH:O_2) oxidoreductases were measured polarographically with a Clark oxygen electrode. Succinate:phenazine methosulfate (PMS) oxidoreductase and NADH:ferricyanide oxidoreductase were assayed spectrophotometrically with a Zeiss spectrophotometer according to the methods of King[16] and King and Howard[17], respectively. Protein content was determined by the method of Lowry *et al.*[18] using crystalline bovine serum albumin as standard.

DNS chloride was obtained from Sigma, TNS chloride was synthesized and generously donated by Dr B. T. Storey, 7-chloro-NBD was synthesized by the method of Boulton *et al.*[19] and was a kind gift of Dr N.C. Price. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a gift of Dr P. Heytler, E. I. duPont de Nemours, Wilmington, Del. All other chemicals were of the purest grades available commercially. Glass redistilled water was used throughout.

RESULTS AND DISCUSSION

Several methods of covalently labeling submitochondrial particles were employed, the one used for the results reported here being the most satisfactory according to the following criteria: (a) mild, rapid reaction with the membrane; (b) easy removal of unreacted and hydrolysed labeling agent; (c) reproducibility both of extent of labeling and of effect on various properties of the submitochondrial membrane.

The three labeling agents used are very insoluble in water, so a means of dispersing them was needed in order to attain a rapid reaction. Using our previous experience with fluorescent labeling of enzymes[20] we first tried using the method of Rinderknecht[21,22] of dispersing the labeling agent on a solid support (celite). This produced a mild rapid reaction, and the unreacted label was easy to remove by low speed centrifugation. However, the results were very difficult to reproduce due to inhomogeneous dispersion of the labeling agent on the celite.

The method of dispersion used in the present studies was by organic solvent. Of the readily available water-miscible organic solvents, dimethyl formamide is the least damaging to submitochondrial particles, so this was used throughout these studies. The minimum volume consistent with good dispersion was used (usually 2.5% by vol.). However, this method of dispersion means that it is not so easy to separate unreacted and hydrolysed labeling agent from the labeled particles. Initially bovine serum albumin (approx. 2 mg/ml) was used to absorb any noncovalently bound labels, but this resulted in anomalously enhanced respiratory control ratios, especially for NADH oxidase. Repeated washing of the samples resulted in considerable damage and loss of activity, with no appreciable decrease in contamination (as measured by fluorescence) (Fig 1). We found that the best method of removing excess reagent was to use a fairly large volume of quenching medium and to spin the samples out of this, taking care to wash the pellets thoroughly. This treatment resulted in

samples which upon further washing gave no decrease in fluorescence, indicating that none of the fluorescent label present could be removed by washing. Presumably it was bound covalently.

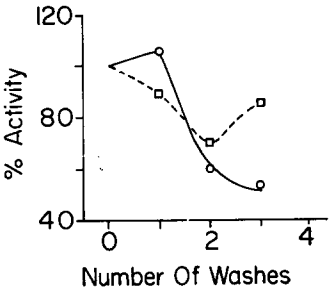

Fig. 1. Effect of washing on particle activity. The particles were diluted to 2.5 mg protein per ml with 0.25 M sucrose and sedimented at 38000 rev./min for 45 min. Part of the pellet was resuspended to a concentration of approx. 20 mg/ml; the remainder was suspended to 2.5 mg/ml (both in 0.25 M sucrose) and sedimented again. This process was repeated twice, and the retained portions were assayed for NADH oxidase (□---□) and succinate oxidase (○—○) activities. The activities were expressed as a percentage of the activity of the starting material. The rates of NADH oxidase and succinate oxidase of the untreated particles were 550 and 240 nmoles/min per mg protein, respectively.

