Biochimica et Biophysica Acta, 636 (1981) 91–97
Elsevier/North-Holland Biomedical Press

BBA 48065

STUDIES ON BEEF HEART UBIQUINOL-CYTOCHROME *c* REDUCTASE

QUANTIFICATION AND DISTRIBUTION OF THE CORE PROTEINS AS DETERMINED BY RADIOIMMUNOASSAY

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(Received October 27th, 1980)
(Revised manuscript received February 16th, 1981)

Key words: Ubiquinol-cytochrome c reductase; Core protein; Radioimmunoassay; Complex III; Respiratory chain; (Bovine heart mitochondria)

Core proteins I (M_r 50 000) and II (M_r 47 000) were isolated from beef heart ubiquinol-cytochrome *c* reductase, and radioimmunoassays were developed for both. Immunoreplica experiments show that antisera against each protein react with a single peptide in both isolated Complex III and in mitochondria. Thus, core proteins are not aggregated forms of smaller peptides as suggested for the yeast protein (Jeffrey, A., Power, S. and Palmer, G., Biochem. Biophys. Res. Commun. (1979) 86, 271–277). Core proteins were quantitated in Complex III and in mitochondria using radioimmunoassay. Approx. 2 mol core protein II per mol core protein I were found. A molar ratio of 1 : 2 : 2 : 1 is suggested for core protein I : core protein II : cytochrome *b* : cytochrome c_1. Radioimmunoassay shows that the antibodies react as extensively with Complex III-bound core protein as with the isolated core proteins. In spite of this, the antibodies do not inhibit electron transport in submitochondrial particles or isolated Complex III, and they have no oligomycin- or uncoupler-like effects on submitochondrial particles oxidizing NADH. The combined results from radioimmunoassay and immunoreplica experiments strongly suggest, however, that core proteins are specifically associated with Complex III in the mitochondria, implying a specific role there.

Complex III from the mitochondrial respiratory chain contains 7–8 peptides [1–2]. Two of these, the so-called core proteins [3], make up 40–50% of the total protein of the Complex [3]. Since core proteins contain no prosthetic groups, it has not been possible to establish their functions. In fact, it was recently concluded that core proteins in yeast Complex III are electrophoretic artifacts [4], and not bonafide, functional members of the ubiquinol-cytochrome *c* reductase.

A major problem in studying core proteins has been the lack of specific probes. To learn more about their structures and locations in beef heart mitochondria, we have turned to immunological methods. In the present paper we demonstrate, with the aid of radioimmunoassay and immunoreplica techniques, that core proteins are present in stoichiometric amounts with the respiratory chain proteins, that they are present in mitochondria as single peptides, not as aggregates, and that they are probably associated only with Complex III. These findings strongly suggest that core proteins have specific associations and, therefore, probably specific functions with the respiratory chain.

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Abbreviation: SDS, sodium dodecyl sulfate.

Methods

Complex III was isolated from beef heart by the method of Rieske et al. [5].

Isolation of core proteins. Complex III (1–1.5 mg proteins) was resolved on 2 mm thick, 11% polyacrylamide gels containing sodium dodecyl sulfate (SDS). The buffer of Laemmli [6] was used. Electrophoresis was carried out in the cold for 20 h at 20 mA/gel. the gels were lightly stained (1% Coomassie blue G-250 in water) and core proteins were sliced from the gels. The slices were diced and extracted four times at 50°C for 2 h with 0.1% SDS. The eluted proteins were concentrated by freeze-drying, and SDS was decreased by dialysis. The protein concentration was determined by the method of Petterson [7]. The purity of the preparations was checked by electrophoresis or isoelectric focusing.

Immunological methods. Antibodies were raised in rabbits by injecting 200 μg protein directly into the popliteal lymph node. A booster of 50 μg was given 1 month after the primary injection and serum was collected 2 weeks after each booster. Complement was inactivated by incubation for 10 min at 56°C. Antibody titers were followed by rocket electrophoresis [8].

