ELSEVIER

Biochimica et Biophysica Acta 1273 (1996) 62–70

BBA Biochimica et Biophysica Acta

Reconstitution of the F_1-ATPase activity from purified α, β, γ and δ or ϵ subunits with glutathione *S*-transferase fused at their amino termini

Yongchol Shin, Ken Sawada, Tadashi Nagakura, Masamitsu Miyanaga, Chie Moritani, Takato Noumi, Tomofusa Tsuchiya, Hiroshi Kanazawa *

Department of Biotechnology, Faculty of Engineering Sciences, Okayama University, Okayama 700, Japan

Received 21 June 1995; revised 19 September 1995; accepted 25 September 1995

Abstract

Systems for overexpression and purification of active α, β and γ subunits of *Escherichia coli* H^+-ATPase were established. The α and β subunits recovered as soluble form were purified by hydroxyapatite column chromatography. Since the γ subunit was overexpressed as the insoluble form, this subunit was purified by polyacrylamide gel-electrophoresis containing sodium dodecyl sulfate. By subsequent denaturation of this subunit with guanidine hydrochloride and renaturation, the active γ subunit for reconstitution of the F_1-ATPase activity with the purified α and β subunit was obtained. The δ and ϵ subunits which were fused to the carboxy terminus of glutathione *S*-transferase (GST) were overproduced and purified by affinity chromatography. These fused proteins (δ-GST and ϵ-GST) were incubated with the purified α, β and γ subunits and applied to affinity chromatography. The $\alpha\beta\gamma\delta$-GST and $\alpha\beta\gamma\epsilon$-GST complex were eluted specifically by addition of glutathione and exhibited high and low ATPase activity, respectively, with a subunit stoichiometry similar to that in the native F_1-ATPase, indicating that active complexes could be reconstituted with the fused proteins. These results suggested that the amino-terminal ends of the δ and ϵ subunits are not involved in formation of the active complex. The fused ϵ-GST bound the γ subunit strongly, and the α subunit weakly. The δ-GST bound the γ subunit significantly, and the α and β subunits very weakly.

Keywords: ATPase, F_1-; ATPase, H^+-; Subunit reconstitution; Glutathione *S*-transferase; Overexpression; Expression plasmid; (*E. coli*)

1. Introduction

The proton translocating ATPase F_1F_0 has a central role in ATP synthesis in mitochondria, chloroplasts and bacterial membranes [1–6]. The enzyme also catalyzes ATP hydrolysis as the reverse reaction. The ATP hydrolytic activity is found in the membrane peripheral portion F_1, which has five non-identical subunits, α, β, γ, δ and ϵ. The membrane integral portion F_0 forms the proton channel and has three subunits, a, b and c in *Escherichia coli*. The ATP hydrolytic activity was reconstituted previously from the purified *E. coli* α, β and γ subunits [7]. The active center of the enzyme is in the β subunit or the interface of the α and β subunits, with the δ and ϵ subunits forming a connecting portion between the $\alpha\beta\gamma$ core-complex and F_0 portion. The ϵ subunit has F_1-ATPase inhibitory activity [1–6].

The unique mechanisms in coupling of the proton flow with the hydrolysis or synthesis of ATP have not been clarified at the molecular level. However, it is believed that protons are not required directly for catalysis but a conformational change in the F_1 portion caused by the access of a proton through the F_0 portion plays a key role in catalysis [5]. To understand this hypothetical conformational change, interactions of the F_1 subunits should be resolved at the amino acid level. X-ray crystallographic data were obtained for the mitochondrial $\alpha\beta\gamma$ complex [8]. In this report, a dynamic rotation model for catalysis was proposed, in which the $\alpha\beta$ core rotates around the γ subunit. However, dynamic interactions of these subunits involved in the rotation may not be resolved only by

Abbreviations: F_1F_0 ATPase, proton translocating ATPase; SDS, sodium dodecyl sulfate; GST, glutathione *S*-transferase; IPTG, isopropyl thiogalactoside; PBS, phosphate-buffered saline.

* Corresponding author. Fax: +81 86 2537399; e-mail: kanazawa@cc.okayama-u.ac.jp.

SSDI 0005-2728(95)00132-8

crystal data which give a static structure of the residues. For determination of dynamic mechanisms, biochemical analysis together with genetic studies of F_1F_0 subunits at their amino acid level is still important.

Not only the dynamic mechanisms of the subunit interactions, but also the assembly process of the subunits is not known. To study such in vitro interactions and assembly process of the F_1-ATPase subunits, in vitro reconstitution of the F_1-ATPase from the purified subunits is useful. Such a system was established by Futai and Dunn for the F_1 complex [7], in which the α, β and γ subunits were purified from the F_1 complex after dissociating the complex into each subunit [7]. However, the preparation of each subunit by these procedures is time-consuming, especially for the γ subunit. Since the γ subunit is contained in the complex at levels stoichiometrically lower than the α and β subunits, it was difficult to obtain a large quantity of this subunit for reconstituting the F_1-ATPase. Overproduction of each subunit in *E. coli* directly by expressing each gene on an expression vector may be convenient for large-scale preparation of the subunits. The β subunit was shown to be overproduced by an expression plasmid in *E. coli* and purified [9]. However, for the α and γ subunits such systems have not been developed. Here, we report a new simple procedure to obtain reconstitutively active α and γ subunits.

For investigation of the subunit interactions and assembly mechanisms using in vitro systems, a simple method for detecting functionally inactive intermediate complexes, if available, is useful. For establishing such a system, we prepared the δ and ϵ subunits fused to glutathione *S*-transferase (GST). Inactive intermediate complexes containing the δ or ϵ subunit fused to GST and other subunits could be fixed to solid beads through glutathione as the ligand and will be released with addition of excess free glutathione. In the present study, we analyzed whether these proteins could reconstitute the F_1-ATPase activity with the purified α, β and γ subunits. Although the amino termini of these proteins were blocked by the fused proteins, they could reconstitute the active ATPase complex. These systems may be convenient in studying the subunit interactions of F_1-ATPase.

