Studies on Beef Heart Ubiquinol-Cytochrome c Reductase. Topological Studies on the Core Proteins Using Proteolytic Digestion and Immunoreplication

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Abstract

The topology of beef heart Complex III has been studied by tryptic and chymotryptic digestion of isolated Complex III, Mg^{2+}-ATP submitochondrial particles, and mitoplasts. Degradation products were detected by the immuno-replica technique using specific antibodies against core protein 1 (50 K) and core protein 2 (47 K). It can be shown that both peptides are digested from the matrix side of the inner membrane. However, no evidence was found that these peptides were digested by trypsin or chymotrypsin from the cytoplasmic side. It is concluded that the beef heart core proteins are membrane-bound peptides containing tryptic and chymotryptic digestion sites only on the matrix surface of the inner membrane. The data also suggest that beef heart core protein 2 contains multiple domains which are inserted into the membrane from the matrix surface. Proteolytic treatment of submitochondrial particles under conditions which digested at least 50% of the core proteins from the matrix surface did not, however, influence NADH oxidation rates or the respiratory control ratios.

Key Words: Ubiquinol-cytochrome c reductase; core proteins; topology; proteolysis; immunoreplica; Complex III; respiratory chain (bovine heart mitochondria).

Introduction

Quinol-cytochrome c reductase (Complex III) is composed of eight peptides (Nelson, 1981; Rieske, 1976; Von Jagow and Sebald, 1980). Two of these, core protein 1 (50 K) and core protein 2 (47 K), together make up about 50% of the total Complex III protein (Silman *et al.,* 1967; Mendel-Hartvig and

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Nelson, 1981). Although the roles which these peptides play in the function of Complex III are not known (see Nelson, 1981, for a review), it is hoped that some clues might be provided by their topological arrangements in the membrane.

The *Neurospora* core proteins have been isolated as soluble peptides (Weiss *et al.,* 1979), which appear to be loosely attached to Complex III on the matrix side of the inner membrane. The situation in beef heart mitochondria is, however, less clear. Core protein 2 has been detected only on the matrix surface (Mendel-Hartvig and Nelson, 1978; Low *et al.,* 1980) or on both the matrix and cytoplasmic surfaces (Bell *et al.,* 1979) of the inner membrane using surface-labeling reagents. Core protein 1 has been assigned either to the matrix (Mendel-Hartvig and Nelson, 1978) or to the cytoplasmic (Bell *et al.,* 1979) surface.

The above combined results might be taken to indicate that the core proteins are integrated into the membrane, and perhaps even transmembranous in nature. The solubility properties of beef heart core proteins support this conclusion to some extent (Rieske, 1976; Silman *et al.,* 1967; Gellerfors and Nelson, 1977). However, assuming that core proteins have similar functions in *Neurospora* and beef heart Complex III, it is striking that the topological organization of these peptides might be so different. In view of this, we have continued our investigations on the core proteins in beef heart (Mendel-Hartvig and Nelson, 1978, 1981; Gellerfors *et al.* 1976). In the present study we report the results of experiments in which proteolysis of the core proteins was investigated on the two surfaces of the inner membrane using specific antibodies as probes and the immunoreplica method for detection of the degradation products.

Methods and Materials

Beef heart mitochondria, Mg^{2+}-ATP submitochondrial particles (Lee and Ernster, 1967), and Complex III (Engel *et al.,* 1980) were prepared as described. Core proteins were purified from dodecyl sulfate polyacrylamide slab gels, and antibodies were raised as reported previously (Mendel-Hartvig and Nelson, 1981). Specificity of the antisera was controlled by immunoreplication. Mitoplasts were prepared as described in (Schnaitman *et al.,* 1967) in a medium described by Schnaitman and Greenawalt (1968). Care was taken to remove the light fluffy layer which formed on top of the mitoplast pellet after the second centrifugation step. This layer contains damaged inner membranes (Schnaitman *et al.,* 1967).

In order to optimize proteolysis, treatment of the different fractions was carried out with varying concentrations of chymotrypsin or trypsin and with

different incubation times. These are described in the legends to the figures and in the text. In all cases proteolytic treatment was conducted at 0°C in a buffer containing 0.25 M sucrose and 10 mM Tris-Cl, pH 7.0. The reactions were stopped by adding sample buffer for electrophoresis (2% SDS, 5% mercaptoethanol, and 10 mM Tris-Cl, pH 6.8) and boiling for 1 min.

