Phospholipid Topology of the Inner Mitochondrial Membrane of Rat Liver†

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ABSTRACT: Mitoplasts from rat liver mitochondria were reacted with trinitrobenzenesulfonate (TNBS), fluorodinitrobenzene (FDNB), isethionyl acetimidate (IA), methyl acetimidate (MA), and methyl picolinimidate (MP) at 4 and 21 °C in order to elucidate the topology of phosphatidylethanolamine (PE). TNBS was found to penetrate the mitoplast membrane rapidly at 21 °C and more slowly at 4 °C. Penetration was not influenced by valinomycin but was inhibited by 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS). Kinetic analysis of the TNBS reaction was required to elucidate the asymmetric arrangement of PE. The fast-reacting PE was considered to be located on the outer membrane surface. The slow-reacting PE may be located on the inner membrane surface. The fraction of PE which did not react with FDNB or MA is believed to be masked by tight binding to membrane proteins. The results suggest that three different pools of PE occur in the membrane, but their location cannot be firmly defined. Our interpretation is that 30–40% of the total membrane PE is located on the outer membrane surface, 45–60% is located on the inner membrane surface, and 10–15% is buried within the membrane. IA labeled only 13% of the total PE at 4 °C at saturation of available sites. Perturbing the membrane by sonication, by treatment with lubrol, and by freeze–thawing has little effect on the extent of labeling of PE by IA. Therefore, a large number of PE molecules are not available to react with IA due to either charge repulsion and/or tight binding to protein. MA, a penetrating probe, at 24 mM concentration labeled about 90% of the total PE molecules in mitoplasts in contrast to 20 mM MP which labeled only 50% of the PE molecules. The labeling of PE by MP was not influenced by disrupting the mitoplasts with lubrol. TNBS, FDNB, IA, and MA have differential effects on the activities of glutamate dehydrogenase (GDH) (matrix enzyme) and ATPase (membrane enzyme). At saturating levels of probe, 20 mM MA at 4 °C inhibited GDH by 20% and inhibited ATPase by 16% whereas 20 mM IA inhibited GDH by only 8% and gave no inhibition of ATPase. TNBS at 2 mM concentration at 4 °C inhibited ATPase by 40% and inhibited GDH by 96%. FDNB at 2 mM concentration at 4 °C inhibited ATPase by 95% and inhibited GDH by 96%.

Several nonpenetrating chemical probes have been used to study membrane architecture. These include formylmethionyl sulfone methyl phosphate (Bretscher, 1972), diazotized sulfanilic acid (Berg, 1969; Tinberg et al., 1974), trinitrobenzenesulfonate (Gordesky & Marinetti, 1973), isethionyl acetimidate (Whiteley & Berg, 1974; Rothman & Kennedy, 1977), and fluorescamine (Hawkes et al., 1976). Gordesky & Marinetti (1973) used TNBS[1] to show that the major part of PE and nearly all the PS were located on the inner surface of the erythrocyte membrane, confirming an earlier report by Bretscher (1972), who postulated an asymmetric arrangement of PE in the red cell ghost. Litman (1973, 1974) showed that PE was localized to a greater extent on the inner surface of PE–PC liposomes. Crain et al. (1978) found an asymmetry of PE in rod outer segment disk membranes. Rothman & Kennedy (1977) reported an asymmetry of PE in the *Bacillus megatherium* membrane. Phospholipid asymmetry appears to be a general property of several biological membranes.

The present study is an extension of preliminary work by Marinetti et al. (1976) which suggested that PE was asymmetrically disposed in the inner mitochondrial membrane, on the basis of the assumption that TNBS did not penetrate the membrane. Nilsson & Dallner (1977), using phospholipase A_2 cleavage, reported that 90% of the PE is located on the outer membrane surface. We will show in this paper that TNBS penetrates both intact mitochondria and mitoplasts but that the asymmetry of PE in the inner mitochondrial membrane can be established by kinetic analysis of the labeling with TNBS. The limitations of the use of imido esters and TNBS are presented.

Materials and Methods

Albino rats (250–300 g, male, Sprague–Dawley) were obtained from Charles River, Boston, MA. 1-Fluoro-2,4-dinitrobenzene (FDNB), 2,4,5-trinitrobenzenesulfonate (TNBS), methyl acetimidate (MA), and isethionyl acetimidate (IA) were purchased from Pierce Chemical Co. Valinomycin and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. Digitonin was from Fisher Scientific Co. and 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid (SITS) was from Nutritional Biochemical Co. 2,4-[3,5-^{3}H]FDNB (14 Ci/mmol) was obtained from New England Nuclear.

Mitochondria were prepared from the livers of five fasted rats according to the method of Schnaitman & Greenawalt (1968). Mitoplasts were prepared from mitochondria by treatment with digitonin (0.11–0.12 mg/mg of mitochondrial protein) for 15 min at 0 °C. Preparations were checked routinely for microsomal, lysosomal, and outer mitochondrial membrane contamination. The integrity of the membrane was checked by measurement of respiratory control and by assay for marker enzymes (Schnaitman & Greenawalt, 1968).

Phosphate analysis was done spectrophotometrically after digestion in 70% perchloric acid (Harris & Popat, 1954).

† From the Department of Biochemistry, The University of Rochester Medical Center, Rochester, New York 14642. *Received September 26, 1978.* This work was supported in part by Grant HLB02063 from the National Institutes of Health. This paper was taken in part from the thesis of R.C.C. submitted in partial fulfillment of the requirements for the Ph.D. Degree, Department of Biochemistry, The University of Rochester, 1978.

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[1] Abbreviations used: MA, methyl acetimidate; IA, isethionyl acetimidate; MP, methyl picolinimidate; TNBS, trinitrobenzenesulfonate; FDNB, fluorodinitrobenzene; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulfonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; TLC, thin-layer chromatography; GDH, glutamate dehydrogenase.

