Novel Insights into the Chemical Mechanism of ATP Synthase

EVIDENCE THAT IN THE TRANSITION STATE THE \( \gamma \)-PHOSPHATE OF ATP IS NEAR THE CONSERVED ALANINE WITHIN THE P-LOOP OF THE \( \beta \)-SUBUNIT

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The chemical mechanism by which the \( \text{F}_1 \) moiety of ATP synthase hydrolyzes and synthesizes ATP remains unknown. For this reason, we have carried out studies with orthovanadate (\( V_i \)), a phosphate analog which has the potential of “locking” an ATPase, in its transition state by forming a MgADP-V\( _i \) complex, and also the potential, in a photochemical reaction resulting in peptide bond cleavage, of identifying an amino acid very near the \( \gamma \)-phosphate of ATP. Upon incubating purified rat liver \( \text{F}_1 \), with MgADP and \( V_i \) for 2 h to promote formation of a MgADP-V\( _i \)-\( \text{F}_1 \) complex, the ATPase activity of the enzyme was markedly inhibited in a reversible manner. When the resultant complex was formed in the presence of ultraviolet light inhibition could not be reversed, and SDS-polyacrylamide gel electrophoresis revealed, in addition to the five known subunit bands characteristic of \( \text{F}_1 \), two new electrophoretic species of 17 and 34 kDa. Western blot and N-terminal sequencing analyses identified both bands as arising from the \( \alpha \)-subunit with the site of peptide bond cleavage occurring at alanine 158, a conserved residue within \( \text{F}_1 \)-ATPases and the third residue within the nucleotide binding consensus \( \text{GX}\text{GKT}(\text{T/S}) \) (P-loop). Quantification of the amount of ADP bound within the MgADP-V\( _i \)-\( \text{F}_1 \) complex revealed about 1.0 mol/mol \( \text{F}_1 \), while quantification of the peptide cleavage products revealed that no more than one \( \beta \)-subunit had been cleaved. Consistent with the cleavage reaction involving oxidation of the methyl group of alanine was the finding that \( [3\text{H}] \) from NaB\( [3\text{H}]_4 \) incorporates into MgADP-V\( _i \)-\( \text{F}_1 \) complex following treatment with ultraviolet light. These novel findings provide information about the transition state involved in the hydrolysis of ATP by a single \( \beta \)-subunit within \( \text{F}_1 \)-ATPases and implicated alanine 158 as residing very near the \( \gamma \)-phosphate of ATP during catalysis. When considered with earlier studies on myosin and adenylate kinase, these studies also implicate a special role for the third residue within the \( \text{GX}\text{GKT}(\text{T/S}) \) sequence of many other nucleotide-binding proteins.

Despite our extensive knowledge about the structure and function of the \( \text{F}_1 \) moiety of ATP synthases (1–5), sufficient information is not available to write a chemical mechanism by which ATP is hydrolyzed and synthesized. In contrast, myosin-ATPase has been successfully studied using orthovanadate, \( V_i \), a phosphate analog which in the presence of MgADP forms a transition state MgADP-V\( _i \)-myosin inhibitory complex (6–8). Irradiation of this complex with uv light results in the modification of the single serine within the nucleotide binding consensus GESGAGKT followed by peptide bond cleavage at this site (Fig. 1A) (8, 9). These studies strongly implicated this serine as contacting directly the \( \gamma \)-phosphate of ATP in the transition state, and were recently confirmed by x-ray structural analysis (10). The reaction pathway of \( \text{F}_1 \)-ATPase is believed to be quite similar to that of myosin-ATPase (11).

