Synthesis of Pyrophosphate and ATP by Soluble Mitochondrial F_1†

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ABSTRACT: Soluble F_1 from beef heart that has been depleted of adenine nucleotides to values of 0.4 mol of ADP and 0.1 mol of ATP/mol of enzyme has the capacity to synthetize about 0.1 mol of ATP/mol of enzyme from medium phosphate in the presence of Mg^{2+} and 30% dimethyl sulfoxide. Under the same conditions, native and adenine nucleotide depleted F_1 can also synthesize pyrophosphate to values that range from 0.03 to 0.05 mol/mol of F_1. The formation of pyrophosphate requires Mg^{2+} and dimethyl sulfoxide. The formed pyrophosphate remains bound to F_1 during filtration through Sephadex centrifugation columns. In all water media, adenine nucleotide depleted, but not native, F_1 can hydrolyze pyrophosphate to values of about 0.2 nmol min^{-1} mg^{-1}. This activity is inhibited or stimulated by agents (adenylyl imidodiphosphate, aurovertin, and methanol) that produce such effects on the ATPase activity of F_1; NaN_3 stimulated the activity. Therefore, F_1 from bovine heart mitochondria has the capacity to catalyze synthesis and hydrolysis of ATP. Synthesis of pyrophosphate by the soluble F_1 appears to follow the same energetic considerations that have been postulated for ATP synthesis by the soluble enzyme [de Meis (1989) *Biochim. Biophys. Acta 973*, 339–349].

The F_1F_o synthase of mitochondria catalyzes the synthesis of ATP using the energy of electrochemical H^+ gradients derived from electron transport. The F_1 portion has six adenine nucleotide binding sites (Wagenwoord et al., 1980; Cross & Nalin, 1982; Lunardi & Vignais, 1982; Issartel & Vignais, 1984; Issartel et al., 1986) and the catalytic machinery for synthesis and hydrolysis of ATP. F_1 can be obtained in soluble form, and in this state it actively hydrolyzes ATP (Penesfky et al., 1960). However, when soluble F_1 is incubated in mixtures that contain Me_2SO,[1] phosphate, and Mg^{2+}, the soluble enzyme can catalyze synthesis of ATP which remains tightly bound to the enzyme (Sakamoto & Tonomura, 1983; Yoshida, 1983; Sakamoto, 1984a,b; Gómez-Puyou et al., 1986; Kandpal et al., 1987; Beharry & Bragg, 1991a,b 1992). To account for the latter observations, it has been proposed that Me_2SO increases the partition of inorganic phosphate into a hydrophobic catalytic site where spontaneous synthesis of ATP takes place (de Meis, 1989; Gómez-Puyou et al., 1986; Al-Shawi & Senior, 1992).

F_1 in the presence of Me_2SO can bind adenine nucleotides, although with characteristics different from those observed in all aqueous media (Beharry & Bragg, 1991a,b, 1992). In conditions for ATP synthesis, these newly bound adenine nucleotides do not yield ATP; instead, the species that is phosphorylated is an ADP that previously existed in the enzyme (Beharry & Bragg, 1991a). These findings agree with observations that showed that, in the presence of Me_2SO, ADP that endogeneously existed in the native enzyme could be phosphorylated (Gómez-Puyou et al., 1986; Kandpal et al., 1987). Therefore, synthesis of ATP occurs at the expense of an ADP that does not easily exchange with medium nucleotides. Mitochondrial F_1 can be largely depleted of adenine nucleotides to values of less than 1 adenine nucleotide/enzyme (Garret & Penesfky, 1975); hence we explored whether, after depletion, the adenine nucleotides that remain in the enzyme become phosphorylated by medium phosphate in the presence of Me_2SO.

However, a principal aim in this work was to study whether F_1 depleted of adenine nucleotides could catalyze the formation of PP_i. The possibility derived from observations that indicate that PP_i can release adenine nucleotides from catalytic sites in F_1 (Kironde & Cross, 1986). Along the same line, Peinnequin et al., (1992) showed that F_1 can bind PP_i to values as high as three per enzyme; earlier, this group (Issartel et al., 1987; Michel et al., 1989) described that PP_i could interact with the same amino acids that interact with the α and β phosphate groups of ADP. Moreover, Baltscheffsky et al., (1987) suggested that there may be structural and functional similarities between inorganic pyrophosphatases and H^+-ATP synthases. In addition, Garboczi et al., (1988) synthetized a peptide with the sequence β 141–190 that is part of the nucleotide binding site of F_1 and interacts with ATP, ADP, and PP_i.