Core proteins and protein A were radioiodinated as described [9] using Iodogen as the catalyst. Conditions were adjusted to give a specific activity of $(2–3) \cdot 10^9$ cpm/mg core protein I and II, or $1.9 \cdot 10^6$ cpm/mg protein A. The iodinated proteins were separated from free ^{125}I on Sephadex G-50, and the purity of the iodinated preparations was checked by electrophoresis and autoradiography.

Radioimmunoassays were developed in which iodinated core proteins were incubated with increasing concentrations of unlabeled antigen in 0.3 ml buffer containing 50 mM potassium phosphate, pH 7.0/150 mM NaCl/0.5 mM EDTA/0.5% Triton X-100/0.5 mg azide/ml/0.25% bovine serum albumin and antibodies. The ratio of antibody to iodinated core protein was adjusted to give 50% precipitation of the latter. Incubations were carried out for 18–20 h at 4°C and the antigen-antibody complex was absorbed with fixed *Staphlococcus aureus* membranes. The precipitates were washed three times with 3 ml buffer and the radioactivity was counted. Preimmune sera was tested in each experiment. Nonspecific binding was negligible (<45%).

Immunoreplica experiments were conducted as described previously [10]. Core proteins, Complex III, and mitochondria were subjected to electrophoresis in the presence of SDS. The gels were incubated overnight at 4°C with a 1% agarose overlay containing antibodies to either core protein I or core protein II. The agarose gel was removed, washed and decorated with ^{125}I-labeled protein A. The decorated gels were washed, dried and exposed to Kodirex film. The film was scanned with a densitometer.

Electrophoresis. SDS-polyacrylamide gel electrophoresis was run on slab gels using the buffer of Laemmli [6]. One-dimensional isoelectric focusing was carried out in flat bed gels containing 5% acrylamide as support [11]. The buffer contained 2% Triton X-100, 6 M urea and a mixture of Ampholines (2.7%, pH 3.5–10; 0.2%, pH 4–6; 0.2%, pH 5–7; and 0.4%, pH 1–11). Two-dimensional electrophoresis was carried out using the methods of O'Farrell [12].

Quantitation of core proteins by dye binding. Increasing concentrations of core protein I, core protein II and Complex III were run on the same slab gels. The gels were stained with Coomassie blue G-250 and the individual bands removed along with pieces of the same volume which contained no protein. The latter served as blanks. Coomassie blue was eluted by incubating the gel slices in 2% SDS and 10 mM Tris-HCl, pH 6.8 at 50°C. Incubation continued until no further extraction occurred (approx. 3 h). Coomassie blue was measured in the extract at 620 nm. The isolated core proteins served as standards. Absorbancy at 620 nm was linear to 10 μg of pure core protein (Fig. 2).

Results

No cross-contamination of purified core proteins I (M_r 50000) and II (M_r 47000) was detected by Coomassie blue staining of SDS gels (Fig. 1). No additional heterogeneity was observed upon electrophoresis of core proteins or holo-Complex III in the two-dimensional system of O'Farrell [12]. The pK_I values calculated from seven such experiments were 6.3 ± 0.1 and 8.5 ± 0.3 for core protein I and II, respectively. One-dimensional isoelectric focusing in 5% acrylamide gels containing Triton X-100 and urea revealed a substantial microheterogeneity of both core proteins (6–8 bands) which is eliminated during separa-

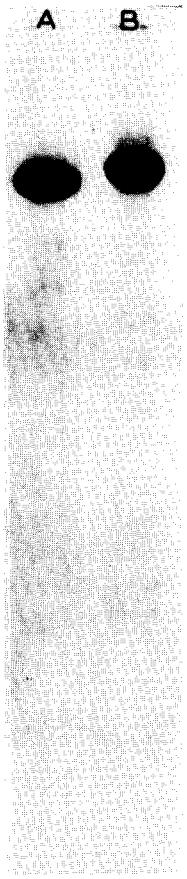

Fig. 1. Electrophoresis of purified core proteins. Core protein II (A) and core protein I (B) were isolated from beef heart Complex III as described in Methods. Electrophoresis was conducted on 11% polyacrylamide gels in the presence of SDS. Each track was loaded with 30 μg protein.

tion in the second dimension of the O'Farrell method. The significance of this microheterogeneity is not clear, however artifacts have not been excluded.

