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The Role of the Amino-Terminal β-Barrel Domain of the α and β Subunits in the Yeast F_1-ATPase

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The crystal structure of mitochondrial F_1-ATPase indicates that the α and β subunits fold into a structure defined by three domains: the top β-barrel domain, the middle nucleotide-binding domain, and the C-terminal α-helix bundle domain (Abrahams *et al.*, 1994); Bianchet *et al.*, 1998). The β-barrel domains of the α and β subunits form a crown structure at the top of F_1, which was suggested to stabilize it (Abrahams *et al.* 1994). In this study, the role of the β-barrel domain in the α and β subunits of the yeast *Saccharomyces cerevisiae* F_1, with regard to its folding and assembly, was investigated. The β-barrel domains of yeast F_1 α and β subunits were expressed individually and together in *Escherichia coli*. When expressed separately, the β-barrel domain of the β subunit formed a large aggregate structure, while the domain of the α subunit was predominately a monomer or dimer. However, coexpression of the β-barrel domain of α subunit with the β-barrel domain of β subunit, greatly reduced the aggregation of the β subunit domain. Furthermore, the two domains copurified in complexes with the major portion of the complex found in a small molecular weight form. These results indicate that the β-barrel domain of the α and β subunits interact specifically with each other and that these interactions prevent the aggregation of the β-barrel domain of the β subunit. These results mimic *in vivo* results and suggest that the interactions of the β-barrel domains may be critical during the folding and assembly of F_1.

KEY WORDS: F_1-ATPase; β-barrel domain; mitochondria; assembly; yeast; *Saccharomyces cerevisiae*.

INTRODUCTION

The ATP synthase is found in the mitochondrial inner membrane, the bacterial cytoplasmic membrane, and in the chloroplast thylakoid membrane. It is composed of three parts: a membrane-spanning sector, F_0, an extrinsic multisubunit complex, F_1, and a stalk region, which connects the F_0 and F_1 portions. The F_1 comprises the active site of the enzyme and is composed of five different subunits in a stoichiometry of $\alpha_3\beta_3\gamma\delta\varepsilon$ (Walker *et al.*, 1985).

The high-resolution crystal structure of the bovine (Abrahams *et al.*, 1994) and rat liver (Bianchet *et al.*, 1998) mitochondrial F_1 provide atomic details on the arrangement of the subunits. The three α and three β subunits are arranged alternatively in a ring structure encircling the γ subunit. Although the α and β subunits are only weakly homologous to each other, they are folded into an almost identical three-domain structure. The top domain is formed by the first 90 amino acids and folds into a six-stranded β-barrel domain structure. The central nucleotide-binding domain contains nine α helixes and nine-associated β strands, where the noncatalytic and catalytic nucleotide-binding sites are located. The final 100 amino acids of the C terminal of the α and β subunits form α-helical domains in which the α subunit has seven and the β subunit has six α-helixes.

The N-terminal β-barrel domain is formed by six β strands and lies about 50 A away from the catalytic

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site. Hydrogen bonds between the adjacent subunits of the β-barrel domains stabilize a quaternary structure that was referred to as a "crown" (Abrahams *et al.*, 1994). The crown does not directly form the nucleotide-binding domain, but rather was postulated to be important in stabilizing the enzyme (Abrahams *et al.*, 1994). This study was initiated to study the role of the β-barrel domain in the stability of the F_1-ATPase, but the results suggest a role of the domains in the folding and assembly of the enzyme.

The expression and reasssembly of the β-barrel crown could provide a means to determine quantitatively the amount of stabilization that the crown contributes to the F_1-ATPase. Instead, it was determined that when the β-barrel domain of the β subunit is expressed in the absence of the β-barrel domain of the α subunit, it is aggregated into large complexes and this aggregation could be prevented by the coexpression of the β-barrel domain α subunit. This behavior mimics the behavior for the α and β subunits when they are expressed in yeast in the absence of the β or α subunit, respectively (Ackerman and Tzagoloff, 1990). This indicates that the β-barrel domain may be at least partially responsible for the aggregation of the α or β subunits observed *in vivo*. Further, the β-barrel domain of the α subunit can specifically interact with that of the β subunit, forming specific complexes that prevent the aggregation of the β subunit domain. As such, the β-barrel domain of the α subunit has a chaperonelike activity.

MATERIAL AND METHODS

Construction of Plasmids for Heterologous Expression of the β-Barrel Domains

Standard procedures for plasmid DNA manipulation were used (Maniatis *et al.*, 1982). Four plasmids, pet3aΔRVαH, pet3aΔRVβH, pet3aΔRVβF, and pet3aΔRVαH/βF were constructed in this study to overexpress the yeast β-barrel domains in *E. coli*. The DNA encoding the yeast β-barrel domains were amplified by PCR[4] using Vent DNA polymerase (New England Biolabs). To construct plasmids pet3aΔRVαH and pet3aΔRVβH, two pairs of primers, αH36/α125 and his-4/β110, were used in a PCR reaction (Table I). The forward primers αH36 and his-4 include a *NheI* restriction site GCTAGC followed by six His codons (CAC) and by the sequence of coding the regions of the ATP1 or ATP2 genes. The downstream reverse primers, α125 and β110, encode the C-terminal sequence of the domains with the addition of a stop codon *CTA* (italic) and a *BamHI* recognition sequence. The PCR products were digested by *NheI* and *BamHI* and ligated into the *NheI–BamHI* sites of expression vector pET3aΔRV (Mukhopadhyay *et al.*, 1992), a derivative of pet3a (Studier *et al.*, 1990). The DNA sequence of the cloned DNA was determined to ensure the correct ligation and the absence of any mutations. The resultant plasmids are referred to as pet3aΔRVαH (α-subunit domain) and pet3aΔRVβH (β-subunit domain). The proteins expressed from pet3aΔRVαH and pet3aΔRVβH are referred to as α-His and β-His, respectively, and constitute the β-barrel domains with a (His)6 tag at the amino end of the protein.

