Accelerated Publication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 37, Issue of September 15, pp. 28353–28355, 2000
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Printed in U.S.A.

Dimerization of Bovine F_1-ATPase by Binding the Inhibitor Protein, IF_1*

Received for publication, June 30, 2000, and in revised form, July 25, 2000
Published, JBC Papers in Press, July 28, 2000, DOI 10.1074/jbc.C000427200

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In mitochondria, the hydrolytic activity of ATP synthase is regulated by a natural inhibitor protein, IF_1. The binding of IF_1 to ATP synthase depends on pH values, and below neutrality, IF_1 forms a stable complex with the enzyme. Bovine IF_1 has two oligomeric states, dimer and tetramer, depending on pH values. At pH 6.5, where it is active, IF_1 dimerizes by formation of an antiparallel α-helical coiled-coil in its C-terminal region. This arrangement places the inhibitory N-terminal regions in opposition, implying that active dimeric IF_1 can bind two F_1 domains simultaneously. Evidence of dimerization of F_1-ATPase by binding to IF_1 is provided by gel filtration chromatography, analytical ultracentrifugation, and electron microscopy. At present, it is not known whether IF_1 can bring about the dimerization of the F_1F_0-ATPase complex.

The F_1F_0-ATP synthase complex (also called F_1F_0-ATPase) plays a central role in energy transformation in most living organisms. It is composed of two major domains, a globular F_1 catalytic domain and a membrane-bound F_0 proton-translocating domain linked together by a central stalk. The synthesis of ATP requires an electrochemical proton gradient across the inner mitochondrial membrane, which is driven by the transport of protons back into the matrix through the F_0 domain. When a cell is deprived of oxygen, the electrochemical gradient across the inner membrane collapses, and the enzyme switches its catalytic activity from ATP synthesis to ATP hydrolysis. Under these conditions, F_1-ATPase, the catalytic domain of ATP synthase, catalyzes the hydrolysis of ATP to ADP and phosphate. In mitochondria, this hydrolytic activity is regulated by a natural inhibitor protein, IF_1. In bovine mitochondria, IF_1 is a basic protein of 84 amino acids long (1). The binding of IF_1 to ATP synthase depends on pH values and, below neutrality, its inhibitory capacity increases (2). Recently, we have shown that bovine IF_1 has two oligomeric states, tetramer and dimer, favored by pH values above and below 6.5, respectively (3). Activation is accompanied by a decrease in IF_1 helicity relative to the inactive form between residues 35 and 47, which are involved in the formation of the inactive tetramers. At a pH value of about 6.5, IF_1 forms an active antiparallel dimer, and this arrangement places the inhibitory N-terminal regions in opposition. At higher pH values, two dimers associate into the inactive tetramer. An important implication of this model is that dimeric IF_1 is capable of binding to two F_1 domains simultaneously.

In this paper, we describe experiments using gel filtration chromatography, analytical ultracentrifugation, and electron microscopy that validate this prediction.

MATERIALS AND METHODS

Purification of F_1-ATPase and the IF_1-F_1 Complex—The purification of bovine F_1-ATPase (4) and recombinant bovine IF_1 (3) were carried out as described previously. The IF_1-F_1 complex was prepared as follows: purified F_1-ATPase (25 mg), stored as an ammonium sulfate precipitate, was collected by centrifugation (31,000 × *g*) at 4 °C, re-dissolved in minimal buffer (20 mM MOPS[1]-NaOH, pH 6.6, 1 mM EDTA, 10% glycerol, 0.001% (w/v) phenylmethylsulfonyl fluoride), and desalted on a PD-10 column (Amersham Pharmacia Biotech). The enzyme was mixed with a 5-fold molar excess of IF_1 over F_1 and kept for 20 min at 37 °C. A portion of MgATP was added at 5, 10, and 15 min to give a final concentration of 0.5 mM. Then, the mixture was loaded onto a HiLoad 26/60 Superdex 200 column to separate the IF_1-F_1 complex from F_1-ATPase and free IF_1.

Gel Filtration Chromatography and SDS Polyacrylamide Gel Electrophoresis—Protein samples (5 mg/ml; 100 μl) prepared as described above were chromatographed at room temperature at a flow rate of 0.2 ml/min on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) pre-equilibrated in buffer containing 50 mM MOPS-NaOH, pH 6.5, 10 mM magnesium sulfate, 1 mM EDTA, 0.02% sodium azide,10% glycerol, and 0.001% phenylmethylsulfonyl fluoride. The absorbance of the eluant was monitored at 280 nm. Thyroglobulin, ferritin, catalase, and aldolase (molecular masses of 669, 440, 232, and 158 kDa, respectively) were used to calibrate the column. Its void volume (V_0) was determined using blue dextran 2000. The K_{av} for the individual proteins was calculated from the following expression: $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_t and V_e are the elution volume for the protein and the total bed volume, respectively. A plot of K_{av} *versus* log(molecular weight) with standards gave a straight line with a correlation coefficient of 0.99. Linear regression gave the equation log(molecular weight) = −2.239 K_{av} + 3.427, which was used to calculate the apparent molecular weight.