The method used for the results presented here is as follows: the particle preparation was diluted to a concentration of 5 mg protein per ml in a medium consisting of 150 mM sucrose and 50 mM sodium phosphate, pH 7.5, and incubated at 25 °C in a shaking waterbath. Reaction was initiated by adding dimethyl formamide and labeling agent dissolved in dimethyl formamide (to a total of 0.1 ml dimethyl formamide per 4 ml of sample) and mixing thoroughly. After incubation for 15 min at 25 °C the reaction was quenched by mixing the sample with 12 ml cold medium (150 mM sucrose 30 mM Tris–acetate, pH 7.5). The sample was then spun down (Spinco Model L2-65B, 40 rotor, 38000 rev./min for 45 min). The pellet was rinsed well with 0.25 M sucrose, and resuspended in 0.25 M sucrose to a concentration of 15–20 mg protein per ml. In order to study several labeling agent concentrations it was convenient to initiate (and quench) the reaction of a series of samples at 30-s intervals.

Effect of labeling on respiratory chain activities

DNS chloride. As can be seen in Fig. 2A, covalent labeling of submitochondrial particles with DNS chloride resulted in a marked inhibition of both succinate and NADH oxidases. The succinate oxidase was more susceptible, being 75% inhibited when NADH oxidase was only 50% inhibited and when 67 nmoles DNS were bound per mg of membrane protein. The scatter of data at low DNS levels results mainly from the uncertainty in estimating the amount of DNS incorporated at low concentrations. Clearly the majority of the DNS is bound non-specifically, the observed effects being caused by a small percentage of the bound labels. These data indicate that the major sites of action for DNS chloride are on the section of the respiratory chain wich is common to the two oxidases. The higher susceptibility of succinate oxidase suggested that the primary succinate dehydrogenase was also affected by

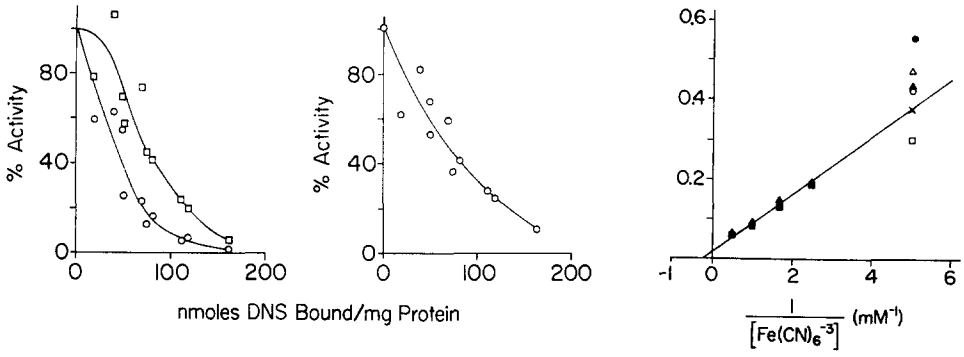

Fig. 2. (A) Effect of DNS on the oxidase activities. The reaction mixture consisted of 150 mM sucrose, 30 mM Tris–acetate, pH 7.5, and 0.5–2 mg of protein of sample. The reaction was started by addition of 3.3 mM Tris–succinate (○—○) or 200 μM NADH (□—□). Total volume: 3.0 ml, temperature: 25 °C. The activities are expressed as percentages of the activities of unlabeled particles (succinate oxidase and NADH oxidase 320 and 790 nmoles/min. per mg protein respectively). (B) Effect of DNS on succinate–PMS reductase activity. The reaction mixture consisted of 50 mM sodium phosphate, pH 7.5, 150 mM sucrose, 33.3 mM succinate, 0.2% bovine serum albumin, 1.7 mM KCN, 100 mM dichlorophenol indophenol and 15–150 μM PMS. Total volume: 3.0 ml, temperature: 25 °C. Reaction was initiated by the addition of 0.4 mg of protein of sample. V for each sample was obtained by extrapolation of a (velocity)$^{-1}$ *vs* [PMS]$^{-1}$ plot, and the activity plotted here is V as a percentage of V for unlabeled particles. V (for PMS) for unlabeled particles was 230 nmoles/min per mg protein. (C) Effect of DNS on NADH–ferricyanide reductase activity. The reaction mixture consisted of 150 mM sucrose, 50 mM sodium phosphate, pH 7.5, 0.2–2 mM potassium ferricyanide, 0.43 μM rotenone and 0.07 mg of protein of sample. Total volume: 3.0 ml. temperature: 25 °C. Reaction was initiated by addition of 233 μM NADH. Samples contained 0 (x—x); 22 (○—○); 33 (△—△) 48 (□—□); 76 (▲—▲) and 111 (●—●) nmoles DNS/mg protein. The straight line is drawn for unlabeled sample. V (for ferricyanide) was 83 μmoles/min per mg protein.