Plasmid pet3aΔRVβF was made by a similar scheme with primers β-fg/β110 (Table I). This plasmid also expresses the β-subunit domain, but instead of a (His)6 tag, has the Flag epitope tag at the amino end of the protein. The Flag tag, consists of the peptide sequence DYKDDDDK and is recognized by the M_2 anti-Flag antibody (Chiang and Roeder, 1993). The β-subunit domain expressed from this plasmid is referred to as β-Flag.

Plasmid pet3aΔRVαH/βF allows the coexpression of α-His with β-Flag in *E. coli*. This plasmid was derived from pet3aΔRVαH and pet3aΔRVβF. Vector pet3aΔRVαH was digested with *BglII* and made blunt with the Klenow fragment of DNA polymerase. After digestion with *EcoRI*, the plasmid was ligated with the *EcoRI/EcoRV* fragment of pet3aΔRVβF, which contained the gene encoding β-Flag.

Induction and Purification of the β-Barrel Domain Proteins in Cells BL21(DE3)

For expression, the plasmids were transformed into *E. coli* BL21 (DE3) (Studier *et al.*, 1990). A preculture of *E. coli* BL21 (DE3) bearing the plasmid was inoculated into fresh LB medium containing ampicillin (100 μg/ml). The cell culture was grown at 37°C until the OD_{600} reached 0.6–0.8, at which time expression was induced by the addition of 0.7 mM IPTG

[4] The abbreviations used are: PCR, polymerase chain reaction: α-His, the β-barrel domain of α subunit with (His)6 tag; β-His, β-barrel domain of β-subunit with (His)6 tag; β-Flag, β-barrel domain of β-subunit with Flag epitope; CD, circular dichroism; IPTG, isopropyl-β-thiogalactopyranoside; RI, refractive index; LB medium, 1% tryptone, 0.5% yeast extract, and 1% NaCl.

Table I. Oligonucleotide Primers Used for PCR[a]

Primer	Sequence	Description
αH36	TAATTGCTAGC(CAC)6ACCAAGGCACAACCC	Complementary to coding strand of ATP1 gene at positions 112–126
α125	TAATTGGATCC*CTA*ATTACCGGTTCTCTT	Complementary to noncoding strand of ATP1 gene at positions 375–390
his-4	AATATGCTAGC(CAC)6GCTGCTCA ATCTACTCCAA	Complementary to coding strand of ATP2 gene at positions 100–118
β110	TAATTGGATCC*CTAG*ATAGGGCCA CCAGTGTCAA	Complementary to noncoding strand of ATP2 gene at positions 323–342
β-fg	CTGCTGCTAGC(GACTACAAGGACGACGAT-GACAAG)GCTGCTCAATC TACTCCAATCA	Complementary to coding strand of ATP2 gene at positions 100–118, contains codons for β-Flag

[a] Primer pairs αH36/α125 and his-4/β110 were used for PCR amplification of the β-barrel domain coding region of the α and β subunits (α-His or β-His), respectively. The β-fg/β110 primers were used to introduce the Flag epitope on the N-terminus of the β-barrel domain of β subunit (β-Flag). The forward primers, αH36 and his-4, start with a *NheI* site (underline) followed by 6 His codons (CAC) and the sequence of the ATP1 (α-subunit) or the ATP2 (β-subunit) gene. In the primer β-fg, codons for the Flag tag DYKDDDDK (in parenthesis) were inserted after the *NheI* site. Reverse primers, α125 and β110, have a *BamH1* site (underline) at their 5′ ends followed by a stop codon (*italic*) and sequence for the ATP1 or ATP2 gene. The A in the initiating ATG codon is designated as number 1. The coding strand is defined as the DNA strand that serves as the template for transcription.

(for α-His, β-His, β-Flag). For the coexpression of α-His/β-Flag, no IPTG was added to the culture. The cultures were shaken at 37°C for another 4 h, the cells were harvested by centrifugation, and the pellet was stored at −80°C.

The frozen cell pellets were thawed at room temperature and resuspended in 1/20 of the original cell culture volume in resuspension buffer (50 mM, pH 8.0 Tris–Cl, 0.1% Triton X-100). Lysozyme (20 mg/100 ml) was added to the sample and the cells were digested at 30°C for 30 min with gentle shaking. The cells were broken by sonication, the cell lysate was centrifuged at 30,000 rpm for 20 min at 4°C, and the resultant supernatant was filtered through a 0.7-μm filter.