In addition, within the catalytic sites of both enzymes resides the nucleotide binding consensus G\( _X\)G\( _Z\)G\( _K\)T\( (\text{T/S}) \) (P-loop), which in the \( \beta \)-subunit of \( \text{F}_1 \) (GGAGVGKT), contains alanine in place of the internal \( \gamma \)-serine sensitive serine characteristic of the myosin consensus GESGAGKT. As this serine in myosin is known to contact the \( \gamma \)-phosphate of ATP in the transition state (10), it seemed reasonable to assume that in \( \text{F}_1 \), a nearby serine residing outside the consensus region or the terminal threonine within the consensus region may serve this role in the transition state. Alternatively, the third position within the consensus region of \( \text{F}_1 \), although containing an alanine, may play an important role in the transition state of \( \text{F}_1 \) as does the serine in the same position in myosin. Studies described below were carried out both to define optimal conditions for trapping \( \text{F}_1 \)-ATPase in a MgADP-V\( _i \)-\( \text{F}_1 \) inhibitory transition state complex, and to establish in this state the identity of an amino acid residue near the \( \gamma \)-phosphate of ATP. Both goals were accomplished and provided novel insights into the chemical mechanism of ATP synthases.

EXPERIMENTAL PROCEDURES

Materials

Rats (Harlan Sprague-Dawley, white males) were obtained from Charles River Breeding Laboratories. ATP, ADP, MgCl\(_2\), MOPS, CAPS, sodium orthovanadate, phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase were obtained from Sigma. SDS, acrylamide, and bisacrylamide were from Bio-Rad. Ammonium sulfate and potassium phosphate were from J. T. Baker Chemical Co. Tuberculin syringes and Sephadex G-50 used in nucleotide binding assays were from Becton-Dickinson Co. and Pharmacia Biotech Inc., respectively. PVDF membranes were obtained from Millipore and Western blot reagents from Amersham. A polyclonal antibody against the rat \( \text{F}_1 \)-\( \beta \)-subunit was raised in rabbits using the synthetic peptide KIGLFGGAGVGKT.

\[ [\text{H}]\text{ADP and NaB}[\text{H}]_4 \] were from NE Life Science Products. All other reagents were of the highest purity commercially available.

Methods

Purification of Rat Liver \( \text{F}_1 \)-ATPase—The enzyme was purified by the procedure of Catterall and Pedersen (13) with the modification

\[ \text{V}_i \] The abbreviations used are: \( \text{V}_i \), orthovanadate; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(N-morpholino)propanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; PVDF, polyvinylidene difluoride; uv, ultraviolet.

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described by Pedersen et al. (14). The purified enzyme, in 250 mM KP, and 5.0 mM EDTA, was divided into 100-μl aliquots, lyophilized to dryness, and stored at −20 °C. Prior to use, aliquots (150–250 μg) of lyophilized F₄₅ were dissolved at 25 °C in 100 μl of water and precipitated twice with 3 mM ammonium sulfate, 5 mM EDTA, redissolving between precipitations in 200 mM K₂SO₄, 10 mM Tris-Cl, pH 7.5, or 50 mM MOPS, pH 8.0.

Preparation of Orthovanadate (V₅) Solution—To minimize the presence of polymeric species the following protocol was followed: Na₃VO₄ powder was dissolved in water and the pH adjusted with HCl to pH 10 (orange color). The solution was boiled for 2 min at which time the solution became clear. The pH was readjusted to pH 10 and the previous boiling repeated 2 times. After the optical density was determined at 265 nm, the V₅ concentration was calculated using the molar extinction coefficient of 2925 M⁻¹ cm⁻¹. The stock solutions used in this study were 155 mM and were covered with aluminum foil and stored until use at −80 °C.

Prior Treatment of F₁ with V₅—F₁ (50 μg) was primarily incubated in a 100- or 200-μl system containing 50 mM MOPS, pH 8.5, 10% glycerol (v/v) and, where indicated, also V₅, V₅ + ADP, V₅ + ADP + MgCl₂, V₅ + ATP + MgCl₂, or MgCl₂, all at concentrations indicated in legends. Prior incubations were carried out at 25 °C for the indicated times. In experiments where photoactivation of vanadate was induced with uv light (320 nm), the incubation mixture in an open Eppendorf tube was placed under a 100 watt, long wavelength mercury spot lamp (BLAK- RAY Model B 100A, 115 V, 2.5 amperes; San Gabriel, CA) at a distance of 7.8 cm. The time in the presence of the light source varied as indicated in the figure legends.