Precipitating antibodies against core proteins I and II do not cross-react (Fig. 3). Furthermore, the antibodies react with single peptides in both intact beef heart mitochondria and in isolated Complex III (Fig. 3). An estimate of the core protein content of mitochondria and isolated Complex III can be obtained from the areas under the curves in Fig. 2, plus the amounts of proteins used in each experiment. These calculations indicate that core proteins are enriched 15–20-fold during preparation of Complex III from mitochondria, which is in line with the extent of

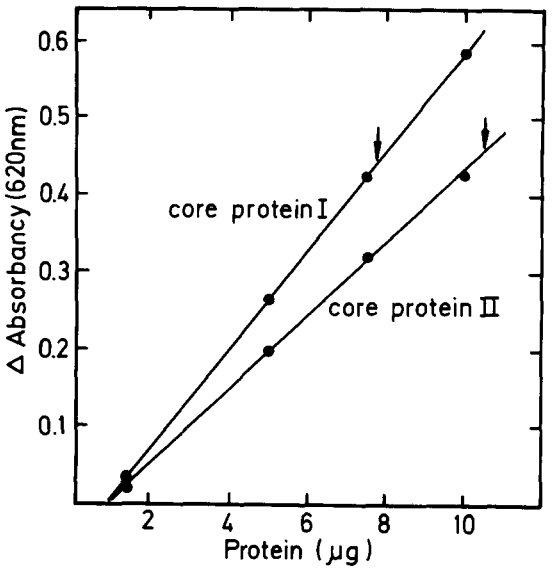

Fig. 2. Quantitation of core proteins by dye binding. Core protein I and II (1.3–10 μg protein) were resolved on slab gels and stained. Coomassie blue was extracted and measured at 620 nm as described in Methods. Complex III (40 μg protein) was run on the same gel. Coomassie blue absorbancy of the two core proteins in Complex III are marked by the arrows.

purification usually expected for this complex [13]. Thus, most of the mitochondrial core protein can be accounted for by that in Complex III.

Radioimmunoassays were developed for core proteins I and II (Fig. 4). Competition curves are shown in which binding of iodinated core protein I (Fig. 4A) or core protein II (Fig. 4B) to their specific antibodies was inhibited by unlabeled antigen. The unlabeled antigens used in this experiment were core proteins, isolated Complex III and mitochondria. Several salient features arise from these curves. The first is that binding of iodinated core proteins is completely inhibited by the unlabeled antigens present in SDS-denatured Complex III, native Complex III, and mitochondria. Secondly, the curves are essentially parallel, indicating that the affinity constants for the antigenic sites do not differ significantly in purified core proteins and in core proteins associated with isolated Complex III or mitochondria. The most reasonable explanation for this complete immunological identity of the different preparations of antigens is that the SDS-treated core proteins resume a more native state when diluted in the Triton X-100-con-