Regarding the energetics of PP_i synthesis by soluble F_1, it is relevant that the catalytic site of F_1 that accounts for ATP synthesis is hydrophobic (de Meis, 1989; Al-Shawit & Senior, 1992) and that the equilibrium constant of PP_i hydrolysis is drastically affected by the solvation of substrates and products (de Meis, 1984). Thus as Me_2SO favors the partition of phosphate into the catalytic site (de Meis, 1989, Gómez-Puyou et al., 1986; Al-Shawi & Senior, 1992), a priori in the presence of the cosolvent there would be no energetic barriers for synthesis of PP_i. Therefore the question could in fact be whether F_1 has the catalytic capacity to carry out such a reaction. We made the first experiments with F_1 depleted of adenine nucleotides; these revealed that this enzyme could indeed synthetize PP_i. Subsequent studies showed that native F_1 also exhibited this property.

MATERIALS AND METHODS

Soluble F_1 was prepared from bovine heart mitochondria as described elsewhere (Tuena de Gómez-Puyou & Gómez-Puyou, 1977); this enzyme was stored as an ammonia sulfate

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[1] Abbreviations: AMPPNP, adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetate; Me_2SO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PP_i, pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetate.

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suspension at 4 °C. F_1 was depleted of adenine nucleotides following the procedure of Garrett and Penesfky (1977); the depleted enzyme was kept at 4 °C in 50% glycerol, 100 mM Tris-HCl, pH 8.0, and 4 mM EDTA. The specific ATPase activity of several preparations of native and adenine nucleotide depleted enzymes ranged between 80 and 100 μmol min^{-1} mg^{-1}.

The content of adenine nucleotides was measured in native F_1 and the adenine nucleotide depleted enzymes according to Issartel et al. (1986). Native F_1 was precipitated two times with 1.5 volumes of saturated ammonia sulfate. The precipitate was dissolved at a concentration of 5 mg/mL of a solution that contained 250 mM sucrose, 50 mM Tris–acetate, pH 7.4, and 1.5 mM $MgCl_2$. Subsequently, four volumes of 1 mM EGTA, pH 9.0, were added and the suspension was placed in a boiling water bath for 4 min. The content of nucleotides in the supernatant was measured by HPLC by two methods. In one, nucleotides were separated with a Radial-Pak cartridge column obtained from Waters (SAX P 308201) whose dimensions were 8 mm × 10 cm; elution was carried out at a flow rate of 1.5 mL/min with a linear gradient that went from 0.007 M KH_2PO_4 and 0.007 M KCl, pH 4.0, to 0.25 M KH_2PO_4 and 0.25 M KCl, pH 8.0. In a second method, nucleotides were separated with a reverse-phase μBondapak TM/C_{18} 3.9-mm × 300-mm steel column (Waters) at a flow rate of 1 mL/min with 6.0 mM tetrabutylammonium, 100 mM KH_2PO_4, pH 6.8, and 15% methanol. The content of adenine nucleotides was calculated with external standards of ADP and ATP.

Synthesis and Quantitation of Pyrophosphate and ATP Labeled with [^{32}P]Phosphate. The uptake of [^{32}P]phosphate was measured in a standard reaction mixture that contained 50 mM MOPS–KOH, pH 7.0, 1 or 2 mM [^{32}P]phosphate (2×10^5 cpm/nmol), 10 mM $MgCl_2$, and 30% Me_2SO (unless otherwise stated); the concentration of F_1 was 1 mg/mL of reaction mixture. The volume of the reaction mixture was 0.2 mL, except in experiments in which samples (0.2 mL) from a common reaction mixture were withdrawn at different times. As F_1 depleted of adenine nucleotides was kept in a glycerol mixture, when this enzyme was used, the reaction mixture for [^{32}P]phosphate uptake also contained 6.5–10% glycerol. For the calculation of the molar concentration of enzymes a molecular mass of 357 000 was used. The incubations were carried out at 30 °C in a shaking water bath. For the assay of [^{32}P]phosphate uptake into ATP and PP_i, two procedures were followed:

(1) The reaction was arrested with 1.5 N HCl; this was followed by the addition of 0.5 mM nonradioactive ATP and PP_i. After elimination of protein by centrifugation, 0.5 mL of 3.3% ammonium molybdate in 3.7 N H_2SO_4 was added: the mixture was extracted seven times with water-saturated 2-butanol–benzene (1:1). In each extraction, 20 μL of 20 mM phosphate was included. The radioactivity of the last water phase was measured in a scintillation counter. This was considered as the total amount of [^{32}P]phosphate taken up. Blanks without enzyme were run in each experimental condition. The measured radioactivity in the blanks ranged between 500 and 1000 cpm, whereas in conditions in which [^{32}P]phosphate was taken up to the extent of about 0.1 mol/mol of F_1, the measured radioactivity was in the range of 10 000 cpm. The difference between the blanks and experimental samples was used for the calculation of total phosphate uptake.

(2) In another procedure, at desired incubation times, several 100-μL aliquots of the reaction mixture were filtered by centrifugation in Sephadex G-50 columns equilibrated with 30% Me_2SO and 40 mM MOPS–KOH, pH 7.0. The filtrates were received in 1.5 N HCl. Several (four to five) identical filtrates were pooled. To 0.5 mL of the filtrates, 0.225 mL of 60 mM ammonium molybdate in 10 mM HCl was added, followed by the addition of 0.5 mM nonradioactive ATP and PP_i. Inorganic phosphate was extracted with 2-butanol–benzene as described above. As filtration through the Sephadex columns removed most of the radioactive inorganic phosphate, three extractions with 2-butanol–benzene sufficed to remove radioactive phosphate. For the experiments shown, in each of the extractions, 20 μL of 100 mM phosphate was added; the extractions were repeated (generally three times) until the water extract was free of ammonium molybdate. Afterward the mixtures were taken to pH 7.0–7.5 and divided into two equal portions; to one of the portions, yeast inorganic pyrophosphatase (Sigma I-4503 lyophilized powder) was added, and the mixture was incubated for 30 min. At this time, 0.5 mL of 3.3% ammonium molybdate in 3.7 N H_2SO_4 was added and the mixture was extracted three times with 2-butanol–benzene. The portion that did not receive yeast inorganic pyrophosphatase was treated in the same form. Radioactivity in the two samples was determined. The difference in radioactivity between the two samples was considered to correspond to PP_i formed by F_1. It is noted that in this procedure the amount of [^{32}P]phosphate taken up corresponds to that which remained bound to the enzyme after filtration in the Sephadex columns.

Hydrolysis of Pyrophosphate. Pyrophosphatase activity of F_1 was measured by incubating the native or adenine nucleotide depleted enzyme in a reaction medium that contained 100 mM sucrose, 100 mM MOPS–KOH, pH 6.5, 1.5 mM $MgCl_2$, and the indicated concentrations of [^{32}P]PP_i (5000 cpm/nmol). The reaction was arrested with 6.0% trichloroacetic acid that contained 1 mM phosphate. [^{32}P]Phosphate produced was determined after its extraction as a phosphomolybdate complex into 2-butanol–benzene. In some experiments inorganic phosphate was also determined by a colorimetric method (Sumner, 1944).

ATPase activity was determined in the presence of pyruvate kinase and lactate dehydrogenase by following the absorbance changes of NADH at 340 nm as described elsewhere (Pullman et al., 1960).

Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

RESULTS

Uptake of [^{32}P]Phosphate by Adenine Nucleotide Depleted F_1. Following the procedure of Garrett and Penefsky (1975), F_1 was depleted of adenine nucleotides to values of around 0.4 mol of ADP and less than 0.1 mol of ATP/mol of enzyme. Similarly to native F_1 (Sakamoto & Tonomura 1983; Gómez-Puyou et al., 1986; Kendpal et al., 1987; Beharry & Bragg, 1991b), the adenine nucleotide depleted enzyme catalyzed the uptake of [^{32}P]phosphate through a process that depended on the concentration of Me_2SO (Figure 1). Uptake was clearly observed with 30% and 40% Me_2SO; with 50% Me_2SO, phosphate uptake decreased markedly (not shown).