0006-2960/79/0418-2407$01.00/0

Protein was estimated by a biuret method (Gornall et al., 1949). ATPase was measured by colorimetric analysis of phosphate released (Senior, 1971). Glutamate dehydrogenase was assayed by following formation of NADH at 340 nm according to the method of Beaufay et al. (1959) as modified by Schnaitman & Greenawalt (1968). Acid phosphatase was measured by hydrolysis of *p*-nitrophenyl phosphate in citrate buffer, pH 4.4. Monoamine oxidase was measured by the method of Schnaitman et al. (1967). Glucose 6-phosphatase was assayed by the method of Hubscher & West (1965).

Reaction of Chemical Probes with Mitoplasts and Mitochondria. Mitoplasts (5–10 mg of protein) or mitochondria (10–20 mg of protein) were suspended in buffer (70 mM sucrose, 200 mM mannitol, 40 mM $KHCO_3$, and 1 mM succinic acid), pH 8.5, at 4 or 21 °C. This buffer was used in all subsequent studies. The reaction was initiated by addition of buffer, pH 8.5, containing various amounts of TNBS, FDNB, IA, MA, or MP. The final volume was 20 mL. The reaction was stopped by addition of 5 mL of acidified buffer (to bring the final pH to 7.0) and centrifugation for 10 min at 9000*g*. The supernatants were discarded and the pellets rinsed once. The pellets were homogenized in 300 μL of buffer, and the lipids were extracted by the method of Folch et al. (1957) as modified by Crain et al. (1978). TNP-PE and DNP-PE were separated from unreacted PE by thin-layer chromatography, visualized by their yellow color, and quantitated spectrophotometrically (Gordesky & Marinetti, 1973; Gordesky et al., 1972). Unreacted PE and *N*-acetimidoyl-PE or *N*-picolinimidoyl-PE separated by thin-layer chromatography were visualized by treatment with iodine vapors and quantitated by phosphate analysis after perchloric acid digestion (Crain & Marinetti, 1978). In some experiments GDH and ATPase activities were determined on duplicate samples of labeled membranes.

Effect of Pretreatment of Mitoplasts with TNBS on Labeling by [^{3}H]FDNB. Mitoplasts (5 mg of protein) were reacted at 4 °C with 6 mM TNBS in 20 mL of buffer. Controls were incubated at 4 °C without TNBS. Lubrol (3 mg/10 mg of protein) was added to half of the samples. After 30 min, 0.5 μCi of [^{3}H]FDNB in methanol (final concentration 2 mM FDNB and 0.5% methanol) was added to each. The reaction was stopped after 1 h at 4 °C by acidification. Lubrol was added to samples which had been reacted in the absence of detergent. The inner mitochondrial membranes were spun down at 100000*g* for 90 min. The supernatants (matrix proteins) were collected and the protein was precipitated by treatment with Cl_3AcOH (final concentration 5%). The precipitated matrix proteins were spun down for 10 min at 2000*g*, washed once with 5% Cl_3AcOH, and dissolved in 2 mL of 5% $NaDodSO_4$. Radioactivity was measured on a Searle Delta 300 liquid scintillation spectrometer (Model 6890).

Results

Reaction of Mitoplasts with TNBS and FDNB. The integrity and purity of the mitoplast preparations were checked by enzyme marker assays and by respiratory control. As seen in Table I, the mitoplasts had little contamination of outer mitochondrial membrane and microsomes as detected by monoamine oxidase and glucose 6-phosphatase activities. Acid phosphatase activity, a marker enzyme for lysosomes, was also lower than that of intact mitochondria or outer mitochondrial membranes.

Using the value obtained by Vignais et al. (1971) for acid phosphatase activity in a purified lysosomal fraction (prepared by injection of Triton WR1339 into rats several days before membrane fractionation), we calculated the contamination of mitoplasts by lysosomes to be less than 1%. Though we did not analyze for plasma membrane contamination, there is good evidence that there is little plasma membrane contamination in inner mitochondrial membranes plus matrix (Vignais et al., 1971). We have independent evidence that very little plasma membranes were in our mitoplast preparations. In lipid analyses of mitoplasts, we found that less than 0.1% of the total phospholipid is made up of PS. Rat liver plasma membranes contain about 15% of the total phospholipid as PS (Emmelot et al., 1974). Respiratory control (the rate of oxidation in the presence of ADP divided by the rate in the absence of ADP) was measured for mitochondria in the presence and absence of Mg^{2+} and for mitoplasts in the absence of Mg^{2+}. Mg^{2+} has been found to irreversibly uncouple oxidative phosphorylation in mitoplasts (Schnaitman & Greenawalt, 1968). Mitochondria incubated in the presence of Mg^{2+} yielded values for respiratory control approaching 7 while those incubated in the absence of Mg^{2+} yielded values of about 3. Our mitoplast preparations had an average respiratory control ratio of 2.8, in good agreement with Schnaitman & Greenawalt (1968).

Table I: Marker Enzyme Activities of Rat Liver Subcellular Fractions[a]

membrane fraction	acid phosphatase [nmol/(min mg)]	glucose 6-phosphatase [nmol/(min mg)]	monoamine oxidase (% total act.)	respiratory control
mitochondria	45	3.3	100	5–7
mitoplasts	11 ± 2	0.6 ± 0.6	8 ± 8	2.8 ± 0.8
outer membranes	130		90–95	
microsomes	14.5	71		

[a] Mitochondria and mitoplasts were prepared as described under Materials and Methods. The supernatant after centrifugation of mitochondria was spun at 30000*g* for 20 min to remove damaged mitochondria and cell debris. The microsomal fraction was isolated by centrifugation of the resulting supernatant at 100000*g* for 90 min. The outer mitochondrial membrane fraction was prepared by centrifugation of the postmitoplast supernatant at 100000*g* for 90 min. Enzyme activities were measured as described under Materials and Methods. Values with standard deviations represent the mean of determinations of three to four separate preparations. Values without standard deviations represent the average of duplicate determinations of two separate preparations.

