Circular Dichroism and Nucleotide and Phosphate-Induced Conformational Changes of Mitochondrial Adenosinetriphosphatase†

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ABSTRACT: The conformational changes induced by the binding of different effectors on F_1-ATPase are investigated by using circular dichroism and are related to enzyme activity. The hydrophilic part of the terminal enzyme of oxidative phosphorylation, F_1-ATPase, solubilized from the pig heart mitochondrial membrane contains both regulatory and catalytic sites which can bind nucleotides and phosphate. The circular dichroic spectra of F_1-ATPase in the absence or in the presence of ADP, Mg^{2+}, phosphate, and the substrate analogue guanosine 5'-(β,γ-imidotriphosphate) [GMP-P(NH)P] were recorded and analyzed in terms of secondary structure. The most significant result is a sizable increase from 35% to 42% of the α-helix content when the enzyme is incubated with all the effectors. Since the kinetic study showed that GMP-P(NH)P is a competitive inhibitor of MgATP with or without preincubation of the enzyme with ADP and phosphate, it was concluded that the catalytic and regulatory sites can be simultaneously occupied by ADP and GMP-P(NH)P. The increase of α-helix content is then interpreted by a conformational change that occurs only after occupation of both types of sites.

The terminal enzyme of oxidative phosphorylation is a reversible adenosine-5'-triphosphatase (ATPase)[1]–ATP synthase which catalyzes the synthesis of ATP from ADP and phosphate. The hydrophilic part of this complex, F_1-ATPase solubilized from the mitochondrial membrane, exhibits only the ATPase activity and contains the nucleotide and phosphate binding sites [see the reviews of Penefsky (1979) and Senior (1979)].

Recent studies from our laboratory (Di Pietro et al., 1980) have shown that preincubation of pig heart mitochondrial F_1-ATPase with ADP leads to a binding of ADP that induced a progressive hysteretic inhibition of MgATP hydrolysis. This binding occurs at regulatory sites specific to adenine nucleotides (Harris et al., 1978; Baubichon et al., 1981; Di Pietro et al., 1981) and efficiently prevents the inactivation of the enzyme by trypsin (Di Pietro et al., 1983). The binding of ADP therefore induced a change in the conformation of the enzyme that became resistant to trypsin. A hypothesis has been advanced to explain why an inhibition was progressively established after the addition of MgATP to F_1-ATPase preincubated with ADP. According to this hypothesis, the addition of the substrate MgATP or substrate analogue could induce a second conformational change of the enzyme from a highly active to a partially active state (Di Pietro et al., 1980). In the present paper, the conformation of the enzyme is more directly studied with circular dichroism. The use of this technique demonstrated that, when the regulatory site(s) of F_1-ATPase was (were) occupied by ADP and the catalytic sites by a nonhydrolyzable substrate analogue, GMP-P(NH)P, a second conformational change was induced in the presence of phosphate. This change was shown by an increase of the α-helix content of the enzyme.

Experimental Procedures

Enzyme Preparation. Pig heart mitochondria were obtained at 0–4 °C as previously described (Gautheron et al., 1964). The mitochondrial F_1-ATPase was purified by the method of Penin et al. (1979) and stored frozen at −70 °C in 100 mM Tris–H_2SO_4–5 mM EDTA–50% glycerol, pH 8.0. Before use, an aliquot was rapidly thawed in a water bath at 30 °C and kept 30 min as such. The ATPase activity, which increased by about 20% during this treatment, remained stable at least for a day after.

The protein content of enzyme solutions was estimated by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard.

Assay of ATPase Activity. ATPase activity was routinely determined at 30 °C with about 1 μg of enzyme in 0.62 mL of 50 mM Tris–H_2SO_4 buffer, pH 8.0, 10 mM sodium bicarbonate, 3.3 mM ATP, and 3.3 mM $MgSO_4$ (unless otherwise indicated) by using an ATP regenerating system (phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, and NADH). The rate of NADH disappearance was followed with a spectrophotometer at 340 nm [Pullman et al. (1960) as modified by Baubichon et al. (1982)]. Nucleotides were purchased from Boehringer Mannheim.

