An Azido Analogue of Quinacrine: Its Synthesis and Utilization as a Photoaffinity Probe with Submitochondrial Particles†

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ABSTRACT: The sites of interaction of quinacrine (QA) with submitochondrial membranes have been labeled with the use of an azido analogue of quinacine, 3-azido-9-{[4-(diethylamino)-1-methylbutyl]amino}-7-methoxyacridine (QA-N_3). QA-N_3 was prepared in a seven-step synthesis and shown to be similar in chemical and spectroscopic properties to QA. In the presence of submitochondrial membranes, the fluorescence of QA and QA-N_3 decreased the same extent upon energization of the membranes. Irradiation of QA-N_3 in the presence of either energized or nonenergized membranes resulted in covalent reaction of QA-N_3 with membrane components; however, (1) with 3.75 μM QA-N_3, the dye covalently bound upon irradiation to nonenergized and energized membranes with yields of 40% and 60%, respectively, (2) the dye covalently bound to the energized membranes retained an energy-linked fluorescence response while the dye labeled to the nonenergized membranes did not, and (3) the fluorescence excitation spectrum of the dye cross-linked to the membranes under energized and nonenergized conditions differed in the 440-nm region. The fluorescence excitation spectrum of the extracted lipid products retained this spectral difference while the delipidated membrane proteins did not. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed that there were primarily three peptides labeled, with corresponding molecular weights of 54K, 33K, and 30K. Enzymatic activity profiles indicated there were at least two distinct sites of interaction of the dye with the membrane. One site was in the vicinity of succinate dehydrogenase while the other, which was less susceptible, was along the electron-transport chain on the oxygen side of NADH dehydrogenase. Furthermore, the latter site was more susceptible to photoinduced inactivation when the membrane was in the nonenergized state as compared to the energized state. Our results indicate that there are specific sites or regions of interaction of QA-N_3 with the energized membranes which are distinct from those in the nonenergized membranes. These sites are in a hydrophobic environment and are probably dominated by the lipid constituents of the membrane.

Quinacrine (QA)[1] was introduced by Kraayenhof (1970, 1971) as a fluorescent probe for chloroplast membranes. The fluorescence intensity of QA was shown to decrease 90% upon energization of the membranes. Later the use of QA was extended to bacterial membranes (Eilerman, 1970), chromatophores (Gromet-Elhanan, 1972), and submitochondrial particles (SMP) (Lee, 1971; Azzi et al., 1971). Thus, there appears to be some common feature of these different membrane systems which causes the fluorescence quenching of QA.

The chemiosmotic hypothesis (Mitchell, 1961, 1966a,b, 1977) provided a common property which could be used to explain the fluorescence quenching of QA. The probe was suggested to accumulate inside the vesicle due to the establishment of the ΔpH across the membrane (Azzi et al., 1971; Gromet-Elhanan, 1971, 1972; Schuldiner & Avron, 1971; Schuldiner et al., 1972). However, this postulate is not generally accepted. A number of other hypotheses, which include a role for the membrane, have been suggested to explain the energy-linked fluorescence quenching of QA or other aminoacridines: (1) stacking of the dye molecules on the membrane surface (Dell'Antone et al., 1971a,b; Massari et al., 1974); (2) protonation of the acridine ring nitrogen within the membrane phase (Lee, 1971, 1972; Huang et al., 1977); and (3) interaction with negative surface changes (Kraayenhof & Arents, 1976; Searle & Barber, 1978; Schapendonk et al., 1980). There are two possibilities which may distinguish the nature of the acridine dye interactions with the energized and nonenergized membranes: either new sites of interaction are developed upon energization or the physical properties of preexisting sites are altered. Whatever the changes are, they are directly related to the ability for the membrane to sustain an energy pool.

The present study examines the identity of these sites and whether they differ between the energized and nonenergized membranes. We have synthesized an azido photoaffinity analogue of QA, QA-N_3, which differs from QA only in the substitution of a chloride group with the azide. Since the azide is considered a pseudohalide (Treinin, 1971), this derivative was predicted and, indeed, observed to have very similar properties to QA and thus serves as an effective, sensitive photochemical probe of QA interactions with the membrane.

We report here, in full experimental detail, the synthesis of QA-N_3, its properties as a fluorescent probe of SMP, and its utility as a photoaffinity reagent for the binding sites of QA with the membrane. Portions of this work have been communicated in preliminary form (Mueller et al., 1979a,b, 1981; Mueller, 1980).

Materials and Methods

Membrane Preparations. Malonate- and oligomycin-treated EDTA submitochondrial particles (MOESP) from heavy beef heart mitochondria were prepared as described by Lee &

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[1] Abbreviations: QA, quinacrine; QA-N_3, 3-azido-9-{[4-(diethylamino)-1-methylbutyl]amino}-7-methoxyacridine; DCIP, 2,6-dichlorophenolindophenol; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)-phenylhydrazone; IR, infrared; MOESP, malonate- and oligomycin-treated EDTA submitochondrial particles; mp, melting point; NADH, nicotinamide adenine dinucleotide (reduced); CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PMS, phenazine methosulfate; S_{13}, 3-*tert*-butyl-5,2′-dichloro-4′-nitrosalicylamide; NaDodSO$_4$, sodium dodecyl sulfate; SMP, submitochondrial particles; EDTA, ethylenediaminetetraacetic acid.

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Ernster (1967) and Lee (1979). The particles were treated with oligomycin (1 μg/mg of protein) and malonate, suspended, and stored in 0.25 M sucrose at a protein concentration of 25 mg/mL. The oxidase activities of the particles were measured at 25 °C in a medium consisting of 150 mM sucrose and 30 mM phosphate, pH 7.4, with a Clark oxygen electrode with either 1 mg of NADH or 1.66 mM sodium succinate as the substrate. Succinate–DCIP and NADH–$Fe(CN)_6^{3-}$ reductase activities were measured spectrophotometrically as described by King (1967) and Mackler (1967) except that the medium consisted of 150 mM sucrose and 30 mM phosphate, pH 7.4.

Labeling Experiments. Photolysis of QA-N_3 was performed in a reaction mixture consisting of 150 mM sucrose, 30 mM phosphate, pH 7.4, and 3.33 mM KSCN, with varying dye concentrations from 3.75 to 30 μM with or without 1.66 mM succinate. The MOESP were added to the reaction mixture to a final protein concentration of 0.5 mg/mL and incubated at room temperature, in the dark, for 1 min prior to the irradiation. The incubation mixture was placed in a water-jacketed cylindrical Pyrex chamber (diameter of 2.6 cm) which was positioned in the center of a Rayonet photochemical reactor equipped with 16 3500-A lamps, and the samples were irradiated for 1 min. This was sufficient to completely photodecompose the azide group. The samples were then centrifuged at 105000*g* for 40 min at 4 °C. The supernatant (S-1) was collected and the pellet washed in 50 volumes of 0.25 M sucrose, centrifuged as above, and resuspended in 0.25 M sucrose to a final protein concentration of 4–8 mg/mL.

