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Photoaffinity labelling of submitochondrial membranes with the 3-azido analogue of 9-amino-3-chloro-7-methoxyacridine

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9-Amino-3-azido-7-methoxyacridine has been synthesized and shown to be a suitable photoaffinity probe for the site(s) of interaction of 9-amino-3-chloro-7-methoxyacridine with submitochondrial membranes. Both the excitation and emission spectra of the azido analogue covalently bound to membranes in the energized state display distinctive differences from the spectra of labelled, non-energized membranes (i. e., in the absence of oxidizable substrate, or its presence when uncoupler (FCCP) is also present during photolysis). Enzymatic analyses indicate that the probe interacts with the ATPase and the respiratory chain enzymes; energization appears to afford some protection against inactivation. Electrophoresis of the labelled membranes and isolation of their lipid and protein components indicate that the spectral differences are attributable to differing interactions with the lipid components of energized, relative to non-energized, membranes. Similar results have been obtained with the 3-azido analogue of quinacrine (Mueller, D.M., Hudson, R.A. and Lee, C.P. (1982) Biochemistry 21, 1445–1453), which differs significantly, however, in the extent of its interactions with the enzymes of the respiratory chain and the ATPase. These results indicate that the energy-linked fluorescence responses of 9-aminoacridines with submitochondrial membranes arise from direct interactions with membrane components and may involve redistribution of the probe molecules and/or alteration of their microenvironments upon energization.

Introduction

The 9-aminoacridine dyes and their derivatives represent a class of highly fluorescent compounds which interact with a variety of macromolecular systems in such a manner as to yield potentially invaluable information as to the structural and functional relationships which exist among their various components. Indeed, 9-aminoacridine (9AA), 9-amino-3-chloro-7-methoxyacridine (9ACMA, may also be read as 9-amino-6-chloro-2-methoxyacridine), and, especially, quinacrine (QA), among others, have been used extensively to probe such systems, e.g., as an intercalating dye with DNA [2–6], as a fluorescent, local anesthetic probe of electroplaque preparations [7,8] and mammalian axonal membranes [9], and to report on energy-linked changes associated with the generation of a proton gradient in chromatophores [10], chloroplasts [11], and submitochondrial particles [12,13]. The complex nature of many of these systems, coupled with the environmental sensitiv-

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Abbreviations FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl-hydrazone; 9AA, 9-aminoacridine; 9ACMA, 9-amino-3-chloro-7-methoxy-acridine (may also be read as 9-amino-6-chloro-2-methoxyacridine); QA, quinacrine; PMS, phenazine methosulphate.

ity of the dyes' fluorescence properties, has, in many cases, complicated the interpretations of the probes' responses and the elucidation of the sites and mechanisms of interaction.

9AA, 9ACMA and QA, of which only QA possesses a bulky side chain at the 9-position of the acridine nucleus, when associated with energy-transducing membranes display characteristic, significant fluorescence decreases upon membrane energization. The mechanism(s) responsible for this energy-linked fluorescence response has been the subject of numerous hypotheses [14–17]. Evidence accumulated in this laboratory indicates that with submitochondrial membranes the exact mechanism differs with the molecular structure of the probe. Quinacrine appears to monitor an intramembrane H^+ transfer associated with energization of the membrane; the fluorescence decrease stems from protonation of monovalent quinacrinium ions yielding the less fluorescent, divalent species [16]. 9AA and 9ACMA, however, appear to interact with negatively charged membrane components, generated upon energization, with the resultant formation of non-fluorescent complexes [18]. Recently, a photoaffinitity analogue of QA, QA-N_3 has been synthesized and characterized [19] and its interactions with submitochondrial membranes, in both the energized and non-energized states, have been examined [1]. The results provide further support to our earlier contention that QA probes the H^+ content in the hydrophobic regions of energized membranes. In this paper, we report on work done with the 3-azido analogue of 9ACMA, 9-amino-3-azido-7-methoxyacridine (9AAMA), including its synthesis and characterization, as well as its utilization as a photoaffinity reagent for the site(s) of interaction of 9ACMA with submitochondrial membranes. Portions of these results have been reported briefly [20–22].

Materials and Methods

Submitochondrial particles prepared in the presence of EDTA (ethylenediaminetetraacetic acid) were treated with malonate or both malonate and oligomycin as described by Lee [23,24]. These preparations can be stored in liquid N_2 with virtually no change in enzymatic activity as compared with freshly prepared particles.

Photolabelling with 9AAMA was performed essentially as described previously with the analagous QA azide (QA-N_3) [1] in a medium consisting of 150 mM sucrose/30 mM phosphate (pH 7.4). The submitochondrial membranes (0.5 mg protein/ml) were incubated, at room temperature and in the dark, with various concentrations of 9AAMA (1.88–30.0 μM), in the presence of 1.66 mM NaSCN. The membranes were labelled in both the non-energized and energized states; energization was effected by the addition of succinate to 3.33 mM. After 60 s, the mixtures were irradiated by 12 Rayonet 350 nm lamps for a period of 90 s. Except in experiments designed to measure the extent of binding, photolysis was immediately followed by the addition of bovine serum albumin, in buffer, to 10 mg/ml to adsorb both noncovalently bound dye and, possibly, long-lived radicals. The labelled membranes were centrifuged at 105 000 $\times$ g for 40 min at 4°C and the pellets homogenized into 20 ml of ice-cold sucrose-phosphate medium containing 6 mg bovine serum albumin/ml. Centrifugation was repeated, followed by two washings with sucrose-phosphate medium. The final suspension was into 0.25 M sucrose at 6–8 mg/ml followed by storage in liquid N_2 before analyses.

