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Does Pyrophosphate Bind to the Catalytic Sites of Mitochondrial F_1-ATPase?†

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ABSTRACT: The interactions between the pyrophosphate (PP_i) binding sites and the nucleotide binding sites on mitochondrial F_1-ATPase have been investigated, using F_1 preparations containing different numbers of catalytic and noncatalytic nucleotide-binding sites occupied by ligands. In all cases, the total number of moles of bound nucleotides and PP_i per mole of F_1 was less than or equal to six. F_1 preparations containing either three or two filled noncatalytic sites and no filled catalytic sites (referred as $F_1[3,0]$ and $F_1[2,0]$) were found to bind 3 mol of PP_i/mol of F_1. Tight binding of ADP–fluoroberyllate complexes to two of the catalytic sites of F_1 converted the three heterogeneous PP_i-binding sites into three homogeneous binding sites, each exhibiting the same affinity for PP_i. The addition of PP_i at saturating concentrations to F_1 containing GDP bound to two catalytic sites ($F_1[2,2]$) resulted in the release of 1 mol of GDP. Furthermore, the addition of PP_i to F_1 filled with ADP–fluoroberyllate at the catalytic sites resulted in the release of 1 mol of tightly bound ADP/mol of F_1. Taken together, these results indicate that PP_i binds to specific sites that interact with both the catalytic and the noncatalytic nucleotide-binding sites of F_1.

Despite recent advances in the understanding of the mechanism by which the catalytic sector F_1 of H^+-ATPase[1] undergoes ATP hydrolysis, there remains a number of unsolved problems, some of which concern the status of the nucleotide-binding sites. The presence of six nucleotide-binding sites located in the three α and the three β subunits of mitochondrial, bacterial, and chloroplastic F_1 has been demonstrated (Dunn & Futai, 1980; Ohta et al., 1980; Wagenwoord et al., 1980; Cross & Nalin, 1982; Lunardi & Vignais, 1982; Issartel & Vignais, 1984; Gromet-Elhanan & Khananshvili, 1984; Perlin et al., 1984; Issartel et al., 1986; Hisabori et al., 1986). Three of these sites rapidly exchange their bound ADP and ATP with added nucleotides (Cross et al., 1982; Gresser et al., 1982; Melese & Boyer, 1985), and they are competent for catalysis. When prepared by the method of Knowles and Penefsky (1972), beef heart mitochondrial F_1 contains three

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[1] Abbreviations: F_1-ATPase, catalytic sector (soluble) of the beef heart mitochondrial H^+-ATPase complex; $F_1[x,y]$, F_1 with x noncatalytic and y catalytic sites occupied by nucleotides; AdN, adenine nucleotide; PP_i, pyrophosphate.

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tightly bound nucleotides, two of which are bound to noncatalytic sites and the other one is bound to a catalytic site (Garrett & Penefsky, 1975; Kironde & Cross, 1986). This type of F_1 is referred to as [2,1]; the first and second numbers designate the number of nucleotides bound to the noncatalytic sites and the catalytic sites, respectively (Kironde & Cross, 1986). In addition to the nucleotide-binding sites, F_1 contains binding sites for P_i and PP_i [for review see Issartel et al. (1987)]. PP_i appears to be an interesting ligand because it binds to specific sites on F_1, and in some respects, it mimics ADP (Issartel et al., 1987). Millimolar concentrations of PP_i have been determined in the matrix of liver mitochondria [for review see Mansurova (1989)]; a regulatory or metabolic function of PP_i on the mitochondrial F_1 activity is therefore not unlikely. On the other hand, a membrane-bound pyrophosphatase has been demonstrated to catalyze the energy-dependent synthesis of PP_i in *Rhodospirillum rubrum* (Strid et al., 1986). The presence of PP_i-binding sites in mitochondrial F_1 might be a relic of a primitive catalyst in cell energetics, and comparative studies on the topography of these sites and those of the *Rh. rubrum* pyrophosphatase may have interesting developments. A potentially fruitful approach to study PP_i binding, which has not been extensively used so far, consists of using preparations of F_1 whose nucleotide-binding sites are filled to different extents with either ADP or ATP (Cunningham & Cross, 1988; Lunardi et al., 1988). The experiments described in the present paper deal with the PP_i-binding properties of such preparations of F_1. The results show that PP_i binds to specific sites, which interact with both the catalytic and the noncatalytic sites.

