STRUCTURAL STUDIES ON THE ORGANIZATION OF PROTEINS IN MITOCHONDRIAL MEMBRANES USING PROTEOLYTIC ENZYMES

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Proteolytic enzymes, pronase and trypsin, digest protein in ETP and in SU-particles (devoid of the soluble ATPase) at similar rates and to the same extents for intact and lipid-depleted membranes, showing that lipids do not constitute a barrier to the action of the proteases. The rates and extents of hydrolysis are slightly depressed when membranes are reconstituted from lipid-depleted particles and phospholipids. The hydrolysis rates for the various particles are not greatly enhanced by detergent solubilization nor by other denaturing treatments, indicating that the rates measured in absence of treatments are maximal under the conditions used. The circular dichroism spectra of pronase treated ETP are noticeably altered showing modification of the original conformation. Moreover, enzymic activities of mitochondria and submitochondrial particles are progressively affected by proteases according to their localization at, or near to, a given surface of the membrane. The matrix enzyme, malate dehydrogenase, is not apparently released from mitochondria during the initial incubation period. The results are tentatively discussed in terms of organization of lipids and proteins in the mitochondrial membrane.

Previous investigations from our laboratory (1–3) have shown that the proteins of the inner mitochondrial membrane bind phospholipids hydrophobically in reconstitution experiments. Indirect reasons have suggested that the proteins which are bound hydrophobically in the intact membrane are those which may be considered "intrinsic" and are water-insoluble when separated from the lipids (4). Superficial "extrinsic" proteins (4) which are water-soluble when detached, may be bound by polar forces to the membrane continuum (5).

The type of binding alone may not be a sufficient tool to evaluate the organization of proteins and lipids in the inner mitochondrial membrane. For example, hydrophobic binding between lipids and proteins is compatible with different membrane models, although the macromolecular organization is quite different in these models. Membrane proteins may be hydrophobically bound to phospholipids according to any one of the following possibilities: (a) penetration of phospholipid fatty acyl chains from the lipid bilayer into superficial protein layers (6); (b) partial penetration of protein side residues between lipid molecules in the bilayer (7); (c) extensive penetration of protein molecules into interstices of the lipid bilayer, like in some mosaic models recently proposed (8–10); (d) extensive interdigitation of proteins and lipids to produce a novel lipoprotein complex distinguishable from the original components (11). Possibilities (a) and (b) are not in contrast with the Danielli model (12) as for general membrane organization.

Proteolytic dissection of a membrane may be a useful tool, together with phospholipases action on the lipids, to study the spatial arrangement of lipids and proteins within the membrane. Studies of lipid-protein organization by use of proteolytic enzymes and protein reagents have been directed to solve the structure of soluble lipoproteins (cf 13). There is a growing interest in this technical approach to investigate membrane structure; many published papers concern red cell membranes either in intact erythrocytes or in ghosts prepared by hypotonic lysis (cf 14–17), while fewer detailed studies are available for other membranes (cf 18–20).

This kind of study may be of interest for investigating two related problems: (a) the effect of lipids on the availability of membrane proteins to protein reagents or proteolytic enzymes; (b) the topological distribution of the individual proteins within the membrane, considering for each membrane three possible locales for proteins (outer surface, the membrane in its width, inner surface).

In this investigation we have been mainly concerned with the first aspect related to the inner mitochondrial membrane. Parallel studies are being followed on the effect of phospholipases. Preliminary results of this investigation have been presented elsewhere (21, 22).

METHODS

Beef heart mitochondria (BHM)* were prepared by the method of Smith (23). From BHM, submitochondrial particles ETP were obtained by sonic oscillation (24); SU-particles were prepared according to Racker and Horstman (25). HCl extraction of "extrinsic" proteins has been described elsewhere (1). Different particles were delipidated using the acetone-ammonia procedure of Fleischer and Fleischer (26): after extraction of the lipids the P content of the particles decreased from about 20 μg to 1–3 μg/mg protein. Lipid binding to lipid-depleted mitochondrial membranes was carried out as described elsewhere (1, 2) using soybean phospholipid aqueous vesicles (Asolectin) dispersed by sonication (26); the phospholipid content was restored to values very close to the original values for intact particles (18–20 μg/mg protein). Protein concentration was determined with a biuret method (27); in mitochondrial membranes this method gives values about 20% higher than those calculated from the values of total nitrogen as determined with a micro-Kjeldhal method. Lipid phosphorus was determined according to Marinetti (28). Enzymic activities were assayed according to the following references: succinoxidase (29); ATPase (30); malate dehydrogenase (31).