Electrophoresis was carried out on slab gels in the presence of sodium dodecyl sulfate using the buffer of Laemmli (1970). Detection of core proteins and their proteolytic digestion products was done using the immunoreplica technique as before (Mendel-Hartvig and Nelson, 1981). The SDS-polyacrylamide gels were overlayed with an agarose gel containing specific antibodies or with diazobenzyloxymethyl (DBM)–activated paper (Renart et al., 1979) to which antibodies were later bound. Diazobenzyloxymethyl-activated paper was prepared by the method of Alwine et al. (1979). The overlays were decorated with ^{125}I protein A and then exposed to X-ray film.

Monoamine oxidase was measured using benzamine as the substrate (Schnaitman et al., 1967). The cytochrome cc_1 content of mitoplasts was measured spectrally at 550 nm minus 540 nm assuming a millimolar extinction coefficient of 19.1. Protein was determined with the biuret reagent (Gornall et al., 1949) or by the method of Peterson (1977).

Results

In order to treat the cytoplasmic surface of the inner membrane with proteolytic enzymes, it was necessary to remove the outer membrane without damaging the inner membrane. This was achieved by digitonin treatment (Fig. 1). Conditions were established in which the inner membrane retained cytochrome cc_1 and remained impermeable to NADH. The residual oxidation
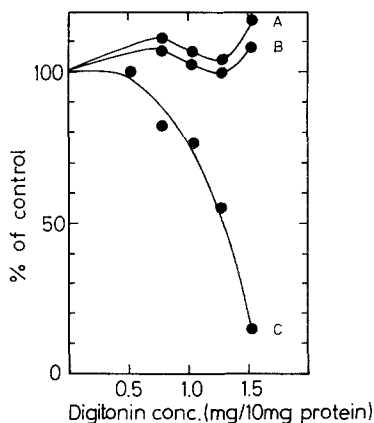

Fig. 1. Preparation of mitoplasts from beef heart mitochondria. Beef heart mitochondria were treated with varying concentrations of digitonin as in Schnaitman et al. (1967, 1968). Treated mitochondria were pelleted by centrifugation and the following enzyme measurements were made. The integrity of the inner membrane was monitored by: (A) retention of cytochrome cc_1, and (B) oxidation of external NADH. Removal of the outer membrane was monitored by the retention of monoamine oxidase (C). The 100% values were: NADH oxidation (9.6 nmol/min/mg protein), monoamine oxidase (2.1 nmol/min/mg protein), and cytochrome cc_1 (0.42 nmol/mg mitoplast protein).

of NADH by digitonin particles is about 2–3% of that normally found in submitochondrial particles. Thus, the concentration of digitonin chosen for further experiments (0.15 mg/mg protein) removes nearly all of the outer membrane without disturbing the permeability of the inner membrane.

Figure 2 shows the effects of chymotrypsin on isolated core protein 2 (47 K), isolated Complex III, submitochondrial particles, and mitoplasts prepared as above. The digestion products were detected by the immunoreplica technique with core protein 2 specific antiserum. Several digestion products are observed in chymotrypsin-treated submitochondrial particles (Fig. 2, tracks 6–8). Although the same bands are also weakly labeled in mitoplasts preparations (Fig. 2, tracks 9–11), this is due to the presence of small amounts of disintegrated mitoplasts and the subsequent exposure of the matrix surface of the inner membrane to chymotrypsin. This conclusion is supported by the fact that identical results are obtained even in the absence of digitonin (Fig. 2, track 11), indicating that removal of the outer membrane barrier was not necessary for chymotrypsin digestion to occur. Furthermore, it should be mentioned that no additional effect of chymotrypsin was observed in mitoplasts in spite of the more drastic digestion conditions used. It can, thus, be concluded that core protein 2 is digested by chymotrypsin on the matrix surface but not on the cytoplasmic surface of the inner membrane. A similar conclusion is drawn from the same type of experiment in which trypsin was used in place of chymotrypsin (Fig. 3). In this case, two digestion products were resolved in trypsin-treated submitochondrial particles (tracks 5–8), whereas none were observed in treated mitoplasts (tracks 9–11).

