Lowered Temperature or Binding of Pyrophosphate to Sites for Noncatalytic Nucleotides Modulates the ATPase Activity of the Beef Heart Mitochondrial F_1-ATPase by Decreasing the Affinity of a Catalytic Site for Inhibitory MgADP†

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ABSTRACT: Lineweaver−Burk plots for ATP hydrolysis catalyzed by bovine heart mitochondrial F_1-ATPase (MF_1) at 30 °C are biphasic, whereas they are linear at 15 °C. The rate of inactivation of the enzyme at 23 °C by 5′-[(*p*-fluorosulfonyl)benzoyl]adenosine (FSBA), which derivatizes noncatalytic nucleotide binding sites, is about 4 times faster when loss of activity is monitored at 15 °C as opposed to 30 °C. This suggests that maximal loss of ATPase monitored at 15 °C is observed when a single noncatalytic site is derivatized, whereas maximal inactivation at 30 °C requires modification of three noncatalytic sites. Prior incubation of MF_1 depleted of endogenous nucleotides (nd-MF_1) with pyrophosphate (PP_i) stimulates ATPase activity 2-fold when assayed at 30 °C and pH 8.0. This stimulation correlates with binding of [^{32}P]PP_i to the second and third binding sites for PP_i to be filled. Prior binding of PP_i to nd-MF_1 increases the rate of inactivation of the enzyme by FSBA at 23 °C about 4-fold when loss of activity is monitored at 30 °C and pH 8.0, whereas it does not affect the rate of inactivation when loss of ATPase is monitored at 15 °C or loss of ITPase is monitored at 30 °C. This indicates that the accelerated rate of inactivation induced by PP_i when assays are conducted at 30 °C is not due to an increased rate of derivatization of noncatalytic sites. After 85% inactivation with FSBA, nd-MF_1 retains the capacity to bind 2.8 mol of [^{32}P]PP_i per mole. However, on modification of approximately one noncatalytic site with FSBA, the stimulatory effect induced by PP_i is lost. Prior incubation of nd-MF_1 with PP_i eliminates labeling of noncatalytic sites with 2-N_3-[^{3}H]ADP and decreases labeling of catalytic sites by about 35%. Moreover, the range of concentration of PP_i which prevents labeling of noncatalytic sites by 2-N_3-[8-^{3}H]ADP is similar to the range that stimulates ATPase activity. These results firmly support the contention that PP_i stimulates the ATPase activity of nd-MF_1 by binding to noncatalytic sites which in turn promotes dissociation of inhibitory MgADP from a catalytic site. The variations in the kinetic properties of ATP hydrolysis noted at 15 and 30 °C raise the possibility that the break in the Arrhenius plot at 18 °C for ATP hydrolysis originally described by Harris et al. [Harris, D. A., et al. (1981) *Biochim. Biophys. Acta 635,* 412−428] is caused by entrapment of inhibitory MgADP in a catalytic site of the enzyme during turnover at temperatures above 18 °C.

The ATP synthases of energy-transducing membranes are comprised of an integral membrane protein complex, F_o, which mediates proton translocation, and a peripheral protein complex, F_1, which, in its soluble form, catalyzes ATP hydrolysis (Hatefi, 1993). F_1-ATPases[1] are composed of five gene products in a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. The complexes have molecular weights of about 380 000 and contain six nucleotide binding sites per mole (Pedersen & Amzel, 1993). In the case of MF_1, three binding sites exchange nucleotides with medium readily and are, therefore, potentially catalytic for ATP hydrolysis (Cross, 1992). In contrast, the other three binding sites exchange bound nucleotides slowly. Since a defined physiological function has yet to be assigned to them, they are called noncatalytic sites. Nevertheless, the hydrolytic activity of F_1-ATPases *in vitro* depends on the nature of nucleotides bound to noncatalytic sites (Di Pietro et al., 1980; Milgrom et al., 1991; Jault & Allison, 1993, 1994a). Catalytic sites are mostly located on the β subunit. However, in the recently deduced crystal structure of MF_1, the side chain of an arginine residue arising from an adjacent α subunit forms part of the catalytic site (Abrahams et al., 1994). The major part of the noncatalytic binding sites is in a domain in the α subunit. However, a tyrosine residue which is contributed from an adjacent β subunit interacts with the adenine moiety of the ATP or ADP bound to noncatalytic sites (Cross et al., 1987; Allison et al., 1992; Abrahams et al., 1994). Consequently, irradiation of MF_1 after loading catalytic sites with 2-N_3-AD(T)P leads to derivatization of tyrosine-β345, whereas when irradiation follows loading of noncatalytic sites, tyrosine-β368 is labeled (Cross et al., 1987; Jault et al., 1994).

Selective modification of catalytic or noncatalytic sites is also achieved when the enzyme is inactivated with FSBI or FSBA, respectively. Maximal inactivation by FSBI is caused by derivatization of tyrosine-345 in a single copy of the β subunit of MF_1 (Bullough & Allison, 1986b). On the other

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[1] Abbreviations: F_1-ATPase, soluble moiety of the ATPase ATP synthase; MF_1, bovine heart mitochondrial F_1-ATPase; nd-MF_1, MF_1-ATPase depleted of nucleotides; HPLC, high-performance liquid chromatography; FSBA, 5′-[(*p*-fluorosulfonyl)benzoyl]adenosine; PP_i, pyrophosphate; DTT, dithiothreitol.

