Topography of succinate dehydrogenase in the mitochondrial inner membrane

A study using limited proteolysis and immunoblotting

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The arrangement of the large (70 000- M_r) and small (30 000- M_r) subunits of succinate dehydrogenase in the mitochondrial inner membrane was investigated by immunoblot analysis of bovine heart mitochondria (right-side-out, outer membrane disrupted) or submitochondrial particles (inside-out) that had been subjected to surface-specific proteolysis. Both subunits were resistant to proteinase treatment provided that the integrity of the inner membrane was preserved, suggesting that neither subunit is exposed at the cytoplasmic surface of the membrane. The bulk of the small subunit appears to protrude into the matrix compartment, since the 30 000- M_r polypeptide is degraded extensively during limited proteolysis of submitochondrial particles without the appearance of an immunologically reactive membrane-associated fragment; moreover, a soluble 27 000- M_r peptide derived from this subunit is observed transiently on incubation with trypsin. Similar data obtained from the large subunit suggest that this polypeptide interacts with the matrix side of the inner membrane via two distinct domains; these are detected as stable membrane-associated fragments of 32 000 M_r and 27 000 M_r after treatment of submitochondrial particles with papain or proteinase K, although the 27 000- M_r fragment can be degraded further to low- M_r peptides with trypsin or α -chymotrypsin. A stable 32 000–34 000- M_r fragment is generated by a variety of specific and non-specific proteinases, indicating that it may be embedded largely within the lipid bilayer, or is inaccessible to proteolytic attack owing to its proximity to the surface of the intact membrane, possibly interacting with the hydrophobic membrane anchoring polypeptides of the succinate:ubiquinone reductase complex.

INTRODUCTION

The simplest mitochondrial component that catalyses the conversion of succinate to fumarate is succinate dehydrogenase (SDH, EC 1.3.99.1), an active fragment of the succinate:ubiquinone reductase complex (complex II) of the mitochondrial respiratory chain isolated by treatment of intact complex II with chaotropic agents.

Bovine heart SDH consists of two non-identical hydrophilic subunits: a small 30 000- M_r iron-sulphur protein (S) and a larger 70 000- M_r polypeptide (L) that contains one molecule of covalently attached FAD (Walker & Singer, 1970; Davis & Hatefi, 1971). Amino acid sequencing of the bovine heart small subunit (Yao *et al.*, 1986) in conjunction with similar information on the structural genes for SDH from *Escherichia coli* (Wood *et al.*, 1984; Darlison & Guest, 1984) and *Bacillus subtilis* (Phillips *et al.*, 1987), deduced from the DNA sequence of the cloned genes, has revealed the presence of three conserved cysteine arrangements capable of accommodating all three distinct iron-sulphur centres on the small subunit.

SDH as isolated requires the employment of artificial electron acceptors such as ferricyanide or dichlorophenol-indophenol for measurement of activity. It utilizes physiological electron acceptors, ubiquinone or menaquinone, only as part of the native succinate:ubiquinone reductase complex, in which it is bound to two small hydrophobic polypeptides with M_r values of 15 500 and 13 500 in bovine heart (Hatefi & Galante, 1980). Complex II preparations also contain approx. 1 mol of *b*-type haem bound to these latter proteins. The equivalent hydrophobic subunits of the bacterial succinate:ubiquinone reductase complexes from *E. coli* (Wood *et al.*, 1984) and *B. subtilis* (Magnusson *et al.*, 1986) have been cloned and sequenced as well as the corresponding

components of the analogous fumarate reductase complex from *E. coli* (Cole, 1982; Cole *et al.*, 1982; Grundström & Jaurin, 1982). Examination of the predicted amino acid sequences of these two polypeptides indicates the presence of three hydrophobic segments in each, long enough to form transmembrane α -helices with these integral proteins serving as anchoring sites for the catalytic subunits.