Assay for ATPase Activity—The spectrophotometric procedure was used in which ADP formed was coupled to the pyruvate kinase and lactate dehydrogenase reactions (13). The reaction mixture contained the following in a volume of 1 ml at pH 7.5 and 25 °C: 0.2 mM ATP, 65 mM Tris-Cl, pH 7.5, 0.2 mM MgCl₂, 25 mM KP, 0.4 mM NADH, 0.6 mM phosphonozyrupovic acid, 5 mM KCN, 2 h of the reaction. Following the uv and oxygen-dependent reactions, which result in peptide bond cleavage at two places in the serine residue, the amino group of the serine is covalently attached to the preceding amino acid in the protein, the side chain is oxidized to form an aldehyde and the additional carbonyl group is attached to the oxalyl moiety which remains at the N terminus of the subsequent amino acid. (See Ref. 27 for a more detailed description of the chemistry.) B, amino acid sequence from the rat liver F₁ subunit deduced from cDNA showing the Walker A and B nucleotide binding consensus sequences (boxed). Tryrosine 163 which terminates the A consensus is depicted with an asterisk. The predicted catalytic base, glutamate 188, which lies between the A and B consensus regions, is underlined. The first N-terminal amino acid detected in the β subunit of isolated rat liver F₁ is alanine. (See underlined region.)

RESULTS AND DISCUSSION

F₁ Forms an Inhibitory Complex in the Presence of MgCl₂, ADP, and V₅—To promote formation of an inhibitory MgADP·V₅·F₁ transition state complex, several precautions were taken. First, the V₅ stock solution was priorly treated exactly as described under “Experimental Procedures” and maintained at pH 10 in the dark at −80 °C until use to prevent formation of polymeric species. Second, the zwitterionic buffer MOPS was used in all experiments as it both stabilizes F₁ and prevents or minimizes inhibition by MgCl₂ and the product MgADP (or ADP). Third, a pH of 8.5 was used in all experiments, as this pH is near optimal for the hydrolysis of ATP catalyzed by rat liver F₁. Finally, equimolar amounts of MgCl₂, ADP, and V₅ were used to promote formation of MgADP·V₅ at the active site of F₁.

Fig. 2A summarizes results of an experiment where F₁ was priorly incubated in the presence of 200 μM each of MgCl₂, ADP, and V₅ for the indicated times and then assayed for ATPase activity as described under “Experimental Procedures.” Here it is clear that under these conditions, which are optimal for forming a MgADP·V₅·F₁ transition state complex, F₁-ATPase activity is markedly inhibited. Half-maximal inhibition (50%) is reached in about 45 min and maximal inhibition (~80%) is reached in about 2 h. In control experiments with F₁ alone, F₁ + V₅, F₁ + ADP, and F₁ + MgADP; inhibition at 2 h is, respectively, ~1, 10, 20, and 20% under conditions described in the figure legend. In an experiment not presented, F₁ prior incubated with MgCl₂ alone, was not inhibited after 2 h. Finally, Fig. 2B shows that when F₁ is prior incubated with 200 μM each of MgCl₂, ADP, and V₅, but in the presence of light (320 nm) and atmospheric oxygen, an essentially identical inhibi-
Inhibition of F-ATPase Activity by MgCl₂ + ADP + V₁ Is Reversible in the Absence of UV Light but Not in Its Presence—If formation of a MgADP-V₁-F₁ transition state complex is responsible for the inhibition observed in the presence of MgCl₂ + ADP + V₁, this state should be reversible. Results presented in Fig. 3A show that this is the case. Thus, following maximal inhibition of F₁-ATPase activity in the presence of 200 μM each of MgCl₂, ADP, and V₁ (Fig. 3A, left panel), an aliquot was removed and subjected to two dilution/wash cycles in the presence of 50 mM MOPS, 10% glycerol, pH 8.5. This resulted after two such cycles in the restoration of the original activity to a level near 90% (Fig. 3A, right panel). Significantly, reversal of ATPase activity could not be achieved (Fig. 3B, right panel) following inhibition of F₁-ATPase activity under identical conditions but in the presence of UV light (Fig. 3B, left panel) and with longer incubation times. The reason for this becomes clear in the description of other experiments described below.