reaction with DNS chloride. That this is the case is demonstrated in Fig. 2B. The enzyme was assayed as succinate–PMS reductase, the percentage activity being obtained from the maximum velocities extrapolated from a double reciprocal plot. Although the primary dehydrogenase is affected, it behaves in these plots more like NADH oxidase than succinate oxidase (*cf.* Figs 2A and 2B). Unlike succinate dehydrogenase, the primary NADH dehydrogenase is unaffected by treatment with DNS chloride (Fig. 2C). The enzyme was assayed as NADH–ferricyanide reductase, and the results are presented as a double reciprocal plot. The straight line is drawn for the unlabeled enzyme.

TNS chloride. As might be expected, the results for TNS chloride (Fig. 3) are qualitatively the same as those for DNS chloride. Both NADH and succinate oxidase are inhibited (Fig. 3A), though the difference between them is much more marked: when succinate oxidase is 75% inhibited, NADH oxidase is only 25% inhibited and 50 nmoles TNS are bound per mg membrane protein. It is also worth noting that at any given level of labeling succinate oxidase is more inhibited and NADH oxidase less inhibited than with DNS. The curve obtained for succinate–PMS reductase (Fig. 3B) is almost identical with that obtained with DNS and falls between the two oxidase curves for TNS. There is also no inhibition of NADH–ferricyanide reductase (Fig. 3C). Thus TNS chloride also gives a large amount of unspecific labeling, but

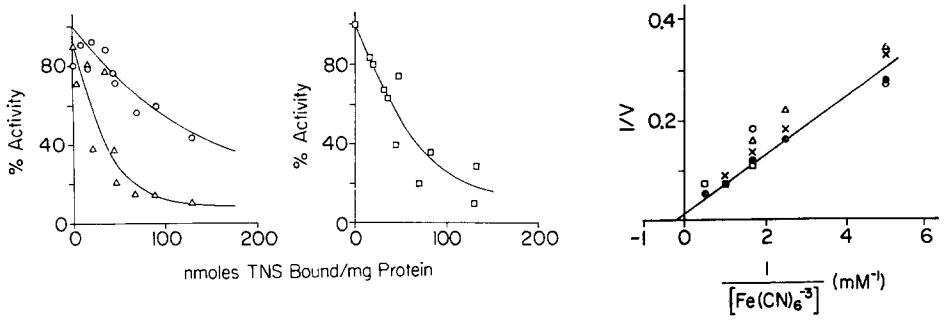

Fig. 3. Effect of TNS on: (A) the oxidase activities; (B) succinate–PMS reductase activity; (C) NADH–ferricyanide reductase activity. Samples contained 0 (●—●); 15 (○—○); 34 (□—□); 43 (x—x); 69 (△—△) nmoles TNS per mg protein. The straight line is drawn for unlabeled sample. Other conditions were the same as in Fig. 2. The rates of NADH oxidase and succinate oxidase of the unlabeled particles were 833 and 235 nmoles/min per mg protein, respectively. V (for PMS) was 240 nmoles/min per mg protein for succinate–PMS reductase; and V (for ferricyanide) was 83 μmoles/min per mg protein for NADH–ferricyanide reductase of the unlabeled particles.

the preferential labeling is much more evenly distributed between the primary succinate dehydrogenase and the common pathway than is the case for DNS chloride.