"Hysteretic" Inhibition of F_1-ATPase Induced by Preincubation with ADP. The method of Di Pietro et al. (1980) was modified as follows: F_1-ATPase (0.5–1 mg of protein/mL) was preincubated for 30 min at 30 °C in 50 mM Tris–H_2SO_4, pH 8.0, 30% glycerol, 1 mM EDTA, 0.2 mM ADP, and 2.5 mM $MgSO_4$. When indicated, 5 mM potassium phosphate was included, and the pH was adjusted to 7.0. The solution was used as such for circular dichroism studies. An aliquot (1–2 μL) was transferred into the spectrophotometer cuvette to measure the ATPase activity as described above. Under these conditions, the enzyme activity remained constant during all the CD measurements.

CD Measurements. Spectra were taken on a Jobin-Yvon Mark IV dichrograph, using cuvettes of 0.01–0.5-cm path length. The measurements were made at room temperature since F_1-ATPase is cold labile. The spectra were recorded

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[1] Abbreviations: ATPase, adenosine-5'-triphosphatase; F_1, pig heart mitochondrial F_1-ATPase prepared according to the procedure of Penin et al. (1979); ADP, adenosine 5'-diphosphate; ATP, adenosine 5'-triphosphate; GMP-P(NH)P, guanosine 5'-(β,γ-imidotriphosphate); EDTA, ethylenediaminetetraacetic acid; NADH, reduced nicotinamide adenine dinucleotide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; CD, circular dichroism.

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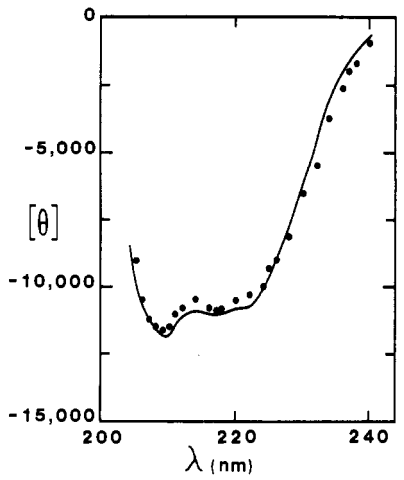

FIGURE 1: Circular dichroism spectra of F_1-ATPase without effector; conditions were as described under Experimental Procedures. F_1-ATPase (0.68 mg of protein/mL) was preincubated for 30 min at 30 °C in 50 mM Tris–H_2SO_4, pH 8, 30% glycerol, and 1 mM EDTA. The solution was then transferred into the circular dichroism cell, and the spectra were recorded. (●) Experimental data. The theoretical curve (—) was calculated according to the model of Chen et al. (1972).

between 200 and 250 nm at a rate of 5 nm·min^{-1}.

Molar ellipticities were obtained from the mean of three or more spectra recorded on one sample, and each experiment was repeated at least 3 times.

The ellipticities were calculated in degrees centimeter squared per decimole on the basis of a molecular weight of 380 000 (Di Pietro et al., 1975) and a mean weight per amino acid of 115. The calculations were made at 1-nm intervals.

The analysis of these spectra was performed on a computer using a multilinear least-squares program which is not constrained; i.e., the sum of the amounts of secondary structure can equal any value. Several models were used for the calculations of the fractions of the different conformations, and the results were compared. In general, the sum of the percentages of all the components was not very far from 100%; this fact seems a useful criterion for judging the validity of the procedure, according to Brahms & Brahms (1980).

In a few cases, the sum of the percentages is between 80 and 90%; the fit of the experimental data was then improved by an iterative modification of the percentages obtained directly from this program, in order to obtain a sum of 100 for all the conformations. It should be noted that, in this case, the percentage of α-helix obtained directly from the program is affected slightly or not at all.

