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The Use of Amphipathic Maleimides to Study Membrane-Associated Proteins

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Abstract

A series of amphiphilic polymethlyenecarboxymaleimides has been synthesized for use as sulfhydryl reagents applicable to membrane proteins. Physical properties of the compounds which are relevant to their proposed mode of action have been determined. By comparing rates of reaction in aqueous and aprotic solvents, the compounds have been shown to react exclusively with the thiolate ion. The effects of the reagents on three membrane-associated proteins are reported, and in two cases a comparative study has been made of the effects on the proteins in the absence of membranes. A mechanism is proposed whereby the reagents are anchored at the lipid/water interface by the negatively charged carboxyl group, thus siting the reactive maleimide in a plane whose depth is defined by the length of the reagent. Supporting evidence for this model is provided by the inability of the reagents to traverse membranes, and variation of their inhibitory potency with chain length when the proteins are embedded in the membrane, but not when extracted into solution. As examples of general use of the reagents to probe sulfhydryl groups in membrane proteins, the reagents have been used to (a) determine the depths in the membrane at which two populations of sulfhydryl groups occur in the mitochondrial phosphate transporter; (b) locate a single sulfhydryl associated with the active site of D-β-hydroxybutyrate dehydrogenase in the inner mitochondrial membrane; (c) examine sulfhydryl groups in the D-3-glyceraldehyde phosphate dehydrogenase associated with the human red blood cell membrane.

Key Words: Sulfhydryl reagents; chemical modification; membrane proteins; phosphate transporter; D-β-hydroxybutyrate dehydrogenase; D-3-glyceraldehyde phosphate dehydrogenase.

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Introduction

With the exception of the few membrane proteins that have been crystallized and for which there is X-ray analysis data (Findlay and Pappin, 1986; Allen *et al.*, 1988), the molecular organization of proteins within membranes is an area of almost total ignorance, particularly for those peptide sequences that are embedded within the hydrophobic regions of the bilayer (Ikemoto, 1982; Jorgenson, 1982; Chasteen, 1983; Jennings, 1989; Rees *et al.*, 1989). Presently, using conventional chemical reagents and enzymes, it is only possible to localize the position of chemical functional groups of membrane proteins to either the hydrophilic surface region, or to the bulk hydrophobic region of the membrane. The precision of these techniques is low for those functional groups located on the surface of the membrane, and nonexistent for those groups in hydrophobic regions.

We have developed a strategy to provide information on the distribution of thiol groups within the hydrophobic regions of biological membranes. To this end, we have synthesized a series of polymethylenecarboxymaleimides (acid maleimides; AM*n*,[5] see structure 1 in Griffiths *et al.*, 1981). It was anticipated that these amphipathic molecules would penetrate biological membranes and orient with the maleimide moiety inserted in the membrane and the polymethylene chain parallel to the hydrocarbon sidechains of the lipids in the bilayer regions of the membrane. The penetration of the maleimide moiety would be controlled by the length of the polymethylene chain, since it is improbable that the ionized carboxyl group would penetrate the polar surface of the membrane. Thus the thiolate ions that can react with the maleimide moiety of the AM molecules would be limited to those at set depths within the plane of the membrane.

In this paper, we present results of experiments in which the action of the AM on three membrane-associated proteins was studied. These were (a) the phosphate transporter located in the inner mitochondrial membrane, (b) the D-β-hydroxybutyrate dehydrogenase also located in the inner mitochondrial membrane, and (c) the D-3-phosphoglyceraldehyde dehydrogenase associated with the membrane of mammalian red blood cell ghosts. The data are most easily explained in terms of the AM molecules acting in the way that was anticipated, and illustrate the value of this approach to the analysis of membrane proteins. Preliminary accounts of some of these experiments have appeared (Churchill *et al.*, 1982; Moore and Beechey, 1984).

[5] Abbreviations: AM*n*, polymethylenecarboxymaleimide with *n* methylene groups; MA, maleamic acid; 3-PGDH, 3-phosphoglyceraldehyde dehydrogenase.

Methods and Materials

Chemical Syntheses

Preparation of AM. These compounds were made by a two-step procedure; first, synthesis of the appropriate maleamic acid (MA), and second, ring closure to yield AM. Each of the compounds prepared was characterized by elemental analysis, ^{1}H NMR, and IR specta. The essential details are presented in Griffiths (1984).

Synthesis of the MA. Maleic anhydride (10–200 mmol) was dissolved in tetrahydrofuran. An equivalent of the appropriate ω-carboxy-*n*-alkylamine was added and the resulting suspension was stirred overnight at room temperature. The solvent was removed under vacuum, and the residue was recrystallized from boiling methanol, save for *N*-(ω-carboxymethyl)maleamic acid, where methanol/water, 8 : 2 (v/v), was used. Yields were in the range 70–85%.