The linearity of the absorbances of photolyzed 9AAMA solutions (1.88 to 30.0 μM) allowed for the estimation of the extent of labelling, calculated from the differences between the absorbances of the dye photolyzed alone and those of the corresponding first supernatants from non-albumin treated, photolyzed mixtures of 9AAMA and oligomycin pre-treated membranes [1]. Non-covalent association of photolyzed 9AAMA was similarly estimated from identical incubations of oligomycin pretreated submitochondrial membranes, non-energized and energized, with solutions of 9AAMA that had been photolyzed for 90 s in the absence of membranes. Alternatively, covalent attachement was estimated from the absorbances of labelled, bovine serum albumin-treated membranes with respect to a standard curve obtained with photolyzed dye in the presence of membranes.

Absorbance data were acquired with an Aminco DW-2 dual wavelength spectrophotometer. Fluorescence spectra were recorded with SLM's Model 4000 fluorescence spectrophotometer interfaced

with a Hewlett-Packard 9815S data processor. Fluorescence excitation and emission spectra were corrected for both the intensity of the excitation light (with a concentrated solution of rhodamine in propylene glycol) and particle, protein or lipid background as required.

Enzyme assays were performed in a medium of 150 mM sucrose/30 mM Tris-SO_4 (pH 7.5), except ATPase, 150 mM sucrose/2.5 mM Tris-SO_4/5 mM $MgCl_2$ (pH 7.4). NADH oxidase was measured spectrophotometrically at 340 nm, while succinate oxidase was determined polarographically [1,25]. NADH-ferricyanide reductase and succinate-PMS reductase activities were measured spectrophotometrically as described by Mackler [26] and King [27]. ATPase activity was measured by monitoring the rate of H^+ production resulting from the hydrolysis of 5 mM ATP [28].

Lipids were extracted from labelled and unlabelled membranes with chloroform/methanol according to Folch et al. [29], taken to dryness by evaporation and resuspended to 0.5 μmol PO_4^{3-}/ml in 1:1 $CHCl_3/CH_3OH$ for fluorescence measurements. Total lipid phosphorous was assayed according to Parker and Peterson [30]. Proteins were separated from lipid components by an initial extraction of labelled membranes with hexane/isopropanol [31]. The proteins were then dissolved in 0.33% sodium dodecyl sulfate (SDS), to 0.3 mg/ml, and extracted with 0.5 vol. of $CHCl_3/CH_3OH$ (2:1).

SDS polyacrylamide gel electrophoresis of intact membranes was done as described by Neville [32]. Protein was determined by the method of Lowry et al. [33] with bovine serum albumin as the standard.

Glass double distilled water was used throughout this study; all chemicals were reagent grade. Unless otherwise specified, experiments were done at ambient temperature (23–25°C).

Synthesis of 9AAMA

In the dark, 3-azido-7-methoxy-9-chloroacridine [19] (250 mg, 0.878 mmol) was added to 0.9 g of phenol and heated, with stirring, at 100–110°C in a single-neck, round bottom flask equipped with a $CaCl_2$ drying tube. After 1 h, $(NH_4)_2CO_3$ (240 mg, 2.5 mmol) was added, all at once, and stirring of the reaction mixture continued for 3 h. Acetone was then added to the cooled mixture. The crude product was filtered, washed with acetone and dried under vacuum. Purification was effected by converting the salt to the free base by the addition of 1 M NaOH to a stirred solution of the product in boiling ethanol. Addition of cold H_2O precipitated the free base which was collected by filtration, washed with water, dissolved in boiling methanol and recrystallized, to yield yellow crystals, by the addition of water until the solution became slightly turbid, followed by slow cooling. Recrystallization was repeated and the product, in CH_3OH, converted to the HCl salt with concentrated HCl, filtered and dried over P_2O_5. Repetition of the purification procedure yielded the final product, 9-amino-3-azido-7-methoxyacridine: calculated for $C_{14}H_{11}N_5O \cdot HCl \cdot 1/2H_2O$: C, 54.11; H, 4.22; N, 22.54; O, 7.72; Cl, 11.41; found: C, 54.23; H, 4.18; N, 22.56; Cl, 11.59; IR 2100 cm^{-1}. Microanalyses were performed by Galbraith Laboratories, Knoxville, TN, U.S.A.

Results

9AAMA as a Photoaffinity Analogue of 9ACMA

Replacement of the chloro-group of 9ACMA with the azido-group was expected to yield a suitable analogue for investigating the nature of the interactions of 9ACMA with submitochondrial membranes via photoaffinity labelling. On the basis of the pseudo-halogenic nature of the azido-group [34], 9AAMA would be expected to have chemical properties similar to those of 9ACMA. Changes in the absorbance spectrum of 9AAMA in the pH range of 6.0–11.0 reflect the distribution of the neutral and protonated species of the molecule in aqueous solution (with an isosbestic point near 373 nm). The pK_a value of 9.3 derived for 9AAMA [20] compares favorably to the value of 9.1 obtained under identical conditions for 9ACMA (data not shown).

Fig. 1 illustrates the photosensitivity of 9AAMA to exposure to 350 nm light at pH 7.4. The inset plot shows the first-order kinetics of the photodecomposition reaction and yields a $t_{1/2}$ of approx. 4 s. In addition, an isosbestic point is observed in the photodecomposition spectra near 445 nm. Like 9ACMA, when associated with oligomycin