F_1 depleted of adenine nucleotides is highly labile (Garret & Penefsky, 1975); therefore, in order to estimate whether enzyme inactivation took place during incubation in mixtures for [^{32}P]phosphate uptake, aliquots were withdrawn at different times, and the ability of F_1 to hydrolyze ATP was assayed in all water media. In mixtures that contained 40% and 50% Me_2SO, adenine nucleotide depleted F_1 was almost completely inactivated in a 90-min incubation time. With 30% Me_2SO, inactivation of about 20% took place in 3 h of

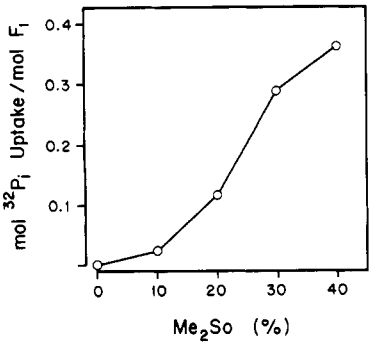

FIGURE 1: Uptake of [^{32}P]phosphate by adenine nucleotide depleted F_1 at various concentrations of Me_2SO. The standard conditions for measurement of [^{32}P]phosphate uptake were used (see Materials and Methods), except that the concentration of Me_2SO was varied as indicated. After 90 min the reaction was arrested with HCl and [^{32}P]orthophosphate was extracted. After extraction radioactivity that remained was used to calculate phosphate uptake.

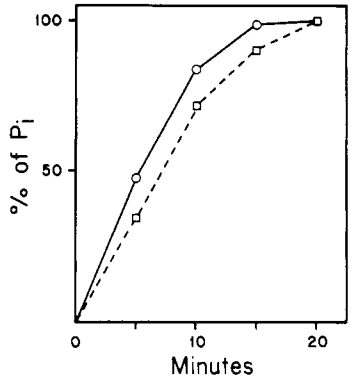

FIGURE 2: Identification of pyrophosphate as a product of phosphate uptake by F_1 depleted of adenine nucleotides. In the experiments the standard mixture for [^{32}P]phosphate uptake was used; the concentration of Me_2SO was 30%. After extraction of orthophosphate and molybdate, PP_i was precipitated under alkaline conditions in the presence of 1.0 M $MgCl_2$ (de Meis, 1985). The precipitate was dissolved in HCl, neutralized, and supplemented with a mixture of 3.0 mM nonradioactive PP_i, 0.5 mM $MgCl_2$, and 40 mM MOPS, pH 7.4. Hydrolysis of PP_i was started by the addition of yeast inorganic pyrophosphatase. At the indicated times the reaction was arrested with 6.0% trichloroacetic acid. Total orthophosphate (circles) was determined colorimetrically; [^{32}P]orthophosphate (squares) was determined from the radioactivity of phosphomolybdate complexes extracted into 2-butanol–benzene.

incubation. Therefore, all experiments on phosphate uptake by F_1 were carried out in 30% Me_2SO.

In more than 30 experiments with four different preparations of adenine nucleotide depleted F_1 incubated in 30% Me_2SO, the enzymes catalyzed the uptake of [^{32}P]phosphate to values that ranged between 0.15 and 0.3 mol/mol of F_1. However, we had a preparation that had 0.4 mol of ADP/mol of enzyme, no detectable ATP, and an ATPase activity of about 100 μmol min^{-1} (mg of protein)$^{-1}$. This preparation lost most of its ATPase activity in less than 5 min of incubation in the standard mixture for [^{32}P]phosphate uptake (30% Me_2SO). This enzyme catalyzed uptake of [^{32}P]phosphate, but to total values of about 0.05 mol/mol of F_1 into both ATP and PP_i (see below).