Pronase (Calbiochem B grade, 45,000 P.U.K./g) and trypsin (Difco, 1:250) were used to hydrolyze membrane proteins according to both the following procedures.

Procedure (a). Ten mg of membrane protein were homogenized in a total volume of 6 ml of 0.15 M NaCl and allowed to equilibrate at 37° in the cell of a pH-Stat automatic titrator Radiometer; after equilibration at pH 8.3 for trypsin and 8.0 for pronase, a small volume containing 1 mg of the proteolytic enzyme in water was added to the cell and hydrolysis was recorded for at least one hour. The amount of 0.01 N NaOH required to keep the solution at constant pH was taken as a measure of hydrolysis of peptide bonds.

*Abbreviations used are the following: BHM, beef heart mitochondria; ETP, sonic submitochondrial particles; SU-particles, ETP treated with Sephadex and urea; CD, circular dichroism; LD-ETP and LD-SU, lipid-depleted ETP and SU-particles respectively; SDS, sodium dodecyl sulphate; TFE, trifluoroethanol.

Since the average pKa's for amino groups in peptides is 7.6 (13), we can assume that a general correspondence exists between number of protons liberated and number of peptide bonds hydrolyzed.

Procedure (b). Five to ten mg of membrane protein were homogenized in 0.01 M sodium bicarbonate at pH 8.2; either pronase or trypsin were then added according to ratios specified in the legends of Tables and Figures, and the mixtures were incubated at 37°; aliquots were withdrawn at different times and assayed for free amino nitrogen with the method of Moore and Stein (32).

CD Studies. The circular dichroism spectra were recorded in a Cary-60 spectropolarimeter, fitted with a model 6001 circular dichroism attachment, at wavelength range 300–190 nm. The sample concentrations, determined according to a biuret method, were 2.5 mg prot./ml and 1.4 mg prot./ml for ETP and pronase treated ETP, respectively. The spectra of the membrane suspensions were run using a cell whose pathlength was 0.1 mm. The spectra were corrected for light scattering using the "Pseudoreference state" approach described by Urry et al. (33). CD and absorption of the pseudo reference state were run using membrane suspensions dissolved with SDS and diluted 1:4 with trifluoroethanol (TFE). Accordingly, the spectra were recorded using a 0.5 mm cell.

RESULTS

Rate of Proteolytic Digestion

The action of pronase and trypsin has been investigated in submitochondrial particles ETP and in submitochondrial particles devoid of the F_1–ATPase (SU-particles). The rate of hydrolysis has been followed with the automatic titrator (see Methods). It is shown in Fig. 1 that the rate of proteolysis is about the same for pronase or trypsin in intact and in lipid-depleted ETP. Since the ETP particles have on their surface the typical projections (knobs) containing the Mg++-dependent ATPase, which is water soluble when detached from the membrane (34), we have examined SU-particles, which are devoid of this

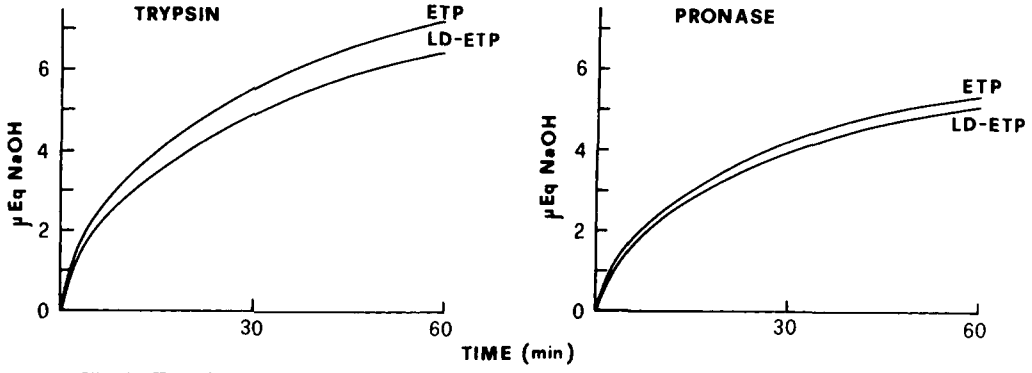

Fig. 1. Trypsin and pronase digestion of intact and lipid-depleted ETP. (Redrawn from pH-Stat recordings.) One mg of proteolytic enzyme was added to 10 mg of particle protein in 10 ml of 0.15 M NaCl. Hydrolysis was followed at 37° by titrating to an end point of pH 8.3 for trypsin and 8.0 for pronase with 10 mM NaOH in a pH-Stat (Radiometer) as described in the Methods section. LD-ETP = lipid-depleted ETP.