Materials and Methods

[^{14}C]ADP, [^{3}H]ADP, [^{3}H]GDP, and [γ-^{32}P]ATP were purchased from Amersham, and [^{32}P]sodium pyrophosphate was obtained from Du Pont–New England Nuclear. They were diluted with the corresponding unlabeled chemical to a specific radioactivity of 100–150 dpm/pmol. The purity of [^{32}P]PP_i was checked by thin-layer chromatography as described by Issartel et al. (1987). After autoradiography, the radioactive areas were scratched and counted. The only contaminant found was [^{32}P]P_i, and its amount was less than 2% of that of [^{32}P]PP_i. F_1-ATPase was prepared from beef heart mitochondria according to the method of Knowles and Penefsky (1972) modified by Klein et al. (1982). F_1 was stored at 4 °C, at a concentration of 5–10 mg/mL, as an ammonium sulfate precipitate in 50 mM Tris-HCl/250 mM sucrose/4 mM ATP/2 mM EDTA/2.1 M $(NH_4)SO_4$, final pH 8.0. In routine assays, the ATPase activity of F_1 was measured using a coupled NADH oxidation assay in an ATP-regenerating medium (Chang & Penefsky, 1973). Tightly bound nucleotides were released after heat denaturation (Issartel et al., 1986) and assayed by a luminescence test (Lundin et al., 1976). This test, which is specific for adenine nucleotides, does not detect guanine nucleotides. The radioactivity of labeled ligands was measured by liquid scintillation counting (Patterson & Greene, 1965). Protein concentrations were determined using bovine serum albumin as standard, as described by Bradford (1976). The molecular mass of 371 000 Da for F_1 was used for the calculations (Walker et al., 1985).

Prior to binding assays, F_1 was desalted by two sequential centrifugation–filtrations through short Sephadex columns made with tuberculin syringes and filled with 1 mL of Sephadex G50 fine grade equilibrated with the appropriate medium, according to the method described by Penefsky (1977), slightly modified in our laboratory (Issartel et al., 1987). Different types of medium were used, which resulted in F_1 preparations with catalytic sites and noncatalytic sites filled to different extents with ADP plus ATP (Kironde & Cross, 1986). F_1[2,1] which designates the mitochondrial ATPase with two nucleotides bound to noncatalytic sites and one nucleotide bound to a catalytic site (Kironde & Cross, 1986) was obtained as follows. In a first centrifugation–filtration, the Sephadex column was equilibrated with a magnesium-free medium consisting of 250 mM sucrose and 50 mM Tris–acetate, final pH 7.5 (STA buffer). For the second centrifugation–filtration, the column was equilibrated in a sucrose–saline medium consisting of 150 mM sucrose, 50 mM Tris, 30 mM NaCl, 3 mM $MgCl_2$, and 10% glycerol (w/v) with the pH being adjusted at 8.0 with HCl (STNMg buffer). The effect of glycerol was to partially deplete F_1 of bound nucleotides. In our hands, this F_1 preparation contained 2.7–3.1 mol of bound ADP plus ATP/mol of F_1, among which one-third was exchangeable as measured in 10 different preparations of F_1.

F_1[3,0], with the three noncatalytic sites occupied with adenine nucleotides and the three catalytic sites empty, was prepared as described by Cunningham and Cross (1988), with the following modifications. F_1[2,1] was incubated at 20 °C at the concentration of 6 μM in 150 mM sucrose/10 mM K–Hepes, 1 mM KPi/1 mM $MgCl_2$, final pH 8.0 (SHP_1Mg buffer) in the presence of 8 mM MgATP for 2 min. The enzyme was then subjected to centrifugation–filtration through Sephadex G50 equilibrated in SHP_1Mg buffer. This was followed by incubation of F_1 with 4 mM MgGDP for 30 s and then by centrifugation–filtration on Sephadex G50 equilibrated in $SHP_{50}Mg$ buffer. $SHP_{50}Mg$ buffer differs from SHP_1Mg buffer by an increase in the P_i concentration to a value of 50 mM and a pH of 7.0. After 10 min of incubation, the nucleotides displaced by P_i were removed by filtration through a third column of Sephadex G50 equilibrated in $SHP_{50}Mg$ buffer. This treatment was repeated twice. F_1[3,0] was finally recovered after centrifugation through a column of Sephadex G50 equilibrated in STNMg buffer. It was verified that the bound nucleotides were released only after heat treatment. In six different preparations of F_1, the amount of bound nucleotides released upon heating was between 2.8 and 3.1 mol/mol of F_1, a result consistent with the conclusion that after this treatment F_1 contains three noncatalytic sites occupied by adenine nucleotides (Cunningham & Cross, 1988). F_1[2,0] which is referred as F_1 with three catalytic sites empty and only two out of the three noncatalytic sites filled with adenine nucleotides was prepared following the same procedure as that described for F_1[3,0] except that the initial incubation with 8 mM MgATP was omitted. The amount of bound ADP plus ATP released by heat treatment was somewhat more variable than that measured with F_1[3,0], ranging between 1.5 and 2.1 mol/mol of F_1.

