H^+-ATP Synthase from Rat Liver Mitochondria. A Simple, Rapid Purification Method of the Functional Complex and Its Characterization†

Yutaka Yoshihara, Hideki Nagase, Takeshi Yamane, Hideki Oka, Isamu Tani, and Tomihiko Higuti*
Faculty of Pharmaceutical Sciences, The University of Tokushima, Shomachi, Tokushima, 770, Japan
Received September 24, 1990; Revised Manuscript Received April 24, 1991

ABSTRACT: A novel, simple, and rapid preparative method for purification of rat liver H^+-ATP synthase by anion-exchange HPLC was developed. The H^+-ATP synthase purified had higher ATPase activity in the absence of added phospholipids than any preparation reported previously, and this activity was completely inhibited by oligomycin. When reconstituted into proteoliposomes, the H^+-ATP synthase showed an ATP-dependent 8-anilinonaphthalene-1-sulfonate response and ATP–P_i exchange activity, both of which were also completely inhibited by oligomycin and an uncoupler, indicating the intactness of the H^+-ATP synthase. An immunochemical study and a labeling experiment with *N,N'*-[^{14}C]dicyclohexylcarbodiimide ([^{14}C]DCCD) demonstrated the presence of chargerin II (a product of mitochondrial A6L DNA) and DCCD-binding protein (subunit c) in the complex. The subunits of the complex were separated into 11 main fractions by reverse-phase HPLC, and 3 of them and the δ subunit in F_1 were partially sequenced. A search for sequence homologies indicated that these components were subunit b, coupling factor 6, subunit δ, and subunit ϵ. This is the first report of the existence of subunit b, factor 6, and chargerin II in H^+-ATP synthase purified from rat liver mitochondria.

H^+-ATPase synthase, which is also called F_oF_1ATPase, H^+-ATPase, ATP synthase, or complex V, is a multisubunit complex that utilizes a transmembrane proton gradient ($\Delta\mu_{H^+}$) to form ATP (Mitchell, 1961; Kagawa, 1978). H^+-ATP synthase is composed of two domains: a hydrophilic part called F_1, which is the catalytic site of ATP synthesis, and a membranous domain called F_o, which is responsible for energy transduction (Fillingame, 1980; Cross, 1981; Hatefi, 1985; Futai et al., 1989). However, it is still unknown how this enzyme converts $\Delta\mu_{H^+}$ into energy for ATP synthesis.

Previously we proposed a charge movement coupling mechanism for the action of H^+-ATP synthase (Higuti, 1984a,b; Higuti et al., 1989). According to this hypothesis, one or two of the subunits of F_o, which has an unbalanced charge(s) in its hydrophobic sequence, may have an essential role in energy transduction between F_o and F_1. We suppose that $\Delta\mu_{H^+}$ causes cyclic movement of the unbalanced charge(s) 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 P_i bound to the β subunit without the input of energy [cf. review by Slater (1987)]. Consequently, F_1 releases the bound ATP into the medium, completing the energy transduction from $\Delta\mu_{H^+}$ to the phosphate potential.

To obtain direct evidence for this model, it is essential to develop a simple and rapid method for purification of H^+-ATP synthase with activity for energy transduction. Therefore, in the present work, we developed a simple, rapid method for purification of H^+-ATP synthase from rat liver mitochondria by anion-exchange high-performance liquid chromatography (HPLC).[1]

A preliminary account of this work has appeared (Higuti et al., 1988b).

Experimental Procedures

Materials. HTG and HFBA were purchased from Wako Chemicals, Osaka, Japan. Oligomycin was obtained from Sigma Chemical Co., St. Louis, MO. Digitonin was obtained from Wako Chemicals and purified as described (Higuti et al., 1980b). A Bio-Rad silver stain kit was used. [^{14}C]DCCD was purchased from Amersham Japan. Soybean asolectin (a product of Associate Concentrates, Woodside, NY) was a gift from Dr. Hajime Hirata (Jichi Medical School) and was partially purified as described (Kagawa & Racker, 1966).

Rat liver mitochondria, mitoplasts, and submitochondrial particles were isolated by the method of Pedersen and coworkers (Cham et al., 1970; Williams et al., 1984) except that mitochondria were treated with digitonin at a concentration of 0.04 mg/mg of protein during the preparation of mitoplasts.

Purification of H^+-ATP Synthase. Step 1. Solubilization of H^+-ATP Synthase from Submitochondrial Particles. The submitochondrial particles were thawed and added at a concentration of 5 mg of protein/mL with gentle stirring to PEG buffer [20 mM potassium phosphate, 5 mM EDTA, and 20% (v/v) glycerol (pH 7.4)] at 0 °C. Then 1% HTG was added, and the mixture was stirred gently and stood for 5 min. The suspension was diluted with an equal volume of PEG buffer and centrifuged at 105000*g* (Beckman 60Ti rotor, 32000 rpm) for 30 min. The resulting supernatant was concentrated in an Amicon, Model 402, Diaflo cell equipped with a YM-5 Diaflo membrane under 3.7 kg/cm^2 pressure of N_2 gas, and then the solvent was changed by adding an equal volume of PEG buffer and concentrating the solution to its original volume in the Diaflo cell. This procedure was repeated 3 times. All procedures in step 1 were carried out at 0 °C.

† This work was supported by Grants-in-Aid for Scientific Research 63480502, 01880022, and 02204009 from the Ministry of Education, Science and Culture of Japan and by a grant from the Takarazuka Research Center for Sumitomo Chemical Co.
* To whom correspondence should be addressed.

