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Interconversion Between Dimers and Monomers of Endogenous Mitochondrial F_1-Inhibitor Protein Complexes and the Release of the Inhibitor Protein. Spectroscopic Characteristics of the Complexes

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The F_1-inhibitor protein complex (F_1-IP) was purified from heart submitochondrial particles. Size exclusion chromatography of the endogenous complex showed that it contains dimers (D) and monomers (M) of F_1-IP. Further chromatographic analysis showed that D and M interconvert. At high protein concentrations, the interconversion reaction is shifted toward the D species. The release of the inhibiting action of IP is faster at low than at high protein concentrations. During activation of F_1, the M species accumulates through a process that is faster than the release of IP from F_1. These findings indicate that the activation of F_1-IP involves the transformation of D into M, which subsequently loses IP. The spectroscopic characteristics of D, M, and free F_1 show that the binding of IP and dimerization modifies the fluorescence intensity of tyrosine residues and that of the single tryptophan of F_1 which is far from the IP binding site.

KEY WORDS: Mitochondrial ATPase inhibitor protein; mitochondrial ATP synthase; dimerization; monomer–monomer interactions; mitochondrial F_1-inhibitor protein complexes; mitochondrial ATPase activation.

INTRODUCTION

The F_oF_1 synthase is the enzyme complex that harnesses the electrochemical energy generated by electron transport and catalyzes the synthesis of ATP from ADP and inorganic phosphate. The complex has a protein moiety called F_1 which has the catalytic machinery for ATP synthesis and hydrolysis; it can be isolated in a soluble form. The ATP synthase also has a membrane portion called F_o which is responsible for ion translocation. It is currently believed that the ATP synthase catalyzes the synthesis and hydrolysis of ATP according to the binding change mechanism (Boyer, 2001), and that this process involves the rotation of subunits γ, ε, and c relative to subunits α, β, δ, a, and b (Noji *et al*., 1997). The F_oF_1 complex from different organisms has between 15–17 different subunits (Velours and Arselin, 2000); bovine F_oF_1, however, has subunits that do not exist in enzymes from other organisms, such as bacteria. One of these supernumerary subunits is the inhibitor protein (IP). This protein was originally described by Pullman and Monroy (1963) as a heat-resistant, nondialyzable protein of about 10 kDa

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Key to abbreviations: Bis-Tris, bis-[2-hydroxyethyl]iminotris [hydroxymethyl]-methane; D, dimer of two F_1 and two inhibitor proteins; EDTA, ethylenediamine tetraacetic acid; IP, natural ATPase inhibitor protein; M, monomer of F_1 with one inhibitor protein; Mes, (2 [*N*-morpholino]ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride.

that inhibits ATP hydrolysis and confers cold-resistance to soluble F_1. The binding of the inhibitor protein to F_1 requires ATP hydrolysis; this is because IP binds to the enzyme when it is in the MgADP form (Di Pietro *et al*., 1988; Milgrom, 1991). It is also known that binding of IP is faster at a relatively acid pH (Lebowitz and Pedersen, 1993). The binding constant is estimated to be 50 and 200 nM for particulate and soluble F_1, respectively (Power *et al*., 1983; Lippe and Harris, 1988a,b; Van Heeke *et al*., 1993). The inhibitor protein binds to F_1 with a stoichiometry of 1 to 1 (Klein *et al*., 1980; Wong *et al*., 1982; Jackson and Harris, 1983).

Attempts have been made to ascertain the residues of the inhibitor protein that interact with F_1, and if a portion of the inhibitor protein has the capacity to inhibit ATP hydrolysis (for review see Green and Grover, 2000). It has been shown for example, that a peptide that contains residues 22–46 is a potent inhibitor of ATPase activity (de Chiara *et al*., 2002); however, under different conditions, the potency of this peptide was not confirmed (van Raaij *et al*., 1996). Thus, from the available data is not yet possible to define precisely which residues in the inhibitor protein are responsible for the inhibition of the activity of F_1. The problem became more complex when Cabezón *et al*. (2000a) showed that F_1 reconstituted with the inhibitor protein exists as a dimer of two F_1-IP complexes. Additional data showed that at acid pH, two inhibitor proteins associate to form a dimer, and that at alkaline pH, the protein associates into a tetrameric structure. Histidine 49 has been shown to be crucial for this pH-dependent regulation (Schnizer *et al*., 1996; Cabezon *et al*., 2000b, 2001).

NMR and X-ray studies of the dimer indicate that the two inhibitor proteins are linked by residues 44–84 in a coiled-coil arrangement in which there is a relative abundance of hydrophobic amino acids. More recently, the crystal structure of the dimeric F_1-IP complex was reported (Cabezón *et al*., 2003). In the structure, the amino acid terminal regions of two inhibitor proteins interact with two F_1. The dimeric structure is stabilized by the coiled-coil arrangement of the two inhibitor proteins. In consonance with these data, we found that the extraction and purification of F_1 in complex with the inhibitor protein from bovine heart submitochondrial particles yielded dimers of two F_1-IP complexes (Dominguez-Ramirez *et al*., 2001). However, it was also observed that this endogenous F_1-IP complex has a substantial portion of monomers of F_1 in complex with an inhibitor protein. Because the stoichiometry of F_1 to inhibitor protein is 1:1 (Klein *et al*., 1980; Wong *et al*., 1982; Jackson and Harris, 1983), the existence of endogenous F_1-IP monomers implies that in the monomers one inhibitor protein interacts with one F_1.

It was shown several years ago that in submitochondrial particles from bovine heart, ATP synthesis during oxidative phosphorylation does not proceed until the inhibitory protein has been released from its inhibitory site in F_1 (Gómez-Puyou *et al*., 1979; Harris *et al*., 1979; Schwerzmann and Pedersen, 1986). The release is induced by the proton motive force that is established during electron transport. Therefore, in regard to the mechanisms that operate in oxidative phosphorylation, it is important to determine the significance of the monomeric and dimeric forms of the F_1-inhibitory protein complexes in relation to the release of the inhibitory protein from its inhibitory site. Here we characterized the release of the inhibitory protein from monomers and dimers of F_1-IP. We also explored if during the release of IP from F_1, there is interconversion between the two species, and some of the factors that control such process. In addition, we determined some of the spectroscopic characteristics of the monomeric and dimeric forms of F_1 in complex with the inhibitor protein.