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hand, inactivation by FSBA is associated with derivatization of tyrosine-β368 or histidine-β427 in a mutually exclusive manner (Bullough & Allison, 1986a). However, the number of sites derivatized on maximal inactivation by FSBA depends on the activity measured. Maximal loss of ATPase activity at 30 °C requires derivatization of 3 mol of tyrosine-β368 plus histidine-β427 per mole of MF_1 whereas only 1 mol/mol must be derivatized when ITPase activity is monitored (Bullough & Allison, 1986a). It was thought earlier on the basis of kinetic experiments that noncatalytic sites are selective for adenine nucleotides (Schuster et al., 1975; Harris et al., 1978). Therefore, to explain the differential sensitivity of the ATPase and ITPase activities to modification of MF_1 by FSBA, it was postulated that binding of ATP to unmodified noncatalytic sites on partially derivatized MF_1 partially restores ATPase activity, whereas no restoration is possible during ITP hydrolysis. However, contradictory to this postulate, recent studies have shown that noncatalytic sites are not selective for adenine nucleotides (Milgrom et al.,1991; Milgrom & Cross, 1993). Therefore, a study was initiated to provide more information on the differential sensitivity of the ATPase and ITPase activities of MF_1 to inactivation by FSBA. In the course of these studies, it was found that the rate of inactivation of MF_1 at 23 °C was faster when ATPase activity was monitored at 15 °C than when monitored at 30 °C. It was also found that saturation of the noncatalytic sites with PP_i considerably accelerates the rate of inactivation of the enzyme by FSBA when the loss of ATPase activity is monitored at 30 °C. In both cases, most of the ATPase activity is lost when about one noncatalytic site is modified per mole of MF_1. These results are discussed in terms of factors contributing to apparent noncatalytic site to catalytic site cooperativity.

EXPERIMENTAL PROCEDURES

Unless stated otherwise, biochemicals were purchased from Sigma Chemical Co. Bicinchoninic acid was purchased from Pierce Chemical Co. MF_1 was prepared from bovine heart mitochondria with a previously described modification (Esch & Allison, 1978) of the method of Knowles and Penefsky (1972). The enzyme was depleted of nucleotides by passing it twice through a column of Sephadex G-50 which was equilibrated and eluted with 100 mM Tris–sulfate, pH 8.0, containing 4 mM EDTA and 50% glycerol (v/v) as described by Garrett and Penefsky (1975). The nd-MF_1 used in this study hydrolyzed 80–100 μmol of ATP per minute per milligram of protein and contained less than 0.2 mol of ATP and no ADP as determined by HPLC of samples denatured with perchloric acid (Bullough et al., 1988). ATPase and ITPase activities were determined spectrophotometrically at 30 or 15 °C, with an assay medium containing 50 mM Hepes/KOH, pH 8.0, 30 mM KCl, 4 mM phosphoenolpyruvate, 0.4 mM NADH, 21 μg/mL lactate dehydrogenase, 42 or 82 μg/mL pyruvate kinase, respectively, for ATPase or ITPase activity (both enzymes obtained from Boehringer Mannheim as solutions in 50% glycerol), and $MgCl_2$ in 1 mM excess over the concentration of nucleotide unless specified otherwise. Low concentrations of ATP are hydrolyzed in three distinct kinetic phases. An initial burst decelerates in about 15 s to a slow, intermediate phase, which in turn accelerates to a final rate that approaches the initial rate. Therefore, rate measurements were made between 20 and 40 s after initiation assays. At low ATP concentrations, this interval corresponds to the initial rate of the intermediate phase (Jault & Allison, 1993). ATP and ITP concentrations were determined by the absorbance at 259 nm (ϵ = 15 400 M^{-1} cm^{-1}) and 248 nm (ϵ = 12 300 M^{-1} cm^{-1}), respectively. Protein concentrations were determined by the bicinchoninic acid method described by Smith et al. (1985). [^{32}P]PP_i (21.5 Ci/mmol, Du Pont–New England Nuclear) was diluted with the nonradioactive reagents to the desired specific radioactivity.

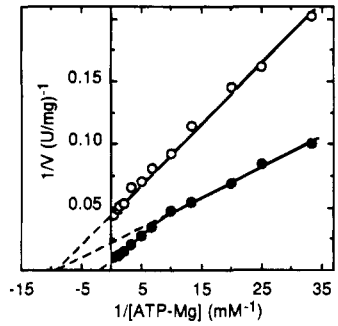

FIGURE 1: Lineweaver–Burk plots for ATP hydrolysis catalyzed by nd-MF_1 at 30 and 15 °C. A solution of 0.7 mg/mL nd-MF_1 in 90 mM Tris–sulfate, pH 8.0, containing 0.4 mM EDTA/10% (w/v) glycerol was incubated at 23 °C for 15 min. Samples of 2 μL were assayed either at 30 °C (○) or at 15 °C (●) at the concentrations of ATP specified.

Binding of [^{32}P]PP_i to nd-MF_1 was performed as described previously (Jault & Allison, 1993). Synthesis of 2-N_3-[8-^{3}H]ADP was performed as described previously (Jault & Allison, 1994b). The concentration of 2-N_3-[8-^{3}H]ADP was determined in 0.1 M HCl at 274 nm (ϵ = 15 200 M^{-1} cm^{-1}; Czarnecki, 1984). Radioactivity was detected with a Packard 1600 TR liquid scintillation counter using Ecoscint from National Diagnostics.

Irradiation, trypsin digestion, and separation of tryptic peptides by HPLC were carried out as described previously (Jault & Allison, 1994a). The C_4 reversed-phase column (Brownlee Labs, 220 × 4.6 mm) was connected to a guard column (Brownlee Labs, 30 × 4.6 mm) and developed with a gradient of CH_3CN in 0.1% HCl as previously described (Jault & Allison, 1994a).