For the determination of the topographical distribution of PE in the inner mitochondrial membrane, labeling studies were performed by using TNBS and FDNB. TNBS was chosen as the vectorial probe because under specified conditions it has been found to be impermeable to a number of membranes and because it yields a derivative which is easily isolated and quantitated. FDNB, a hydrophobic, penetrating probe was used to determine the PE available on both surfaces of the membrane.

The reaction of PE of mitoplasts with TNBS and FDNB was examined as a function of the concentration of the probe at 21 and 4 °C (Figure 1). FDNB was found to react with slightly more PE at 4 °C as compared to TNBS. However, at 21 °C, labeling with either TNBS or FDNB saturated at about 85% of the total PE. Addition of ethanolamine to the reaction mixture at the termination of the incubations showed that both TNBS and FDNB were present in excess.

Valinomycin, which has been shown to cause penetration of TNBS into red cells (Marinetti et al., 1978) as measured by the labeling of hemoglobin, was found to have no effect on the labeling of PE by TNBS at 4 °C (data not shown). Three explanations may be offered for these data. First, if TNBS is not penetrating the membrane, 80–85% of the total PE is

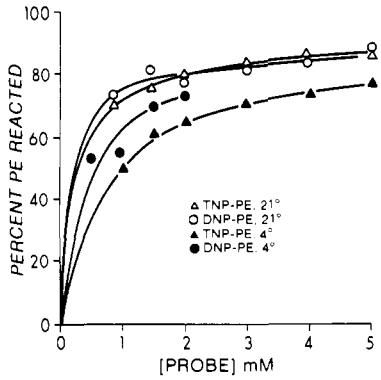

FIGURE 1: Reaction of PE of mitoplasts with TNBS or FDNB at 4 and 21 °C as a function of the concentration of each probe. Mitoplasts (5–10 mg of protein) were suspended in 20 mL of buffer, pH 8.5, at 4 or 21 °C. The reaction was initiated by addition of ice water or 21 °C buffer, pH 8.5, containing various amounts of TNBS or FDNB (FDNB was added in methanol to give a final concentration of 0.5% methanol). Methanol was added to control samples and samples containing TNBS to give a final concentration of 0.5%. The final volume was 20 mL and the final concentration of the probe was 0.5–5.0 mM. The reaction was stopped after 2 h at 4 °C and 1 h at 21 °C by acidification to pH 7.0 and centrifugation at 9000*g* for 10 min. The pellets were suspended in 0.2 mL of water, and the lipids were extracted with chloroform–methanol as described previously by Crain et al. (1978). The lipid-containing chloroform phase was brought to dryness under nitrogen and quantitatively applied to thin-layer plates. TLC was carried out in chloroform–methanol–water (65:25:4 v/v). The yellow TNP-PE and DNP-PE (trinitrophenylated PE and dinitrophenylated PE) bands were scraped out, eluted in methanol, and quantitated spectrophotometrically. The percent PE reacted was determined by comparison with values for total PE derived from unreacted mitoplasts. The points presented for 4 °C represent the average of four experiments on four different mitoplast preparations. The points presented for 21 °C are the average of duplicate determinations on one preparation.

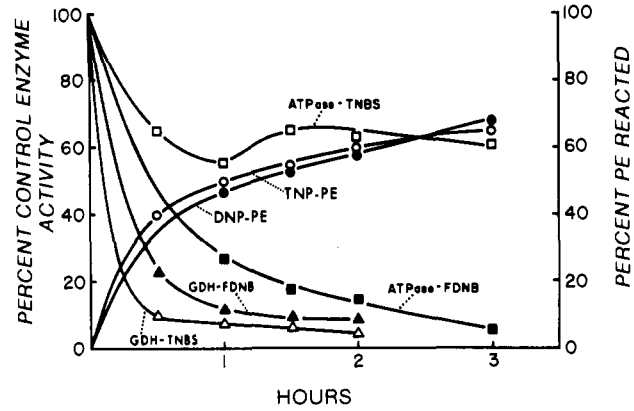

FIGURE 2: Effect of TNBS and FDNB on GDH and ATPase activities of rat liver mitoplasts and correlation with the labeling of PE. Mitoplasts (5–10 mg of protein) were added to 20 mL of buffer, pH 8.5, at 4 °C, containing TNBS or FDNB (added in methanol). The final concentration of the probe was 2.0 mM. The reaction was stopped by acidification to pH 7.0 at specified time intervals (0.5–3 h) and centrifugation at 9000*g* for 10 min. Labeling of PE was measured as given in Figure 1. At each time point, ATPase and GDH activities were also measured after homogenization of the pellets in 1 mL of water. Each value represents the average of duplicate determinations.

located on the exterior surface. The remaining 15–20% of PE is unavailable for reaction with either TNBS or FDNB, presumably due to masking by membrane proteins. A second explanation is that PE undergoes rapid translocation across the membrane. This is unlikely in light of the results of Rousselet et al. (1976), who reported no PC translocation in the inner mitochondrial membrane. A third possibility is that TNBS is permeable to the mitoplast. This latter possibility must be known in order to make any conclusions regarding the asymmetry of PE in the inner mitochondrial membrane from the data using TNBS.

In order to find out if TNBS penetrates the mitoplast membrane, we measured the effect of labeling on two internal enzymes. First, ATPase-catalyzed hydrolysis of ATP was measured. This activity is due to the F_1 portion of the ATPase complex and has clearly been shown to be localized on the inner membrane surface, attached by a stalk to the membranes. Tinberg et al. (1974), using diazobenzenesulfonate (a "nonpenetrating" probe which reacts with histidine, tyrosine, and lysine groups), found no inhibition of ATPase activity when this chemical was reacted with intact mitochondria while they found 80% inhibition when it was reacted with inside-out inner membranes. This study supports the use of ATPase as a measure of TNBS penetration. Second, GDH-catalyzed reduction of NAD^+ was measured. This enzyme has been clearly demonstrated to be localized in the matrix space of the mitochondria (Matlib & O'Brien, 1975; Sottocasa et al., 1967). The activities of these two enzymes were studied as a function of time of reaction with TNBS or FDNB and correlated with the amount of PE labeled (Figure 2). The 35–40% inhibition of the ATPase activity and the 91–94% inhibition of the GDH activity by TNBS indicated that penetration was occurring. FDNB gave a similar inhibition of GDH activity; however, it gave much more inhibition of ATPase activity than did TNBS. Therefore, although both probes penetrate, they have a differential effect on the ATPase. The extent of inhibition of GDH and ATPase by FDNB and TNBS is correlated temporally with the extent of reaction of PE with these probes. However, the nature of the correlation between the labeling of PE and the activities of these two enzymes is not clear since GDH is inhibited much more rapidly by TNBS than is ATPase.