SDH appears to be exposed exclusively on the matrix side of the mitochondrial inner membrane. Such an arrangement is inferred from the facts that antimycin-insensitive succinate:ferricyanide reductase activity, inhibition of enzymic activity by anti-SDH antibodies and labelling of SDH by the membrane-impermeant probe diazobenzene[35 S]sulphonate are observed only in submitochondrial particles, where the matrix side of the inner membrane is accessible to exogenously added reagents (Klingenberg & Buchholz, 1970; Merli *et al.*, 1979).

In the present study we have investigated the topographical arrangement of the large and small subunits of SDH in the mitochondrial inner membrane in greater detail, employing immune-replica analysis in combination with surface-specific proteolysis to detect membrane-associated or released proteolytic fragments of the two subunits.

MATERIALS AND METHODS

Materials

Low- M_r marker proteins were obtained from Pharmacia. Nitrocellulose paper (0.45 μ m pore size) was from Schleicher und Schüll, Dassel, Germany.

Phenylmethanesulphonyl fluoride, leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal), Protein A, papain (type III), tosyl-L-lysyl-

Abbreviations used: SDH, succinate dehydrogenase; SMP, submitochondrial particle.

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chloromethane ('TLCK')-treated α -chymotrypsin (type VII) and tosyl-L-phenylalanylchloromethane ('TPCK')-treated trypsin (type XIII) were obtained from Sigma Chemical Co. Proteinase K was from Boehringer Mannheim.

Iodogen was purchased from Pierce and Warriner, Chester, U.K., Na¹²⁵I (carrier-free) was obtained from Amersham International, and X-ray film (X-Omat S or XAR-5) was from Kodak. All other reagents were of the highest grade available commercially.

Preparation of antisera

Bovine heart SDH was purified as described by Clarkson *et al.* (1987). Antisera to the purified holoenzyme and its constituent large and small subunits (anti-L and anti-S sera respectively) were raised by using previously described regimes (De Marcucci *et al.*, 1985; Hunter & Lindsay, 1986).

Isolation of bovine heart mitochondria

Bovine hearts were obtained from a local abattoir, transported to the laboratory on ice, and used within 2 h of animal death. Mitochondria were isolated by the method of Smith (1967), with the following modifications. Tris base (2 M, 3 ml/300 g of starting material) was added to the suspension of minced tissue, which was then blended for 15 s in a Philips food blender. The material was reblended for 5 s after a second identical addition of Tris base, and, after adjustment of the pH to 7.8, the procedure was continued as described previously (Smith, 1967). Mitochondria were either suspended at a protein concentration of 4 mg/ml and used within 24 h of isolation or stored in pellet form at -80°C until required.

Preparation of submitochondrial particles (SMPs)

Mitochondria were thawed from -80°C , resuspended in 50 mM-sodium phosphate, pH 7.5 (phosphate buffer), to approx. 10 mg/ml, and repelleted by centrifugation for 15 min at 6500 *g* at 4°C . All further manipulations were performed at 4°C . The resuspension/centrifugation step was repeated once, and the resulting pellet was resuspended in phosphate buffer at approx. 10 mg/ml.

To generate SMPs, the mitochondrial suspension was subjected to three 15 s bursts of sonication, with 15 s cooling intervals, with an MSE sonicator at amplitude setting 3 (high power). Unbroken mitochondria were removed by centrifugation at 10000 *g* for 10 min; then the supernatant fraction was centrifuged at 100000 *g* for 1 h.

The final pellet was resuspended in phosphate buffer at 4 mg of protein/ml concentration and stored in 1.0 ml portions at -80°C .

Protease treatment of mitochondria and SMPs

Portions of a suspension of freshly isolated mitochondria were incubated for 60 min at 30°C with α -chymotrypsin or trypsin at a proteinase/mitochondrial protein ratio of 4% (w/w). A control incubation (no proteinase added) was performed in parallel. Samples (1 mg of mitochondrial protein) were taken at 0, 15, 30 and 60 min, and these were dissociated in an equal volume of hot (70°C) Laemmli sample buffer [62.5 mM-Tris/HCl, pH 6.8, containing 2% (w/v) SDS, 10% (w/v) sucrose, 10 mM-dithiothreitol and 0.001% (w/v) Pyronin Y]. The dissociated samples were boiled immediately for 5 min before analysis by SDS/PAGE and immunoblotting. An identical protocol was followed for proteinase treatment of freeze-thawed mitochondria.