The α-His and β-His proteins were purified by affinity chromatography using a Ni–agarose resin (Qiagen). Soluble cell lysate was bound to a Ni–agarose affinity column equilibrated with 20 mM Tris–Cl, 0.3 M NaCl, and 5mM imidazole, pH 8.0. The column was washed with 20 mM Tris–Cl, 0.3 M NaCl, and 60 mM imidazole, pH 8.0 (WB). The protein was eluted with a gradient of imidazole (0–0.5 M) in WB. The fractions that contained the protein were pooled, the protein was precipitated with 70% saturation ammonium sulfate, and stored at 4 °C.

The β-Flag was purified on anti-Flag M_2 antibody column (Kodak). The cell lysate was bound to an anti-Flag M_2 column equilibrated with 0.1 M glycine–HCl, pH 3.5, and 50 mM Tris–HCl, and 150 mM NaCl, pH 7.4 (TBS). The column was washed with TBS and the protein was eluted with 0.1 M glycine–HCl, pH 3.5. The fractions (1 ml) were immediately neutralized in Tris base (0.02 ml). The fractions were pooled and concentrated by ultrafiltration with a YM3 membrane (Amicon). The concentrated protein solutions were aliquoted and stored at −20°C.

Analysis of Molecular Mass of Proteins by Gel Filtration FPLC Chromatography and by On-Line Light Scattering

A Superose 12 size-exclusion column (Pharmacia) was used for molecular mass analysis and to separate the protein complexes. A Waters 410 refractometer (Millipore) and PD2000 laser Raleigh light scattering detector (Precision Detector, Inc.) were connected in-line with the column allowing detection and molecular mass determinations. The column was equilibrated with 20 mM Tris–SO_4, 100 mM KCl, and 1 mM EDTA, pH 7, and the protein was dissolved in, or dialyzed against, the same buffer at 0.5 to 2 mg/ml. Samples (200–400 μl) were loaded onto the column and eluted at flow rate 0.5 ml/min. The elution profile of the protein was monitored by on-line refractive index and light scattering (90 degrees). The molecular mass of the proteins was calculated using PDI 2000 software (Precision Detector, Inc.). The molecular mass was also determined by the retention time of the protein relative to that of a set of standard proteins:

bovine serum albumin (BSA) (67 kD), carbonic anhydrase (31 kD), lysozyme (14.4 kD), and aprotinin (6.5 kD). The relative amount of protein in each fraction was determined by refractive index, Bradford protein assay (Stoscheck, 1990), and staining using Coomassie blue after separation by SDS–gel electrophoresis.

Circular Dichroism (CD) Spectroscopy

Protein used for CD analysis was dialyzed overnight against 10 mM phosphate, pH 7.5, at 4°C or room temperature. Spectra were collected with a JA700 spectropolarimeter (JASCO) and were corrected by subtracting the absorbance of the buffer. The constituent secondary structures were calculated by deconvolution of the spectra with the convex constraint algorithm (Perczel *et al.*, 1992).

Other Methods

Electroporation was used for transformation of plasmid DNA into *E. coli* competent cells according to the manufacturer's instruction (Bio-Rad). Double-strand DNA sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) with a sequencing kit Version 2.0 (Amersham) and α-[^{35}S]dATP (Amersham). Protein concentration was determined by a modified Bradford procedure (Stoscheck, 1990). SDS–Tris polyacrylamide gel electrophoresis was carried out as described by Laemmli (Laemmli, 1970), except that SDS was omitted in the stacking and separating gels. This allowed the resolution of α-His from β-His and β-Flag. Tricine–SDS polyacrylamide gel electrophoresis was performed as described (Schagger and Von Jagow, 1987). The gels were stained either with Coomassie Brilliant blue R-250 or silver and, in some cases, transferred to nitrocellulose for Western blot analysis (Towbin *et al.*, 1979). Anti-Flag BioM$_2$ monoclonal antibody (Kodak) was used to detect the Flag epitope in conjunction with the avidin/biotinylated horseradish peroxidase complex (ABC Kits, Vector Laboratories, CA) for the Western blot analysis. The peroxidase activity was assayed by light emission using enhanced chemiluminescence (ECL, Amersham Life Science) and detected on X-ray film.

RESULTS

Expression and Purification of the β-Barrel Domains of the α and β Subunits

The α and β-subunits of bovine F_1 fold into nearly identical three-domain structures (Abrahams *et al.*, 1994). The first 90 amino acids in the α subunit and the first 80 amino acids in the β subunit, form a six-stranded β-strand barrel domain. However, the first 18 amino acids in the α subunit and the first 8 amino acids in the β subunit are not seen in crystal structure and thus may fold into an unordered structure.

The β-barrel domain regions of α and β subunits in yeast F_1-ATPase can be predicted by the primary amino acid sequence comparison between the bovine and yeast subunits. Figure 1 shows a comparison of the primary sequence of the β-barrel domains of bovine and yeast enzyme. The comparison indicates that the yeast domains are highly homologous to the bovine domains with 68 and 72% of the residues identical within the α- and β-subunit domains, respectively. This percentage identity is slightly less than that seen of the rest of the molecule with the α and β subunits having 75 and 81% identical residues, respectively, between yeast and bovine.