Denaturing SDS-PAGE gels containing a 12–22% (w/v) acrylamide gradient separating gel and a 4% (w/v) stacking gel (acrylamide:*N,N′*-methylenebisacrylamide), 30:0.8 (w/w) were cast in 10-cm × 10-cm × 0.6-mm format and run in the buffer system of Laemmli (5).

Sedimentation Velocity—Sedimentation velocity runs were performed at 20.0 °C and at various rotor speeds (finally using 40,000 rpm for F_1-ATPase and 30,000 rpm for the IF_1-F_1 complex) in a Beckman AN-60Ti rotor and a Beckman XL-A ultracentrifuge. The samples were dissolved in buffer consisting of 50 mM MOPS-NaOH, pH 6.5, 10 mM magnesium sulfate, 1 mM EDTA, 0.02% sodium azide, and 0.001% phenylmethylsulfonyl fluoride. Scans were taken as frequently as possible (*i.e.* with a zero-interval setting, which gives ~4.5 min with 3 cells in the rotor). Data were analyzed initially by plotting g(s^*) against s^*, where g(s^*) is the fraction of material sedimenting between s^* and (s^* + δs^*) (6, 7), using the DCDT+ software package (Version 1.05) (8). This software was also used for direct fitting of simple Gaussian functions to d*c*/d*t* *versus* *s* curves (9) to test for the number of components

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§ Supported during part of this work by an European Molecular Biology Organization fellowship and by a Training and Mobility of Researchers Marie Curie research training grant from the European Community.

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[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

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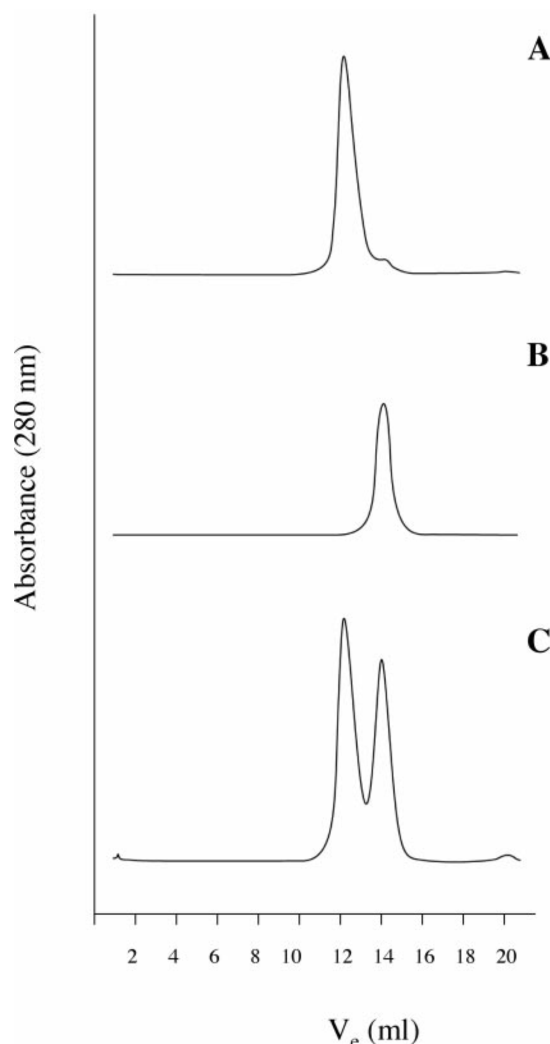

FIG. 1. **Gel filtration chromatography of F_1-ATPase and of the IF_1-F_1 complex.** *A* and *B*, elution profiles of the IF_1-F_1 complex and F_1-ATPase, respectively. *C*, separation of a mixture of the IF_1-F_1 complex and F_1-ATPase.