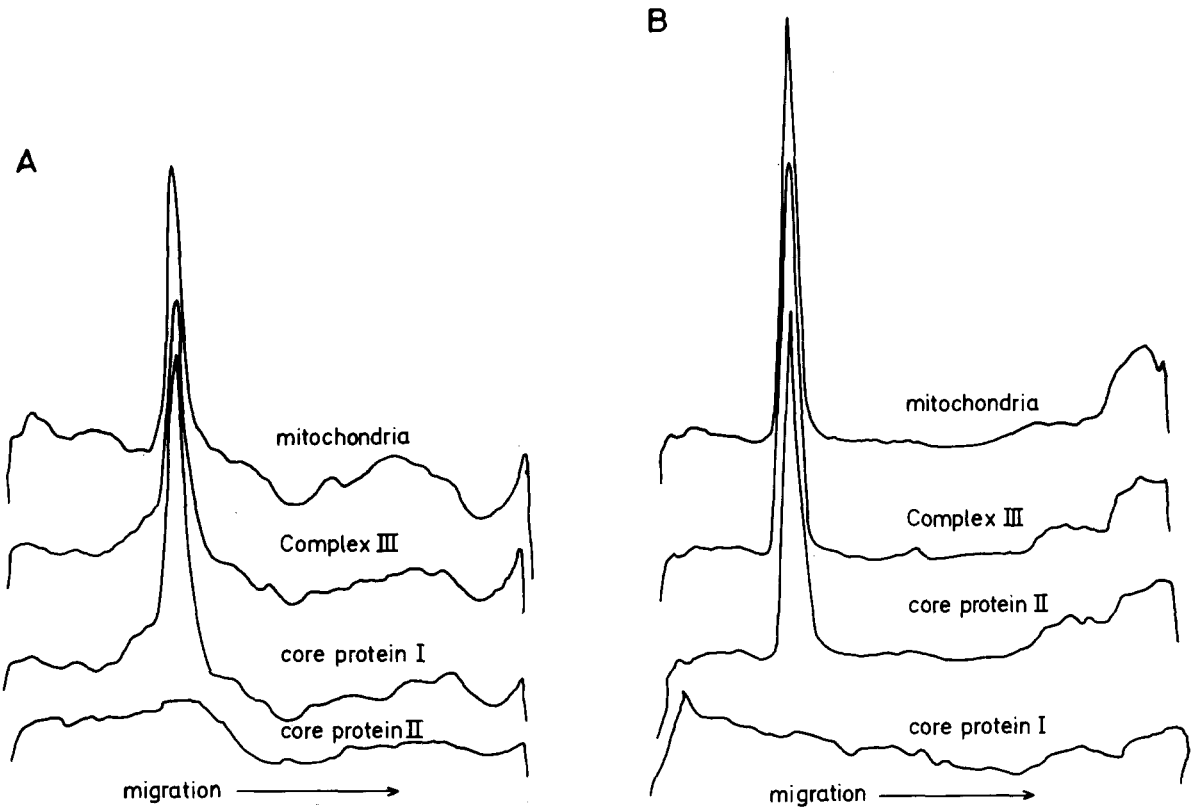

Fig. 3. Detection of core proteins in Complex III and mitochondria by immunoreplica. The SDS-polyacrylamide gels contained 0.5 μg core protein I and core protein II, 2.5 μg Complex III and 25 μg beef heart mitochondria. The overlay contained antiserum against: (A) core protein I and (B) core protein II. The overlay was decorated with ^{125}I-labeled protein A as described in Methods. Autoradiographs were scanned with a densiometer.

taining assay buffer, or in the adjuvant prior to injection. This is supported by the finding that antisera to holo-Complex III cross-react extensively with core proteins isolated in the presence of SDS (not shown). The trivial explanation that core proteins are either denatured and/or dissociated from the complex during incubation can be excluded on the basis of the following unpublished findings: (1) specific antibodies to core proteins I and II precipitate radioiodinated Complex III as an intact unit. (2) core proteins and their proteolytic digestion products are not removed by washing Complex III with high salt, indicating tight binding, and (3) effects of SDS on radioimmunoassays have been tested and found to have no influence below 0.25%.

The above characteristics make it possible to use these antisera for radioimmunoassay of core proteins in both mitochondria and isolated Complex III. Quantitative data calculated from competition curves shown in Fig. 4 are summarized in Table I. Core protein I constitutes about 0.6% of the total mitochondrial protein and 18% of the total Complex III protein. Core protein II comprises 1.3% of the mitochondrial protein and 26% of Complex III protein. In agreement with earlier studies [3], core proteins comprise 45–50% of the total Complex III protein.