Synthesis of the AM. For the shorter-chain AM (n = 1–4), the ring closure was effected using the procedure described by Bose *et al.* (1958). Approximately 20 mmol of the appropriate MA was dissolved in 250 ml dry toluene. Triethylamine (36 mmol) was added and the mixture was refluxed for 1 h, the water produced being removed by azeotropic distillation in a Dean and Stark apparatus. The solvent was then removed under vacuum and the residue dissolved in water and sufficient HCl to reduce the pH to 1–2. The mixture was extracted with 2 × 40 ml of ethyl acetate. The combined extracts were dried overnight with anhydrous magnesium sulfate. The solution was filtered and concentrated to a residue that was recrystallized from either chloroform or a mixture of diethyl ether and petroleum ether, pb 46–60°C. The yields varied between 40 and 50%.

For the longer-chain AM (n = 5–11), the method of Searle (1948) was followed with some modification. The appropriate MA (20 mmol) was added to a mixture of 15 mmol fused sodium acetate and 70 mmol acetic anhydride. The temperature was raised to 70°C and stirring was continued for 1 h. After cooling to room temperature, 10 ml of distilled water was added. The mixture was poured into chloroform/water 2 : 3 (v/v), and stirred for 45 min. The chloroform layer was removed and the mixture was extracted with a further 100 ml for 45 min. The combined chloroform extracts were washed with iced distilled water and dried overnight with magnesium sulfate. After filtration, the solution was concentrated to yield a brown oil. This was dissolved in 10 ml of acetone, and chromatographed on a silica column (15 μm, 2 × 15 cm). The required material was eluted with acetone/hexane 1 : 1 (v/v). The presence of the compound was detected by TLC on silica gel plates using the same solvent. The fractions containing the compound were pooled,

decolorized with charcoal, and filtered. Solvent was removed by vacuum distillation, and the residue was recrystallized from either acetone/isopropyl ether mixtures or from aqueous methanol. The yields were about 20%. *N-n*-octylmaleamic acid was prepared as described by Tsou *et al.* (1955). It was converted into the final product as described by Heitz *et al.* (1968).

Preparation of Mitochondria and Submitochondrial Particles

Rat liver mitochondria were prepared by the method of Schnaitman and Greenawalt (1968). Rat liver submitochondrial particles were made by resuspending intact mitochondria in a medium containing 250 mM sucrose, 50 mM Tris–HCl, and 1 mM EDTA, pH 7.6, at a protein concentration of 20–30 mg ml^{-1}. The suspension was disrupted for 4 × 15 sec in an MSE sonicator. The sonicate was centrifuged at 10,000 *g* for 10 min. The pellet was discarded, and the supernatant centrifuged at 100,000 *g* for 30 min. Sedimented vesicles were resuspended in the above medium and stored at −20°C. Bovine heart mitochondria were prepared on a large scale as described by Blair (1967) with the modifications described by Bock and Fleischer (1974). They were stored at −80°C. Submitochondrial particles were prepared from these mitochondria by nitrogen compression, decompression, and shear using a Parr bomb (Fleischer *et al.*, 1974).

Measurement of Phosphate Transport

Measurement of Passive Transport. The passive transport of phosphate was measured by a modification of the techniques of Chappell and Crofts (1966) and Tyler (1980). Mitochondria, 5 mg of protein, were mixed with 100 μl of either 250 mM sucrose, 50 mM Tris-HCl, and 1 mM EDTA, pH 8.0, or 200 mM sucrose, 10 mM HEPES-KOH, and 15 mM KCl, pH 8.0. They were then reacted with the appropriate maleimide for 1 min. The reaction was terminated by the addition of β-mercaptoethanol to 0.5 mM. The mitochondria were then resuspended in 180 mM sucrose, and 9 mM HEPES, pH 7.4, to a final volume of 2.8 ml. A 50 μl portion of 1 M ammonium phosphate were added with rapid mixing, and the extent and rate of the passive swelling were determined spectrophotometrically at 546 nm.

Assay of Energy-Dependent Transport. The method of Tyler (1969), as modified by Klingenberg *et al.* (1974), was used. Antimycin A (1 μg mg^{-1} protein), rotenone (2 μg mg^{-1} protein), and FCCP (2 nmol) were added as alcohol solutions. The energy-dependent swelling was initiated by addition of ATP to a final concentration of 2 mM.

D-β-Hydroxybutyrate Dehydrogenase

Purification and Assay. Enzyme was purified according to the procedure of Bock and Fleischer (1974, 1975). The specific activity of the preparation used in these studies was 90 μmol NAD^+ reduced min^{-1} mg^{-1} protein at 37°C, when activated with mitochondrial phospholipid. Activity was measured spectrophotometrically by following the reduction of NAD^+ with D-β-hydroxybutyrate as a substrate. When submitochondrial particles were used, antimycin A was added to block the reoxidation of NADH by the electron transport system. The activity of the purified enzyme was measured after the formation of a complex with mitochondrial phospholipids (Table III). An aliquot of the complex was added to a cuvette containing 1 ml of assay medium (10 mM potassium phosphate, 0.5 mM EDTA, 5 mM NAD^+, 20 mM DL-β-hydroxybutyric acid, 0.3 mM dithiothreitol, 0.4 mg ml^{-1} albumin, and 1.27% (v/v) ethanol) which had been preincubated at 37°C.