2. Materials and methods

2.1. E. coli strains and culture conditions

E. coli strains KM230 [10], BL21 (DE3) [11] or KY7485 [10] carrying a transducing phage λ (λ asn-5) [12] encoding the entire ATPase operon were used for overexpression of F_1 subunits or F_1F_0 complex. *E. coli* cells were grown in synthetic M9ZB medium [11] or Tanaka medium [10] supplemented with glucose or glycerol as the carbon source at 37°C or at the desired temperature with vigorous shaking.

2.2. Construction of expression plasmids for the α, β, γ, δ-GST or ϵ-GST protein

The α and β subunit expression plasmids based on the pET expression plasmid [13] were constructed previously [11,14]. The γ subunit expression plasmid was also constructed with the pET plasmid in this study, from which the γ subunit gene is transcribed by T7 polymerase. The coding sequence of the γ subunit gene was prepared by amplification of the wild-type DNA from KM230 as the template for polymerase chain reaction (PCR) as described previously [15]. Primer oligonucleotides GEF-N3 (5′-TACTCCATGGCCGGCGCAAAAGAG) and GER-C2 (5′-GACTGGATCCTGTTTAAACCGCGGC) were used with Pfu polymerase (Stratagene) and a programmable incubator (Driblock PHC-1, Techne, London). GEF-N3 and GER-C2 contain the amino-terminal and the carboxy-terminal portions of the γ subunit together with a recognition sequence for restriction endonucleases *Nco*I and *Bam*HI at their 5′ ends, respectively. The amplified DNA was separated from free nucleotides by agarose gel electrophoresis and the desired DNA was eluted from the gel electrophoretically. The DNA was then digested by the restriction endonucleases *Nco*I and *Bam*HI and the coding sequence was inserted between the *Nco*I and *Bam*HI sites in the expression vector pET3d. [13]

The expression plasmids of the fused ϵ-GST or δ-GST genes were constructed as follows: primer nucleotides DEF-N2 for the amino-terminal end of the δ subunit (5′-TTTGGATCCATGTCTGAATTTATTACGGTA), DER-C1 for the carboxy-terminal end of the δ subunit (5′-CCTTGGATCCCCTTAAGACTGCAA), EEF-N2 for the amino-terminal end of the ϵ subunit (5′-TTTTG-GATCCATGGCAATGACTTACCACCTG), and EER-C1 for the carboxy-terminal end of the ϵ subunit were synthesized for PCR. All oligonucletides had the *Bam*HI recognition sequence at the 5′ end. Using these primers and wild-type DNA from KM230, the DNAs for the δ and ϵ genes were amplified with Pfu polymerase under the previously described conditions [11]. The amplified DNAs were digested by *Bam*HI and the desired DNA fragments were purified by agarose gel electrophoresis. These fragments were introduced into the unique *Bam*HI site at the carboxy-terminal end of the GST gene in the fusion vector plasmid GEX-2T [10]. The resultant chimeric plasmids were introduced into host cells, *E. coli* BL21(DE3), by the cold $CaCl_2$ method [11]. The nucleotide sequences of the cloned γ, δ and ϵ subunit genes were confirmed by nucleotide sequencing.

2.3. Overexpression of the α, β, γ, δ-GST or ϵ-GST proteins

E. coli BL21(DE3) carrying the expression plasmids for the α, β, γ, δ-GST or ϵ-GST proteins were cultured in

M9ZB medium at 30°C and their growth were monitored by turbidity of the culture using a photometer at 600 nm. When the culture reached an A_{600} of 0.4, gene expression was induced by adding 0.4 mM IPTG. After incubation of 1 liter of culture at 30°C for 3 h, cells were harvested, washed in phosphate-buffered saline (PBS) once and then suspended in PBS containing 6 mM benzamidine.

2.4. Purification of the α, β or γ subunit

From the cells containing overproduced α, β or γ subunit, membranes and cytoplasmic fractions were prepared as described previously [7] after disrupting the cells with a French pressure system (5700 lb/inch^2). The α and β subunits in the cytoplasmic fraction were concentrated by ammonium sulfate precipitation (40% saturation). After dialysis of the concentrated materials to remove excess ammonium sulfate, the samples were applied to hydroxyapatite column chromatography as described previously [7]. In cases where the purity was not adequate, eluted materials were rechromatographed on phenyl-Sepharose columns [7]. From 1 liter of typical culture, 16.3 mg of α (2.2 g wet cells), 17.3 mg of β (2.1 g wet cells) were purified.

Since the γ subunit was recovered completely in the membrane fraction or the precipitate fraction after low centrifugation of the disrupted cells (3000 × *g* for 10 min), this subunit was purified after denaturating the insoluble materials once by SDS-polyacrylamide gel electrophoresis (12.5% acrylamide). The protein band corresponding to the γ subunit was visualized by soaking the gel in 4 M sodium acetate and then eluted from the gel slice containing the γ subunit. From 0.34 g of wet cells, 520 μg of the purified γ subunit was obtained.

2.5. Purification of the fused δ-GST and ϵ-GST proteins

In a typical experiment for ϵ-GST preparation, 2.6 g of cells containing overproduced ϵ-GST from 1 liter of culture was suspended in 6 ml of PBS with 2% Triton X-100 and disrupted by sonication as described previously [12]. The supernatant fraction obtained after centrifugation for 60 min at 105000 × *g* containing 170 mg protein was applied directly onto the affinity chromatography column containing 3 ml of glutathione-Sepharose resin (Pharmacia Biolabs) equilibrated with PBS plus 2% Triton X-100. The loaded column was washed with 30 ml PBS and the fused proteins were eluted with 15 ml PBS containing 10 mM glutathione, and 49.9 mg of purified ϵ-GST was thus obtained. For the fused δ-GST, a similar scale of preparation was performed and similar recovery of the proteins at each step was observed.

