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ORIENTATION OF CHARGERIN II (A6L) IN THE ATP SYNTHASE OF RAT LIVER MITOCHONDRIA DETERMINED WITH ANTIBODIES AGAINST PEPTIDES OF THE PROTEIN*

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SUMMARY: Previous studies suggested that the hydrophobic protein chargerin II, which is encoded in the unidentified reading frame A6L of mitochondrial DNA (URFA6L), may have a key role in the energy transduction by mitochondrial ATP synthase because an antibody against chargerin II inhibited ATP synthesis and ATP-Pi exchange, in an energy-dependent fashion. In the present work, the orientation of chargerin II in F_0 of the ATP synthase of rat liver mitochondria was examined using antibodies against peptides of chargerin II. Results showed that its N-terminal region (about 8 amino acid residues) was exposed on the surface of the C-side of F_0, but its C-terminal and charge-cluster regions were buried in F_0.

ATP synthase is a multi-subunit complex that utilizes a transmembrane proton gradient ($\Delta\mu H^+$) to form ATP (1). ATP synthase is composed of two domains: a hydrophilic portion called F_1, which is the catalytic site of ATP synthesis, and a membranous domain called F_0, which is responsible for energy transduction (2-4). However, it is still unknown how this enzyme converts $\Delta\mu H^+$ into energy for ATP synthesis.

Previously we proposed a charge-transfer coupling mechanism for the action of ATP synthase (5). According to this hypothesis, one of the subunits of F_0, which has unbalanced charges in its sequence, may have an essential role in energy

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transduction between F_o and F_1. We suppose that a membrane potential causes electrophoretic movement of these unbalanced charges in F_o. The resulting conformational change of the protein in turn causes a conformational change of the β-subunit of F_1 and a decrease in its binding constant for ATP, which is formed from ADP and Pi bound to the β-subunit without the input of energy [cf. review by Slater (6)]. Consequently, F_1 releases bound ATP into the medium.

Recently we purified a hydrophobic protein named chargerin II from rat liver mitochondria (7), which was encoded by the unidentified reading frame URFA6L of mitochondrial DNA (8,9). Although chargerin II is hydrophobic and soluble in a mixture of chloroform and methanol (2:1), it has unbalanced positive charges in its sequence. Chargerin II is one of the subunits of F_o of ATP synthase (10-12). Furthermore, an antibody against chargerin II inhibited energy transduction by ATP synthase in mitoplasts in an energy-dependent fashion, suggesting that energization of ATP synthase causes a conformational change in chargerin II (11).

These unique features of chargerin II suggest that it has an essential role in the energy transduction by mitochondrial ATP synthase, in good accord with the charge-transfer coupling hypothesis (5)

In the present work, the orientation of chargerin II in F_o of the ATP synthase of rat liver mitochondria was examined using antibodies against peptides synthesized based on the sequence of chargerin II.

MATERIALS AND METHODS

Rat liver mitochondria, mitoplasts, submitochondrial particles (inverted inner membrane vesicles), and urea particles (F_1-depleted submitochondrial particles) were isolated by the methods of Pedersen and his coworkers as described in (12).

Synthesis of peptide fragments of chargerin II: Three peptides with the sequences of the N-terminus, hydrophilic portion, and C-terminus of chargerin II were synthesized and designated as N8P(C), H11P(C), and C12P(C), respectively. Their sequences were as follows: N8P(C), MPQLDTST; H11P(C), PPSPKTMATEK; C12P(C), TKIYLPLSLPPQ.

C12P(C) was synthesized by the solid-phase method (13). N8P(C) and H11P(C) were kindly synthesized by Pharmacia LKB Biotechnology using a peptide synthesizer, LKB Biolynx, model 4175 and were purified by reverse-phase HPLC.

The purities of the preparations of N8P(C), H11P(C), and C12P(C) were confirmed by reverse-phase HPLC and amino acid analysis.

Preparation of antibodies against peptide fragments of chargerin II: Purified N8P(C)(2.5 mg), H11P(C)(2.7 mg), and C12P(C)(9.8 mg) were each dissolved in 10 ml of 0.14 M potassium phosphate buffer, pH 7.0. Samples of keyhole limpet hemocyanin, 5 times the weight of the peptide fragments, were dissolved in 10 ml of the same buffer and centrifuged at 10,000xg for 15 min; and then the supernatants obtained were mixed with the peptide solutions. Crosslinking of each peptide fragment to hemocyanin was carried out by adding 1.5 ml of 3% glutaraldehyde in 0.5 ml aliquots at 10 min intervals. The reaction was allowed to proceed at room temperature for an additional 90 min after the last addition of glutaraldehyde. The preparations were dialyzed overnight against 2 liters of 25 mM potassium phosphate

buffer containing 0.14 M NaCl at 4°C and then concentrated in an Amicon 402 Diaflo Cell equipped with a Diaflo membrane under 2 kg/cm^2 pressure of N_2 gas.