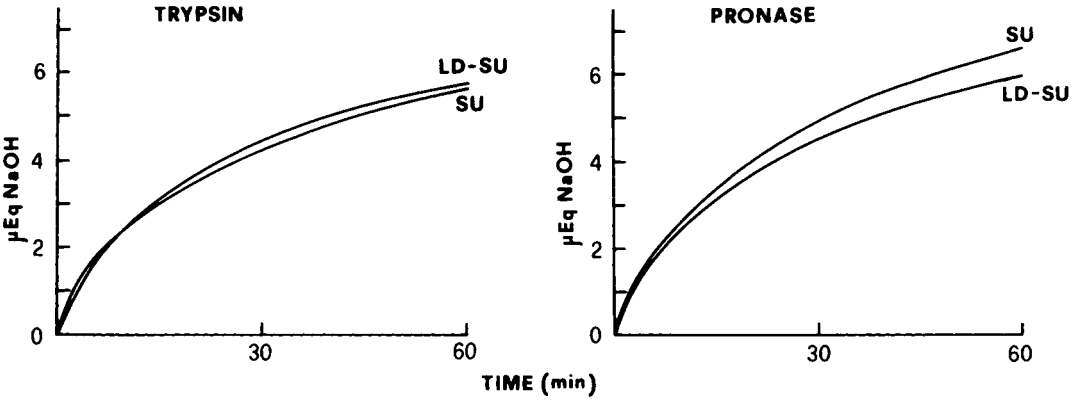

Fig. 2. Trypsin and pronase digestion of intact and lipid-depleted SU-particles. The experimental conditions were the same as in Fig. 1. LD-SU = lipid-depleted SU-particles.

soluble ATPase while keeping intact oxidative activities (25). As shown in Fig. 2, the rate of hydrolysis also in these particles is independent of the presence of the lipids in the membrane. Table I reports the calculated rates of hydrolysis from the curves in the two figures and from other digestion experiments. No major differences due to membrane delipidation are apparent. The rates of pronase digestion of ETP, lipid-depleted ETP, and lipid-reconstituted ETP are compared in Table II. The reincorporation of phospholipids into lipid-depleted membranes slightly depresses the rate of hydrolysis.

TABLE I. Rate of Proteolytic Digestion of Submitochondrial Particles

Exp.	Enzyme	ETP	LD–ETP	SU	LD–SU	Albumin
		nEq NaOH/min*				
1	Trypsin	183	167	137	147	165
	Pronase	143	140	177	167	197
2	Trypsin	153	147			
	Pronase	140	127	160	150	
3	Trypsin	173	197	137	140	
	Pronase	143	123	213	213	

*The data are calculated from the curves in Figs. 1 and 2, and from similar experiments, from the amounts of NaOH consumed during the initial 30 min.

TABLE II. Pronase Hydrolysis of Lipid-Depleted and Reconstituted Particles

Exp.	Particle	Rate of hydrolysis	
		nEq NaOH/min*	%**
1	ETP	107	97
	LD–ETP	111	100
	LD–ETP + Asolectin†	88	79
2	ETP	114	104
	LD–ETP	109	100
	LD–ETP + Asolectin†	105	96

*Calculated as described in the legend of Table I.

**Assuming 100 for the rate of hydrolysis of LD–ETP.

†Reconstitution was accomplished according to ref. 1; after incubation of LD–ETP and Asolectin at 30° for 30 min, the samples were washed and resuspended in the medium for pronase hydrolysis (see Methods).

TABLE III. Effect of Detergents on the Rate of Pronase Digestion of Submitochondrial Particles

Particle	Addition or treatment	nEq NaOH/min*
ETP	–	86
	SDS 0.1%	112
	SDS 1%	106
LD–ETP	–	81
	SDS 0.1%	99
	SDS 1%	88

*Experimental conditions were the same described in the legend of Table I.

TABLE IV. Tryptic Digestion of Heated ETP in Comparison with Albumin

Particle	Treatment	nEq NaOH/min*
ETP	–	170
	1 min at 100°	171
Albumin	–	172
	1 min at 100°	222

*Calculated as described in the legend of Table I.