Results

Analysis of CD Spectra of Pig Heart Mitochondrial F_1-ATPase. Figure 1 shows a typical CD spectrum of pig heart mitochondrial F_1-ATPase. The determination of the contribution of the various secondary structures to the overall conformation has been calculated by using four different models. The results are summarized in Table I. The models of Chen et al. (1972) and of Chang et al. (1978) gave a sum of the different contributions closer to 100% than that of the two other models. However, it seems surprising to observe a percentage of β-pleated sheets as low as 1%; therefore, the model of Chen et al. (1972) was preferred. In Figure 1, the theoretical curve calculated with this model conforms reasonably well with the experimental data.

Influence of Effectors on the CD Spectra of F_1-ATPase. The same model has been used to study the influence of various effectors on the CD spectrum of F_1-ATPase. The results are presented in Table II. Preincubation of the enzyme with ADP and Mg^{2+} or with ADP + Mg^{2+} + P_i has little effect on the calculated percentages. However, the fit obtained directly from the unconstrained program is not quite satisfactory, and an improvement could be made by iterative adjustment in order to obtain a sum of the different contributions equal to 100%.

The substrate analogue GMP-P(NH)P added before or after preincubation of the enzyme with ADP and Mg^{2+} seems to slightly decrease the α-helix content, but this effect may be within the limits of the errors of the technique.

However, a very important and significant effect was observed when the enzyme preincubated with ADP and Mg^{2+} was mixed with GMP-P(NH)P and phosphate. The latter could be added either to the ADP solution or to the substrate analogue solution without causing a difference in the spectrum. The experimental data obtained for experiment 6 (Table II) are displayed in Figure 2 and compared with curves calculated either in the presence or in the absence of phosphate (experiments 5a and 5b, Table II). There is an obvious difference between the two sets of curves. It should also be noted that, from comparison of experiments 5a and 5b, there is almost

Table I: Comparison of Calculated Secondary Structures from Different Models for F_1-ATPase in the Absence of Effectors[a]

	% of secondary structure				
model	α form	β form	β turn	unordered form	sum
Greenfield et al. (1969)	26 (3)	20 (8)		31 (0.9)	77
Chen et al. (1972)	35 (0.8)	21 (3)		40 (5)	96
Chang et al. (1978)	28 (1.8)	1 (7)	26 (5)	41 (9)	96
Bolotina et al. (1980)	31 (1)	6 (3)		43 (7)	80

[a] The indicated percentages were obtained directly from the unconstrained program without further adjustment. The conditions are the same as in Figure 1. Standard deviations are indicated in parentheses.

Table II: Influence of the Presence of Various Effectors on the Percent of Secondary Structure of F_1-ATPase Estimated by CD[a]

		% of secondary structure			
expt	effectors	α form	β form	unordered form	sum
1		35 (0.8)	21 (3)	40 (5)	96
2a	ADP + Mg^{2+}	38 (0.7)	14 (2)	40 (3)	92
2b	ADP + Mg^{2+}	38	13	49	100
3a	ADP + Mg^{2+} + P_i	38 (0.7)	13 (2)	33 (5)	84
3b	ADP + Mg^{2+} + P_i	36	16	48	100
4	Mg^{2+} + GMP-P(NH)P	33 (0.5)	16 (2)	45 (3)	94
5a	ADP + Mg^{2+} + GMP-P(NH)P	32 (1)	19 (4)	39 (7)	90
5b	ADP + Mg^{2+} + GMP-P(NH)P	32	19	49	100
6	ADP + Mg^{2+} + P_i + GMP-P(NH)P	42 (1)	18 (4)	39 (6)	99

[a] Conditions were as described under Experimental Procedures. The enzyme was preincubated for 30 min in the absence of effector or in the presence of 0.2 mM ADP, 2.5 mM $MgSO_4$, and 5 mM potassium phosphate, pH 7.0, and for 15 min with 0.5 mM GMP-P(NH)P before the recording of the spectra. Calculations were made according to Chen et al. (1972); for experiments 2b, 3b, and 5b, the fit of the experimental data to the calculated curve was improved by a modification of the percentage obtained directly from the program, in order to obtain a sum of 100 for the three conformations. Standard deviations are indicated in parentheses.