In order to provide evidence that the inhibition of ATPase and GDH was due to labeling of the interior of the mitoplast, we performed an additional experiment. Mitoplasts were first labeled with 6 mM TNBS for 30 min at 4 °C. The labeling of matrix proteins by [^{3}H]FDNB was then measured and compared to that of control mitoplasts. It was found that TNBS treatment inhibits by 30% the labeling of matrix protein by [^{3}H]FDNB (data not shown). Lubrol has little effect on the labeling pattern (data not shown). It was therefore concluded that TNBS was permeable to the mitoplast membrane.

Reaction of Mitochondria with TNBS. One possible explanation for the leakiness of mitoplast preparations to TNBS was that during the preparation, the membrane was altered. In order to test this possibility we treated intact mitochondria for 1 h with different amounts of TNBS at 4 °C. The fraction of PE reacted and the inhibition of ATPase activity were measured as a function of TNBS concentration (Table II). Of the total PE, 75–80% reacted with 4.5 mM TNBS, and this was correlated with about 50% inhibition of the ATPase activity. From these data it is clear that intact mitochondria as well as isolated mitoplasts are leaky to TNBS. Therefore, other chemical probes were examined as possible vectorial probes.

Reaction of Mitoplasts with MA and IA. IA was used as a nonpenetrating probe in the human erythrocyte by Whiteley & Berg (1974). It was examined as a possible vectorial probe in the mitoplast. MA was used to measure the total available PE molecules on both membrane surfaces.

Because of the rapid rate of hydrolysis of imido esters, it is believed necessary either to use sequential additions of probe or to use very high initial concentrations (20–30 mM). We

Table II: Reaction of PE with TNBS and Its Correlation with the Inhibition of ATPase Activity in Intact Mitochondria[a]

TNBS concn (mM)	PE (% reacted)	protein labeling [OD × 10^3/(mg mL)]	ATPase act. (% control)
	(a) Reaction at 0 °C for 1 h		
0.4	23.3	2.7	92
0.8	36.9	5.3	83
1.5	51.2	10.3	82
3.0	71.1	19.7	66
4.5	79.5	25.9	54
	(b) Reaction at 21 °C for 15 min		
0.4	28.2	6.8	93
0.8	37.9	7.3	82
1.5	52.2	11.8	81
3.0	69.5	19.0	62
4.5	75.5	24.1	52

[a] Mitochondria (20 mg of protein) were suspended in a total volume of 20 mL of buffer, pH 8.5, containing 0.5–4.5 mM TNBS. The reaction was run for 15 min at 21 °C or 60 min at 4 °C and stopped as described under Materials and Methods. The percent of PE reacted, the labeling of protein (measured after solubilization of delipidated protein in 10 mL of 5% $NaDodSO_4$), and the ATPase activity were determined. Each value represents the average of duplicate determinations.

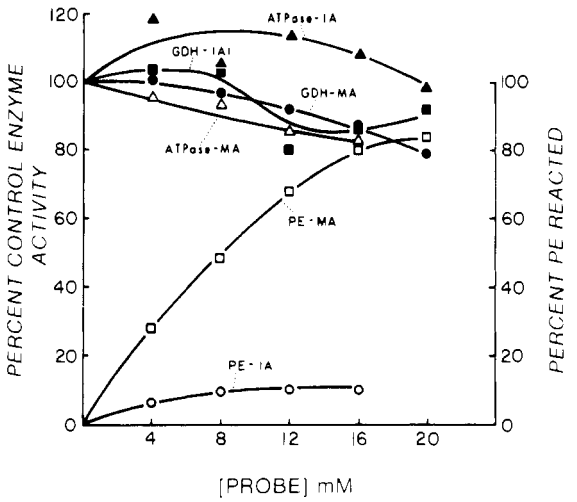

FIGURE 3: Effect of MA and IA on GDH and ATPase activities of rat liver mitoplasts and correlation with the labeling of PE. Mitoplasts (8 mg of protein) were suspended in buffer, pH 8.5, at 4 °C, containing 4–20 mM methyl acetimidate or isethionyl acetimidate. The final volume was 20 mL. The reaction was stopped after 2 h as described in the text, and the pellet was homogenized in 1 mL of distilled water. Aliquots were removed for protein determination and measurement of GDH and ATPase activities. The percent of the total PE which reacted was measured as given in Figure 4. Values represent the average of duplicate determinations.

found that sequential additions of either IA or MA did not significantly improve the total labeling of PE compared to a single addition at any total concentration of probe added. Therefore, saturation of available PE was achieved by using a single addition of 20–30 mM probe. As was suggested by Whiteley & Berg (1974) for studies on the red cell, neither MA nor IA had a marked inhibitory effect on enzyme activities. The "penetrating" probe MA gave about 20% inhibition of ATPase and GDH activity while the "nonpenetrating" probe IA gave little inhibition of GDH and no inhibition of ATPase activity (Figure 3).