Vi Induces Inhibition of F₁-ATPase Activity under Turnover Conditions—It is expected that if a MgADP-V₁-F₁ transition state is formed in the presence of MgCl₂, ADP, and Vi as implicated from the above studies, it would be formed also under turnover conditions, i.e. when ATP, the substrate for ATP hydrolysis is present. For this reason, F₁ was incubated exactly as described above but with ATP replacing ADP in the prior incubation mixture. Thus, the final prior incubation mixture contained 200 μM each of MgCl₂, ATP, and Vi. After 1 h under these turnover conditions, aliquots were removed and assayed for ATPase activity. Results presented in Fig. 4 show that F₁-ATPase activity is inhibited about 50% in the absence of UV light and about 70% in its presence. Although after 1 h the degree of inhibition is not as great as that achieved when prior incubation is carried out with ADP rather than ATP in the prior incubation mixture. Thus, the final prior incubation mixture contained 200 μM each of MgCl₂, ATP, and V₁. After 1 h under these turnover conditions, aliquots were removed and assayed for ATPase activity. Results presented in Fig. 4 show that F₁-ATPase activity is inhibited about 50% in the absence of UV light and about 70% in its presence. Although after 1 h the degree of inhibition is not as great as that achieved when prior incubation is carried out with ADP rather than ATP in the prior incubation mixture, this is to be expected. Thus, under the latter conditions ATP must first undergo hydrolysis before ADP is available for formation of the MgADP-V₁-F₁ complex and MgATP competes with ADP for binding to F₁-ATPase active sites.

Polypeptide Chain Cleavage Occurs within the MgADP-V₁-F₁ Complex in the Presence of UV Light—As indicated earlier,

Fig. 2. Time dependent loss of F₁-ATPase activity upon incubation of F₁ with MgADP-V₁ in the absence (A) and presence of UV light (B). Prior incubation was carried out as indicated under “Experimental Procedures” in a 0.1-ml system containing F₁ alone ( ), and where indicated, F₁ + 0.2 mM V₁ ( ), F₁ + 0.2 mM ADP + 0.2 mM V₁ ( ), F₁ + 0.2 mM MgCl₂ + 0.2 mM ADP ( ), or F₁ + 0.2 mM each of MgCl₂, ADP, and V₁ ( ). At the indicated times, a 3-μl aliquot (1.5 μg F₁) was withdrawn and assayed for ATPase activity exactly as described under “Experimental Procedures.” The results presented are representative of more than five different experiments.

Fig. 3. Demonstration that the inhibition of F₁-ATPase activity by MgCl₂ + ADP + V₁ is reversible in the absence of UV light (A) and irreversible in its presence (B). Prior incubations were carried out with F₁-ATPase in the absence ( ) or presence ( ) of MgCl₂, ADP, and V₁ exactly as described in the legend to Fig. 2 and under “Experimental Procedures.” After inhibition of ATPase activity had reached a maximal level (left panels, see arrows), a 0.1-ml aliquot was withdrawn and diluted 6-fold in 50 mM MOPS, 10% glycerol, pH 8.5. The diluted solution was filtered through Amicon’s Microcon 100 Filtration Unit at 25 °C by centrifugation at 500 × g for 15 min. The filtrate was discarded and the retentate was diluted to 0.1 ml and assayed for F₁-ATPase activity as described under “Experimental Procedures” (right panels, Cycle 1). The dilution, washing, assay procedures were then repeated (right panels, Cycle 2). The entire experiment was repeated with essentially identical results.
vanadate is photoreactive and has been shown in the case of the MgADPV_i-myosin complex to modify an active site serine residue within contact distance of V_i, and, in the presence of light and atmospheric oxygen, to induce peptide bond cleavage at this site (8, 9) (Fig. 1A). For this reason F_i was priorly incubated in the presence of 200 μM each of MgCl_2, ADP, and V_i in the absence and presence of uv light exactly as described for Figs. 2 and 3 and then subjected to SDS-PAGE. When the resultant SDS-PAGE profiles (Fig. 5, A and B) of the two incubation mixtures (absence and presence of light) are compared, it is clear that only in the latter case has polypeptide bond cleavage occurred. Thus, in addition to Coomassie-stained bands corresponding to the known F_1 subunits α, β, γ, δ, and ε, bands distinct from these subunits with apparent molecular masses of 17 and 34 kDa appear (Fig. 5B, lane 6). Polypeptide bond cleavage within the F_1 molecule is highly specific, and is not observed in the absence of uv light under any condition tested (Fig. 5A), and is observed in the presence of uv light with MgCl_2 + ADP + V_i (Fig. 5B, lane 6). Fig. 5C shows that the appearance of the 17- and 34-kDa peptide fragments increases with time as expected and levels off after about 2 h (lane 9). These findings are consistent with polypeptide bond cleavage within the nucleotide-binding consensus region (GGAGVGVKT) of the 51.5-kDa β-subunit, as this would give rise to two fragments with molecular masses near the experimentally determined values of the 17- and 34-kDa bands (Fig. 5C).