7-Chloro-NBD. The obvious difference between this and the previous two labels is that it is much more specific (Fig. 4), the level of labeling necessary for inhibition being approximately 20-fold lower than for DNS chloride or TNS chloride. Both oxidases are inhibited (Fig. 4A), succinate oxidase being 75% inhibited when NADH oxidase is only 35% inhibited and where 3 nmoles NBD are bound per mg membrane protein. As with DNS chloride the inhibition curve for succinate–PMS reductase is more like the curve for NADH oxidase (Figs 4A and 4B). Once again the NADH–ferricyanide activity is unaffected by incorporation of these levels of NBD (Fig. 4C). Thus for all three labels studied the major site of action appears to be on the common

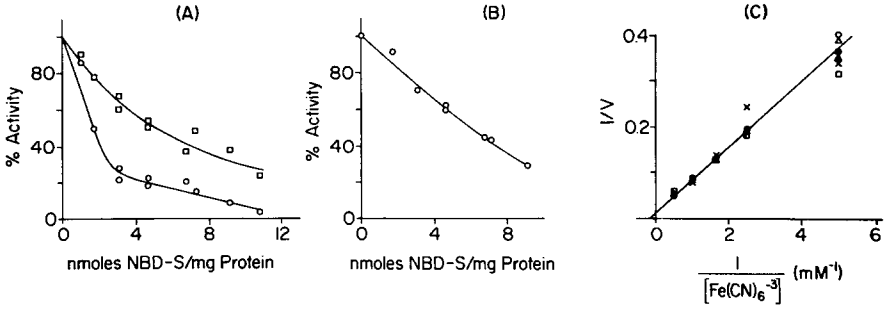

Fig. 4. Effect of NBD on: (A) the oxidase activities; (B) succinate–PMS reductase; (C) NADH–ferricyanide reductase activity. Samples contained 0 (●—●); 3.0 (x—x); 4.6 (□—□); 7.2 (▲—▲); 9.1 (△—△); 10.7 (○—○) nmoles NBD-S per mg protein. The straight line is drawn for unlabeled sample. Other conditions were the same as in Fig. 2. The rates of NADH oxidase and succinate oxidase of the unlabeled particles were 870 and 240 nmoles/min per mg protein, respectively. V (for PMS) was 230 nmoles/min per mg protein for succinate–PMS reductase; and V (for ferricyanide) was 83 μmoles/min per mg protein for NADH–ferricyanide reductase of the unlabeled particles.

pathway (*i.e.* to the oxygen side of the flavoproteins) with varying effects on the primary succinate dehydrogenase.

In principle 7-chloro-NBD offers two distinct advantages over DNS chloride and TNS chloride which should make it a good fluorescent label. When it reacts with nucleophiles (-NH_2 or -SH groups) new absorption bands appear which have maxima characteristic of the type of group with which the 7-chloro-NBD has reacted. Thus, it is possible (i) to determine what groups have been labeled, and whether they are essential for enzymatic activity and (ii) to follow the progress of the labeling reaction continuously, and thus to analyse the kinetics of the reaction. Both of these approaches were used here.

It was found that the absorption peak of NBD in the labeled particles overlapped that of the Soret region of the cytochromes. This made determination of the degree of labeling very difficult. The values used in Fig. 4 were therefore obtained by running parallel experiments in a Johnson Foundation dual wavelength spectrophotometer and measuring the change in absorption resulting from reaction of different concentrations of 7-chloro-NBD with a fixed concentration of EDTA submitochondrial particles. This was done both at 420 nm (the NBS-S peak) and at 480 nm (the NBD-NH peak), and the amounts of the two types of adducts were calculated using the published extinction coeffients. In practice, the amounts of NBD-NH resulting from initial reaction were negligable, so a calibration curve (Fig. 5) was constructed assuming NBD-S was the only adduct. This was used to estimate the amounts of incorporated NBD used in Fig. 4.

