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STOICHIOMETRY OF CHARGERIN II (A6L) IN THE H^+-ATP SYNTHASE OF RAT LIVER MITOCHONDRIA[1]

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Summary: **Previous studies suggested that the hydrophobic protein chargerin II, which is encoded in the A6L of mitochondrial DNA, may have a key role in the energy transduction by mitochondrial H^+-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 contents of chargerin II in the H^+-ATP synthase purified from rat liver mitochondria and in submitochondrial particles were determined by radioimmunoassay. Results showed that the H^+-ATP synthase contained chargerin II in a molar ratio of one to one. This is the first report on the stoichiometry of the A6L-product in mitochondrial H^+-ATP synthase.**

H^+-ATP synthase is a multi-subunit complex that utilizes a trans-membrane proton gradient ($\Delta\mu H^+$) to form ATP (1). H^+-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 H^+-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 transduction between F_0 and F_1. We suppose that a membrane potential causes $\Delta\mu H^+$-dependent movement of these unbalanced charges in F_0. 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 the bound ATP into the medium, completing the energy transduction from $\Delta\mu H^+$ to the phosphate potential.

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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_0 of H^+-ATP synthase (10-12). We found that the N-terminal region (about 8 amino acid residues) of chargerin II 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 (13). Furthermore, an antibody against chargerin II inhibited energy transduction by H^+-ATP synthase in mitoplasts in an energy-dependent fashion, suggesting that energization of H^+-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 H^+-ATP synthase, in good accord with the charge-transfer coupling hypothesis (5).

In the present work, the contents of chargerin II in the H^+-ATP synthase and submitochondrial particles were determined by radioimmunoassay.

Materials and Methods

Chargerin II and H^+-ATP synthase were purified from rat liver mitochondria as described by Higuti et al. (7) and Yoshihara et al. (12), respectively. Submitochondrial particles were isolated by the method of Pedersen and coworkers (14), except that mitochondria were treated with digitonin at a concentration of 0.04 mg per mg protein during the preparation of mitoplasts.

Amino acid analysis of chargerin II was carried out in a Waters PICO TAG HPLC system as described in the manual. The amounts of chargerin II and H^+-ATP synthase were estimated from the determined total amount of amino acids.

Antibodies against the C-terminus of chargerin II (TKIYLPLSLPPQ), which was designated as C12P(C), were prepared as described previously (13).

Electrophoresis was performed in a 17.5 % or 10-20 % polyacrylamide gel overlaid by a 1-cm stacking gel by a modification of the procedure of Laemmli (38) in an Atto Rapidus, model AE-6200 apparatus. The separation gel also contained 8 M urea. After electrophoresis at 4.5 mA for about 17 hours, proteins in one half of the gel were stained with Coomassie Blue and then with Bio-Rad silver stain; and proteins in the other half of the gel were transferred electrophoretically to a nitrocellulose filter (Bio-Rad), which was presoaked in the electrophoresis buffer (pH 8.0) containing 25 mM Tris, 192 mM glycine, and 0.1 % (w/v) sodium dodecyl sulfate, in a Sartorius semi-dry-electroblotter at about 6 V for 4 hours. The filter was dried and then baked at 80°C for 2 hours. Chargerin II was identified radioimmunochemically with the antibody against C12P(C) and [^{125}I]-protein A (Amersham IM-144) as described previously (7). The filters were exposed to X-ray film (Kodak, XRA-5) in a Kodak X-Omatic cassette at -85°C for about 8 hours or the described duration.

For the dot-immunoblotting assay, chargerin II, submitochondrial particles, and H^+-ATP synthase were dissolved in 80 % formic acid, and then they were diluted to 50 % formic acid with water. The resulting solutions (200 µl) were blotted on the nitrocellulose filter using the Bio-Dot Slot Format blotting apparatus (Bio-Rad).

The cytochrome c oxidase content of the submitochondrial particles was determined in a Hitachi, model 556, two-wavelength and double-beam spectrophotometer by measuring the absorbance of the "dithionite-reduced" form minus the "oxygen-oxydized" form at the wavelength pair of 605 nm minus 630 nm (absorbance coefficient, 16.5 $mM^{-1}\,cm^{-1}$) (17).

Other methods were as described previously (7, 12).