[1] Abbreviations: HPLC, high-performance liquid chromatography; HTG, *n*-heptyl β-thioglucoside; ANS, 8-anilinonaphthalene-1-sulfonate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; TFA, trifluoroacetic acid; HFBA, heptafluorobutyric acid; SDS, sodium dodecyl sulfate; OSCP, oligomycin sensitivity conferring protein.

0006-2960/91/0430-6854$02.50/0

The use of 250 mM potassium phosphate, instead of 20 mM described above, increased the yield of H+-ATP synthase from 23% (Table I) to 65%. However, this high-yield method also increased the contaminants.

Step 2. Anion-Exchange HPLC. The solution from step 1 (10 mg of protein) was applied to a column (8 mm × 7.5 cm or 21.5 mm × 15 cm) of TSK DEAE-5PW that had been equilibrated with the PEG buffer. The column was attached to a Waters M600 multisolvent delivery system, a Waters 710B sample processor, an ISCO V4 absorbance detector, an LKB 2211 Superrac, and a Shimazu C-R4A Chromatopak. Elution was carried out with a linear gradient of 0–100% 250 mM potassium phosphate, 5 mM EDTA, 500 mM KCl, and 20% glycerol at pH 7.4 for 60 min as described below. The bottles of PEG and elution buffers were cooled to about 2 °C in ice–water. Fractions containing H+-ATP synthase were collected.

Step 3. Centrifugation in the Presence of Glycerol. The solution from step 2 was centrifuged at 155000*g* (Beckman 70.1 Ti rotor, 41 000 rpm) for 2 h at 20 °C, and the precipitate obtained was suspended in a minimum volume of PEG buffer.

Purification of F_1. F_1 of the rat liver mitochondria was also purified by anion-exchange HPLC. Submitochondrial particles were shaken with 0.5 volume of neutralized chloroform as described (Williams et al., 1984). The aqueous layer was centrifuged at 155000*g* for 45 min, and the resulting supernatant was concentrated to about 5 mg of protein/mL in an Amicon, Model 402, Diaflo cell equipped with a YM-5 Diaflo membrane under 3.7 kg/cm^2 pressure of N_2 gas. The concentrate (10 mg of protein) was applied to a column (8 mm × 7.5 cm) of TSK DEAE-5PW that had been equilibrated with PE buffer containing 20 mM potassium phosphate and 5 mM EDTA at pH 7.4. Elution was carried out with a linear gradient of 0–100% 250 mM potassium phosphate and 5 mM EDTA at pH 7.4 for 60 min. The fractions containing ATPase activity were collected. All procedures were carried out at room temperature (about 25 °C).

Electrophoresis. Electrophoresis was performed in a 17.5% polyacrylamide gel overlaid with a 1-cm stacking gel by a modification of a reported procedure (Laemmli, 1970) 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 h, proteins were stained with Coomassie Blue and then with Bio-Rad silver stain.

ATPase Assay. ATPase activity was measured essentially as described (Stiggall et al., 1978) in a Hitachi Model 556 dual-wavelength, double-beam spectrophotometer using a wavelength pair of 340 nm minus 400 nm. The reaction mixture contained, in a volume of 2 mL at pH 8.2, 25 mM $KHCO_3$–Tris, 300 mM sucrose, 2 mM $MgCl_2$, 1.5 mM phosphoenolpyruvate, 0.25 mM NADH, 4 mM ATP, 5 units of pyruvate kinase, and 5 units of lactic acid dehydrogenase. The ATPase activity was calculated from the stable kinetic phase of ATP hydrolysis by using the absorbance change caused by the addition of 10 μL of 0.665 mM ADP as a standard.

Reconstitution of H+-ATP Synthase into Proteoliposomes. H+-ATP synthase purified as described above was reconstituted into proteoliposomes by a reported method (Muneyuki et al., 1987).

ATP-P_i Exchange and ANS Response. ATP-P_i exchange was measured as described (Joshi et al., 1979) except that $MgSO_4$ was used in place of $MgCl_2$, and the reaction time was 20 min. The ANS response was measured by a reported method (Muneyuki et al., 1987).

Detection of DCCD-Binding Protein. H+-ATP synthase (200 μg) was resuspended in 10 mM Tris-HCl buffer (pH 7.5) containing 250 mM sucrose and 1 mM EDTA at a concentration of 3 mg of protein/mL. Then [^{14}C]DCCD (Amersham CFA 662, 55 mCi/mmol) was added to the solution at a ratio of 20 nmol of [^{14}C]DCCD/mg of protein, and the mixture was incubated for 1 h at 4 °C. The resulting precipitate was washed twice with 30% trichloroacetic acid and subjected to electrophoresis as described below. The distribution of radioactivity in the gels was monitored by fluorography (Bonner & Lanskey, 1974) except that DPO (2,5-diphenyloxazole) was used at a concentration of 20% (w/w). The gel was exposed to X-ray film (Kodak, XRA-5) in a Kodak X-Omatic cassette at −85 °C for about 24 h.

Isolation of Subunits from H+-ATP Synthase. H+-ATP synthase (500 μg of protein) was incubated in a solution containing 6 M guanidine hydrochloride and 0.1% HFBA at a concentration of 5 mg of protein/mL for 2 h at 4 °C. Then the mixture was applied to a column (6 mm × 25 cm) of Asahipac ODP-90 that had been equilibrated with 20% acetonitrile containing 0.1% HFBA. Elution was carried out with a linear gradient of 20–60% acetonitrile containing 0.1% HFBA for 60 min. The absorbances of the proteins eluted were monitored at 214 and 280 nm.

Other Methods. The contents of cytochrome *a*, *b*, and *c* + c_1 and flavins were determined as described (Chance, 1957) in a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer.

