Chemical Mechanism of ATP Synthase

MAGNESIUM PLAYS A PIVOTAL ROLE IN FORMATION OF THE TRANSITION STATE WHERE ATP IS SYNTHESIZED FROM ADP AND INORGANIC PHOSPHATE

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The chemical mechanism by which ATP synthases catalyze the synthesis of ATP remains unknown despite the recent elucidation of the three-dimensional structures of two forms of the F1 catalytic sector (subunit stoichiometry, α3β3γ6) (8, 9). Lacking is critical information about the chemical events taking place at the catalytic site of each β-subunit in the transition state. In an earlier report (Ko, Y. H., Bianchet, M. A., Amzel, L. M., and Pedersen, P. L. (1997) J. Biol. Chem. 272, 18875–18881), we provided evidence for transition state formation in the presence of Mg2+, ADP, and orthovanadate (V₅), a photoactive phosphate analog with a trigonal bipyramidal geometry resembling that of the γ-P of ATP in the transition state of enzymes like myosin. In the presence of ultraviolet light and O₂, the MgADP-V₅-F₁ complex was cleaved within the P-loop (GGAGVGKT) of a single β-subunit at alanine 158, implicating this residue as within contact distance of the γ-P of ATP in the transition state. Here, we report that ADP, although facilitating transition state formation, is not essential. In the presence of Mg2+ and V₅ alone the catalytic activity of the resultant MgV₅-F₁ complex is inhibited to nearly the same extent as that observed for the MgADP-V₅-F₁ complex. Inhibition is not observed with ADP, Mg₂⁺, or V₅ alone. Significantly, in the presence of ultraviolet light and O₂, the MgV₅-F₁ complex is cleaved also within the P-loop of a single β-subunit at alanine 158 as confirmed by Western blot analyses with two different antibodies, by N-terminal sequence analyses, and by quantification of the amount of unreacted β-subunits. These novel findings indicate that Mg₂⁺ plays a pivotal role in transition state formation during ATP synthesis catalyzed by ATP synthases, a role that involves both its preferential coordination with P₁ and the repositioning of the P-loop to bring the nonpolar alanine 158 into the catalytic pocket. A reaction scheme for ATP synthases depicting a role for Mg₂⁺ in transition state formation is proposed here for the first time.

ATP synthases are involved in the synthesis of ATP from ADP and P₁ by oxidative phosphorylation in both aerobic bacteria and in the mitochondria of eukaryotic cells (1–4) and by photosynthetic phosphorylation in chloroplast plant cells (5). In all cases, the ATP synthase involved is comprised of two basic units, a water-soluble catalytic moiety called F₁, which binds ADP and P₁ and synthesizes ATP, and a detergent-soluble unit called F₀, which delivers the energy from an electrochemical proton gradient to the F₁-ATP complex to induce the release of ATP. The F₁ unit consists of five different subunit types in the stoichiometric ratio αβγδεX (6, 7). Two recently derived three-dimensional structures of F₁ (8, 9) show that it consists of a hexagonal array of alternating α- and β-subunits with a centrally located γ-subunit, which has been shown to rotate during catalysis (10–12). Much evidence supports the view that ATP synthesis at the quaternary structural level of F₁ occurs by a binding change mechanism (13, 14), whereby energy from the electrochemical proton gradient, transmitted via the rotating γ-subunit, induces the release of tightly bound ATP on one of the three β-subunits while promoting ATP synthesis from ADP and P₁ on a second β-subunit and binding of ADP and P₁ to a third. Following rotation of the γ-subunit a full 360°, each of the three β-subunits has bound ADP and P₁ synthesized ATP, and released ATP.