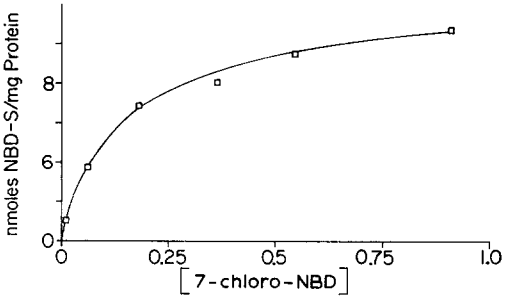

Fig. 5. Calibration curve for NBD binding. The reaction mixture consisted of 150 mM sucrose, 50 mM sodium phosphate, pH 7.5; 15 mg of particle protein. Total volume: 3.0 ml, temperature: 25 °C. To this were added different volumes of 37.5 mM 7-chloro-NBD dissolved in dimethyl formamide to initiate the reaction. The increase in absorbance at 420–550 nm was followed with a Johnson Foundation dual wavelength spectrophotometer. The amounts of NBD bound were calculated using an extinction coefficient of $E_{420-550\ nm} = 12\ mM^{-1}$ for NBD-S (ref. 13). Abscissa: concentration in mM.

The small amount of NBD required to bring about inhibition suggested that specific essential -SH or -NH_2 groups were being labeled. However, preliminary kinetic analysis reaction curves used to construct Fig. 5 showed that no kinetically similar sets of groups could be identified as being responsible for loss of activity.

The results presented here, while being qualitatively the same as those reported earlier[14] do differ in detail. These differences are due mainly to the fact that the preliminary report used particles which had been over-washed, and which consequently were somewhat damaged, as well as being inhibited by the labeling process.

Fluorescence spectra

The fluorescence spectra of the DNS- and TNS-labeled particles were very similar to those obtained with the non-covalently bound probes[20,23] (*i.e.* excitation maxima at 340 and 332–370 nm, and emission maxima at 505 and 445 nm for DNS-EDTA submitochondrial particles and TNS-EDTA submitochondrial particles, respectively). The spectra for NBD-EDTA submitochondrial particles are shown in Fig. 6. The excitation peak is distorted by the xenon lamp line spectrum of the instrument, but an experiment using a dual wavelenght fluorimeter designed in the Johnson Foundation which uses a tungsten lamp seems to indicate that the true excitation peak is at 480 nm. This is in apparent contradiction of the absorption data

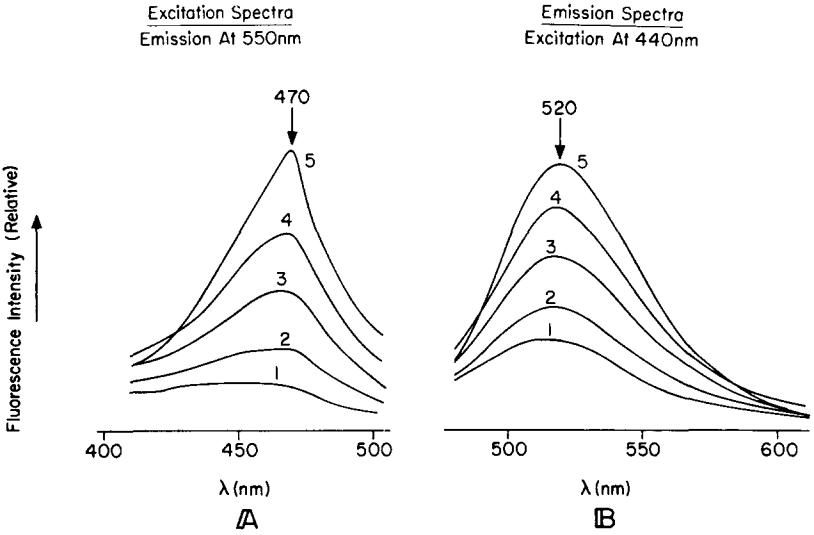

Fig. 6. Fluorescence spectra of NBD-labeled particles. Samples of labeled particles were diluted to 0.5 mg protein per ml in a medium of 150 mM sucrose, 30 mM Tris–acetate, pH 7.5, temperature 25 °C. Emission spectra were recorded using 440 nm for excitation; excitation spectra were recorded using 550 nm for emission. In both cases, bandwidths of 6 nm were used in both emission and excitation beams. The appropriate amounts of NBD bound were: (1) 1.7; (2) 3.0; (3) 4.3; (4) 7.0; (5) 10.0 nmoles/mg protein.