The amount of dye covalently bound to the membranes was estimated from the absorbance (A) of the supernatant (S-1) at 440 nm and the absorbance (A_0) of QA-N_3 irradiated in the absence of membranes. The difference ($A_0 - A$) represented the amount of dye covalently bound to the membrane.

Lipid Extraction and Identification of the Covalently Labeled Proteins. The lipids were extracted from the labeled particles by using a modified method of Folch et al. (1957). The labeled SMP were centrifuged as above, resuspended in 0.34% $MgCl_2$ to a protein concentration of 5–10 mg/mL, and extracted with 5 volumes of $CHCl_3$–CH_3OH. The rapidly vortexed suspension was filtered through a glass wool filter and the upper layer removed from the clear biphasic solution which formed. The bottom layer (lipid extract) was washed with CH_3OH–water (1:1) and the bottom layer saved for the spectral studies. The filtered protein was collected and homogenized in water. A sample was removed, lyophilized, dissolved in the electrophoresis sample buffer, and run on the discontinuous polyacrylamide gel system described by Neville (1971). When electrophoresis was complete, the gel was photographed for the fluorescence by illuminating under the gel with a long-wavelength ultraviolet light box with a Corning CS-7-54 glass filter. The emission of the light exposing the film was filtered with a Wratten No. 8 filter. Kodak Royal pan film was exposed for 3 min, developed with D76, and fixed with Rapid Fix. The gel was then stained with Coomassie blue, destained (Weber & Osborn, 1969), and photographed with a Polaroid film system. The lipid-extracted protein was determined by the method of Lowry et al. (1951) in the presence of 0.55% $NaDodSO_4$.

Carbonic anhydrase (M_r 28 840), ovalbumin (M_r 42 660), creatine phosphokinase (M_r 39 800), bovine serum albumin (M_r 67 600), and cytochrome *c* from beef heart (M_r 12 500) were dissolved to a concentration of 2 mg/mL and used as electrophoresis standards. The F_1-ATPase was isolated by the method of Senior & Brooks (1970) and had a specific activity of 56 units. The F_1-ATPase was stored at −10 °C in its mother liquor, after precipitation with saturated ammonium sulfate, and prepared for electrophoresis by centrifuging a sample and dissolving the pellet in sample buffer to a protein concentration of 2 mg/mL. The mobilities were calculated relative to bromophenol blue.

Spectrophotometric Measurements. Absorption spectra were recorded with an Aminco DW-2 dual-wavelength spectrophotometer. Fluorescence emission and excitation spectra were recorded with a SLM fluorescence spectrophotometer (Model 4000) interfaced with a data processor (HP 9815A). The spectra were routinely obtained with an excitation slit of 4 nm and an emission slit of 8 nm. Photodecomposition of QA-N_3 during these measurements was not detected. All fluorescence spectra were corrected for the light output with a concentrated solution of rhodamine in propylene glycol while corrections for the efficiency of the phototube were not made. Difference spectra were calculated and plotted with the spectrum processor. Generally, the spectra were corrected for the amount of dye covalently bound to the membranes and/or the amount of protein present during the recording. The fluorescence spectra for the labeled membrane proteins were obtained after dissolving the delipidated membranes in 5% $NaDodSO_4$ and diluting it to 0.33% $NaDodSO_4$ with water. The spectra of the labeled lipids were obtained by taking the lipid extract to dryness with a benzene azeotrope in vacuo and dissolving the residue in $CHCl_3$–CH_3OH (1:1). The IR spectra were recorded with a Perkin-Elmer spectrophotometer with all samples but QA-N_3 recorded as a Nujol mull. QA-N_3 was recorded in a chloroform solution. The absorption bands were reported in reference to polystyrene (1600 cm^{-1}).

Chemicals. Glass double distilled water was used in the preparation of all solutions. All chemicals were reagent grade unless otherwise noted. Melting points were uncorrected. Microanalyses were performed by Galbraith Laboratories, Knoxville, KY.

Synthesis of 3-Azido-9-{[4-(diethylamino)-1-methylbutyl]amino}-7-methoxyacridine (QA-N_3). The scheme for the synthesis is shown in Figure 1. All of the azides are indefinitely stable if stored in the dark in solution or dry in a vacuum desiccator. The 9-chloroacridine (VIII) was stored over KOH to neutralize any HCl which might catalytically decompose the acridine.

5-Nitro-4'-methoxydiphenylamine-2-carboxylic Acid (III). A modified version of the synthesis of the ethoxy derivative was used (Albert & Gledhill, 1942). In a 250-mL round-bottom flask, 2-chloro-4-nitrobenzoic acid (1.2 g, 5.0 mmol), *p*-methoxyaniline (0.640 g, 5.6 mmol), Cu powder (0.25 g), anhydrous sodium carbonate (0.52 g), and dry butanol were refluxed with rapid mixing for 5 h. The solution was cooled to room temperature, water (100 mL) added, and the butanol steam distilled. After all of the butanol was removed, the product was precipitated by the addition of 5 N H_2SO_4. The precipitate was collected, washed with water, and dissolved in water (40 mL) containing sodium carbonate (0.27 g). The solution was continuously extracted with benzene until no more color was extracted and made acidic with 5 N H_2SO_4, and the product was collected and dried: yield 74%; mp 235–237 °C (CH_3OH); λ_{max} 276 and 427 nm in ethanol.