As shown in Figure 4 at either 4 or 21 °C, about 90% of the PE reacted with MA at a probe concentration of 24 mM. The extent of reaction was the same at pH 8.5 and 9. The

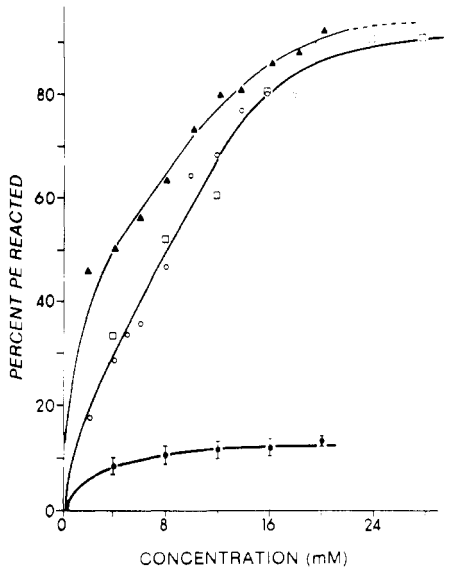

FIGURE 4: Reaction of PE of mitoplasts with MA and IA as a function of probe concentration. Mitoplasts (5–10 mg of protein) were suspended in 20 mL of buffer containing 2–30 mM imido ester. The reaction conditions were as follows: (a) mitoplasts reacted with MA (○) or IA (●) in buffer, pH 8.5, at 4 °C for 2 h; (b) mitoplasts reacted with MA (□) in buffer, pH 9.0, at 4 °C for 2 h; (c) mitoplasts reacted with MA (▲) in buffer, pH 8.5, at 21 °C for 1 h. The reactions were stopped by acidification, and the lipids were extracted, applied quantitatively to silica gel G thin-layer plates, and developed in chloroform–methanol–concentrated ammonium hydroxide (65:25:4) as described previously (Crain & Marinetti, 1978). The reacted derivatives and unreacted PE were scraped and quantitated by phosphate analysis after elution and perchloric acid digestion. The curves are based on values obtained on four separate mitoplast preparations.

reaction of nearly all the PE is expected since MA penetrated the membrane. However, at saturating concentrations of the nonpenetrating probe IA, only 13% of the total PE reacted (Figure 4). This would indicate that the majority of the PE in the mitoplast was not accessible to the hydrophilic IA. In order to determine if the inaccessibility of the majority of PE to labeling by IA was due to the localization of PE on the inner membrane surface or to the masking of PE on either surface, we did labeling under a variety of conditions to perturb the membrane.

Mitoplasts reacted with 20 mM IA during soniciation showed no appreciable alteration in the amount of labeling (data not shown). Furthermore, as seen in Table III, reaction in the presence of lubrol (which causes lysis of the membrane and release of matrix proteins) increased the reaction only slightly, labeling a maximum of 36% of the total PE at 25 mM IA at 21 °C. Freezing and thawing also had no significant effect on the extent of labeling of PE by IA (data not shown). These experiments indicate that the limited reaction of PE with IA is not due to an asymmetric arrangement of PE, with most being localized on the inner membrane surface, but rather to some membrane property which makes the bulk of the PE unavailable to IA.

Kinetics of Penetration of TNBS into the Mitoplast. We have shown that TNBS, when reacted to saturation of available sites at one time interval, cannot be used as a vectorial probe in the mitoplast membrane because of penetration. Furthermore, we have shown that IA has limitations as a vectorial probe of PE asymmetry in the inner mitochondrial membrane because it can only react with a small fraction of the available PE molecules located on either surface of the membrane. In order to measure the asymmetry of PE in the inner mitochondrial membrane, we considered the kinetic analysis of the

Table III: Reaction of Methyl Acetimidate and Isethionyl Acetimidate with Mitoplast PE in the Presence and Absence of Lubrol[a]

probe	deter-gent	temp (°C)	PE (% reacted)
25 mM methyl acetimidate	−	0	87 ± 1.9
25 mM methyl acetimidate	+	0	83
25 mM methyl acetimidate	−	21	99 ± 2.1
25 mM methyl acetimidate	+	21	100
25 mM isethionyl acetimidate	−	0	11 ± 1.4
25 mM isethionyl acetimidate	+	0	19
25 mM isethionyl acetimidate	−	21	31 ± 2.2
25 mM isethionyl acetimidate	+	21	36

[a] Mitoplasts (5 mg of protein) were incubated 1 h in 20 mL of buffer, pH 8.5, containing 25 mM MA or IA. The reaction was carried out at 0 or 21 °C in the presence and absence of lubrol (1 mg). The reaction was stopped by addition of acidified buffer to bring the pH to 7.4. Samples containing lubrol were centrifuged at 100000*g* for 90 min. Samples reacted in the absence of lubrol were centrifuged at 10000*g* for 10 min. The lipids were extracted and the labeled PE was quanitated as described under Materials and Methods. Samples without detergent added are the mean of triplicate determinations plus or minus the standard deviation. Samples with detergent added are the average of duplicate determinations.

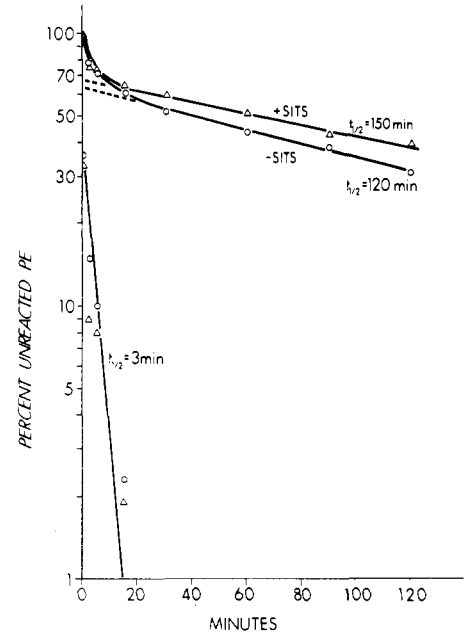

FIGURE 5: Reaction of PE of mitoplasts with TNBS at 4 °C and the effect of preincubation with SITS. Mitoplasts (5–10 mg of protein) were suspended in 20 mL of buffer, pH 8.5, at 4 °C, containing 2 mM TNBS. Half the samples were preincubated 40 min in 10 mL of buffer, pH 8.5, at 4 °C, containing 2 mM SITS. The other samples were incubated in buffer without SITS. The reactions were stopped after 2–120 min by acidification to pH 7.0 and centrifugation for 10 min at 10000*g*. The percent PE reacted was determined as described in Figure 1. Points for the percent of PE reacted after preincubation with SITS (△) are the average of duplicate determinations of one mitoplast preparation. Points for the percent PE reacted without preincubation with SITS (○) are the average of duplicate determinations (2–15 min) or the mean of three determinations done in duplicate on three separate preparations of mitoplasts (30–120 min).

reaction of TNBS with PE since the PE molecules on the outer membrane surface should react faster than the PE molecules on the inner membrane surface if the rate of penetration of TNBS through the membrane is rate limiting. If this is not the case, one cannot determine whether the slower reacting PE is localized on the inner membrane surface.