MATERIAL AND METHODS

Mitochondria prepared from bovine heart were the starting material for the preparation of Mg-ATP submitochondrial particles. The particles were prepared as described elsewhere (Gomez Puyou *et al*., 1986); they were stored at -70°C. The particles exhibited an ATPase activity of about 1 μmol/(min mg). When they were incubated under conditions that cause release of the action of the inhibitor protein (see below), their activity increased to 8–9 μmol/(min mg). Soluble F_1 in complex with the inhibitor protein was prepared as described previously (Dominguez-Ramirez *et al*., 2001). Briefly, Mg-ATP particles were suspended in 250 mM sucrose and sonicated for 30 min; care was taken that during sonication the temperature remained between 10 and 15°C. At the end of the sonication, 10 mM Mes-Tris and 200 μM ADP (pH 6.8) were added. The mixture was centrifuged at 45,000 rpm for 30 min at 4°C. The supernatant was kept at 4°C. The pellet was suspended in 250 mM sucrose and sonicated for 15 min at 10–15°C; Mes-Tris (10 mM pH 6.8) and 200 μM ADP were added afterwards. The mixture was centrifuged for 30 min at 45000 rpm; the supernatant was mixed with the first supernatant and centrifuged again for 60 min at 45000 rpm at 4°C. The supernatant was loaded into a column of Sepharose-hexylammonium previously equilibrated with 250 mM sucrose, 10 mM Mes-Tris, and 200 μM ADP (pH 6.8) and washed with the same buffer. The F_1-inhibitor protein complex was eluted with the same media that in addition contained 500 mM KCl. The fractions containing the complex were precipitated

with 50% saturation ammonium sulfate. The suspension was centrifuged and the pellet dissolved to a concentration of 10–15 mg protein per milliliter in 250 mM sucrose, 10 mM Mes, and 200 μM ADP (pH 6.8) and stored at -70°C. F_1 free of inhibitor protein was prepared as described elsewhere (Tuena De Gomez-Puyou and Gomez-Puyou, 1977). Its specific activity was between 90 and 100 μmol/(min mg).

Separation and Analysis of Dimeric and Monomeric F_1-Inhibitor-Protein

For the separation of dimeric and monomeric F_1-IP complexes, between 0.15 and 0.2 mL of the aforementioned solution of F_1-IP were applied to a Superdex 200 FPLC column (Pharmacia), and eluted with 50 mM phosphate pH 6.5 that in addition contained 0.001% PMSF. Fractions of 0.5 mL were collected. The elution of the proteins was recorded by following their absorbance at 280 nm (see Fig. 1). The relative abundance of monomeric and dimeric F_1-IP was calculated from the elution profile according to

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

The equation involves a nonlinear Gaussian fit where y is absorbance, a_0 = area, a_1 = center of the area, a_2, = width, and x is the elution volume, using the Jandel Scientific PeakFit software. These calculations are commonly used to determine the relative population of two or more species (Dominguez-Ramirez *et al*., 2001).

The fractions enriched in each of the two species were collected and concentrated to 5–10 mg/mL. These fractions were used for experiments in which the characteristics of the dimeric and monomeric F_1-IP were studied. The fractions were also used to assess some of the factors that could induce the interconversion between F_1-IP dimers and monomers. To this end, the two species were incubated in the conditions and at the concentrations indicated in the Results section, and thereafter analyzed by HPLC in a SEC4000 column (Phenomenex). The column was eluted with 50 mM phosphate buffer pH 6.5 and 0.001% PMSF. The elution profile was obtained by absorbance of the eluate at 280 nm. From the data, the relative abundance of monomeric and dimeric F_1-IP was calculated as described earlier.

Activation of F_1-Inhibitor Complexes and Activity Measurements

To release F_1 from the action of the inhibitor protein, F_1-IP complexes at the indicated concentrations were incubated at 40°C in 25 mM tris-sulfate, 25 mM Bis-tris, 2 mM EDTA, and 10 mM ATP, and 100 mM KCl at the indicated pH. At different times of incubation, samples were withdrawn in order to determine their ATPase activity by a coupled assay system.

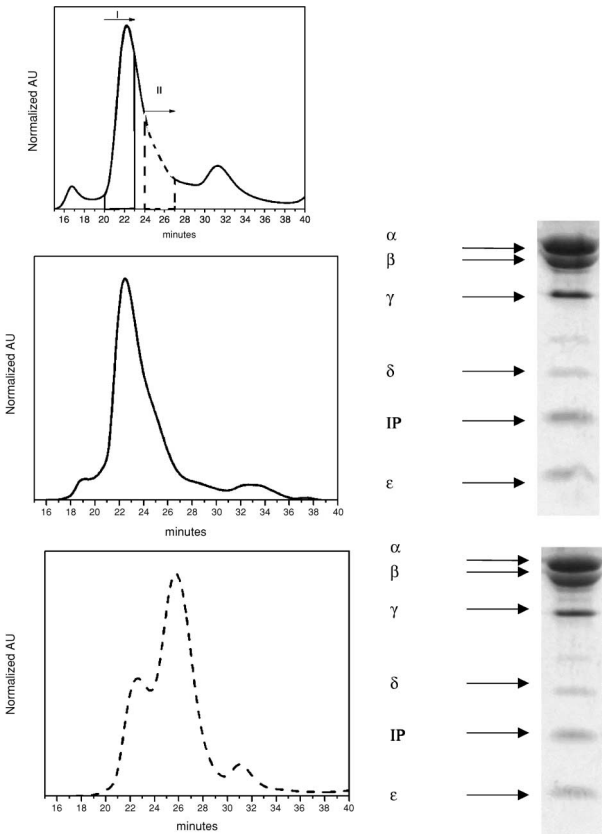


Fig. 1. Size exclusion chromatography of dimers and monomers of F_1-IP. A standard preparation of endogenous F_1-IP was dissolved in 50 mM phosphate buffer pH 6.5 and PMSF 0.001% at room temperature. The complex was applied to size exclusion FPLC and eluted with the same buffer at a flow rate of 0.5 mL/min. Fractions of 0.5 mL were collected. The elution profile as monitored by absorbance at 280 nm showed proteins with molecular weights that corresponded to those of a dimer (solid line) and monomer of F_1-IP (dashed line). The fractions shown in solid and dashed lines were pooled, concentrated to about 5–10 mg of protein per milliliter of the aforementioned buffer and stored at -70°C. Aliquots of the latter fractions at a concentration of 1 mg/mL were analyzed again by size exclusion chromatography; the resulting profiles are shown. The fractions were also analyzed by SDS-PAGE.