SMPs were subjected to incubation with α -chymotrypsin, trypsin, papain or proteinase K. Incubations were performed at 30°C for periods of up to 90 min. Specific conditions for each experiment are given in the appropriate Figure legend.

In some cases, proteolysis was terminated by mixing samples with 0.33 vol. of $2\times$ Laemmli sample buffer containing 0.8 mg of leupeptin/ml (for inactivation of papain) or 2 mM-phenylmethanesulphonyl fluoride (for inactivation of α -chymotrypsin, trypsin or proteinase K). After 5 min at room temperature, samples were boiled for 5 min before analysis by SDS/PAGE and immunoblotting. Alternatively, samples of proteinase-treated SMPs were diluted with 3 vol. of ice-cold 20 mM-phosphate buffer containing 0.15 M-NaCl and 0.8 mg of leupeptin/ml or 2 mM-phenylmethanesulphonyl fluoride. Subsequent manipulations were performed at 4°C . The samples were centrifuged at 27000 *g* for 20 min, then the pelleted membranes were each resuspended in 0.5 ml of the same buffer and re-centrifuged as above. The wash procedure was repeated once with buffer, once with distilled water, and the final pellets were dissociated in Laemmli sample buffer and boiled for 5 min.

Other methods

Procedures for SDS/PAGE and immunoblotting were described previously (Clarkson *et al.*, 1987, and references cited therein).

Protein concentrations were determined by a modification of the Lowry procedure, with BSA as a standard (Markwell *et al.*, 1978). ¹²⁵I-labelled Protein A and low-*M_r* markers were prepared by the method of Salacinski *et al.* (1981), as described by De Marcucci *et al.* (1985).

RESULTS

It has been shown previously that in preparations of freshly isolated bovine heart mitochondria the cytoplasmic surface of the inner membrane is exposed to labelling agents such as lactoperoxidase, indicating that the outer membrane has been disrupted by the isolation procedure (Boxer, 1975). A high degree of removal of the outer membrane is also indicated by absence of monoamine oxidase activity, a marker for the outer membrane. Therefore, by immunoblot analysis of proteinase-treated fresh mitochondria, it was possible to probe for exposed regions of subunits of SDH on the cytoplasmic surface of the inner membrane.

Samples of freshly isolated mitochondria were incubated in the absence of proteinase or after addition of 4% (w/w) α -chymotrypsin or trypsin. The immunoblot obtained by probing these samples with anti-SDH serum (Fig. 1*a*) shows that both subunits of SDH were essentially stable during a control incubation [(i), lanes 2–5]. Inclusion of α -chymotrypsin [(ii), lanes 6–9] or trypsin [(iii), lanes 10–13] caused no significant change in the stability of the 70000-*M_r* and 30000-*M_r* polypeptides. Similar results were obtained with proteinase K and Pronase (results not shown).

To test the possibility that SDH in its membrane environment is inherently resistant to proteolysis under the conditions described above, the experiment was repeated with freeze-thawed mitochondria, where fracturing of the inner membrane has occurred (Pettit *et al.*, 1978). Both polypeptides were again stable during a control incubation [Fig. 1*b*, (i), lanes 2–5], but were rendered proteinase-sensitive (at least partially) by the freeze-thaw procedure. In the presence of α -chymotrypsin, the large subunit was degraded with the concomitant appearance of a 34000-*M_r* proteolytic fragment [(ii), lanes 6–9] and a weakly reacting species that migrated with an *M_r* value of 17000. In contrast, there was no discernible decrease in the amount of intact small subunit.

