Transverse-plane Topography of Long-chain Acyl-CoA Synthetase in the Mitochondrial Outer Membrane*

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Transverse-plane topography of mitochondrial outer-membrane long-chain acyl-CoA synthetase was investigated using proteases as probes for exposure of crucial domains, i.e. domains containing the active site or otherwise required for enzymatic activity. Incubation of intact mitochondria with the nonspecific proteases proteinase K and subtilisin resulted in a time-dependent loss of 90% or more of the long-chain acyl-CoA synthetase activity compared to control incubations. The integrity of the outer membrane before and during this treatment was shown by cytochrome c oxidase latency as well as the stability of adenylate kinase activity in the presence of protease. After a 15-min incubation in these conditions, site-specific proteases such as trypsin and chymotrypsin had only a limited inhibitory effect (29 and 58% loss of activity, respectively); however, treatment of hypotonically disrupted mitochondria with these proteases resulted in increased (71 and 77%, respectively) loss of activity. Exposure of trypsin-sensitive crucial domains on the inner surface of the membrane was directly demonstrated by incubation of trypsin-loaded outer-membrane vesicles. Together, these results suggest that mitochondrial long-chain acyl-CoA synthetase is a transmembrane enzyme, possessing crucial domains on both sides of the outer membrane. However, the cytosolic exposure of the enzyme does not appear to be affected by a change in the medium ionic strength as seen previously for other outer-membrane enzymes. In an experiment investigating the topography of the active site of the enzyme, an immobilized substrate analog, desulfo-CoA-agarose, was preincubated with intact mitochondria. This resulted in up to a 42% loss of the activity of long-chain acyl-CoA synthetase, consistent with a cytosolic exposure for at least the CoA-binding domain of the active site.

Recent studies have shown the mitochondrial outer membrane to have several important functions within the cell. As the external boundary of the mitochondrion, the outer membrane interacts with other organelles, the cytoskeleton, as well as the cytosol (1, 2). The vast majority of mitochondrial proteins are nuclear-coded, are synthesized extramitochondrially, and must be imported into the mitochondrion during its biogenesis in a process controlled by the outer membrane (3, 4). Also, the outer membrane, through the selective permeability of a pore protein or "porin" (5), is capable of maintaining a composition in the intermembrane space of the mitochondrial that is distinct from that of the cytosol.

The outer membrane is also active metabolically primarily as a site of lipid biosynthesis. Indeed, it is at the level of this membrane that the metabolic fate of cellular fatty acids is largely determined. Long-chain fatty acids are activated to acyl-CoA by the activity of outer-membrane long-chain acyl-CoA synthetase (EC 6.2.1.3) (6). Fatty acyl-CoAs are then utilized for the sequential acylation of glycerol 3-phosphate by outer-membrane glycerol-3-phosphate acyltransferase (7) and acyl-CoA:sn-glycerol-3-phosphate acyltransferase (8) to form phosphatidic acid. The phosphatidic acid synthesized in the outer membrane is thought to serve as a precursor for cellular glycerolipid (9-11) or cardiolipin biosynthesis in the mitochondrial inner membrane (12). Alternatively, long-chain acyl-CoAs are utilized catabolically during β-oxidation in the mitochondrial matrix after transloaction across the inner membrane by the carnitine shuttle.