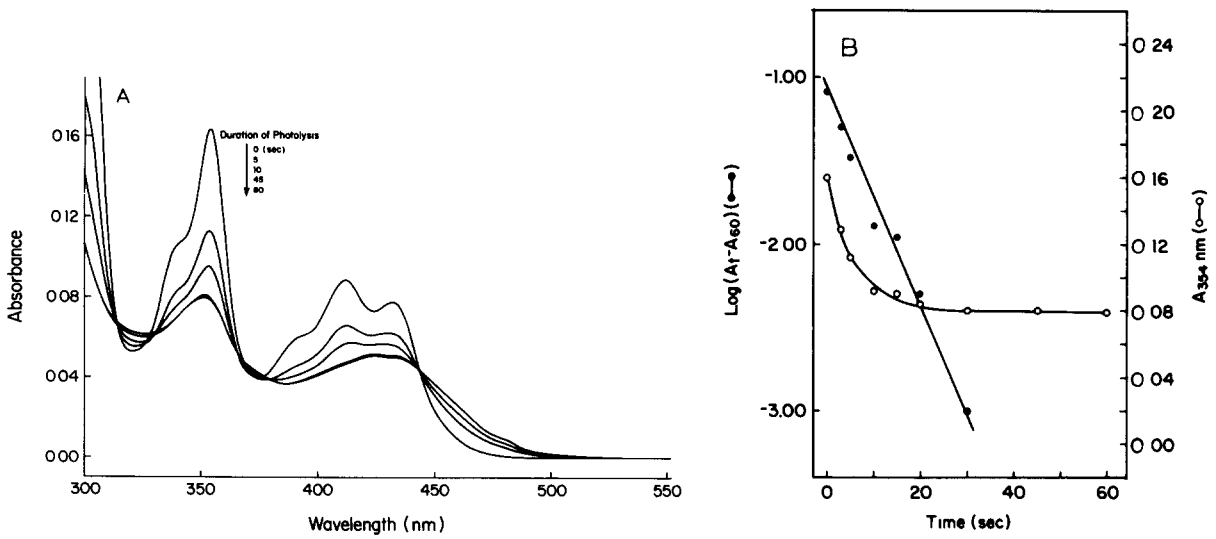

Fig 1 (A) Visible absorption spectra for the photodecomposition of 15 μM 9AAMA in 30 mM phosphate buffer (pH 7 4), irradiation by 4 Rayonet RPR-350 nm lamps for the indicated periods (B) The plots illustrate the absorbance at 354 nm (○) and the logarithm of the A_{354} observed at time, t, minus the A_{354} at $t = 60$ s (●) versus time

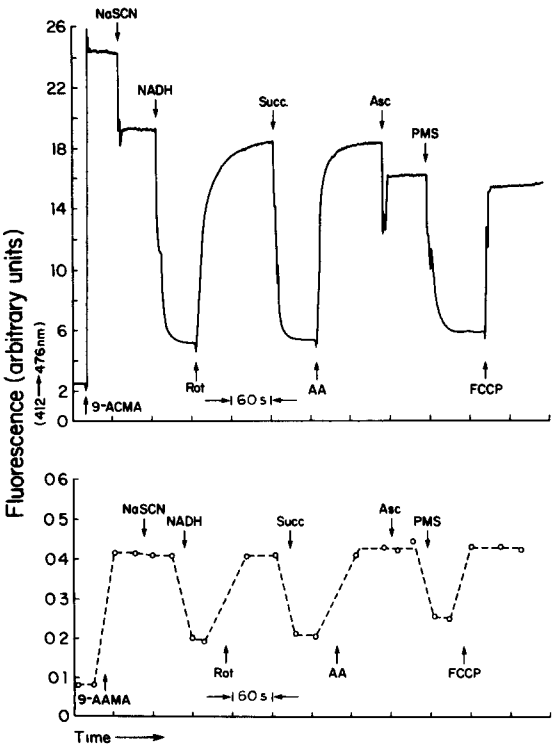

Fig 2 The energy-linked fluorescence responses of 9ACMA (top) and its 3-azido analogue, 9AAMA (bottom). The reaction mixture consisted of 150 mM sucrose/30 mM phosphate (pH 7 4)/0 25 mg oligomycin-pretreated membranes per ml; other additions are: 7 5 μM dye (9ACMA or 9AAMA), 1 7 mM NaSCN, 150 μM NADH, 3 3 μM rotenone (Rot) 6 6 mM succinate (Succ), 1 7 μg/ml antimycin A (AA), 3 3 mM ascorbate (Asc), 1.4 μM phenazine methosulfate (PMS) and 1 7 μM FCCP To prevent photodecomposition, for 9AAMA the excitation shutter was kept closed except at the points indicated

pretreated submitochondrial membranes, 9AAMA exhibits a significant fluorescence decrease upon energization of the membranes with substrates capable of supporting respiration, e.g., NADH, succinate and ascorbate-phenazine methosulfate. The fluorescence is restored by the addition of uncoupler (FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone) or electron-transport inhibitors, e.g., rotenone and antimycin A in the case of NADH oxidase or antimycin A in the case of succinate oxidase, indicating that the nature of the response of 9AAMA is the same as that of 9ACMA (Fig. 2).

These results indicate that 9AAMA is well-suited to serve as a photoaffinity analogue of 9ACMA and its interactions with submitochondrial membranes.

Photo-Labelling of Submitocohondrial Membranes with 9AAMA

Estimations of the extent of covalent attachment, upon photolysis, of 9AAMA to energized

and non-energized, oligomycin pretreated submitochondrial membranes as a function of dye concentration are presented in Fig. 3. These results were obtained from measuring the amounts of photodecomposition products remaining in the first supernatants from the non-albumin-treated photolyses of 9AAMA plus membranes. Binding is a linear funtion of dye concentration up to 30 μM 9AAMA. Also illustrated is the degree of non-covalent association of the photodecomposition products of 9AAMA, which did not differ with the energy state of the membranes. This significant amount of non-specific binding necessitated the washing of labelled membranes with bovine serum albumin (cf. Methods section); fluorescence measurements indicated that the vast majority of the non-covalently bound photodecomposition products were removed in this manner. Fluorescence due to (free) decomposition products in the supernatant from the final washing was approx. 10% that of the first supernatant, and approached background levels. All reported data were obtained with labelled particles that had been substantially freed of non-covalently bound photodecomposition products. Results obtained when the extent of incorporation was estimated from the absorbances of the labelled, oligomycin pretreated particles (albumin-washed) themselves, with respect to a standard curve constructed for free, photolyzed dye in association with membranes again indicate that binding is a linear function of azide concentration: 10.7 and 20.2 nmoles/mg for non-energized membranes at 7.5 and 15.0 μM 9AAMA, respectively, with the coresponding values for energized membranes being 6.1 and 11.9.