TABLE V. Pronase and Trypsin Digestion of Submitochondrial Particles in the Presence of 3 M Urea

Particle	Pronase	Trypsin
	nEq NaOH/min*	
ETP	103	128
LD–ETP	92	112
SU	84	102
LD–SU	99	100

*Calculated as described in the legend of Table I. The digestion was accomplished by adding to the suspension an equal volume of freshly prepared 6 M urea immediately before digestion.

Pronase activity on ETP and LD-ETP is enhanced by SDS solubilization only to a limited extent (Table III); this result, together with hydrolysis of heat-denatured membranes (Table IV) or hydrolysis in presence of urea (Table V) suggests that the rate of proteolysis in intact ETP is close to the maximal rate at which the same proteins are hydrolyzed when the membrane is disorganized by a variety of means.

Extent of Proteolytic Digestion

Submitochondrial particles have also been subjected to pronase hydrolysis in buffered solutions, and the extent of hydrolysis was measured by the increase of free amino groups. Table VI shows the extent of hydrolysis of intact and lipid-depleted submitochondrial particles by pronase and trypsin. The hydrolysis proceeds to similar extents in both types of particles, in accordance with what was shown by alkali titration. The percent extent of hydrolysis can be calculated from these data. For example, the increase in amino

TABLE VI. Proteolytic Digestion of Intact and Lipid-Depleted Submitochondrial Particles*

Set of experiments	Particle	Hydrolysis time (hours)	Pronase*		Trypsin**	
			μg amino N†			
I	Albumin	12	542	351		
	ETP	12	348	309	384	372
	LD–ETP	12	310	307	333	366
	SU	12	324	300		327
	LD–SU	12	357	345		330
II	ETP	0	96			
		24	380			
		48	414			
	LD–ETP	0	56			
		24	288			
		48	352			

*The digestion was accomplished in bicarbonate buffer as described in Methods.
**The amount of enzyme added was 10% for set of experiments I and 2% for set of experiments II. In set I, each column refers to a different experiment.
†The values are referred to the whole mixture containing 10 mg of protein determined with the biuret method (27); content of amino N in the undigested membrane ranged from 5 to 7 μg/mg of particle protein. Each column refers to a different mitochondrial preparation.

TABLE VII. Effect of Pronase Digestion on Protein and Phospholipid Content of ETP*

	ETP	Pronase-digested ETP	% loss of protein
Total protein, mg	10.0	5.6	44
P/protein	18.7	37.7	50**
Total phospholipid P, μg	187	211	

*ETP were digested with 10% pronase as described in Table VI and the mixture was centrifuged at 105,000 g for 60 min. Protein and P were assayed in the pellet resuspended in 0.25 M sucrose containing 10 mM Tris-HCl.
**Calculated assuming that no phospholipid was lost in the supernatant after centrifugation.

TABLE VIII. Trypsin and Pronase Hydrolysis of Lipid-Depleted and Reconstituted Particles*

Enzyme	Particle	μg amino N	%**
Trypsin	ETP	355	106
	LD–ETP	335	100
	LD–ETP + Asolectin	270	80
	Albumin	400	
Pronase	ETP	412	92
	LD–ETP	445	100
	LD–ETP + Asolectin	331	74
	Albumin	542	

*Experimental conditions are those described in the legends of Table VI. See also Table II for details on reconstitution. Hydrolysis time was 12 hours; enzyme to protein ratio was 10%.
**Assuming 100 for the extent of hydrolysis of LD–ETP.

nitrogen content after pronase hydrolysis for 12 hours averages 300 μg for 10 mg of biuret protein. Using a coefficient of 6.25 for the conversion of N to protein, and considering that the biuret determination gives values 20% higher than a micro-Kjeldhal method (see Methods), we can calculate that about 25% of the peptide bonds are digested under our experimental conditions in both intact and lipid-depleted particles (compared with 40% for albumin). However, pronase determines a much higher loss of peptide material from the membrane, as determined either by centrifugation of the membranous material and determination of the protein in the pellet or by enrichment in lipid P in the membranous fraction (Table VII).

Lipid-reconstituted particles are hydrolyzed to a lesser extent than lipid-depleted ETP (Table VIII), a finding in accordance with that reported for hydrolysis rates in Table II.

Effect of Pronase Digestion on Protein Conformation of ETP

In order to achieve meaningful CD data, correction for the distortions arising from the particulate nature of the samples must be applied first. The samples treated with SDS/TFE were chosen as the pseudo reference state since they were optically clear. However, in the region below 200 nm, this dissolving system as well as other detergents proved unsuitable for obtaining a pseudo reference state. The corrections were therefore applied in the spectral region 240–206 nm using the following equation:

$$[\Theta] = \frac{[\Theta] \text{ suspension}}{Q_A^2 \, Q_\sigma}$$

where Q_A is the absorption flattening quotient, and Q_σ is the absorption obscuring quotient derived from light scattering (33).