Occupancy of two catalytic sites of F_1 with ADP–fluoroberyllate was achieved by incubation of 10 μM native F_1 at 20 °C for 20 min in STNMg-SO_4 buffer, pH 8.0 (similar to STNMg buffer, except that Cl^- was replaced by SO_4^-), supplemented with 100 μM [^{3}H]ADP or [^{14}C]ADP and 1 mM $MgCl_2$. Then, 100 μM $BeCl_2$ and 3 mM NaF were added to the mixture. The mixture was incubated until the ATPase activity was completely inhibited, and the reaction was terminated by filtering the sample through a Sephadex G50 column equilibrated in STNMg-SO_4 buffer. In the absence of $BeCl_2$, no inhibition was observed. Exchangeable [^{3}H]ADP or [^{14}C]ADP present on the enzyme, i.e., ADP which was not trapped in the catalytic sites in the form of ADP–fluoroberyllate, was displaced from its binding sites by incubation with 100 μM ATP for 15 min and removed by precipitation

Table I: Effect of PP_i on the Distribution of Nucleotides Bound to F_1 Fully Inhibited by Incubation with [^{3}H]ADP–Fluoroberyllate[a]

added [^{32}P]PP_i (μM)	total AdN[b] (mol/mol of F_1)	bound [^{3}H]ADP (mol/mol of F_1)	endogenous AdN[c] (av value; mol/mol of F_1)	bound [^{32}P]PP_i (mol/mol of F_1)	total bound ligands[d] (av value; mol/mol of F_1)
0	3.8–4.0	1.9–2.3	1.8		3.9
100	2.6–2.8	1.7–1.9	0.9	2.9	5.6

[a] F_1 (final concentration 3 μM) with two catalytic sites occupied with [^{3}H]ADP–fluoroberyllate was incubated for 15 min at 30 °C in the presence of [^{32}P]PP_i in STNMg buffer, pH 8. Assays of total adenine nucleotides and F_1-bound [^{3}H]GDP and [^{32}P]PPi were carried out after separation of free and bound ligands by centrifugation–filtration on Sephadex G50. The values given correspond to two different titration experiments. [b] Total nucleotides refer to adenine nucleotides which were titrated using the luciferine–luciferase test; e.g., both added[^{3}H]ADP and endogenous tightly bound nucleotides. [c] The amount of endogenous AdN is obtained by subtracting the amount of bound [^{3}H]ADP (column 2) from the amount of total adenine nucleotides (column 1). [d] Total bound ligands refer to the sum of endogenous adenine nucleotides (column 3), [^{3}H]ADP–fluoroberyllate (column 2), and [^{32}P]PP_i (column 4).

with 2.1 M $(NH_4)_2SO_4$. Fully inhibited F_1 contained two [^{3}H]ADP–fluoroberyllate locked into the catalytic sites (Garin, 1989; Issartel et al., 1991).

For binding studies, F_1[3,0] and ADP–fluoroberyllate-inhibited F_1 at a concentration of 3 μM were incubated with increasing concentrations of [^{14}C]ADP, [^{3}H]ADP, [^{3}H]GDP, or [^{32}P]PP_i in 0.1 mL of STNMg-Cl buffer final pH 8.0. After a 15-min incubation at 30 °C, the samples were subjected to centrifugation–filtration through a Sephadex G50 (fine grade) column to remove unbound ligands according to the method described by Penefsky (1977) and modified by Issartel et al. (1987).