9-Chloro-7-methoxy-3-nitroacridine (IV). A mixture of III (0.5 g, 164 mmol) in phosphorus oxychloride (2.5 mL) was refluxed for 2 h in an oil bath at 130 °C. After the mixture was cooled to room temperature, the excess $POCl_3$ was removed in vacuo. The red residue was dissolved in a minimal amount of chloroform and added slowly to a rapidly mixed

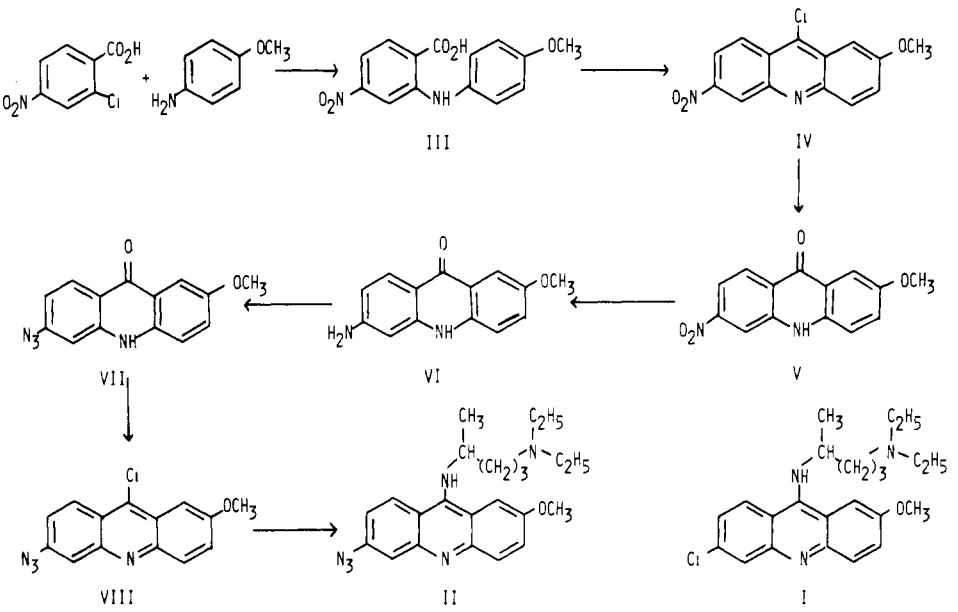

FIGURE 1: Scheme for the synthesis of QA-N_3 (II) and the chemical structure of quinacrine (I).

solution of ammonia (10%) on ice. Ammonia was added continuously to keep the solution alkaline to litmus paper, until the precipitate turned a light tan or yellow. The product was filtered, washed with water, and dried in a vacuum desiccator over P_2O_5. The product was recrystallized from chloroform or benzene, giving yellow needles: yield 80%; mp 217–218 °C (lit. mp 215 °C) (Mendenwald, 1956).

7-Methoxy-3-nitroacridone (V). A mixture of IV (0.5 g, 1.85 mmol) and 10% HCl (15 mL) was refluxed for 2 h or until the product turned bright orange. The product was filtered and recrystallized from concentrated acetic acid, or, alternatively, it remained in the hydrochloric acid solution for the next step: yield 95%; mp >300 °C (lit. mp 320 °C) (Mendenwald, 1956); λ_{max} 269 and 445 nm in ethanol.

3-Amino-7-methoxyacridone (VI). To a solution of V (0.44 g, 1.85 mmol) in 5% HCl was added $SnCl_2 \cdot 2H_2O$ (1.43 g, 1.1 equiv) dissolved in concentrated HCl (1.40 mL) with rapid mixing. The solution was refluxed for 3 h after which the yellow product was filtered, washed with water, and dried in an oven at 100 °C. The aminoacridone was recrystallized from pyridine–water: yield 90%; mp 294 °C (lit. mp 290 °C) (Mendenwald, 1956); λ_{max} 269 nm at pH 2.2 and 264 nm at pH 7.4.

3-Azido-7-methoxyacridone (VII). The 3-amino-7-methoxyacridone (0.5 g, 1.88 mmol) was added to 2.3% aqueous HCl (30 mL). The solution was cooled in an ice–saline bath with rapid mixing while $NaNO_2$ (0.15 g, 1.29 equiv) was added in small portions. The diazotization was allowed to proceed for 1 h, and then NaN_3 (0.26 g, 2.1 equiv) in water (2 mL) was added slowly to the deep violet solution. All operations from this point were performed in the dark under a fume hood. The reaction caused considerable effervescence. Foaming which occurred was controlled with additions of ether. The solution was stirred for 1 h in the ice bath and then for a second hour at room temperature. To the solution was added NaOH (1.3 g in 10 mL of water), and the alkaline solution was filtered and washed with water. The product was dried in a vacuum desiccator over P_2O_5 and recrystallized from pyridine–water: mp 200 °C dec; IR 2120 cm^{-1}; λ_{max} 282 and 414 nm in ethanol. Anal. Calcd for $C_{14}H_{10}N_4O_2$: C, 63.15; H, 3.79; N, 21.04. Found: C, 63.15; H, 3.69; N, 20.51.

3-Azido-7-methoxy-9-chloroacridine (VIII). In the dark, a solution of 3-azido-7-methoxyacridone (0.47 g, 1.70 mmol) was heated in an oil bath at 100–110 °C with $POCl_3$ (2.5 mL) which contained 3% concentrated HCl (v/v) for 3 h. The solution was cooled, and the excess $POCl_3$ was removed in vacuo. The red residue was dissolved in chloroform and added to a rapidly mixed solution under the same conditions as those for the synthesis of IV. After the product was dried over P_2O_5, it was dissolved in chloroform and filtered. The filtrate was evaporated to a volume of 5 mL and passed through a silica gel column (10 mL) poured with chloroform. The product, which was the first to elute with chloroform–ether (45:5), was collected and recrystallized from chloroform–methanol to give yellow needles: yield 50%; mp 161–162 °C dec; IR 2120 cm^{-1}; λ_{max} 337, 386, and 406 nm in ethanol. Anal. Calcd for $C_{14}H_9N_4ClO$: C, 59.06; H, 3.19; N, 19.68; Cl, 12.45. Found: C, 59.04; H, 3.22; N, 19.52; Cl, 12.40.

Table I: Absorption Maxima (nm) and Molar Absorptivities (mM^{-1} cm^{-1}) of the Monoprotonated and Diprotonated Forms of QA and QA-N_3

QA·H^+	342 (4.79)	359 (5.37)	419 (7.94)
QA-N_3·H^+	353 (8.19)	368 (9.45)	420 (7.94)
QA·$2H^+$	345 (5.50)	425 (9.12)	445 (8.71)
QA-N_3·$2H^+$	359 (13.18)	425 (9.12)	444 (8.32)

3-Azido-9-{[4-(diethylamino)-1-methylbutyl]amino}-7-methoxyacridine (II, QA-N_3). In the dark, 3-azido-7-methoxy-9-chloroacridine (VIII) (0.15 g, 0.527 mmol) was added to phenol (0.56 g) and heated in an open vessel with 2-amino-5-diethylaminopentane (0.113 g, 1.33 equiv) for 3 h at 100 °C. The solution was cooled, ether added (10 mL), dry HCl bubbled into the solution, and the solution filtered. The product was washed with ether and dissolved in a minimal amount of methanol and the HCl salt reprecipitated with the addition of ether. The dihydrochloride was recrystallized from methanol: mp 173 °C dec; IR 2110 cm^{-1}; λ_{max} 281 and 359 nm at pH 5.0. Anal. Calcd for $C_{23}H_{29}N_6O \cdot 2HCl \cdot H_2O$: C, 55.53; H, 6.89; N, 16.89; Cl, 14.24. Found: C, 55.37; H, 6.43; N, 16.44; Cl, 13.78.