Reconstitution of the ATPase, affinity chromatography and assays. For reconstitution of the ATPase activity, the purified α, β and γ subunits were mixed at a molar ratio of 3:3:1 to 3:3:3 and dialyzed against reconstitution buffer (50 mM succinate-Tris (pH 6.0), 5 mM ATP, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA and 10% glycerol) at room temperature for 8 h. For reconstitution of the ATPase with the fused proteins, δ-GST or ϵ-GST was added to the α, β and γ subunit mixture. To test formation of the ATPase complex with fused proteins, the mixtures of subunits and the fused proteins were applied onto the glutathione-Sepharose column as described in the purification section for the fused proteins. The ATPase activity and content of the protein in an aliquot of the dialyzed or eluted materials was assayed as described previously [15].

Binding of the subunits to GST fusion proteins. 15 μg of δ-GST, ϵ-GST or GST was mixed with 20 μl glu-

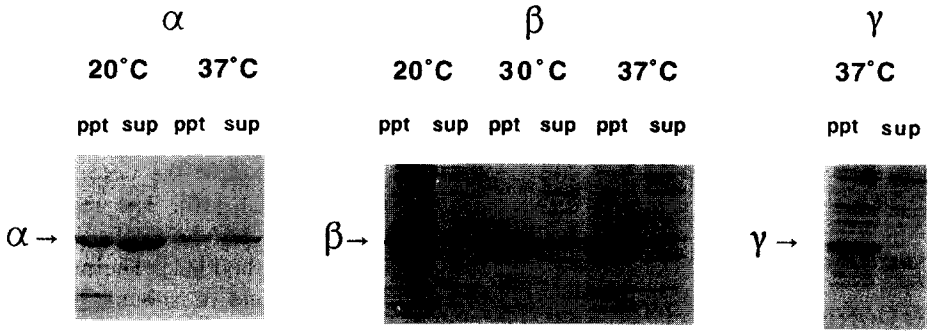

Fig. 1. Effects of temperature on overexpression and distribution of the α, β and γ subunits. 15-ml cultures of *E. coli* BL21 (DE3) carrying expression plasmids for the α, β or γ subunit gene were incubated at 37°C until culture turbidity determined at $A_{600\,nm}$ reached 0.1. Then, the culture was shifted to the various temperatures shown at the top of the panels in the presence or absence of inducer IPTG and further incubated for 10 h at 20°C, for 5 h at 30°C or for 2 h at 37°C. Membrane (ppt) and cytoplasmic fractions (sup) were obtained after centrifugation for 60 min at 105000 × *g* from cells disrupted by sonication. The membrane fraction was suspended in buffer (50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 10% glycerol, 6 mM benzamidine). 1 μg of cytoplasmic protein, and membrane protein, which amount was equivalent to those calculated from 1 μg of cytoplasmic protein in the same ratio of total amounts of the cytoplasmic vs. membrane fractions, were applied to SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue as described previously [12].

Table 1
Reconstitution of the $\alpha\beta\gamma$ complex using various γ subunit preparations

Preparation	Reconstituted ATPase activity (units/mg)
Guanidinium HCl 2.5 M	14.5
Guanidinium HCl dialysis	3.5
Electroelution	0.5

The α (10 μg), β (10 μg), and γ (2 μg) subunits were mixed and dialyzed against reconstitution buffer at room temperature for 8 h. The γ subunit was electroeluted from the gel matrix after SDS-polyacrylamide gel electrophoresis (Electroelution). Electroeluted γ subunit was concentrated by cold acetone and then dissolved in 2.5 M guanidium HCl. Aliquots of the subunit preparations were used for reconstitution after removing guanidium HCl by dialysis. An aliquot of the reconstituted sample was used for the ATPase assay.

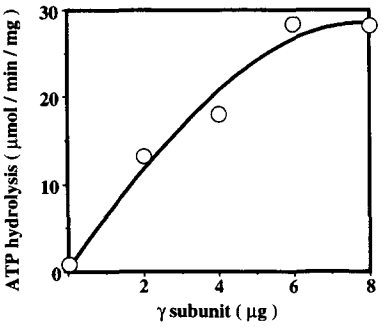

Fig. 2. Effects of the purified γ subunit on reconstitution of the ATPase with the purified α and β subunits. 10 μg of the α or β subunit was mixed with various amounts of the γ subunit and dialyzed against reconstitution buffer as described in Materials and methods.

tathione-Sepharose (final volume 400 μl) and incubated for 30 min at 4°C. The bound Sepharose beads were washed with 100 μl reconstitution buffer three times to remove unbound fusion proteins. These beads were incubated for 20 h at 4°C with 5% (w/v) skimmed milk to block nonspecific binding of proteins. After washing out excess skimmed milk with PBS, 20 μg of α, β or γ subunit was added to the beads in 400 μl of reconstitution buffer. After incubation of the mixed solution at room temperature for 8 h, the beads were washed three times with reconstitution buffer (200 μl). The beads were then suspended in SDS sample buffer (70 μl) and boiled for 10 min at 100°C [16]. Proteins released from the beads were applied to SDS-polyacrylamide gel elctrophoresis, and the proteins were visualized by staining with Coomassie brilliant blue [12].

Immunological detection of the proteins. 2 μg of the purified δ-GST or ϵ-GST protein was applied to SDS-polyacrylamide gel-electrophoresis and blotted onto GVHP filters [12]. The blotted filters were blocked with BSA and then skimmed milk to avoid nonspecific binding of the antibodies and incubated with rabbit polyclonal antibodies against the α, β, γ or GST which were prepared in this study. The reacted materials were visualized using an ABC Vectastain kit as described previously [12].

DNA sequencing and modification of DNAs. Cloned DNAs encoding the γ, δ or ϵ subunits were sequenced by the dideoxy chain termination method using ^{35}S-α-dCTP (37 TBq/μmol) [17]. Preparation of various plasmids, digestion of DNAs with restriction enzymes, ligation of DNAs with T4 ligase and other procedures for manipulation of DNA were performed according to the published procedures [18].