At 224 nm, the distortions arising from light scattering effects are of paramount importance and Q_A can be taken as being virtually equal to 1. The CD spectra of ETP and pronase-treated ETP as recorded as well as the corrected spectra are shown in Fig. 3. The ellipticity values at 224 and 208 nm are summarized in Table IX. The observed ellipticity at 222–224 nm of ETP is in good agreement with the data published by Zahler et al. (35) who gave a value of 10,800 at 222 nm. The corrected value 1.53×10^4, of course, shows an improvement in the ellipticity magnitude of the first extremum. In order to assess the extent of the conformational changes brought about by the enzymic digestion, it was decided to use the method proposed by Greenfield and Fasman (36), taking poly-L-

TABLE IX. Molar Ellipticities of ETP and Pronase-Treated ETP*

System	$[\Theta] \times 10^{-4}$			
	$[\Theta]^{224}$	$[\Theta]_c^{224}$	$[\Theta]^{208}$	$[\Theta]_c^{208}$
ETP	1.08	1.53	1.18	2.14
Pronase-treated ETP	0.96	1.2	1.0	1.54

*Calculated on the final protein content in the case of pronase-treated ETP.

$[\Theta]^{\lambda}$: molar ellipticity at the wavelength of interest.

$[\Theta]_c^{\lambda}$: molar ellipticity corrected for light scattering.

TABLE X: Percent Content* of Recognized Protein Conformational States of ETP and Pronase-Treated ETP

Conformations	α-helix	β-structure	Disordered
ETP	54	6	40
Pronase-treated ETP	∿39	–	∿60

*Calculated according to Greenfield and Fasman (36).

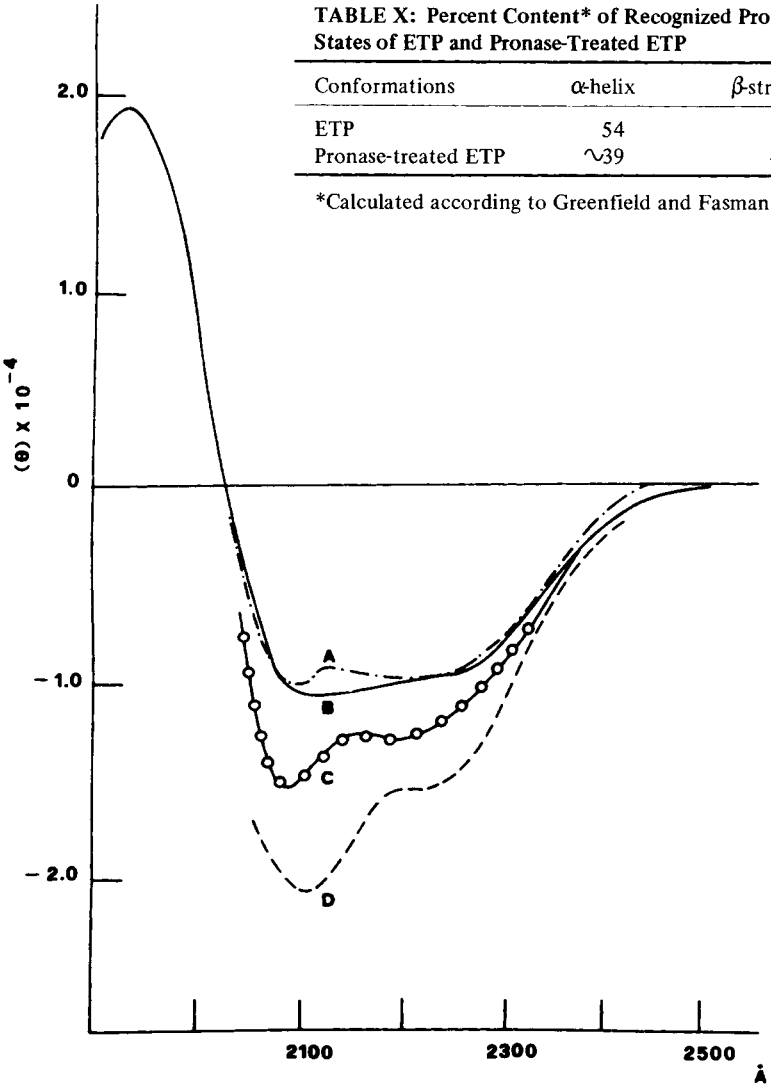

Fig. 3. Effect of pronase digestion on the CD spectra of ETP. Uncorrected CD spectra of ETP (A) and pronase-treated ETP (B). After correction for light-scattering, the CD pattern of ETP is represented by curve D, while curve C refers to pronase-treated ETP.

lysine as a model. The 240–208 nm spectral region, chosen by the above mentioned authors for the assignements in α-helix, β-structure and random coil contents of the homopolymer, is indeed the region where the data afford us a rather good correction for the distortions of CD spectra.