Since compositionally distinct compartments can be maintained on either side of the outer membrane, the positioning of the fatty acid-activating and -utilizing enzymes within the transverse plane of this membrane determines the milieu of these reactions as well as the accessibility of the products of these reactions to their ultimate subcellular destination. Hence, it is necessary to determine the transverse-plane topography of these enzymes in the outer membrane to more completely understand outer-membrane lipid metabolism. In a previous study we have determined that one of these enzymes, glycerophosphate acyltransferase, spans the transverse plane of the outer membrane and the exposure of the cytosolic domain of the enzyme is modulated by the ionic strength of the medium (13). In this report we extend our investigation of the topography of the outer membrane to include long-chain acyl-CoA synthetase. The results of these experiments indicate that acyl-CoA synthetase possesses one or more protease-sensitive essential ("crucial") domains on the cytosolic surface of the membrane, in addition to having trypsin-sensitive sites on the inner surface of the membrane. Also, the use of an immobilized substrate analog for acyl-CoA synthetase suggests that at least a portion of the active site of the enzyme is accessible on the cytosolic surface of the mitochondrion.
outer membrane. Thus, similar to glycerophosphate acyl-
transferase, mitochondrial long-chain acyl-mitochondria syn-
thetase appears to be a transmembrane enzyme. However,
unlike glycerophosphate acyltransferase, the results show the
exposure of the cytosolically exposed domain(s) to be not
significantly affected by change in the ionic strength of
the medium.

EXPERIMENTAL PROCEDURES

Materials—[9,10-3H]Palmitic acid (23.5 Ci/mmol) was obtained
from Du Pont-New England Nuclear. Coenzyme A (lithium salt,
reference standard grade) and palmitic acid (free acid) were purchased
from Eastman Organic Chemicals; Diethyl ether from Fisher Scientific;
T. Baker Chemical Co. Desulfo-coenzyme A-agarose, dithiothreitol, and
Triton WR-1339 (tyloxapol) were purchased from Sigma. Cross-
linked 4% agaro (Sepharose CL-4B) was obtained from Pharmacia
I.KR Biotechnology Inc. Male Sprague-Dawley rats were purchased from
Taconic Farms, Germantown, NY. The sources of all other
materials were as described previously (13, 14).

Analytical Methods—Long-chain acyl-CoA synthetase was assayed
by quantitating the conversion of [3H]palmitic acid into a non-ether-
extractable product, [3H]palmityl-CoA. The method used was a mod-
fication of that described by Suzue and Marcel (15). The 0.2 ml
reaction mixture contained a final concentration of 500 mm Tris-HCl
(pH 7.4), 8 mm MgCl2, 0.5 mm CoA, 10 mm ATP, 1 mg/ml Triton WR-1339,
and 30 mm [3H]palmitic acid (specific radioactivity = 6 × 106 cpm/nmol).
The assay mixture was cooled in 15 × 100 mm Teflon-lined screw-capped
culture tubes. The reaction was initiated by the addition of the mitochondrial fraction (3-5 μg of protein) which had been diluted with 100 mm
Tris-HCl (pH 7.4), 1 mg/ml dithiothreitol. The assay mixture was incubated
for 4 min at 37 °C and stopped by the addition of 0.8 ml of
1% perchloric acid. Excess [3H]palmitic acid was removed by extract-
ing the aqueous assay mixture four times with 5 ml of water-saturated
diethyl ether. Aliquots of 0.2 ml of the washed aqueous phase were
counted with 2.8 ml of Biofluor liquid-scintillation mixture in a
Packard Instrument Co. Tri-Carb 3255 liquid-scintillation spectrom-
eter. Residual [3H]palmitic acid in the aqueous phase was determined
using control reactions in which the reactants (ATP and CoA) were
omitted. The incorporation of [3H]palmitic acid into aqueous-soluble
product was linear with time and protein concentration. Specific
activities presented are expressed as nanomoles of [3H]palmitic acid
incorporated into aqueous-soluble product per min/mg mitochondrial
protein and are the mean of duplicate assays. In Fig. 4, freshly
prepared mitochondrial fraction (3-5 μg of protein) was preincubated
in the acyl-CoA synthetase assay medium minus the CoA, [3H]
palmitic acid, and Triton WR-1339 for 30 min at 37 °C with the
indicated volumes of cross-linked 4% hepar agaro containing 0.2
mmol/mg packed gel. Per ml of packed gel, the stirring bar
incubations received the same volumes of cross-linked 4% agaro
without desulfo-CoA. Both gels were washed four times in 100 mm
Tris-HCl (pH 7.4), 1 mg/ml dithiothreitol before assay. Assay of acyl-
CoA synthetase activity was then performed as previously described
after initiation of the reactions by the sequential addition of CoA (to
1.5 mm) and [3H]palmitic acid into Triton WR-1339. Glycerophosphate
acyltransferase was assayed by following the incorporation of an-[2-
3H]glycerol 3-phosphate into butanol-extractable phospholipids as
membrane lysis in samples of the mitochondrial fraction before and
after treatment with protease were made by determining the latency
estimation. Adenylate kinase (EC 2.7.4.3) (17), monoamine oxidase
membranes. The method of Lowry et al. (18) was used for protein
assay. Cytochrome c oxidase was assayed using spectrophotometry
(16). The ratio of the specific activities in unactivated versus fully activated
mitochondria in low ionic isotonic medium was adjusted by the
addition of 4 M KCl as previously described (13). Aliquots of the mitochondrial fraction were preincubated for 5 min at 30 °C, and
then proteolytic enzyme was added and incubation continued for 15
min unless otherwise indicated. The proteinase K, subtilisin, and
chymotrypsin reactions were stopped by the addition of phenylmeth-
ysulfonyl fluoride at 1 μg/ml. Trypsin reactions were stopped by adding
soybean trypsin inhibitor to 0.12 mg/ml. The samples were immedi-
ately chilled and then stored at -70 °C until they were used for
enzyme activity analyses within 1 week.