Because TNBS is present in greater than 10-fold excess of the total membrane amino groups, pseudo-first-order kinetics will result if all the PE molecules have the same reactivity and accessibility. Under these conditions, a semilogarithmic plot of the time course of reaction will be linear and will yield a single component. Mitoplasts were reacted with 2 mM TNBS at 4 °C for various lengths of time, and the percent of the total PE reacted was determined. A semilogarithmic plot of the percent unreacted PE vs. the time of reaction was not linear and yielded two components (Figure 5). By curve peeling, this curve may be divided into two linear components, a fast and slow component. This curve is described by the exponential equation $f(t) = f_1e^{-k_1t} + f_2e^{-k_2t}$ where f_1 and k_1 are the kinetic parameters corresponding to the rapid PE labeling while f_2 and k_2 are the kinetic parameters corresponding to the slower PE labeling. The latter can be analyzed at times at least 5 times greater than the half-time of the rapid labeling. From Figure 5, the half-time of the slow component ($0.693/k_2$) was calculated to be 120 min, and the y intercept (f_2) was calculated as 64%. The half-time of the fast component ($0.693/k_1$) was calculated as 3 min, and the y intercept (f_1) was calculated as 36%. We have interpreted the fast-reacting component of PE to be that which is located externally while the remainder is localized internally or is not accessible due to protein masking. However, it is also possible that the slower reacting PE represents a different pool of PE which reacts differently due to some environmental factor. The location of this pool of PE may or may not be on the inner membrane surface.

The slow labeling of a fraction of the PE may be due to penetration of TNBS or to membrane rearrangement and exposure of more PE molecules. In order to distinguish between these, we repeated the time course of reaction of PE with TNBS under conditions which alter the rate of penetration of TNBS. SITS has been shown to inhibit anion transport in the red blood cell (Knauf & Rothstein, 1971). It also may compete for anion transport in mitochondria since Wehrle et al. (1978) have demonstrated phosphate transport in mitochondria. Treatment of mitoplasts for 40 min with 2 mM SITS was followed by reaction with TNBS. A semilogarithmic plot of the percent unreacted PE vs. the time of reaction still shows a fast and slow component (Figure 5). The fast component accounts for 33% of the total PE and reacts with a half-time for reaction of 3 min. The major effect of SITS is to increase the half-time for reaction of the slow component from 120 to 150 min. The effect of SITS on the kinetics of TNBS reaction with PE is consistent with the hypothesis that the fast-labeling component is due to TNBS reacting with PE on the outer membrane surface, while the slow-labeling component is due to the penetration of TNBS and labeling of PE on the inner membrane surface. SITS does not affect the fast reaction of TNBS with the exterior PE but does affect the rate of labeling of the slow component by inhibiting TNBS penetration. This is supported by our finding that SITS also protects the ATPase activity in mitochondria from the inhibitory effect of TNBS (Table IV). SITS also decreases the labeling of PE and membrane protein by TNBS.

Valinomycin has been shown to increase penetration of TNBS into red cells (Marinetti et al., 1978). Valinomycin was found to have no effect on the kinetics of TNBS reaction with mitoplasts at 4 °C (data not shown). This result may be explained by the rigidity of the mitoplast membrane at 4 °C, which might restrict valinomycin penetration through the membrane. The effect of valinomycin on membranes is temperature dependent (Krasne et al., 1971). Freeze–thawing mitoplasts caused a marked decrease in the half-time of reaction of the slow component at 4 °C from 120 to 56 min

Table IV: Effect of SITS on the Labeling of Mitochondrial PE and Membrane Protein by TNBS and on the Inhibition of ATPase by TNBS[a]

TNBS (mM)	SITS	ATPase (% control)	PE (% reacted)	protein (% reacted)[b]
0		100	0	
0.38	−	92	32	16
0.75	−	77	46	26
1.5	−	68	63	45
3.0	−	63	75	67
4.5	−	43	80	77
0		100	0	
0.38	+	97	25	10
0.75	+	95	39	16
1.5	+	84	55	27
3.0	+	64	69	47
4.5	+	61	72	67

[a] Mitochondria were incubated 15 min at 37 °C in the presence and absence of 1 mM SITS. Then TNBS was added, and after reaction for 15 min, at 21 °C, mitochondria were spun down and washed with buffer. An aliquot was assayed in duplicate for ATPase activity. An aliquot was extracted, and the percent PE reacted was determined as described in Figure 1. The protein precipitate was dissolved in 1% $NaDodSO_4$ with heating, and the absorbance at 337 nm was measured. This value includes both labeled membrane proteins and labeled matrix proteins. [b] The percent protein labeled was determined by dividing the absorbance at 337 nm of membranes reacted with different amounts of TNBS by the absorbance at 337 nm of $NaDodSO_4$-solubilized membranes reacted with TNBS.