Polypeptide Chain Cleavage within the MgADPV_i-F_1 Complex Induced by UV Light Does Not Alter the Oligomeric State of F_1 and Occurs Only within the β-Subunit—Results of native PAGE experiments presented in Fig. 6A, lanes 1–5, show that, under conditions which result in cleavage of a polypeptide chain within the MgADPV_i-F_1 complex in the presence of uv light and atmospheric oxygen, the oligomeric state of the F_1 molecule remains intact. In fact, the F_1 molecule remains intact in uv light under all conditions tested (i.e. alone or with V_i, MgCl_2 + ADP, ADP + V_i, or MgCl_2 + ADP + V_i; Fig. 6A, lanes I–5, respectively). Specifically, as it applies to the uv light and V_i-dependent cleavage of a polypeptide chain within the MgADPV_i-F_1 complex (Fig. 5B, lane 6), these results are consistent with a very localized reaction which is otherwise without deleterious effect on the remaining part of the F_1 molecule.

Identification of the β or “catalytic” subunit within F_1 as the source of the 17- and 34-kDa bands was derived from two separate experiments. In the first, SDS-PAGE gels of the uv-light-treated MgADPV_i-F_1 complex, after transfer to PVDF membranes, were probed with a polyclonal, antibody raised against the synthetic peptide KIGLFGGAGVGKCT, containing the GXGKKT consensus region of the rat liver β subunit. As shown in Fig. 6B, lane 6, only the intact β subunit and the 17-kDa band cross-react with the antibody. This is the expected result if polypeptide bond cleavage occurs within the nucleotide binding consensus region of the β subunit, as the epitope reactive with the antibody would be largely retained at the C terminus of the 17-kDa fragment, but missing from the 34-kDa fragment. In the second experiment, N-terminal sequence analysis (Fig. 6C) which identified the 7-amino acid stretch APKAGTA confirmed the β subunit (Fig. 1B) as the origin of the 17-kDa fragment. (The first six amino acids at the N terminus of the 17-kDa fragment are not present in the isolated protein.) N-terminal sequence analysis of the 34-kDa fragment also proved possible by carrying out the transfer from SDS-PAGE to PVDF membranes for 2 rather than 1 h, and at 25 °C rather than 4 °C (see “Experimental Procedures”). This modification in the transfer procedure was done to promote removal of an oxalyl group (HO-CO-CO-) predicted to be at the N terminus of the 34-kDa fragment following the V_i-dependent cleavage reaction (Fig. 1A). Significantly, the N-terminal sequence obtained, GVGKTVLIMELINN (Fig. 6D), not only confirmed the 34-kDa frag-

**FIG. 4.** Demonstration that V_i induces inhibition of F_1-ATPase activity under turnover conditions. Prior incubation was carried out at 25 °C with F_1-ATPase in the absence or presence of 200 μM each of MgCl_2, ATP, and V_i. After 1 h, 3-μl aliquots (1.5 mg F_1) were withdrawn and assayed for ATPase activity as described under “Experimental Procedures.” Where indicated a control was also carried out under identical conditions but with ADP rather than ATP in the prior incubation mixture. Dark bars, absence of light; shaded bars, presence of light. The data presented are averages of duplicate determinations.