Circular Dichroism and Intrinsic Fluorescence Spectra

CD spectra from 185 to 260 nm at 20°C were obtained on a Jasco J-715 spectropolarimeter using a cell of 0.1 cm. The cell contained 20 mM phosphate pH 6.5 and 1 mg/mL

of the indicated proteins. The spectra shown are the average of three different preparations minus the spectra of their respective blanks. The emission fluorescence spectra of the proteins were obtained in a ISS Photon Counting Spectrofluorometer using a 0.5 mm cell that contained 20 mM phosphate buffer pH 6.5 at 20°C. The excitation wavelengths were either 280 or 295 nm. Blanks without protein were recorded and their spectra were subtracted from the experimental.

Other Assays

The proteins were also analyzed by denaturing gel eletrophoresis as described by von Jagow (Schagger *et al.*, 1988). Protein was determined with the BCA assay system (Pierce) using bovine serum albumin as standard.

RESULTS

Many studies with F_1 in complex with the inhibitor protein have been made with F_1 that had been reconstituted with purified inhibitor protein (Di Pietro *et al.*, 1988; Jackson and Harris, 1988; Milgrom, 1991; Rouslin and Broge, 1996; Schnizer *et al.*, 1996; van Raaij *et al.*, 1996; Cabezón *et al.*, 2003). However, it has been shown that MgATP submitochondrial particles which have their ATPase/ATP synthases controlled by the endogenous inhibitor protein can be used to prepare and study the characteristics of the naturally occurring complex of F_1 with the inhibitor protein (Gomez Puyou *et al.*, 1986). More recently, we found that these preparations are a mixture of F_1-inhibitor protein complexes that have the molecular weight of monomers and dimers of F_1-IP. The existence of dimers is in consonance with data that showed that F_1 reconstituted with the inhibitor protein is a dimer of two noncovalently linked F_1-IP complexes (Cabezon *et al.*, 2000a).

Figure 1 shows that the two populations of endogenous F_1-IP complexes can be distinguished by their elution profile in size exclusion chromatography (Dominguez-Ramirez *et al.*, 2001). The data suggested that by selecting the appropriate elution fractions, it would be possible to have preparations that would predominantly have F_1-IP dimers or monomers. However, we found that the fractions that presumably would have a clear enrichment of one of the species still exhibited a mixed population of dimers and monomers when analyzed in a second run of size exclusion chromatography (Fig. 1). The failure to obtain preparations that have only dimers of monomers of F_1-IP suggested that there is a spontaneous interconversion between the two species, and that due to this inherent property of F_1-IP, it is not possible to have preparations that contain exclusively monomers or dimers. Nonetheless, the data of Fig. 1 indicate that it is possible to have F_1-IP preparations that are enriched in one of the species. In this regard, it is noted that we have isolated the fractions that contain dimers and monomers (as shown in Fig. 1) in 30 different experiments. When these samples, at a concentration of 1 mg/mL, were analyzed again by size exclusion chromatography, it was found that in the fractions that corresponded to the dimer, the average ratio of dimers to monomers was 1.5 ± 0.15, whereas in those that contained monomers, the ratio of was 0.5 ± 0.05.

In SDS-PAGE, the two preparations exhibited only the protein bands that correspond to F_1, including that of the inhibitor protein (Fig. 1). It is also relevant that under conditions that lead to release of the inhibitory protein (Feinstein and Moudrianakis, 1984), the monomers and dimers of F_1-IP expressed the activity of F_1 free of inhibitor protein (see below), indicating that in the two species their hydrolytic activity is controlled by the inhibitor protein. The preparations of F_1-IP enriched in dimers (D) or monomers (M) were used to explore the factors that affect the interconversion between the two species, their susceptibility to release the inhibitor protein, and some of their spectroscopic characteristics.

Interconversion Between F_1-IP Dimers and Monomers

In order to probe the factors that may affect the distribution of D and M, we incubated the D and M preparations in conditions in which F_1 remained in control by the inhibitor protein. Thereafter, we determined the content of F_1-IP dimers and monomers by size-exclusion chromatography. In a first approach, a D preparation that had 60% dimers and 40% monomers was incubated at 4°C in media with a low salt concentrations at pH 6.5; this is a condition in which the detachment of the inhibitor protein from F_1 is very low (see later). In 2 h of incubation, there was a rather modest transformation of D into M (Table I, exp. A). On the other hand, when the same experiment was carried out in media that had been supplemented with 100 mM KCl, it was observed that within 1 min, the population of dimers exhibited a significant decrease, whereas that of monomers increased (Table I, exp. B). The conversion continued, and after 1 h, the ratio of D to M was 0.25. When an M preparation (33% dimers and 67% monomers) was incubated in the latter conditions, there was also a rapid shift from D to M giving a ratio of about 1 D per 10 M in one min of incubation (Table I, exp. C). The overall

Table I. Stability of Dimeric and Monomeric F_1I

Experiment	Starting preparation	Time	% Dimer	% Monomer
A	Dimer	0	60 ± 2	40 ± 3
		1 min	54 ± 2	46 ± 3
		1 h	49 ± 4	51 ± 3
		2 h	47 ± 3	53 ± 2.7
B	Dimer	0	60 ± 2	40 ± 3
		1 min	41 ± 1.5	58 ± 2
		1 h	20 ± 2.1	79 ± 2
		2 h	19 ± 1.7	80 ± 1
C	Monomer	0	33 ± 4	67 ± 3
		1 min	9.7 ± 0.7	90 ± 1
		1 h	18 ± 1.7	91 ± 1.6
		2 h	10 ± 1.2	89 ± 1.7