To determine the stoichiometry of incorporation of [^{3}H]-SBA into βY368, during the inactivation of nd-MF_1 with [^{3}H]FSBA, samples were removed from inactivation mixture, quenched by dilution with 50 mM dithiothreitol, and then processed essentially as described by Bullough and Allison (1986a) except that the δ and ϵ subunits were removed by precipitating the α, β, and γ subunits by dialyzing the protein samples, after denaturation by addition of 6 M guanidinium chloride, against distilled water (Knowles & Penefsky, 1972). FSBA and [^{3}H]FSBA were synthesized as described elsewhere (Allison et al., 1986; Bullough & Allison, 1986b).

RESULTS

The Lineweaver–Burk plot in Figure 1 shows that the ATPase activity of nd-MF_1 measured at 15 °C does not display apparent negative cooperativity. Only a single K_m of 125 μM is extrapolated with an associated V_{max} of 280 s^{-1}. This is similar to the first K_m of 120 μM extrapolated when assays are conducted at 30 °C. The second apparent K_m of about 440 μM observed at 30 °C which is associated with slow binding of ATP to noncatalytic sites (Jault & Allison, 1993) is absent at 15 °C.

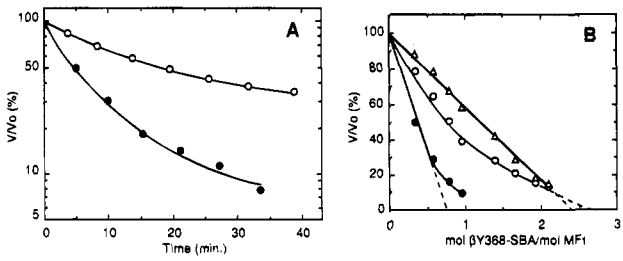

FIGURE 2: Sensitivity of ATPase activity of nd-MF_1 to inactivation by FSBA at 30 and 15 °C. (A) A solution of 0.63 mg/mL nd-MF_1 in 50 mM Tris−sulfate at pH 8.0, containing 0.4 mM EDTA and 11.2% (w/v) glycerol, was incubated at 23 °C for 15 min. FSBA was then added to a final concentration of 0.8 mM. Samples, 2 μL each, were withdrawn from the reaction mixture at the times indicated and assayed with 2 mM ATP at 30 (○) or 15 °C (●). (B) A 4 mL solution of 0.53 mg/mL nd-MF_1 in 50 mM Tris−sulfate at pH 8.0, containing 0.3 mM EDTA and 5% (w/v) glycerol, was incubated at 23 °C for 15 min. Then [^{3}H]FSBA was added to a final concentration of 0.36 mM. At 2, 5, 9, 15, 28, 40, 55, and 70 min, 475 μL fractions were removed from the reaction mixture and mixed with 25 μL of 1 M DTT. Samples, 2 μL each, were removed from the fractions for assays with 2 mM ATP at 30 °C. To determine the stoichiometry of incorporation of [^{3}H]FSBA, the remaining material was treated as described under Experimental Procedures. The loss of ATPase activity at 30 °C (○) was plotted against the moles of β-Y_{368} per mole of MF_1 derivatized by FSBA. For inactivations monitored at either 15 °C [(●) in panel A] or 30 °C with 50 μM ATP (not shown), the moles of β-Y_{368} modified by FSBA per mole of enzyme were calculated from the curve obtained for inactivation of enzyme monitored with 2 mM ATP at 30 °C. (△) loss of ATPase activity monitored with 50 μM ATP at 30 °C; (●) loss of ATPase activity monitored with 2 mM ATP at 15 °C.

The rate of inactivation of nd-MF_1 by FSBA at 23 °C differs when the loss of activity is monitored at 15 and 30 °C as shown in Figure 2A. Clearly, the apparent rate of inactivation is much faster when monitored at 15 °C. This indicates that the ATPase activity at 15 °C is influenced to a greater extent by derivatization of noncatalytic sites than activity measured at 30 °C. Figure 2B shows that this is indeed the case. Derivatization of a single noncatalytic site is sufficient to abolish most of the ATPase activity monitored at 15 °C, whereas greater than two noncatalytic sites must be modified to observe 85% inactivation when residual ATPase activity is assayed at 30 °C. However, when inactivation at 23 °C is monitored by hydrolysis of 2 mM ATP at 30 °C, the correlation of inactivation with the number of sites modified is nonlinear as shown by the open circles in Figure 2B. It was previously shown that MF_1 is less sensitive to inactivation by FSBA when loss of activity is monitored at low ATP concentration than when monitored at saturating ATP concentration [see Jault and Allison (1993); Figure 2A]. Therefore, it was of interest to correlate the extent of inactivation with the number of sites modified when the enzyme is inactivated with FSBA at 23 °C and the loss of activity is monitored by hydrolysis of 50 μM ATP at 30 °C. This analysis is also illustrated in Figure 2B. In this case, loss of ATPase activity is linearly correlated with derivatization of noncatalytic sites, and full inactivation extrapolates to the modification of about 2.4 noncatalytic sites.