Results and Discussion

Figure 1 clearly indicates that antibodies against the C-terminus of chargerin II [C12P(C)] specifically reacted only to chargerin II in the submitochondrial particles, showing that the antibodies against C12P(C) can be used for the identification of chargerin II in the submitochondrial particles and also H^+-ATP synthase.

Figure 2 demonstrates that chargerin II was one of the subunits of the F_o of the H^+-ATP synthase purified from rat liver mitochondria by our recently developed procedure (12). The preparation of H^+-ATP synthase used in the present work was essentially devoid of cytochrome c oxidase, cytochrome c, the cytochrome $b\,c_1$ complex, NADH dehydrogenase, and succinate dehydrogenase, as described in ref. (12). The preparation had high ATPase activity (14.5 units per mg protein) in the absence of added phospholipids, which was completely inhibited by oligomycin. The H^+-ATP synthase reconstituted into proteoliposomes showed an ATP-dependent

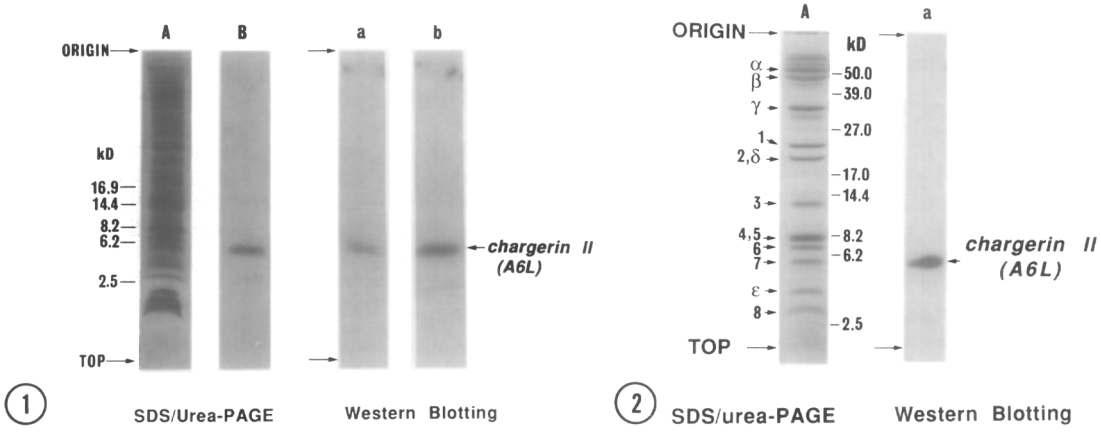

Fig. 1 Sodium dodecyl sulfate/urea-gel electrophoresis on polyacrylamide gel of submitochondrial particles (*lane A*) and chargerin II (*lane B*) and detection of the chargerin II in them by Western immunoblotting (*lanes a* and *b*). Electrophoresis was performed in a 10-20 % linear gradient polyacrylamide gel. The molecular mass standards used were standards from BDH Limited (England) [16.9 kD, myoglobin; 14.4 kD, myoglobin (Fragment I + II); 8.2 kD, myoglobin (Fragment I); 6.2 kD, myoglobin (Fragment II); 2.5 kD, myoglobin (Fragment III)]. Proteins in the gel were doubly stained with Coomassie Blue and then with a Bio-Rad silver staining kit (*lanes A* and *B*). *Lanes A* and *a* were loaded with 50 μg of submitochondrial particles. *Lanes B* and *b* were loaded with 0.7 μg of chargerin II. The other conditions were as described under "Materials and Methods". *SDS/Urea-PAGE*, sodium dodecyl sulfate-polyacrylamide/urea gel electrophoresis.

Fig. 2 Sodium dodecyl sulfate/urea-gel electrophoresis on polyacrylamide gel of the purified rat liver H^+-ATP synthase (*lane A*) and detection of chargerin II in the complex by Western immunoblotting (*lane a*). Electrophoresis was performed in a 17.5 % polyacrylamide gel. The molecular mass standards used were pre-stained standards from Bio-Rad (50.0 kD, ovalbumin; 39.0 kD, carbonic anhydrase; 27.0 kD, soybean trypsin inhibitor; 17.0 kD, lysozyme) and standards from Sigma [14.4 kD, myoglobin (Fragment I + II); 8.2 kD, myoglobin (Fragment I); 6.2 kD, myoglobin (Fragment II); 2.5 kD, myoglobin (Fragment III)]. *Lanes A* and *a* were loaded with 30 μg of the purified rat liver H^+-ATP synthase. Proteins in the gel were doubly stained with Coomassie Blue and then with a Bio-Rad silver staining kit (*lane A*). The other conditions were as described under "Materials and Methods".

anilino-naphthalene sulfonate (ANS)-response and ATP-Pi exchange activity, both of which were also completely inhibited by oligomycin and uncouplers. Thus, the H^+-ATP synthase used in the present work was highly purified and remained intact.