Note. Dimeric and monomeric F_1-IP (D and M, respectively) as obtained in Fig. 1 were used for the experiments. The distribution of F_1 in D and M in the two preparations is shown (time zero). The preparations were incubated at 4°C in 50 mM phosphate buffer pH 6.5 (Experiment A), or in 50 mM phosphate buffer (pH 6.5) + 100 mM KCl (Experiments B and C). At the indicated times, aliquots of the mixtures were withdrawn and analyzed by size exclusion HPLC at room temperature. From the elution diagram, the distribution of F_1 into D and M was calculated. The average of two different experiments is shown; ± indicates the difference in the two experiments.

data of Table I indicate that, except for a small increase in dimers when the monomers were incubated for 1 h, there was always a shift toward the monomeric species.

In connection to the data of Table I, it is important to note that at the beginning of the experiments of Table I, the activities of the D and M preparations were about 2 μmol/(min mg), and that at the end of experiment, their activities were respectively, 16 ± 1 and 17 ± 2 μmol/(min mg). Since after full activation of the F_1-inhibitor complexes, the activity is around 100 μmol/(min mg), the data indicate that the changes in the distribution of D and M took place in a population of F_1 in which most of the enzymes remained inhibited by the inhibitor protein. Hence, the data essentially reflect the interconversion between dimers and monomers. A noteworthy point of the data of Table I is that in media that had KCl, an important shift of dimers to monomers occurred within 1 min of incubation, regardless of whether the starting preparation was dimeric or monomeric F_1-IP (Table I).

Distribution of Monomeric and Dimeric F_1-IP at High and Low Protein Conentrations

Because dimers and monomers interconvert and the formation of dimers from monomers is a bimolecular reaction, it would be expected that according to the law of mass action the ratio of D/M would be larger at high than at low protein concentrations. Therefore, to study the effect of protein concentration on the interconversion reaction, we determined by size exclusion chromatography the distribution of D and M in samples that were incubated at a concentration of 10 and 1 mg/mL (Fig. 2). It is important to indicate that in order to probe solely the interconversion reaction, the experiments were carried out in media in

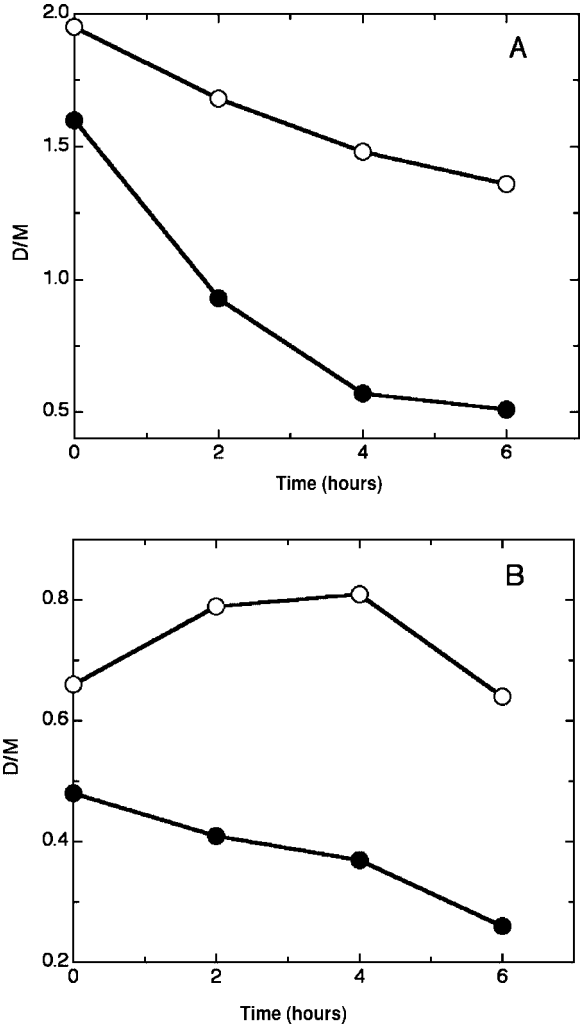


Fig. 2. Distribution of dimers and monomers at concentrations of 1 and 10 mg of protein per milliliter. Dimeric (panel A) and monomeric fractions (panel B) of F_1-IP were obtained as in Fig. 1. The protein concentrations of the two fractions were adjusted to concentrations of 10 mg/mL (open circles) and 1 mg/mL (closed circles) in 50 mM phosphate buffer pH 6.5 at room temperature. The fractions were analyzed by size exclusion chromatography at time zero, and after incubation for the indicated times at 25°C. From the chromatographic profiles, the ratio of dimers to monomers (D/M) was calculated.

which only a relatively small activation took place. For example, in the experiment shown in Fig. 2 which lasted for 6 h, the activity of the preparations was about 5 μmol/(min mg) at time zero and 17 μmol/(min mg) at the end of the experiment.

In both, the D and M preparations, the ratio of D/M was higher when the sample injected into the column was at a concentration of 10 mg/mL than when it was at a concentration of 1 mg/mL (time zero in the Figures). The samples were again analyzed after 2, 4, and 6 h. At the two protein concentrations, dimers were progressively converted into monomers; it is relevant however, that the D to M conversion was much faster at the lower protein concentration (Fig. 2).