Results

Binding Parameters of [^{32}P]PP_i to F_1[3,0] and F_1[2,0]. The two preparations of F_1 used, namely F_1[3,0] and F_1[2,0], incubated with [^{32}P]PP_i under saturation conditions were found to bind at least 3.3 mol of [^{32}P]PP_i and 3.1 mol of [^{32}P]PP_i, respectively, suggesting the presence of three PP_i binding sites for F_1 and possibly some unspecific PP_i binding. The Scatchard plots illustrated in the case of F_1[3,0] in Figure 1 were curvilinear, indicating either the occurrence of cooperative interactions between the three binding sites or the presence of three independent sites with different binding affinities. A graphical treatment of the Scatchard plots, using the method of Rosenthal (1967), yielded a K_d value of 0.9 μM for one site and a K_d of 8 μM for the other two sites, in the case of F_1[3,0]. In the case of F_1[2,0], a K_d value of 0.3 μM for one site and another one of 5 μM for the other two sites were determined. The amount of bound adenine nucleotide released when PP_i at a saturating concentration was added to F_1[3,0] was small (0.1–0.2 mol/mol of F_1) compared to the amount of adenine nucleotide released (1 mol/mol of F_1) when PP_i was added to F_1[2,1] (Issartel et al., 1987).

Effect of Occupancy of the Catalytic Sites of F_1 on the Binding Parameters of [^{32}P]PP_i. In the following PP_i-binding experiments, the catalytic sites of F_1 were filled either with fluoroberyllate in the presence of [^{3}H]ADP (Lunardi et al., 1988) or with [^{3}H]GDP (Cunningham & Cross 1988). In the first experiment, the ATPase activity of F_1 was fully inhibited by the tight binding of two [^{3}H]ADP–fluoroberyllate to catalytic sites. The resulting F_1, termed F_1[2,2], supplemented with a large excess of [^{3}H]GDP (100 μM), a specific ligand of mitochondrial F_1 catalytic sites (Cunningham & Cross, 1988), could not bind more than 0.2 mol of [^{3}H]GDP/mol of F_1. This F_1 preparation was titrated with [^{32}P]PP_i. The results are illustrated in the Scatchard plots of Figure 1. Upon extrapolation of the rectilinear plots on the ordinate, a maximal value of 3.2 mol of [^{32}P]PP_i bound/mol of F_1 was determined. This led us to conclude that ADP–fluoroberyllate-inhibited F_1[2,2] possesses three homologous PP_i-binding sites with a K_d of 0.5 μM. In other words, tight binding of ADP–fluoroberyllate to two catalytic sites of F_1 alters the binding properties of the three PP_i-binding sites of F_1 in such a way

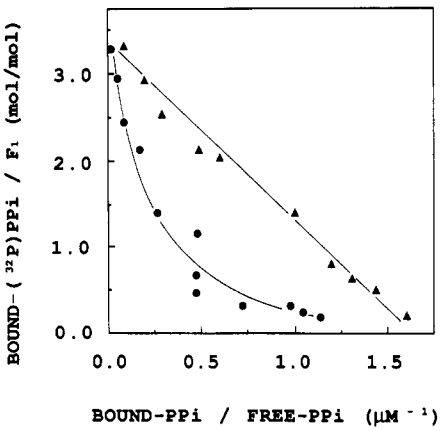

FIGURE 1: Scatchard plots of the [^{32}P]PP_i binding to mitochondrial F_1[3.0] and to mitochondrial F_1 fully inhibited with ADP–fluoroberyllate complexes. F_1[3.0] and a fully inhibited form of F_1[2,2] with ADP–fluoroberyllate bound to two catalytic sites were obtained as described in Materials and Methods. Mitochondrial F_1 was incubated at a final concentration of 3 μM with an increasing concentration of [^{32}P]PP_i up to 120 μM (F_1[3,0]) or 100 μM (F_1[2,2]) in STNMg buffer, pH 8.0. After a 15-min incubation at 30 °C, bound and free ligands were separated by centrifugation–filtration on Sephadex G50 columns and the radioactivity of the filtrates corresponding to F_1-bound [^{32}P]PP_i was measured. The curvilinear Scatchard plots illustrated in the figure were treated by the method of Rosenthal (1967) (see text): (●) F_1[3.0]; (▲) fully inhibited form of F_1.