It was previously reported that binding of PP_i to MF_1 accelerates inactivation of nd-MF_1 by FSBA (Jault & Allison, 1993) and inactivation of MF_1 by 8-N_3-FSBA in the dark (Zhuo et al., 1992). Figure 3B shows a semilogarithmic plot of the fractional ATPase activity remaining as a function of time when nd-MF_1 is treated with FSBA with and without

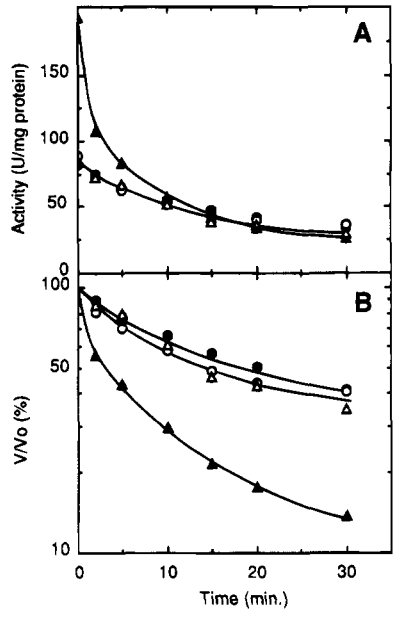

FIGURE 3: Effect of prior incubation with PP_i on inactivation of nd-MF_1 with FSBA. Two solutions of 750 μL containing 0.44 mg/mL nd-MF_1, 90 mM Tris−sulfate at pH 8.0, 0.2 mM EDTA, and 10% (w/v) glycerol were incubated at 23 °C for 15 min in the presence (▲, △) or absence (●, ○) of 2 mM PP_i plus 2.5 mM $MgCl_2$. FSBA was added to the solutions to a final concentration of 0.36 mM. At the times indicated in the figure, 100 μL of each reaction mixture was mixed with 2 μL of 0.5 M DTT. Then 2 μL samples of the quenched reaction mixtures were assayed for residual ATPase activity (▲, ●). The remainder of each quenched reaction mixture was then passed through a centrifuge column of Sephadex G-50 which was equilibrated with 50 mM Tris−sulfate at pH 8.0, containing 0.5 mM EDTA and 10% (w/v) glycerol. After incubating for 2 h at 23 °C, the ATPase activity of the gel-filtered samples was determined at 30 °C with 2 mM ATP (△, ○).

prior incubation of the enzyme with PP_i. The rate of inactivation is about 4 times faster after prior treatment with PP_i. However, when specific activity is plotted versus time of inactivation (Figure 3A), a different picture emerges. After an initial, rapid loss in the specific activity of MF_1 during the first 10 min, the rate of loss of activity parallels that of the control sample not treated with PP_i. This suggests that the primary effect of FSBA on the PP_i-treated enzyme is to abolish that part of the ATPase activity induced by binding PP_i. Therefore, the faster rate of inactivation of nd-MF_1 with PP_i bound to the enzyme might not be due to more rapid derivatization of the noncatalytic sites by FSBA. To test this hypothesis, MF_1 treated with PP_i was inactivated with FSBA at 23 °C, but in this case, bound PP_i was removed from partially inactivated samples prior to assay at 30 °C. This was accomplished by passing aliquots of inactivation mixtures through centrifuge columns of Sephadex G-50 (Penefsky, 1978) and then incubating the gel-filtered samples for 2 h to allow dissociation of bound PP_i before conducting assays. In a control experiment, it was shown that the specific activity of nonderivatized nd-MF_1 treated with PP_i was identical to nd-MF_1 which had not been treated with PP_i 2 h after gel-filtration. After dissociation of bound PP_i, the specific activity of each inactivated sample treated with PP_i was similar to the specific activity of inactivated samples not treated with PP_i (Figure 3A,B). This shows that increased sensitivity of the ATPase activity of nd-MF_1 treated with PP_i does not reflect increased reactivity of the enzyme with FSBA, but rather reflects rapid loss of the accelerated activity induced by the binding of PP_i when a noncatalytic

Table 1: Prior Incubation of nd-MF_1 with PP_i Stimulates Inactivation by FSBA When Monitored at 30 °C

	time of half-inactivation by FSBA (min)	
	MF_1	MF_1 treated with PP_i
ATPase activity, 30 °C	18	5
ATPase activity, 15 °C	5	4
ITPase activity, 30 °C	4	4

[a] Two solutions of 0.73 mg/mL nd-MF_1 in 95 mM Tris-sulfate at pH 8.0 containing 0.42 mM EDTA/10.5% (w/v) glycerol were incubated at 23 °C for 15 min, one in the presence and the other in the absence of 2 mM PP_i plus 2.5 mM Mg^{2+}. FSBA was then added to a final concentration of 0.39 mM. Samples of the reaction mixtures were removed as a function of time to monitor inactivation at 30 °C. The times of half-inactivation were determined from Guggenheim plots (1926).

Table 2: Partial Inactivation of nd-MF_1 by FSBA Prevents Stimulation of ATPase Activity by PP_i without Affecting Binding of [^{32}P]PP_i[a]

% inactivation by FSBA	mol of [^{32}P]PP_i/mol of MF_1	k_{cat} before PP_i treatment (s^{-1})	k_{cat} after PP_i treatment (s^{-1})	% stimulation (+) or % inhibition (−) by PP_i
0	2.85 ± 0.05	607	1380	+227
50		304	228	−25
85	2.85 ± 0.013	92		

[a] Two solutions of 1.75 mg/mL nd-MF_1 in 50 mM Tris−sulfate at pH 8.0 containing 0.8 mM EDTA and 12.6% (w/v) glycerol were incubated for 15 min at 23 °C. Then FSBA in Me_2SO was added to one to a final concentration of 1.42 mM, and an identical volume of Me_2SO was added to the other which was used as a control. Inactivation of the ATPase was monitored at 30 °C with 2 mM ATP. Samples were withdrawn from the reaction mixture at 50% and 85% inactivation which were quenched by addition of dithiothreitol to a final concentration of 10 mM. The quenched samples were passed through centrifuge columns of Sephadex G-50 as described in Figure 3. Aliquots of the eluants were either incubated for 15 min with 2 mM PP_i plus 2.5 mM $MgCl_2$ which was followed by determination of ATPase activity or incubated with 1 mM [^{32}P]PP_i plus 2.5 mM $MgCl_2$ for 15 min before determining the amount of bound [^{32}P]PP_i as described in Figure 4. The standard deviation was determined for three different samples.