Reagents and enzymes. Restriction endonucleases, T4 DNA ligase, Pfu DNA polymerase, and T7 DNA polymerase were purchased from Toyobo, Bethesda Research

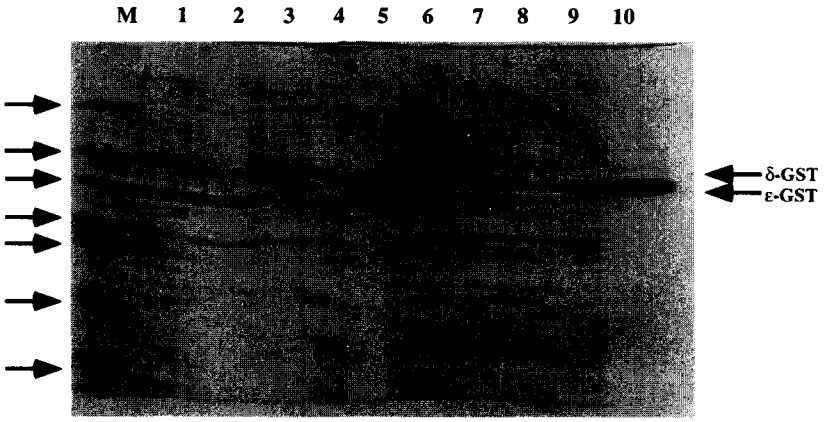

Fig. 3. Induction of the fused δ-GST and ϵ-GST proteins and their purified samples after affinity chromatography. 500 ml of BL21 (DE3) cells carrying the expression plasmid of the fused δ-GST or ϵ-GST was cultured and the proteins were induced as described in Materials and methods. Cells were disrupted by sonication, and the membrane and cytoplasmic fractions were prepared. 5 μg of the membrane or cytoplasmic fraction was applied to SDS-polyacrylamide gel electrophoresis and protein bands were visualized by Coomassie brilliant blue staining. For whole-cell protein, an amount equivalent to 2.0 $A_{600\ nm}$ units in 25 μl was applied to electrophoresis. M, molecular markers. The arrows indicate M_r 66000, 45000, 36000, 29000, 24000, 20100 and 14200 from top to bottom. Lanes 1 to 5 and lanes 6 to 10 show δ-GST and ϵ-GST, respectively. Lanes 1 and 6, whole-cell protein without IPTG induction; lanes 2 and 7, whole-cell protein with IPTG induction; lanes 3 and 8, cytoplasmic fraction; lanes 4 and 9, membrane fraction; lanes 5 and 10, purified samples.

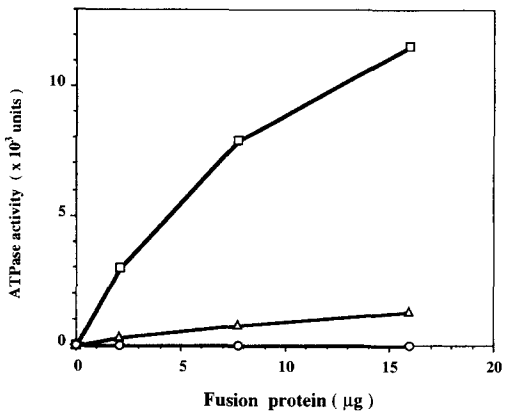

Fig. 4. ATPase activity in the purified δ-GST and ϵ-GST fractions. ATPase activities in the major fractions eluted by GST affinity chromatography (Fig. 3) were assayed. Open squares, δ-GST; open triangles, ϵ-GST; open circles, GST.

Labs., New England Biolabs or Takara. The oligonucleotides were synthesized with a DNA synthesizer (Applied Biosystems Model 380A). [^{35}S]-α-dCTP used for sequencing was obtained from Dupont/NEN Research Products. Other materials were of the highest grade commercially available.

3. Results

3.1. In vitro reconstitution of the αβγ complex using the subunits overproduced in E. coli expression system

We constructed expression plasmids carrying the α, β or γ subunit gene from *Escherichia coli* under the promoter for T7 phage RNA polymerase and overexpressed these proteins in *E. coli* BL21 (DE3) cells. The T7 phage RNA polymerase is integrated into the chromosome of BL21 under the control of the *lac* operon promoter. With addition of the inducer IPTG, the α, β or γ subunit was overexpressed. When the proteins were induced at 37°C, a half of the induced α subunit was recovered in the soluble fraction, while less than half of the β subunit and essentially no γ subunit were recovered in the supernatant fraction after centrifugation of the total cell extracts (Fig. 1). To increase the recovery of the proteins in the supernatant, the effects of temperature on the induction were tested. Recoveries of the α and β subunits in the supernatant fraction were more than that in the precipitate fraction, when the induction temperature was decreased from 37 to 30 or 25°C. However, the γ subunit was not found in the supernatant fraction, even at 25°C (data not shown). Since the cell growth and induction rate were lower at 25°C, the induction of these subunits were performed at 30°C in the following experiments. The α and β subunits in the soluble fraction were purified by hydroxyapatite column chromatography as described previously [7].

Since the γ subunit was mainly recovered in the membrane fraction, even with centrifugation at lower speed, inclusion bodies might be formed. Therefore, the γ subunit was purified by SDS-polyacrylamide gel electrophoresis and was eluted from the gel matrix electrophoretically. The eluted proteins were precipitated with cold acetone and then treated with guanidine hydrochloride. When the γ subunit dissolved in 2.5 M guanidine hydrochloride were mixed with the α and β subunits and then guanidine hydrochloride was removed by dialysis during the reconstitution process, high ATPase activity was reconstituted (Table 1). However, the γ subunit preparation in which guanidine hydrochloride was removed before incubation with the α and β subunits, and also the electro-eluted γ subunit gave lower activity (Table 1). It has been reported that the highest ATPase activity was reconstituted from the α, β and γ subunits with 3:3:1 stoichiometry, which