**FIG. 5.** SDS-PAGE gels demonstrating that a polypeptide chain is cleaved when the MgADPV_i-F_1 complex is exposed to uv light. A, absence of light (control). F_1 was priorly incubated for 2 h exactly as described in the legend to Fig. 2 either alone (lane 2) or in the presence of 0.2 mM V_i (lane 3), ADP + MgCl_2 (lane 4), ADP + V_i (lane 5), MgCl_2 + ADP + V_i (lane 6). Molecular weight markers are depicted in lane 1. B, presence of light. Conditions are identical to A except samples were subjected to uv light (see “Experimental Procedures”) during the 2-h prior incubation period. C, time dependence of the polypeptide cleavage reaction in the presence of MgADPV_i. Conditions are identical to those described in the legend to the Fig. 2 for ADP + MgCl_2 + V_i. Prior incubation was carried for 17, 23, 33, 49, 61, 71, 83, and 114 min, respectively, for lanes 2–9; lane 10, control without prior incubation; lane 1, molecular weight standards.
FIG. 6. A, the oligomeric state of F₁-ATPase is not altered by light activation of the MgADP-V₁-F₁ complex. After prior incubation for 2 h of F₁ alone or with the components indicated in legends to Figs. 2 and 3, in the presence of uv light, the incubation medium was subjected to native PAGE exactly as described under “Experimental Procedures.” Lane 1, F₁ alone; lane 2, F₁ + V₁; lane 3, F₁ + MgCl₂ + ADP; lane 4, F₁ + ADP + V₁; lane 5, F₁ + MgCl₂ + ADP + V₁. B, Western blot analysis of the cleavage products. Prior incubation was carried out for 2 h with F₁ alone or with the components indicated in legends to Figs. 2 and 3, in the presence of uv light. Following SDS-PAGE, Western blot analysis using an anti-β subunit antibody was carried out exactly as described under “Experimental Procedures.” Lane 2, F₁ alone; lane 3, F₁ + V₁; lane 4, F₁ + MgCl₂ + ADP; lane 5, F₁ + ADP + V₁; lane 6, F₁ + MgCl₂ + ADP + V₁. C and D, N-terminal sequence analysis of the cleavage products. E, representative of more than five different experiments.

The Ratio of the Number of β Subunits Cleaved/F₁ to the Number of β Subunits Binding ADP Is Near 1—To determine the extent of involvement of the 3 β subunits of F₁ in the formation of the MgADP-V₁-F₁ transition state complex, and in the formation of the 17- and 34-kDa cleavage products, both ADP-binding studies and densitometric analysis of Coomassie-stained bands were carried out. Fig. 6E shows that under the conditions used in this study, rat liver F₁ binds only about 1 mol of ADP/mol of F₁ and that V₁ and MgCl₂ have little or no affect on this stoichiometric ratio. When MgCl₂, ADP, and V₁ are added together, each at a concentration of 200 μM to favor formation of the MgADP-V₁-F₁ complex (Fig. 2A), the stoichiometry of ADP binding remains constant. Thus, formation of the transition state complex appears to be restricted predominantly to the involvement of 1 β subunit. This correlates well with the estimated number of β subunits involved in formation of the 17- and 34-kDa cleavage products when the transition state complex is formed in the presence of uv light. Here, a loss of 33% of the total F₁ β subunit staining intensity results which is fully recovered by the sum of the staining intensities of the 17- and 34-kDa products (Fig. 6F).

Studies with Sodium Borohydride Provide Further Evidence That an Alanine Residue Is Oxidized when the MgADP-V₁-F₁ Complex Is Treated with UV Light—Studies described above provide rather compelling evidence that, in the presence of atmospheric oxygen, uv light-induced cleavage of a single β subunit within the MgADP-V₁-F₁ complex occurs at alanine