site is modified with FSBA. The results shown in Table 1 are consistent with this conclusion. The rate of inactivation of ATPase activity of nd-MF_1 by FSBA at 23 °C is not affected by PP_i when loss of activity is monitored at 15 °C. Similarly, the rate of inactivation of ITPase activity by FSBA at 30 °C is not affected by PP_i when loss of activity is monitored at 30 °C. One interpretation of these results is that derivatization of the first noncatalytic site by FSBA abolishes binding of PP_i to the enzyme, thus preventing stimulation of ATPase activity. However, Table 2 shows that after inactivation of nd-MF_1 by 85% with FSBA, full capacity of the enzyme to bind [^{32}P]PP_i (2.8−2.9 mol/mol) is retained. In contrast, after about 50% inactivation, residual ATPase activity of partially inactivated nd-MF_1 is inhibited rather than stimulated by binding of PP_i as shown in Table 2. The inhibition observed might reflect binding of PP_i to that fraction of noncatalytic sites derivatized with FSBA.

The relationship between PP_i binding and stimulation of ATPase activity has been investigated in more detail. Figure 4A shows that maximal stimulation is reached after prior incubation of nd-MF_1 with approximately 500 μM PP_i for 10 min. About 3 mol of [^{32}P]PP_i/mol of MF_1 was retained when the samples were submitted to gel permeation chromatography as illustrated in Figure 4B. Correlation of binding of PP_i with activation of MF_1 shown in the inset of

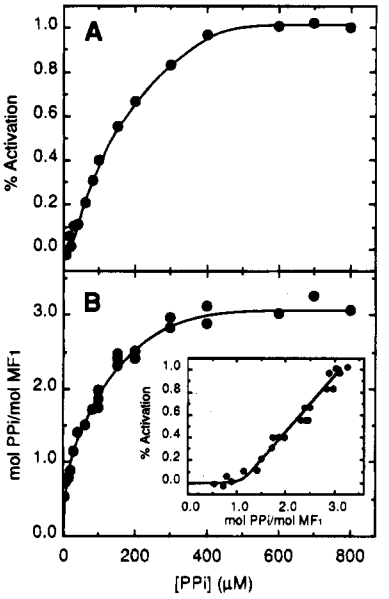

FIGURE 4: Correlation of binding of PP_i and activation of nd-MF_1. (A) Solutions of 0.53 mg/mL nd-MF_1 in 90 mM Tris−sulfate, pH 8.0, containing 0.24 mM EDTA/12.6% (w/v) glycerol, were incubated at 23 °C for 10 min in the presence of 2.5 mM $MgCl_2$ and the concentrations of PP_i specified in the figure. Then 2 μL samples were assayed for ATPase activity at 30 °C. Percent activation is with reference to the activity of an enzyme solution prepared in the absence of PP_i and $MgCl_2$. (B) The conditions were the same as in (A) except that the PP_i was replaced with [^{32}P]-PP_i. After the 10 min incubation, the samples were passed through centrifuge columns of Sephadex G-50 as described previously (Jault & Allison, 1993). The inset represents the percent activation determined in (A) versus the amount of bound PP_i determined in (B).

Figure 4 reveals that the first mole of PP_i bound does not participate in the activation process. Activation correlates with the binding of the second and third mole of PP_i per mole of MF_1. This analysis might indicate that activation is only observed for that fraction of MF_1 with three PP_i binding sites filled. In these activations, 2.5 mM Mg^{2+} was present when the enzyme was incubated with PP_i prior to assay, and therefore was present in excess of PP_i in most cases. Control experiments showed that prior incubation of nd-MF_1 with 2.5 mM Mg^{2+} for 10 min has no effect on the activity of the enzyme. Similar observations were reported by Drobinskaya et al. (1985). Furthermore, when nd-MF_1 was incubated with 2.0 mM PP_i and 1.5 mM Mg^{2+}, the enzyme was activated to the same extent as observed when it is incubated with 2.0 mM PP_i and 2.5 mM Mg^{2+}. Therefore, free Mg^{2+} does not contribute to the activation observed in the presence of PP_i and excess Mg^{2+}.

The effects of prior binding of PP_i on labeling of nucleotide binding sites of nd-MF_1 have been examined using 2-N_3-[^{3}H]ADP as a probe. Two major radioactive peptides are resolved when a tryptic digest of nd-MF_1 is submitted to reversed-phase HPLC after photoinactivation of the enzyme with 2-N_3-[^{3}H]ADP as shown in Figure 5A. The tryptic peptide eluting at about 61 min under these conditions contains derivatized tyrosine-β368 (Jault & Allison, 1994a). This residue is labeled when the photoaffinity label binds to noncatalytic sites (Cross et al., 1897). The asymmetric, radioactive peak eluting at about 80−85 min is contributed by tryptic peptides in which tyrosine-β345 (Garin et al., 1986; Cross et al., 1987) is derivatized to contain either an [8-^{3}H]-ADP or an [8-^{3}H]AMP moiety (Jault & Allison, 1994b).