Despite our extensive knowledge about the events involved in ATP synthesis at the quaternary structural level of F₁, our knowledge (15–17) is limited about the chemical events occurring at the active site of each β-subunit in the transition state, where chemical bond formation/breakage occurs between ADP and P₁ to produce ATP and H₂O. As a major step in this direction, we recently showed, in the presence of Mg₂⁺, ADP, and orthovanadate (V₅), a photoactive phosphate analog, that an inhibited MgADP-V₅-F₁ transition state-like complex is formed (16), similar to that reported for myosin (18–20) where the predicted trigonal bipyramidal geometry of V₅ has been visualized by X-ray analysis of the MgADP-V₅-myosin complex (21). Significantly, photocleavage of the protein backbone in the presence of uv light and O₂ of both the MgADP-V₅-F₁ and the MgADP-V₅-myosin transition state complexes occurs at the third position in the P-loop region (GXXXYGKT) (16, 22). In F₁, a conserved alanine (158 in the animal enzymes) occupies this position (Fig. 1A). The chemistry involved in the photocleavage events has been described (23). Specifically, as these earlier results relate to the mechanism of the ATP synthase reaction, they indicate that alanine 158, which in the two reported crystal structures of F₁ (8, 9) is near but not within the catalytic pocket (residing, respectively, >6.0 Å and >4.5 Å away from the β and γ-P atoms of MgADP and MgATP (8)), moves within the catalytic pocket in the transition state (Fig. 1A).

Here, we report the novel finding that transition state formation in the ATP synthase reaction does not require the presence of ADP and occurs in the presence of only Mg₂⁺ and V₅. As V₅ alone is without effect on transition state formation, these findings have rather profound implications for the role of Mg₂⁺ in the reaction mechanism of ATP synthases as described below.

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Experimental Procedures

Materials

The source of rats (Harlan Sprague-Dawley, white males) for the preparation of the F₁, moiety of the ATP synthase was Charles River Breeding Laboratories. Two different polyclonal antibodies against the F₁-β-subunit were raised in rabbits using the synthetic peptides RKLFLGAGVQKCT (antibody 1) and YVPADLTDPAAPATFFAH-DAC (antibody 2). Antibody 1 has been shown in our laboratory to recognize only the subunit KGLFGQG in the present protein, whereas orthovanadate (V₁), and PVDF membranes were obtained, respectively, from Amer- sham Pharmacia Biotech, Sigma, and Millipore.

Methods

Purification and Assay of the F₁, Moiety of Mitochondrial ATP Synthase—F₁ was prepared from rat liver mitochondria by the procedure of Catterall and Pedersen (6) with the modification described by Pedersen et al. (24) and then stored and processed for the studies described here as exactly as outlined by Ko et al. (16). ATPase activity was assayed by a spectrophotometric procedure in which ADP-formed was coupled to the pyruvate kinase and lactic dehydrogenase reactions (6).

Prior Treatment of F₁ with Orthovanadate (V₁) and F₁ Ligands—The V₁ solution used for these studies was carefully prepared exactly as described previously (16) to minimize the presence of polymeric species and forms other than V₁. The V₁ concentration was determined by measuring the optical density at 265 nm and using the extinction coefficient 2925 M⁻¹ cm⁻¹. F₁ (50 μg) was prior incubated for the times indicated in the figure legends to Figs. 1 and 2 in a 100- or 200-μl system containing 50 mM MOPS, pH 8.5, 10% glycerol (v/v), and where indicated, also V₁, V₁ + ADP, V₁ + ADP + MgCl₂, and V₁ + MgCl₂. Concentrations are provided in the figure legends to Figs. 1 and 2.

Photoactivation with Ultraviolet (uv) Light—Where indicated, the incubation mixture in an open Eppendorf tube was placed under a 100-watt, long wavelength mercury spot lamp at a distance of 7.8 cm for the times indicated in the figure legends to Figs. 1 and 2.

SDS-PAGE and Western Blot Analysis—SDS-PAGE was carried out according to the procedure of Laemmli (25) in 15% acrylamide using a Bio-Rad Mini-Protein dual slab cell. The Coomassie-stained bands were subjected to densitometric analysis using a Fujifilm Bas-1500 phosphorimager and MacBas (version 2.31) software. For Western blot analysis, proteins, after SDS-PAGE, were transferred electrophoretically onto a PVDF membrane and then probed with two different F₁ antibodies (see "Materials") exactly as described previously (16) using horseradish peroxidase-conjugated anti-rabbit IgG as a secondary antibody and the enhanced chemiluminescence (ECL) system for detection.