The activities of NADH and succinate dehydrogenases were determined by measuring the amount of reduced potassium ferricyanide in a Hitachi, Model 556, two-wavelength, double-beam spectrophotometer with a wavelength pair of 470 nm minus 500 nm. The reaction mixture contained 2 mg of purified H+-ATP synthase, 20 mM potassium ferricyanide, 10 mM NADH or 10 mM succinate, 5 mM $MgCl_2$, 2 mM EDTA, 15 mM KCl, 50 mM sucrose, 25 mM Tris-HCl buffer, and 20 mM KH_2PO_4—K_2HPO_4 at pH 7.4 in 2 mL.

Automated sequence analyses of the subunits were performed in an Applied Biosystems Model 470A protein sequencer equipped with a 120A PTH analyzer.

Other methods were as described (Higuti et al., 1988a).

Results

Solubilization of Rat Liver H+-ATP Synthase from Submitochondrial Particles by HTG. HTG was developed (Tsuchiya & Saito, 1984; Shimamoto et al., 1985) as a mild detergent for solubilization of membrane proteins. It has the additional advantage that it can easily be removed from media becuase its critical micellar concentration is high (30 mM). Figure 1A shows that solubilizaton of H+-ATP synthase from submitochondrial particles increased gradually with an increase in the amount of HTG added, reaching a maximum with 1.5% HTG. The oligomycin sensitivity of the ATPase activity of the H+-ATP synthase solubilized from submitochondrial particles with 1.0% HTG was about 95%, but gradually decreased on increasing the HTG concentration above 1.25%, as shown in Figure 1A. The sensitivity of the H+-ATP synthase to oligomycin seemed to be a good marker of its "intactness". On the basis of these results, we used 1% HTG to solubilize H+-ATP synthase from submitochondrial particles in as intact a form as possible. Figure 1B shows the effects of HTG concentration on the solubilization of cytochromes, flavins, and proteins. The solubilizations of all these compounds except cytochrome *c* oxidase increased with an increase in the concentration of HTG: the solubilization of cytochrome

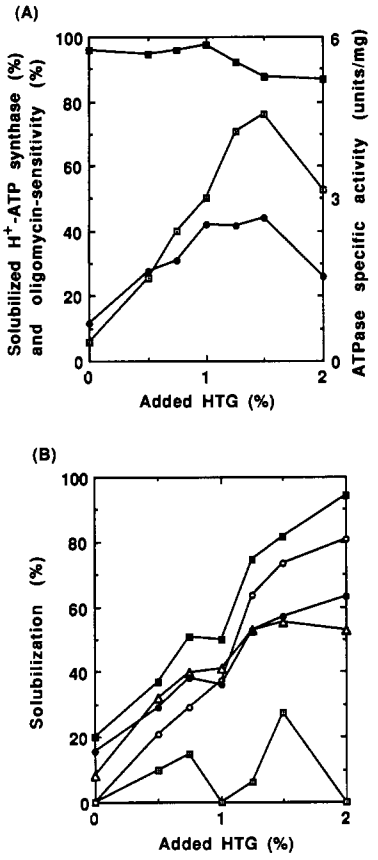

FIGURE 1: Dose–response curves for the solubilization of rat liver H^+-ATP synthase (A) and cytochromes, flavins, and proteins (B) from submitochondrial particles by HTG. Conditions were as described for step 1 of the purification of H^+-ATP synthase under Experimental Procedures, except that 25 mg of submitochondrial particles was treated by HTG at the concentrations indicated in 5 mL of PEG buffer. The suspensions obtained were diluted with an equal volume of PEG buffer and centrifuged at 105000*g* for 30 min. The resulting supernatants were used for assay of ATPase activity and the contents of cytochromes, flavins, and proteins. Other conditions were as described under Experimental Procedures. (A) Dose–response curve for the solubilization of H^+-ATP synthase: (□) total activity of solubilized H^+-ATP synthase; (■) oligomycin sensitivity of ATPase activity of solubilized H^+-ATP synthase; (●) ATPase specific activity of solubilized H^+-ATP synthase. (B) Dose–response curves for the solubilization of cytochromes, flavins, and proteins: (□) cytochrome *c* oxidase; (○) cytochrome *b*; (■) cytochromes $c + c_1$; (△) flavins; (●) proteins.

c oxidase was not dose-dependent. Fortunately, no cytochrome *c* oxidase was solubilized with 1% HTG in the present conditions.

Purification of H^+-ATP Synthase by Anion-Exchange HPLC. As shown in Figure 2, H^+-ATP synthase was eluted from a TSK DEAE-5PW column as a main peak with a retention time of 62.5 min. The ATPase activity of this material was almostly completely inhibited by oligomycin. This is the first report of the use of HPLC for purification of H^+-ATP synthase. The H^+-ATP synthase was eluted from the column with medium containing chloride, but could not be eluted with medium containing other anions such as phosphate or sulfate. The fractions in the shoulder with a retention time of about 58 min, which showed lower oligomycin sensitivity of ATPase activity of about 92%, seem to contain a small amount of F_1 (ATPase), since F_1 was easily removed by subsequent centrifugation in the presence of 20% glycerol (v/v).

A MonoQ-HR column (Pharmacia) could be used in place of the TSK DEAE-5PW column for purification of H^+-ATP synthase, but a higher concentration of chloride salt was required for elution of the enzyme from this column.