Release of the Inhibitory Action of the Inhibitor Protein in Monomers and Dimers of F_1-IP

Before it was discovered that F_1-inhibitor protein complexes can exist as monomers and dimers of F_1-IP, it had been observed that the action of the inhibitory protein can be released from the F_1-IP complex by exposing it to temperatures of 40°C, alkaline pH, and a relatively high salt concentration (Feinstein and Moudrianakis, 1984). We now know that these preparations are formed by monomers and dimers of F_1-IP; thus, the early data indicated that both, D and M can become free of the action of the inhibitor protein. We have now examined if the two preparations behave differently with respect to loss of control by the inhibitor protein. To this end, we examined the kinetics of activation of the preparations enriched in D or M by incubating them at a concentration of 1 mg/mL of media that is known to induce loss of the inhibitory action of the protein at pH 6.5, 7.0, and 8.0. In agreement with published data (Feinstein and Moudrianakis, 1984), we observed that the rate and extent of activation was high at pH 8.0, lower at pH 7.0 and still lower at pH 6.5 (Fig. 3). It is noteworthy however, that at the three pH, the activation profiles of the D and M preparations were markedly similar (Fig. 3).

Changes in the Distribution of Dimers and Monomers of F_1-IP During the Activation Reaction

The markedly similar activation curves of the D and M preparations (Fig. 3) could indicate that the two species are equally prone to release the inhibitor protein. However, since the two species interconvert, there is the possibility that only one the species is capable of releasing the inhibitor protein; in this case, the interconversion reaction would be central in the release of the inhibitor protein

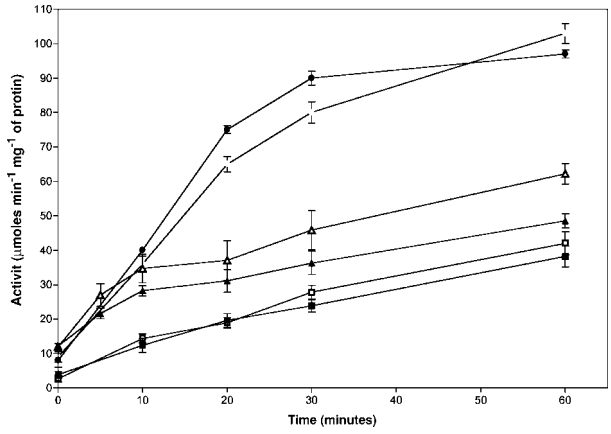


Fig. 3. Activation of dimeric and monomeric F_1-inhibitor protein complexes. Preparations of F_1-IP dimers (60% dimer and 40% monomers) and monomers (33% dimers and 67% monomers) were obtained as described in Fig. 1. The fractions at a concentration of 1 mg/mL were incubated at 40°C in 25 mM Bis-tris, 25 mM tris, 2 mM EDTA, 10 mM ATP, and 100 mM KCl at pH 6.5, 7.0 and 8.0. At the indicated times, aliquots were withdrawn to measure ATPase activity. Open and closed symbols indicate data with monomeric and dimeric F_1-IP, respectively. Squares, pH 6.5; triangles, pH 7.0; circles, pH 8.0.

from the whole enzyme population. Therefore, we measured the changes that occur in the population of D and M during the course of formation of free F_1. To this end, we incubated D and M preparations at a concentration of 1 mg/mL under conditions identical to those of Fig. 3. After 10 and 60 min of incubation the content of dimers and monomers was estimated by size-exclusion chromatography. Figure 4 shows the chromatographic profiles of the preparations at time zero, and after they were incubated at 40°C in media at pH 8.0. Please note that in our chromatographic system, it is not possible to distinguish between monomeric F_1-IP and F_1 free of inhibitor protein, or other species that may be formed during the activation reactions and that have a Stokes radii similar to M or free F_1, for example, one F_1 in complex with a dimer of two IP (see Discussion section).

For the experiments, we used a D preparation that had 60% of the total amount of F_1 as D and 40% as M. After 10 min of incubation in activating conditions, the population of D had decreased to 33% (Fig. 4). At this time, the ATPase activity was about 40% of the activity that was reached when all enzymes became free of inhibitor protein (Fig. 3). In other words, after 10 min of incubation, 60% of the overall population of F_1 remained to be activated, and yet the amount of D was only 33%. The same experiment was performed with a preparation of F_1-IP monomers that at time zero had 33 and 66% of the total population of F_1 as D and M, respectively. In 10 min

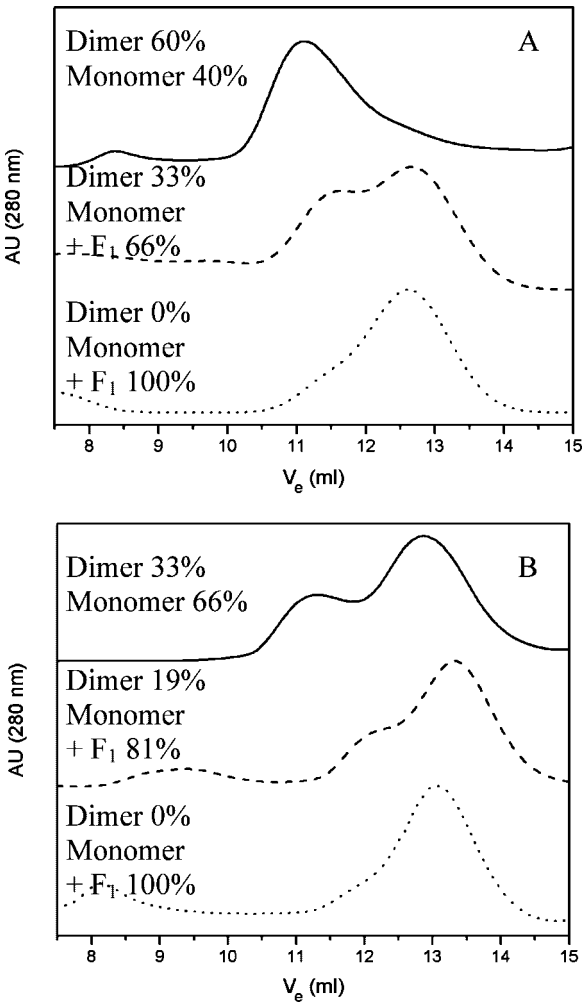


Fig. 4. Distribution of F_1-IP in dimers or monomers during activation. Preparations of dimers (A) or monomers (B) of F_1-IP as obtained in Fig. 1 were incubated in the conditions of Fig. 3 at pH 8.0 at a concentration of 1 mg/mL. After 10 and 60 min of incubation, the mixtures were analyzed by size exclusion FPLC using 50 mM phosphate buffer pH 8.0 PMSF 0.001%. The solid lines show the elution profile of the starting preparations at time zero. The dashed and dotted lines show the profiles after 10 and 60 min of incubation, respectively. At the side of the traces, the % distribution of D and M is shown; it was assumed that the starting preparations had no free F_1. Note that during activation, free F_1 is formed, and that it elutes together with M. Thus, the relative abundance of F_1 in the M peak is shown as $M + F_1$. The M peak could also contain a species with a Stokes radii similar to that of F_1 or monomeric F_1-IP (see text).