that these sites now behave as homogeneous sites. The combined effect of PP_i and ADP–fluoroberyllate resulted in the release of 0.9 mol of endogeneous nucleotide/mol of F_1 (Table I, column 3). As noncatalytic sites of F_1 are involved in the tight binding of nucleotides, this suggests that the PP_i-binding sites interact with noncatalytic sites in F_1. On the other hand, the release of bound [^{3}H]ADP induced by addition of PP_i was more modest, amounting to 0.3 mol/mol of F_1. This is consistent with the observation that PP_i, unlike ADP or GDP, does not promote inhibition of F_1 by fluoroberyllate (Lunardi et al., 1988), indicating that PP_i does not completely mimic ADP or GDP at the catalytic sites.

In the second experiment, F_1[3,2G] with two bound [^{3}H]-GDP at exchangeable sites was used. F_1[3,0] was incubated with a saturating concentration of [^{3}H]GDP. Upon addition of [^{32}P]PP_i, [^{3}H]GDP was released (Figure 2). The amount of released [^{3}H]GDP was in proportion with the concentration of PP_i used, reaching a value of 0.9 mol/mol of F_1 for a saturating concentration of PP_i. A detailed balance sheet of bound ligands including [^{3}H]GDP, [^{32}P]PP_i, and endogenous nucleotides was obtained with two different preparations of F_1[3,0] incubated with 105 μM [^{3}H]GDP and 100 μM [^{32}P]PP_i (Table II). The total amount of bound [^{3}H]GDP (0.9 mol/mol of F_1) and [^{32}P]PP_i (2.0 mol/mol of F_1) when [^{3}H]GDP and [^{32}P]PP_i are added together was 2.9 mol/mol of F_1 (fourth row, column 3 of Table II), compared to a

Table II: Distribution of Bound [^{3}H]GDP and [^{32}P]PP_i and Endogenous AdN following Incubation of F_1 with [^{3}H]GDP and [^{32}P]PP_i[a]

added ligand	bound [^{3}H]GDP (mol/mol of F_1)	bound [^{32}P]PP_i (mol/mol of F_1)	bound [^{32}P]PP_i + [^{3}H]GDP (av value; mol/mol of F_1)	endogenous AdN (mol/mol of F_1)	total bound ligands[b] (av value; mol/mol of F_1)
no addition				2.8–3.1	3.0
[^{32}P]PP_i		2.5–2.7	2.6	2.7–2.9	5.4
[^{3}H]GDP	1.7–2.1		1.9	2.5–2.7	4.5
[^{32}P]PP_i + [^{3}H]GDP	0.9–1.0	1.9–2.1	2.9	2.1–2.3	5.1
difference between rows 4 and 1 (av value)	+0.9	+2.0	+2.9	−0.8	+2.1

[a] F_1[3,0] (final concentration 3 μM) was incubated for 15 min at 30 °C in STNMg buffer, pH 8, in the presence of either 100 μM [^{32}P]PP_i (row 2) or 105 μM [^{3}M]GDP (row 3) or both of them (row 4). A control in the absence of these ligands was carried out (row 1). Assays of bound [^{3}H]GDP, bound [^{32}P]PP_i, and bound adenine nucleotides were carried out on the filtrates recovered after centrifugation–filtration on Sephadex G50. The values given correspond to two different titration experiments. [b] Total bound ligands refer to the sum of endogenous adenine nucleotide (column 4) and bound [^{32}P]PP_i and [^{3}H]GDP (column 3).