Results

QA-N_3: A Photoaffinity Analogue of Quinacrine. Table I compares the absorption bands of QA and QA-N_3 in the monoprotonated and the diprotonated species. The monoprotonated species of QA and QA-N_3 share an absorption

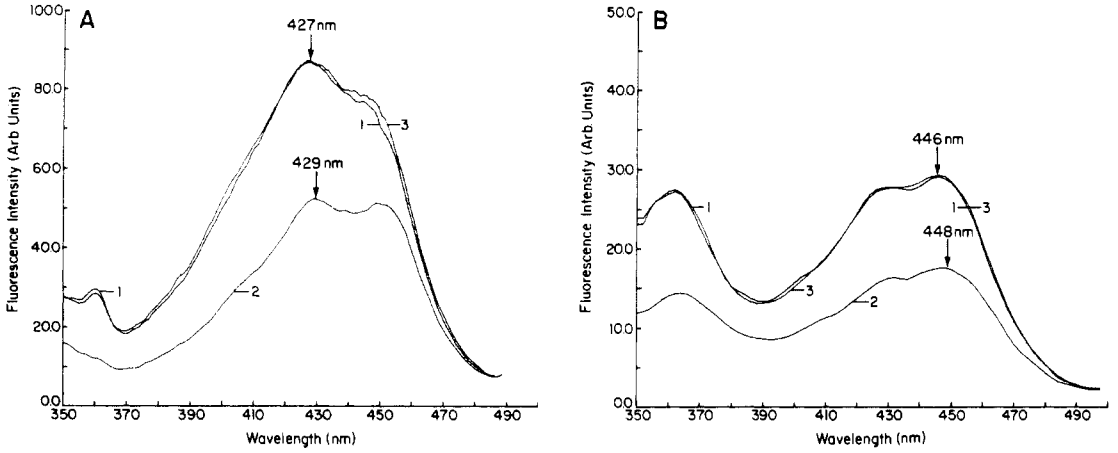

FIGURE 2: Fluorescence excitation spectra of QA and QA-N_3. The reaction mixture consisted of 150 mM sucrose, 30 mM phosphate buffer, pH 7.5, 0.9 mg of MOESP protein, and 3.3 μM dye. (A) QA (with emission at 500 nm); (B) QA-N_3 (with emission at 510 nm). (1) No further addition; (2) with 3.3 mM succinate; (3) with 3.3 mM succinate and 1 μM FCCP.

maximum with identical molar absorptivities at 419 and 420 nm. The maxima at 342 and 359 nm of QA were shifted to 353 and 368 nm for QA-N_3 with moderately large increases in molar absorptivities. The diprotonated species of QA has absorption maxima at 425 and 445 nm which are almost identical with those observed for QA-N_3 as were the corresponding molar absorptivities. The low-wavelength absorption maximum for the diprotonated QA was, however, shifted 14 nm to the red upon substituting the azide for the chloride group. More apparent was the large change in the extinction coefficient from 5.5 mM^{-1} for QA to 13.2 mM^{-1} for QA-N_3. This large change was predicted, however, since the azide group is known to increase the ^{1}La band (Reiser & Wagner, 1971) and the azide group is also known to red shift the absorption bands.

Figure 2 shows the fluorescence excitation spectra of QA and QA-N_3 under identical conditions, associated with energized and uncoupled membranes while actively oxidizing succinate and with nonenergized membranes. A number of points are clearly illustrated. (1) The fluorescence intensity of QA associated with the membranes was greater than 2 times that of QA-N_3; however, the percentage of the fluorescence decrease produced by the energization of the membrane was nearly identical. The fluorescence intensity at the 429-nm maximum of QA associated with the energized membranes was 39% less than that of the 427-nm maximum of QA associated with the nonenergized membranes. Similarly, the fluorescence intensity of the 448-nm maximum for QA-N_3 associated with the energized membranes was also 39% less than that of the corresponding 446-nm maximum of QA-N_3 associated with the nonenergized membranes. (2) The fluorescence spectrum of QA and QA-N_3 associated with the energized membranes returned to that of the spectrum with the nonenergized membranes upon the addition of the uncoupler FCCP. The fluorescence quenching was therefore reversible. The response has previously been shown to be supported by any of the substrates of the electron-transport chain (Mueller et al., 1979a,b). Thus, QA-N_3 can be used to monitor the energy state of submitochondrial membranes and was clearly similar to that of QA.

Photolabeling Submitochondrial Membranes with QA-N_3. The azido derivative of quinacrine, QA-N_3, has been shown previously to be very photosensitive to 350-nm light (Mueller et al., 1981). The extent of covalent labeling of QA-N_3 to the energized and nonenergized membranes was determined at varying dye concentrations with succinate as the energy-yielding substrate. Figure 3 shows that the dye covalently

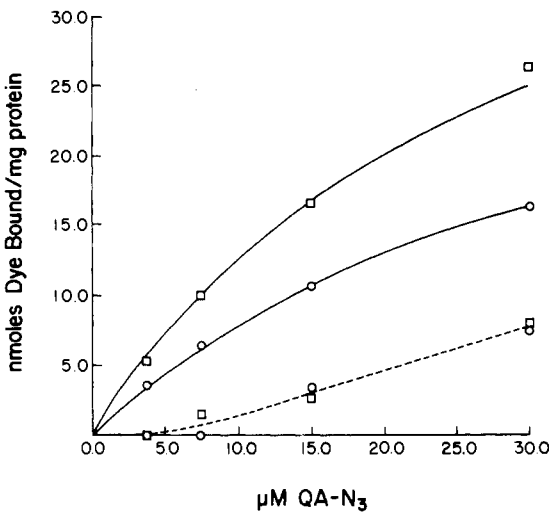

FIGURE 3: Dependence of the amount of covalent labeling to the membrane (—) on the concentration of QA-N_3. Conditions are as described under Materials and Methods. The control (- - -) shows the amount of dye associated with the membrane if the dye was first irradiated and then the membranes were incubated with the dye solution for 1 min. The membranes were in the energized (□) or nonenergized (○) state.

bound to the membrane but consistently with greater yield to the energized membranes. In neither case was the labeling saturated at the highest dye concentration used. The extent of labeling to uncoupled membranes (membranes not treated with oligomycin) did not differ between membranes actively oxidizing succinate and those without succinate (data not presented). Thus, the apparent greater binding to the coupled membranes could not be attributed to an absorption artifact due to the presence of succinate.