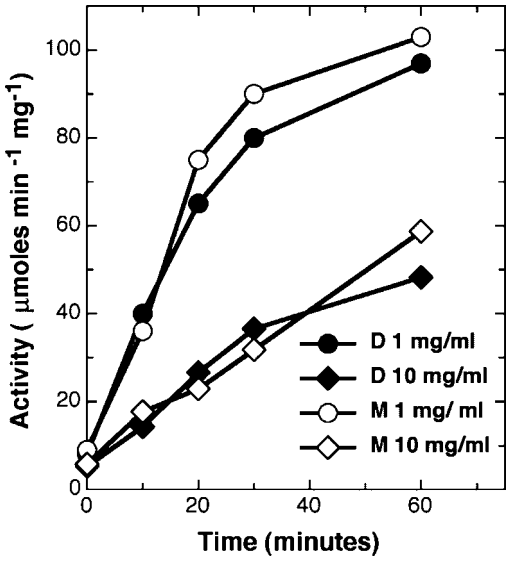


Fig. 5. Activation of F_1-IP at concentrations of 1 and 10 mg/mL. The fractions of dimeric and monomeric F_1-IP obtained as described in Fig. 1 were adjusted to concentrations of 1 and 10 mg/mL, and incubated at 40°C as in Fig. 3 at pH 8.0. At the indicated times aliquots were withdrawn for assay of ATPase activity.

of incubation, D decreased to 19% and about 60% of the population remained to be activated (see Figs. 3 and 4).

A salient feature of the data with the D and M preparations is that the decrease in D was accompanied by an increase in the protein peak that corresponded to M. For example, after 10 min of incubation in activating conditions, the M peaks were 66 and 81% when the starting preparations were D and M, respectively. However, it is noted that during activation, the absorbance of the M peak could represent the sum of the absorbance of $M + F_1$, or $M + F_1$ + the absorbance of other species that may have been formed during activation.

Formation of Free F_1 at Low and High Protein Concentrations

The activation of the two species of F_1-IP at a concentration of 1 and 10 mg/mL was also determined (Fig. 5). In both, the D and M preparations, the release of the inhibition by the inhibitor protein was much faster at the lower protein concentration. Since at high protein concentrations the interconversion reaction is shifted toward the dimeric species (Fig. 2), the faster rate of formation of free F_1 at low protein concentrations suggests that the species that losses the inhibitor protein is monomeric F_1-IP.

Spectroscopic Characteristics of Monomers and Dimers of F_1-IP

In regard to the characteristics of monomers and dimers of F_1-IP, it was considered of interest to ascertain if M and D preparations have distinct spectroscopic characteristics. Accordingly, the far UV CD spectra of D and M were determined and their spectra compared to that

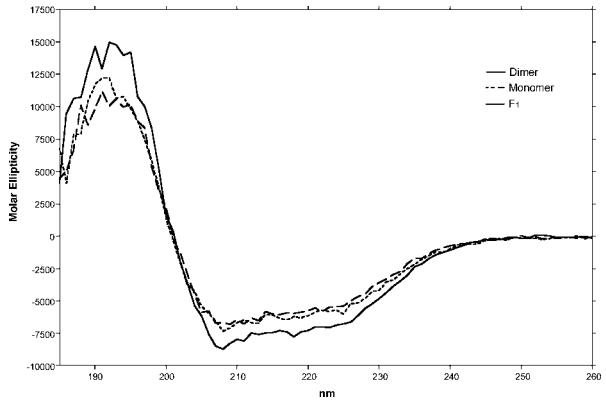


Fig. 6. Circular dichroism spectra of dimers and monomers, and free F_1. Dimeric F_1-IP (solid line), monomeric F_1-IP (dotted line) as obtained in Fig. 1, and free F_1 (dashed line) at a concentration of 1 mg/mL of 20 mM phosphate buffer pH 6.5.

of free F_1. The spectra of free F_1 and M were almost indistinguishable (Fig. 6), and slightly different from that of D. A deconvolution of the spectra with the program CDNN (Bohm *et al*., 1992) indicated that the differences were not significant (not shown). Thus, the interaction of the inhibitor protein with F_1, or its dimerization did not induce gross alterations of its overall secondary structure.

On the other hand, the intrinsic fluorescence spectra of F_1 and the D and M preparations were markedly different. At an excitation wavelength of 280 nm, the fluorescence intensity of D was higher than that of M, which in turn was higher than that of free F_1 (Fig. 7(A)). At 280 nm, tyrosines and tryptophans are excited. F_1 has 98 tyrosines and 1 tryptophan, whereas the inhibitor protein has 1 tyrosine and no tryptophans. In view of the abundance of tyrosines, the differences in intrinsic fluorescence at an excitation wavelength of 280 nm most likely indicate differences in the environment of tyrosines.

At an excitation wavelength of 295 nm, the emission fluorescence spectra reflect exclusively changes in the environment of the single tryptophan of F_1. Therefore, it is noteworthy that at this wavelength the fluorescence of the D preparations were higher than those of M, and that the fluorescence of the latter species was almost equal to that of free F_1 (Fig. 7(B)).

Taken together, the fluorescence data indicate that the interaction of the inhibitor protein with F_1 and dimerization are accompanied by conformational changes in the environment of the intrinsic fluorophores. In this context, it is stressed that our preparations of F_1-IP dimers and monomers are a mixture of the two species. Therefore, it is very likely that the observed fluorescence differences between species are underestimated.