Identification of ^{32}P-Labeled Components Formed by Soluble F_1-ATPase. The following experiments were designed to ascertain if the uptake of medium [^{32}P]phosphate by native and adenine nucleotide depleted F_1 occurred solely into ATP or whether it also gave rise to PP_i. Radioactive PP_i was identified as product of a reaction catalyzed by F_1 from [^{32}P]-phosphate with the use of yeast inorganic pyrophosphatase (See Materials and Methods).

Table I: Identification of [^{32}P]Phosphate-Labeled Components Formed by F_1[a]

	mol of [^{32}P]-X/ mol of F_1
total	0.12
+ pyrophosphatase	0.04
+ F_1	0.07

[a] Adenine nucleotide depleted F_1 was incubated in the standard incubation for [^{32}P]phosphate uptake for 60 min. The reaction was arrested with 1.5 N HCl, and this was followed by the addition of nonradioactive ATP and PP_i and centrifugation. [^{32}P]Phosphate was extracted and excess molybdate was removed as described in Materials and Methods. Subsequently the extract was neutralized and divided into three equal portions. Where shown, yeast inorganic pyrophosphatase (1 unit) and F_1 (100 μg) were added. After 30 min the reaction was arrested with 3.3% ammonium molybdate in 3.7 N H_2SO_4 and extracted with 2-butanol–benzene (three times). The radioactivity in the last water phase was determined and the amount of [^{32}P]-labeled ATP and PP_i hydrolyzed by the indicated enzymes was calculated.

In the experiment of Figure 2, the [^{32}P]-labeled components formed by adenine nucleotide depleted F_1 that precipitated with added nonradioactive PP_i under alkaline conditions were incubated with yeast inorganic pyrophosphatase. The formation of orthophosphate (assayed colorimetrically) and [^{32}P]-orthophosphate (measured by radioactivity extracted as phosphomolybdate into 2–butanol–benzene) were measured as a function of time. It may be observed (Figure 2) that the time curves of orthophosphate production as measured by either of the two methods were almost superimposable. It is noted that no hydrolysis of ATP was detected in presence of inorganic pyrophosphatase [in accordance with Issartel et al. (1987)]. Therefore, these findings indicate that PP_i could be formed by F_1. In addition it was observed that pyrophosphatase-insensitive radioactivity yielded [^{32}P]orthophosphate when the extracts were incubated in all water media with soluble F_1 (Table I). Thus the enzyme catalyzes the synthesis of both ATP and PP_i.

Nelson and Racker (1973) described that incubation of ATP with inorganic phosphate in the presence of Me_2SO without enzymes led to formation of PP_i. Although no phosphate uptake took place in the absence of enzymes (blanks in all experiments), it was necessary to ascertain if [^{32}P]PP_i formation took place via a noncatalytic phosphorolytic reaction in which [^{32}P]orthophosphate split either endogenous enzyme-bound ATP or [γ-^{32}P]ATP that had been formed in F_1. Hence, the whole protocol for incorporation of [^{32}P]phosphate was carried out in the absence of the enzyme but in the presence of nonradioactive ATP. No uptake of [^{32}P]phosphate took place. If phosphorolysis had occurred, [^{32}P]phosphate uptake and formation of [^{32}P]PP_i should have been detected.

In the experiments that follow, [^{32}P]phosphate uptake sensitive to inorganic pyrophosphatase was considered to correspond to the amount of [^{32}P]PP_i synthetized. The difference between the latter value and total [^{32}P]phosphate taken up was equal to the amount of ATP formed.

Characteristics and Location of [^{32}P]Pyrophosphate Formed by Native and Adenine Nucleotide Depleted F_1. The experiments of Figure 1 and Table I indicated that adenine nucleotide depleted F_1 could form ATP and PP_i. Hence, in a time course experiment, it was explored if native F_1 formed PP_i. It was found that synthesis of [^{32}P]phosphate, PP_i, and ATP ran almost in parallel (Figure 3). In 62 experiments with native and adenine nucleotide depleted F_1 incubated in 30% Me_2SO, pyrophosphatase-sensitive radioactivity varied between 30% and 60%. As two phosphates yield one PP_i, PP_i synthesis was always lower than that of ATP.