D-3-Phosphoglycerate Dehydrogenase

Preparation of Red Cell Ghosts. The method of Steck and Kant (1974) was adapted. Packed human erythrocytes were suspended to a hematocrit of 50% in 5 mM phosphate buffer, and 150 mM sodium chloride, pH 8.0. After 10 min, the cells were sedimented by centrifugation at 3000 *g* for 10 min. The supernatant and the buffy coat were removed by aspiration. The washing procedure was performed three times. Washed cells were finally suspended to a packed cell volume of 50%.

Preparation of Right-Side-Out Sealed Ghosts. Hemolysis was initiated by the addition of 1 ml washed cell suspension to 35 ml phosphate and 1 mM magnesium sulfate, pH 8.0. The ghosts were sedimented by centrifugation at 22,000 *g* for 15 min. They were then washed three times in the same solution. The washed cells were finally resuspended in 2.0 ml of the magnesium/phosphate solution. The resealed ghosts contained 1.9% of the original hemoglobin. On the addition of Triton X-100 to a final concentration of 0.2% (v/v), the 3-PGDH activity was increased 15-fold to a specific activity of 1.6 μmol min^{-1} mg^{-1} protein.

Unsealed Ghosts. These were prepared by the procedure used to produce sealed ghosts, except that magnesium sulfate was omitted from the hemolysis solution. The membrane preparations did not contain any detectable haemoglobin, and had a 3-PGDH activity of 2.5 μmol min^{-1} mg^{-1} protein. Intact red cell ghosts were also rendered permeable by addition of 0.1% (v/v) Triton X-100. Control experiments showed that this treatment left the membranes apparently intact under the microscope, and totally relieved the latency of 3-PGDH.

Preparation of Soluble 3-PGDH. Unsealed ghosts were incubated with 25 ml of a solution containing 100 mM sodium chloride, 0.1 mM EDTA, 10 mM Tris, and 0.1% saponin (w/v), pH 7.4, at 22°C. The membranes were sedimented by centrifugation at 37,000 *g* for 30 min. The supernatant was used as "membrane-free" 3-PGDH. This is a modification of a method first described by Kilman and Steck (1980).

Assay of 3-PGDH Activity. Red blood cell membranes (2–10 μg protein in 10–100 μl) were incubated for 1 min with an equal volume of 5 mM phosphate buffer, pH 8.0, with or without 0.2% (v/v) Triton X-100. The volume was made up to 0.82 ml by the addition of a solution containing 30 mM sodium pyrophosphate, pH 8.4, and 4 mM cysteine hydrochloride added immediately before use. A 30-μl portion of 0.4 M sodium arsenite and 50 μl of NAD (final concentration 20 mM) were then added. The reaction was initiated by the addition of DL-3-phosphoglyceraldehyde. The progress of the reaction was followed at 30°C by measuring the change in optical density at 340 nm.

Results

Properties of the AM

When dissolved in a phosphate buffer, pH 6–8, the AM showed a maximum absorbance at 300 nm ($\varepsilon = 620$ liter mol^{-1}). This is comparable to the value reported for *N*-ethylmaleimide in aqueous media by Gregory (1955). The absorbance at 300 nm disappeared on reaction with thiolate ions, and was therefore used to monitor the rates of reaction of the maleimides. It must be emphasized that the maleimides will only react with thiolate ions and not with thiols. No reaction occurs between the maleimides and β-mercaptoethanol in anhydrous *n*-octanol. However, when the β-mercaptoethanol was converted to a thiolate ion, with lithium 18-crown-6-ether clathrate as a counterion, the reaction with maleimides in *n*-octanol was too rapid to measure by the conventional methods described in this paper. This observation emphasises the possibility of thiolate ions existing in hydrophobic regions of membranes, provided that there is a suitable nucleophilic residue present to facilitate ion-pairing (Gray and Matthews, 1984).

It can be seen from the data in Table I that (1) at pH 8, AM 1–10 were more soluble in water than in the hydrophobic phase; (2) that the calculated pK_a values (Rich *et al.*, 1975) indicate that at pH 8, no more that 0.1% of the AM would exist with the carboxyl group in the protonated form; (3) that there is little variation in the value for the second-order rate constants for the reaction of AM with β-mercaptoethanol in an aqueous medium, pH 6.8. The value of the rate constant for the reaction of β-mercaptoethanol with

Table I. Properties of the *N*-(Polymethylenecarboxy)maleimides[a]

Compound	Abbreviation	Partition coefficient	Length[b] (nm)	pK_a	$k \times 10^{-3}$ ($M^{-1}\,s^{-1}$)
$R\text{–}CH_2COOH$	AM1	0.03	0.59	2.88	0.43
$R\text{–}(CH_2)_2COOH$	AM2	0.03	0.71	4.18	0.42
$R\text{–}(CH_2)_3COOH$	AM3	0.03	0.83	4.56	0.62
$R\text{–}(CH_2)_4COOH$	AM4	0.03	0.96	4.62	0.61
$R\text{–}(CH_2)_5COOH$	AM5	0.05	1.08	4.72	0.56
$R\text{–}(CH_2)_7COOH$	AM7	0.09	1.32	4.90	0.56
$R\text{–}(CH_2)_{10}COOH$	AM10	0.34	1.71	4.90	0.72
$R\text{–}(CH_2)_{11}COOH$	AM11	2.84	1.83	4.90	0.75
$R\text{–}CH_2CH_3$	NEM	3	—	—	0.65