The results of the calculations are shown in Table X. It can be observed in Fig. 3 that pronase digestion modifies the CD spectra of ETP. It is worthy to notice that treatment of the particles with the protease results in a loss of protein in the washes, as demonstrated by the enrichment in lipid phosphorus shown by pronase-treated ETP (cf Table VII). The data shown in Table X suggest a noticeable change in the protein conformation of the submitochondrial vesicles. It is difficult to ascertain if the observed

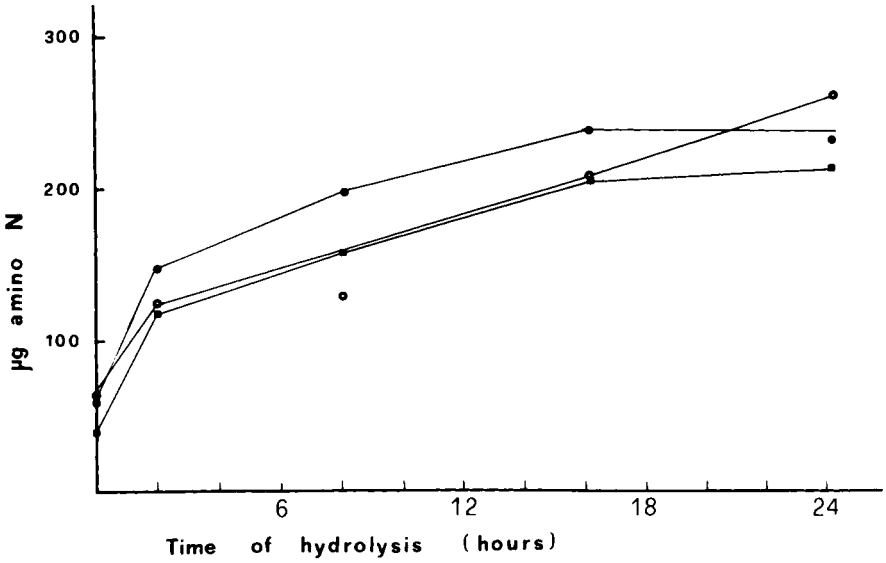

Fig. 4. Pronase digestion of mitochondria and ETP. ●—●, ETP; ○—○, LD-ETP; □—□, BHM hypotonically swollen. Each membrane was added to the incubation medium in a quantity of 10 mg on a protein basis. The ratio of pronase to membrane protein was 2%. The hydrolysis was measured by assaying free amino groups at the times indicated. See text for experimental details.

conformational changes are due to a structural rearrangement of the proteins within the membranes or the increase in random coil content after enzymic treatment is merely due to a preferential digestion of proteins in the α-helical conformation, that are being better available to pronase. Very little content in β-structure, instead, is observed both in ETP and pronase-treated ETP.

Comparison of Proteolytic Digestion of Mitochondria and ETP

It is well known that sonic submitochondrial particles ETP are inside-out with respect to the orientation of the cristae in intact mitochondria (37). A unique possibility is therefore given by this system to investigate the effect of proteolytic enzymes on both sides of the inner mitochondrial membrane.

BHM hypotonically swollen in 0.015 M KCl in order to break their outer membrane, leaving an extended normally oriented inner membrane (38), have been digested with pronase and the results compared with those of sonic particles ETP. The extents of hydrolysis at different times are shown in Fig. 4, together with that of LD-ETP for comparison. Some differences are indeed present between the regularly oriented and the inside-out inner membranes; they do not necessarily depend on the environment of the proteins exposed at the two surfaces, but may be the result of differences in the nature and conformation of the proteins as well as of particle shape and size. Nonetheless the results are of the same order of magnitude for BHM and ETP.