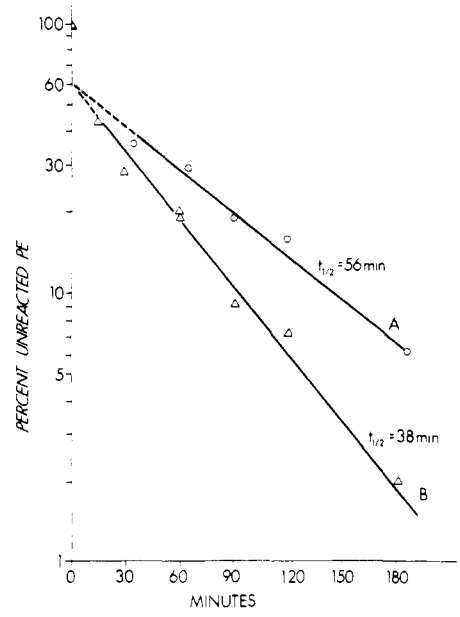

FIGURE 6: Effect of freezing on the reaction of PE in mitoplasts with TNBS. (A) represents the time course of reaction of frozen and thawed mitoplasts with 2 mM TNBS in buffer, pH 8.5, at 4 °C as described in Figure 1. (B) represents the time course of reaction of mitoplasts reacted with 2 mM TNBS in buffer, pH 8.5, at 21 °C. Each point in both (A) and (B) represents the average of duplicate determinations. Lipid analyses were carried out as given in Figure 1.

(Figure 6). It is known that freezing and thawing rat liver mitoplasts causes loss of respiratory control, presumably by damaging the membrane and causing leakiness to H^+. It would also be expected to cause leakiness to other ions and might therefore cause an increased rate of penetration of TNBS. Besides an increased rate of labeling of PE by TNBS, freeze–thawed mitoplasts showed about a twofold increase in the inhibition of ATPase activity. Though the possibility of other membrane perturbations caused by freeze–thawing which might change the accessibility of masked PE cannot be ruled out, it appears that a change in the permeability of TNBS is most likely responsible for the change in the rate of labeling of PE as well as the increased inhibition of ATPase activity.

We conclude that the PE which reacts rapidly with TNBS at 4 °C (Figure 5) is localized on the outer (cytoplasmic) surface of the inner mitochondrial membrane. This constitutes 30–40% of the total PE and is not changed by treatment with SITS or by freeze–thawing. An additional 10–15% of the total PE is concluded to be present in a less accessible configuration and may be localized on the inner or outer membrane surface. This conclusion is based on the labeling of 80% of the total PE at saturation by the penetrating hydrophobic probe FDNB and on the labeling of 90% of the total PE at saturation by the penetrating hydrophilic probe MA. The remaining 45–60% of the total PE which is labeled slowly by TNBS is considered to be localized on the inner surface of the membrane or represents a different pool of PE whose location in the membrane cannot be defined by the available data.

Reaction of Mitoplasts with Methyl Picolinimidate. MP was tested as another imido ester probe in order to see how it compared with methyl acetimidate. At saturation of sites, only 50% of the total PE reacts with MP. Furthermore, treatment of mitoplasts with lubrol has no effect on the extent of reaction of PE with MP. MP provides another example of how the nature of a chemical probe may influence the extent of its reaction with membrane PE. For sake of brevity, data using MP are not shown.

Discussion

Membrane function is determined by the types of components present and how they are arranged. Mitochondria require a very special array of respiratory components to accomplish energy transduction (Tinberg et al., 1973). It has been shown that the lipid is important in the functioning of this membrane. In lipid-depleted mitochondria (Fleischer et al., 1962) or mitochondria containing an altered fatty acid or lipid composition (Haeffner & Privett, 1975; Haslam & Fellows, 1975; Solov'eva & Yasaitis, 1974), function is impaired. The role of lipids in mitochondrial energy coupling has been suggested from temperature studies in different functional states coupled with the use of spin-labeled probes of the lipid environment (Tinberg et al., 1972).

The arrangement of cardiolipin in mitochondria has been investigated. Antibodies against cardiolipin do not react with intact mitochondria (Guarnieri et al., 1971; Aho et al., 1973), suggesting that most of the cardiolipin occurs on the inner membrane surface. However, a maximum of 30% of the total cardiolipin can react after aging or sonication (Guarnieri et al., 1971). Cardiolipin is a major acidic phospholipid in the inner mitochondrial membrane and has been shown to bind tightly to cytochrome oxidase (Awasthi et al., 1971).

In this paper we have studied the topology of PE, which comprises 35–40% of the total phospholipid in the inner mitochondrial membrane. TNBS has been found to be nonpenetrating in the erythrocyte (Gordesky et al., 1975) and in artificial liposomes (Litman, 1974). Under certain conditions TNBS was found to penetrate the erythrocyte membrane (Bonsall & Hunt, 1971) and the *B. megatherium* membrane (Rothman & Kennedy, 1977). Earlier work by Marinetti et al. (1976) using TNBS with mitoplasts indicated an asymmetric arrangement of PE with 65% being localized on the outer surface. This work assumed that TNBS did not penetrate the membrane. However, as shown in the present study, TNBS does penetrate the mitoplast membrane, and therefore the earlier conclusion on the asymmetry of PE has

to be modified.

The present study has shown from the inhibition of GDH and ATPase activities that TNBS penetrates the mitochondrial membrane. Furthermore, we found that in intact and disrupted mitoplasts, PE reacted with IA to a maximum extent of 30–36% at 21 °C. This extent of reaction is similar to the results of Whiteley & Berg (1974), who found only 31% of the total amino phospholipid to be labeled by IA in red cell ghosts. It is known that the limited reaction of PE in the mitoplast and red cell does not result from a characteristic of the reaction of PE with IA because PE in organic solution or PE present in PC–PE liposomes reacts nearly to completion with the probe (Crain & Marinetti, 1978). Furthermore, the limited reaction of PE in mitoplasts is not a characteristic of other membranes. Reaction of IA with rod outer segment disk membranes results in the labeling of over 70% of the PE and over 50% of the PS (Crain et al., 1978).

We believe that the inaccessibility of the major portion of the amino phospholipid in the inner mitochondrial membrane and the red cell membrane to labeling by IA is due in part to the negatively charged sulfonic acid group of this probe. Data using IA as a vectorial probe of phospholipid asymmetry must therefore be interpreted with caution.