Molar concentrations of core proteins were calculated using the data from both immunoassay and the apparent molecular weights determined in SDS-polyacrylamide gel electrophoresis [12] (Table I). Core protein content of Complex III determined by dye binding is also shown. In mitochondria there are approx. 2 mol core protein II for each mol of core protein I. In Complex III the ratio of core II : core I

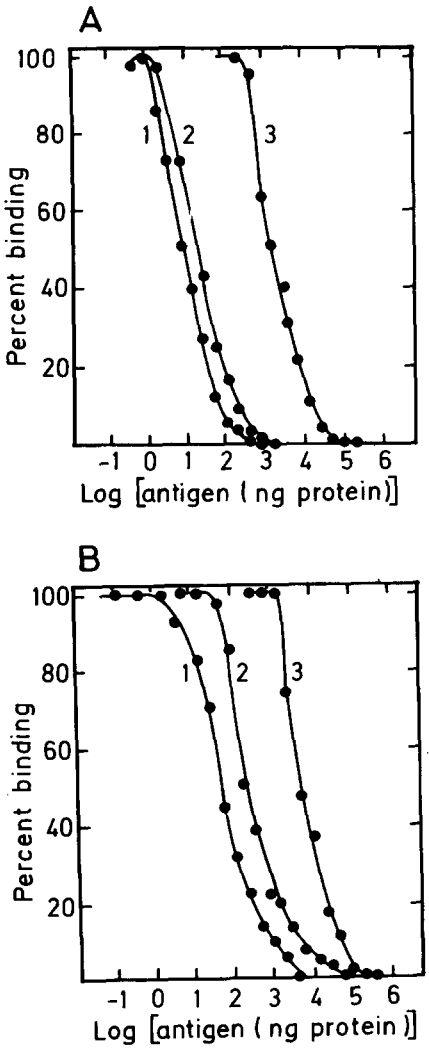

Fig. 4. Competitive radioimmunoassay of core proteins. (A) The assay contained ^{125}I-labeled core protein I (5000 cpm) and 50 μl of 1000-fold diluted antiserum against core protein I. The competing, unlabeled antigens were: core protein I (curve 1), Complex III (curve 2) and mitochondria (curve 3). (B) The assay contained ^{125}I-labeled core protein II (9000 cpm) and 50 μl of 250-fold diluted antiserum to core protein II. The competing, unlabeled antigens were: core protein II (curve 1), Complex III (curve 2) and mitochondria (curve 3).

is also greater than 1 and closer to 2. The combined data for mitochondria and isolated Complex III suggests a molar ratio of 1 : 2 : 2 : 1 for core protein I : core protein II : cytochrome b : cytochrome c_1, respectively.

This result, along with immunoreplica experiments

TABLE I

RADIOIMMUNOASSAY OF CORE PROTEINS IN BEEF HEART MITOCHONDRIA AND COMPLEX III

Cytochrome b and cytochrome c_1 concentrations are expressed on a heme basis. cyt., cytochrome.

Preparation	(nmol/mg protein)			
	core I	core II	cyt. b	cyt. c_1
Mitochondria	0.12	0.29	0.30	–
Complex III	3.6	5.6	7.4	4.0
	(3.6) *	(5.9) *	7.4	4.0

* Determined by Coomassie blue binding.

(Fig. 3), strongly suggest that most of the mitochondrial core proteins are associated with Complex III.

We have attempted to use specific antibodies as probes of core protein function. This is justified in view of the extensive reaction of the antibodies with core protein in both Complex III and mitochondria (Fig. 4). The results show, however, that, in spite of this extensive cross-reaction, neither NADH oxidation by submitochondrial particles or duroquinol-cytochrome c reductase activity of Complex III are inhibited. Furthermore, we have tested the effects of antibodies on respiratory control in submitochondrial particles, but could not demonstrate either an oligomycin- or an uncoupler-like effect on NADH oxidation. This suggests that core proteins probably do not form proton conducting channels which can be blocked in an open or closed position by specific antibodies.