Figure 2 (tracks 3–5) also shows that chymotrypsin treatment of isolated Complex III leads to the formation of at least six digestion products of core
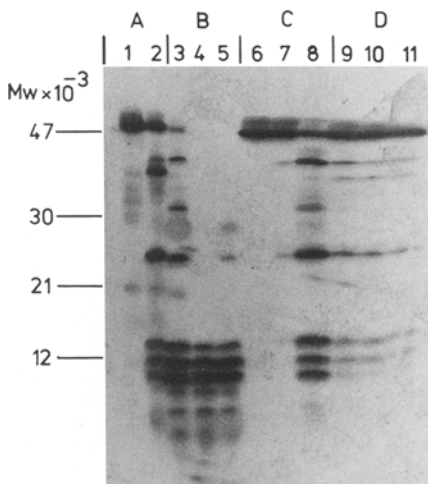

Fig. 2. Chymotryptic digestion of core protein 2. Purified core protein 2, Complex III, Mg^{2+}-ATP submitochondrial particles, and mitoplasts were incubated with chymotrypsin prior to electrophoresis. Digestion products were detected by immunoreplication using core protein 2 specific antiserum. The apparent double band in the region of the undigested peptide is due to shrinkage of the agarose overlay during the replication. Autoradiographs are shown. (A) core protein 2 (13 $\mu g/ml$) was incubated in the absence (track 1) or presence (track 2) of chymotrypsin (20 $\mu g/ml$) for 3 hr. (B) Complex III (1.6 mg/ml) was treated with chymotrypsin (80 $\mu g/ml$) for 5, 30, or 180 min (tracks 3–5, respectively). (C) Mg^{2+}-ATP submitochondrial particles (5 mg/ml) were treated with chymotrypsin (0.8, 8, or 80 $\mu g/ml$, tracks 6–8, respectively) for 30 min. (D) mitoplasts (5 mg/ml), prepared with 1.5, 0.75, or 0.00 mg digitonin/10 mg protein (tracks 9–11, respectively), were treated with chymotrypsin (100 $\mu g/ml$) for 30 min.
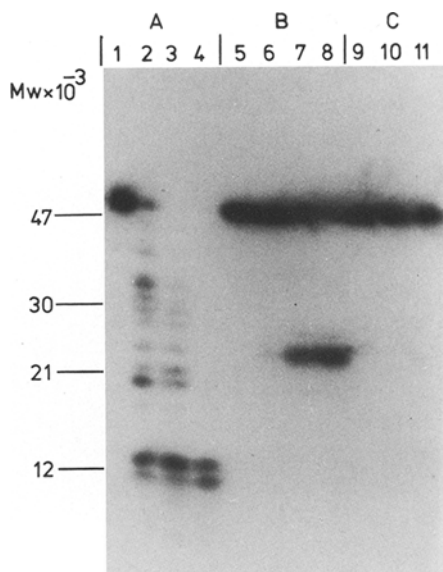
Fig. 3. Tryptic digestion of core protein 2. Complex III, Mg^{2+}-ATP submitochondrial particles, and mitoplasts were treated with trypsin exactly as described in Fig. 2 for chymotrypsin. An autoradiograph of the immunoreplica is shown. (A) Complex III (1.6 mg/ml) was treated with trypsin (80 μg/ml) for 0, 5, 30, or 180 min (tracks 1–4, respectively). (B) Mg^{2+}-ATP submitochondrial particles (5 mg/ml) were treated with 0, 0.8, 8, or 80 μg trypsin/ml (tracks 5–8, respectively) for 30 min. (C) mitoplasts (5 mg/ml) were prepared with 1.5, 0.75, or 0.00 mg digitonin/10 mg protein (tracks 9–11, respectively) and incubated with trypsin (100 μg/ml) for 30 min.

protein 2. These peptides (MW = 12,000 and below) appear to be stable end-products of digestion. The results indicate the presence of a minimum of five proteolytic sites on core protein 2 which are exposed in the isolated complex. In contrast, only three or four of these peptides (a minimum of two to three cutting sites) are present in chymotrypsin-treated submitochondrial particles (Fig. 2, tracks 6–8). This raises the possibility that certain domains of core protein 2 are exposed in the isolated complex but are protected in submitochondrial particles by surrounding proteins or by lipids. This suggestion received additional support from experiments in which Complex III and submitochondrial particles were treated with trypsin (Fig. 3). In the latter experiments, the tryptic digestion pattern obtained with submitochondrial particles (Fig. 3, tracks 5–8) is considerably simpler than that obtained with isolated Complex III (Fig. 3, tracks 1–4).

The topology of core protein 1 (50 K) was also studied using trypsin (Fig. 4) and chymotrypsin (Fig. 5). These studies were slightly complicated by the quality of the antisera, however, since one preparation of antiserum appeared to weakly cross-react with core protein 2 (Fig. 4), and the other, although specific for core protein 1, gave a high, non specific background on the replica plate (Fig. 5).