N-terminal Sequence Analysis—The 17- and 34-kDa peptide fragments of the F₁-β-subunit were transferred onto PVDF membranes as described previously (16) and then subjected to N-terminal sequencing using an Applied Biosystems 475A Protein Sequencing System (26).

Protein Determination—Protein was determined by Pierce’s Coomassie dye binding assay protocol using bovine serum albumin as standard.

Results and Discussion

The F₁, Moiety of Mitochondrial ATP Synthase Forms an Inhibitory Complex in the Presence of Only Mg²⁺ and V₁—In a previous report (16), we showed that when incubated with Mg²⁺, ADP, and V₁, the F₁, moiety of mitochondrial ATP synthase forms a MgADP-V₁-F₁ inhibitory complex, and that under the same incubation conditions, V₁, Mg²⁺, ADP, MgADP, and V₁ + ADP have little or no effect on catalytic activity. These findings, when taken together with the additional findings made, i.e. that inhibition of F₁ by MgADP-V₁ occurs under turnover conditions, is reversible and results in specific cleavage at alanine 158 of the P-loop region (Fig. 1A) of a single β-subunit (catalytic subunit) in the presence of uv light and O₂, provided evidence that the MgADP-V₁-F₁ complex represents a transition state-like intermediate. These findings also implicated movement of alanine 158 into the catalytic pocket (Fig. 1A). Significantly, results presented in Fig. 1B show that when F₁ is prior incubated in the presence of Mg²⁺ and V₁ alone, the catalytic activity of F₁, is inhibited to nearly the same extent as that observed in the presence of Mg²⁺, ADP, and V₁, although the formation of the inhibited complex is faster when ADP is present. In control studies, prior incubation of F₁ with Mg²⁺ alone (16), V₁ alone, ADP + V₁, or ADP + Mg²⁺ had little or no inhibitory capacity (Fig. 1C). These findings indicated that Mg²⁺ plays an important role in transition state formation in the ATP synthase reaction. Therefore, the Mg²⁺-F₁ complex was studied in greater detail.

In the Presence of UV Light and O₂, the Mg²⁺-F₁-inhibited Complex Is Cleaved in the Third Position (Alanine 158) of the P-loop Region (158) of the F₁ β-subunit—Our previous study showed that when the MgADP-V₁-F₁ complex is coupled to uv light (320 nm) and O₂ (atmospheric conditions), the ATP synthase β-subunit is cleaved at alanine 158 of the P-loop into a 17-kDa fragment and a 34-kDa fragment (16). It is expected that if the Mg²⁺-F₁ inhibitory complex mimics a transition state-like intermediate in the ATP synthase reaction pathway, then the cleavage site in the presence of uv light and O₂ will be identical to that observed previously for the MgADP-V₁-F₁ com-
FIG. 2. A, demonstration by SDS-PAGE that F₃ is cleaved in the presence of light (no ligands), Mg²⁺, ADP, and V₃. Conditions are exactly as described under "Methods." Lane 1, control, F₃ treated for 1 h in the absence of light; lane 2, control, F₃ treated for 1 h in the presence of light (no ligands); lanes 3–7, F₃ treated, respectively, for 0, 15, 30, 45, and 60 min in the presence of Mg²⁺, ADP, V₃, and light (320 nm); lanes 8–12, F₃ treated, respectively, for 0, 15, 30, 45, and 60 min in the presence of Mg²⁺, V₃, and uv light. B, diagram depicting the relative regions within the F₃-β-subunit where the epitopes reside for the antibodies used in the experiments described in C. Epitope 1 is KIGLFGG and the last 2 amino acids of which are the first 2 amino acids of the P-loop. Epitope 2 is YYPADDLTDPAPATFAHLDA. C and D, Western blot analyses of the samples in A with either the antibody raised against β-subunit epitope 1 (C) or β-subunit epitope 2 (D). Lane 1, control, F₃ incubated for 1 h in the presence of Mg²⁺, ADP, and V₃; lane 2–6, F₃ treated, respectively, for 0, 15, 30, 45, and 60 min in the presence of Mg²⁺, ADP, and V₃; and lanes 7–11, F₃ treated, respectively, for 0, 15, 30, 45, and 60 min in the presence of Mg²⁺, V₃, and light; and lane 12, control, F₃ incubated for 1 h in the presence of light (no ligands). Conditions are exactly as described under “Methods.”