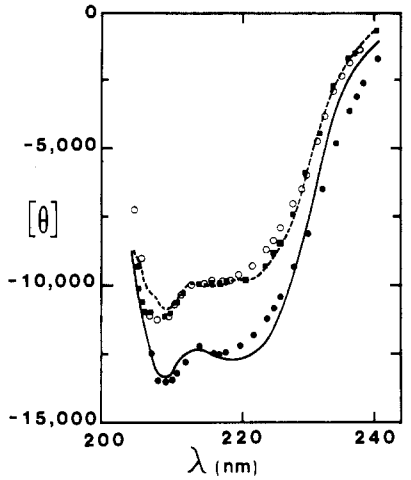

FIGURE 2: Circular dichroism spectra of F_1-ATPase, the catalytic and regulatory sites of which are occupied by nucleotides and phosphate. F_1-ATPase was preincubated for 30 min at 30 °C in the presence of 0.2 mM ADP–2.5 mM $MgSO_4$ with (●) or without (○) 5 mM potassium phosphate, pH 7.0, and for 15 min with 0.5 mM GMP-P(NH)P before the recording of the spectra. (● and ○) Experimental data. The theoretical curves with (—) or without (---) phosphate (■) were calculated according to the model of Chen et al. (1975) and correspond to the analysis made in experiments 5a (---), 5b (■) and 6 (—) of Table II.

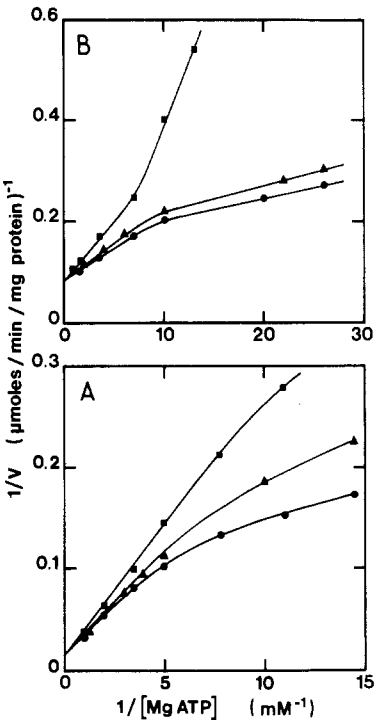

FIGURE 3: Inhibition by GMP-P(NH)P of MgATP hydrolysis by pig heart mitochondrial F_1-ATPase either preincubated or not preincubated with ADP and phosphate. F_1-ATPase (0.1 mg) was preincubated for 30 min at 30 °C in 0.1 mL of buffer containing 50 mM Tris–H_2SO_4, pH 7.0, 30% glycerol, 1 mM EDTA, and 2.5 mM $MgSO_4$ either in the absence (A) or in the presence (B) of 5 mM potassium phosphate and 0.2 mM ADP. An aliquot (1–2 μL) was transferred into the spectrophotometer cuvette containing the ATPase assay medium as described under Experimental Procedures except that the MgATP concentration was varied from 0.02 to 2 mM and that GMP-P(NH)P was present in the following concentrations: (●) no GMP-P(NH)P; (▲) 100 μM; (■) 580 μM.

no difference between the values for the α-helix or β-pleated sheet contents obtained directly from the program and those obtained after correction of the sum to 100°. A significant increase in the α-helix content was also consistently observed when experiments 5 and 6 were analyzed to determine the percentages of secondary structure from the models of Greenfield et al. (1969), Chang et al. (1978), or Bolotina et al. (1980).

Influence of GMP-P(NH)P and Phosphate on the Rate of ATP Hydrolysis Catalyzed by F_1-ATPase either Preincubated or Not Preincubated with ADP. In order to correlate the CD spectra to the kinetic properties of F_1-ATPase, the effects of GMP-P(NH)P and phosphate were studied. The presence of phosphate together with ADP in the preincubation medium modified the kinetics of hysteretic inhibition in such a way that the inhibition was established at a stable rate immediately after mixing the preincubated enzyme with the ATPase assay medium. The inhibited rate had the same final value as the one that slowly appeared in the absence of added phosphate (not shown). Consequently, the initial rate of MgATP hydrolysis could be easily measured. Figure 3A,B shows that GMP-P(NH)P produced a complex inhibition pattern of MgATP hydrolysis. However, whether the enzyme was preincubated (Figure 3B) or not (Figure 3A) with ADP and phosphate, the inhibition was competitive at high MgATP concentration.