FIG. 7. UV light-dependent incorporation of tritium from NaB³[H] into F₁-ATPase following the enzymes inactivation by MgADP-V₁. A, predicted steps in the oxidation by V₁ and oxygen of the methyl side chain of alanine. Note: one of the intermediates is predicted to be an aldehyde which can be reduced by NaBH₄. B, incorporation of [³H] from NaB³[H] into F₁-ATPase. F₁ was prior incubated with 0.2 mM of MgCl₂, ADP, and V₁ in a 1.0-ml system containing 50 mM MOPS, 10% glycerol, pH 8.5, at 25 °C for 30 min, and then treated with uv light as indicated under “Experimental Procedures.” At the times indicated 100-μl aliquots were withdrawn and subjected to column centrifugation as described under “Experimental Procedures” to remove excess V₁, MgCl₂, and ADP. The resultant eluate was treated with 90 μM NaB³[H] (25 μCi/ml) at 25 °C for 45 min. The excess NaB³[H] was removed by column centrifugation. The entire eluate was then subjected to liquid scintillation counting. The radioactivity of [³H] at the zero time point was subtracted from that obtained at each time point and the difference (ΔCPM) was plotted as a function of time. Similar results were obtained in two additional experiments.
that such an incorporation does occur in very significant hydrolysis at the active site of F1 is proposed. Here, formation presented in this paper in mind, a tentative pathway for ATP S1 complex (10). With these facts, and the extensive data been shown to be within contact distance (2.6 Å) of the V i due, serine 181, in respectively, in a light-dependent incorporation of tritium [3H] from 3H-labeled oxygen atoms in the x-ray structure of the MgADP z atom of alanine 158 and the γ-phosphorus atom of ATP in the pre-hydrolysis state (left panel), the transition state (center panel), and post-hydrolysis state (right panel). Distances in the left and right panels were obtained from the coordinates of the x-ray structure of bovine heart F1 (5) kindly provided by Dr. J. E. Walker. .B represents an unknown base, involved either in the abstraction of a proton from water (5) or in stereochemically orienting and polarizing the attacking water without net proton abstraction (28). The hatched line between the Cβ carbon of alanine 158 and the γ-P group of ATP in the transition state indicates that they are very near one another, not that there is a direct chemical interaction, although this possibility cannot be excluded. B, comparison of the amino acid sequences of the Walker A motif within myosin (rabbit muscle), adenylate kinase (chicken muscle), and F1-ATPase (rat liver). See Refs. 8 and 26, respectively, for myosin and adenylate kinase and refer to Fig. 1B of this paper for F1-ATPase.

158. If the β-methyl group of this alanine residue is oxidized, it is expected to proceed through a series of reactions resulting first in the formation of serine, then an aldehyde (Fig. 7A), and finally other intermediates before peptide bond cleavage finally occurs (see Fig. 1A and Refs. 22 and 27). Therefore, it should be possible, via reduction of the aldehyde, to demonstrate a uv light-dependent incorporation of tritium [3H] from 3H-labeled sodium borohydride into F1-ATPase following the enzyme’s inactivation by MgADP-Vi. Results presented in Fig. 7B show that such an incorporation does occur in very significant amounts over control levels obtained in the absence of MgADP-Vi. Incorporation reaches a maximal level in about 7 min, and then declines as expected because the aldehyde is a transient intermediate in the overall oxidation process.

Implications for the Chemical Mechanism of ATP Hydrolysis by F1—Studies reported here strongly implicate alanine 158 in the third position of the GGA(GVKT consensus of the rat liver F1 β-subunit as residing very near the γ-phosphate group of ATP in the transition state. Significantly, alanine in this position is conserved in all F1-ATPases (23), and in Escherichia coli, F1 mutations in this position to valine or proline result, respectively, in a >90% loss or a 2-fold activation of catalytic substrate specificity (see also Refs. 24 and 25). Along these lines, it is interesting to note that Vi in the presence of uv light and oxygen also cleaves adenylate kinase at the third position (proline 17) within the nucleotide binding consensus GGPGSKGT (26). Thus, in support of the view proposed here, three different enzymes, myosin, F1-ATPase, and adenylate kinase are all cleaved at the same third position despite the fact that the amino acid occupying this position is very different in all cases (Fig. 2B), but conserved within its specific enzyme class.