The DNA encoding the β-barrel domains of the yeast α and β subunits were cloned into a T7 expression vector, pet3a, and expressed in *E. coli*. In addition, an affinity tag, either a (His)6 tag or Flag tag, was

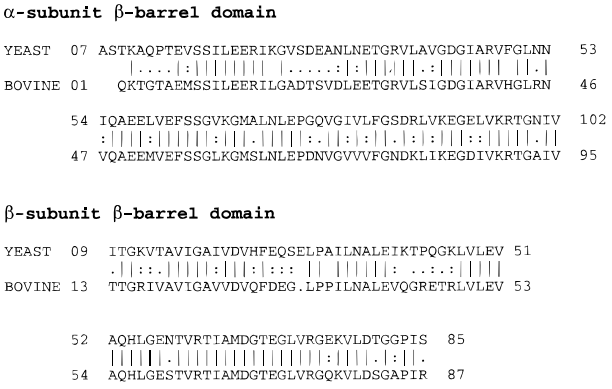

Fig. 1. Homology of the β-barrel Domains in the α and β Subunits in Yeast and Bovine F_1-ATPase. The numbering system is based on the sequence of the mature subunit. The solid lines and double single dashes indicate identical or similar residues as determined by BESTFIT (Genetics Computer Group, 1996). The analysis indicates that β-barrel domains of the α and β-subunits are 68 and 72% identical and 77 and 85% similar, between yeast and bovine, respectively.

placed on the N terminus of the domain to facilitate protein purification by Ni–agarose affinity or by an anti-Flag M_2 antibody column. As a result, three peptides, the β-barrel domain of α subunit with a (His)6 tag (α-His), the β-barrel domain of β subunit with a (His)6 tag (β-His), and the β-barrel domain of β subunit with a Flag epitope tag (β-Flag) were expressed. These proteins contained 101, 88, and 103 amino acids, respectively, with corresponding molecular weight of 10.8, 9.3, and 11.0 kD.

The initial studies centered on the His tagged proteins, α-His and β-His. The expression and purification of these proteins is shown in Fig. 2. The affinity tags at the amino end of the proteins allowed a one-step purification of the soluble-extracted proteins in *E. coli*. For α-His and β-His, the soluble portion of cell lysate was directly loaded onto a Ni–agarose column for affinity purification. The samples were bound to the Ni–agarose column, the column was washed, and the proteins eluted with a linear concentration gradient of imidazole. The major portion of the desired protein eluted out at imidazole concentration of 0.2 M, as determined by SDS gel electrophoresis (Fig. 2B, lanes 1 and 2).

In later studies, β-Flag was used after expression in *E. coli* and purification using the M2 monoclonal antibody column (Fig. 2B, lane 3). The bound protein was eluted with glycine–HCl, pH 3.5 and immediately neutralized with 1 M Tris base. The protein fractions were pooled and concentrated by ultrafiltration. In studies that will be discussed later, β-Flag was coexpressed in *E. coli* with α-His and the β-Flag/α-His complex was copurified using the Ni–agarose affinity column (Fig. 2B, lane 4).

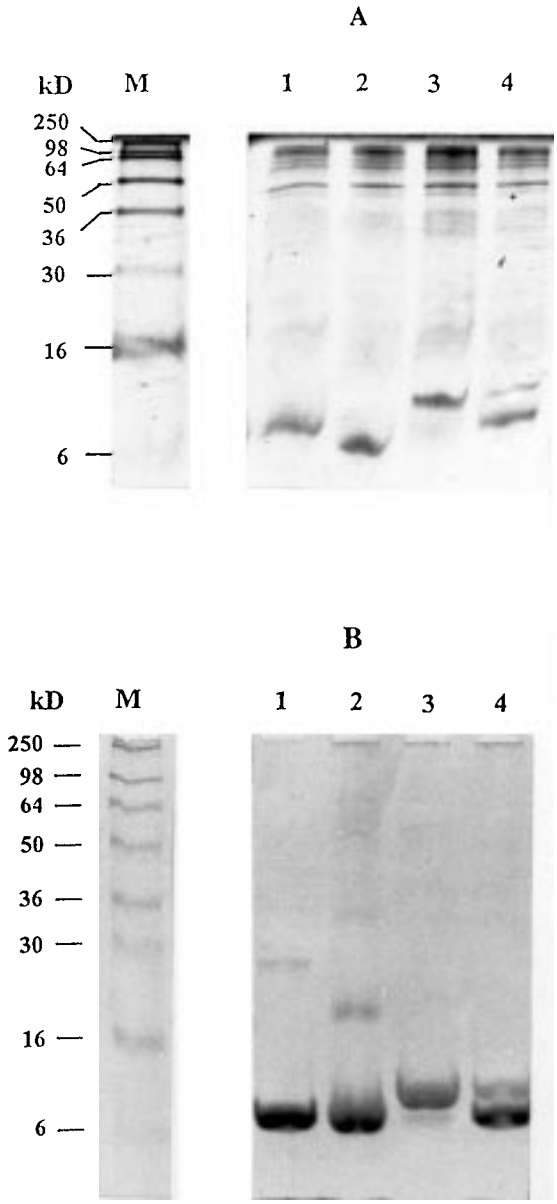

Fig. 2. Heterologous Expression and Purification of the β-Barrel Domains of the α and β Subunits. (A). Total cellular proteins were separated by tricine-SDS gel analysis (Material and Methods) and stained with Coomassie Brilliant blue R-250. Lane 1, α-His (10 μl); lane 2, β-His (10 μl); lane 3, β-Flag (40 μl); lane 4, α-His/β-Flag coexpressed (60 μl). (B). SDS-PAGE analysis of purified proteins (8 μg) after affinity column purification; lane 1, α-His; lane 2, β-His, lane 3, β-Flag; lane 4, α-His/β-Flag. α-His, β-His, and α-His/β-Flag were purified by Ni–agarose chromatography while β-Flag was purified by an M2 monoclonal antibody column. The molecular weight markers (M) are in the lanes.