The control experiment in Figure 3 shows the amount of dye associated with the membrane if the dye was first irradiated and subsequently incubated with the membranes for 3 min at room temperature. The nonspecific association of the photoproducts with membranes was small at the lowest concentrations but increased dramatically at the higher dye concentrations. The exact cause for this association has not been determined, but it was stable to high salt (0.5 N), alkaline pH (pH 10.0), sonication, or any combination of these treatments (data not shown). The extent that this apparent nonspecific binding occurred in the presence of membranes was not known since the environment of the dye during the irradiation might have differed from that in the absence of membranes and thus may have resulted in different photo-

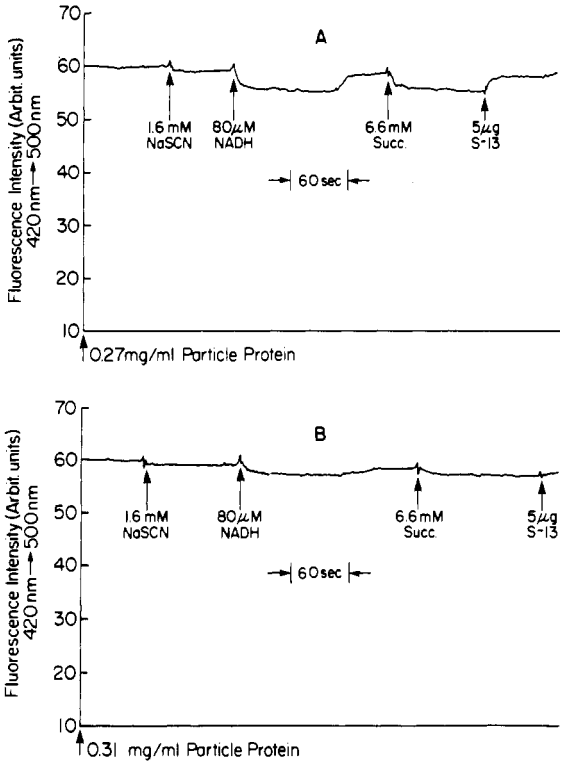

FIGURE 4: Energy-linked fluorescence responses of the covalently bound dye; the membranes were labeled with 30 μM QA-N_3 while in the energized (A) and nonenergized (B) state. The reaction mixture consisted of 150 mM sucrose and 30 mM phosphate buffer, pH 7.5. Other additions were as indicated.

products. Furthermore, covalently labeled membranes may not adsorb the same amount of dye as the nonlabeled membranes. However, this control may be considered to approximate the nonspecific "binding", and it was only significant at the higher dye concentrations.

Clearly, over the range of dye concentrations tested, the labeling of the energized membranes was at least 50% greater than that to the nonenergized membranes with the greatest percentage of binding at the lowest dye concentrations. More surprisingly, however, was the rather large yield of covalent labeling to the membrane, which was 40% and 60% for the nonenergized and the energized membranes, respectively, when 3.75 μM QA-N_3 was used. At the highest dye concentration, the binding decreased to just 23% and 37%, respectively.

The covalently bound dye still retained an energy-linked fluorescence response if the dye was bound to the membrane while in the energized state. Figure 4 shows that the fluorescence of the dye covalently bound to the membrane while in the energized state exhibited a fluorescence quenching with either NADH or succinate as the substrate, and this quenching was released after exhaustion of NADH or by the addition of the uncoupler S_{13}. This same response was not seen if the membrane was labeled while in the nonenergized state. Although the percentage of fluorescence quenching of the dye bound to the energized membrane was small, it was considerably higher than that of the photoproducts of QA-N_3 which exhibited only a 3% fluorescence quenching in response to the energization of the membrane (not shown). Some of the dye covalently bound to the energized membranes, therefore, may be locked into the site responsible for the fluorescence response.

Fluorescence Spectra of the Labeled Membrane and Its Components. The fluorescence spectra of the covalently bound dye were dependent on the energy state of the membrane at the time of irradiation (Figure 5). The much larger maximum at 440 nm of the excitation spectrum and the shift in the

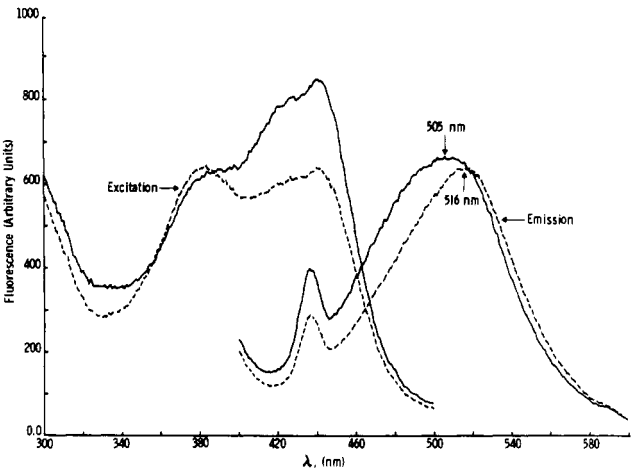

FIGURE 5: Fluorescence excitation (emission at 520 nm) and emission (excitation at 380 nm) spectra of the dye covalently bound to the membranes while in the energized (---) and the nonenergized (—) state. The membranes were labeled with 7.5 μM QA-N_3 (cf. Figure 3), and the spectra were normalized with respect to the amount of dye present.

emission maximum from 516 to 505 nm for the dye bound to the nonenergized membranes indicated either that the covalent products differed between the membranes labeled while in the energized and nonenergized state or that the dye was covalently bound at a different microenvironment. The fluorescence excitation spectrum of the dye covalently bound to the energized membranes *minus* the spectrum of the dye covalently bound to the membrane while in the nonenergized state showed that the difference was greatest at the lowest concentration of QA-N_3, and this difference steadily decreased with increasing QA-N_3 concentration (Mueller et al., 1979a,b). Thus, if the selective labeling was measured by the excitation spectral differences, then increasingly nonspecific labeling occurred at the higher dye concentrations. This was not surprising since QA has an optimal response to membrane energization at about 5 μM. Unlike the differences in the excitation spectra, the emission spectral shift of the energized compared to the nonenergized membrane was not dependent on the dye concentration.