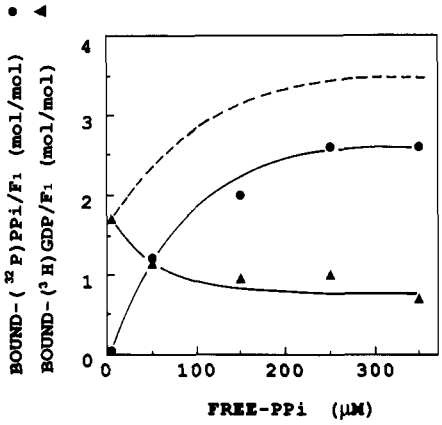

FIGURE 2: Competition between [^{32}P]PP_i and [^{3}H]GDP for binding to mitochondrial F_1[3.0]. F_1[3.0] was incubated at a final concentration of 3 μM for 15 min at 30 °C in STNMg buffer in the presence of 105 μM [^{3}H]GDP and increasing concentrations of [^{32}P]PP_i up to 350 μM. Bound and free ligands were separated by centrifugation–filtration on Sephadex G50 columns as described in Materials and Methods. The amount of bound [^{3}H]GDP in the absence of added [^{32}P]PP_i was 1.7 mol/mol. The radioactivity of the filtrates, corresponding to the bound ligands was measured: (●) [^{32}P]PP_i; (▲) [^{3}H]GDP; (---) total ligands ([^{32}P]PP_i + [^{3}H]GDP).

theoretical mean value of 4.5 mol/mol of F_1, corresponding to the sum of bound [^{3}H]GDP (1.9 mol/mol of F_1) and [^{32}P]PP_i (2.6 mol/mol of F_1) determined in separate [^{3}H]-GDP- and [^{32}P]PP_i-binding assays, as shown in row 3, column 1, and row 2, column 2, of Table II, respectively. This means that PP_i-binding decreases the binding capacity of catalytic sites for GDP. [^{32}P]PP_i (row 2) or [^{3}H]GDP (row 3) added separately to F_1 hardly affected the release of endogenous adenine nucleotides (column 4). In contrast, the simultaneous addition of [^{3}H]GDP and [^{32}P]PP_i (row 4) resulted in the release of about 0.8 mol of adenine nucleotide/mol of F_1 (column 4). Taken together, these results indicate that PP_i affects the release of nucleotides from both catalytic and noncatalytic sites. The synergistic effect of a nucleotide bound to a catalytic site (here GDP) and PP_i on the release of adenine nucleotides from noncatalytic sites of mitochondrial F_1 is noteworthy.

Discussion

The demonstration of the presence of saturable PP_i-binding sites on mitochondrial F_1-ATPase raises the question of the nature and the specificity of these sites. Kironde and Cross (1986) first reported that PP_i was able to empty the catalytic nucleotide-binding sites of bound nucleotides. Issartel et al. (1987) described a number of ADP-mimicking effects of PP_i: (a) PP_i competes with ADP, but not with P_i, for binding to F_1; (b) like ADP and in contrast to P_i, PP_i enhances the fluorescence of F_1-bound aurovertin, a fluorescent antibiotic ligand of the β subunit of F_1; (c) PP_i prevents the photolabeling of the catalytic site of the β subunit of F_1 with 2-azido-ADP. On the other hand, Kalashnikova et al. (1988) suggested that PP_i interacts with noncatalytic sites. However, these authors were unable to distinguish a possible interaction of PP_i with anion-binding sites from an interaction with regulatory noncatalytic sites.

In the present work, we have determined the parameters of PP_i binding to different mitochondrial F_1 preparations, each characterized by a well-defined nucleotide content. The results will be discussed on the basis of the following observations: (a) Although PP_i mimics ADP or GDP in some respects, it is unable to substitute for these nucleotides to form a stable fluoroberyllate complex. (b) Binding of ADP–fluoroberyllate to the catalytic sites of F_1 modifies the PP_i-binding parameters. The three heterogeneous binding sites present on F_1[2,1] are converted into three homogeneous binding sites in the presence of two ADP–fluoroberyllate bound to F_1. (c) PP_i competes with GDP for binding to F_1 under conditions in which GDP is assumed to interact essentially with the catalytic sites. Its effects extend to the noncatalytic sites, since PP_i binding leads to the release of a significant amount of endogenously bound nucleotides trapped in the noncatalytic sites. (d) The total number of species (nucleotides and PP_i) bound to the different F_1 preparations never exceeds a value of 6 mol of ligand/mol of F_1. As shown in Table II, F_1[3,0] incubated in presence of PP_i and GDP retained 5.5 mol of PP_i and AdN/mol of F_1. Likewise, F_1 fully inhibited by ADP–fluoroberyllate complexes and incubated in the presence of PP_i was able to bind 5.6 mol of AdN and PP_i/mol of enzyme.