[a] Partition coefficients were measured by adding 50 μl of a 20 mM solution of the relevant compound in dimethylsulfoxide to 1 ml of *n*-octanol and 1 ml of phosphate buffer, pH 8. The mixture was vortexed for 60 s after which the emulsion was broken by centrifugation. The concentration of the maleimide in each phase was measured by HPLC on a reversed phase-bonded silica column, Lichrosorb RP2, eluted with methanol/50 mM potassium phosphate buffer, pH 7.4, 3 : 2 (v/v) at a flow rate of 1 ml min^{-1}. Kinetic constants for the reaction of AM with β-mercaptoethanol were calculated from measurement of the decline of absorption at 300 nm when equal volumes of both reactants in 50 mM phosphate buffer, pH 6.8, were mixed at 25°C to give final concentrations of 200 μM. The values of pK_a were calculated as described by Rich *et al.* (1975).

[b] Measured from the reactive maleimide olefin bond to the carbon atom of the carboxyl group.

N-ethylmaleimide also falls within this range. Other experiments showed that as the pH increased to 8, there was an approximately tenfold increase in the value of the rate constant. The significance of these results is that any variation in the rates of reaction of the AM with the biochemical systems which we have studied cannot be explained in terms of either variation of reactivity with length of polymethylene chain, or through the presence of a carboxylate anion.

The Effects of AM on Soluble Enzymes

Soluble 3-PGDH was incubated with AM for 5 min at 30°C at a final concentration of 8 μM, followed by quenching of the unreacted maleimides by addition of excess β-mercaptoethanol. Subsequent assay of 3-PGDH activity (Table II) shows that all of the AM were effective inhibitors, as was the uncharged *n*-octylmaleimide. There was no apparent variation in the inhibitory potency with the length of polymethylene chain. The data also show that the presence of 7 mM D-3-phosphoglyceraldehyde during pre-incubation with the AM significantly reduced the levels of inhibition. We conclude that the maleimides are acting at one or more of the catalytic centers in the tetrameric enzyme complex.

The time course of inhibition of apo-D-β-hydroxybutyrate dehydrogenase is shown in Table III. The apoenzyme was incubated with a 12.5

Table II. The Effects of the Maleimides on the Activity of Soluble 3-PGDH from Red Blood Cells[a]

Maleimide	Inhibition of soluble 3-PGDH activity (%)	
	No substrate present	Substrate present
NOM[b]	100	15
AM1	92	27
AM2	90	0
AM5	93	12
AM7	100	9
AM10	98	38

[a]Soluble 3-PGDH (8 μg protein in a final volume of 0.2 ml) was incubated for 5 min at pH 7.4 and 30°C with maleimides at a final concentration of 8 μM in the presence of either 7 mM D-3-phosphoglyceraldehyde or 5 mM phosphate buffer. A 1.5 molar excess of β-mercaptoethanol was then added and the enzymic activity of the preparation was determined as described in the Materials and Methods section.
[b]NOM = *N*-octylmaleimide.

Table III. Inactivation by Maleimides of D-β-Hydroxybutyrate Dehydrogenase[a]

Maleimide	Half-time for inactivation (s)	
	Water-soluble aponenzyme	Preformed enzyme–phospholipid complex
AM3	33	944
AM4	16	972
AM5	30	474
AM7	17	145
AM10	19	15
AM11	14	21
NEM	42	204

[a]*Inactivation of apodehydrogenase.* Maleimides to a final concentration of 24 μM were added to 0.5 mg of apo-D-β-hydroxybutyrate dehydrogenase (equivalent to 15.9 μM, final volume was 0.15 ml) in 5 mM HEPES-KOH and 0.1 mM EDTA, pH 7.0 at 0°C. Aliquots of 10 μl were removed at various time intervals and added to 35 μl 5 mM HEPES, 0.1 mM EDTA, and 10 mM dithiothreitol. After 5 min, 25 μl of sonicated mitochondrial phospholipids (21 μg phosphorus ml^{-1}) were added and the mixture was incubated at 30°C to allow the formation of the enzyme phospholipid complex. 10 μl of this solution was then assayed for D-β-hydroxybutyrate dehydrogenase activity. *Inactivation of phospholipid-activated enzyme.* Maleimides (final concentration of 90 μM) were added to a 7.2 μM solution of preformed D-β-hydroxybutyrate–mitochondrial phospholipid complex in 4 mM HEPES and 0.1 mM EDTA, pH 7.0. Aliquots were removed at various times and activity assayed as described in the Materials and Methods section except that the assay contained 10 mM dithiothreitol.

molar excess of maleimide for 5 min at 30°C. Aliquots were removed for assay at various times, and unreacted maleimide was quenched by the addition of dithiothreitol. The remaining enzyme activity was elicited with mitochondrial phospholipid. It can be seen that there was little variation in the rates of inactivation of D-β-hydroxybutyrate dehydrogenase by the different AM in this monophasic aqueous system. Thus any variation in the rates of inactivation of the enzyme by the AM in a biphasic membrane-bound system cannot be interpreted in terms of inherent differences in the rates of reaction of the AM with the soluble enzyme. These results also show that both charged and uncharged maleimides have a high affinity for a thiolate group which is essential for the activity of D-β-hydroxybutyrate dehydrogenase. Evidence will be presented that the thiolate group at which the AM are acting is close to the active site of the enzyme, but is not part of the catalytic process (Dalton *et al.*, 1984).