Secondary Structural Analysis

The purified proteins were studied by circular dichroism to determine if the domains have folded into the correct secondary structure, in this case, into the β-barrel structure. The CD spectra are shown in Fig. 3. The spectrum of the β-barrel domain of the β-subunit (β-His) has a minimum at 217 nm, a maximum between 190 and 195 nm, and a negative to positive transition near 198 nm. This is a typical spectrum for a protein folded into a β-structure (Brahms, and Brahms, 1980), thus reflecting a large secondary structure consisting of β-sheets. This result suggests that the β-barrel domain of the β subunit folds into the

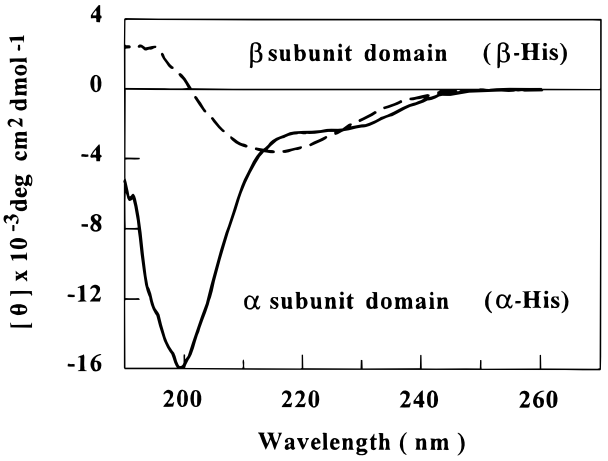

Fig. 3. CD Spectra of the β-Barrel Domains of α-His and β-His. The proteins α-His (solid line) and β-His (dashed line) were dissolved in 10 mM phosphate, pH 7.5, at 10 μM protein. The recorded CD spectra were converted to mean residue ellipticity (deg-cm^2/dmol $\times$ 10^{-3}).

secondary structure predicted from the crystal structure of the bovine F_1.

In contrast, α-His exists in solution predominantly in a coiled state. The CD spectrum of the β-barrel domain of the α subunit (α-His) is characterized by a strong negative absorption band near 200 nm and weak negative absorption band near 220 nm (Fig. 3). This result suggests that α-His is not folded in the final β-barrel conformation. Upon deconvolution of the CD spectra using the convex constraint algorithm (CCA) (Perczel *et al.*, 1992), the secondary structure of β-His was calculated to be 50% β strand/turn conformation and 40% coil, consistent with the crystal structure of bovine F_1. In contrast, the α-His spectrum was calculated to be 58% coil, 30% β-sheet, and a trace amount of α-helix, again suggesting that α-His is not folded into the conformation observed in the crystal structure of bovine F_1.

Analysis of Molecular Mass of Purified Protein

While it appeared that β-His, but not α-His, was correctly folded, it is possible that the final folded structure of α-His requires the presence of β-His. In order to test this, experiments were performed to analyze the interactions of the β-barrel domains. In this study, both the homotrophic and heterotrophic interactions of β-barrel domains of α and β subunits were investigated.

This question was first addressed by determining the apparent molecular weights, by size-exclusion chromatography coupled with on-line light scattering of α-His, β-His, and a mixture of α-His and β-His. If homotrophic or heterotrophic interactions occurred, then the apparent molecular weights would be higher than the calculated monomeric molecular weights. Typical elution profiles of α-His and β-His from a Superose 12 column are shown in Fig. 4. The α-His eluted with two peaks: an early minor peak and a later major peak with corresponding molecular masses of 60 and 16 kD, as calculated by light-scattering measurements (Fig. 4A). This indicated that the predominant form of the α-His was either a monomer (10.8kd) or dimer but it was also present as a hexamer. In contrast, the protein peak of β-His eluted at the void column volume indicating that β-His (9.8kd monomer) was aggregated into a large complex. This conclusion was supported by the light-scattering measurements, which indicated that the molecular mass of the β-His was over 1 million daltons.

The molecular weights of the proteins were also determined based on a comparison to those of the standard proteins (Fig. 4 C), using the peak profiles shown in Fig. 4A and B. This analysis gave results consistent with those determined from light-scattering measurements.

Many conditions were tried to fold β-His into a form that was not aggregated, including, extraction of the proteins in buffers with low (10 mM), or high (200 mM) salt in the absence or in the presence of 10% glycerol, low and high pH, and denaturation followed by slow or fast renaturation. In no case tested were we able to get β-His to fold into a β-sheet (as judged by CD analysis) and not in the aggregated form.