Preparation and Incubation of Trypsin-loaded Outer-membrane
Vesicles—Trypsin-loaded outer-membrane vesicles were prepared from
the mitochondrial fraction by a modification of the method of
Lowry et al. (20) as described previously (13) in which trypsin was
added (0.5 mg/ml) before sonication or, to control aliquots, after
sonication or not at all. Following sonication, soybean trypsin
inhibitor was added (1 mg/ml) to inhibit externally accessible trypsin.
Aliquots of the sonicated mitochondria were incubated at 30 °C for
the indicated time and then the reaction was stopped by the addition
of phenylmethylsulfonyl fluoride.

RESULTS

Fig. 1 documents the results of time-course experiments in
which the nonspecific proteases, proteinase K (Fig. 1a) or subtilisin (Fig. 1b), were used to probe for cytosolically ex-
plored crucial domains of several outer-membrane enzymes.
During digestion of preparations of intact rat liver mitochondria with either protease, long-chain acyl-CoA synthetase was the
most rapidly inactivated outer-membrane enzyme enzyme
studied in these experiments. Under these conditions, maxi-
mal inactivation (90%) of acyl-CoA synthetase occurred
within 5 min of incubation. Proteolysis by either proteinase K or subtilisin caused a moderately rapid and extensive degree
of inactivation of mitochondrial glycerophosphate acyltransferase, in keeping with results presented previously for 15-
min proteolysis under these conditions (13). Rotenone-insen-
sitive NADH-cytochrome c reductase, a marker enzyme for the
outer surface of the outer membrane (21), was inactivated by
these proteases at an initial rate exceeding that of glycer-
ophosphate acyltransferase. In either experiment, both of
these enzymes were not maximally inactivated until 20 min

Fig. 1. Time course of effect of nonspecific proteases on
activity of mitochondrial outer-membrane enzymes and aden-
ylate kinase. Aliquots of mitochondria, suspended in low ionic
isotonic incubation medium at a protein concentration of 4 mg/ml,
were treated with either proteinase K (a) or subtilisin (b) at 40 μg/ml
as described under "Experimental Procedures" for the indicated
time periods. Resulting specific activities are expressed as percentage
of that of similarly treated control (no protease) incubations. The
enzymes assayed and the range of specific activities (nanomoles/min/mentho-
lysomal control) are as previously described, long-chain acyl-CoA syn-
thetase, 46-53; O, glycerophosphate acyltransferase, 3.3-4.5; D, roten-
one-insensitive NADH-cytochrome c reductase, 272-293; □, mono-
amine oxidase, 11.0-12.5; A, adenylate kinase, 392-418.