The Inability of the AM to Penetrate Membranes

The Red Blood Cell Membrane. An experiment was designed to compare the abilities of the AM and uncharged maleimides to penetrate the red blood cell membrane and inhibit the 3-PGDH activity which is associated with the inner face of the membrane. Resealed red blood cell ghosts were incubated at pH 8 with the various maleimides at a level of 20 nmol mg^{-1} of protein for 5 min at 30°C. Unreacted maleimide was destroyed by addition of excess mercaptoethanol and the vesicles were rendered permeable by addition of Triton X-100 prior to assay of 3-PGDH activity. The data in Table IV demonstrate that the only maleimides capable of inhibiting intravesicular 3-PGDH were those carrying no net charge (*N*-*n*-octylmaleimide and *N*-(4′-dimethylamino-3,5-dinitrophenyl)-maleimide). *N*-(4′-trimethylaminobenzylamine)-maleimide, which carries a net positive charge, and the AM which are negatively charged at the pH of the experiment, were ineffective as inhibitors of the internalized enzyme activity. We attribute this loss of inhibitory potency to the inability of the charged maleimides to cross the red blood cell membrane.

The Inner Mitochondrial Membrane. Succinic acid dehydrogenase is located on the inner surface of the inner mitochondrial membrane. When submitochondrial particles are prepared from mitochondria by treatments which pinch off the cristae, the orientation of the membrane is reversed, i.e., the enzyme is located on the outer surface of the vesicles and is exposed to the bulk aqueous phase. Succinate dehydrogenase activity has been shown to be inhibited by maleimides when associated with submitochondrial particles (Feldberg and Hollocher, 1972; Kenney, 1975).

Rat liver submitochondrial particles were preincubated with 25 nmol AM mg^{-1} protein at 37°C for 4 min in oxygen-free 50 mM sodium phosphate

Table IV Inhibition of Membrane-Bound 3-PGDH by Maleimides[a]

Maleimide[b]	Inhibition of 3-PGDH activity		
	Resealed ghosts	Unsealed ghosts	Triton X-100 treated
NOM	95	94	97
QM	90	79	80
NAM	8	96	94
QM1	11	76	77
AM1	13	24	32
AM2	12	92	98
AM5	18	67	60
AM7	11	44	38
AM10	7	65	62

[a]Suspensions of either unsealed or resealed red blood cell ghosts or red blood cell ghosts rendered leaky by treatment with 0.1% (v/v) Triton X-100 (140 μg protein) were incubated in 200 μl of a solution containing 5 mM phosphate, 1 mM magnesium sulfate, pH 8.0, and maleimide at 20 nmol mg^{-1} protein, at 30°C for 5 min. The excess maleimide was quenched by addition of 1 μl β-mercaptoethanol. In the case of resealed ghosts, 200 μl of 2% (v/v) Triton X-100 was added to permeabilize the membrane prior to assay, while 200 μl of buffer was added to leaky preparations which had been treated with Triton during preincubation. After 1 min a solution containing 30 mM sodium pyrophosphate and 4 mM cysteine, pH 9.0, was added to bring the volume to 0.82 ml. 3-PGDH activity was assayed as described in the Materials and Methods section.

[b]NOM = *N*-octylmaleimide; QM = *N*-(4′-dimethylamino-3,5-dinitrophenyl)maleimide; QM1 = *N*-(4′-trimethylaminobenzylamine)maleimide; NAM = 2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid.

buffer at pH 8.0. The succinate dehydrogenase activity was determined spectrophotometrically in the presence of rotenone and KCN (Veeger *et al.*, 1969; LeQuoc *et al.*, 1981). There was a graded response of the enzyme activity to these treatments; AM1 inhibited the activity by 50%, while AM10 and AM11 caused 80% inhibition. This is in marked contrast to the effects of the AM on the succinate dehydrogenase activity of intact mitochondria. Treatment of rat liver mitochondria with up to 50 nmol AM mg^{-1} protein had no effect on uncoupled respiration with succinate as substrate. Treatment of mitochondria with the membrane-penetrant reagent *N*-ethylmaleimide, however, resulted in greater than 80% inhibition of uncoupled succinate respiration, in agreement with the results of LeQuoc *et al.* (1981). It is apparent that the charged AM molecules cannot pass through the inner mitochondrial membrane to react with molecules located on the inner face of the membrane or in the matrix of the mitochondrion.