Formation of Hetero-Domain Complex in Vivo

The aggregation of α-His and β-His is reminiscent of the aggregation of the yeast α subunit in the absence of the β subunit and the aggregation of the β subunit in the absence of the α subunit (Ackerman, and Tzagoloff, 1990). Thus, one hypothesis is that the α subunit might prevent the aggregation of the β subunit. Similarly, the presence of α-His might prevent the aggregation of β-His. In order to test this hypothesis, it was necessary to provide a means to coexpress the α and β-subunit β-barrel domains in *E. coli*, and then be able to independently purify the domains. To achieve this, the β-barrel domain of the β subunit was

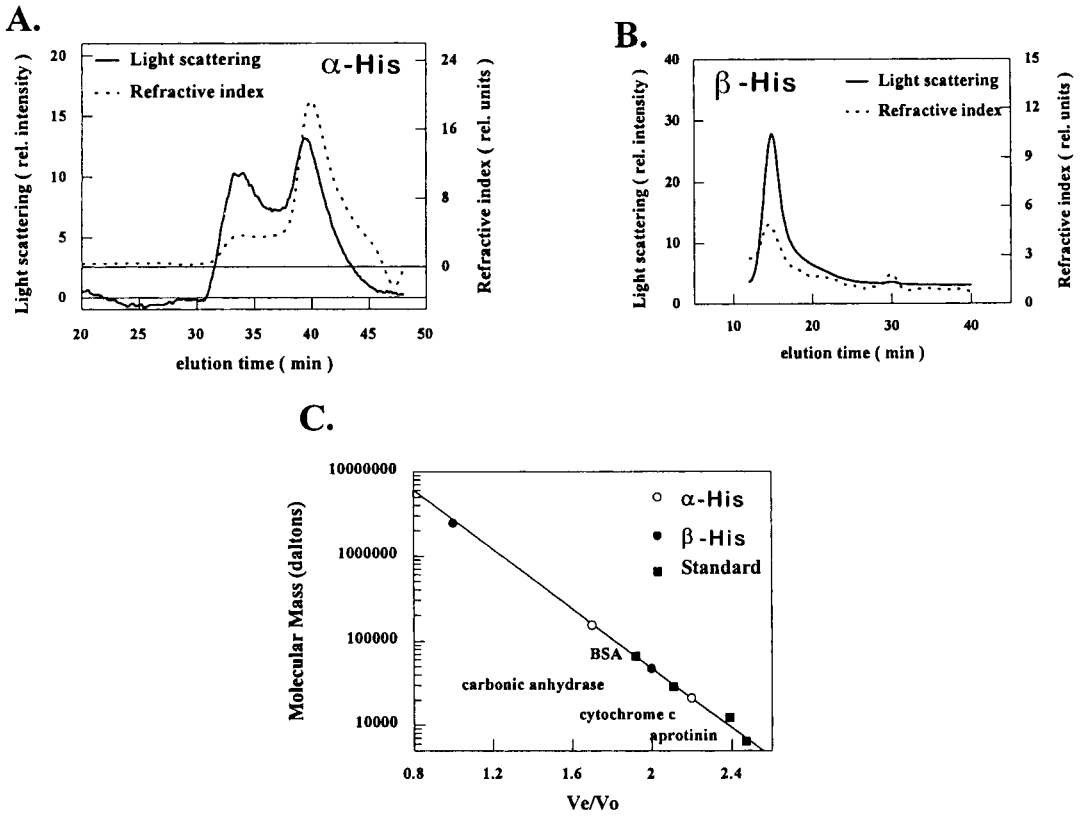

Fig. 4. Size-exclusion analysis of α-His and β-His on a Superose 12 column. The α-His (A) and β-His (B) proteins were dissolved in 20 mM Tris–SO_4, 100 mM KCl, and 1 mM EDTA, pH 7.5 buffer. The sample (200 μl of 1 mg/ml) was separated on a Superose 12 (30 × 10) size-exclusion FPLC column equilibrated with the same buffer. The elution profiles were monitored with on-line light scattering (solid line) and refractive index (dashed line). The molecular mass of the protein was determined from the light scattering as well as the position of protein in the elution profiles. (C). Molecular mass determined from the standard curve. To calibrate the column, standard proteins (100–200 μl of 0.5 mg/ml), indicated on the figure, were run under the same conditions. Blue Dextran was used to determine V_0.

tagged with the Flag epitope (β-Flag) (see Material and Methods). This allowed the purification of β-Flag by using a monoclonal antibody affinity column and it also allowed the detection of β-Flag using the M2 monoclonal antibody. Purification of α-His was still performed by Ni–agarose affinity chromatography. In addition, α- His and β-Flag were coexpressed in *E. coli* on a single vector that used identical transcriptional promoters, translational elements, and transcriptional terminators. Coexpression in *E. coli* of the α and β subunit domains would allow protein–protein interactions to occur, which may be necessary for the folding of α-His. Furthermore, coexpression in *E. coli* would also allow the heat-shock proteins, e.g., groEL and groES, help in the assembly and folding of the proteins.

Coexpresssion of α-His and β-Flag did indeed have an effect on the physical state of these proteins. The first evidence for this was revealed by the quite different conditions required for the stable expression of these proteins. When expressed individually, α-His, β-His, and β-Flag, were all induced by the addition of IPTG. However, when coexpressed, significant amounts of α-His and β-Flag were present in the cell only if the cells were grown in the absence of IPTG (Fig. 2A, lane 4). This was despite the fact that the plasmid and the transcriptional and translational elements were the same as those used to express the domains individually (cf. Fig. 2A, lanes 1 and 2). This suggests that the proteins have different stabilities when coexpressed as compared to expressed individually and this may reflect different conformations or states of the proteins.