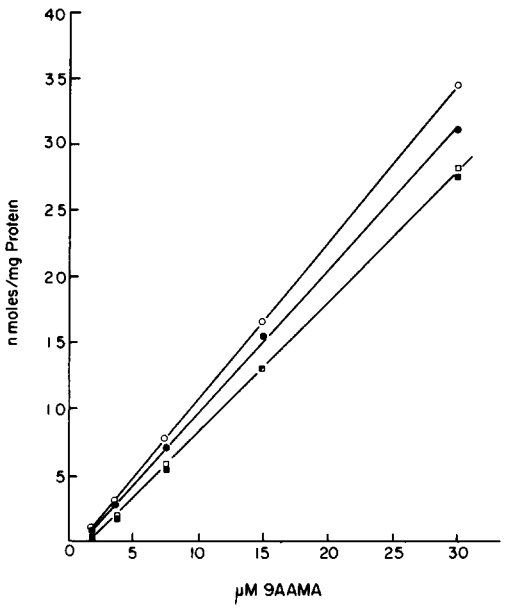

Fig. 3. Dependence of the extent of covalent labelling of non-energized (○) and energized (●) oligomycin-pretreated membranes on the concentration of 9AAMA. Also illustrated is the degree of nonspecific, non-covalent association of photolyzed 9AAMA to non-energized (□) and energized (■) membranes. Conditions as described in Materials and Methods.

Both methods indicate that the degree of covalent incorporation is greater for non-energized than for energized submitochondrial membranes. These measurements can, however, only approximate upper and lower limits for the extent of covalent attachment of 9AAMA, since neither method can adequately account for differences in extinction coefficients, quantum yields and/or adsorption properties, relative to the energy state of the membranes and the status of the photolyzed dye (free/covalently attached/non-specifically bound). More precise determination of 9AAMA binding parameters can be assessed with the use of radioactively labelled 9AAMA in the future.

Fig. 4 presents the fluorescence excitation and emission spectra of 9AAMA covalently bound to oligomycin pretreated submitochondrial membranes. The membranes were labelled in the pres-

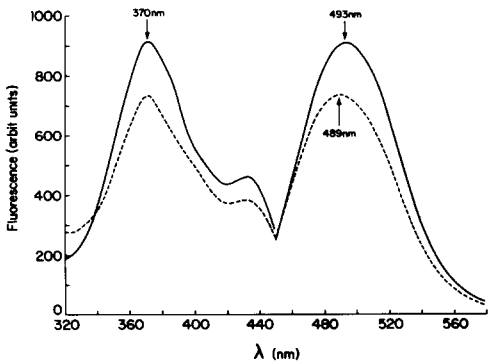

Fig. 4 Fluorescence excitation (emission at 485 nm) and emission (excitation at 370 nm) spectra of 9AAMA covalently bound to non-energized (------) and energized (———) oligomycin pretreated membranes; 0.3 mg labelled membranes/ml of 150 mM sucrose, 30 mM phosphate (pH 7.4). Particles were labelled at 15 μM 9AAMA and washed with bovine serum albumin as described in Materials and Methods.

ence of 15.0 μM 9AAMA in both the non-energized and energized states. Both the excitation and the emission spectra differ with the energy state of the membranes at the time of photolysis. The location of the excitation peak, 370 nm, is independent of both the dye concentration (not shown) and the energy state of the membranes, however, a distinct shoulder is visible near 385 nm in the excitation spectrum of the dye covalently bound to energized membranes. The emission peak for 9AAMA covalently attached to energized membranes is red-shifted approx. 4 nm with respect to that of the dye bound to non-energized membranes. In contrast, the excitation and emission spectra of photolyzed 9AAMA itself, under the same conditions, display excitation and emission peaks near 365 nm and 503 nm, respectively. Furthermore, these spectra do not change upon either the addition of membranes or their subsequent energization [21].

The spectral differences (energized-minus-non-energized) of covalently bound 9AAMA dependent upon the energy state of the membranes are shown in Fig. 5. The most obvious feature of the difference excitation spectrum is the broad peak extending from about 350–390 nm, which results from the increased peak height at 370 nm and the shoulder at 385 nm present in the excitation spectrum of 9AAMA bound to energized, oligomycin

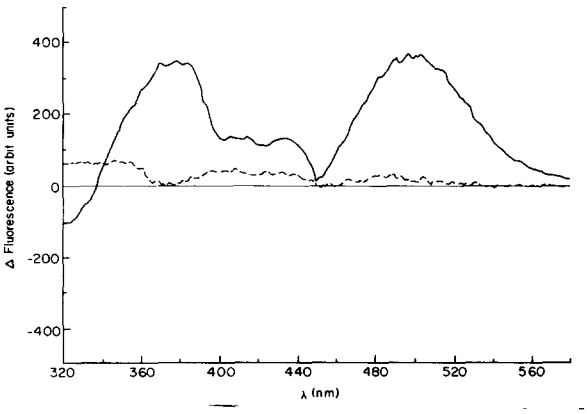

Fig. 5. Difference fluorescence excitation and emission spectra (energized-minus-nonenergized) of 9AAMA covalently bound to oligomycin-pretreated membranes (———); conditions as in Fig. 4. Also illustrated (- - - - -) are the difference spectra for membranes labelled with 9AAMA in the presence of the uncoupler, FCCP (1.7 μM).

pretreated membranes (cf. Fig. 4). Other differences consist of a much smaller peak near 435 nm and a valley at the short-wavelength region of the figure. The difference emission spectrum, with its peak near 500 nm, reflects the increased emission and red-shift seen in the spectrum of 9AAMA bound to energized, compared to non-energized, membranes. Fig. 5 also illustrates that the presence of the uncoupler, FCCP, during photolysis completely abolishes the spectral differences observed with labelled, coupled membranes. The excitation and emission spectra of 9AAMA bound to energized and non-energized membranes, in the presence of FCCP, are virtually identical to each other and to those of the dye covalently bound to non-energized membranes in the absence of FCCP (not shown).