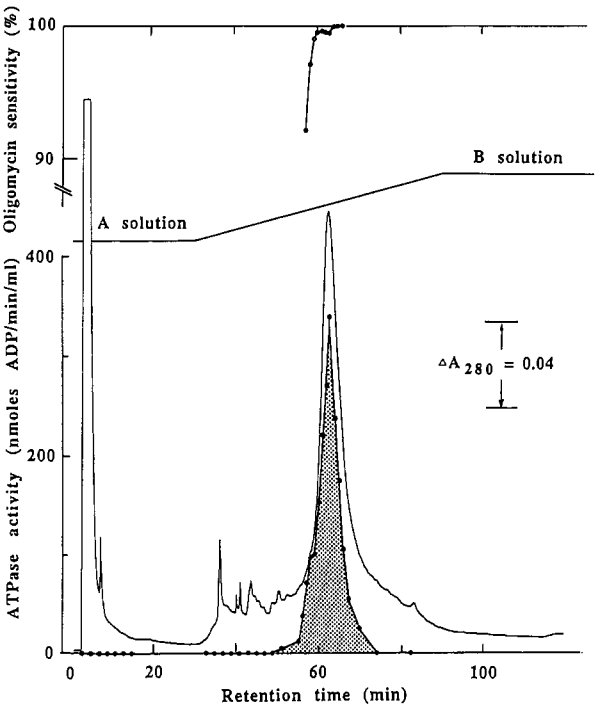

FIGURE 2: Anion-exchange HPLC on a TSK DEAE-5PW column of the solubilized rat liver H^+-ATP synthase. The solution from step 1 (10 mg of protein) was applied to a column (8 mm × 7.5 cm) of TSK DEAE-5PW (+ guard column) that had been equilibrated with PEG buffer. Other conditions were as described under Experimental Procedures.

Table I: Summary of Purification of Rat Liver H^+-ATP Synthase[a]

fraction	ATPase sp act. (μmol mg^{-1} min^{-1})	oligomycin sensitivity (%)	purification (*x*-fold)	total protein (mg)
mitochondria	0.40	100	1	3019
mitoplasts	0.52	100	1.3	2325
SMP[b]	3.00	92	7.5	600
HTG extract	2.95	95.2	7.4	350
HPLC	13.2	99.7	33	51.7
centrifugation	14.5	100	36.3	29.1

[a] All procedures were carried out as described under Experimental Procedures. [b] Submitochondrial particles.

Centrifugation in the Presence of Glycerol. H^+-ATP synthase eluted from the TSK DEAE-5PW column was easily precipitated by centrifugation at 155000*g* for 2 h in the presence of glycerol.

Table I summarizes typical results obtained in the purification of H^+-ATP synthase by the present method. As can be seen, the HPLC step was very effective for increasing the specific activity of the ATPase. The final preparation of H^+-ATP synthase had an ATPase activity of 14.5 units/mg of protein in the absence of added phospholipids. This activity is higher than those reported previously. This activity was completely inhibited by oligomycin, indicating that the purified H^+-ATP synthase was intact. The activity of the oligomycin-sensitive ATPase was stable for several days at 0–25 °C and for several months at −85 °C. Furthermore, in support of its intactness, the H^+-ATP synthase showed energy-dependent responses of ANS fluorescence and ATP–P_i exchange activity when it was reconstituted into proteoliposomes, as described below.

Reconstitution of Purified H^+-ATP Synthase into Proteoliposomes. The purified H^+-ATP synthase was reconstituted

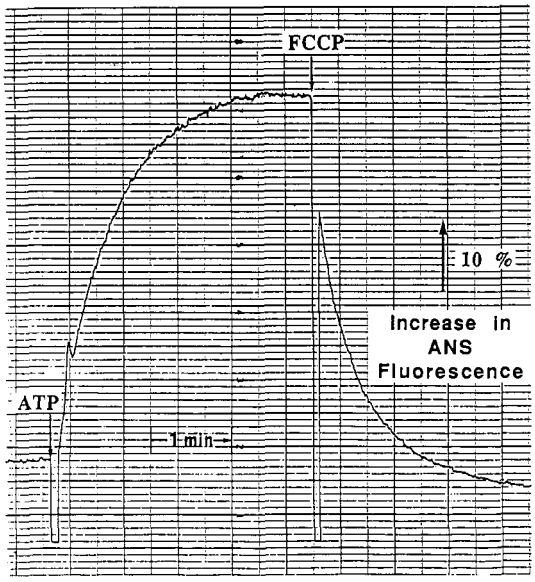

FIGURE 3: Enhancement of fluorescence intensity of ANS on addition of ATP to proteoliposomes containing rat liver H+-ATP synthase. The amounts of ATP and FCCP were 1.5 mM and 0.73 μg/mL, respectively. Other conditions were as described under Experimental Procedures.

Table II: ATP-P_i Exchange Activity of Proteoliposomes Containing Purified Rat Liver H+-ATP Synthase[a]

addition	ATP-P_i exchange act. (%)
none	100[b]
oligomycin (2 μg)	0.0
CCCP (2 μg)	0.0
ANS (100 μM)	60.0
ANS (200 μM)	36.1
ANS (300 μM)	18.4
ethidium (500 μM)	93.5

[a] Conditions were as described under Experimental Procedures. [b] The exchange activity was 87 nmol (mg of protein)$^{-1}$ min^{-1}. ATPase activity was 13.0 μmol (mg of protein)$^{-1}$ min^{-1} in reconstituted proteoliposomes.

into proteoliposomes. Figure 3 shows the energy-dependent response of ANS fluorescence in the reconstituted proteoliposomes. Addition of ATP increased the ANS fluorescence, and this effect was completely reversed by an uncoupler (FCCP) or oligomycin (data not shown), indicating that the sidedness of the reconstituted vesicles was the same as that of submitochondrial particles, in which F_1 is located on the outside of the membrane. Table II also shows that the reconstituted vesicles showed ATP-P_i exchange activity and that this activity was completely inhibited by oligomycin or the uncoupler. This indicates that the purified preparation of H+-ATP synthase retained its intactness as a H+-ATP synthase complex. The exchange activity was also inhibited by ANS, which is an anisotropic inhibitor of energy transduction in rat liver submitochondrial particles and spinach chloroplasts by acting from the F_1 side of F_o, presumably by inhibiting charge movement in F_o (Higuti, 1984a,b; Higuti et al., 1989). However, ethidium (500 μM), which is a positively charged anisotropic inhibitor of energy transduction, caused only slight decrease in ATP-P_i exchange activity in the vesicles as expected (Higuti et al., 1978a,b, 1979, 1980, 1983, 1984).