quoted above which indicated the presence of NBD-S only (and hence absorption and fluorescence excitation maxima at 420 nm). Because of this, the absorption spectra of NBD-EDTA submitochondrial particles were redetermined. It seems that especially at high NBD concentrations, a long wavelength shoulder appears in the absorption spectrum on standing (Fig. 7). This could be due to S→N transfer of NBD of the type reported by Birkett *et al.*[13]. This would partially explain the anomalous fluorescence excitation spectra since the NBD-N compounds are much more fluorescent than the corresponding NBD-S derivatives[13].

All these labels quenched protein tryptophan fluorescence (up to 80%, Figs 8–10). In the cases of DNS and TNS this quenching is at least in part due to energy transfer since the quenching was accompanied by an enhanced label fluorescence excited at 270 nm (Figs 8–10). Since energy transfer[24] is observed, the maximum tryptophan–label distance can be estimated as 50 Å. A more precise determination

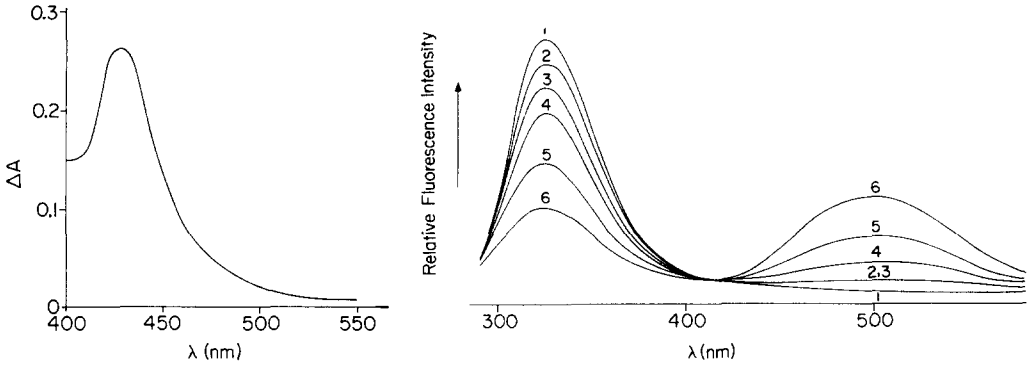

Fig. 7. Absorption spectrum of NBD-labeled particles. The sample consisted of 5 mg protein per ml medium (150 mM sucrose, 30 mM Tris–acetate, pH 7.5). Temperature: 25 °C. The spectrum was measured in a Johnson Foundation spilt beam spectrophotometer. A base-line (medium–medium) was run, then the requisite volume of labeled particles was added to the sample cuvette and a second spectrum was scanned. The difference between these is plotted here. Amount of NBD bound = 3.3 nmoles/mg protein.

Fig. 8. Fluorescence emission spectrum of DNS particles. Samples were diluted to 0.1 mg protein per ml in 150 mM sucrose, 30 mM Tris–acetate, pH 7.5, temperature 25 °C. The spectra were excited at 270 nm with excitation and emission slit-widths of 8 and 4 nm, respectively. Extents of labeling were: (1) 0; (2) 22; (3) 33; (4) 48; (5) 76; (6) 111 nmoles DNS/mg protein.