During incubation of freeze-thawed mitochondria with trypsin [(iii), lanes 10–13] degradation of both subunits was paralleled by the appearance of large fragments of *M_r* 32000, as well as a transient 27000-*M_r* immunoreactive polypeptide. The stable

Fig. 1. Immunoblot analysis of the effect of proteinases on SDH in bovine heart mitochondria

Portions of freshly isolated (a) or freeze-thawed (b) mitochondria were incubated for 60 min at 30 °C with α -chymotrypsin or trypsin [4% (w/w) proteinase/mitochondrial protein] or with no proteinase addition (control incubation). Samples were removed from each incubation at the indicated times, mixed with 0.33 vol. of $2 \times$ Laemmli sample buffer and subjected to electrophoresis on 10% (w/v) SDS/polyacrylamide-slab gels. Resolved polypeptides were electrophoresed on to nitrocellulose paper for detection of immunoreactive species with anti-SDH serum and ^{125}I -labelled Protein A. Lanes 1 and 14, ^{125}I -labelled low- M_r markers; (i) lanes 2–5, control samples; (ii) lanes 6–9, incubation with α -chymotrypsin; (iii) lanes 10–13, incubation with trypsin. Volumes corresponding to 40 μg of total mitochondrial protein were loaded in lanes 2–13 inclusive.

trypsin-derived 32000- M_r species appeared to be less antigenic than the corresponding fragment, M_r 34000, observed after incubation with chymotrypsin, although this was rather variable from experiment to experiment. The 27000- M_r intermediate was shown subsequently (Fig. 2b) to be a product of the small subunit, which was no longer associated with the inner membrane. Taken together, the above data suggest that both subunits of SDH are exposed exclusively on the matrix side of the inner membrane.

Bovine heart SMPs generated by sonication of mitochondria are predominantly vesicles of inner membrane with an inverted orientation (Godinot & Gautheron, 1979; Smith & Ragan, 1980). Therefore the exposure of SDH on the matrix side of the inner membrane was investigated by immunoblot analysis of control (i) or proteinase-treated [(ii) and (iii)] SMPs (Fig. 2) with either anti-L serum (Fig. 2a) or anti-S serum (Fig. 2b). Results similar to those observed in freeze-thawed mitochondria were obtained, indicating that disruption of the inner membrane by sonication in the preparation of SMPs does not radically alter

Fig. 2. Immunoblot analysis of the effect of proteinases on the large and small subunits of SDH in bovine heart SMPs

SMPs were incubated for up to 60 min in the absence of proteinase or in the presence of α -chymotrypsin or trypsin as described in Fig. 1 legend. Samples were processed in an identical manner for detection of immunologically reactive fragments with subunit-specific anti-[large subunit (L)] (a) or anti-[small subunit (S)] (b) sera. Lanes 1 and 14, ^{125}I -labelled low- M_r markers; (i) lanes 2–5, control incubations; (ii) lanes 6–9, digestion with α -chymotrypsin; (iii) lanes 10–13 digestion with trypsin.

the accessibility of SDH to surface-specific proteolysis. Degradation of the large subunit with either α -chymotrypsin (ii) or trypsin (iii) yields stable immunologically reactive 34000- M_r and 32000- M_r species respectively, but rapid degradation of the small subunit once again is achieved only with trypsin, yielding a transient 27000- M_r polypeptide. The minor chymotryptic product of the large subunit, M_r 17000, is again also just detectable.