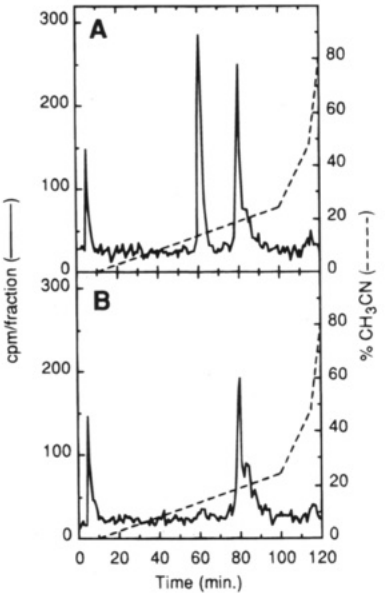

FIGURE 5: PP_i protects noncatalytic sites of nd-MF_1 from labeling with 2-N_3-[8-^{3}H]ADP. A solution of 1.05 mg/mL nd-MF_1 in 90 mM Tris−sulfate at pH 8.0, containing 0.48 mM EDTA/12.6% (w/v) glycerol, was incubated at 23 °C for 15 min in the presence (B) or absence (A) of 2 mM PP_i plus 2.5 mM $MgCl_2$. Then 2-N_3-[8-^{3}H]ADP was added to a final concentration of 250 μM. The incubation was then continued for an additional 15 min at which time the samples were irradiated and digested with trypsin as described under Experimental Procedures. Radioactive peptides in the tryptic digest were resolved on a C_4 reversed-phase column equilibrated with 0.1% HCl and eluted with the gradient of acetonitrile illustrated. The collected 1 mL fractions were submitted to liquid scintillation counting.

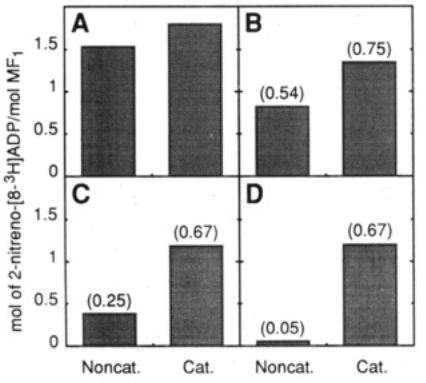

FIGURE 6: Effect of PP_i concentration on labeling of catalytic and noncatalytic sites with 2-N_3-[8-^{3}H]ADP. The experimental conditions were the same as in Figure 5 except that the concentration of 2-N_3-[8-^{3}H]ADP was 125 μM and the concentrations of PP_i were (A) 0, (B) 20 μM, (C) 100 μM, and (D) 1 mM. The numbers in parentheses represent the ratio of 2-N_3-[8-^{3}H]ADP incorporated into the tryptic peptide in the presence of PP_i to that incorporated in the absence of PP_i.

Tyrosine-β345 is present at catalytic sites. Figure 5B shows that binding of PP_i to nd-MF_1 completely prevents covalent modification of noncatalytic sites by 2-N_3-[^{3}H]ADP, whereas derivatization of catalytic sites is only moderately affected. When nd-MF_1 is irradiated in the presence of 2-N_3-[8-^{3}H]-ADP after prior incubation with increasing concentrations of PP_i, photolabeling of tyrosine-β368 decreases as shown in Figure 6. Concentrations of PP_i which abolish labeling of noncatalytic sites by 2-N_3-[^{3}H]ADP are similar to those that stimulate ATPase activity (Figure 4). Labeling of the catalytic site by 2-N_3-[^{3}H]ADP is also reduced by about 35% after preincubation with PP_i. Diminished labeling of catalytic sites might reflect direct competition of PP_i and the ADP analog for catalytic sites or lowered affinity of catalytic sites for the analog caused by a conformational change induced when PP_i binds to noncatalytic sites.

DISCUSSION

Modulation of the ATPase Activity of MF_1 by Temperature. It is clear from the results presented that the properties of MF_1 are quite different when ATPase activity is assayed at 30 °C opposed to 15 °C. Whereas pronounced negative cooperativity is exhibited when the enzyme hydrolyzes ATP at 30 °C, it is not observed at 15 °C. Similar behavior has been reported for the yeast mitochondrial F_1-ATPase by Tuena de Gómez-Puyou et al. (1978). They found that F_1 from *Saccharomyces cerevisiae* mitochondria does not display apparent negative cooperativity at 12 °C, but exhibits pronounced apparent negative cooperativity at 30 °C. The ATPase activity of MF_1 responds differently when assayed at the two temperatures as the enzyme is inactivated with FSBA at 23 °C. Maximal inactivation is observed on modification of a single noncatalytic site when inactivation is monitored at 15 °C. In contrast, maximal inactivation is observed when greater than two noncatalytic sites are modified when inactivation is monitored at 30 °C.