Composition. The preparation was essentially devoid of cytochrome *c* oxidase, cytochrome *c*, and the cytochrome *b*–c_1 complex, but contained 0.2 nmol of cytochrome *b*-559 (Higuti et al., 1975) per milligram of protein. However, this contaminating cytochrome *b*-559 gave only a faint band at an

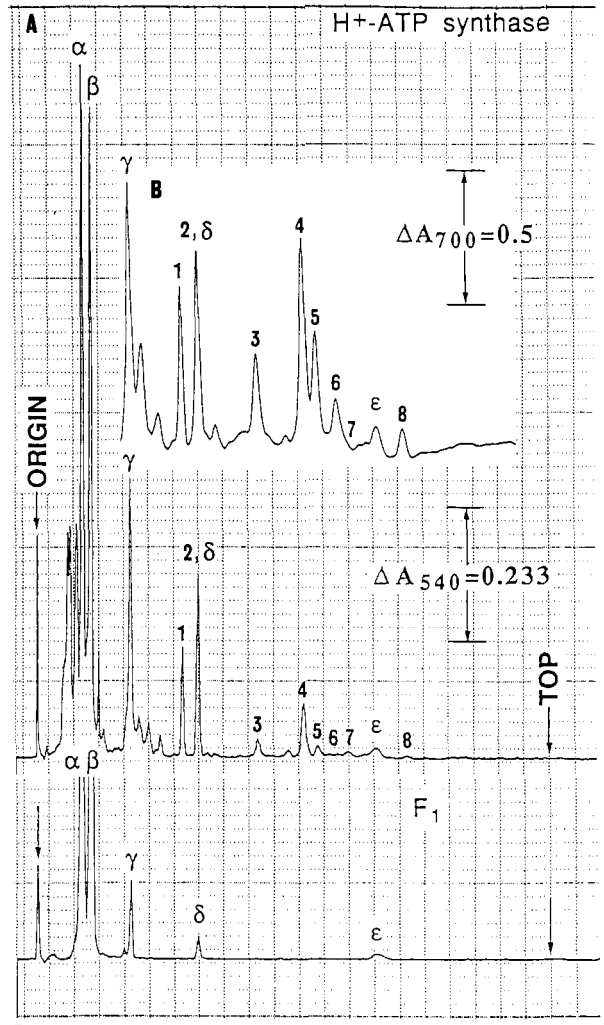

FIGURE 4: SDS/urea gel electrophoresis on a polyacrylamide gel of purified rat liver H+-ATP synthase and F_1. Conditions were as described under Experimental Procedures. The molecular mass standards used were prestained standards from Bio-Rad (50.0 kDa, ovalbumin; 39.0 kDa, carbonic anhydrase; 27.0 kDa, soybean trypsin inhibitor; 17.0 kDa, lysozyme) and standards from Sigma [14.4 kDa, myoglobin (fragment I + II); 8.2 kDa, myoglobin (fragment I); 6.2 kDa, myoglobin (fragment II); 2.5 kDa, myoglobin (fragment III)]. (A) SDS/urea–polyacrylamide gel electrophoretograms of purified rat liver H+-ATP synthase (30 μg) and F_1 (20 μg), stained with Coomassie Blue only, were scanned with a Shimazu, model CS-9000, two-wavelength flying-spot scanner at 540 nm. (B) SDS/urea–polyacrylamide gel electrophoretogram of purified rat liver H+-ATP synthase (30 μg) stained with Coomassie Blue and then with a Bio-Rad silver staining kit was scanned at 700 nm.

apparent molecular weight of 28 800 on SDS–polyacrylamide gel electrophoresis (Figure 4). The activity of succinate dehydrogenase was 0.008 μmol (mg of protein)$^{-1}$ min^{-1}, indicating that contamination with the dehydrogenase was less than 1%. No NADH dehydrogenase activity was detected in the preparation.

Subunit Composition of Purified H+-ATP Synthase. Figure 4 shows densitometric traces of the SDS/urea–polyacrylamide gel electrophoretograms of purified H+-ATP synthase and F_1. In the present work, rat liver F_1 was also purified by anion-exchange HPLC as described under Experimental Procedures. F_1 was eluted as a single peak on HPLC (data not shown) and was composed of five polypeptides named subunits α, β, γ, δ, and ε (Figure 4A).

For estimation of contamination of purified H+-ATP synthase, the bands were doubly stained with Coomassie Blue and silver. (Silver staining is known to be a more sensitive method

Table III: Polypeptide Composition of H^+-ATP Synthases Purified from Rat Liver and Bovine Heart[a]

rat liver H^+-ATP synthase				bovine heart H^+-ATP synthase	
according to Pedersen's group		present work			
app molecular mass (kDa)	polypeptides	app molecular mass (kDa)	polypeptides	app molecular mass (kDa)	polypeptides
62.5	α^b	53.0	α	53.0	α^b
57.0	β^b	49.0	β	50.0	β^d
33.8	γ^b	34.7	γ	33.0	γ^d
28	$(1')^{c,j}$	22.4	subunit b (1)	24.0	subunit b[e]
23	$(1)^c$	19.5	(2)	21.0	OSCP[f]
20	$(2)^e$	19.5	δ	19.0	subunit a[g]
<20	$(3)^c$	11.7	(3)	19.0	subunit d[e]
13.5	δ^c	7.9	(4)	17.0	δ^d
13.5	$(4)^c$	7.1	factor 6 (5)	10.0	inhibitor[g]
11	$(4')^{c,j}$	6.1	chargerin II (6)	9.0	factor 6[f]
8–10	subunit c $(5)^c$	5.3	subunit c (7)	<9.0	A6L[g]
7	ϵ^b	4.4	ϵ	8	subunit c[h,i]
7	$(5')^{c,j}$	3.6	(8)	7.5	ϵ^d