Results presented in Fig. 2 show that this is indeed the case. Thus, Fig. 2A, which compares the SDS-PAGE patterns obtained after the two different inhibitory complexes had been subjected to uv light and O₂ for the time period ranging from 0 min to 1 h, clearly shows in both cases (lanes 3–7 for the MgADP, F₃ complex and lanes 8–12 for the MgV₃, F₃ complex) the appearance of two new bands, 34 and 17 kDa, relative to control F₃ (lanes 1 and 2) depicting the five different F₃ subunits (α, β, γ, δ, and e). When the SDS-PAGE gels were subjected to Western blot analysis using one β-subunit antibody with its epitope (KIGLFGG, residues 151–157) overlapping with the P-loop region and a second β-subunit antibody with its epitope (YVPADDLTDPAPATTFAHLDA, residues 311–331) in the C-terminal region (Fig. 2B), the appearance of the 17-kDa band and the 34-kDa band could be observed throughout the course of the cleavage reaction as shown in Fig. 2, C and D.

Thus, alanine 158 must be the amino acid residue that was photo-oxidized and cleaved at the junction between the end of the 17-kDa fragment and the beginning of the 34-kDa fragment. The N-terminal sequence (GVGKTVL) of the 34-kDa fragment (Fig. 3B) commences at glycine 158, one residue after alanine 158 within the P-loop. Thus, alanine 158 must be the amino acid residue that was photo-oxidized and cleaved at the junction between the end of the 17-kDa fragment and the beginning of the 34-kDa fragment.

Only One of Three β-Subunits of the MgV₃, F₃-Inhibited Complex Is Cleaved in the Presence of UV Light and O₂—Fig. 3C summarizes results obtained by quantifying both the staining intensities of the 17- and 34-kDa bands and the β-subunit bands following SDS-PAGE of the untreated MgV₃, F₃ inhibitory complex and the uv light treated complex (Fig. 2A). These data show that 67% of the F₃ β-subunit band remains, while the lost 33% is accounted for by the appearance of the 17- and 34-kDa cleavage products (Fig. 3C, right column). Thus, only one of the three β-subunits has undergone cleavage, a result respectively. Consistent with the finding presented in Fig. 1 showing that the MgADP, V₃, F₃ inhibitory complex is formed faster than the MgV₃, F₃ complex, results presented in Fig. 2, C and D, show that the former complex is also cleaved faster in the presence of uv light and O₂ than the latter complex (compare lanes 2–6, MgADP, V₃, F₃, with lanes 7–11, MgV₃, F₃).