The Use of AM to Probe the Location of Thiolate Ions in Membranes

Inhibitory Effects of AM on the mitochondrial Phosphate Translocator. We have previously shown that the transport of phosphate from the matrix space through the inner mitochondrial membrane is optimally inhibited by

AM4 and AM10, with intermediate levels of inhibition by the other members of the AM series (Griffiths *et al.*, 1981). These results were obtained using the energized swelling technique and are reproduced in Fig. 1a. The results were confirmed using a passive swelling technique (Chappell and Crofts, 1966). However, it is possible for mitochondrial volume changes to be caused by other effects than osmosis (Holton and Beechey, 1959). Therefore, the effects of the AM on the extrusion of phosphate from the matrix space was measured directly, following the procedure of Tyler (1980). The method relies on the generation of intramitochondrial phosphate by an uncoupler-stimulated ATPase activity. ATP is transported to the ATPase by the mitochondrial ATP/ADP translocator. The relative ability of compounds to inhibit efflux of phosphate is then reflected by the internal phosphate content of the treated mitochondria. The results in Fig. 1b show that AM4 and AM10 optimally inhibit the extrusion of internal mitochondrial phosphate, in agreement with our earlier observations. These results confirm our previous conclusion that there are two populations of sulfhydryl groups associated wtih the phosphate translocator, and that these lie at different depths within the inner mitochondrial membrane.

Inactivation of Membrane-Bound 3-PGDH Activity. The ability to inhibit the activity of 3-PGDH which was associated with the interior face of the red blood cell membrane was assayed by either unsealed ghosts, or by rendering the red blood cell permeable with Triton X-100. The results are presented in Table IV. It can be seen that identical results were obtained with unsealed and Triton-treated ghosts. There was a marked variation in the ability of the AM to inhibit 3-PGDH activity. Under the conditions used in this experiment, AM1 caused 25% inhibition. In contrast, AM2 completely inhibited the activity of Triton-treated ghosts, while the activity of unsealed ghosts was inhibited by 92%. There was a progressive diminution of the inhibitory potency of the AM as the chain length increased from 2 to 7. There was a small but reproducible increase in inhibition for AM10 relative to AM7. It is clear that the interaction of AM with 3-PGDH is significantly affected by the association of the enzyme with the cell membrane.

Inactivation of D-β-Hydroxybutyrate Dehydrogenase. The time course of inactivation of D-β-hydroxybutyrate dehydrogenase by AM is illustrated in Fig. 2a. Incubation of submitochondrial particles with AM2-7 for periods up to 12 min did not lead to significant inhibition. AM10 and AM11, however, gave almost complete inhibition within 5 and 8 min respectively. It has been shown that there are two thiolate groups which react with maleimides (Dubois, Trommer, McIntyre, and Fleischer, unpublished). One of these groups is at the active site, while the other is located nearby but does not play in the catalytic mechanism (Dalton *et al.*, 1984). The data in Fig. 2b suggests that AM10 and AM11 are reacting with these two thiolate ions since the presence of either the substrate NADH or the substrate analogue

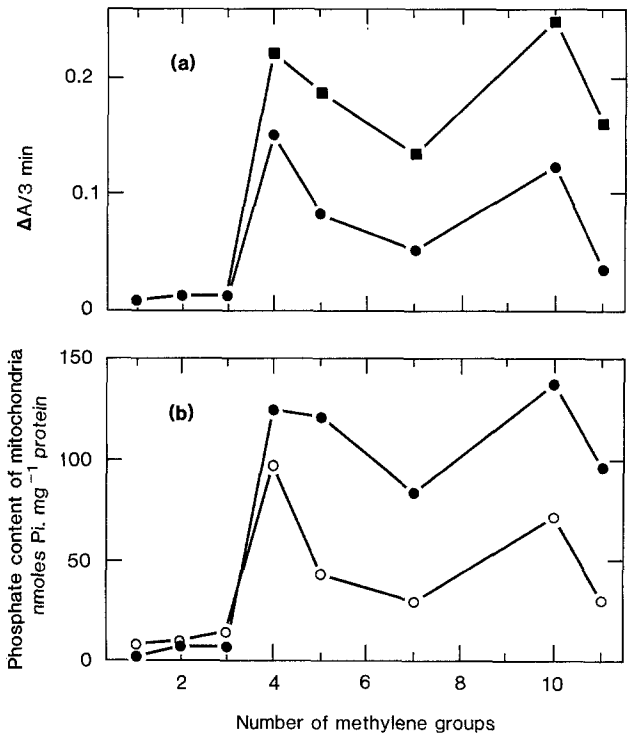

Fig. 1. Inhibition of mitochondrial phosphate transport by maleimides. (a) The effect of AM on ATP-dependent swelling of rat liver mitochondria. Data from Griffiths *et al.*, (1981). (b) The effect of AM on the phosphate content of rat liver mitochondria. Mitochondria (5 mg) in 3 ml of a solution containing 250 mM sucrose, 50 mM Tris, and 1 mM EDTA, pH 7.6, were incubated with the maleimides at 20 nmol mg^{-1} protein (○), or 30 nmol mg^{-1} protein (●) for 1 min at 4°C. The reaction was terminated by addition of a twofold excess of β-mercaptoethanol. ATP-dependent swelling was induced by the addition of 0.5 μM FCCP and 2 mM ATP. After 1 min, carboxyatractylate was added to 6 μM, and oligomycin to 2 nmol mg^{-1} protein. A 1-ml sample was removed immediately and centrifuged at top speed in an Eppendorf microfuge for 3 min. A second sample was taken 1 min later and pipetted into cold 10% trichloroacetic acid solution. This was centrifuged as described above. The phosphate contents of the supernatant solution and in the undenatured mitochondrial pellet were measured by the method of Tausky and Schorr (1953).