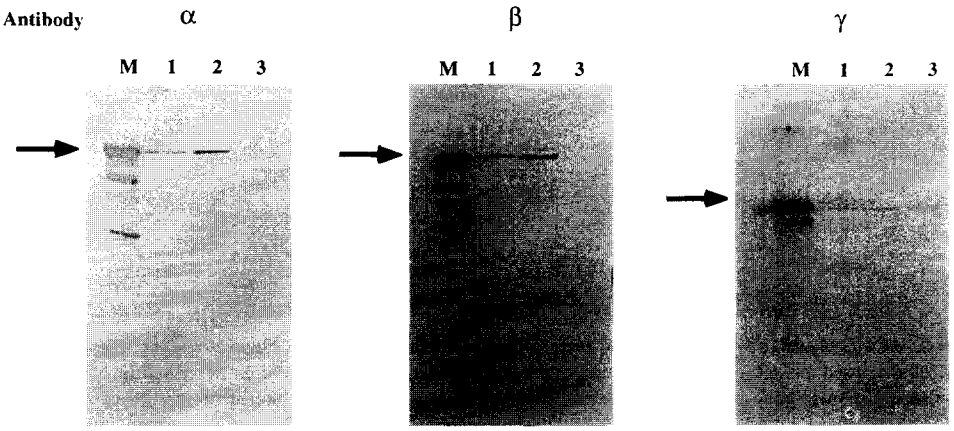

Fig. 5. Western blotting analysis of the immunologically cross-reactive materials in the purified fusion proteins. 2 μg of the purified samples of α, β, γ, δ-GST, ϵ-GST and GST were applied to SDS-polyacrylamide gel electrophoresis and blotted onto GVHP filters. The blotted filters were incubated with the polyclonal antibodies against each subunit shown at the top. The arrows indicate the positions of the α, β, and γ subunits from left to right. Lane M, control α, β, or γ subunit; Lanes 1, 2 and 3 are δ-GST, ϵ-GST and GST, respectively.

subunits were purified from dissociated F_1-ATPase [7]. With the γ subunit prepared in the present method, the highest activity was obtained using the α, β and γ in molar ratios of 3:3:2 to 3:3:3 (Fig. 2). These results suggested that some of the γ subunit were not restored to the native conformation or that the purified subunit fraction was in some way impure. In the later experiments, a molar ratio of the α, β and γ in 3:3:2 was adopted. Although we attempted to obtain active β subunit from the membrane fraction by the same procedure as for the γ subunit, the ATPase could not be reconstituted with the α and γ subunits (data not shown).

3.2. Preparation of the δ and ϵ subunits fused to the carboxy terminus of GST

The entire DNA encoding the gene for the δ or ϵ subunit was prepared and fused to the carboxy-terminal end of the GST gene in the expression plasmid. Both fusion proteins were overproduced in *E. coli* BL21 (DE3) (Fig. 3) and purified by affinity column chromatography with glutathione as the ligand. The fraction eluted after addition of glutathione contained a major peptide corresponding in size to the δ-GST or ϵ-GST fusion protein (Fig. 3). A few minor bands which were larger or smaller were observed on SDS-PAGE (Fig. 3) for δ-GST. These protein bands were immunoreactive to a specific serum against GST, suggesting that these were aggregated or degraded proteins of the fused δ-GST. Degradation may occur during cell culture or preparation of the protein. For the ϵ-GST fusion protein, a major protein with the molecular size expected for the fusion protein was observed without such degraded materials found for the δ-GST fusion protein.

Since we expected that the both fusion subunits could assemble with the other F_1 subunits, the presence of the α, β and γ subunits in the fraction eluted by glutathione was tested by measuring the ATPase activity and western blotting with antiserum against these subunits. For δ-GST and ϵ-GST, in the major fraction eluted by glutathione we observed the ATPase activity (Fig. 4), while no such activity was observed for the fraction from the control cell extract with GST instead of δ-GST or ϵ-GST (Fig. 4). The presence of α, β and γ subunits in the fusion protein fraction was detected by immunoblotting analysis (Fig. 5). These subunits showed similar stoichiometry to those in the native F_1-ATPase, indicating that the fusion subunits could assemble in the in vivo configuration with the α, β and γ subunits to form an F_1 like complex.

3.3. In vitro reconstitution of the $\alpha\beta\gamma\delta$-GST and $\alpha\beta\gamma\epsilon$-GST complex

We tested in vitro reconstitutability of the purified δ-GST or ϵ-GST proteins with the α, β and γ subunits. After incubation of the α, β and γ subunits with the fused proteins to reconstitute the ATPase, the ATPase activity was measured. Addition of the δ-GST to the α, β and γ subunits increased the ATPase activity formed by the α, β and γ alone by up to 139%. This result suggests that the δ-GST increased the activity by changing the conformation of the $\alpha\beta\gamma$ complex on binding (data not shown). The maximal enhancement was obtained in the presence of δ-GST at a molar ratio of 2 to 1 α or β subunit, which ratio is larger than that in the native F_1. Since the purified δ-GST contained degraded δ-GST, this ratio may be larger. In fact, the $\alpha\beta\gamma\delta$-GST purified by affinity chromatography contained close to 1 mol of δ-GST (Fig. 7). The fusion protein with excess amount (molar ratio of 5 to the α and β subunits) slightly decreased the reconstituted ATPase activity (data not shown). GST alone increased the ATPase activity up to 116%, which increase was also observed for BSA instead of GST (data not shown), suggesting a non-specific stabilizing effect of these proteins in the reconstitution reaction. ϵ-GST decreased the reconstituted activity to 50% of the control with equal amounts of the fusion protein to the α and β subunit, indicating that the ϵ-GST inhibited the activity of $\alpha\beta\gamma$ complex as expected.