Rabbits were immunized with 0.5 mg of each crosslinked peptide fragment of chargerin II in Freund's complete adjuvant. Booster injections of 0.25 mg of the crosslinked peptide fragment in Freund's incomplete adjuvant were given 4 or 5 times at 2 week intervals. Antisera were tested for appropriate antibodies by a dot immunoblotting assay as previously described (7).

Determination of amounts of antibodies against peptide fragments of chargerin II bound to mitoplasts, submitochondrial particles, and urea particles: Skim milk (Snow Brand Nyugyou Co.) was dissolved at 1%(w/v) in a solution of 0.89 mM $MgCl_2$, 0.36 mM EDTA, 32 mM sucrose, and 4.5 mM Tris-HCl at pH 7.4 and centrifuged at 15,000xg for 10 min, and the resulting supernatant was used as the binding medium.

Mitoplasts (0.1 mg protein) were suspended in 1 ml of binding medium at 25°C in 1.5 ml Eppendorf centrifuge tubes. Then the indicated amounts of antisera against the peptide fragments of chargerin II or control serum (preimmune serum) were added with mixing. The tubes were incubated for 2 hours at 25°C and then centrifuged at 8000xg for 4 min at 4°C in an Eppendorf, model 3200, microcentrifuge. The supernatants were discarded, and the precipitates were suspended in 1 ml of binding medium by pipeting with a 1-ml disposable syringe with a 26G needle. The suspensions were incubated for 10 min at 25°C and centrifuged, and the precipitates were suspended in 0.5 ml of binding medium as described above. Then 0.5 ml of binding medium containing 0.06 μCi of [^{125}I]-protein A (Amersham) and 0.03 nmol of cold protein A (ICN ImmunoBiologicals) was added, and the tubes were incubated for 1 hour at 25°C, and centrifuged in the same way as before. The precipitates were suspended in binding medium and incubated for 10 min at 25°C. Finally, the precipitates were washed once more and suspended in 1 ml of binding medium and the radioactivities of 0.9-ml aliquots of the mixture were measured in an Aloka, Multi-Mode Scaler, γ-counter.

Submitochondrial particles and urea particles were incubated in 0.3 ml of binding medium in 10-ml centrifuge tubes for 3 min at 25°C. Then antisera (30 μl) against the peptide fragments of chargerin II or control serum [preimmune serum before injection of N8P(C)] were added, and the mixtures were incubated for 2 hours at 25°C. Other procedures were as described above for mitoplasts except that the centrifugations were carried out at 100,000xg for 30 min at 2°C in a Beckman ultracentrifuge, model L8-M, equipped with a 70.1Ti rotor.

Other methods were as described (7,12).

RESULTS

Binding of antibodies against three peptide fragments of chargerin II to mitoplasts: Peptides corresponding to the N-terminus, hydrophilic portion, and the C-terminus of chargerin II, designated as N8P(C), H11P(C), and C12P(C), respectively, were synthesized and purified as described under Materials and Methods.

Figure 1 shows that antibodies against N8P(C), H11P(C) and C12P(C) bound specifically to the corresponding peptide fragments of chargerin II. These antibodies specifically bound only to chargerin II in submitochondrial particles and H^+-ATP synthase; antibody against C12(C) was useful for determining the content of chargerin II in H^+-ATP synthase (Muraguchi, M., Yoshimura, Y., and Higuti, T., in preparation).

First we examined the binding of these antibodies to mitoplasts, in which the C-side of F_o is present on the outer surface of the inner membrane. The antibodies against C12P(C) (Fig. 2) and H11P(C) (data not shown) did not bind to mitoplasts. However, as seen in Fig. 3(A), antibodies against N8P(C) bound to mitoplasts; and the dose-response curve showed saturation, indicating that the antibodies bound to a specific component exposed on the surface of the C-side of the mitochondrial inner