2-methylmalonate provides substantial protection against inhibition by these maleimides.

If the purified apo-D-β-hydroxybutyrate dehydrogenase is activated by incorporation into phospholipid vesicles prior to incubation with AM, the pattern of differential sensitivity to AM (Table III) is similar to that found with submitochondrial vesicles, although in this reconstituted system AM7 shows appreciable inhibition of the dehydrogenase. It is also significant that NEM, which is a membrane-permeable molecule, reacts at a much slower rate

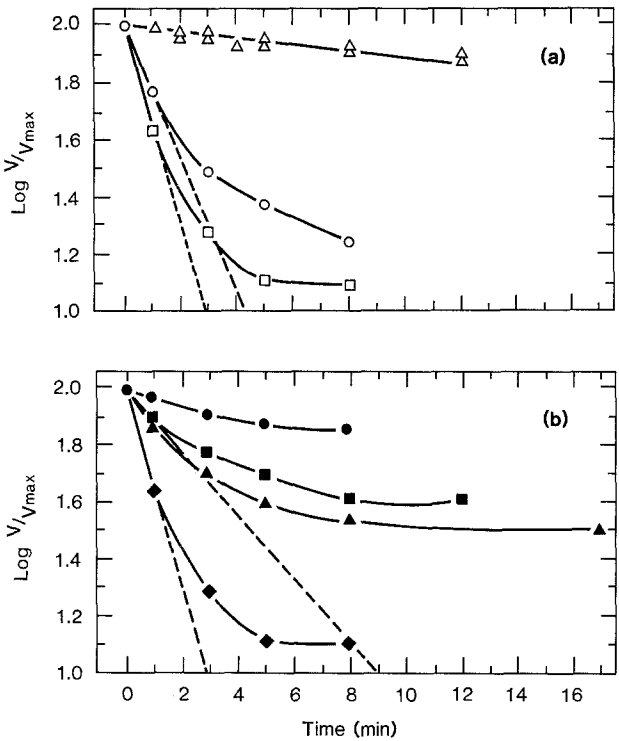

Fig. 2. Effects of maleimides on D-β-hydroxybutyrate dehydrogenase activity. (a) Differential inactivation of activity by AM. A 0.4 ml portion of a suspension of submitochondrial particles (1 mg ml^{-1}) in 5 mM HEPES, 0.1 mM EDTA, and 50 μg antimycin A, 7.0, was incubated at 30°C with AM to give final concentrations of 1.25 nmol AM mg^{-1} protein. Samples were removed at the indicated times and D-β-hydroxybutyrate dehydrogenase activity assayed in a reaction mixture supplemented with 10 mM dithiothreitol. (△), AM2, AM5, AM7; (□), AM10; (○), AM11. (b) Protection against inactivation. The basic reaction mixture was the same as in (a), except that 5.0 mM NADH and 10 mM methylmalonate was added (●, ■). AM10 was used at either 1.25 nmol mg^{-1} protein (■, ◆), or at 0.625 nmol mg^{-1} protein (▲, ●).

than either AM10 or AM11. Since the water-soluble apoenzyme is inhibited at the same rate and to the same extent by all of the AM and by *N*-ethylmaleimide, the variation in the response of membrane-bound activity must be due to the inclusion of the protein within the phospholipid bilayer. In our model, the slower reaction of NEM would be predicted, as the uncharged maleimide, being freely diffusible, can react with thiolates throughout the membrane bilayer, whereas the AM are confined to diffusion in one plane. D-β-Hydroxybutyrate dehydrogenase is an intrinsic membrane protein, with the active center accessible only from the internal face of the inner mitochondrial membrane. We propose that when the enzyme is associated with a membranous structure, the thiolates adjacent to the active

site are embedded 1.3–1.7 nm below the phase boundary (see Table I). This is in good agreement with the results of Dalton *et al.* (1984). These workers used lecithin molecules containing spin labels at different positions in the *sn*-2 fatty acyl chain. They concluded that thiolate groups in the vicinity of the active site are located 0.8–1.0 nm from the aqueous domain.

Discussion

The results presented in Table I show that *N*-alkylmaleimides and the polymethylene-carboxymaleimides (structure 1, $n = 1$–11) react with mercaptoethanol at similar rates, demonstrating that the inherent reactivity of the maleimide moiety is not affected significantly either by the length of the polymethylene chain or by the presence of an ionized carboxyl group. Since the AM failed to interact with 3-PGDH when associated with the inner face of the red blood cell membrane, or with succinic dehydrogenase on the matrix side of the inner mitochondrial membrane, we conclude that these reagents cannot traverse either of these membranes. In contrast, uncharged maleimides were effective inhibitors of both activities when added to the intact cell or organelle. These molecules must diffuse through the membranes to achieve inhibition. It is apparent that the presence of a terminal ionized carboxyl group on the polymethylene chain prevents transmembrane movement of the AM. This conclusion is supported by the observation that the positively charged QM1 has no inhibitory effect on 3-PGDH in intact red blood cells, while the uncharged QM is a good inhibitor (Table IV). It has been shown that the carboxylate ions of *n*-alkanoic acids do not penetrate the hydrophobic phase of membranes at appreciable rates (Schaper, 1982).