[a] Apparent molecular masses of polypeptides were estimated from the SDS–polyacrylamide gel electrophoretograms of H^+-ATP synthases. [b] Soper et al. (1979). [c] McEnery et al. (1989). [d] Walker et al. (1985). [e] Walker et al. (1987b). [f] Walker et al. (1987a). [g] Fearnley and Walker (1986). [h] Garf and Sebald (1978). [i] Sebald and Hoppe (1981). [j] Faint bands.

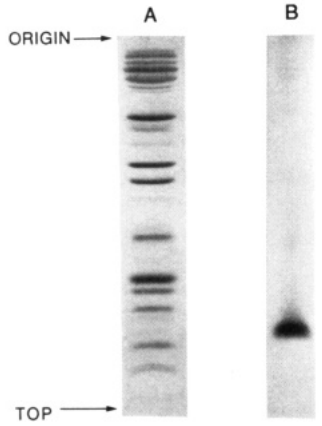

FIGURE 5: Fluorogram of H^+-ATP synthase labeled with [^{14}C]DCCD. Purified rat liver H^+-ATP synthase [30 μg (lane B)] labeled with [^{14}C]DCCD was subjected to electrophoresis and fluorography as described under Experimental Procedures. Lane A, SDS/urea–polyacrylamide gel electrophoretogram of purified rat liver H^+-ATP synthase (30 μg) doubly stained with Coomassie Blue and a Bio-Rad silver staining kit.

for detecting polypeptides in the gel.) Seven main bands and one minor band of DCCD-binding protein (subunit c) (Figure 5) are seen in addition to five bands of F_1 subunits. Since only band 7 was labeled with [^{14}C]DCCD (Figure 5), this band was considered to be subunit c. Subunit c (band 7) was faintly stained with Coomassie Blue but not stained with silver (Figure 4 B). Band 6 was chargerin II, which is encoded by the mitochondrial A6L gene (Uchida et al., 1987; Higuti et al., 1981, 1985, 1988a; Oda et al., 1989; Muraguchi et al., 1990), that was determined from a radioimmunogram of a Western blot (Muraguchi et al., 1990). As seen in Figure 4A,B, the band of chargerin II was hardly stained with Coomassie Blue, but it was clearly stained with silver, in good accord with our previous finding (Higuti et al., 1988a). The addition of urea was helpful for obtaining sharp bands in the gel, especially of molecules of lower molecular weight, as reported (Merle & Kadenbach, 1980).

Faint bands were detected between β and γ, between γ and band 1, and between bands 2 and 3 (Figure 4). The thickness of a stained band does not always reflect the amount of polypeptide, but from their apparent molecular weights and thicknesses relative to those of the bands of F_1 subunits close to them, we concluded that these faint bands were those of

```
                    1       9
Rat band δ          AQAAASPAP
Bovine δ subunit    AEAAAAQAP
                    1                  18                  36
Rat band ε          VAYWRQAGLSYIRFSQI-AKAVRDALKTEFKANAEK
Bovine ε subunit    VAYWRQAGLSYIRYSQICAKAVRDALKTEFKANAMK
                    1                                  34
Rat band 5          NKELDPVQKLFLDKIREYKAKRLASGGPVDTGPE
Bovine factor 6     NKELDPVQKLFVDKIREYRTKRQTSGGPVDAGPE
                    1              27
Rat band 1          PLPPLPEYGGKVRLGLIPEEFFQFLYP
Bovine subunit b    PVPPLPEHGGKVRFGLIPEEFFQFLYP
```

FIGURE 6: Partial amino acid sequences of subunits δ and ϵ, factor 6, and subunit b purified from rat liver H^+-ATP synthase and their homologous sequences of bovine H^+-ATP synthase. Conditions were as described under Experimental Procedures and also in the text.

contaminants. Thus, the F_o of H^+-ATP synthase purified in the present work could be composed of eight different polypeptides.

Subunit Separation and Partial Sequence. The components of purified H^+-ATP synthase were separated into 11 main peaks on a reverse-phase column of Asahipak ODP-90 (data not shown). The partial sequences of peaks 1, 2, and 10 were determined in an Applied Biosystems sequenator (Figure 6). A search for sequence homologies showed that the sequence of the 35 N-terminal amino acids of peak 1 was also highly homologous with that of the bovine ϵ subunit (Vinas et al., 1990), 33 of 35 amino acids being identical. On automated Edman degradation of the intact ϵ subunit, no PTH-amino acid was detected at cycle 18. However, from cycle 19, PTH-amino acids were consistently detected. The amino acid at position 18 could be cysteine, because cysteine is the only amino acid decomposed during Edman degradation, and position 18 of the bovine ϵ subunit was deduced to be cysteine from its cDNA sequence. The sequence of the 34 N-terminal amino acids of peak 2 (band 5 in Figure 4) was highly homologous with that of bovine coupling factor 6 (Fang et al., 1984; Walker et al., 1987a), 29 of 34 amino acids being identical. The sequence of the 27 N-terminal amino acids of peak 10 (band 1 in Figure 4) was highly homologous with that of subunit b of bovine heart deduced from the sequence of its cDNA (Walker et al., 1987b), 24 of 27 residues being identical. The sequence of the nine N-terminal amino acids of band δ, which was recovered electrophoretically from the gel after SDS/urea–polyacrylamide gel electrophoresis of F_1, was also highly homologous with the amino acid sequence of the bovine δ subunit of F_1, showing identity of six of nine residues (Walker et al., 1985).