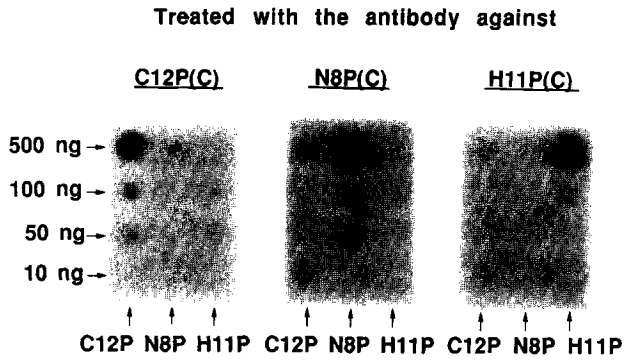

Fig. 1 Dot-immunoblotting assay of antibodies raised against C12P(C), N8P(C), and H11P(C), Samples of 10, 50, 100, and 500 ng of C12P(C), N8P(C), and H11P(C) were dotted onto three nitrocellulose filters as indicated. These filters were treated with antibodies raised against C12P(C), N8P(C), and H11P(C). Other conditions were as described under "Materials and Methods".

membrane. The maximum amount of antibodies bound to mitoplasts was 67 pmoles per mg protein [Fig. 3(B)].

Binding of the antibodies against peptide fragments of chargerin II to submitochondrial particles and urea particles: Next we examined the bindings of the antibodies against the three peptide fragments of chargerin II to submitochondrial particles, which are inside-out relative to the membranes of mitoplasts and have F_1s on the outer surface of the M-side of the mitochondrial inner membrane. None of the three antibodies bound to submitochondrial particles. This result could be due to

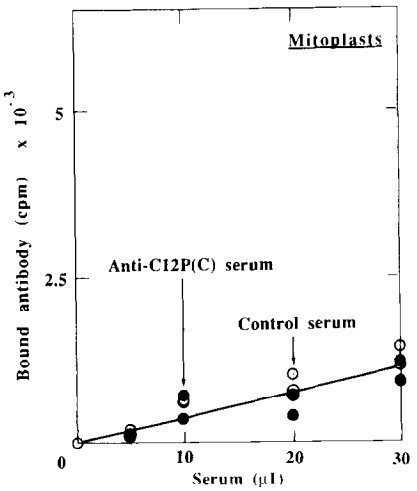

Fig. 2 Binding of anti-C12P(C)-antibodies to mitoplasts. Mitoplasts were treated with anti-C12P(C) serum or control serum (preimmune serum) as described under "Materials and Methods".

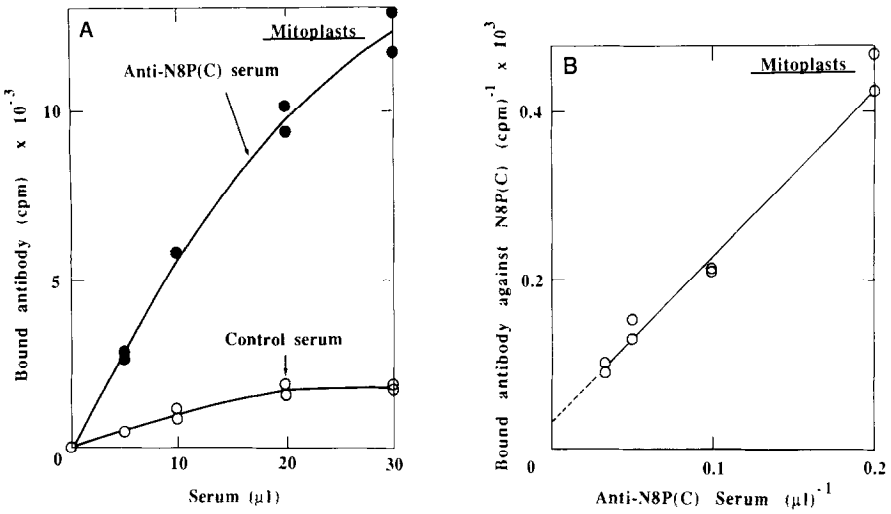

Fig. 3 (A) Dose-response curve of the binding of anti-N8P(C)-antibodies to mitoplasts. Mitoplasts were treated with anti-N8P(C) serum or control serum (preimmune serum) as described under "Materials and Methods" except that the reaction volume was 0.3 ml. (B) Reciprocal plot of the dose-dependence of the binding of anti-N8P(C)-antibodies to mitoplasts. Values were calculated from the data in (A).

steric hindrance by F_1, so we next examined the binding of antibodies to urea particles, which are submitochondrial particles treated with 3.2 M urea to remove F_1s from their inner membrane. The ATPase activity of the urea particles used was about 15% that of the submitochondrial particles, indicating that about 85% of the F_1 had been removed. Figure. 4 shows that none of the three antibodies bound to the membranes of urea particles.

