Equilibrium between monomeric and dimeric mitochondrial F1–inhibitor protein complexes

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Abstract Mg-ATP particles from bovine heart mitochondria have more than 95% of their F1 in complex with the inhibitor protein (IF1). The F1–IF1 complex was solubilized and purified. The question addressed was if this naturally occurring complex existed as monomers or dimers. Size exclusion chromatography and electron microscopy showed that most of the purified F1–IF1 complex was a dimer of two F1–IF1. As determined by the former method, the relative concentrations of dimeric and monomeric F1–IF1 depended on the concentration of protein that was applied to the column. Apparently, there is an equilibrium between the two forms of F1–IF1. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Dimerization; F1-ATPase; Inhibitor protein; Endogenous complex

1. Introduction

In the membranes of mitochondria the ATP synthase catalyzes the synthesis of ATP from ADP and inorganic phosphate with the energy of electrochemical H^+ gradients. It consists of a membrane moiety, Fo, that allows the passage of H^+ across the membrane, and F1, which is a multisubunit protein that has the catalytic machinery for ATP synthesis and hydrolysis. F1 can be detached from the membrane as a soluble protein; it is formed by five different subunits α_3, β_3, γ, δ and ε (for review see [1,2]). The mitochondrial ATP synthase also has a 84 residue protein [3] that regulates the catalytic properties of the enzyme; this protein is known as naturally occurring ATPase inhibitor [4]. When particles devoid of inhibitory protein or soluble F1 are incubated with the inhibitory protein (IF1) at relatively acid pH and ATP, IF1 binds to F1 and inhibits its ATPase activity. In these reconstitution experiments, the stoichiometry of bound IF1 to F1 is 1 [5]. IF1 also inhibits ATP synthesis in submitochondrial particles [6–8]; in this case, the inhibitory action of IF1 is removed by electrochemical H^+ gradients, and thereby, allows ATP synthesis.

The structure of IF1 has been extensively studied [9–11]. A characteristic of IF1 is that at acid pH, two IF1 interact and form a homodimer [12]. Other experiments have shown that the incubation of soluble F1 with IF1 at acid pH and ATP yields a dimeric enzyme formed by two F1–IF1 complexes [13]. The authors suggested that IF1 dimerizes through antiparallel α-helical coiled-coil portions of their C-terminal region, and that their opposite N-terminal regions interact with F1. In yeast mitochondria, it has also been shown that the FoF1 ATP synthase may exist in dimers through a process that is dependent on subunits *e, g*, and *k* [14,15]. However, it is not known if in the mitochondrial membrane F1 in complex with IF1 is a dimer formed by two F1–IF1 complexes.

The F1–IF1 complex may also be obtained from submitochondrial particles that have their F1 already in complex with IF1 [16,17]. Thus, it was relevant to the mechanism of action of IF1, and in consequence to the regulation of the catalytic properties of FoF1, to examine if the naturally occurring F1–IF1 complex in its soluble form is also a dimer. The results of the studies showed that the solubilized form of the naturally occurring F1–IF1 is a dimer that exists in equilibrium with monomeric F1–IF1.

2. Materials and methods

Mitochondria from bovine hearts and Mg-ATP submitochondrial particles were prepared as described in [18,19]. F1–IF1 was prepared as in [19], except that the complex obtained from the Sepharose–hexylammonium column was applied to a Superdex 200 HR FPLC column and eluted with 50 mM Tris–SO_4 pH 6.5. The eluted F1–IF1 complex was concentrated in Centricon filters to between 5 and 10 mg/ml, and stored at −70°C. To determine the extent of control of the hydrolytic activity of soluble and particulate F1 by IF1, the preparations were incubated in 50 mM Tris–SO_4 pH 8.0, 2 mM EDTA, 100 mM KCl, and 10 mM ATP at 40°C. At different incubation times aliquots were withdrawn for assay of ATPase activities. The maximal activities reached, in relation to the initial activities were considered an index of control of either soluble or particulate F1 by IF1 [17]. The basal activities of Mg-ATP particles and soluble F1–IF1 were around 0.4 and 2–3 μmol/min/mg, respectively. At the maximal point of activation, activities were of the order of 7 and 70 μmol/min/mg for particulate and soluble F1, respectively. Soluble F1, free of IF1 was prepared as described [20].