In spite of this complication, it is clear from Fig. 4 that core protein 1 is not digested by trypsin in either submitochondrial particles (tracks 5–8) or mitoplasts (tracks 9–11), but is extensively degraded in isolated Complex III (tracks 1–4). The minor digestion products observed in submitochondrial particles (tracks 7 and 8) appear to be due to cross-reaction of the antiserum
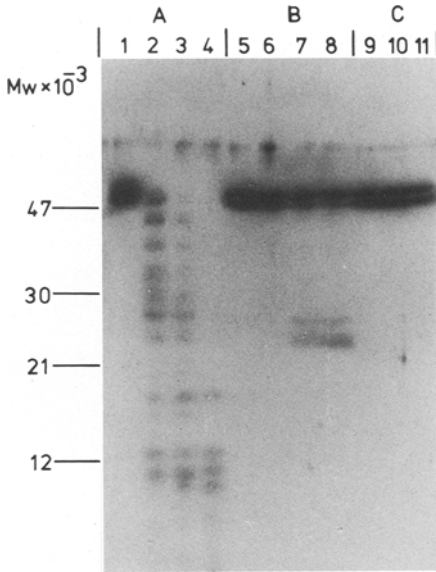
Fig. 4. Tryptic digestion of core protein 1. All conditions were exactly as described in Fig. 3 with the exception that immunoreplication was run using an antiserum against core protein 1. See Fig. 3 for a description of the symbols.

with core protein 2 (cf. Fig. 3, tracks 7 and 8). Two conclusions can be drawn from these experiments: (1) core protein 1 is not digested by trypsin on either surface of the inner membrane, and (2) many of the tryptic sites on core protein 1 which are exposed in isolated Complex III are inaccessible in the intact membrane and are presumably buried.

The effect of chymotrypsin on core protein 1 is shown in Fig. 5. A number of digestion products are weakly detected in chymotrypsin-treated submitochondrial particles (tracks 1–3). This result suggests that core protein 1 is exposed to the matrix surface of the membrane and that the exposed domain probably lacks trypsin-specific sites. The presence or absence of chymotrypsin
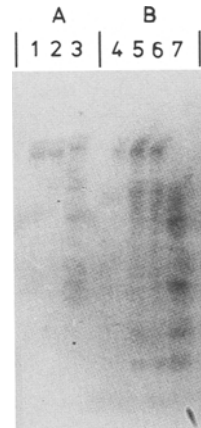

Fig. 5. Chymotryptic digestion of core protein 1. Immunoreplication was run using core protein 1 specific antiserum. (A) Mg^{2+}-ATP submitochondrial particles (5 mg/ml) treated 30 min with chymotrypsin (0.8, 8, and 80 μg, tracks 1–3, respectively), and (B) Complex III (1.6 mg/ml) treated with chymotrypsin (80 μg/ml) for 0, 5, 30, and 180 min (tracks 4–7, respectively).

digestion products in mitoplasts could not be decided due to the weak antibody reaction. However, in agreement with the data obtained with trypsin (Fig. 4), a more complex chymotryptic digestion pattern is obtained with Complex III (Fig. 5B) than with submitochondrial particles (Fig. 5A), indicating that certain domains of core protein 1 are protected in the intact membrane.

The binding properties of core protein 2 to the inner membrane have also been investigated with the aid of the immunoreplica method. This peptide did not appear in the supernatant after washing submitochondrial particles with 1 M NaCl (Fig. 6), but remained with the intact membrane upon centrifugation. Similar results were obtained by washing with varying concentrations of
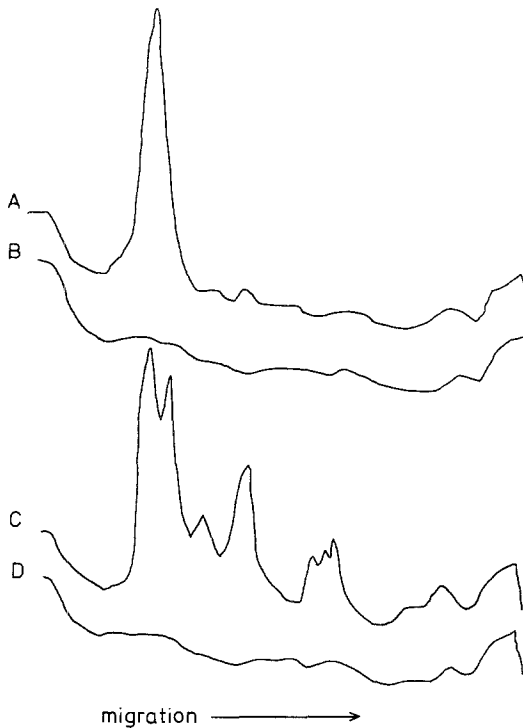