If the differences in the fluorescence excitation spectra were due to different covalent products, rather than differences in the labeling environment, then the spectra should still differ after the membranes were dissolved. Thus, the lipids of the labeled membranes were extracted with $CHCl_3$–CH_3OH (2:1), taken to dryness, and dissolved in $CHCl_3$–CH_3OH (1:1), while the delipidated proteins were dissolved in a 0.33% $NaDodSO_4$ solution. The fluorescence spectra of the labeled lipid products are shown in Figure 6A, and those of the labeled protein products are shown in Figure 6B. Except for the magnitude, the spectra were not greatly different at any of the dye concentrations tested. Two points were immediately apparent: (1) the fluorescence spectral differences for the energized and nonenergized labeled membranes (cf. Figure 5) were very similar to those observed for their lipid products but quite distinct from those observed for the extracted protein products; (2) the fluorescence excitation and emission spectra of the labeled proteins were not dependent on the energy state of the membrane at any of the dye concentrations, and the spectra resembled those of the dye labeled to the intact membrane if labeled while in the nonenergized state. Thus, the selectivity of the insertion reaction appeared to be in the lipid-soluble phase of the membrane. Apparently, at the lower dye concentrations, the fluorescence spectrum of the membrane labeled while in the energized state represented mainly that of

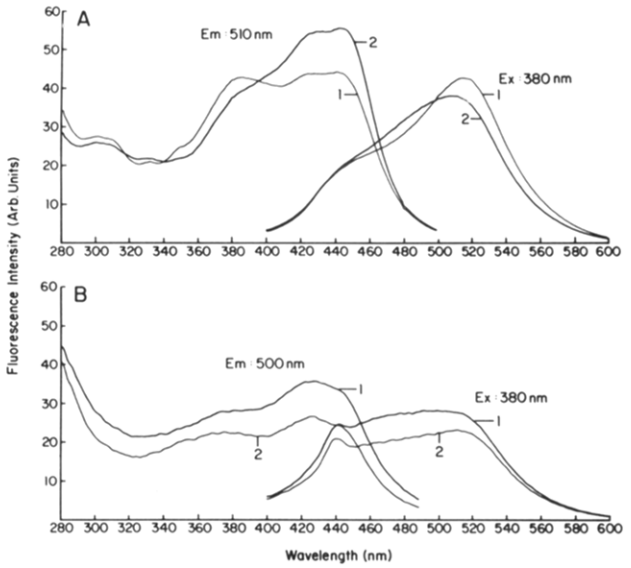

FIGURE 6: Fluorescence spectra of the lipid (A) and the protein (B) components derived from the membranes labeled with 15 μM QA-N_3 (cf. Figure 3) while in the energized (1) and nonenergized (2) state.

the labeled lipid products. At the higher dye concentrations, the fluorescence of the labeled proteins overwhelmed the spectrum. The contributions to the fluorescence spectrum of the membranes labeled while in the nonenergized state were more difficult to evaluate since the fluorescence excitation spectra of the labeled lipids and proteins were similar. The membranes were analyzed with $NaDodSO_4$–polyacrylamide gel electrophoresis to determine if there was any selectivity in the labeling to the proteins.

Covalent Labeling of the Membrane Proteins and Lipids. Figure 7 shows the Coomassie blue stain and the corresponding fluorescence photograph of the total delipidated proteins of the labeled membranes in the energized and nonenergized states with varying dye concentrations after separation by $NaDodSO_4$–polyacrylamide slab gel electrophoresis. There were three bands which were primarily labeled with corresponding molecular weights of 54K, 33K, and 30K. The 54K band was evident by its large mass relative to that of the total protein, and it migrated identically with the α and β subunits of the F_1-ATPase (not shown). When electrophoresis was run on a different system, the α and β subunits separated with the fluorescence, suggesting that both the α and β subunits were labeled. The 33K protein migrated with the γ subunit of the F_1-ATPase. The δ subunit does not appear to have been labeled. The peptide labeled with the greatest intensity corresponded to a molecular weight of 30K. This labeled peptide may be the adenine nucleotide translocator protein, apocytochrome *b*, and/or the uncoupler binding protein [cf. DePierre & Ernster (1977)]. While the total labeling of the membrane proteins was greater if the membranes were in the nonenergized state, there was no large variation in the labeling pattern or in the relative proportions of labeling for each of the peptides. These results further suggest that the membrane proteins do not participate, to a great extent, in the membrane and dye interactions which are specific for the energized membranes.

Preliminary analysis of the labeled lipid products (Mueller, 1981) was not entirely conclusive. Since there are many more dye than lipid molecules (ca. >70:1) at the dye concentrations where there was specific labeling, the analysis of the labeled lipid products was very difficult. In order to conclusively determine which lipids were derivatized, the lipids of the membrane, as well as QA-N_3, must be radioactively labeled.

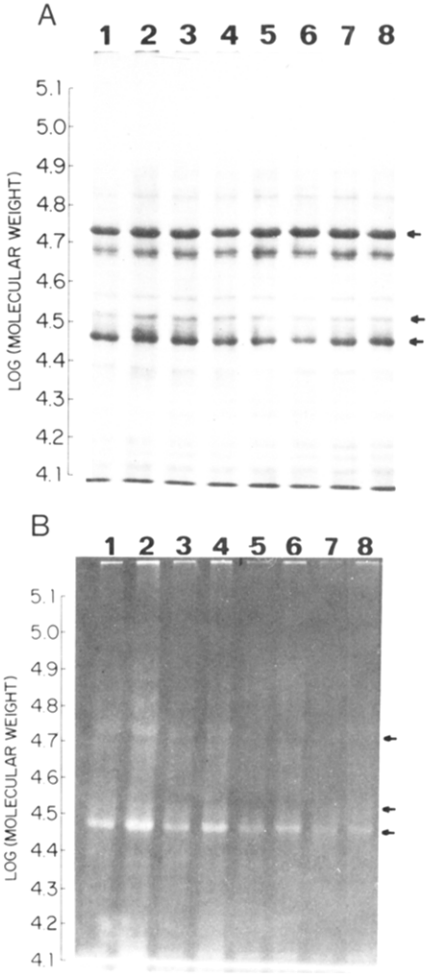

FIGURE 7: Coomassie blue stain (A) and the fluorescence photograph (B) of the labeled membrane proteins (80 μg/lane) after separation by $NaDodSO_4$ gel electrophoresis. The samples correspond to labeling while the membranes were in the energized (1, 3, 5, and 7) and nonenergized (2, 4, 6, and 8) states with QA-N_3 concentrations of 34 (1 and 2), 17 (3 and 4), 8.5 (5 and 6), and 4.3 μM (7 and 8). The arrows indicate the membrane proteins which have fluorescence associated with them.

Experiments of this type are now being considered.

