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INTERACTION OF ANTI-IRON-SULFUR PROTEIN AND ANTI-UBIQUINONE BINDING PROTEIN ANTIBODIES WITH COMPLEX III OF BEEF HEART MITOCHONDRIA

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Summary: Ubiquinol-cytochrome c reductase activity of Complex III was substantially inhibited by anti-iron-sulfur protein antibody, whereas it was not affected by anti-ubiquinone binding protein antibody. Enzyme-linked immunosorbent assay indicated that anti-ubiquinone binding protein antibody do not bind to the complex, but that it binds to Complex III of which iron-sulfur protein and phospholipids have been depleted. These results indicate that some of the antigenic sites of the iron-sulfur protein are located on the surface of Complex III, while the antigenic sites of the ubiquinone binding protein are inaccessible to antibody owing to the interaction with iron-sulfur protein and/or phospholipids in the complex.

Complex III (cytochrome bc_1 complex) of beef heart mitochondria, which catalyzes electron transfer from ubiquinol to cytochrome c in the mitochondrial electron transport system (1), consists of ten different polypeptides (2,3). Among them, cytochrome b and cytochrome c_1 were established as essential redox-active components for catalytic function of the complex, and ISP[1] has recently been demonstrated to be essential for the catalytic function (4,5). More recently, it has been found that QP exists in Complex III (6-9), and this protein is thought to act as an essential component in the catalysis by the complex (8).

In the course of our study on Complex III of beef heart mitochondria, we developed the methods for purification of the ISP (5) and QP (9), and obtained antibodies directed against these proteins (9,10). Here, we have studied interaction between these antibodies and Complex III by immunoinhibition and ELISA.

[1]The abbreviations used are: ISP, iron-sulfur protein; QP, ubiquinone binding protein; ELISA, enzyme-linked immunosorbent assay; PBS, phosphatebuffered saline; BSA, bovine serum albumine.

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Materials and Methods

Materials ----- Complex III was prepared from beef heart mitochondria by the method of Rieske et al. (11), and the ISP-depleted Complex III was prepared as described previously (5). The ISP (5,10) and QP (9) of Complex III were purified and rabbit antisera directed against these proteins were obtained as described previously. Antibodies were purified from these antisera by ammonium sulfate fractionation (50% saturation) and disolved in 20 mM Tris-Cl, pH 7.5, containing 150 mM NaCl. After the resulting solution was dialyzed against the above buffer at 4°C overnight, the insolble material formed was removed by centrifugation. Cytochrome c (type III) and soybean phospholipid mixture (containing 22% L-α-phosphatidylcholine) were purchased from Sigma, St. Louis ; a phospholipid mixture (5 mg/ml) was prepared as described previously (5). Ubiquinone-2 was supplied from Eisai Co., Ltd., Tokyo, and ubiquinol-2 was prepared as described by Rieske (12). Alkaline phosphatase-labeled affinity purified anti-rabbit IgG antibody was obtained from Kirkegaad and Perry Laboratories Inc., Gaithersburg, Maryland, and 96-well microtiter plates from Nunc.

Immunoinhibition of activity of Complex III-----Complex III (20 µg of protein) was incubated at 4°C overnight with varying amounts of IgG (0-550 µg) obtained from anti-ISP or anti-QP antiserum in 100 µl of 20 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 0.2% Tween 20. Ubiquinol-cytochrome c reductase activity in the incubation mixture was assayed as described previously (5).