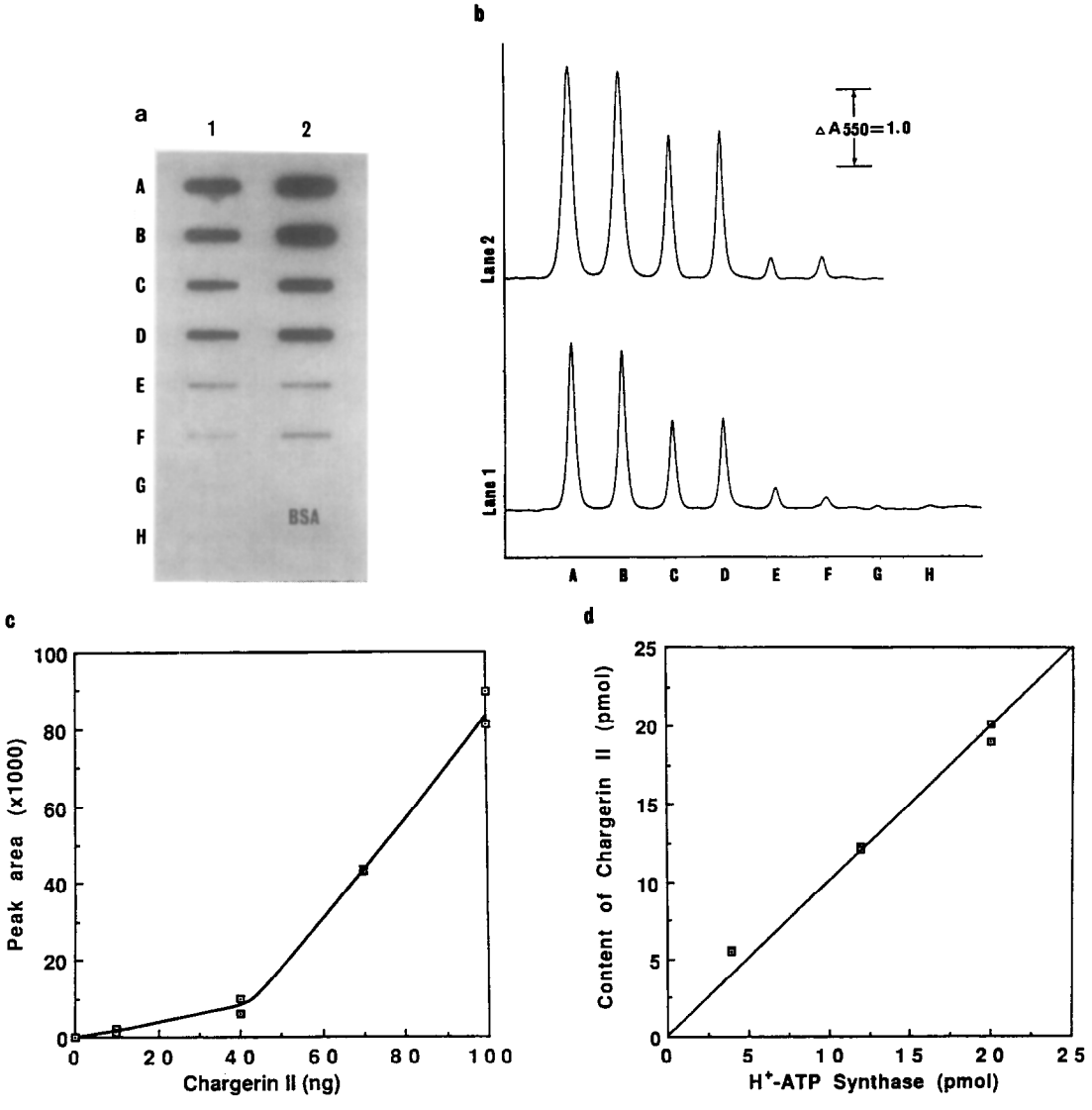

Fig. 3 Determination of the content of chargerin II in the H^+-ATP synthase purified from rat liver mitochondria. (a) Radioimmunogram of chargerin II (*lane 1*) and H^+-ATP synthase (*lane 2*). Dotted amounts of chargerin II (ng of protein): A and B, 100; C and D, 70; E and F, 40; G and H, 10. Dotted amounts of the H^+-ATP synthase (μg of protein): A and B, 10; C and D, 6; E and F, 2. G of *lane* 2, 10 μg of bovine serum albumin. The filter was exposed to X-ray film for about 20 hours at -85°C. (b) Chromato-scanning of the radioimmunogram. The radioimmunogram was scanned with a Shimazu, model CS-9000, two-wavelength flying spot scanner at 550 nm (band size, 1.0 x 1.0 mm), and the peak area were calculated automatically. (c) Dose-response curve of the peak area of the densitogram and the dotted amount of chargerin II. The data obtained from (b) were plotted. (d) Content of chargerin II in the H^+-ATP synthase. Data were calculated using the data in (b) and (c) and the molecular weights of chargerin II (7633) and H^+-ATP synthase (500,000). The other conditions were as described under "Materials and Methods".

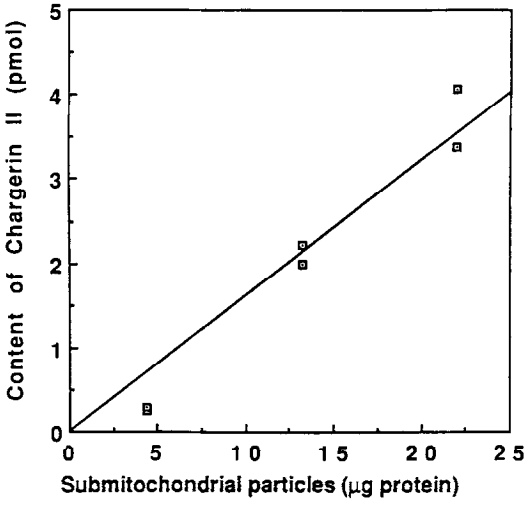

Fig. 4 Determination of the content of chargerin II in submitochondrial particles. Methods were the same as described in the legends of Fig. 3 except that submitochondrial particles in place of the H^+-ATP synthase were dotted on the filter in the amounts of 22, 13.2, and 4.4 μg of protein. The content of cytochrome c oxidase in the submitochondrial particles was 0.340 nmol per mg protein.