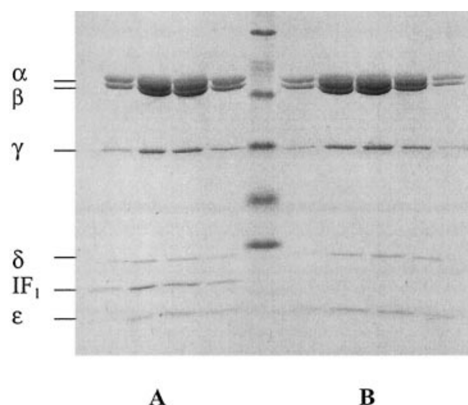

FIG. 2. **Analysis of column fractions by SDS-PAGE.** *Panels A* and *B*, fractions from peaks *A* and *B*, respectively, in Fig. 1. The apparent molecular masses of the standard proteins are: 83, 62, 47.5, 32.5, 25, and 16.5 kDa.

giving a "best fit" to the data. Sedimentation coefficients were converted from s^* to $s_{20,w}$, taking values of 0.759 ml/g for the partial specific volume, 1.00213 g/ml for the solvent density, and 1.0103 (entipoise) for the viscosity, calculated with SEDNTERP (Version 1.03) (10). All plots were produced from the data using the program ProFit (QuantumSoft). For plots of the original scans, scans at equal time intervals were

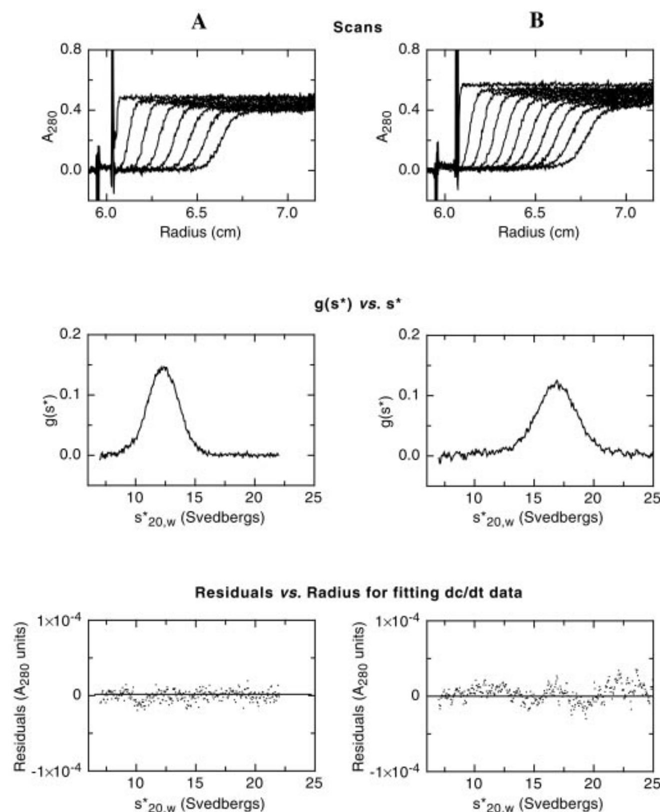

FIG. 3. **Sedimentation velocity analysis of F_1-ATPase and of the purified IF_1-F_1 complex.** The data are shown (at equal time intervals for each sample) as A_{280} against radius for each sample. Analysis of the data is shown as plots of g(s^*) against s^* (showing the distribution of the sample with sedimentation coefficient) and also as the residuals (in A_{280} against $s^*_{20,w}$) for fitting of a model with a single component, distributed as a Gaussian function, to the dc/dt curves. *Panels A* and *B*, F_1-ATPase and IF_1-F_1 complex, respectively.

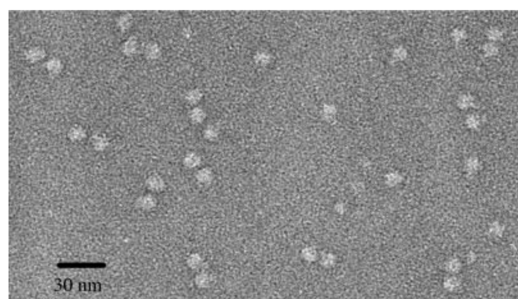

FIG. 4. **Electron microscopy of IF_1-F_1 complexes in negative stain.** Samples were diluted at 0.01 mg/ml in gel filtration buffer and stained with a solution of 4% (w/v) methylamine tungstate in water (pH 6.5).

selected (630 s for F_1 alone and 666 s for F_1-IF_1complex), to give visual separation between the traces drawn.

Electron Microscopy—Grids were prepared by evaporating carbon onto 400-mesh copper/rubidium grids (Maxtaform, Graticules, Tombridge, U.K.). They were covered by a thin film of a 0.5% Formvar solution and then washed with chloroform. Grids were glow-discharged for 30 s to make the carbon film hydrophilic. A solution of the purified IF_1-F_1 complex (5 μl, 0.01 mg/ml) was applied to the grid and left for 2 min. Then the grid was washed with three drops of gel filtration buffer and stained with a solution of 4% (w/v) methylamine tungstate (Agar, Stansted, U.K.) in water (pH 6.5).