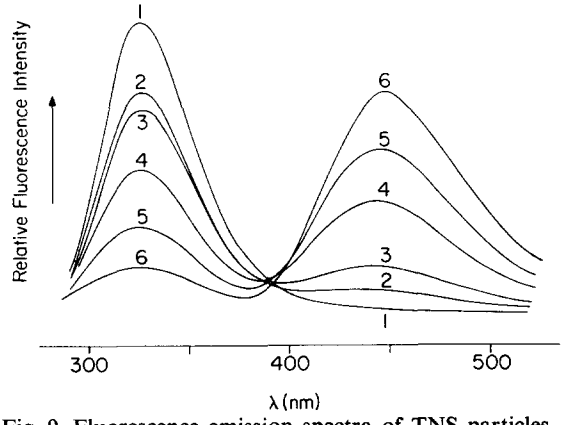

Fig. 9. Fluorescence emission spectra of TNS particles. Conditions were the same as in Fig. 8. Extents of labeling were: (1) 0; (2) 15; (3) 34; (4) 43; (5) 69; (6) 129 nmoles TNS/mg protein.

will be possible when fluorescence life time data have been obtained. However, the percentage quenching is a good measure of the amount of label bound to the particles, and gives much less scatter in activity plots like Figs 2–4. Unfortunately it is not easy to quantitate the data in terms of actual amounts bound so we had to resort to the methods discussed above.

The data reported above give some indication of the nature of the binding sites of these labels. DNS and TNS are binding in hydrophobic regions (as judged from their emission maxima) and are quite close to membrane proteins (energy transfer from tryptophan). These sites appear to be very similar to those occupied by

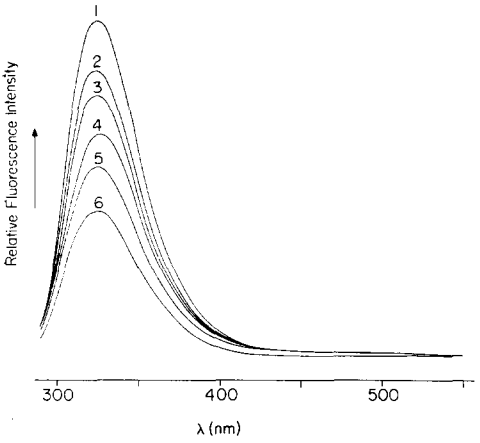

Fig. 10. Fluorescence emission spectra of NBD particles. Conditions were the same as in Fig. 8 except that a concentration of 0.5 mg protein per ml was used. Extents of labeling were: (1) 0; (2) 3.0; (3) 4.6; (4) 7.2; (5) 9.1; (6) 10.7 nmoles NBD-S per mg protein.

ANS and TNS when they bind non-covalently to submitochondrial particles[5,23]. This leads us to expect that covalently bound TNS should exhibit an energy-dependent response provided that probe movement is unimportant in the energy-dependent response. However, no energy-dependent response could be detected with either NADH or succinate as substrates, for any of the three labels, under conditions where the particles were at least 50% active and where labeled particles showed a normal response with non-covalently bound ANS. Under these conditions a small rapid response was observed similar to that reported by Chance *et al.*[25].

In order to investigate the origin of this rapid response a small cuvette was constructed to fit the normal 10 mm × 10 mm one. A DNS–bovine serum albumin solution was placed in the inner cuvette, while this inner cuvette was surrounded by a suspension of submitochondrial particles. When the particles were energized (by succinate or NADH) the DNS–bovine serum albumin fluorescence underwent a small rapid enhancement similar to that observed with labeled particles (*i.e.* under conditions where there was no contact between the probe and the particles). This indicates that the rapid response is an artifact caused by changes in absorption of the particles at the emission and/or excitation wavelength used.

This absence of an energy dependent response could be the result of a number of things, for example: (1) The covalent labels bind in the wrong place to be able to detect the energy-dependent changes. (2) As with all other chemical modification studies, the binding could be heterogeneous. Since the measured effect is an average, it is quite possible that 50% inhibition represents a situation where 50% of the particles are labeled and 100% inhibited while 50% are unlabeled and 100% active. If this were the case it is unlikely that the covalently bound probe would be able to detect the energy dependent change.