Enzymatic Activities of the Labelled Membranes

Figs. 6–8 present the enzymic activities of labelled membranes relative to those of control membranes, treated identically to labelled membranes except for the absence of 9AAMA during photolysis. Included for comparison in Figs. 6 and 7 are plots of the corresponding data for QA-N_3 obtained in a previous study [1]. Figs. 6A and 7A show that both NADH and succinate oxidases exhibit gradually decreasing activities over the range of 9AAMA concentrations (0–30 μM) present during photolysis. The maximal level of inhibition of NADH oxidase (Fig. 6A) was 50% with no apparent difference between labelling in either the energized or non-energized state. Succinate oxidase activity (Fig. 7A), however, was approx. 20% higher for labelled, energized, as opposed to labelled, non-energized membranes, maximal levels of inhibition being 50% and 70%, respectively. Determination of the NADH-ferricyanide reductase (Fig. 6B) and succinate-PMS reductase activities (Fig. 7B) permitted a localization of the sites of interaction of bound 9AAMA with the electron-transport assembly. The NADH ferricyanide reductase was virtually unaffected by the covalent binding of 9AAMA (Fig. 6B), while the succinate-PMS reductase was nearly unaffected in the case of energized membranes, but inhibited at an almost constant level of approx. 20% in labelled, non-energized membranes (Fig. 7B). Apparently, the slight protection against partial inactivation

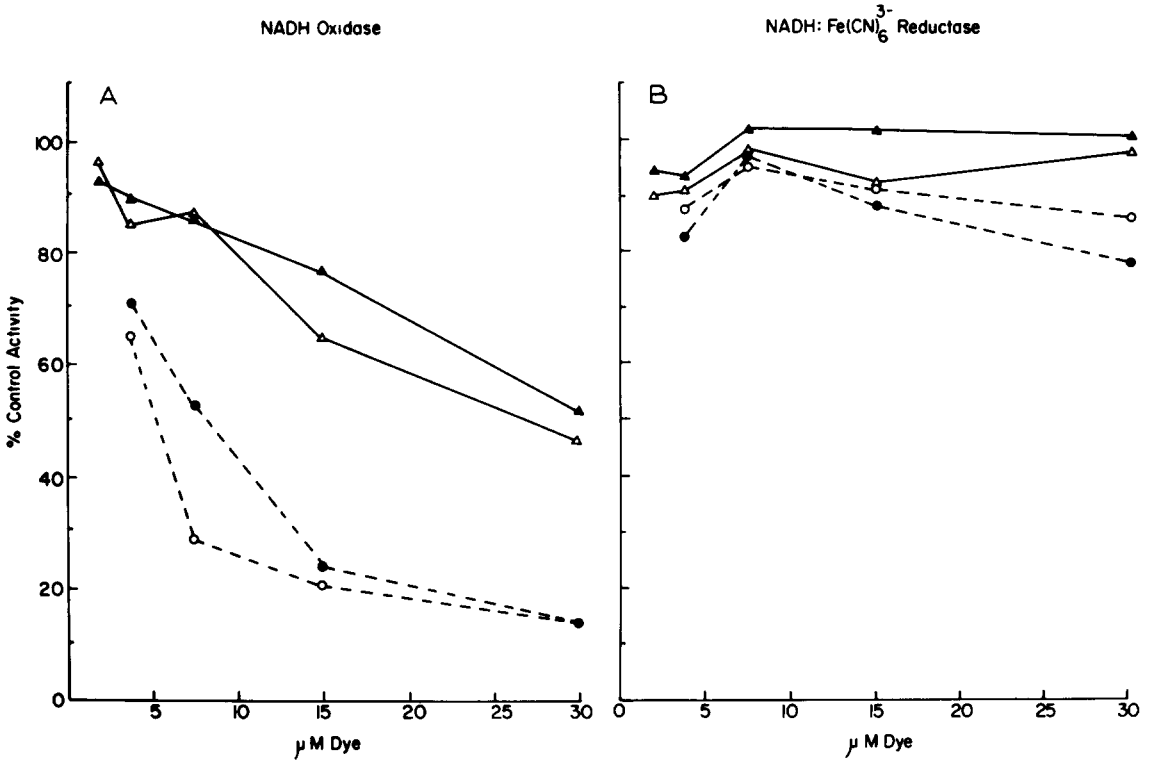

Fig. 6 (A) NADH oxidase and (B) NADH-ferricyanide reductase activities of 9AAMA (———) and QA-N_3 (- - - - - -) labelled, oligomycin-pretreated membranes. Particles were labelled in the non-energized (△, ○) or energized (▲, ●) states; data for QA-N_3 taken from Ref. 1 Activities are expressed as percentages of those of control membranes treated exactly as labelled membranes except for the absence of azide during irradiation. Conditions as in Materials and Methods.