This is the first report of the presence of subunit b, factor 6, and chargerin II in H+-ATP synthase of rat liver mitochondria.

Discussion

In the present work, we developed a simple and rapid method for purification of H+-ATP synthase from rat liver mitochondria by HPLC. H+-ATP synthase could be purified from submitochondrial particles in a single day. This method was confirmed to be reproducible by repeated trials by several of us. For obtaining highly active H+-ATP synthase, it was essential to prepare active submitochondrial particles from intact mitoplasts. To prepare intact mitoplasts, we used purified digitonin and decreased the amount of digitonin used for the treatment of mitochondria to 0.04 mg/mg of protein, which did not affect the yield of mitoplasts appreciably.

The ATPase activity of the purified H+-ATP synthase was 12–15 units/mg of protein in the absence of phospholipids, which was higher than the activities reported previously (Serrano et al., 1976; Rott & Nelson, 1981; Galante et al., 1981; Hughes et al., 1982, 1983; Penin et al., 1982; McEnery et al., 1983, 1989), and this activity was completely inhibited by oligomycin. On reconstitution of the H+-ATP synthase into proteoliposomes, an ATP–P_i exchange activity of 70–90 nmol (mg of protein)$^{-1}$ min^{-1}, which was completely inhibited by oligomycin or an uncoupler, was observed in repeated experiments. This exchange rate is comparable with the ATP–P_i exchange activities of other detergent-solubilized preparations of H+-ATP synthase (Serrano et al., 1976; Galante et al., 1979; Rott & Nelson, 1981; McEnery et al., 1983, 1989), but lower than that of H+-ATP synthase purified using lysolecithin (Hughes et al., 1982, 1983; Penin et al., 1982). Further studies are required to clarify the reason for this discrepancy between detergent-solubilized and lysolecithin-solubilized preparations.

The preparation of H+-ATP synthase purified in the present work seemed to contain eight different polypeptides as F_o subunits beside the five subunits of F_1 (Table III). Subunit b, factor 6, chargerin II, and subunit c were identified as subunits of F_o from rat H+-ATP synthase in the present work. Further studies are required to identify the other possible constituents of F_o. Table III also shows the polypeptides of H+-ATP synthase purified from rat liver mitochondria by Pedersen and his colleague and those of H+-ATP synthase purified from bovine heart mitochondria. The discrepancies in apparent molecular masses of polypeptides in these preparations of H+-ATP synthase could be due to differences in the standard proteins used and in the experimental conditions for gel electrophoresis.

The present simpie and rapid preparative method for purification of H+-ATP synthase with the ability for energy transduction from rat liver mitochondria should be useful for studies on the molecular architecture (Tsurumi et al., 1990; Higuti et al., 1990) and also for study of the mechanism of energy transduction by mitochondrial H+-ATP synthase, especially to measure the charge movement in F_o driven by an electrochemical potential of protons (Higuti, 1984a,b; Higuti et al., 1989).

Acknowledgments

We thank Drs. Hideo Ohkawa, Kenji Oeda, and Masatoshi Shimizu (Takarazuka Research Center, Sumitomo Chemical Co. Ltd.) for protein sequencing and Drs. Eiro Muneyuki (Saitama University) and Hajime Hirata (Jichi Medical School) for suggestions on the method for reconstituting H+-ATP synthase into lipid vesicles.