Discussion

The results presented in this paper demonstrate that the simultaneous occupation of catalytic and regulatory sites by nucleotides and phosphate induced an important change in the overall conformation of F_1-ATPase shown by an increase of the α-helix content.

Schuster et al. (1975) have shown that GMP-P(NH)P is a competitive inhibitor of MgGTP hydrolysis and gave complex inhibition patterns with MgATP as the substrate using beef heart or rat liver mitochondrial F_1-ATPase. With the pig heart mitochondrial enzyme, we see that the inhibition pattern was also complex (Figure 3), and at high MgATP concentration, the inhibition was competitive as for the beef heart enzyme. But we show here that this competition between MgATP and GMP-P(NH)P could be observed whether F_1-ATPase had been preincubated with ADP and phosphate or not (panel B vs. panel A of Figure 3). This means that GMP-P(NH)P could occupy the catalytic site(s) of the enzyme in all cases even when ADP occupies regulatory sites. Indeed, previous studies from our laboratory have shown that the preincubation of F_1-ATPase with ADP induced the binding of ADP to regulatory site(s) and a concomitant hysteretic inhibition (Di Pietro et al., 1980) and that GMP-P(NH)P did not modify the binding of ADP to the regulatory site(s) (Baubichon et al., 1981). All these results demonstrate that the catalytic and regulatory sites can be simultaneously occupied by ADP and GMP-P(NH)P. The ADP-induced inhibited rate could be observed immediately after mixing the preincubated enzyme with the reaction mixture when phosphate was present in the preincubation medium. This might indicate that the binding of phosphate is involved in this inhibition. This observation may be related to the binding of phosphate to F_1-ATPase described by Penefsky (1977). The relationship between the binding of phosphate and the ADP-induced inhibition will be further investigated.

On CD spectra, the most significant modification observed is an increase of the α-helix content from 35% (F_1-ATPase without effector) to 42% [F_1-ATPase + ADP + Mg^{2+} + P_i + GMP-P(NH)P]. Since the sum of the percentages for the different secondary structures was close to 100%, with an unconstrained program, the value of the contribution of the α-helix may be accepted with confidence. When an adjustment to 100% seemed necessary, only the percentage of the unordered form was affected, while the α-helix content was

barely modified. This fact further confirms the significant difference between the percentage of the α-helix observed when all the effectors were present or when one effector was missing. The increase in the α-helix content implies a more compact conformation of the enzyme when both catalytic and regulatory sites are occupied by nucleotides and phosphate.

The limits of precision of this technique did not enable us to ascertain whether a conformational change occurred upon addition of ADP + Mg^{2+}. However, it has been clearly demonstrated previously (Di Pietro et al., 1983) that the only addition of ADP + Mg^{2+} protected the enzyme from trypsin proteolysis. In this case, circular dichroism could not clearly demonstrate the expected conformational change.

In conclusion, in the model presented previously (Di Pietro et al., 1980) to explain the inhibition of F_1-ATPase by ADP, two conformational changes were postulated: the first, occurring upon preincubation of the enzyme with ADP and Mg^{2+}, has been demonstrated by the trypsin effect (Di Pietro et al., 1983); the second, occurring after occupation of this catalytic site(s), is now demonstrated by this CD study.

Acknowledgments

We thank Professor D. C. Gautheron for stimulating discussions during the course of this work, F. Penin for the preparation of the F_1-ATPase, R. Lambrech for improving the English version of the manuscript, C. Van Herrewège for the drawings, and L. Bonafuente for typing the manuscript.

Registry No. ATPase, 9000-83-3; GMP-P(NH)P, 34273-04-6; ADP, 58-64-0; P_i, 14265-44-2.

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