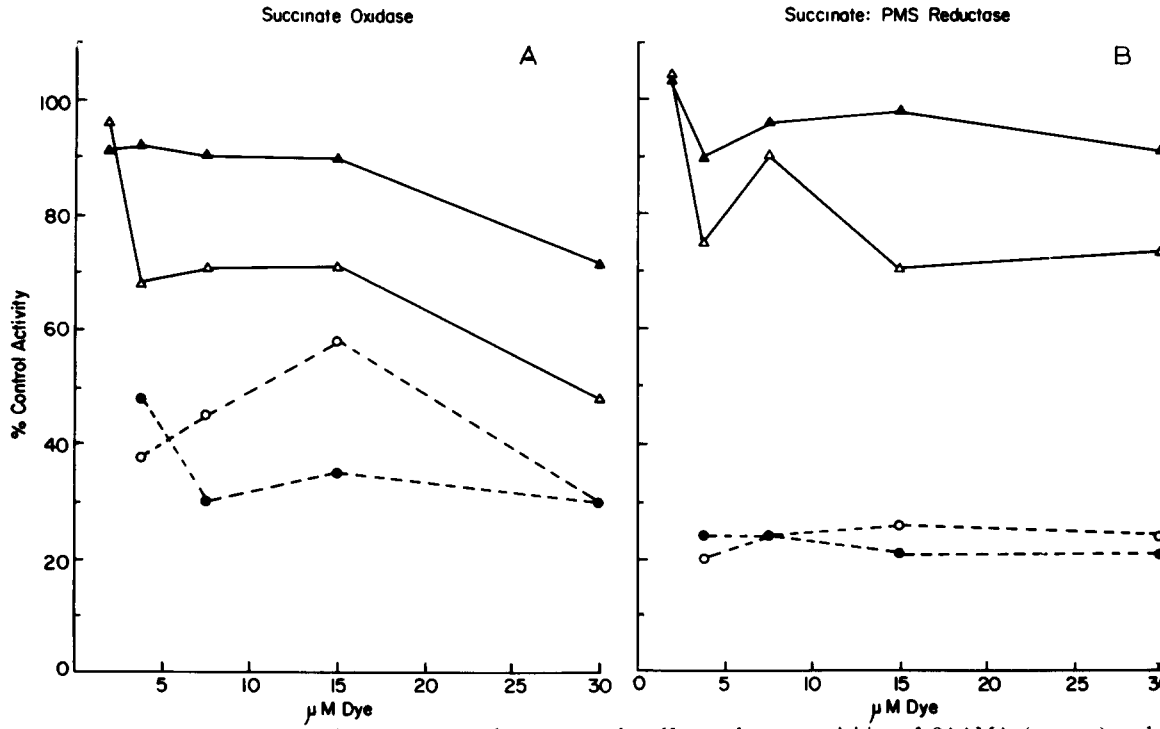

Fig. 7. (A) Succinate oxidase and (B) succinate-phenazine methosulfate reductase activities of 9AAMA (———) and QA-N_3 (- - - - - -) labelled, oligomycin-pretreated membranes. Membranes were labelled in the non-energized (△, ○) or energized (▲, ●) states; remainder of legend as for Fig. 6.

afforded the succinate oxidase activity of labelled, energized membranes results from this lower level of inhibition of the dehydrogenase. The inhibition noted in the NADH oxidase activity, and the difference between the inhibition levels of the succinate oxidase and succinate-PMS reductase activities, may both result from additional interaction at a site(s) along the electron-transport chain on the oxygen side of the NADH and succinate dehydrogenases.

Fig. 8 illustrates the effect of covalent labelling with 9AAMA and QA-N_3 on the ATPase activity of submitochondrial membranes (non-oligomycin pretreated). Similar to the oxidases, ATPase activity in 9AAMA labelled membranes is diminished; particles labelled at 30 μM 9AAMA display about half the control activity. Again, however, the energy state of the membranes at the time of photolysis appears to affect the extent of inhibition. The ATPase activity of membranes (non-oligomycin pretreated) labelled while in the energized state is about 15% higher than that of membranes labelled while non-energized, over the entire range of 9AAMA concentrations studied.

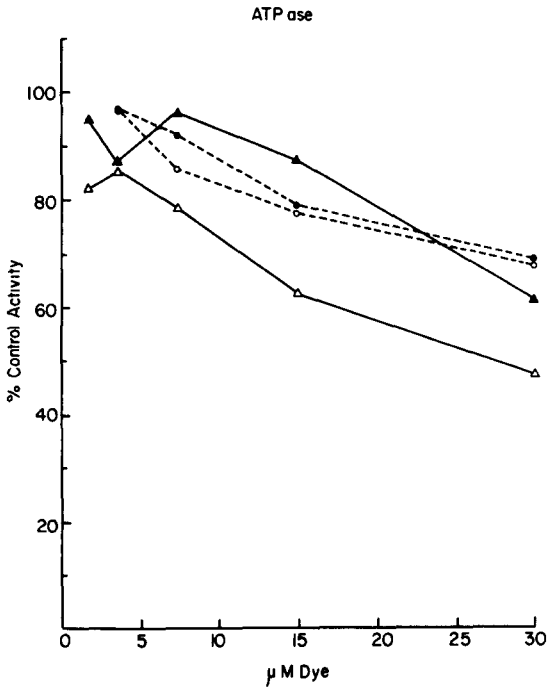

Fig. 8. ATPase activities of 9AAMA (———) and QA-N_3 (- - - - -) labelled, non-oligomycin-pretreated membranes. Membranes were labelled in the non-energized (△, ○) or energized (▲, ●) states; remainder of legend as for Fig 6

In light of a recent report by Laikind and Allison [35] that quinacrine inhibits and quinacrine mustard inactivates the F_1-ATPase from bovine heart, and to extend our earlier work with the analagous azido derivative of quinacrine [1], Fig. 8 also presents the effect of covalent labelling of submitochondrial membranes with QA-N_3 on ATPase activity. The magnitude of the inhibition is similar to that seen with 9AAMA, though the energy state of the membrane has no effect on the extent of the inhibition. It should be noted that the dye concentrations utilized by Laikind and Allison were approx. 100-times greater than in the present study. They reported a K_I for quinacrine of 440 μM, and binding of the majority of the label to the β subunit of the isolated F_1-ATPase. Covalent attachment to the α, β and γ subunits of the F_1-ATPase, along with the labelling of a peptide of approx. 30 kDa, dominated the interactions of QA-N_3 with the protein components of both energized and non-energized submitochondrial membranes [1].