It became apparent that for the examination of lipid asymmetry using TNBS, a kinetic analysis of PE labeling by TNBS would be required as has been done previously (Rothman & Kennedy, 1977; Crain et al., 1978). Using this type of analysis, we find that 30–40% of the PE is localized on the external surface. Although the localization of the other PE molecules is less certain, we conclude that 45–60% is localized on the internal surface and 10–15% is less available to reaction with TNBS. These latter values are estimates which can represent different pools of PE in the membrane but whose location remains to be determined. Our results are not in agreement with the findings of Nilsson & Dallner (1977). Using phospholipase A_2 treatment, they concluded that 90% of the total PE was localized on the external membrane surface. They also have studied phospholipid asymmetry in other subcellular organelles by using phospholipase A_2. They found 90% of the PE and 85% of the PS to be external in the endoplasmic reticulum and Golgi membranes. Their results have been challenged by Sundler et al. (1977), who found a nearly symmetrical distribution of PE and PS in the same membranes. These discrepancies may be due in part to the problems encountered by using phospholipases for the study of phospholipid asymmetry (Martin et al., 1975) or to different experimental conditions.

The reaction of mitoplasts with MP presents an enigma since only 50% of the total PE reacts at saturation of available sites. MP, like MA, is considered to be a penetrating probe, yet MA reacts to a greater extent with PE than does MP. The extent of reaction of MP and IA with PE at 21 °C is not appreciably influenced by disrupting mitoplasts with lubrol. Therefore, these probes have a limited accessibility to all the PE molecules, even in disrupted membranes. It appears that different chemical probes which vary in hydrophobicity, charge, and size sense different populations of PE molecules and proteins in cell membranes. Other evidence for heterogeneous domains of PE in the red cell membrane has been reported elsewhere (Marinetti & Crain, 1978). Further insight into the finer structure of cell membranes may be attained by judicious use of these probes.

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Synthesis of a Spin-Labeled Analogue of Nicotinamide Adenine Dinucleotide Phosphate and Its Interaction with Dihydrofolate Reductase†

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ABSTRACT: A spin-labeled analogue of NADP has been prepared and characterized. Adenosine 2′,3′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy) has been prepared by coupling adenosine 2′,3′-phosphate 5′-phosphoromorpholidate with 4-phospho-2,2,6,6-tetramethylpiperidinyl-1-oxy and isolated by ion-exchange chromatography. The cyclic phosphate was cleaved by treatment with dilute acid to give a mixture of the 2′- and 3′-phosphate isomers. Incubation with a 3′-nucleotidase and subsequent ion-exchange chromatography gave the desired 2′-phosphate isomer, adenosine 2′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy). The spin-labeled analogue of NADP inhibits dihydrofolate reductase isoenzyme II from *Streptococcus faecium* var. *durans* Strain A. The inhibition is competitive with respect to NADPH with $K_{is} = 7.7 \pm 1.4$ μM. This is consistent with the dissociation constant for the inhibitor complex, $K_D = 5.1 \pm 0.8$ μM, as measured by the effect of binding on the EPR signal of the ligand. The electron paramagnetic resonance (EPR) measurements also show that the number of binding sites for the ligand on the reductase is 1.1 ± 0.1. The EPR signal shape at high ratios of the analogue to enzyme is unchanged from the signal in the absence of enzyme, but at low ratios of analogue to enzyme there is a marked broadening of the signal. Analysis of the enzyme-bound signal gave a correlation time of 5 ns, which is somewhat shorter than the rotational correlation of the enzyme [20 ns; Cocco, L., Blakley, R. L., Walker, T. E., London, R. E., & Matwiyoff, N. A. (1978) *Biochemistry 17*, 4285–4290]. The analogue does not appear to inhibit glutamate dehydrogenase, isocitrate dehydrogenase, or glucose-6-phosphate dehydrogenase.

The structure of dihydrofolate reductase (EC 1.5.1.3) is the subject of intensive study because of its clinical relevance (Kisliuk & Brown, 1979; Matthews et al., 1978, 1979), the reductase being the target in cancer chemotherapy utilizing the drug methotrexate (Blakley, 1969). As part of continuing investigations of dihydrofolate reductase structure, we are using NMR spectroscopy of ^{13}C-labeled dihydrofolate reductase (Blakley et al., 1978; Cocco et al., 1978, 1979), which can yield information about motion of side chains, protein tumbling, conformational transitions, and ligand binding. In order to extract all the information from the NMR spectra, resonances need to be assigned to specific amino acid residues in the protein sequence.

A possible first step in making such assignments is the identification of resonances corresponding to residues near the active site. This would help to distinguish spectral changes reflecting conformational transitions from those due to direct interaction between the ligand and labeled residues. One tool for the identification of such resonances is the spin-labeled analogue of the ligands (Krugh, 1976; Morrisett, 1976). Resonances of ^{13}C nuclei near the bound spin-labeled ligand will be broadened, and in favorable cases the distance between the spin label and the ^{13}C nucleus can be calculated. In the present paper we report on the synthesis and characterization of a spin-labeled analogue of NADP to be used for this purpose. Some of these results have been presented in a preliminary form (Cocco & Blakley, 1978; Cocco et al., 1979). The corresponding spin-labeled analogue of NAD has been synthesized by Weiner (1969).

Experimental Procedure

Materials

4-Hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo-OH)[1] was obtained from the Aldrich Chemical Co. 2-

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[1] Abbreviations used: Tempo-OH, 4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy; PEI, poly(ethylenimine); DEAE, diethylaminoethyl; DCC, dicyclohexylcarbodiimide; cPADP-SL; adenosine 2′,3′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy); PADP-SL, adenosine 2′-phosphate 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy); Mes, 2-(*N*-morpholino)ethanesulfonic acid; ADP-SL, adenosine 5′-diphospho-4-(2,2,6,6-tetramethylpiperidinyl-1-oxy); TLC, thin-layer chromatography; BSA, bovine serum albumin; ADPR, adenosine 5′-diphosphoribose.

0006-2960/79/0418-2414$01.00/0