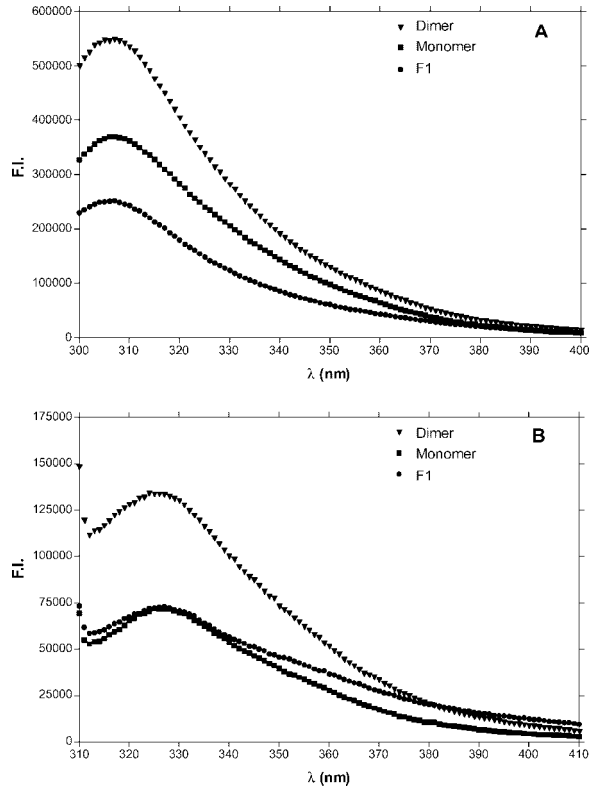


Fig. 7. Intrinsic fluorescence spectra of dimers and monomers, and free F_1. The spectra of mixtures that contained 1 mg/mL of 20 mM phosphate buffer at pH 6.5 were recorded after exciting at 280 nm (A) or 295 (B). Dimers (inverted triangles), monomers (squares), and F_1 (circles).

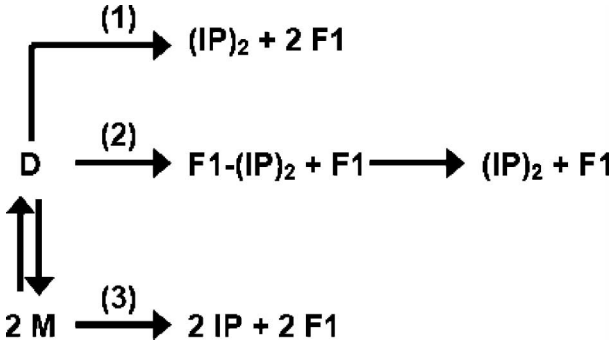
DISCUSSION

In confirmation of previous data (Dominguez-Ramirez *et al*., 2001), we found that F_1-IP complexes extracted and purified from submitochondrial particles that have their ATP synthases controlled by the inhibitor protein exist as monomers and dimers of F_1-IP. Under conditions that induce release of the inhibitory protein, the specific hydrolytic activity of either of the two species increases from around 2–5 μmol/(min mg) to about 100 μmol/(min mg), which is the activity of F_1 free of inhibitor protein. Thus, the ATPase activity of monomeric and dimeric F_1-IP is controlled by the inhibitor protein. Because it has been shown that the stoichiometry of F_1 to inhibitor protein is 1:1, it may be concluded that in F_1-IP monomers extracted from submitochondrial particles, the activity of one F_1 is controlled by one inhibitor protein, and that in the endogenous dimer, a dimer of two IP controls the activity of two F_1.

The expression of the hydrolytic and synthetic activities of the ATP synthase requires displacement of the

inhibitor protein from its inhibitory site in F_1 (Gómez-Puyou *et al*., 1979; Harris *et al*., 1979; Feinstein and Moudrianakis, 1984; Schwerzmann and Pedersen, 1986). Therefore, it is mechanistically important to determine the pathways through which F_1 becomes free of inhibitor protein. In this regard, a central question is if the displacement of the inhibitor protein from F_1 occurs equally well from both, monomers and dimers of F_1-IP. Along this line, perhaps the most relevant observation is that the rate of formation of free F_1 is slower when the interconversion reaction is shifted toward the dimeric species (Fig. 5). Hence, F_1-IP dimers and monomers are not equally susceptible to loss of the inhibitor protein.

Accordingly, we considered the various routes that could account for formation of free F_1 from F_1-IP monomers and dimers. These are shown in the following scheme in which $(IP)_2$ is a dimer of two inhibitor proteins. D represents the dimer of two F_1 joined by $(IP)_2$. M is one F_1 in complex with one IP. In the scheme, the only reversible step is the interconversion reaction.



In route 1, D is directly converted into 2 free F_1 and $(IP)_2$. This route would not seem to be compatible with experiments that showed that a shift in the interconversion reaction toward the D species as induced by high protein concentrations slows down the rate of activation of F_1. In route 2, D initially dissociates into free F_1 and $F_1(IP)_2$, and in a second step, the latter species yields another free F_1 and $(IP)_2$. The formation of free F_1 in the first step of route 2 would not be consistent with the results that showed that a shift in the direction of the interconversion reaction toward the D species is accompanied by a decrease in the rate of formation of free F_1. Likewise, it was observed that under conditions in which no activation of F_1 took place, the species that accumulates is M.

On the other hand, the experimental findings would seem to fit quite well with route 3. This is because (i) the rate of F_1 activation is faster when the interconversion reaction between D and M is shifted toward M, (ii) the conversion reaction of D to M is faster than the rate at which the whole population of F_1 becomes free of inhibitor protein (Table I, exps. B and C), (iii) and during activation, a species that peaks in the region of monomeric F_1-IP accumulates. Although in the latter case, the amount of M could not be precisely determined, it is relevant that under conditions in which free F_1 is not formed, (Table I), there is an accumulation of the M species.