Interactions with Protein and Lipid Components of the Membrane

The electrophoretic profiles of those peptides of oligomycin pretreated membranes that are covalently labelled by 9AAMA are presented in Fig. 9 alongside those obtained under similar conditions by QA-N_3 [1]. It is apparent that the major peptides labelled with 9AAMA and QA-N_3 are identical, and independent of the energy state of the membranes at the time of photolysis. Separate experiments (not shown) indicate that 9AAMA labelled, non-oligomycin pretreated membranes display identical electrophoretic patterns with respect to fluorescence and that covalent attachment of these acridine molecules does not alter the mobility of submitochondrial peptides. Significant levels of fluorescence are associated with peptides having molecular weights of approx. 100000, 55000, 30000 and 15000 (the fluorescence at 70 kDa is intrinsic to that peptide). As with QA-N_3, the highest level of fluorescence is associated with a peptide in the vicinity of 30 kDa. It is not possible to ascertain, from this data alone, whether or not the site(s) of attachment to the same peptide dif-

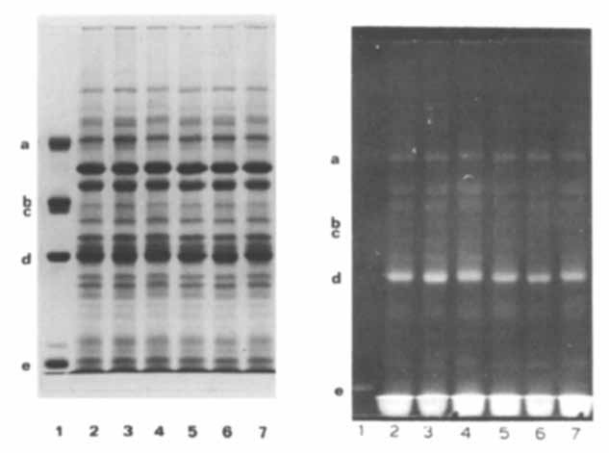

Fig 9. Coomassie blue stain (left) and the fluorescence photograph before staining (right) of oligomycin pretreated membranes covalently labelled with 9AAMA or QA-N_3, after sodium dodecyl sulfate electrophoresis. Lane 1, standards (a, bovine serum albumin, 68 kDa; b, ovalbumin, 43 kDa; c, creatine phosphokinase, 40 kDa; d, carbonic anhydrase, 29 kDa; e, cytochrome *c*, 12.5 kDa; lane 2, 1:1 mixture of 9AAMA and QA-N_3 labelled, non-energized membranes; lanes 3 and 4 9AAMA and QA-N_3 labelled, non-energized membranes, respectively; lane 5, 1.1 mixture of 9AAMA and QA-N_3 labelled, energized membranes, lanes 6 and 7, 9AAMA and QA-N_3 labelled, energized membranes, respectively. Dye concentration during labelling was 15 μM; 60 μg labelled membrane protein per lane

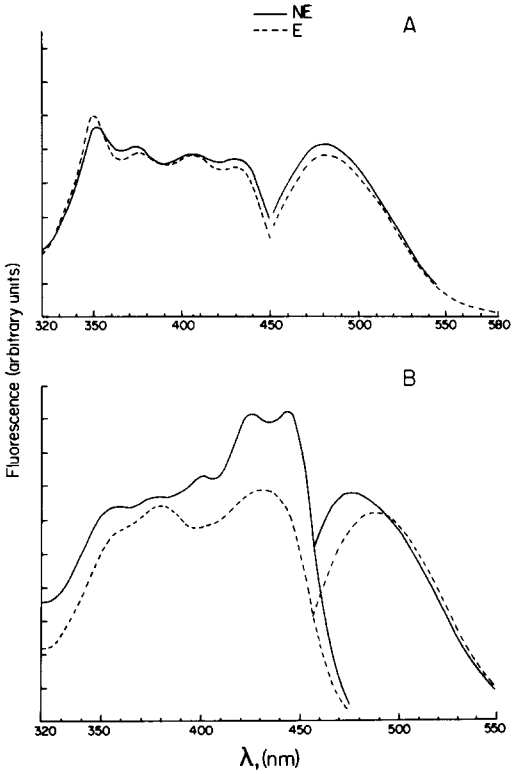

Fig. 10. Fluorescence excitation (emission at 485 nm) and emission (excitation at 370 nm) spectra of the protein (A) and lipid (B) components isolated from oligomycin pretreated membranes labelled at 15 μM 9AAMA in the non-energized (———) or energized (- - - - -) states; 0.3 mg protein/ml of 0.33% SDS (A) or 0.5 μM PO_4^{3-} in 1:1 $CHCl_3/CH_3OH$ (B), extracted as in Materials and Methods

fers between the energy states of the membranes for either dye or, additionally, if the site(s) of attachment differs between QA-N_3 and 9AAMA for membranes in the same energy state.

Obviously, the fluorescence excitation and emission spectra of the covalently labelled membranes, and the differences noted therein, reflect the net contributions of the labelled protein and lipid components. Fig. 10 presents the fluorescence spectra of the protein (A) and lipid (B) components derived from oligomycin pretreated submitochondrial membranes labelled at 15.0 μM 9AAMA. Complementing the electrophoresis data, the spectra of the bulk proteins from both labelled, energized and non-energized membranes are identical, suggesting that the interactions with protein components are not responsible for the differences in the spectra of labelled, intact membranes.

Fluorescence spectra of the labelled lipid components, however, differ significantly with the energy state of the membranes at the time of photolysis. In particular, the excitation spectrum of lipids derived from labelled, energized membranes shows a distinct peak near 380 nm, while the corresponding emission spectrum is considerably red shifted (10–15 nm) with respect to that of lipids derived from labelled, non-energized membranes. These differences correspond closely to those noted in the fluorescence spectra of intact, labelled membranes (cf. Fig. 4). Additionally, the excitation spectra of the labelled lipids display other differences, most notably in peak height and band structure from 400–450 nm. These observations indicate that the specific lipids to which 9AAMA binds and/or the specific location of the binding sites on the same lipids differ significantly, dependent on the energy state of the membranes. Either condition could result from a change in the

microenvironment of 9AAMA upon membrane energization. The red shift in the emission spectrum of 9AAMA bound to intact, energized membranes indicates an altered hydrophobicity of the microenvironment from that of dye bound to non-energized membranes. This would imply a redistribution of dye molecules and/or gross structural changes in portions of the membrane upon energization.