References

Bonner, W. M., & Lanskey, R. A. (1974) *Eur. J. Biochem. 46*, 83–88.
Cham, T. L., Greenwalt, J. W., & Pedersen, P. L. (1970) *J. Cell. Biol. 45*, 201–305.
Chance, B. (1957) *Methods Enzymol. 4*, 273–329.
Cross, R. L. (1981) *Annu. Rev. Biochem. 50*, 681–714.
Fang, J. K., Jacobs, J. W., Kanner, B. I., Racker, E., & Bradshaw, R. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6603–6607.
Fearnley, I. M., & Walker, J. E. (1986) *EMBO J. 5*, 2003–2008.
Fillingame, R. H. (1980) *Curr. Top. Bioenerg. 11*, 35–106.
Futai, M., Noumi, T., & Maeda, M. (1989) *Annu. Rev. Biochem. 58*, 111–136.
Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1979) *J. Biol. Chem. 254*, 12372–12378.
Galante, Y. M., Wong, S.-Y., & Hatefi, Y. (1981) *Arch. Biochem. Biophys. 211*, 643–651.
Graf, T., & Sebald, W. (1978) *FEBS Lett. 94*, 218–222.
Hatefi, Y. (1985) *Annu. Rev. Biochem. 54*, 1015–1069.
Higuti, T. (1984a) *Mol. Cell. Biochem. 61*, 37–61.
Higuti, T. (1984b) *Seikagaku 56*, 151–183.
Higuti, T., Mizuno, S., & Muraoka, S. (1975) *Biochim. Biophys. Acta 396*, 36–47.
Higuti, T., Yokota, M., Arakaki, N., Hattori, A., & Tani, I. (1978a) *Biochim. Biophys. Acta 503*, 211–222.
Higuti, T., Arakaki, N., Yokota, M., Hattori, A., & Tani, I. (1978b) *FEBS Lett. 87*, 87–91.
Higuti, T., Arakaki, N., & Hattori, A. (1979) *Biochim. Biophys. Acta 548*, 166–171.
Higuti, T., Arakaki, N., Niimi, S., Nakasima, S., Saito, R., Tani, I., & Ota, F. (1980a) *J. Biol. Chem. 255*, 7631–7636.
Higuti, T., Niimi, S., Saito, R., Nakasima, S., Ohe, T., Tani, I., & Yoshimura, T. (1980b) *Biochim. Biophys. Acta 593*, 463–467.
Higuti, T., Ohe, T., Arakaki, N., & Kotera, Y. (1981) *J. Biol. Chem. 256*, 9855–9860.
Higuti, T., Arakaki, R., Kotera, Y., Takigawa, M., Tani, I., & Shibuya, M. (1983) *Biochim. Biophys. Acta 725*, 1–9.
Higuti, T., Takigawa, M., Kotera, Y., Oka, H., Uchida, J., Arakaki, R., Fujita, T., & Ogawa, T. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1331–1335.
Higuti, T., Negama, T., Takigawa, M., Uchida, J., Yamane, T., Asai, T., Tani, I., Oeda, K., Shimizu, M., Nakamura, K., & Ohkawa, H. (1988a) *J. Biol. Chem. 263*, 6772–6776.
Higuti, T., Yoshihara, Y., Kimura, M., Oda, T., Muraguchi, M., Tani, I., Futaki, S., & Kitagawa, K. (1988b) *Bioenergetics 14*, 96–97.
Higuti, T., Nagase, H., Yoshihara, Y., Tani, I., & Inoue, T. (1989) *Seikagaku 61*, 1101.
Higuti, T., Osaka, F., Yoshihara, Y., Tsurumi, C., Kawamura, Y., Tani, I., Toda, H., Kakuno, T., Sakiyama, F., Tanaka, K., & Ichihara, A. (1990) *Biochem. Biophys. Res. Commun. 171*, 1079–1086.
Hughes, J., Joshi, S., Torok, K., & Sanadi, D. R. (1982) *J. Bioenerg. Biomembr. 14*, 287–295.
Hughes, J., Joshi, S., & Sanadi, D. R. (1983) *FEBS Lett. 153*, 441–446.
Joshi, S., Hughes, J. B., Shaikh, F., & Sanadi, D. R. (1979) *J. Biol. Chem. 254*, 10145–10152.
Kagawa, Y. (1978) *Biochim. Biophys. Acta 505*, 45–93.
Kagawa, Y., & Racker, E. (1966) *J. Biol. Chem. 241*, 2461–2466.

Laemmli, U. K. (1970) *Nature 227*, 680–685.
McEnergy, M. W., Buhle, E. L., Jr., Aebi, U., & Pedersen, P. L. (1983) *J. Biol. Chem. 259*, 4642–4651.
McEnery, M. W., Hullihen, J., & Pedersen, P. L. (1989) *J. Biol. Chem. 264*, 12029–12036.
Merle, P., & Kadenbach, B. (1980) *Eur. J. Biochem. 105*, 499–507.
Mitchell, P. (1961) *Nature 191*, 423–427.
Muneyuki, E., Ohno, K., Kagawa, Y., & Hirata, H. (1987) *J. Biochem. 102*, 1433–1440.
Muraguchi, M., Yoshihara, Y., Tsunemitu, T., Tani, I., & Higuti, T. (1990) *Biochem. Biophys. Res. Commun. 168*, 226–231.
Oda, T., Futaki, S., Kitagawa, K., Yoshihara, Y., Tani, I., & Higuti, T. (1989) *Biochem. Biophys. Res. Commun. 165*, 449–456.
Penin, F., Godinot, C., Comte, J., & Gautheron, D. C. (1982) *Biochim. Biophys. Acta 679*, 198–209.
Rott, R., & Nelson, N. (1981) *J. Biol. Chem. 256*, 9224–9228.
Sebald, W., & Hoppe, J. (1981) *Curr. Top. Bioenerg. 12*, 2–64.
Serrano, R., Kanner, B. I., & Racker, E. (1976) *J. Biol. Chem. 251*, 2453–2461.
Shimamoto, T., Saito, S., & Tsuchiya, T. (1985) *J. Biochem. 97*, 1807–1810.
Slater, E. C (1987) *Eur. J. Biochem. 166*, 489–504.
Soper, J. W., Decker, G. L., & Pedersen, P. L. (1979) *J. Biol. Chem. 254*, 11170–11176.
Stiggall, D. L., Galante, Y. M., & Hatefi, Y. (1978) *J. Biol. Chem. 253*, 956–964.
Tsuchiya, T., & Saito, S. (1984) *J. Biochem. 96*, 1593–1597.
Tsurumi, C., Yoshihara, Y., Osaka, F., Yamada, F., Tani, I., Higuti, T. Shimizu, M., Oeda, K., Ohkawa, H., Toda, H., Kakuno, T., Sakiyama, F., Kumatori, A., Tanaka, K., & Ichihara, A. (1990) *Biochem. Biophys. Res. Commun. 169*, 136–142.
Uchida, J., Takigawa, M., Yamane, N., & Negama, T., Tani, I., & Higuti, T. (1987) *Biochem. Biophys. Res. Commun. 146*, 953–958.
Vinas, O., Powell, S. J., Ranswick, M. J., Iacobazzi, V., & Walker, J. E. (1990) *Biochem. J. 265*, 321–326.
Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., & Tybulewicz, V. L. J. (1985) *J. Mol. Biol. 184*, 677–701.
Walker, J. E., Gay, N. J., Powell, S. J., Kostina, M., & Dyer, M. R. (1987a) *Biochemistry 26*, 8613–8619.
Walker, J. E., Runswick, M. J., & Poulter, L. (1987b) *J. Mol. Biol. 197*, 89–100.
Williams, N., Amzel, L. H., & Pedersen, P. L. (1984) *Anal. Biochem. 140*, 581–588.