Enzymatic Activities of the Labeled Membranes. The irradiation of the membranes, in the absence of dye, in either the energized or the nonenergized state, had no effect on the enzyme activities tested. However, there was a clear effect on the enzyme activities when irradiation was done in the presence of QA-N_3. Figure 8B shows the inhibition of NADH oxidase as a function of the amount of dye covalently bound to the membrane. The activity was sharply decreased with increasing covalent labeling, reaching 85% inhibition at 16 nmol of dye bound/mg of protein for the nonenergized membranes and at 26 nmol of dye bound/mg of protein for the energized membranes. Thus, the inhibition of the nonenergized membranes was greater than that of the energized membranes. However, NADH–$Fe(CN)_6^{3-}$ reductase activity was not significantly affected at any of the dye concentrations used (Figure 8A). Contrary to this, succinate–PMS reductase activity was inhibited at a constant level of 70% over all of the dye concentrations used, independent of the energy state of the membrane (Figure 9A). The activity of succinate oxidase (Figure 9B) appeared to be limited by the dehydrogenase activity even at 5 nmol of dye bound/mg of protein.

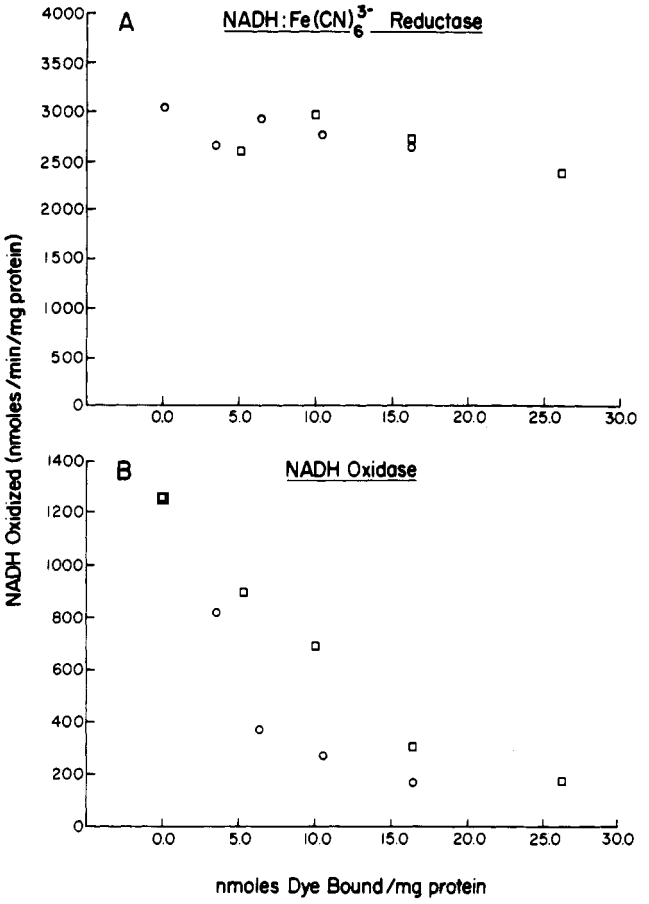

FIGURE 8: Specific activities of NADH–$Fe(CN)_6^{3-}$ reductase (A) and NADH oxidase (B) of the membranes labeled while in the energized (□) and in the nonenergized (○) state.

Discussion

The similarities of the absorbance and fluorescence spectra of QA-N_3 and QA indicated that there was little change in the electron distribution in the chromophore after the azide was substituted for the chloride group. This was expected since the azide radical belongs to a class of inorganic radicals referred to as pseudohalides (Treinin, 1971). The pK_a of the pyridinium nitrogen of QA-N_3 was previously shown to be quite close to that of QA (Mueller et al., 1979a,b). Furthermore, the energy-linked fluorescence decrease of QA-N_3 was to the same extent as was that of QA. The photosensitivity of QA-N_3 together with the above observations supported its use as a photoaffinity probe of the sites of interaction of QA with the membrane.

The azide group has been widely used in photolabeling studies because of the relatively large percentage of insertion reactions of the aryl nitrene. The diazoacyl group introduced by Westheimer (Singh et al., 1962; Shafer et al., 1966; Hexter & Westheimer, 1971a,b) produced a carbene upon irradiation, but only about 3% of the probe reacted via the intramolecular carbene insertion reaction. This low yield is generally attributed to extensive intramolecular rearrangements, e.g., Wolff rearrangements, and to solvent reactions (Knowles, 1972). The aryl nitrene, however, does not rearrange to such a great extent (Doering & Odum, 1966; Wentrup & Crow, 1970), but it does still react quite readily with the solvent. The solvent reactions generally restrict insertion reaction yields to not more than 20–30%. Based on the studies of photolabeling of phospholipid vesicles with phenyl azide, Bayley & Knowles (1978) concluded that nonpolar nitrene compounds were unsatisfactory reagents for lipids or other hydrophobic regions

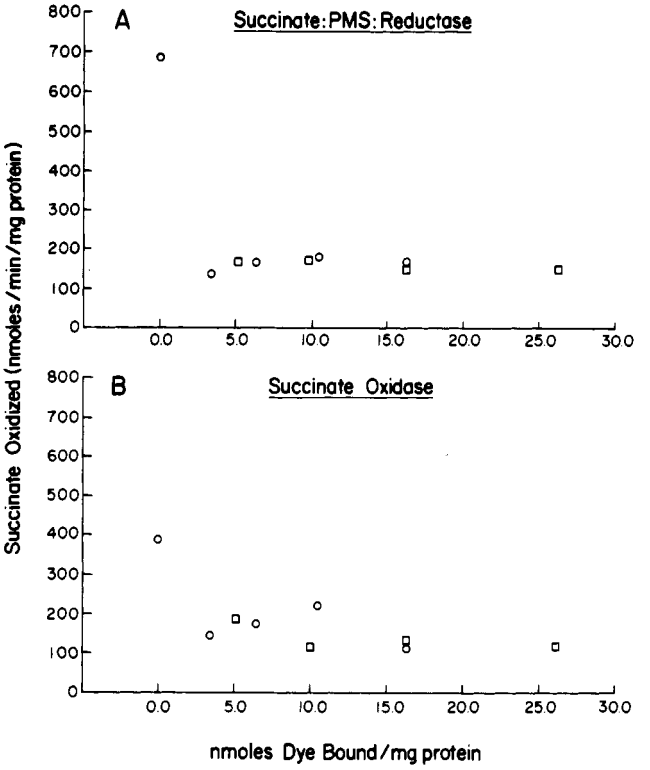

FIGURE 9: Specific activities of succinate–PMS reductase (A) and succinate oxidase (B) of the membranes labeled while in the energized (□) and in the nonenergized (○) state.

of membranes. However, other studies have suggested that generation of lipophilic nitrenes in a hydrophobic environment may increase the yield of the insertion reaction to 70% or more. For instance, the hydrophobic probe 1-azidonaphthalene and 1-azido-4-iodonaphthalene cross-linked in excess of 70% yield to the sarcoplasmic reticulum membranes (Klip & Gitler, 1974; Bercovici & Gitler, 1978). Labeling of a membrane-enriched rhodopsin preparation with 1-azidonaphthalene resulted in a 30% insertion reaction (Klip et al., 1970). The same probe, when used to label bovine serum albumin, however, resulted in only 12% of the probe inserting into the protein (Klip & Gitler, 1974). In the present study, 40% insertion into the nonenergized membranes and 60% insertion into the energized membranes indicated that the probe interacted in a hydrophobic environment of the membrane.