The properties of ATPase activity at 15 °C resemble those of ITPase activity at 30 °C which has the following characteristics: (a) apparent negative cooperativity is not exhibited (Schuster et al., 1975), and (b) maximal inactivation is observed when a single noncatalytic site is modified with FSBA (Bullough & Allison, 1986). What appears to distinguish ITP hydrolysis at 30 °C and ATP hydrolysis at 15 °C from ATP hydrolysis at 30 °C is lack of entrapment of inhibitory MgIDP or ADP in a catalytic site during hydrolysis of low concentrations of substrate. From several reports, it is clear that inhibitory MgADP is trapped in a catalytic site when MF_1 hydrolyzes 1−200 μM ATP (Vasilyeva et al., 1982; Drobinskaya et al., 1985; Milgrom & Boyer, 1990). Consequently, when low concentrations of ATP are assayed in the presence of a regenerating system, a burst of ATP hydrolysis is initially observed which rapidly decelerates to a slower rate. Slow binding of ATP to noncatalytic sites then leads to substantial recovery of the initial activity by promoting release of inhibitory MgADP from the affected catalytic site (Jault & Allison, 1993). In contrast, a linear rate is observed when MF_1 hydrolyzes low concentrations of ITP (Vasilyeva et al., 1982), suggesting that MgIDP is not trapped in a catalytic site during turnover. However, after saturating noncatalytic sites with ADP, nd-MF_1 is hysteretically inhibited during subsequent ITP hydrolysis (Jault & Allison, 1994). This suggests that inhibitory MgIDP is trapped in a catalytic site of MF_1 when noncatalytic sites are filled with ADP. It is possible that saturation of noncatalytic sites of MF_1 with ADP alters the conformation of the enzyme which allows entrapment of inhibitory MgIDP in a catalytic site during turnover. MgIDP appears also to be trapped in a catalytic site of the β-Q_{170}Y point mutant of the F_1-ATPase from *Schizosaccharomyces pombe*. Compared to the wild-type enzyme, the mutant yeast F_1-ATPase exhibits increased affinity for nucleoside diphosphates in a catalytic site (Jault et al., 1993). When the mutant enzyme hydrolyzes low concentrations of ITP, an initial burst of hydrolysis rapidly decelerates to an intermediate rate that slowly accelerates to a final rate (J.-M. Jault, unpublished experiments) reminiscent of hydrolysis of low concentrations

of ATP, but not ITP, by MF_1 (Jault & Allison, 1993). Slow recovery of activity probably accompanies slow binding of ITP to noncatalytic sites. This is consistent with the observations of Milgrom and Cross (1993), who have reported that GTP, but not GDP, binds to noncatalytic sites of MF_1. As will be discussed below, certain oxyanions stimulate the ATPase activity of MF_1 (Ebel & Lardy, 1976) by promoting dissociation of inhibitory MgADP from a catalytic site of MF_1. Consistent with the arguments presented above, oxyanions do not stimulate ITP hydrolysis by MF_1 but do stimulate ITP hydrolysis by the β-$Q_{170}Y$ mutant yeast enzyme (Jault et al., 1993).

The different catalytic properties observed for MF_1 at 15 °C as opposed to 30 °C suggest that inhibitory MgADP is not entrapped in a catalytic site during turnover at the lower temperature. This is consistent with the observation that bicarbonate only stimulates the ATPase activity of yeast F_1 enzyme above 17 °C (Jault et al., 1991) and dinitrophenol, which can be considered as an activating anion, only stimulates ATPase activity of the bovine heart enzyme at temperatures above 18 °C (Harris et al., 1981). The dependence of ATPase activity catalyzed by MF_1 on temperature was first examined in detailed by Harris et al. (1981). They found that the Arrhenius plot for ATP hydrolysis catalyzed by MF_1 shows a sharp break at 18 °C, indicating a change in the rate-determining step. They postulated that release of product ADP is rate-limiting above 18 °C, whereas a hydrolytic step is rate-limiting at lower temperatures. However, the steady-state parameters of MF_1 suggest that, under saturating conditions, ATP binding is rate-limiting at 30 °C. Given a k_{cat} of about 700 s^{-1} and a K_m of 120 μM for MF_1 under saturating conditions at 30 °C, k_{cat}/K_m is 5.8×10^6 M^{-1} s^{-1}. This value is equal to the second-order rate constant determined for ATP binding to MF_1 during unisite (Grubmeyer et al., 1982) and bisite (Cunningham & Cross, 1988) catalysis. The contention of Harris et al. (1981) that ADP release is rate-limiting was based on inhibition of MF_1 by ADP. Although the details of the kinetic experiments were not described, it seems likely that either ADP induced hysteretic inhibition, which leads to entrapment of MgADP in a catalytic site, or inhibition caused by direct binding of MgADP to a catalytic site was observed by Harris et al. (1981). Recently, Milgrom and Cross (1993) have provided evidence suggesting that binding of inhibitory MgADP to a single catalytic site of MF_1 abolishes activity at the two unmodified catalytic sites. Therefore, the biphasic Arrhenius plot for MF_1 may reflect partial enzyme inactivation caused by the entrapment of inhibitory MgADP in a catalytic site during turnover at temperatures greater than 18 °C.

Pyrophosphate Stimulates the ATPase Activity of nd-MF_1 by Binding to Noncatalytic Sites. Activating anions appear to prevent entrapment of inhibitory MgADP in a catalytic site during turnover. This is accomplished by including high concentrations of bicarbonate or certain other oxyanions in the assay medium (Ebel & Lardy, 1975), or by prior binding of PP_i to nd-MF_1 (Kalashnikova et al., 1988). We report here that the ATPase activity of nd-MF_1 is strongly stimulated by prior binding of PP_i when assayed at 30 °C but not at 15 °C. Approximately 3 mol of [^{32}P]PP_i/mol binds to nd-MF_1 at 23 °C which is not removed by a single pass through a centrifuge column of Sephadex G-50 (Jault & Allison, 1993). Kalashnikova et al. (1988) reported that PP_i promotes dissociation of inhibitory MgADP loaded into a catalytic site by prior incubation of nd-MF_1 with stoichiometric ADP in the presence of Mg^{2+}. They also observed that PP_i increases the affinity of native MF_1 for Pi, which presumably binds to catalytic sites. From these observations, they concluded that PP_i interacts with noncatalytic sites of nd-MF_1. The results presented here provide direct support for this contention. Saturation of three PP_i binding sites in nd-MF_1 blocks labeling of noncatalytic sites by 2-N_3-[^{3}H]-ADP. Therefore, it is likely that the three binding sites for [^{32}P]PP_i detected on nd-MF_1 in this and an earlier study (Jault & Allison, 1993), which are associated with stimulation of ATPase activity, are noncatalytic nucleotide binding sites. It is interesting that activation is associated with PP_i binding to the second and third noncatalytic sites to be filled and not with the filling of the first noncatalytic site. Activation of nd-MF_1 by prior incubation of the enzyme with MgATP in the presence of a regeneration system is also associated with the filling of the second and third noncatalytic sites (Jault & Allison, 1993).