Immunobinding assay for ISP by ELISA-----The following procedures were carried out at room temperature (22°C) unless otherwise stated. The purified ISP was dialyzed against 10 mM Tris-Cl, pH 7.5, containing 150 mM NaCl and 20% glycerol at 4°C for 2 days. Wells of 96-well microtiter plates were coated by incubating with a 100 µl solution of the ISP (1 µg/ml) in PBS at 4°C overnight. After residual protein-binding sites on the wells were blocked by incubation with PBS containing 1% BSA for 1 h, the wells were washed with PBS containing 0.1% Tween 20. The plates were used for immunobinding assay for ISP as follows. IgG from anti-ISP antiserum (56 µg) was incubated at 4°C overnight with varying amounts of Complex III (0.122-125 µg of protein) or ISP (0.012-12.5 µg) in 50 µl of 10 mM Tris-Cl, pH 7.5, containing 1% BSA and 0.1% Tween 20. The mixtures were diluted 1,000-fold with PBS containing 1% BSA, and 100 µl of the diluted solutions were added into the wells of the microtiter plate treated as above. After incubation for 2 h, the wells were washed three times with PBS containing 0.1% Tween 20, and incubated for 1.5 h with 100 µl of alkaline phosphatase-conjugated anti-rabbit IgG goat antibody (diluted 50-fold with PBS containing 1% BSA). Then they were washed as above and color was developed by incubation with 100 µl of alkaline phosphatase substrate solution containing 1 mg/ml of p-nitrophenyl phosphate, 1 mM $MgCl_2$, and 1 M diethanolamine, pH 9.8. After 20 min, the reaction was terminated by addition of 40 µl of 4 M NaOH. The absorbance at 408 nm was measured with Immuno Reader NJ-2000 (Japan Spectroscopic Co., Ltd., Hachioji, Japan).

Immunobinding assay for QP by ELISA-----The immunobinding assay for QP was carried out by essentially the same method as used for the assay for ISP. Wells of microtiter plates were coated by incubation with 2 µg/ml of QP. IgG (6.4 µg) obtained from anti-QP antiserum was incubated with varying amounts of Complex III (0.012-12.5 µg of protein), ISP-depleted Complex III (0.012-12.5 µg), or QP (0.61-625 ng) in 35 µl of 10 mM Tris-Cl, pH 7.5, containing 1% BSA. After incubation at 4°C overnight, 15 µl of a phospholipid mixture (5 mg/ml) were added and incubated at 30°C for 30 min. The addition of phospholipids averted the additional binding of antibody-QP complex to the wells during the incubation. The incubation mixture was diluted 25-fold and 100 µl of the diluted solution was processed as described for immunobinding assay for ISP.

Analytical method-----Protein was determined by the Lowry method modified by Hartree (14) using BSA as a standard.

Results and Discussion

Immunoblot analysis of electron-transfer particles of beef heart mitochondria showed that the anti-ISP and anti-QP antisera used in the present study gave only one band at the position corresponding to ISP and QP, respectively (data not shown), indicating that the antisera can be used as specific probes to study the interaction of the antibodies with Complex III.

When Complex III was incubated with varying amounts of IgG obtaind from anti-ISP antiserum, ubiquinol-cytochrome c reductase activity of the complex was inhibited dose-dependently (Fig. 1). Nearly 80% of the activity was inhibited by the addition of more than 20 μg of IgG per μg of protein of the Complex III. On the other hand, a parallel experiment using IgG obtained from normal serum indicated that IgG do not affect the activity of Complex III. These findings suggest that binding of anti-ISP antibody with the ISP in Complex III causes the inhibition of catalytic activity of the complex. A similar observation was previously reported for the interaction of antibody directed against yeast ISP with Complex III of yeast mitochondria (14). In order to know how much of the surface of ISP molecule is exposed in Complex III, absorption of anti-ISP antibody was assessed with Complex III and ISP by ELISA (Fig. 2). Complex III was found to absorb the antibody to some degree;

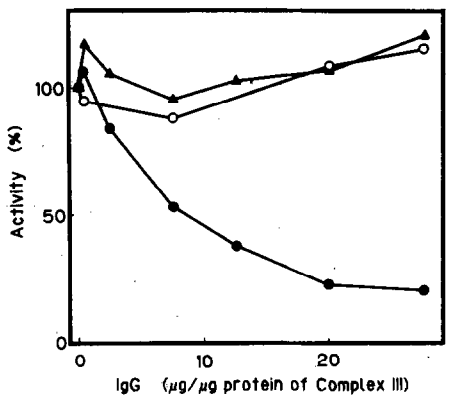
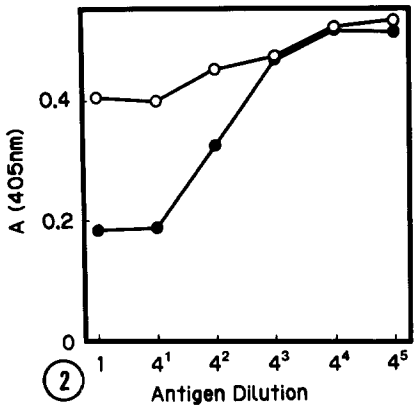