Discussion

Stoichiometry of the core proteins has been determined in beef heart mitochondria and Complex III. The molar ratio of core protein I : core protein II is 1 : 1.5 in isolated Complex III and 1 : 2.4 in mitochondria. Thus, it seems reasonable that there are 2 mol core protein II/mol core protein I in Complex III, and probably also in mitochondria. However, a maximum value of 3 might apply to the mitochondria. These findings suggest a molar ratio of 1 : 2 : 2 : 1 for cytochrome c_1 : cytochrome b : core protein II : core protein I.

This conclusion supports the earlier suggestion of

Silman et al. [3] that each Complex III contains three and possibly four core proteins. Similar values have also been reported by Weiss and Juchs [14] for *Neurospora* Complex III. A 1 : 1 stoichiometry reported earlier for beef heart core proteins [20] is probably due to overlap in the densiometric tracings of the core proteins peaks. This problem was avoided in the present study by removing the stained peptides and extracting Commassie blue (see Methods and Fig. 2).

The Rieske preparation of Complex III [5] is a monomer of molecular weight 260 000–290 000 [15]. This agrees well with the present values of 278 000 obtained assuming the following stoichiometry: one core protein I (50 000), two core proteins II (47 000), one cytochrome c_1 (30 000 [16]), two *b*-cytochromes (27 000 [17]), one iron sulfur protein (25 000 [18]), and one each of the three small peptides (12 500, 10 000 and 5600 [19,20]). Recently, von Jagow [21] reported a molecular weight of 200 000 for the monomer of beef heart Complex III. This preparation lacks, however, two peptides, including the iron sulfur peptide. It is not known if the extra core protein II reported here is present.

The topological organization of core proteins within the membrane has recently been studied [22–25]. In this respect, the isoelectric point of core protein II ($pK_I = 8.5$) is of interest since it is more characteristic of a soluble or peripheral protein [26] than of an integral protein. *Neurospora* core proteins are apparently soluble peptides which attach to the matrix surface of the inner membrane [22]. Beef heart core protein II is also exposed to the matrix surface [23–25]. However, in contrast to the *Neurospora* protein, beef heart core protein II has been reported to extend through the membrane [24].

Establishing a functional role for core proteins in Complex III has proven difficult since both peptides lack prosthetic groups [3]. In view of this lack of information, one might question if these peptides are bonafide members of Complex III. However, the present findings that core proteins are present in stoichiometric amounts with the cytochromes of Complex III, that they are apparently associated only with Complex III in the mitochondria, and that there are no low molecular weight forms of the peptide, all decrease the likelihood that these peptides are isolation artifacts [4].

The lack of effect of specific antibodies on Complex III is somewhat surprising in view of the extensive reaction of the antibodies with the native complex (Fig. 4). No evidence was found for a role of core proteins in the catalytic activity of either mitochondria or isolated Complex III. Furthermore, the antibodies do not exert either an oligomycin-like or an uncoupler-like effect on respiration. The latter experiments were run to investigate the possible roles of core proteins as a proton channel.

Although the above results clearly do not exclude the involvement of core proteins in the catalytic functions of Complex III, they strongly suggest that a search should be directed toward other possibilities. It has been recently shown, for example, that glucagon stimulates respiration in liver mitochondria, and the effect is localized in the cytochrome b–c_1 region [27,28]. We are currently investigating the possibility that core proteins are regulatory proteins which are chemically modified in the presence of glucagon.

Acknowledgement

The authors are greatly indebted to Dr. Pär Askelöf, Statens Bakteriologiska Laboratorium, for preparation of antisera, and to Barbro Persson for technical assistance. This work was supported by grants from the Swedish Cancer Society and The Swedish Natural Science Research Council.

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