The reconstituted materials were applied to GST affinity chromatography to test formation of the active $\alpha\beta\gamma\delta$-GST or $\alpha\beta\gamma\epsilon$-GST complex (Fig. 6). The fraction eluted by addition of glutathione contained the α, β, γ, and the fused proteins, which were assigned by western blotting,

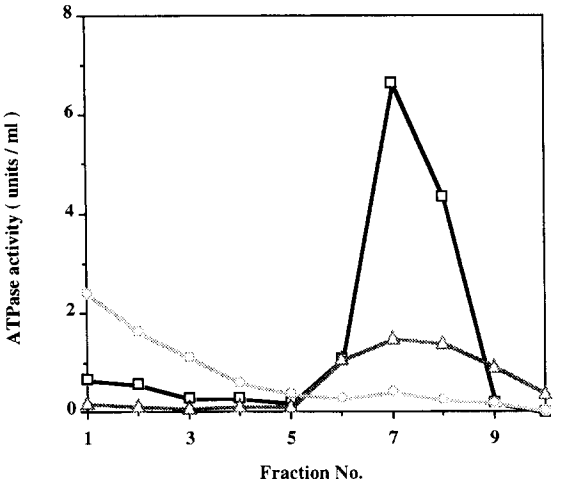

Fig. 6. Affinity chromatography of the reconstituted materials and the ATPase activity. α (180 μg), β (165 μg), γ (100 μg), and δ-GST (82 μg) or ϵ-GST (118 μg) were mixed in 1.2 ml reconstitution buffer and the ATPase was reconstituted by dialyzing the mixture against reconstitution buffer as described in Materials and methods. The reconstituted samples were applied to affinity column chromatography with glutathione-Sepharose under the conditions used for purification of the fused proteins. 500 μl of each eluted sample was collected as a single fraction and the ATPase activity in an aliquot of each fraction was assayed. 10 mM glutathione was added at fraction number 6. 82 μg and 118 μg of proteins were recovered in the eluted fractions 6 to 9 for δ-GST and ϵ-GST, respectively. Open squares, open triangles and open circles indicate δ-GST, ϵ-GST and GST alone, respectively.

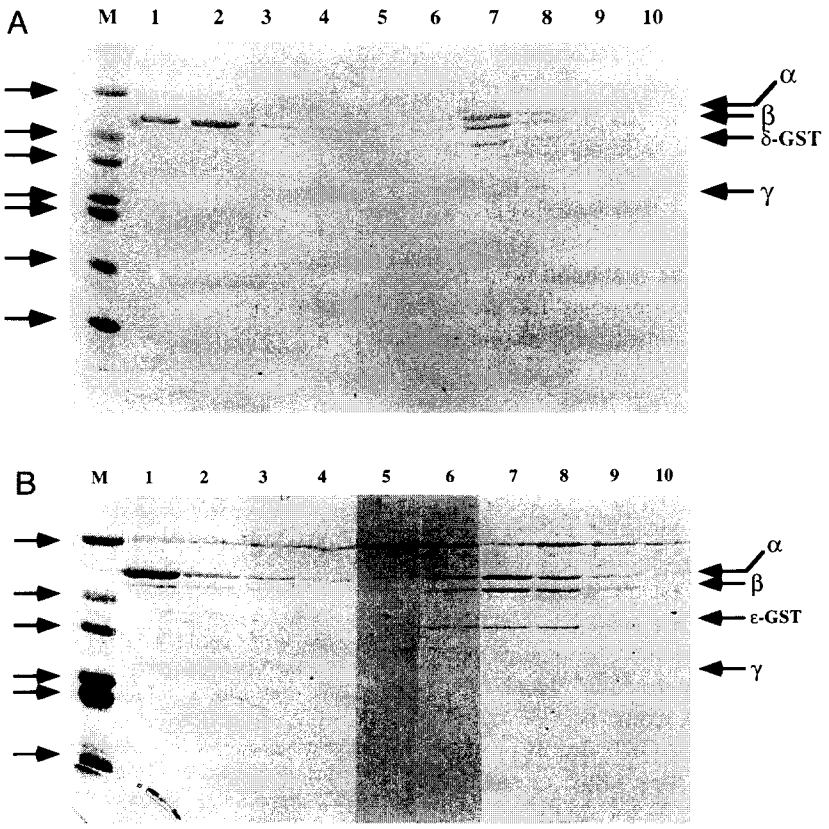

Fig. 7. SDS-polyacrylamide gel electrophoresis of the eluted materials from GST affinity chromatography. 10-μl aliquots of the 500 μl of eluted materials described in Fig. 6 were applied to SDS-polyacrylamide gel electrophoresis. Numbers at the top correspond to the fraction numbers on chromatography in Fig. 6. (A) δ-GST; (B) ϵ-GST. Positions of the molecular markers (M) are the same as in the legend of Fig. 3.

with stoichiometry of each subunit close to that in the native F_1 complex (Fig. 7A, B). The reconstituted complex exhibited ATPase activities with 88.8 units per mg of protein and 25.1 units per mg protein for δ-GST and for ϵ-GST, respectively. The control $\alpha\beta\gamma$ complex indicated 36.2 units per mg of protein. No significant change in the activity was found when GST instead of the fused proteins was added to the $\alpha\beta\gamma$ reconstitution mixture. These results indicated that the δ-GST increased the ATPase activity of the $\alpha\beta\gamma$ complex and that ϵ-GST inhibited the