We have then attempted to investigate the time course of hydrolysis of specific mitochondrial proteins by following the sequential disappearance of enzymic activities when the two forms of the membrane (normal and inside-out) were alternatively exposed to the proteases. This approach takes advantage of the presence of several enzymes in the

TABLE XI. Succinoxidase Activity of Mitochondria and ETP after Treatment with Proteolytic Enzymes*

	Time of digestion (min)	ETP	BHM
		μatoms O_2/min mg	
Pronase (2%)	0	0.097	0.190
	5	0.082	0.170
	10	0.079	0.150
	20	0.055	0.140
	30	0.023	0.040
Trypsin (2%)	0	0.100	–
	5	0.102	0.230
	10	0.089	0.160
	20	0.070	0.120
	30	0.044	0.080
Control		0.178	0.310

*After the time indicated, the samples were rapidly cooled and centrifuged at 105,000 g for 45 minutes. The activity was measured in the insoluble pellets resuspended in 0.25 M sucrose containing 0.05 M Tris-HCl, pH 7.5.

TABLE XII. Effect of Pronase Hydrolysis on Succinoxidase and ATPase Activities of BHM and ETP

Time of hydrolysis (hours)*	BHM				ETP			
	OD at 280 nm**	Succin-oxidase†	ATPase††		OD at 280 nm**	Succin-oxidase†	ATPase ††	
			+ oligo-mycin	– oligo-mycin			– oligo-mycin	+ oligo-mycin
0	0.385	0.100	0.30	0.08	0.460	–	0.16	0.17
0.5	0.450	0.060	0.45	0.26	0.590	0.140	0.15	0.15
1	0.510	0	0.37	0.20	0.665	0.150	0.16	0.17
2	0.558	0	0.26	0.19	0.802	0.080	0.15	0.15
6	0.810	0	0.19	0.08	0.962	0.040	0.15	0.16
Control	0.360	0.170	0.24	0.11	0.295	0.150	1.40	0.30

*Pronase was 2% of membrane protein.
**The optical density of the supernatant obtained after centrifugation at 105,000 g for 45 min is taken as a measure of membrane digestion.
†μatoms of O_2/min. mg.
††μmoles Pi/min. mg.

inner mitochondrial membrane. Another possible approach, i.e., gel electrophoresis of membrane proteins, which has been widely employed to monitor erythrocyte membrane digestion (14–17), appears of more difficult application for mitochondrial membranes, owing to the extremely large number of different proteins present (39).

The action of pronase and trypsin digestion on certain mitrochondrial activities of intact mitochondria and ETP is shown in Tables XI and XII and in Fig. 5. Respiration with succinate as substrate is affected in both types of particles by protease treatment, showing that respiratory components are present at both sides of the membrane. On the contrary, ATPase is early affected in ETP but later in mitochondria, confirming that this enzyme is located at the inner side of the inner membrane (20). Malate dehydrogenase,

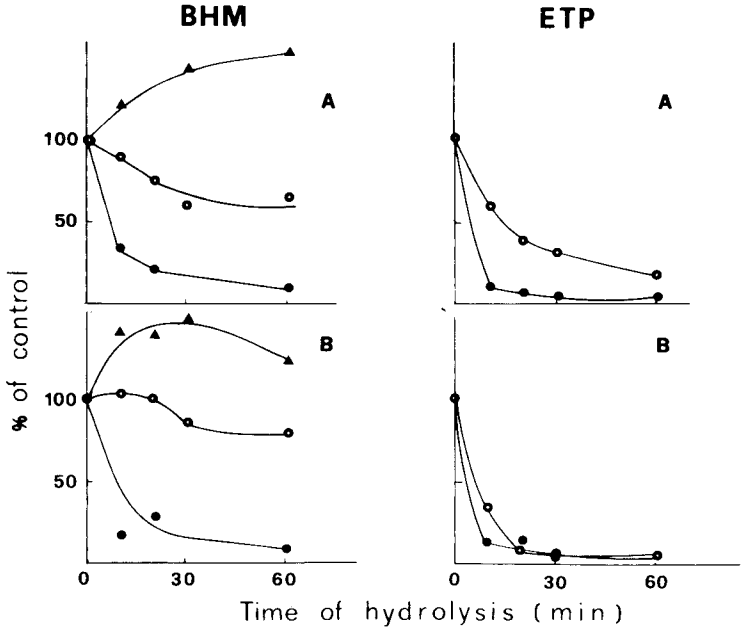

Fig. 5. Effect of trypsin and pronase on enzymic activities of mitochondria and ETP. ○—○, ATPase; ●—●, succinoxidase; ▲—▲, malate dehydrogenase. Activities are indicated as percent of controls at zero time. The ratio of proteolytic enzyme to membrane protein was 2%. The experimental conditions were the same described in the legend of Table IX.

which is an enzyme localized in the mitochondrial matrix, undergoes an initial increase of activity. The lack of a dramatic inactivation in the first 60 min of digestion shows that the matrix is impermeable to the action of the proteolytic enzymes; on the other hand the slight activating effect suggests that there is some increase in membrane permeability to substrate and pyridine nucleotides following protease action.