Fig. 3 illustrates the immunoblot patterns obtained when control or proteinase-treated SMPs were probed with anti-L serum (Fig. 3a) or anti-S serum (Fig. 3b) after being washed with 0.15 M-NaCl to test the interaction of these proteolytic fragments with the mitochondrial inner membrane. In the absence of proteinase, both subunits were stable [Fig. 3(a) and 3(b), lanes 2 and 3, (i)], whereas in the presence of α -chymotrypsin [Fig. 3(a), (ii), lanes 4–7] or trypsin [Fig. 3(a), (iii), lanes 8–11] the large subunit was degraded with the parallel appearance of a membrane-bound fragment of M_r approx. 32000–34000. Incubation with papain generated two large subunit fragments; one exhibited a similar electrophoretic mobility to the α -chymotryptic and tryptic fragments, and the second had an M_r value of approx. 27000 [Fig. 3(a), (iv), lanes 12–15]. Long-term incubation with papain [Fig. 3(a), (iv), lane 15] appeared to affect the integrity of SMPs, which tended not to pellet so readily on centrifugation.

Fig. 3. Immunoblot analysis of salt-washed proteinase-treated submitochondrial particles with anti-[large subunit (L)] and anti-[small subunit (S)] sera

Submitochondrial particles were incubated at 30 °C in the absence of proteinase, or after addition of α -chymotrypsin, trypsin or papain [4% (w/w) protease/particle protein]. In each case, samples were removed at the indicated times, and mixed with 3 vol. of ice-cold phosphate buffer containing 0.15 M-NaCl and 2 mM-phenylmethanesulphonyl fluoride or 0.8 mg of leupeptin/ml. Samples were processed as described in the Materials and methods section, resolved by electrophoresis on 15% (w/v) SDS/polyacrylamide slab gels, and analysed by immunoblotting with anti-[large subunit (L)] (a) or anti-[small subunit (S)] (b) serum. (i) Lanes 2 and 3, control samples; (ii) lanes 4–7, digestion with α -chymotrypsin; (iii) lanes 8–11, digestion with trypsin; (iv) lanes 12–15, digestion with papain. Lanes 1 (a and b) and 16 (b) contain 125 I-labelled low- M_r markers.

Treatment of SMPs with α -chymotrypsin had no discernible effect on the small subunit [Fig. 3(b), (ii), lanes 4–7]. In contrast, both trypsin and papain caused extensive degradation of the 30000- M_r polypeptide [Fig. 3(b), (iii) and (iv), lanes 8–15]. However, no membrane-associated fragment of the small subunit was detected in the trypsin- or papain-treated samples, indicating that the 27000- M_r product of tryptic digestion observed previously was not bound to the inner membrane.

Proteinase K treatment of SMPs also produced a large-subunit fragment pattern similar to that produced by papain (Fig. 4a). Essentially complete digestion of the intact large subunit was achieved during 5 min of incubation with proteinase K; this was accompanied by the appearance of large-subunit fragments of M_r 32000 and 27000 respectively [Fig. 4(a), (ii), lane 4]. Soluble immunologically reactive material corresponding to the residual 11000- M_r region of this polypeptide cleaved from the intact large subunit could be detected at the dye front. During the remainder of a 30 min incubation the ratio of the two fragments remained almost constant [Fig. 4(a), (ii), lanes 5–7]. The 32000- M_r and 27000- M_r species were subsequently shown to remain membrane-

Fig. 4. Immunoblot analysis of proteinase K- or papain and α -chymotrypsin-treated submitochondrial particles with anti-[large subunit (L)] serum

SMPs were incubated for up to 30 min at 30 °C in the presence of proteinase K (a) or papain (b) [4% (w/w) proteinase/particle protein]. In (a), samples were taken at the indicated times, mixed with 0.33 vol. of 2 $\times$ Laemmli sample buffer containing 2 mM-phenylmethanesulphonyl fluoride and boiled for 5 min. Samples were analysed by electrophoresis on a 10% (w/v) SDS/polyacrylamide slab gel followed by immunoblotting with anti-[large subunit (L)] serum. In (b), incubation of SMPs with papain was terminated by the addition of a 5-fold excess of leupeptin (by weight) before further incubation with 4% (w/w) α -chymotrypsin. Samples were then processed as described above. (a) Lane 1, 125 I-labelled low- M_r markers; (i) lanes 2 and 3, control samples; (ii) lanes 4–7, digestion with proteinase K for the indicated times. Equal amounts of protein were loaded in lanes 2–7 inclusive. (b) Lanes 1 and 2, control incubation, 0 and 60 min; lane 3, 30 min digestion with papain showing 32000- M_r and 27000- M_r species; lane 4, as lane 3 plus extra 20 min after papain inactivation; lane 5, as lane 4 plus 10 min incubation with α -chymotrypsin; lane 6, as lane 3 plus 10 min incubation after a second addition of α -chymotrypsin.

associated when proteinase treatment was followed by salt washing (results not shown).