Finally, it should be noted that results presented here, which have focused on alanine 158 of rat liver F1, do not preclude other amino acids near the γ-phosphate of ATP in the transition state. Significantly, in a recent intriguing paper Senior and colleagues (28) have summarized the possible roles of three catalytic site residues in the E. coli F1-ATPase. One of these, lysine 155 (lysine 162 in rat liver F1) is considered to be in the major functional interaction with the γ-phosphate of MgATP in the substrate bound or “ground state,” but to
undergo conformational repositioning during catalysis. Therefore, when taken together with the novel findings from studies reported here, it will be of considerable interest to visualize the precise location and orientation of lysine 162 and alanine 158 in the transition state when the x-ray structure of the MgADP-V$_r$-F$_1$ complex is elucidated.

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REFERENCES
1. Capaldi, R. A., Aggeler, R., Turina, P., and Wilkins, S. (1994) Trends Biochem. Sci. 12, 186–189
2. Pedersen, P. L., and Amzel, L. M. (1993) J. Biol. Chem. 268, 9937–9940
3. Senior, A. E. (1990) Annu. Rev. Biophys. Biophys. Chem. 19, 7–41
4. Bianchet, M., Ysern, X., Hullihen, J., Pedersen, P. L., and Amzel, L. M. (1991) J. Biol. Chem. 266, 21197–21201
5. Abrahams, J. P., Leslie, A. G. W., Lutter, R., and Walker, J. E. (1994) Nature 370, 621–628
6. Goodno, C. C. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 2620–2624
7. Goodno, C. C. (1979) Proc. Natl. Acad. Sci. U. S. A. 79, 21–25
8. Cremo, C. R., Grammer, J. C., and Yount, R. G. (1989) J. Biol. Chem. 264, 6608–6611
9. Cremo, C. R., Long, G. T., and Grammer, J. C. (1990) Biochemistry 29, 7982–7990
10. Smith, C. A., and Rayment, I. (1996) Biochemistry 35, 5404–5417
11. Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982) J. Biol. Chem. 257, 12101–12105
12. Walker, J. E., Saraste, M., Runswick, M. H., and Gay, N. J. (1982) EMBO J. 1, 945–951
13. Catterall, W. A., and Pedersen, P. L. (1971) J. Biol. Chem. 246, 4987–4994
14. Pedersen, P. L., Hullihen, J., and Wehrle, J. P. (1981) J. Biol. Chem. 256, 1362–1369
15. Williams, N., Hullihen, J. M., and Pedersen, P. L. (1987) Biochemistry 26, 162–169
16. Penefsky, H. S. (1977) J. Biol. Chem. 252, 2981–2989
17. Laemmli, U. K. (1970) Nature 227, 285–298
18. Weber, K., and Osborn, M. (1968) J. Biol. Chem. 244, 4406–4412
19. Edman, P. (1955) Acta. Chem. Scand. 4, 283–289
20. Hunkapillar, M. W., and Hood, L. E. (1983) Science 219, 650–659
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
22. Coulston, G. W., Bare, S. R., Kung, K. B., Bethke, G. K., Harlow, R., Herren, N., and Lee, P. L. (1997) Science 275, 191–193
23. Yasen, X., Amzel, L. M., and Pedersen, P. L. (1998) J. Bioenerg. Biomembr. 20, 423–450
24. Takeyama, M., Ibara, K., Moriyama, Y., Nouni, T., Ida, K., Tomioka, N., Itai, A., Mard, M., and Futai, M. (1996) J. Biol. Chem. 271, 21279–21284
25. Tagaya, M., Yamam, T., Nouni, T., Futai, M., Kashi, F., Nakazawa, A., and Futui, T. (1989) J. Biol. Chem. 264, 990–995
26. Cremo, C. R., Lou, J. A., Edmonds, C. G., and Hatlelid, K. M. (1992) Biochemistry 31, 491–497
27. Grammer, J., Lou, J. A., Edmonds, C. G., Cremo, C. R., and Yount, R. G. (1996) Biochemistry 35, 15582–15592
28. Lobau, S., Weber, J., Wilke-Mounts, S., and Senior, A. E. (1997) J. Biol. Chem. 272, 3648–3656

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