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Abbreviations: F1–IF1, mitochondrial F1 in complex with inhibitor protein (IF1)

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2.1. Size exclusion chromatography

The determination of the molecular masses of F1 and F1–IF1 was carried out in SEC4000 column. Soluble F1–IF1 and F1 at the indicated protein concentrations were applied and eluted with 50 mM Tris–SO_4 pH 6.5, 10% glycerol, 0.02 mM sodium azide, 0.001% phenylmethylsulfonyl fluoride, 10 mM $MgSO_4$, and 1 mM EDTA. The flux rate was 1 ml/min; when required fractions were collected every 15 s. The data were analyzed with Microcal Origin 5.0 and Jandel PeakFit. Molecular weights were calculated according to the equation $K_{av} = (V_e - V_0)/(V_t - V_0)$, where V_e, V_0 and V_t are protein elution volume, void volume and total column volume, respectively. The standards were: thyroglobulin, γ globulin, ovalbumin, myoglobin, vitamin-B_{12} (670, 158, 44, 17, and 1.35 kDa, respectively). Plots of K_{av} versus log (molecular weights) were linear with a correlation coefficient of 0.98.

The samples of F1–IF1 for electron microscopy analysis were prepared exactly as described [13], except that the grids were washed with the buffer used in the gel filtration experiments. This step was prior to staining with 4% methylamine tungstate.

Protein was determined by the bicinchoninic acid method [21].

3. Results

Mg-ATP particles from bovine heart mitochondria were the starting material for the preparation of the naturally occurring F1–IF1. The particles had an ATPase activity of 0.4 μmol/min/mg of protein. Their incubation in media with an alkaline pH and salt increased their ATPase activity to a value of 7 μmol/min/mg. This is due to the removal of IF1 from its inhibitory site in F1 [17]. Assuming that the F1–inhibitor complex has no catalytic activity, the data indicate that about 95% of the population of particulate F1 was in complex with IF1. This naturally occurring F1–IF1 complex was solubilized from Mg-ATP particles and purified. In many preparations of F1–IF1, we have seen that in SDS–PAGE, the enzyme exhibits the subunit composition of F1 plus the subunit that corresponds to the inhibitory protein. The basal ATPase activity of soluble F1–IF1 was between 2 and 3 μmol/min/mg. After incubation in the activation media, activity reached a value of 60–70 μmol/min/mg. Thus, particulate and soluble F1–IF1 exhibited the same characteristics.

Size exclusion chromatography of the F1–IF1 preparation showed that the peak of maximal absorbance appeared with 7 ml of elution volume (Fig. 1) that corresponds to a molecular mass of 670 kDa. We also determined the elution volume of free F1. The peak of maximal absorbance was reached with 7.6 ml of elution volume. A mixture of F1 and F1–IF1 exhibited two peaks that appeared with 7 and 7.6 ml. Because the chromatographic system employed would not distinguish between F1 (MW = 360 kDa) and a monomeric form of F1–IF1 (MW = 370 kDa), the elution volume of F1 was the reference of the volume at which monomeric F1–IF1 elutes. Thus, the chromatographic data indicated that the principal component of F1–IF1 was a dimer.

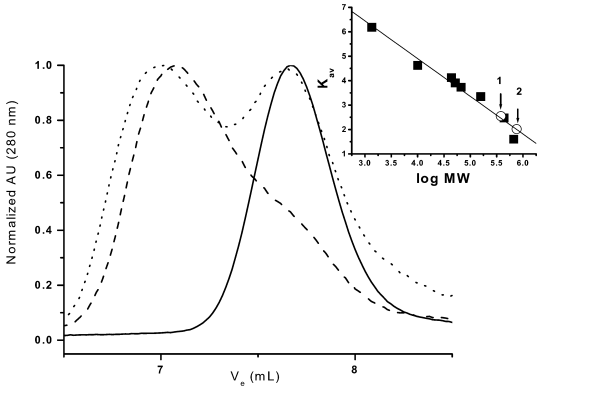
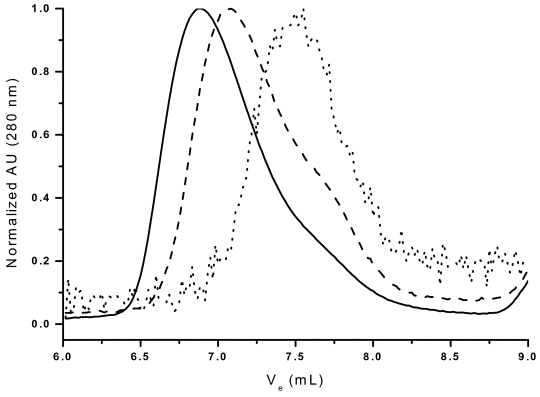


Fig. 1. Elution profiles of soluble F1–IF1, F1 and a mixture of the two enzymes (dashed, solid and dotted lines, respectively). For each of the three samples, the maximal absorbance was normalized to 1. The inset shows plots of K_{av} against the log of the molecular weight of standards and the point of maximal absorbance of (1) F1 and (2) F1–IF1.