To assess the effect of the coexpression on the state of the α-His, it was purified by Ni–agarose affinity chromatography (Fig. 2B, lane 4). Surprisingly, α-His eluted as a complex with β-Flag with about 70%

of the protein eluted from the column as α-His and the remaining as β-Flag (Fig. 2B, lane 4). This is a specific complex between α-His and β-Flag, since they effectively copurify on the Ni–agarose column and β-Flag alone does not bind to the Ni–agarose column. Thus, this elutant may reflect a mixture of α-His as the monomer/dimer and hexamer (as seen in Fig. 4) and α-His with β-Flag in a complex.

The size of the α-His/β-Flag complex was examined by gel filtration chromatography. The α-His/β-Flag protein was separated by gel filtration column and the elution profile was monitored by light scattering and refractive index (Fig. 5A). To determine the composition of protein in each of the fractions, the protein was separated by SDS gel electrophoresis followed silver staining (Fig. 5B) and by Western blot analysis using the M2 antibody directed against the Flag epitope of β-Flag (Fig. 5C). The elution profile is not just a simple sum of the profiles of α-His (Fig. 4A) and β-His (Fig. 4B). Instead, the profile indicates that the α-His/β-Flag complex elutes in three distinct peaks. Peak 1 eluted in the void volume and appeared as the large aggregate seen with β-His (cf. Fig 4B) or β-Flag alone (not shown). However, this peak is much smaller than that observed for β-His or β-Flag alone (not shown) indicating that expression of β-Flag with α-His reduced the aggregation by the β-barrel domain of the β subunit. Analysis of the protein in these fractions by SDS gel electrophoresis followed by silver staining (Fig. 5B) or Western blot analysis (Fig. 5C) indicates that the fractions contain a mixture of α-His and β-Flag. α-His was never observed to elute in such a large molecular weight complex when expressed in the absence of β-Flag, suggesting that it is in a complex with β-Flag. Peak 2 is a new peak and is shifted up from peak 2 of α-His alone (cf. Fig. 4A) and corresponds to a molecular weight of 200 kD. Again, analysis of the protein in this peak indicates that both α-His and β-Flag are present, although the predominate protein is β-Flag. The presence of this new peak also suggests the presence of unique interactions between β-His and β-Flag. Peak 3 eluted at almost the same position as peak 2 of α-His (cf. Fig. 4A) and corresponds to a monomer or dimer of α-His and β-Flag. The refractive index measurements and the SDS gel staining patterns indicate that this peak constitutes the majority of the protein. The protein is a mixture of α-His and β-Flag, again indicating specific association of the proteins. When considering the amount of β-Flag in peaks 2 and 3, the most dramatic feature of the analysis was the reduction of the aggregation state of β-Flag in the mixture. Since the β-Flag copurified with the α-His, this association is probably responsible for the decrease in the aggregation of β-Flag.

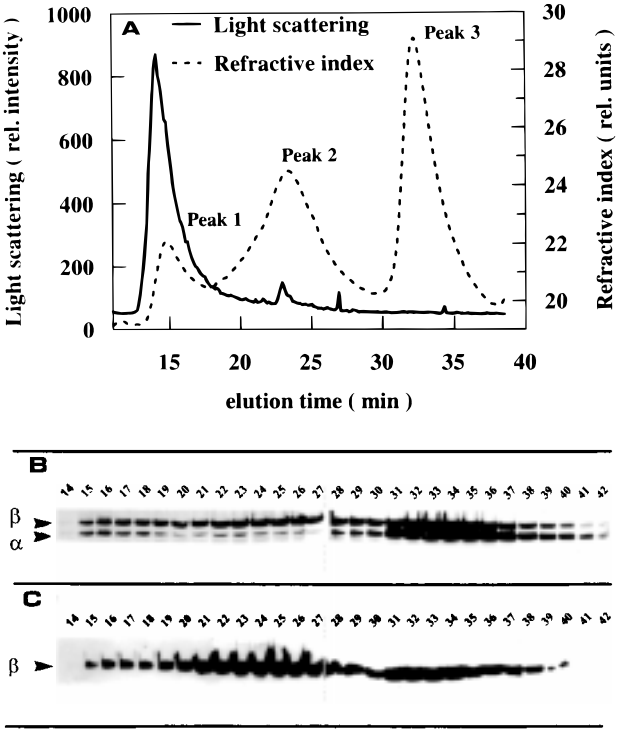

Fig. 5. Size-exclusion analysis of the α-His/β-Flag complex. (A): The α-His/β-Flag complex was purified by Ni–agarose affinity chromatography after coexpression of the proteins in *E. coli*. The purified protein was analyzed gel size-exclusion chromatography on a Superose 12 column. The protein was monitored by refractive index (dashed line) and light scattering (solid line). (B): Equal sample volumes from each fraction were separated on SDS–polyacrylamide gel electrophoresis and visualized by silver staining. The arrows indicate the positions of α-His and β-Flag. (C): The samples shown in (B) were analyzed by Western blot analysis using the anti-Flag $BioM_2$ monoclonal antibody, which reacts with β-Flag (see Material and Methods).