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Fig. 6. Lack of effect of high ionic strength on the removal of core protein 2 and its chymotryptic digestion products from the inner membrane. Mg^{2+}-ATP particles (5 mg/ml) were incubated for 30 min in the absence (A, B) or presence (C, D) of chymotrypsin (80 μg/ml). The membranes were washed with 1 M NaCl and pelleted by centrifugation at 105,000 g for 40 min. The pellets and the dialysed supernatants were electrophoresed, and immunoreplication was run with antisera against core protein 2 as described in Methods. (A, C) Mg^{2+}-ATP particles after washing with NaCl; (B, D) supernatants from the NaCl wash. Densitometric tracings of the film are shown.

Table I. Partial Digestion of Submitochondrial Particles with Trypsin or
Chymotrypsin Does Not Influence NADH Oxidation[a]

Enzyme	Incubation time (min)	NADH oxidase (nmol/min/mg)		RC ratio
		− FCCP	+ FCCP	
Chymotrypsin	0	232	665	2.9
	5	238	627	2.6
	20	238	640	2.7
	40	238	652	2.7
	50	238	652	2.7
Trypsin	0	238	677	2.8
	5	201	602	3.0
	20	250	602	2.4
	40	250	652	2.6
	100	226	577	2.6

[a]Mg^{2+}-ATP particles (5 mg protein/ml) were incubated with either chymotrypsin or trypsin (240 μg/ml). At the appropriate time, samples were removed and NADH oxidase measured at 340 nm in buffer containing 0.167 M sucrose, 50 mM Tris-Cl, pH 7.5 (2 μg oligomycin), and, when present, 2 μM FCCP.

NaCl (0–1 M) or with 0.3 M phosphate buffer in the presence or absence of 50 mM dithiothreitol. Because of the extremely high sensitivity of the immuno-replica technique, it would appear that essentially no core proteins are released by washing with a large range of salt concentrations. Furthermore, the data in Fig. 6 show that the chymotrypsin digestion products of core protein 2 are not removed from submitochondrial particles by washing with high salt. This result suggests that core protein 2 might have multiple sites of attachment to the inner membrane.

Table I shows that the partial digestion of core proteins 1 and 2 with trypsin or chymotrypsin does not alter the ability of submitochondrial particles to oxidize NADH or to produce a protonmotive force which is sufficient to control electron transport. These experiments were done under conditions where the core proteins were judged to be at least 50% digested.

Discussion

A partial picture of the topological organization of core proteins in Complex III and in the inner membrane is starting to emerge. Core protein 1 and core protein 2 are slightly acidic (pK_1 = 6.3) and basic (pK_1 = 8.5) peptides, respectively (Mendel-Hartvig and Nelson, 1981). They are in close proximity within the complex as indicated by cross-linking studies with divalent reagents spanning 6 Å (Smith et al., 1978). Each mole of Complex III contains 1 mole of core protein 1 and 2 moles of core protein 2

(Mendel-Hartvig and Nelson, 1981). Both peptides extend from the matrix surface of the membrane. The latter was shown in the present study by proteolytic digestion with both chymotrypsin and trypsin, and it supports our earlier findings with radiolabeled surface probes (Mendel-Hartvig and Nelson, 1978). Similar conclusions were drawn from structural studies on *Neurospora* Complex III (Weiss *et al.*, 1979). The present study also suggests that both core proteins are inserted into the membrane, since they are more protected from proteases in submitochondrial particles than they are in isolated Complex III. Furthermore, core protein 2 is anchored in the membrane at multiple domains, as indicated by the finding that neither core protein 2 nor its proteolytic digestion products could be removed from submitochondrial particles by salt washes. In this respect the beef heart enzyme differs from the *Neurospora* enzyme (Weiss *et al.*, 1979) in which the core proteins are considered to be water-soluble peptides which bind to the surface of Complex III.

The function of the core proteins is still not known. Binding of specific antibodies (Mendel-Hartvig and Nelson, 1981) or partial proteolysis of the core proteins from the matrix surface do not influence the rate of NADH oxidation by submitochondrial particles or the formation of a protonmotive force which is of sufficient magnitude to inhibit electron transport (Nichols and Bernson, 1977). Thus, it is not clear if the core proteins are required for these functions or if the membrane-bound domains of the peptides can function normally even when the matrix domains are modified by antibody binding or by partial proteolysis.

Acknowledgment

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