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of incubation. At all time points the integrity of the outer membrane was not significantly affected, as shown by the stability of the activity of adenylate kinase in the protease-treated mitochondria. Under conditions in which the outer membrane is lysed, allowing protease access to the inner surface of the outer membrane and the intermembrane compartment, this intermembrane enzyme is readily inactivated (Refs. 21 and 22, and Table I). Also, the stability of monoamine oxidase activity in the experiments presented in Fig. 1 indicates that proteolysis did not lead to exposure of enzymes which are deeply buried within the bilayer and are therefore not protease-accessible (23, 24).

Since maximal inactivation of acyl-CoA synthetase had already occurred by the earliest time point of incubation examined in Fig. 1, the effect on this enzyme of treatment of mitochondria with reduced concentrations of nonspecific protease was examined. At a protease concentration one-fifth that used in Fig. 1, an incubation time-dependent loss of acyl-CoA synthetase activity in either proteinase K or subtilisin treated mitochondria was obtained (Fig. 2). Stability of adenylate kinase activity in the treated samples again demonstrated the maintenance of intact outer membranes during the course of the digestions. Thus, the inactivating effect of these proteases on acyl-CoA synthetase is shown to be due to proteolysis during the course of the incubation, similar to the inactivation of mitochondrial glycero-phosphate acyltransferase and rotenone-insensitive NADH-cytochrome c reductase (Fig. 1). However, much less active proteolytic conditions are necessary to produce comparable rates of inactivation for acyl-CoA synthetase.

Further experiments examined the dependence of proteolytic inactivation of mitochondrial long-chain acyl-CoA synthetase on the ionic strength of the incubation medium. The degree of inactivation observed following a 15-min incubation with nonspecific protease was completely unaffected (40 pg/ml, proteinase K or subtilisin) or only slightly affected (8 pg/ml, proteinase K) when the ionic strength of the incubation medium was increased to physiological levels by the addition of 

### Effect of treatment of mitochondria with various proteases on mitochondrial enzymes and outer-membrane intactness

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<th>Protolytic enzyme</th>
<th>ACS †</th>
<th>GAT †</th>
<th>NCR †</th>
<th>MAO †</th>
<th>AK †</th>
<th>Outer-membrane lysis</th>
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† Long-chain acyl-CoA synthetase.
† Glycero-phosphate acyltransferase.
† Rotenone-insensitive NADH-cytochrome c reductase.
† Monoamine oxidase.
† Adenylate kinase.

The medium was increased to physiological levels by the addition of KCl (Fig. 3). Addition of other salts such as MgCl₂ or (NH₄)₂SO₄ failed to significantly alter the proteolytic inactivation rate of acyl-CoA synthetase. The percentages of control activities remaining following subtilisin digestion at 8 µg/ml in medium containing 0.15 units of additional ionic strength MgCl₂ or (NH₄)₂SO₄ under the conditions described in Fig. 3 increased only 0 and 1%, respectively, over those in unadjusted medium. These results for acyl-CoA synthetase contrast with results of previous experiments conducted under similar conditions showing a marked decrease (52–75%) in the inactivation of mitochondrial glycero-phosphate acyltransferase by these proteases when the ionic strength of the medium was increased by 0.10 or 0.15 unit (12). Also, previous studies have shown that the inactivation of mitochondrial outer-membrane rotenone-insensitive NADH-cytochrome c reductase (21) and GDP-mannose: dolichyl-phosphate mannosyltransferase (25) by protease could be substantially reduced or eliminated by increasing the ionic strength of the

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**Fig. 2. Time course of inactivation of mitochondrial long-chain acyl-CoA synthetase by nonspecific proteases.** Mitochondria (4 mg/ml) suspended in low ionic isotonic medium were treated with proteinase K (■) or subtilisin (○) at 8 µg/ml for the indicated time periods. Protease-treated and control (no protease) samples were assayed for acyl-CoA synthetase (△) and adenylate kinase (□) and the data expressed as percentage of the control incubations. Specific activities (nanomoles/min/mg) in the control samples ranged from 46 to 54 and 384 to 397, respectively.