Results obtained from N-terminal sequence analyses of the 17- and 34-kDa fragments (Fig. 3, A and B, respectively), together with the data obtained with the antibodies, respectively, recognizing epitope 1 (KIGLFGG) and epitope 2, confirm that the MgV₃, photoinduced cleavage site in the F₃-β-subunit lies at alanine 158 within the P-loop (GAGGVKGT). Thus, data in Fig. 3A clearly identify the 17-kDa fragment as commencing from the N terminus of the β-subunit (known sequence = APKAGTA for isolated rat liver F₃), and data in Fig. 3B indicate that this fragment must extend as far as glycine 157, the second amino acid residue within the P-loop. The N-terminal sequence (GVGKTVL) of the 34-kDa fragment (Fig. 3B) commences at glycine 158, one residue after alanine 158 within the P-loop. Thus, alanine 158 must be the amino acid residue that was photo-oxidized and cleaved at the junction between the end of the 17-kDa fragment and the beginning of the 34-kDa fragment.
The importance of Mg\(^{2+}\) play in transition state formation in the ATP synthase-catalyzed reaction. This is suggested here that during ATP synthesis, the role of Mg\(^{2+}\) is greatly aided and justified by the recent availability of crystal structures of two different states of F\(_{1}\) (8, 9), one of which has the nonhydrolyzable MgATP analog, MgAMP-PNP, bound at the active site of one \(\beta\)-subunit at a time (13, 14). The novel studies reported here indicate that Mg\(^{2+}\) plays a pivotal role in the formation of the transition state in the ATP synthase catalyzed reaction. Speculation as to what this role may be is greatly aided and justified by the recent availability of crystal structures of two different states of F\(_{1}\) (8, 9), one which has \(\beta\)-subunits with ADP and Pi bound at the active site but no Mg\(^{2+}\) (9), referred to here as \(\beta\)\(_{\text{DP,Pi}}\) and the other, which has the nonhydrolyzable MgATP analog, MgAMP-PNP, bound at the active site of one \(\beta\)-subunit (8), previously called \(\beta\)\(_{\text{TP}}\). If it is assumed that the \(\beta\)\(_{\text{DP,Pi}}\) and the \(\beta\)\(_{\text{TP}}\)-subunits are representative, respectively, of the substrate and product bound states during ATP synthesis, the role of Mg\(^{2+}\) in transition state formation based on work described here is best depicted as shown in Fig. 4. Thus, considering the fact that within \(\beta\)\(_{\text{DP,Pi}}\) (Fig. 4, top panel) the \(\beta\)-carbon atom of alanine 158 in the P-loop lies at a nonbonding distance of >7 Å from both the \(\beta\)-P atom of ADP and the P atom of P\(_{i}\) (9) but in the transition-like state (Fig. 4, center panel) induced by Mg\(^{2+}\), this amino acid lies sufficiently close to V\(_{i}\) to be oxidatively cleaved, the implication seems clear that a local remodeling of the active site has occurred. It is suggested here that during ATP synthesis Mg\(^{2+}\) induces a conformational change in the \(\beta\)\(_{\text{DP,Pi}}\)-subunit such that the P-loop region containing alanine 158 moves into the active site pocket, and because of its nonpolar nature, displaces a nearby water molecule known to be present (9). Simultaneously, the conformational change induced by the bound Mg\(^{2+}\) results in the proper alignment of ADP and P\(_{i}\) with a catalytic base, most likely glutamic acid 188 (8, 9). While remaining bound to P\(_{i}\), Mg\(^{2+}\) is depicted as facilitating the departure of water, thus resulting in ATP formation. Subsequently, the \(\beta\)-subunit involved relaxes to the \(\beta\)\(_{\text{TP}}\) form (product bound state) in which the \(\beta\)-carbon atom of alanine 158 now lies again at nonbonding distances from both the \(\beta\)-P atom of ATP and from the newly formed \(\gamma\)-P atom (Fig. 4, lower panel). In \(\beta\)\(_{\text{TP}}\), the Mg\(^{2+}\) is known to be coordinated to the \(\beta\) and \(\gamma\) phosphate oxygens, whereas, in the transition state depicted here, it preferentially coordinates to P\(_{i}\) (Fig. 4, center panel), thus implying a positional shift after ATP is formed (Fig. 4, lower panel). It will be noted also that in the final product bound state that two water molecules are known to be present (8). One is likely that derived from the formation of ATP, the other may be the water molecule found originally in the \(\beta\)\(_{\text{DP,Pi}}\) substrate bound state (Fig. 4, top panel), which returns following the exit of alanine 158 from the active site pocket after ATP synthesis.

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