The ability of the AM to react with the inhibit D-β-hydroxybutyrate dehydrogenase and 3-PGDH varies with the location of the enzymes. If either of the enzymes is in a homogeneous aqueous solution, then it is inhibited to the same extent by all of the charged AM and the uncharged *N*-alkylmaleimides. There is little variation in rate of inactivation of D-β-hydroxybutyrate dehydrogenase by the different maleimides (Table III). However, when either of the enzymes is located in a membrane, then the interaction of the AM with the enzymes is restricted (Fig. 2a and b, Tables III and IV). This is also true for the phosphate translocator (Fig. 1a and b). We conclude that the access of the maleimides to the active/sensitive site is restricted by the presence of the membrane. We propose that it is the ionized carboxyl group which prevents the complete entry of the AM molecule into the bilayer region of the membrane. This effectively immobilizes the maleimide moiety of the AM at set depths within the bilayer, defined by the length of the polymethylene

chain. Consequently, the malemide group can maove only in a limited plane within the membrane and can only react with those thiolate ions in that plane. The possibility that the AM adopt a "hairpin" configuration, and so lead to an erroneous assignment of the depth of this plane, must be considered. If this occurred, then it would be expected that all of the AM longer than the minimum required should be equipotent as inhibitors. That such is not the case is demonstrated by (a) phosphate transport across the inner mitochondrial membrane is not significantly affected by AM7 or AM11, while AM4 and AM10 are good inhibitors; (b) AM10 is a better inhibitor of membrane-bound D-β-hydroxybutyrate dehydrogenase than AM11; (c) membrane-bound 3-PGDH activity is inhibited to a greater extent by short-chain AM than by those with longer chains. Indeed, the ability of AM10 and AM11 to give differential inhibition shows that these reagents are capable of fine discrimination. This is remarkable since the depth at which these maleimide moieties are thought to penetrate is regarded as a region of relative mobility for the methylene groups of phospholipids (McConnell and Farland, 1975; MacDonald *et al.*, 1983, 1985).

The molecules are probably indicating significant levels of microheterogeneity in the membrane. The results with D-β-hydroxybutyrate dehydrogenase suggest that the thiolates adjacent to the active center could be embedded in the bulk of the membrane. This conclusion is supported by the finding (Dalton *et al.*, 1984) that spin label maleimides bound close to the active site were located 0.8 to 1.0 nm below the aqueous domain. This implies that the substrates NAD and D-β-hydroxybutyrate must be able to penetrate the membrane to a significant depth in the region of the enzyme, and raises the question as to where exactly in the membrane is the region impenetrable by NAD? Presumably it is not at the aqueous interface for all parts of the membrane surface.

It has been emphasized that the AM will only react with thiolate ions. The extension of this is that thiolate ions must be present in the hydrophobic regions of membranes. The energies of activation in such a milieu will be very high. However, the formation of hydrogen bonds between the thiol sulfur and a carbonyl oxygen in the preceding turn of the α-helix in the protein would impose a degree of polarity which should allow the reaction with maleimides to proceed (Gray and Matthews, 1984).

One conclusion made from the results presented in Fig. 1a and 1b is that there are at least two populations of thiolate ions, at different depths within the membrane, that are associated with the functioning of the phosphate translocator. This situation has been proposed on the basis of many different experimental approaches by Fonyo (1974), Fonyo *et al.* (1975), Hadvary and Kadenbach (1976), Wohlrab (1978), and Houstek *et al.* (1982, 1983). Furthermore, the difference in length between AM4 and AM10 (0.75 nm,

Table I) is very similar to the distance between the two thiols measured by Houstek *et al.* (1983) on the basis of interaction of spin probes.

All of the results presented here are most easily reconciled with a model in which the AM are envisaged as molecules which are immobilized within the structure of the membrane such that the maleimide ring hangs at set depths within the bilayer. The depth of penetration of the maleimide ring is limited by the carboxylate ion which will not enter the hydrophobic region of the membrane, and is defined by the length of the polymethylene chain. It is not anticipated that the lateral movement of the molecules will be impeded. Consequently, the maleimide moiety moves in a relatively narrow plane within the bulk phase of the membrane. The reagents can only react with those thiolate ions which are in that plane. This constraint on movement in the membrane enhances the specificity of reaction. This is emphasized by the very low level of AM10 (5 nmol mg^{-1}) required for inhibition of membrane-bound D-β-hydroxybutyrate dehydrogenase.

These reagents have already been used to demonstrate that there is a thiol group deeply embedded in the mitochondrial membrane which is essential for the translocation of protons across the membrane (Griffiths *et al.*, 1984), and to study the disposition of the glycine decarboxylase system in the inner mitochondrial membrane of *Pisum sativum* (Moore *et al.*, 1984). In these studies the reagents have been used in carefully controlled experiments, and with an understanding of the properties of the compounds. Two other reports have appeared in which the AM have been used (Bacher and Wheeler, 1985; El-Sharif and Banner, 1985). In neither case was attention given to the characteristics of the compounds, and consequently no useful information was acquired, and the conclusions drawn are questionable.

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