**Fig. 3. Effect of different ionic media on the inactivation of mitochondrial long-chain acyl-CoA synthetase by nonspecific proteases.** Aliquots of mitochondria suspended in low ionic isotonic incubation medium at 4 mg of protein/ml were adjusted to the indicated additional ionic strengths with KCl and immediately treated with proteinase K (■) or subtilisin (○) at either 8 µg/ml (△) or 40 µg/ml (□) for 15 min as described under "Experimental Procedures." Protease-treated and control (no protease) samples were assayed for acyl-CoA synthetase activity and the results are expressed as percentage of the control incubations. Control specific activities ranged from 44 to 49 nmol/min/mg.
proteolysis medium to this range.

The above results demonstrate the presence of one or more nonspecific protease-sensitive domains of acyl-CoA synthetase on the outer surface of the mitochondrial outer membrane. To probe the inner surface of the membrane, the effect of limited-specificity proteases (trypsin and chymotrypsin) on both intact and osmotically disrupted mitochondria was studied. Table I presents the effect on the activities of acyl-CoA synthetase as well as other mitochondrial enzymes assayed following proteolytic digestion of mitochondria during suspension in either isotonic or hypotonic medium. As expected, the nonspecific proteases extensively inactivate all three cytosically exposed enzymes in either medium. Similar to glycerophosphate acyltransferase, acyl-CoA synthetase activity was slightly (chymotrypsin) or markedly (trypsin) more susceptible to site-specific protease digestion following osmotic lysis of the outer membrane in the hypotonic medium, suggesting the possibility that additional crucial domains of acyl-CoA synthetase exist on the inner surface of the membrane. However, unlike glycerophosphate acyltransferase, a significant amount of inactivation of acyl-CoA synthetase by these proteases occurred in isotonic medium. This inactivation exceeded by 22% (trypsin) or 53% (chymotrypsin) the percentage of outer membrane lysis as estimated by the cytochrome c oxidase latency. The results of this method of estimating the percentage of the mitochondrial population with disrupted outer membranes showed a close correlation with the percentage of inactivation of adenylate kinase by the various proteolytic enzymes (Table I). Thus, it appears that limited trypsin- and chymotrypsin-sensitive sites of acyl-CoA synthetase exist on the cytosolic surface of the membrane. Therefore, the increased inactivation of the enzyme by these proteases in hypotonic medium may be due to exposure of additional sensitive sites on the inner surface of the membrane, or may instead simply reflect increased exposure of the cytosolic domains.

The experimental approach taken to resolve this question was to measure the activity of acyl-CoA synthetase after direct exposure of only the inner surface of the outer membrane to protease activity. Right-side-out outer-membrane vesicles containing trypsin trapped internally by sonication were prepared as previously described (13) by a modification of the method of Sottocasa et al. (20). Previous studies of permeability as well as protease- or immunoochemical-treated vesicles have shown that virtually all of the outer membrane vesicles prepared by this method have the same "outside-out" surface orientation as the intact mitochondrion (26) and are sealed (26, 27). Also, proteins that are deeply imbedded within the bilayer do not become accessible to protease activity due to this treatment (26). This protocol was previously used for preparation of protease- or lactoperoxidase-loaded outer-membrane vesicles in other topography studies (24, 28). Trypsin-loaded and control outer-membrane vesicles were incubated in the presence of soybean trypsin inhibitor to inactivate any external protease which would have access to the cytosolic membrane surface. The impermeability of these vesicles to molecules greater than 13,000 daltons (27) precludes inactivation of internally trapped trypsin. The activities in the incubated control vesicles which were prepared either without trypsin or with trypsin added after sonication remained stable for the entire incubation period (Table II). In contrast, incubation of the trypsin-loaded vesicles caused a gradual loss of up to 36% of the initial control acyl-CoA synthetase activity after 1 h (Table II). Loss of monoamine oxidase activity following incubation of control or trypsin-loaded vesicles was less than 10% (data not shown), indicating that proteins buried within the bilayer were not exposed by the sonication procedure. Thus, this approach demonstrates the presence of protease-accessible crucial domains of this enzyme on the inner surface of the membrane as well.