These observations are therefore in consonance with the reaction sequence of route 3 in which D are transformed into M, and that this is the species that is susceptible to loose the inhibitor protein. In this reaction sequence, it is kinetically important that in the process of F_1 activation, there is an accumulation of monomeric F_1-IP, which indicates that the rate-limiting step of the overall process is the release of the inhibitor protein from monomeric F_1-IP. These various observations therefore, indicate that route 3 is the predominant pathway through which F_1 becomes free of the inhibitor protein. Nonetheless, it is not possible to conclude that the whole population of F_1 becomes free of the inhibitor protein solely through route 3.

We have also explored some of the factors that may affect the stability of the two F_1-IP species. It was found that low temperatures per se do not affect to an important extent the distribution between D and M (Table I, Exp. A). As hydrophobic interactions are weakened at low temperatures (Privalov *et al*., 1986), the data suggest that interactions between hydrophobic residues do not bear importantly on the dimer to monomer transitions. On the other hand, the rapid salt induced shift of D to M suggests that electrostatic interactions or hydrogen bonding affect strongly the equilibrium between the two species.

In the light of our data on dimerization and monomerization of F_1-IP complexes, it is important to note that Arnold *et al*. (1998) and Schagger and Pfeiffer (2000) clearly showed that in mitochondria the whole ATP synthase is capable of undergoing dimerization through a process in which the participation of e and g subunits is of central importance. Along this line, Tomasetig *et al*. (2002) also reported that in the absence of inhibitor protein, F_1F_o may exist as dimers. Although the physiological significance of the various observations has not been precisely defined, the available data suggest that formation of multi-enzymatic complexes of the inner membrane are indeed instrumental in the function and morphology of mitochondria (Paumard *et al*., 2002; Ko *et al*., 2003). In regard to release of the inhibitor protein from F_1, which is a necessary step for expression of catalysis in soluble and particulate F_1, the present data suggest that release of the inhibitor protein occurs predominantly at the expense of monomeric F_1-IP.

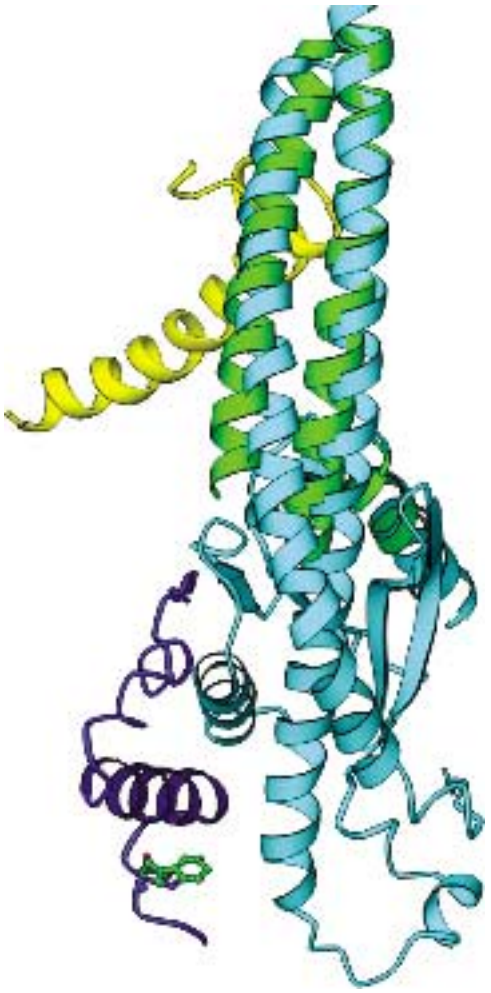


Fig. 8. Structural alignment of free F_1 (pdb ID 1e79) and F_1 reconstituted with the inhibitor protein (pdb ID 1OHH). The γ subunits in free F_1 (blue) and in the complex of F_1-IP (green) were superposed. The best fit involved 10932 atoms and exhibited an RMS of 1.03 Å. The ε subunit with its tryptophan is shown in dark blue. The inhibitor protein (yellow) is at least 48 Å away from the tryptophan. The figure was made with Swiss-PDB viewer (Guex and Peitsch, 1997) and Ribbons (Carson, 1997).

Structural Modifications of F_1 Induced by the Inhibitor Protein and Dimerization

The CD spectra of F_1 and F_1-IP monomers and dimers show that there are no gross differences in the overall secondary structure between these three forms of the enzyme. However, it was found that the intrinsic fluorescence of tyrosines is higher in the dimers than in the monomers, and that the fluorescence of the latter species is higher than in free F_1. Since the fluorescence intensity of tyrosines increases with their level of solvent exposure, the data indicate that the binding of the inhibitor protein to F_1 and dimerization increases the solvent exposure of tyrosine residues. With the present data however, it is not possible to deduce which of the 98 tyrosines of F_1 plus that of IP are responsible for the differences in fluorescence between species.

On the other hand, the data on the fluorescence of the single tryptophan of F_1 free of IP and in the dimers and monomers of F_1-IP provides insight into the structural changes that the binding of the inhibitor protein induces in F_1. In F_1 and in the F_1-IP complexes, there is only one tryptophan; it is located on the ε subunit of F_1 (Fig. 8). In the crystal structure of dimeric F_1-IP, it is far from the site of interaction of the inhibitor protein with the α, β, and γ subunits of F_1 (Cabezon *et al*., 2003). This would be in consonance with the data that show that the intrinsic fluorescence of F_1 and monomeric F_1-IP are almost identical (Fig. 5(B)). However, the fluorescence of the tryptophan in the dimer is nearly twice as high as that of the other two species. In this regard, it is relevant that Solaini *et al*. (1993) reported that the binding of the inhibitor protein to F_1 affects the phosphorescence of the distant tryptophan of F_1 and that the IP can be cross-linked to the γ subunit (Minauro-Sanmiguel *et al*., 2002).

In sum, the data on the intrinsic fluorescence of tyrosines and the sole tryptophan of F_1 indicate that the binding of the inhibitor protein to F_1 and dimerization are accompanied by structural changes that are both close and distant to the binding site. These changes appear to be rather extensive. Indeed, the data of Fig. 8 illustrate that the binding of the inhibitor protein to F_1 changes the geometry of the γ subunit.

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