The first of these difficulties is not easy to get around. We cannot be certain that TNS is bound covalently in the same sort of site as it binds non-covalently (despite the similarity of the fluorescence parameters). However, it would seem

likely that with such heavy labeling (up to 150 nmoles/mg protein) at least a few TNS molecules will be in the right place to see an energy dependent change.

In order to test the second difficulty the extent of reduction of cytochromes *a*, b_K, b_T and *c* was measured both at the steady state, and at anaerobiosis with both succinate and NADH as substrates. No difference could be detected between a sample of TNS-labeled particles which was 50% inhibited (with respect to succinoxidase) and the control particles except that in the presence of oligomycin, cytochromes *b* and b_T were approximately 60% less reduced (by succinate) in the steady state in TNS-labeled particles. This merely indicates that in the presence of oligomycin, the rate limiting step is at the level of succinate dehydrogenase (*cf.* ref. 26). Thus even if some of the respiratory chains are completely inhibited by TNS, all the cytochromes are reducible, presumably *via* chain-branching mechanisms[26-28].

What is the mechanism of the ANS energy-dependent response?

There is considerable disagreement about the mechanism of the ANS energy-dependent response (see Introduction); suggestions ranging from quantum yield changes associated with membrane conformational changes[5,6] through movement of ANS along a membrane gradient[9] to changes in binding parameters[7].

The measurement of binding parameters in a heterogeneous system such as this is difficult. Changes in these parameters due to energization are even more difficult to quantitate since the energized membrane is essentially a transient species. Radda *et al.*[5,6] base their conclusions on binding parameters derived from fluorescence data[5] and confirmed with a filtration technique[20]. On the other hand, Azzi's claim that ANS fluorescence changes are solely the result of binding changes[7] is based on data derived by a centrifugation technique[29] under conditions where it seems likely that what is being measured is merely a difference between aerobic and anaerobic samples. It should be noted that changes in binding parameters or quantum yield will both reflect a change in membrane structure, whereas a movement of ANS along a membrane gradient does not necessarily reflect a membrane structural change.

The data presented here indicate that when TNS is attached to the membrane covalently, it is unable to respond in an energy-dependent manner. This suggests that movement of the probe relative to the membrane (either by a membrane conformational change or by movement of the probe along a membrane gradient) is necessary for the energy-dependent response. The movement of ANS to a new environment (which causes the change in quantum yield) is supported by a large body of evidence[30]. This, together with the evidence presented here, suggests that upon energization ANS moves to its new environment probably as a counter ion along a cation gradient, and that more ANS is bound to the original binding sites (*i.e.* the non-energized ones) to replace that which has moved. Upon de-energization the reverse process takes place. The fast phase of the uncoupler kinetics of the ANS response[5] is much faster than the cation efflux caused by uncoupling[31]. However, the cation efflux following addition of valinomycin is very fast[31] so rapid ion movement is possible, and the observed ANS effects could be associated with it.

Recently Barrett-Bee and Radda[6] observed that the quantum yield change and the change in binding can be separated operationally, and proposed a two-state model for the energization process. Using this model we suggest that ANS movement is essential in the resting→energized A state transition, but that the A→S energized

transition requires a change in the environment to which ANS has moved to produce a change in quantum yield.

The chemical modification aspect of these studies is probably the most interesting in terms of pointers for future research. 7-Chloro-NBD under the correct conditions, will preferentially inhibit succinate dehydrogenase. The course of its reaction may be followed spectrophotometrically, and kinetic analysis of the reaction may yield important information on the type of group which is essential for enzymic activity. It would also be of great interest to label particles in the energized state (*cf.* ref.32) to see if there are any marked differences in reactivity, type or number of groups modified. We have performed preliminary experiments which indicate that great care is needed to avoid side reactions such as reduction of the labeling agent by the energized membrane.

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