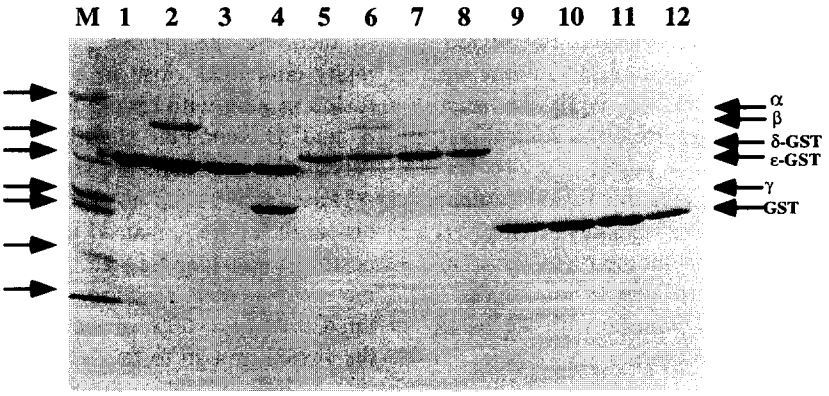

Fig. 8. Binding of δ-GST and ϵ-GST to the α, β or γ subunits. 15 μg of δ-GST, ϵ-GST or GST was bound to glutathione-Sepharose (20 μl). 20 μg of α, β or γ subunit was incubated for 8 h at room temperature with the bound GST fusion proteins in the buffer used for reconstitution of ATPase. After removing unbound proteins in the glutathione-Sepharose by washing the beads, the Sepharose beads were boiled at 100°C. Released proteins were analyzed by SDS-polyacrylamide gel-electrophoresis and subsequent staining with Coomassie brilliant blue. Lane M, molecular size markers as in the legend of Fig. 3. Lanes 1, 5 and 9 are controls of ϵ-GST, δ-GST and GST, respectively; lanes, 2, 6 and 10 are α subunit with ϵ-GST, δ-GST or GST, respectively; lanes, 3, 7 and 11 are β subunit with ϵ-GST, δ-GST or GST, respectively; lanes, 4, 8 and 10 are γ subunit with ϵ-GST, δ-GST or GST, respectively.

activity. These results also suggested that $\alpha\beta\gamma\delta$-GST and $\alpha\beta\gamma\epsilon$-GST complexes with proper topological arrangements of the δ and ϵ as in the native F_1 complex together with the α, β and γ subunits were reconstituted in this experiment.

3.4. Analysis of interaction of the fused proteins with the α, β or γ subunit

As the first step in analyzing the fine mechanisms in formation of the F_1 ATPase complex using the fused δ-GST or ϵ-GST, we studied binding of the fused proteins to the α, β or γ subunit. The ϵ-GST bound the γ subunit with stoichiometry of approximately 1:1 (Fig. 8). ϵ-GST also bound the α and β subunits significantly, although GST alone bound these subunits slightly. δ-GST also bound the α, β and γ subunits. However, a significant binding may be between the δ and γ subunits, because of background binding for the α and β subunits with GST. Thus, all binding except that of the γ and ϵ subunits was weak.

4. Discussion

We established a system for over expression of the α, β and γ subunits of F_1-ATPase based on expression plasmids with each gene under the control of the T7 polymerase promoter. These systems will be useful for obtaining large quantities of the subunits for study of in vitro assembly mechanisms, especially for the γ subunit. Reported procedures [7] include purification of F_1-ATPase, dissociation to its subunits and subsequent purification of the α, β and γ subunits by column chromatography, which is time-consuming compared to the systems and procedures described here. We found that the γ subunit denatured once on SDS-polyacrylamide gel electrophoresis could be renatured by guanidine hydrochloride and dilution during reconstitution. For the highest activity in reconstitution, 2- to 3-times more γ subunits were required compared to the reported amount of the γ subunit, suggesting that some portions of the subunit preparation were not restored to the active form or the γ subunit fraction was in some way impure. Even considering this, however, the present procedure was much easier than the reported procedure to obtain the active γ subunits. This procedure did not work for the insoluble β subunit, which was more than half of the overproduced subunit. The renaturable characteristic of the γ subunit may be due to a unique protein chemical property of this subunit. Thus, it is interesting to note that half of the overproduced α subunit was soluble.

To establish a convenient analytical system of assembly-intermediates of the F_1-ATPase complex, we attempted to reconstitute $\alpha\beta\gamma\delta$-GST and $\alpha\beta\gamma\epsilon$-GST which have the ATPase activity. Formation of these complexes with subunit stoichiometry close to that of the native complex was judged by their specific elution from an affinity column with addition of glutathione, and by their reasonable ATPase activities. The δ-GST stabilized the $\alpha\beta\gamma$ complex and enhanced the activity of the $\alpha\beta\gamma$ complex, while ϵ-GST inhibited the activity of the complex, suggesting that the $\alpha\beta\gamma$ complex with the fused δ and ϵ proteins forms a native F_1 structure.

Since in the fused subunits the amino-terminal portions were blocked, it was suggested that the amino termini of the δ and ϵ subunits are not essential for complex formation. It was reported that the δ subunit may not have residues essential for the function of the F_1F_0 complex throughout the entire molecule, but that residues 149 and 150 are important for the assembly of F_1-ATPase [19,20]. Zieglar et al. reported that Cys-64 and Cys-140 are located in close vicinity and probably place constraints in the tertiary structure of the δ subunit [21]. Hazard and Senior proposed a tertiary structure model of the δ subunit [22], in which residues 7–63 and 91–145 lie in helical domains which form the stalk originally predicted by Sternweis [23], and residues 64–90 may be a loop region, suggesting that the amino- and carboxy-terminal regions are within the central space in the $\alpha_3\beta_3$ core complex which was observed by X-ray crystallography [8]. Hartvig and Capaldi also reported that a peptide fragment containing the amino-terminus binds to F_1 [24]. These observations are not necessarily consistent with the present results. Our findings in this study suggest that the amino-terminal region of the δ subunit is located outside of the $\alpha_3\beta_3$ core complex. Although Hazard and Senior [22] predicted a symmetrical bending structure of the δ subunit within the $\alpha_3\beta_3$ complex, our data may not support this model.

ϵ-GST bound to the γ subunit in an approximately 1:1 molar ratio, which is consistent with the previous observation of ϵ and γ binding [25]. This result supported the notion that interaction of ϵ-GST and γ during reconstitution is the same as in the native topological arrangement of F_1 subunits. The present results are consistent with the proximity of the carboxy-terminal region of the ϵ subunit and the γ subunit [26].