Then we determined the contents of chargerin II in the H^+-ATP synthase as well as submitochondrial particles radioimmunochemically with the antibodies against C12P(C) and [^{125}I]-protein A. The amount of chargerin II determined by the Lowry method (16) was 125.6 % that of chargerin II as measured by amino acid analysis. The amount of H^+-ATP synthase estimated by the Lowry method was almost the same as that of H^+-ATP synthase determined by amino acid analysis. In the present work, we used the amounts of the proteins estimated by the amino acid analysis.

First, chargerin II and the H^+-ATP synthase were dotted on a nitrocellulose filter using a Bio-Rad Blotting apparatus as described under "Materials and Methods". Figure 3(a) shows the radioimmunogram of the dotted chargerin II and the H^+-ATP synthase. This film was scanned with the two-wavelength flying spot scanner [Fig. 3(b)]. Figure 3(c) explains the relationship between the peak area of each dot [*lane 1* in (b)] and the dotted amount of chargerin II, indicating that this method was useful for the determination of chargerin II in H^+-ATP synthase, as shown in Fig. 3(d). Figure 3(d) clearly demonstrates that the H^+-ATP synthase contained chargerin II in a molar ratio of one to one.

Similarly, Fig. 4 indicates that submitochondrial particles contained 0.165 nmol of chargerin II and 0.340 nmol of cytochrome c oxidase per mg protein, in good accord with the molar ratio of 2 molecules of cytochrome c oxidase per one molecule of H^+-ATP synthase in mitochondria (15).

This is the first report on the stoichiometry of the mitochondrial A6L gene-product in the mitochondrial H^+-ATP synthase.

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