Images were recorded at a magnification of 67,000 on a Philips Tecnai 12 electron microscope operating at 100 kV using low dose conditions (approximately 10 $e^-/Å^2$). The quality of the images was checked on an optical difractometer. The defocus was about 1000 nm. The images were collected with a Zeiss-SCAI scanner using a step size

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of 7 μm (pixel size, 1.04 Å). Images were demagnified by linear interpolation on the computer to a pixel size of 5.2 Å.

RESULTS AND DISCUSSION

Apparent Molecular Masses of F_1-ATPase and the IF_1-F_1 Complex in Solution—The apparent molecular masses of F_1-ATPase and IF_1-F_1 complex in solution were determined by gel filtration chromatography (Fig. 1) and sedimentation velocity analysis (see Fig. 3). F_1-ATPase eluted from a Superose 6 column as a single peak of 367 kDa, whereas under the same conditions, the IF_1-F_1 complex eluted at 696 kDa. The small shoulder contains monomeric F_1-ATPase (Fig. 1*A*). Fractions from both peaks were analyzed by SDS-PAGE (Fig. 2). The gels confirmed the presence of IF_1 (9.6 kDa) in the IF_1-F_1 complex. The separation of the F_1 and IF_1-F_1 complexes by gel filtration has been observed before (11).

Sedimentation velocity data for F_1-ATPase and the IF_1-F_1 complex are summarized in Fig. 3. Direct d*c*/d*t* analysis showed that F_1 alone had $s_{20,w} = 12.1\ (\pm\ 0.7)$ S and F_1-IF_1 complex had $s_{20,w} = 17.0\ (\pm\ 0.1)$ S. To test whether there might be more than one component in the boundary, plots of g(s^*) against $s^*_{20,w}$ were also made. As a further test, model fitting of the d*c*/d*t* data with models with either one or two components was carried out. The best fits were given by the single-component model for both data sets, and plots of the fit residuals against radius are shown (Fig. 3). This increase in sedimentation coefficient is compatible with IF_1 binding two F_1-ATPase units in the complex, and this is the only possibility, as any shape change that produced such an increase in sedimentation would have led to a lower apparent molecular mass during elution from the column. Thus all of the hydrodynamic data are only explained by a dimerization of F_1-ATPase on forming a complex with IF_1.

Electron Microscopic Examination of the IF_1-F_1 Complex—Samples of the IF_1-F_1 were diluted in the gel filtration buffer to a protein concentration of 0.01 mg/ml and stained with methylamine tungstate (4%, w/v). Methylamine tungstate, which has a pH value of 6.5 in water solution, has been reported to preserve delicate complexes such as viruses, and pH 6.0–6.5 is optimal for formation of the IF_1-F_1 complex (12).

Electron microscopic analysis of the samples (Fig. 4) revealed the presence of dimeric F_1 complexes. They represented about 70% of the particles present in the fields that were examined, the other particles being mostly monomeric F_1-ATPase. As the starting material was the main peak obtained by gel filtration of the F_1-IF_1 complex, it was devoid of monomeric F_1-ATPase. Therefore, the monomers must have arisen by disruption of the dimeric complex during the dilution and staining procedures. A few particles appear to be trimeric. As there was only evidence for dimer formation by gel filtration and sedimentation analysis, these apparent trimers in the electron microscopy pictures are unlikely to represent biologically significant assemblies. In a control experiment, particles of F_1 alone were examined in the same way. About 94% of them were clearly monomeric.

Implications for Regulation and Quaternary Structure of ATP Synthase—Bovine IF_1 exists in two oligomeric states depending on the pH value. At low pH values, IF_1 forms an active dimer held together by an antiparallel α-helical coiled-coil from residues around 46–84 (3).[2] In this arrangement, the N-terminal inhibitory domains are exposed, and each is capable of interacting with an F_1-ATPase complex, as the results presented here now demonstrate. It is thought that the interaction between IF_1 and F_1 involves the C-terminal region of one or more of the β-subunits (14).

At present, it is not known whether IF_1 can bring about the dimerization of the F_1F_0-ATPase complex and whether it is involved in formation of dimeric complexes of bovine and yeast F_1F_0-ATPases that have been detected by native gel electrophoresis in mild detergents (13, 15). The dimeric yeast enzyme contains membrane bound subunits (e and g) that are not present in the monomer, and they appear to be necessary for dimerization to take place. However, the possible involvement of the inhibitor protein (and of two other proteins known as 9 and 15K proteins, which are both required for the action of the yeast IF_1) have not been investigated. It may be that dimeric F_1F_0-ATP synthase forms specific supramolecular complexes with the respiratory complexes in the inner mitochondrial membrane (13).

Acknowledgments—We thank M. Montgomery for excellent technical assistance.

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