Total protein (μg)	Elution maxima (mL)	Percent of distribution D	M
350	6.9	69 ±10	21 ±10
100	7.0	57 ±10	23 ±10
16	7.5	0	90 ±10

Fig. 2. Elution profile of F1–IF1 at different concentrations. The solid, dashed, and dotted lines indicate the profile when 350, 100, and 16 μg in volumes of 35, 10, and 50 μl respectively, were applied to the SEC4000 column. For each of the three samples, the maximal absorbance observed was normalized to 1. Data of the elution maxima and the relative amounts of dimer (D) and monomer (M) are indicated. The latter was calculated from the elution profile:

$$y = (a_0/\sqrt{2\pi a_2})\exp(-1/2(x-a_1/a_2))^2$$

adjusted to a non-linear Gaussian fit: where y is absorbance, a_0 = area, a_1 = center of the area, a_2 = width, and x is elution volume.

Nonetheless, it is important to note in Fig. 1 that the elution profile of the naturally occurring F1–IF1 was not symmetrical. It exhibited a clear shoulder in the volume at which F1 eluted. The shoulder could be due to a contamination by F1 in the F1–IF1 preparation, or to the existence of a fraction in which F1–IF1 was in the monomeric state. The activity data (see above) indicated that in soluble F1–IF1, the contamination by free F1 was less than 5%. Thus, the clearly apparent shoulder in the elution profile of F1–IF1 suggested that the preparation of soluble F1–IF1 was a mixture of dimeric and monomeric F1–IF1 and that these two forms could be interconvertible.

To assess if there is an equilibrium between dimers and monomers of F1–IF1, three different amounts of F1–IF1 were passed though the size exclusion chromatographic column (Fig. 2). The elution profile of 350 and 100 μg showed that the peak of maximal absorbance appeared with very similar volumes; however, the asymmetry of the elution curve

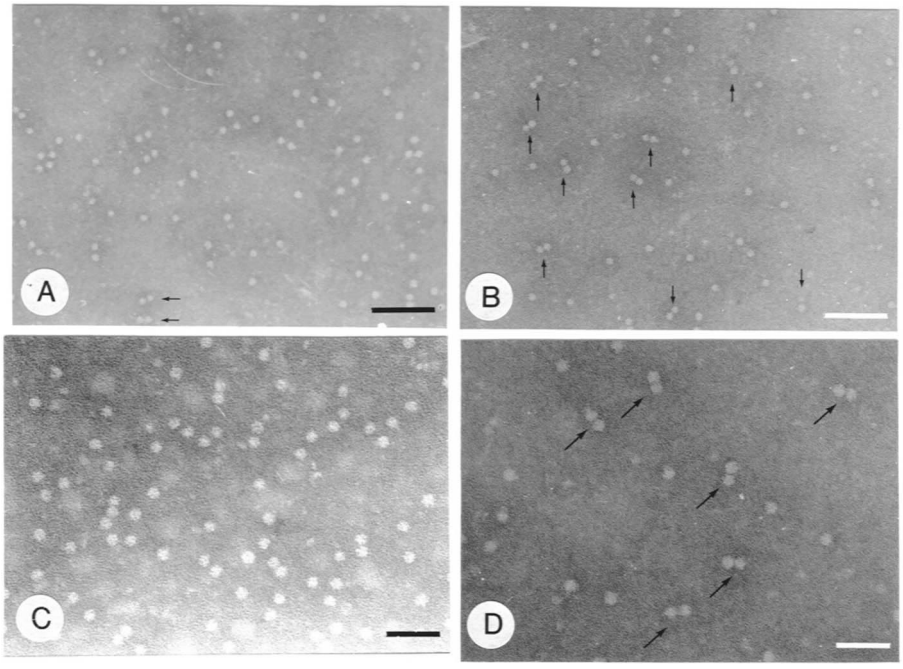


Fig. 3. Electron micrographs of dimeric and monomeric F1–IF1. The fractions of F1–IF1 that eluted from the size exclusion chromatography (Fig. 1) as monomers (A and C) and as dimers (B and D) were analyzed by electron microscopy as described in Section 2. The arrows in B and D indicate dimers. Magnification in A and B was 120 000 and in C and D, 200 000. Bar in A and B = 100 nm, in C and D = 50 nm.