These findings show that none of the three peptide fragments of chargerin II appeared on the surface of the M-side of the mitochondrial inner membrane, even in F_1-depleted submitochondrial particles (urea particles). Furthermore, the data indicate that the membranes of the submitochondrial particles and urea particles used were almost completely inside-out relative to those of mitoplasts.

DISCUSSION

In the present study, we found that the N-terminal part of chargerin II is exposed on the outer surface of the C-side of F_0, and that the C-terminus and the charge-cluster regions of chargerin II are buried in F_0, where antibodies against the central and C-terminal peptide fragments of chargerin II, H11P(C) and C12P(C), could not react with them, even in F_1-depleted submitochondrial particles (urea particles).

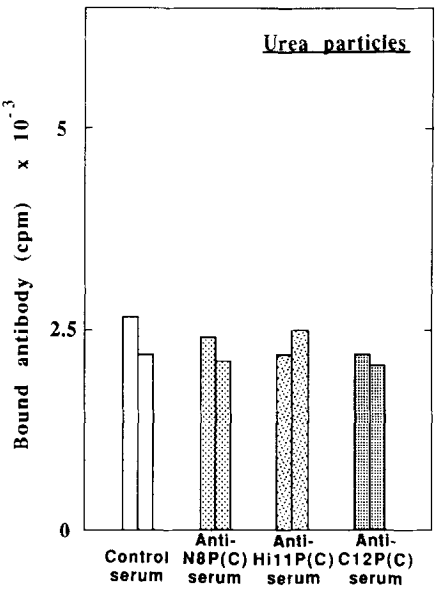

Fig. 4 Binding of antibodies against C12P(C), N8P(C), and H11P(C) to urea particles. Procedures were as described under "Materials and Methods". The activity of ATPase in the urea particles used was about 15% of that in the submitochondrial particles. The amounts of [^{125}I]-protein A bound to urea particles that were not treated with serum were negligible (21 and 37 cpm).

The homologies of the structures and functions of chargerin II (proteins encoded by the A6L gene) and subunit 8 of yeast are unknown. Although only a four amino acid sequence at the N-terminus of subunit 8 is homologous with those of the putative proteins of the URFA6L gene, these proteins are thought to belong to the same family because they both have amino acid sequences containing unbalanced positive charges, their predicted secondary structures show similarities, and each have one transmembrane stem [cf. review by Nagley (14)].

From studies using a non-penetrating amino-reactive reagent, isethionylacetimidate, Velours and Guerin (15) suggested that the C-terminus of subunit 8 is located on the M-side of the mitochondrial inner membrane. As the transmembrane domain of subunit 8 is predicted to extend from residue 15 to residue 35, its N-terminus could be located on the C-side of the mitochondrial inner membrane. Furthermore, the transmembrane domain of chargerin II was predicted by the method of Klein et al. (16) to extend from residue 10 to 26. Therefore, the present results and those of Velours and Guerin suggest that the orientations of chargerin II

and subunit 8 in F_0 are essentially the same. This possibility is consistent with the idea that these two proteins belong to the same family.

It is interesting that only 34% of the C-terminus of subunit 8 (mainly lysine-47) was labeled with the small, hydrophilic reagent isethionylacetimidate, even in F_1-depleted submitochondrial particles. This could be because the C-terminus of subunit 8 is completely embedded in a hydrophobic sphere of ATP synthase. According to the charge-transfer coupling hypothesis (5), for the function of the unbalanced positive charges of these subunits in ATP synthase, it may be essential that they are embedded in a hydrophobic sphere of the enzyme complex. Further studies on this problem are required.

Finally, it should be noted that prokaryotic F_0s are known to consist of only three subunits (a, b, and c) and they do not contain a homologous sequence to chargerin II. However, as previously proposed (5), the prokaryotic subunit b may have a similar function to chargerin II deduced from the charge-transfer coupling model because the subunit b has unbalanced negative charges in the C-terminal region (17) that is known to be essential for ATP-dependent H^+-translocation by *Echerichia coli* ATP synthase and also for the binding of F_1 (18,19). However, mitochondrial subunit b does not conserve such unbalanced charges (20). This may indicate that the mitochondrial F_0-subunits differentiated functionally in the process of evolution. This could be a possible reason why the mitochondrial F_0 is assembled from a much greater number of different polypeptides than the eukaryotic F_0-subunits (12).

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