Taken together, these data indicated that the specific binding sites of PP_i on F_1 differ from the nucleotide-binding sites, either catalytic or noncatalytic. In short, PP_i binds to non-nucleotide-binding sites different from anionic sites since PP_i does not compete with anions such as SO_3^- and SO_4^{2-} (data not shown), and it prevents nucleotide binding, whereas anions do not. Furthermore, the binding of PP_i to any available catalytic or noncatalytic nucleotide-binding site of F_1 does not explain why different forms of F_1 ([2,1], [0,0], [3,0], [2,0], and [2,2]) possess the same number of PP_i-binding sites, namely, three, despite the fact that six sites are available for nucleotide binding. The hypothesis that we favor is that PP_i binds to specific sites of F_1 that interact with both catalytic sites or noncatalytic nucleotide sites. The localization of these sites on the β subunit of F_1 would explain the effects of PP_i on the fluorescence of the F_1-bound aurovertin (Issartel et al., 1987). PP_i-binding sites are likely to interact with catalytic sites as illustrated by the experiment conducted in the presence of GDP for example. On the other hand, PP_i interacts with noncatalytic sites when catalytic sites are locked by ADP–fluoro-

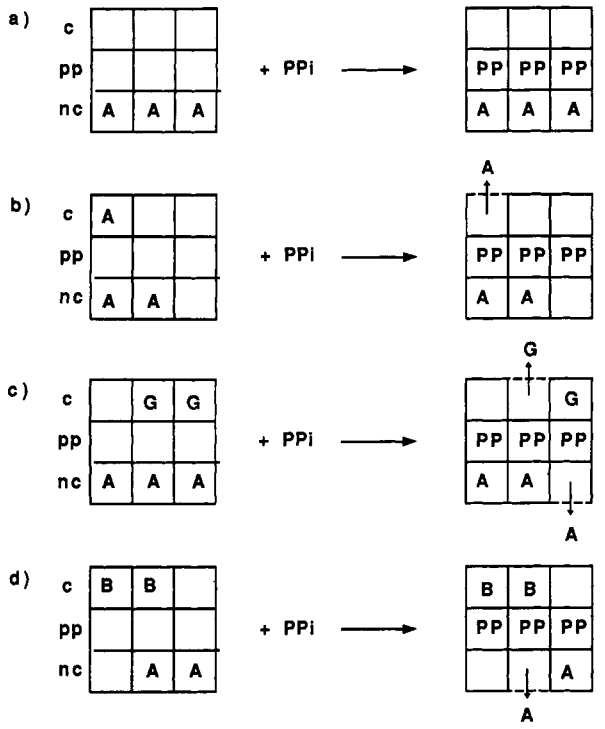

FIGURE 3: Scheme illustrating the occupancy of the nucleotide-binding sites of F_1 in the absence or presence of PPi. Abbreviations: A, adenine nucleotide; G, guanine nucleotide; PP, pyrophosphate; B, ADP–fluoroberyllate complexes; c and nc, catalytic sites and noncatalytic sites, respectively; pp, PP_i-binding sites. The left-hand side of the scheme illustrates the site occupancy in the absence of PP_i, and the right-hand side illustrates the site occupancy in the presence of PP_i. The arrows point to the released bound nucleotides upon PP_i binding to F_1. Panel a, site occupancy of F_1[3,0]; panel b, site occupancy of F_1[2,1] in the presence of added PP_i; panel c, site occupancy of F_1[3,0] with GDP loaded into catalytic sites; panel d, site occupancy of F_1 fully inhibited with two ADP–fluoroberyllate locked into two catalytic sites.

beryllate. The scheme of Figure 3 explains why no more than six ligands (ADP, GDP, ADP–BeF_x, and PP_i) could be detected on the different F_1 preparations. In fact, at any time, a pair of one α and one β subunit would have only two out of its three sites (two nucleotide sites and one PP_i site) filled with ligands. The physical mapping of the PP_i site would be of interest to determine whether the PP_i-binding sites of mitochondrial F_1 are structurally independent or if they share part of the nucleotide-binding sites and whether there is some similarity in the topological features of the PP_i-binding sites of mitochondrial F_1 and those of the membrane-bound pyrophosphatase of *Rh. rubrum* (Strid et al., 1986).

Registry No. ATPase, 9000-83-3; PP_i, 14000-31-8.

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