with the lower concentration was more marked. Moreover, when 16 μg was applied, the peak of maximal elution appeared in the region of 'monomeric F1–IF1'. The deconvolution of the elution curves (inset Fig. 2) showed that at the higher concentrations, the naturally occurring F1–IF1 consisted of dimers and monomers, the concentration of the latter being higher at the lower protein concentrations. With the lowest concentration of F1–IF1 applied, the enzyme eluted essentially as 'monomeric F1–IF1'.

The nature of the protein fraction that elutes as what we have called 'monomeric F1–IF1' deserves a further comment. Dimeric F1–IF1 could have dissociated into two complexes each formed by 1 F1 with 1 IF1. Alternatively, if dimers of IF1 do not dissociate, the fraction could represent a mixture of 1 F1 in complex with dimeric IF1 and free F1. In this respect, it is noted that the specific activity of this protein fraction was around 2 μmol/min/mg and that after activation, it reached a value of at least 70 μmol/min/mg. This indicated that almost the totality of F1 was inhibited by IF1. Therefore, it would seem that dimeric F1–IF1 dissociated into monomeric F1–IF1.

In the work that described that reconstituted F1–IF1 is a dimer, Cabezon et al. [13] included micrographs that showed that about 70% of the population of F1–IF1 was in the dimeric form. With the same methodology we examined the fraction of F1–IF1 that eluted from the size exclusion column as dimers, and that that eluted as monomeric F1–IF1. In the former, the number of structures that appeared as dimers was significantly higher than in the fraction that corresponded to monomeric F1–IF1 (Fig. 3B,D versus A,C). However, the abundance of dimers in this fraction was relatively low, approximately 20% of the total population. It is noted that in the preparation of the samples for electron microscopy, F1–IF1 had to be diluted 1000-fold. According to the data in Fig. 2, at this concentration, F1–IF1 would be essentially in the monomeric form. This could account for the relatively low abundance of dimeric F1–IF1.

4. Discussion

The ATPase activity of Mg-ATP particles is less than 5% of the maximal activity that is reached after removal of the inhibitory action of IF1; thus, in these particles more than 95% of their F1 are in complex with IF1. When this natural form of F1–IF1 is solubilized and purified, the resulting preparation is formed by dimers of F1–IF1 that at pH 6.5 are in equilibrium with F1–IF1 monomers. A central question that arises from these studies is whether the findings are related to the regulation of the catalytic properties of particulate FoF1. It has been estimated that in the mitochondrial membrane the ratio of F1 to IF1 is 1 [5]. In solutions of relatively acid pH, soluble IF1 exists almost exclusively as dimers [13]. If this situation also holds for IF1 in the membrane and the ratio of IF1 monomers to F1 is 1, complete abolition of ATPase activity by IF1 requires that an IF1 dimer interacts with two F1, yielding a dimer of two F1–IF1 complexes. On the other hand, if in the membranes monomeric IF1 is the active species, with a 1:1 stoichiometry of IF1 to F1, full inhibition of F1 could be achieved without obligatory dimerization of two F1–IF1 complexes. In this context, it is noteworthy that a fragment of IF1 that contains the inhibitory residues, but lack the amino acid sequence required for dimerization is a good inhibitor of F1 [22].

As shown here, the monomeric and dimeric forms of F1–IF1 are in equilibrium. Thus, it may also be asked if the proton motive force induced transition of F1–IF1 to the catalytically active form of F1 [6–8] involves a shift in the equilibrium between dimers and monomers of F1–IF1, or whether it merely involves a displacement of monomeric IF1 from its inhibitory site in monomeric F1 [23,24]. Along this line, it is

recalled that IF1 binds to other proteins of the mitochondrial membrane [25,26]. Thus, the findings that FoF1 complexes may dimerize through subunits *e, g,* and *k* [14,15] and that soluble F1–IF1 undergoes reversible dimerization raises new questions as to the contribution of macromolecular complexes to the process of energy conservation during oxidative phosphorylation.

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