No precursor-product relationship could be observed between the 32000- M_r and 27000- M_r fragments in a series of time courses designed to produce controlled degradation of the large subunit with papain or proteinase K. The separate identity of these two membrane-associated fragments was confirmed in double-digestion experiments (Fig. 4b) in which papain-treated SMPs were incubated subsequently with α -chymotrypsin (or trypsin; results not shown). This led to the rapid disappearance of the 27000- M_r polypeptide (lanes 5 and 6), whereas the 32000- M_r component was essentially unaffected by this treatment. Incomplete recovery of samples was often observed after washing and re-centrifugation of SMPs subjected to double proteinase digestions, indicating that significant disruption of the membrane vesicles can occur under these conditions.

The stability of the principal 32000–34000- M_r fragment of the large subunit (L) may result from its protection by the lipid

Fig. 5. Immunoblot analysis of the effect of non-ionic detergent on the stability of the major proteolytic fragment of the large subunit in bovine heart SMPs

SMPs were incubated at 30 °C for 30 or 60 min in the presence of trypsin or α -chymotrypsin as described in Fig. 1 legend. At this point, Triton X-100 was added to some samples (final concentration of 0.1 %, v/v) and incubations were continued for a further 30 min. All samples were then subjected to SDS/PAGE and immuno replica analysis with anti-[large subunit (L)] serum. Lanes 1 and 2, control incubations minus proteinase for 0 and 60 min respectively; lane 3, blank; lane 4, as lane 2 except that 0.1 % (v/v) Triton X-100 was added at 30 min; lanes 5 and 6, incubations for 60 and 30 min with trypsin; lanes 7 and 8, as lanes 5 and 6 except that 0.1 % (v/v) Triton X-100 was added 30 min before termination of the incubation; lanes 9 and 10, as lanes 5 and 6 except incubated with α -chymotrypsin; lanes 11 and 12, as lanes 8 and 9 except incubated with α -chymotrypsin.

bilayer or alternatively relate to its organization into a compact domain structure that is inherently resistant to further proteolysis. To test this latter possibility, the proteolytic sensitivity of the 32000–34000- M_r fragment was tested after solubilization of the inner membrane in 0.1 % (v/v) Triton X-100. As illustrated in Fig. 5, the major tryptic (lanes 5 and 6) and chymotryptic products (lanes 9 and 10) of the large subunit were both degraded rapidly to smaller non-immunogenic fragments (lanes 7 and 8 and lanes 11 and 12 respectively) after solubilization of the submitochondrial particles in non-ionic detergent. Thus a major segment of the large subunit is apparently protected from proteolytic attack by virtue of the intactness of the mitochondrial inner membrane.

DISCUSSION

In this investigation we have confirmed that the large and small subunits of SDH are protected from proteolysis in intact mitochondria, in agreement with previous ferrocyanide interaction, antibody binding and chemical labelling studies (Klingenberg & Buchholz, 1970; Merli *et al.*, 1979). Both polypeptides are sensitive to digestion by a variety of proteinases in 'inside-out' SMPs. The absence of a small-subunit fragment in proteinase-treated salt-washed SMPs suggests that most of this subunit is released from the membrane under these conditions.