Jounouchi et al. [27] and Skakoon and Dunn [28] proposed that the ϵ subunit has a two-domain structure, the amino-terminal half and the carboxy-terminal half, which are separated by a few residues, including Ser-106. In between the two domains, consecutive serine residues at positions 106 to 108 exist. These residues bind to the β subunit at residue 381, and substitution of serine-108 by Cys allows binding to the α subunit, suggesting that these regions interact with the β or $\alpha\beta$ interface. In the amino-terminal half, a Cys introduced at residue 10 interacts with the γ subunit [28]. Skakoon and Dunn [28] reported that their monoclonal antibody ϵ-1 recognizes residue 38 and also residues between 77 and 85, but did not bind to the ϵ subunit in the F_1 complex. Based on these observations, the amino-terminal half appears to form a domain which is

not exposed on the F_1 surface. Our observations indicated that at least the amino terminus is not involved in the interaction with F_1 subunits other than the δ subunit.

A model for in vivo assembly of the F_1F_0 complex, which is as yet unestablished, was proposed previously [29]. While the β subunit plays an essential role in formation of the F_0 portion in this model, observations which contradicted this proposal have been reported [30]. Therefore, the precise assembly mechanism has not yet been clarified [31]. We assumed that the subunit proteins are synthesized and assembled in the order of their genes: i.e., from δ to ϵ for the F_1 portion. In fact, δ-GST could assemble into F_1 with the α, β and γ subunits without the ϵ subunit. However, the δ subunit bound the γ and β subunits weakly and did not bind to the α subunit. These results suggested that, for the association of the δ-GST to the other F_1 subunits, interactions among the other subunits may be required. In this connection, we reported that α-β interaction is essential prior to complete assembly of the F_1 complex [32]. It is also known that the α, β and γ subunits assemble in vitro without the other subunits [7]. Precise analysis of the molecular assembly mechanisms using fusion proteins will provide clues to these questions.

Acknowledgements

The present study was supported by grants-in-aid from the Japanese Ministry of Science, Culture and Education to H.K. Oligonucleotides were kindly prepared by Dr. M. Nakamura, Kurare Institute. The authors thank Ms. H. Kagawa for technical aid in preparing this manuscript.

References

[1] Futai, M., Noumi, T. and Maeda, M. (1989) Annu. Rev. Biochem. 58, 111–136.
[2] Futai, M. and Kanazawa, H. (1983) Microbiol. Rev. 47, 285–312.
[3] Walker, J.E., Saraste, M. and Gay, N.J. (1984) Biochim. Biophys. Acta 768, 164–200.
[4] Senior, A.E. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 7–41.
[5] Fillingame, R.H. (1990) The Bacteria, Vol. XII, 345–391.
[6] Pedersen, P.L. and Amzel, L.M. (1993) J. Biol. Chem. 268, 9937–9940.
[7] Dunn, S.D. and Futai, M. (1980) J. Biol. Chem. 255, 113–117.
[8] Abrahams, J.P., Leslie, A.G.W., Lutter, R. and Walker, J. (1994) Nature 370, 621–628.
[9] Noumi, T., Azuma, M., Shimomura, S. and Futai, M. (1987) J. Biol. Chem. 262, 14978–14982.
[10] Smith, D.B. and Johnson, K.S. (1988) Gene 67, 31–35.
[11] Kanazawa, H., Tamura, F., Mabuchi, K., Miki, T. and Futai, M. (1980) Proc. Natl. Acad. Sci. USA 77, 7005–7009.
[12] Miki, J., Matsuda, T., Kariya, H., Ohmori, H., Tsuchiya, T., Futai, M. and Kanazawa, H. (1992) Arch. Biochem. Biophys. 294, 373–381.
[13] Kanazawa, H., Miki, T., Tamura, F., Yura, T. and Futai, M. (1979) Proc. Natl. Acad. Sci. USA 76, 1126–1130.
[14] Studier, F.W., Rosenberg, A.H. Dunn, J.J. and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[15] Kanazawa, H., Yabuki, M., Miki, J., Fudemoto, T., Ikeda, H., Noumi, T. and Shin, Y. (1995) Arch. Biochem. Biophys. 317, 348–365.
[16] Miki, J., Fujiwara, K., Tsuda, M., Tsuchiya, T. and Kanazawa, H. (1990) J. Biol. Chem., 265, 21567–21572.
[17] Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980) J. Mol. Biol. 143, 161–178.
[18] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
[19] Stack, A. and Cain, B.D. (1994) J. Bacteriol. 176, 540–542.
[20] Hazard, A.L. and Senior, A.E. (1994) J. Biol. Chem. 269, 427–432.
[21] Ziegler, M., Xiao, R. and Penefsky, H.S. (1994) J. Biol. Chem. 269, 4233–4239.
[22] Hazard, A.L. and Senior, A.E. (1994) J. Biol. Chem. 269, 418–426.
[23] Sternweis, P.C. (1977) J. Biol. Chem. 253, 3132–3137.
[24] Mendel-Hartvig, J. and Capalidi, R.A. (1991) Biochim. Biophys. Acta 1060, 115–124.
[25] Dunn, S.D. (1982) J. Biol. Chem. 257, 7354–7359.
[26] Aggeler, R., Haughton, M.A. and Capaldi, R.A. (1995) J. Biol. Chem. 270, 9185–9191.
[27] Jounouchi, M., Takeyama, M., Chairprasert, P., Noumi, T. Moriyama, Y., Maeda, M. and Futai, M. (1992) Arch. Biochem. Biophys. 292, 376–381.
[28] Skakoon, F.N. and Dunn, S.D. (1993) Arch. Biochem. Biophys. 302, 279–284.
[29] Cox, G.B., Downie, J.A., Langmann, L., Senior, A.E., Ash, G., Fayle, D.R.H. and Gibson, F. (1981) J. Bacteriol. 148, 30–42.
[30] Fillingame, R.H., Porter B., Hermolin, J. and White, L.K. (1986) J. Bacteriol. 165, 244–251.
[31] Pati, S. and Brusilow, W.S.A. (1991) Biochemistry 30, 4710–4714.
[32] Miki, J., Kusuki, H. Tsugumi, S. and Kanazawa, H. (1994) J. Biol. Chem. 269, 4227–4232.