In further experiments using nondisrupted mitochondria we investigated the topography of the CoA-binding portion of the active site of outer-membrane long-chain acyl-CoA synthetase using an irreversibly inhibitory immobilized substrate analog for this enzyme. Intact mitochondria were preincubated for 30 min in the presence of cross-linked 4% beaded agarose containing up to 4 nmoles of desulfo-CoA attached covalently with a C6 spacer. Control incubations contained an equal volume of agarose without desulfo-CoA. As shown in Fig. 4, this treatment resulted in the dose-dependent loss of up to 42% of the acyl-CoA synthetase activity in the mitochondrial samples incubated with the desulfo-CoA-agarose. The inhibitory effect was specific for the desulfo-CoA moiety since, at any dose, the activities in the agarose-alone incubations did not differ significantly from the no-agarose control (Fig. 4). These results indicate a cytosolic exposure of the CoA-binding domain of the enzyme on the outer membrane, since the accessibility of the inhibitor during the preincubation was limited to this surface. The pretreatment with the agarose under these conditions did not lead to disruption of the outer membrane as detected by release of adenylate kinase into the medium.

**DISCUSSION**

In the present study we have investigated the transverse-plane topography of long-chain acyl-CoA synthetase within the rat liver mitochondrial outer membrane using, as one approach, proteases as probes for exposed crucial domains of the enzyme. In this study we define crucial domains to include the catalytic domain(s) of the enzyme as well as any regulatory site domains or structural portions of the protein involved in maintaining an active conformation of the enzyme. Crucial
domains therefore may include, but are not limited to, the active site of the enzyme.

Since it is known that exposed membrane enzymes and proteins can display resistance to one or more site-specific proteases, the use of a variety of proteolytic enzymes, preferably of broad specificity, is indicated in this type of membrane topography study (29, 30). The results presented here underline the importance of these criteria. Using both adenylate kinase inactivation and cytochrome c oxidase latency as controls for outer-membrane integrity, digestion of mitochondria using the nonspecific proteases subtilisin and proteinase K clearly demonstrates the exposure of crucial domains of acyl-CoA synthetase on the cytosolic surface of the outer membrane (Figs. 1 and 2, Table I). Extended incubation of intact mitochondria with a substantial amount of trypsin, however, results in only very limited loss of activity of this enzyme (Ref. 31 and Table I). The cytosolic domain of the enzyme is less resistant to chymotrypsin although it does retain substantial activity (>40%) after a similar incubation with this protease (Table I).

The additional inactivation of acyl-CoA synthetase given by these site-specific proteases after osmotic disruption of the outer membrane (Ref. 31 and Table I) and the inactivation of the enzyme during incubation of “right-side-out” outer-membrane vesicles containing trapped trypsin (Table I) provides evidence for the existence of protease-sensitive crucial domains on the inner (noncytosolic) surface of the outer membrane as well. Taken together, the above results indicate that acyl-CoA synthetase spans the outer membrane and is, in fact, a transmembrane protein or polypeptide complex. In light of the results presented here, the previously reported localization of mitochondrial acyl-CoA synthetase entirely to the inner surface of the outer membrane (31) appears to be a consequence of the use of a single site-specific protease.

The inactivating effect of protease treatment on acyl-CoA synthetase presented here corroborates the earlier results of Pande and Blanchaer (32) and DeJong and Hulsen (33) who showed that incubation of isolated rat liver mitochondria with the nonspecific protease Nagarse could virtually completely inactivate long-chain acyl-CoA synthetase without affecting monoamine oxidase activity. Due to lack of controls for the intactness of the outer membranes in their preparations, neither of these studies could firmly conclude a cytosolic exposure of the enzyme on the outer membrane.