An arrangement of the above type is further suggested by the following lines of evidence. Firstly, we were able to detect a soluble 27000- M_r species corresponding to the bulk of the intact small subunit among total tryptic digestion products. Secondly, a hydropathy plot of the amino acid sequences of the small subunit (252 residues) (Yao *et al.*, 1986) indicates that it is generally very hydrophilic. A small hydrophobic region containing several cysteine residues is located between leucine-155 and proline-168 and may provide a non-aqueous pocket for one

of the iron-sulphur centres. Interestingly the C-terminus is hydrophilic in character while the N-terminus consists of a stretch of nine uncharged residues. In light of this evidence, it is possible that the small subunit is associated with the inner membrane via a short N-terminal membrane anchor. A number of potential tryptic-cleavage sites are located in the appropriate region near to the N-terminus of this polypeptide.

Each of the four proteinases used in this study produced a stable large-subunit fragment of M_r value 32000–34000. In each case this species was shown to be tightly bound to the inner membrane, since it was not removed by repeated salt washes. The generation of a common fragment, even by low-specificity proteinases, suggests the presence of an extremely stable membrane-associated domain in the large subunit. As this fragment is rendered susceptible to proteolysis by solubilization of the inner membrane in non-ionic detergents, it is clear that its stability is the result of its close association with an intact lipid bilayer. It is possible that this segment of the large subunit is largely embedded within the lipid bilayer, although a strong interaction with the small hydrophobic anchoring proteins of the native succinate:ubiquinone reductase complex at the membrane surface may be responsible for shielding it from exogenous proteinases. In this regard, there is no evidence for the presence of extensive hydrophobic stretches in the equivalent bacterial subunit (Wood *et al.*, 1984; Phillips *et al.*, 1987), which is similar in size to its mammalian counterpart.

The reproducible appearance of an additional membrane-associated fragment of the large subunit (27000 M_r) in samples of papain- and proteinase K-treated SMPs has two possible explanations. The smaller fragment may be derived from the larger by removal of an additional 5000- M_r fragment from the 32000- M_r membrane-associated domain. Alternatively, it may represent a separate membrane-associated region of the large subunit. In this case, digestion with trypsin or α -chymotrypsin generates additional large-subunit fragments of low M_r value (Fig. 1b) that are released as soluble polypeptides and are only weakly immunogenic (α -chymotrypsin) or non-immunogenic (trypsin). We favour the latter arrangement for two main reasons. Firstly, since the appearance of the 27000- M_r species in proteinase K-treated SMPs is rapid, subsequent conversion of the 32000- M_r species into its 27000- M_r form should also be rapid if the first explanation is correct: such an effect was not observed. A similar precursor-product relationship was not evident either during incubation of SMPs with papain. Secondly, double-digestion experiments, in which papain treatment of SMPs was followed by treatment with trypsin or α -chymotrypsin, suggest that the 27000- M_r fragment is preferentially degraded by these proteinases. This effect indicates that the 27000- M_r domain, although stable to proteinase K and papain, is held in the membrane by a relatively small (non-immunogenic) anchoring region (M_r 10000), since it is susceptible to externally added proteinases of appropriate specificity.

Girdlestone *et al.* (1981) have reported on the intramembrane organization of complex II after incorporation into synthetic phospholipid vesicles in the presence of photoreactive radio-labelled (arylazido) phospholipids. They concluded that the small subunit was partially inserted into the lipid bilayer whereas the large subunit was shielded from or raised above the surface of the membrane. Therefore the proteinase-resistant regions of the large subunit may be bound to the membrane via interactions with the small membrane-anchoring proteins of complex II (Xu *et al.*, 1987) as suggested previously, rather than through direct contact of the large subunit with the hydrophobic interior of the bilayer. However, comparison of data, obtained with photoaffinity probes on complex II incorporated into artificial lipid vesicles, is difficult to reconcile with this immuno-

logical study conducted on intact membranes, which is likely to be of greater physiological relevance. Unfortunately, the amino acid sequence of the large subunit of mammalian SDH is not available as yet to permit our topographical analysis to be compared with its predicted membrane association from hydropathy profiles.

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