DISCUSSION

This study was initially designed to test the role of the β-barrel domains in stabilizing the F_1 molecule. However, this study has provided a unique understanding of the folding and assembly of the β-barrel domains, which mimics what is known about the folding and assembly of the yeast F_1-ATPase. Prior studies have indicated that both the α and β subunits of the yeast F_1-ATPase are stable when expressed in strains with a null mutation in either the gene encoding the

β or α subunit, respectively (Ackerman and Tzagoloff, 1990). Biochemical studies indicated that the β-subunit was in large aggregated structures of high molecular weight in a yeast atp1 (α subunit) null strain. Similarly, the α subunit is aggregated in an atp2 (β subunit) null strain. These results are similar to what is observed after heterologous expression of just the β-barrels domains of the α and β subunits. In this case, there is strong aggregation of the β-barrel domain of the β subunit, which form extremely large complexes in the absence of the α subunit β-barrel domain. The β-barrel domain of the α subunit also aggregates (hexamers) in the absence of the β subunit β-barrel domain, but not nearly as much or as strongly as observed with the β-subunit domain. Further, coexpression of the α- and β-subunit domains provides a heterodomain complexes in which the β-subunit domain is primarily in low molecular weight complexes with the α subunit.

The specific interactions of the β-barrel domains of the α and β subunits after coexpression in *E. coli* is quite clear. The proteins copurify on a Ni–agarose affinity column and this protein is nearly homogeneous for the α- and β-subunit domains. The Ni–agarose column is an affinity column for the (His)6 tag, present on the α-subunit domain and the column does not bind β-Flag to a significant degree. Furthermore, the two proteins coelute off a Superose 12 size-exclusion column in complexes of three different sizes. These complexes are stable since the protein from each peak retain its original elution behavior when rerun on the same column. Thus, the α- and β-subunit β-barrel domains are able to make specific heterodomain complexes.

CD analysis indicates that the β subunit folds into the expected β-sheet structure, while the α subunit was largely in a coil structure. This difference in secondary structure probably accounts for the differences observed in the aggregation of the proteins. Possibly, the β-sheets have hydrophobic faces that cause the aggregation process. In contrast, the α subunit β-barrel domain is folded into a more random conformation, which may be hiding large hydrophobic faces.

While the α-subunit β-barrel domain appears to be largely in the coil structure, it is possible that it is a defined conformational intermediate in the folding pathway. Possibly, this intermediate is necessary to prevent aggregation of the α subunit and still allow specific interactions with the β-subunit domain. This behavior would not be unlike some DNA- and RNA-binding proteins that undergo a coil-to-helix transition or become more ordered upon binding DNA (Spolar and Record, 1994; Blackwell *et al.*, 1994) or RNA (for review, see Frankel and Smith, 1998). Similarly, the α-subunit β-barrel domain may interact with the β-subunit β-barrel domain in a coil conformation and then proceed to the final folded β-barrel conformation.

However, while heterodomain complexes can be made *in vivo*, we have been unable to make heterodomain complexes *in vitro* with the purified proteins. The inability to obtain a specific heterocomplex of the α- and β-subunit β-barrel domains by *in vitro* reconstitution of the purified proteins may also be a direct result of the need for other proteins in the process. It is possible that assembly requires the presence of molecular chaperone(s). In yeast, there are at least four proteins that are important for the folding and assembly of the F_1-ATPase: hsp70, hsp60, atp11p, and atp12p. Protein hsp70 interacts with the precursor peptide on the cytosolic face of the mitochondria (Kang *et al.*, 1990). After entry into the mitochondria, hsp60 interacts with the polypeptide (Ostermann *et al.*, 1989; Cheng *et al.*, 1989). Then, Atp11p and Atp12p are required for the assembly of the ATPase, presumably acting like chaperones (Ackerman and Tzagoloff, 1990; Ackerman *et al.*, 1992). Interestingly, both the α and β subunits are in aggregated complexes in strains with mutations in ATP11 or ATP12. Furthermore, Atp11p is located in the mitochondrial matrix and associated with the α and β subunits of the F_1 (Ackerman *et al.*, 1992). These results indicate that Atp11p and Atp12p are required for the assembly of the ATPase and may prevent the subunits from forming large aggregates.

The results here indicate that the β-barrel domain of the α subunit has a chaperonelike activity—preventing or reducing the aggregation of the β-subunit β-barrel domain. The α subunit of the yeast ATPase has been shown to be required for the import of the β subunit into the mitochondrion (Yuan and Douglas, 1992). Furthermore, the α subunit isolated from chloroplast is active in a chaperonelike assay for the assembly of the β subunit into an active complex (Avni *et al.*, 1991). The results in this study suggest the β-barrel domain of the α subunit may provide a critical role in the assembly of the native complex.

Finally, mitochondrial F_1-ATPase has not been successfully reconstituted from the individual purified subunits, while this has been achieved for the prokaryotic and the chloroplast enzymes (Yoshida *et al.*, 1977; Vogel and Steinhart, 1976; Gao *et al.*, 1995). While it is not known what distinguishes the mitochondrial

enzyme from the prokaryotic enzymes on this issue, an obvious reason is that a chaperone is required in the mitochondrion. However, this only begs the question as to why the mitochondrial enzyme requires proteins for assembly that are not needed by the prokaryotic of chloroplast enzymes. Ultimately, the differences are due to differences in the primary structure of the proteins. In light of the results of this study, an attractive hypothesis is that differences in the β-barrel domains are at least partially responsible for the assembly requirements and demands for the folding of the mitochondrial F_1-ATPase.

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