Concentrations of PP_i that completely eliminate photolabeling of noncatalytic sites with 2-N_3-[^{3}H]ADP also decrease labeling of catalytic sites by about 35%. One possible explanation for this effect is that binding of PP_i to noncatalytic sites reduces the affinity of catalytic sites for 2-N_3-[^{3}H]ADP. Another explanation is that PP_i also binds to catalytic sites of nd-MF_1 but not with sufficient affinity to survive passage through a centrifuge column of Sephadex G-50. The latter explanation is consistent with the observation that a synthetic peptide corresponding to the catalytic nucleotide binding domain of the β subunit is precipitated from solution on addition of PP_i (Garboczi et al., 1988). However, Milgrom and Cross (1993) have reported findings that support the former explanation. They found that PP_i blocks binding of nucleotides to the empty noncatalytic site of MF_1 in which two noncatalytic sites and one catalytic site are filled with nucleotides, whereas it has no effect on the binding of nucleotides to the open catalytic sites. Considering their results with those presented here, it appears that two or three noncatalytic sites must be occupied with PP_i in order to promote dissociation of nucleotides from catalytic sites.

It is interesting that Issartel et al. (1987) reported that PP_i has no effect on the ATPase activity of MF_1 containing endogenous nucleotides at 30 °C. Similarly, Kalashnikova et al. (1988) reported that prior incubation of MF_1 containing an unspecified number of endogenous nucleotides stimulated ATPase activity by only 33% at 25 °C, whereas, as shown here, prior binding of PP_i to nd-MF_1 stimulates ATPase activity 2-fold at 30 °C. On the basis of the observation that MF_1 binds 3 mol of PP_i per mole, irrespective of the occupancy of catalytic sites or noncatalytic sites with nucleotides, Peinnequin et al. (1992) contend that PP_i binds to unique sites that interact with both catalytic and noncatalytic sites. However, binding of PP_i was not correlated with enzyme activity under the various conditions explored by Peinnequin et al. (1992). It is possible that when noncatalytic sites are partially or fully occupied with nucleotides, PP_i might bind to catalytic sites with sufficient affinity to be detected after passage of the enzyme through centrifuge columns of Sephadex G-50 after prior incubation with [^{32}P]-PP_i. From the results presented here, one would expect activation of ATPase activity by PP_i only when at least two noncatalytic sites are free of nucleotides. On the basis of the binding of [^{32}P]PP_i to MF_1 loaded variously with

nucleotides reported by Peinnequin et al. (1992) and the results of this study with nd-MF_1, it appears that occupancy of noncatalytic sites with nucleotides induces additional binding sites for PP_i that either are absent or are of low affinity in nd-MF_1. In support of this interpretation, Tuena de Gómez Puyou et al. (1993) have shown that MF_1 or nd-MF_1 catalyzes synthesis of low amounts of PP_i from Pi in the presence of Me_2SO. It is likely that PP_i synthesis occurs at a catalytic site of the enzyme.

Stimulation of ATPase activity by PP_i appears to be limited to mitochondrial F_1-ATPases when noncatalytic sites are unoccupied. In unpublished experiments, we have observed that TF_1, which is free of endogenous nucleotides as isolated, is not stimulated by PP_i. It should also be noted that the ATPase activity of MF_1 is stimulated to a greater extent when PP_i is bound to noncatalytic sites than when ATP is bound to these sites. Given recent reports that ATP hydrolysis in *Escherichia coli* F_1 is coupled to movement of the γ subunit (Aggeler et al., 1993; Turina & Capaldi, 1994), it is possible that the increased hydrolytic activity observed when PP_i is bound to noncatalytic sites might reflect partial uncoupling by relieving constraints on conformational changes induced by ATP hydrolysis in addition to PP_i promoting dissociation of inhibitory MgADP from a catalytic site.

The observation that PP_i stimulates the initial rate of inactivation of nd-MF_1 by FSBA at 23 °C when loss of activity is monitored at 30 °C with saturating substrate is reminiscent of the biphasic kinetics observed during inactivation of MF_1 under the same conditions (Di Pietro et al., 1979; Bullough & Allison, 1986). The two effects may be distantly related. The results presented here clearly show that the rapid, initial loss of activity observed when nd-MF_1 is treated with FSBA in the presence of PP_i reflects loss of the augmented ATP hydrolysis promoted by PP_i binding to noncatalytic sites. When MF_1 is inactivated with FSBA at 23 °C and loss of activity is monitored at 30 °C with saturating substrate, about 50% inactivation occurs at a fast rate and the remaining activity is lost at a slower rate. It is possible that the faster rate of inactivation is associated with modification of a single noncatalytic site which prevents dissociation of inhibitory MgADP from a catalytic site when ATP binds to the remaining noncatalytic sites. The slower rate of inactivation might then be associated with distortion of the conformation of the enzyme when the two remaining noncatalytic sites are modified that slows catalysis. The lack of equivalence of noncatalytic sites observed during inactivation of MF_1 with FSBA is consistent with the crystal structure of the enzyme recently deduced by Abrahams et al. (1994).

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