Discussion

The minimal chemical and structural changes which result from replacement of the chloro-group of 9ACMA by the azido in 9AAMA yield a fluorescent probe which interacts with submitochondrial membranes in an analagous manner. Additionally, 9AAMA can, through photo-activation, covalently bind to the constituents of its microenvironment allowing for the characterization of the membrane components involved in the energy-linked response of 9ACMA. It has been shown [18] that interactions within the membrane phase are responsible for the energy-linked responses of those 9-aminoacridines (9ACMA, 9AA) which lack a bulky side chain at the 9-amino group of the acridine moiety; these interactions are, however, mechanistically different in nature from those of QA [16].

Not unexpectedly for a complex membrane system, and given the non-specific nature of the reactivity of the nitrene, 9AAMA covalently binds to both protein and lipid components. As evidenced by the excitation and emission spectra, the lipid portion of the membrane reflects the interaction(s) characteristic of the energized state in which the fluorescence response occurs. Similarly, QA-N_3's interaction(s) with energized, oligomycin pretreated submitochondrial membranes results in altered fluorescence spectra, with respect to labelled, non-energized membranes, which are attributable to differing interactions within the lipid phase [1]. Neither study indicates that the direct interaction of aminoacridines with membrane proteins is intimately linked to the fluorescence response.

Though both 9AAMA and QA-N_3 label the same peptides and both appear to differ only in their energy-linked interactions with lipid components, there are several lines of evidence which indicate that these molecules probe different, specific regions of the inner mitochondrial membrane. They also support our earlier contention [18] that, mechanistically, these acridines differ in the specific nature of the interactions responsible for their fluorescence decreases.

(1) The extent of covalent binding of 9AAMA to submitochondrial membranes appears to be a linear function of concentration and shows no indication of saturation of sites, even at 30 μM. In contrast, though of similar magnitude, the binding curve of QA-N_3 at identical dye/protein ratios is hyperbolic, approaching saturation at 30 μM [1]. That the number and nature of binding sites for 9ACMA and QA may differ in submitochondrial membranes is also supported by data from studies with local anesthetics, e.g., the concentration of chlorpromazine required for 50% inhibition of the energy-linked response is an order of magnitude greater for 9ACMA [18] than QA [36] (15 versus 1.5 μM). QA has been shown to bind specifically to axonal membranes and to be displaced by the competitive binding of local anesthetics [9]. Additionally, the extent of incorporation of QA into energized submitochondrial membranes is greater than into non-energized membranes, while for 9AAMA the converse applies: incorporation into non-energized membranes is greater than into energized membranes.

(2) QA-N_3 [1], but not 9AAMA (data not shown), covalently bound to energized, oligomycin pretreated submitochondrial membranes retains the property of exhibiting an energy-linked fluorescence decrease. If it were assumed that both molecules interact with the membrane at identically the same sites, protonation as a mechanism should be equally likely for either dye, while complex formation with charged membrane components should favor the less stearically hindered 9AAMA. The 9-azido derivative of 9ACMA retains its energy-linked fluorescence response when covalently bound to chloroplast membranes [37]. This was suggested to result from small-scale structural rearrangements involving tilting of the acridine nucleus towards the membrane surface. It is difficult to presume that upon de-energization of the submitochondrial particles covalently bound 9AAMA retains its spatial arrangement with the membrane characteristic of the energized state and,

thus, remains in the non-fluorescent form, incapable of further fluorescence decrease. 9AAMA-labelled energized and non-energized submitochondrial membranes are almost equally fluorescent, whereas in the energized state non-covalently bound 9AAMA, and 9ACMA, are almost totally non-fluorescent, presumably via complexation with conjugated π-bond systems in the membrane. Such complexation has been shown to greatly reduce the fluorescence of both 9ACMA and 9AA, but not QA [18]. Attachment through the 3-position of the acridine moiety may involve non-favorable spatial relationships which hinder or prevent the movement required for complexation. Such movement of the dye molecules and/or structural changes involving their microenvironment are supported by the differing effects of covalent attachment on the enzyme activities, dependent on the energy state during photolysis, as well as the red shift observed in the fluorescence emission spectra of both the labelled, intact membranes and the isolated lipid fraction. Alternatively, submitochondrial particles and chloroplasts may differ in the exact nature of their interactions with aminoacridines. It should be mentioned, in any event, that these demonstrations of energy-linked fluorescence decreases with covalently bound acridines exclude the involvement of a transmembrane movement of the probes, and renders the use of such dyes as internal pH indicators questionable [14,38,39]. Such movement is required by those hypotheses of the mechanism which explain the fluorescence decrease as resulting from the accumulation of the dye molecules in the vesicular interiors [10,13,14].

(3) Finally, the interactions of 9AAMA and QA-N_3 with the respiratory chain enzymes differ considerably. Whereas succinate-PMS reductase is inhibited 80% by the binding at 3.75 μM QA-N_3 [1], 30 μM 9AAMA inhibits by only 10 and 25%, in energized and non-energized membranes, respectively. Similarly, both NADH oxidase and succinate oxidase are more severely inhibited by QA-N_3 [1] than 9AAMA. The extent of inhibition of ATPase is independent of the energy state of the membranes with QA-N_3, while energized membranes are substantially less affected, with respect to the inhibition of ATPase activity, than are non-energized membranes when labelled with 9AAMA. Neither probe has any effect on the NADH-ferricyanide reductase activity. That the extent of the inhibition of the F_1-ATPase and succinate dehydrogenase, both located on the outer surface of submitochondrial membranes, is greater for non-energized membranes indicates a redistribution of the 9AAMA molecules upon membrane energization.

If, indeed, specificity of labelling involves the membrane lipid components, this would imply that the enzymatic activities are modulated, in part, by the covalent binding of these probes to the lipids, perhaps via interactions with the enzymes' lipid annuli or localized disruption of membrane structure. Though the specificity could involve the protein components, this seems less likely in view of the identical patterns of labelled peptides detected by SDS gel electrophoresis, and the fluorescence spectra of the labelled proteins. These peptides/proteins may, however, be characteristic of the region(s) of the membrane in which the interactions do occur. Identification of both the labelled lipid and protein components may enable further localization of the sites of interaction of these probes with submitochondrial membranes.

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