Fig. 1. Immunoinhibition of ubiquinol-cytochrome c reductase activity in Complex III. Complex III (20 μg) was incubated with varying amounts of IgG preparations obtained from anti-ISP antiserum (●-●), anti-QP antiserum (o-o), or normal serum (▲-▲). The activity was expressed as percentage of the activity observed in the absence of IgG.



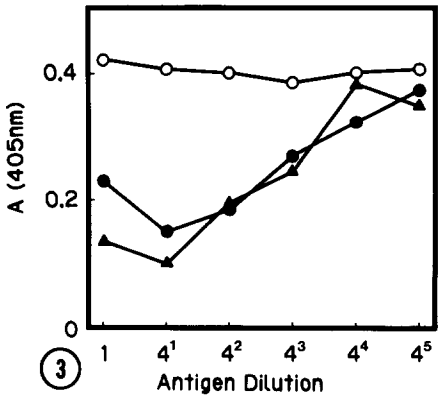


Fig. 2. Immunobinding of anti-ISP antibody with ISP (●-●) and Complex III (o-o). Immunobinding assay for ISP was carried out as described under Materials and Methods. The incubations with undiluted ISP and Complex III contained 12.5 µg and 125 µg, respectively, in 50 µl mixtures. The absorbance observed in the absence of antigen was 0.533.

Fig. 3. Immunobinding of anti-QP antibody with QP (▲-▲), Complex III (o-o), and ISP-depleted Complex III (●-●). Immunobinding assay for QP was carried out as described under Materials and Methods. The incubation with undiluted QP contained 625 ng in a 50 µl mixture, while those with Complex III and ISP-depleted Complex III contained 12.5 µg in 50 µl mixtures. The absorbance observed in the absence of antigen was 0.377.

but, the degree of absorption was much less than that observed with ISP. This indicates that the majority of antigenic sites of ISP are covered with other subunits of the complex and/or phospholipids.

As to the localization in Complex III of subunit VI, which has recently been identified with QP (9), a study on proteolytic digestion of this complex indicated that this subunit could be cleaved with papain (15). Also, a labeling study using [^{35}S]diazobenzenesulfonate showed that subunit VI in Complex III was intensively labeled (16). These observations indicate that at least a part of QP is exposed on the surface of Complex III. We examined whether anti-QP antibody inhibits ubiquinol-cytochrome c reductase activity in Complex III. The result, however, showed that the antibody had no inhibitory effect on the activity. There may be two possibilities to explain this finding: (1) the antibody do not bind to QP in Complex III and (2) the antibody binds to QP in the complex without any inhibitory effect on the activity of the complex. To clarify this point, absorption of anti-QP antibody with Complex III was tested, and it was found that the antibody was not absorbed to any appreciable degree (Fig. 3), indicating that there occurs

no interaction between the antibody and QP in Complex III. Thus, the antigenic sites of QP are apparently not exposed on the surface of Complex III and therefore cannot be recognized by antibody. On the other hand, the experiment using the ISP-depleted Complex III, which is deficient in phospholipids (5), showed that this complex absorbed the antibody to almost the same degree as did QP (Fig. 3). Thus, it is clear from these results that some part of QP interacts with phospholipids or ISP, or both in the structure of Complex III.

In summary, the present immunochemical study on Complex III of beef heart mitochondria demonstrated that in the case of ISP, a substantial part of the surface of this protein molecule is involved in the association with other subunit(s) and/or phospholipids. On the other hand, antibody directed against QP was found not to recognize the surface of this protein in the complex, but to bind to ISP-depleted complex. Therefore, this antibody could be used for studying localization of the part of this protein that interacts with other constituents of Complex III.

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