The fluorescence spectra of the labeled products indicated that the specificity of the labeling was in the lipid portion of the membrane. Previous studies with QA have also suggested that it interacts with the membrane in a hydrophobic environment and that the lipids may be involved. An analysis of the fluorescence quantum yield of QA was taken to suggest that QA interacts in an area of low polarity in the membrane (Massari et al., 1974). Lee first suggested that QA interacts with the membrane phase of energized submitochondrial membranes (Lee, 1971), and this was later supported by the unusual temperature dependence on the fluorescence of QA (Lee et al., 1974; Huang et al., 1977). Furthermore, the fluorescence polarization of QA associated with energized membranes shows a sharp break at the melting point of the membrane (Huang & Lee, 1978; C. S. Huang and C. P. Lee, unpublished experiments). This break in the fluorescence polarization is characteristic of hydrophobic probes which are embedded in the lipid phase of the membrane and has, in fact, been used to measure the viscosity and order of the lipid phase [cf. Schinitzky & Barenholz (1978), Heyn (1979), Livingston & Schater (1980), and Vanderkooi & Chance (1972)].

Although this study has not specified the lipids interacting with QA or QA-N_3, other studies have implicated cardiolipin. Labeling studies with liposomes derived from phosphatidylcholine (PC), phosphatidylethanolamine (PE), and cardiolipin (CL) indicated that, upon irradiation, there were very little insertion reactions of QA-N_3 with PC while there were some with PE and a large amount with CL (Mueller, 1981). In another study, CL was shown to augment the energy-linked fluorescence quenching of QA with submitochondrial membranes as well as to increase QA's binding to the membranes (Lee et al., 1974). The fluorescence quenching of QA when associated with energized membranes increased from 12% to 54% after enrichment of the membranes with CL.

A specific role for membrane proteins participating in the interactions of QA-N_3 with the membrane is not supported here. The apparent greater labeling of both the F_1-ATPase and the 30K peptide to the nonenergized membranes could have resulted from an uptake of the dye to the specific sites (i.e., the hydrophobic region) of the energized membranes. This increased uptake could have reduced nitrene insertion into peripheral proteins. The relative protection of NADH oxidase against photoinactivation while the membrane was in the energized state (cf. nonenergized) suggests such a redistribution of the dye upon energization.

In conclusion, our results indicate that QA-N_3 can serve as a probe for the sites of interaction of quinacrine with the membrane. The dye interacts with specific sites or regions of the energized membranes which are distinct from the sites of the nonenergized membrane. These sites appear to be in the hydrophobic region of the membrane.

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Primary Structure of Two Distinct Rat Pancreatic Preproelastases Determined by Sequence Analysis of the Complete Cloned Messenger Ribonucleic Acid Sequences†

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ABSTRACT: The mRNA sequences for two rat pancreatic elastolytic enzymes have been cloned by recombinant DNA technology and their nucleotide sequences determined. Rat elastase I mRNA is 1113 nucleotides in length, plus a poly(A) tail, and encodes a preproelastase of 266 amino acids. The amino acid sequence of the predicted active form of rat elastase I is 84% homologous to porcine elastase 1. Key amino acid residues involved in determining substrate specificity of porcine elastase 1 are retained in the rat enzyme. The activation peptide of the zymogen does not appear related to that of other mammalian pancreatic serine proteases. The mRNA for elastase I is localized in the rough endoplasmic reticulum of acinar cells, as expected for the site of synthesis of an exocrine secretory enzyme. Rat elastase II mRNA is 910 nucleotides in length, plus a poly(A) tail, and encodes a preproenzyme of 271 amino acids. The amino acid sequence is more closely related to porcine elastase 1 (58% sequence identity) than to the other pancreatic serine proteases (33–39% sequence identity). Predictions of substrate preference based upon key amino acid residues that define the substrate binding cleft are consistent with the broad specificity observed for mammalian pancreatic elastase 2. The activation peptide is similar to that of the chymotrypsinogens and retains an N-terminal cysteine available to form a disulfide link to an internal conserved cysteine residue.

The mammalian exocrine pancreas synthesizes, stores, and secretes approximately 15 enzymes and proenzymes for intestinal digestion. These secretory proteins account for greater than 80% of the protein synthesis of the gland (Jamieson & Palade, 1967; VanNest et al., 1980). A dominant fraction of the pancreatic secretions is a family of at least seven serine proteases: chymotrypsins A and B, two trypsins, elastases 1 and 2,[1] and kallikrein. The members of this gene family are probably related by evolution from a common ancestral protease (Neurath et al., 1967; deHaen et al., 1975) and have retained similar structure, size, and function. As secretory proteins, the serine proteases are expected to be synthesized initially as precursor proteins (Devillers-Thiery et al., 1975; Rutter et al., 1978) with an amino-terminal signal peptide (Blobel & Sabatini, 1971) that specifies the binding of polysomes to the rough endoplasmic reticulum and the subsequent vectorial transport into the cisternae of the reticulum and proteolytic processing of the precursors as the first steps in secretion. In addition, the pancreatic serine proteases are secreted as proenzymes (zymogens) that require selective enzymatic cleavage of an amino terminal "activation peptide" for conversion to the active enzymes. Analysis of the organization of the pancreatic serine protease genes and mRNAs is expected to contribute to an understanding of events in the evolution of this gene family and of the processes involved in the biosynthesis and maturation of the enzymes.

Two members of the pancreatic family of serine proteases are characterized by their ability to hydrolyze elastin. Elastase 1 action is largely limited to Ala–Ala and Ala–Gly bonds (Gertler et al., 1977). Determination of the three-dimensional structure of elastase 1 revealed that its cleavage preference can be explained in part by an occluded substrate binding pocket, relative to other serine proteases, that makes the binding of large amino acid side chains sterically impossible (Shotton & Watson, 1970). In contrast, elastase 2 has a preference for cleavage at amino acids with medium to large

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[1] We use Roman numerals to identify the rat pancreatic elastase mRNAs and the enzymes they encode to indicate that the enzyme activities have not been demonstrated directly. We retain the use of Arabic numerals in the discussion of the purified and well-characterized pancreatic elastases of pig and human.

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