DISCUSSION

Proteolytic or lipolytic enzymes may be useful tools to investigate the microenvironment of proteins and lipids in lipoprotein systems (40). Several investigators have studied the effect of proteases on natural membranes (14–20). Research has been mainly focused both on the localization of specific protein components on the two faces of a given membrane, and to ascertain whether specific components could have a deep location spanning the entire thickness of a membrane.

Other tools towards the same objective have been antibodies against specific protein components (e.g., 20), and nonpenetrating protein reagents or reagents penetrating under controlled conditions, which are expected to hit only (or at least very quickly) targets present on the membrane surface (41–45). The use of these tools has given valuable information on the protein disposition of red blood cell membranes.

The investigation reported in this communication is mainly concerned with the effects of proteolytic digestion on intact and lipid-depleted mitochondrial membranes. The results obtained allow the following considerations: (a) Mitochondrial membranes

are digested by pronase and trypsin at rates and to extents comparable to those of a soluble protein like albumin. (b) Solubilization of the membrane by detergents, or denaturation by urea or by heating, does not significantly enhance the rate of hydrolysis (although heat denaturation slightly enhances the rate of hydrolysis of albumin), showing that digestion is already taking place at the maximal rate in the intact membranes under the conditions used. (c) Hydrolysis of the intact membranes is accompanied by structural changes resulting in noticeable alteration of protein conformation as evidenced by CD studies and by assaying enzymic activities. The loss of enzyme activity is nonetheless gradual and initiates from the side originally exposed to the proteolytic action, indicating that we are studying vesicular membranes and not broken fragments. (d) The central point of this investigation is the observation that pronase and trypsin hydrolyze intact submitochondrial particles at rates and to extents comparable to those obtained when the delipidated membrane proteins are subjected to hydrolysis. The same effect is true for SU-particles, from which water soluble proteins were removed.

Membranes reconstituted from lipid-depleted ETP and phospholipid vesicles usually are hydrolyzed to a lesser extent and at lower rates than are intact ETP containing their original phospholipid complement. It is apparent that reconstitution from LD-ETP and Asolectin does not lead to a membrane having all of the original structural properties. This finding is in accordance with reconstitution experiments from disaggregated *Acholeplasma laidlawii* subunits (46), showing by a variety of physical means that the membranes are incorrectly reassembled.

The possibility must be considered that even the original ETP (which are submitochondrial particles obtained by sonication) do not represent an intact system: NMR studies of erythrocyte membranes (47) have indicated that sonication produces severe damages to the membrane structure, inducing mobilization of both polar groups and hydrocarbon chains of the phospholipids. We are comforted in our conclusions by the maintainance of electron transport and coupled phosphorylation in ETP (24) and by the fact that proteins in nonsonicated mitochondria are hydrolyzed by pronase at similar rates in comparison with ETP.

The similar availability of the peptide bonds of mitochondrial membrane proteins to proteolytic attack independent of the presence of lipids cannot be interpreted in a unique way. However, since lipids do not protect proteins from digestion, a strict and extensive interdigitation of protein chains with individual phospholipid molecules should perhaps be excluded. The results are qualitatively in accord with the Danielli model of membrane structure; according to that model proteins cover the membrane sufaces and at least the proteins on one side should be available to proteolytic attack. The results are also compatible with a mosaic model for mitochondrial membrane structure, since protein penetrated into a discontinuous lipid bilayer would also be progressively available to proteolytic attack.

Obviously the experiments reported here cannot alone clearly discriminate between the two possibilities, but a series of other findings appear to be in favor of the latter: (a) the bonds between the lipids and proteins appear mainly hydrophobic in nature (1–3, 40); (b) phospholipase C attacks mitochondrial membranes and phospholipid vesicles to similar extents (48), so that protein is not a hindrance to lipolytic action; (c) all of the anionic groups of phospholipids in submitochondrial particles or mitochondrial membranes are available for ionic interaction with basic proteins (lysozyme and cytochrome *c*) (49).

Although no definite conclusion can be advanced at the present stage, the concomitant

use of different dissection approaches and of more sophisticated physical means will clarify the many uncertainties existing in the organization of lipids and proteins in membrane structure.

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