Several studies exist in the literature reporting the purification of long-chain acyl-CoA synthetase from rat liver mitochondria. Tanaka et al. (34) have described the purification from both microsomal and mitochondrial fractions of a single 76,000-dalton polypeptide which catalyzed the purification of CoA to Ca2+ fatty acids. The protein purified from both sources was indistinguishable in any molecular or catalytic property examined. In addition, Philipp and Parsons (35) report a purification from the mitochondrial fraction of an approximately 250,000-dalton complex consisting of four subunits, ranging from 55,000 to 135,000 daltons, which activated only C12 to C18 fatty acids. While the relatedness of these purified proteins remains uncertain, it is clear from the solubility requirements reported in these studies (34, 35) that either enzyme is quite hydrophobic in nature and likely contains domains deeply imbedded within the outer membrane, rather than existing as a loosely attached peripheral protein. If several major isoenzyme forms of acyl-CoA synthetase do exist in the outer membrane, the findings presented here showing virtually complete inactivation with nonspecific proteases (Figs. 1 and 2, Table I) indicate that all possess a cytosolic exposure.

The present results show mitochondrial outer membrane long-chain acyl-CoA synthetase to be similar to both the rat liver microsomal (36) and the peroxisomal (37) forms of the enzyme in possessing exposed crucial domains on the cytosolic side of their respective membranes. In all three subcellular fractions these essential domains are accessible for hydrolysis by nonspecific proteases such as proteinase K or subtilisin (Figs. 1 and 2) or Pronase (36, 37) in the absence of disrupted membranes.

Previous studies of outer-membrane topography have defined two categories of integral membrane proteins. One group consists of proteins such as monoamine oxidase (21, 23) and porin (26, 38) which are deeply buried within the membrane and thus completely protected from protease activity. The other group is those enzymes such as glycerophosphate acyltransferase (13), rotenone-insensitive NADH-cytochrome c reductase (21), or GDP-mannose/dolichyl-monomophosphate mannosyltransferase (25) whose accessibility to proteases is dependent upon the ionic strength of the membrane’s environment. Mitochondrial long-chain acyl-CoA synthetase is shown here (Fig. 3) to be an example of a third group of mitochondrial outer-membrane proteins, which possess exposed crucial domains whose protease accessibility is not significantly affected by a shift in the medium ionic strength. This may reflect a more prominent exposure on the membrane such that changes in the microenvironment of the proteins, perhaps due to increases in the membrane fluidity with increasing ionic strength (39), do not lead to masking of crucial domains as for those enzymes whose accessibility changes. The much greater susceptibility of acyl-CoA synthetase to protease activity than glycerophosphate acyltransferase or rotenone-insensitive NADH-cytochrome c reductase (Fig. 1) is also consistent with a greater degree of exposure of acyl-CoA synthetase on the membrane’s cytosolic surface.

The use of immobilized substrates or substrate analogs as active site probes is another investigational approach to the study of membrane enzyme topography. Previous experiments in our laboratory demonstrating the ability of mitochondria with intact outer membranes to use palmityl-CoA-agarose as an acyl donor have provided evidence for a cytosolic exposure of the active sites of both glycerophosphate acyltransferase and acyl-CoA synthetase.
and acyl-CoA-1-acetyl-sn-glycerol-3-phosphate acyltransferase (40). In this study we have taken a similar approach using an immobilized substrate analog for long-chain acyl-CoA synthetase, desulfo-CoA-agarose. This reagent is specifically inhibitory to the enzyme following preincubation with mitochondria with intact outer membranes (Fig. 4). Although further investigation of the topography of the active site is necessary, this result is consistent with a cytosolic exposure for at least the CoA-binding domain of the active site. Thus, it appears that the active sites for long-chain acyl-CoA-generating and -utilizing enzymes in the outer membrane may have a similar cytosolic topographical orientation; an orientation which would allow them to draw directly on cytosolic substrate pools and to be regulated by cytosolic factors.

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