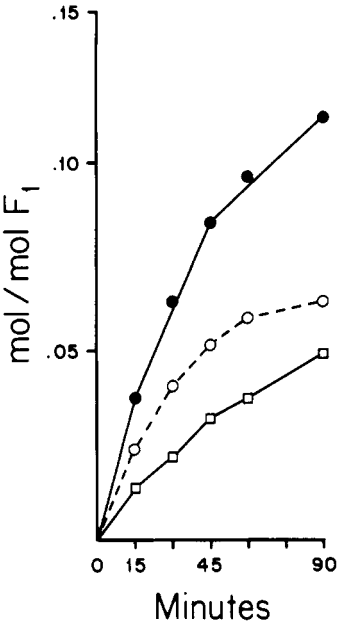

FIGURE 3: Phosphate uptake into pyrophosphate and ATP by native F_1. The incubating conditions were as in Figure 1 with 30% Me_2SO. At the indicated times, five 100-μL aliquots of the reaction mixture were filtered through centrifuge Sephadex columns; the filtrates were received in HCl, pooled, and supplemented with nonradioactive PP_i and ATP (0.5 mM each). After extraction of the filtered orthosphosphate and removal of molybdate (see Materials and Methods), the extracts were neutralized and divided into two portions. To one, 1 unit of yeast inorganic pyrophosphatase was added; after 30 min, 0.5 mL of ammonium molybdate in H_2SO_4 was added to the two mixtures, and these were extracted three times with 2-butanol–benzene. Radioactivity was determined in the last water phase. From the difference in radioactivity of the two samples, the amount of PP_i and ATP formed was calculated. (●) Total uptake; (○) uptake into ATP; (□) uptake into PP_i.

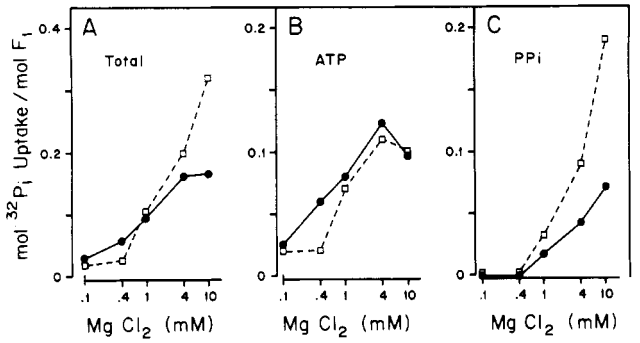

FIGURE 4: Effect of $MgCl_2$ on ATP and pyrophosphate formation by native and adenine nucleotide depleted F_1. The experimental conditions were as in Figure 1 except that the mixtures contained 30% Me_2SO and the indicated concentrations of $MgCl_2$. Incubation time was 1 h. PP_i and ATP were determined by the standard procedure with inorganic pyrophosphatase. The uptake of total phosphate and uptake into ATP and PP_i is shown in panels A, B, and C, respectively. (●) and (□) indicate data with native and adenine nucleotide depleted F_1.

In the experiments of Figure 3, it is pointed out that radioactive ATP and PP_i were determined in filtrates obtained from Sephadex centrifuged columns. The amount of protein recovered in the filtrate varied from 60% to 80%. In these experiments, it is noted that although more than 99% of the radioactive phosphate was retained in the column, the eluate contained radioactive PP_i and ATP. The uptake of phosphate into PP_i in the eluate ranged from 0.04 to 0.14 mol/mol of F_1, whereas that of ATP was 0.04–0.12 mol/mol of F_1. The findings indicate that, similarly to ATP (Gómez-Puyou et al., 1986), the PP_i synthetized was enzyme-bound.

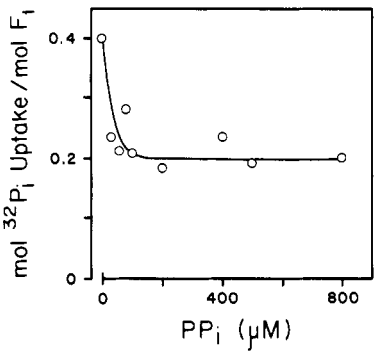

FIGURE 5: Effect of pyrophosphate on phosphate uptake by F_1 depleted of adenine nucleotide. The protocol was as in Figure 1 with 30% Me_2SO.

The uptake of phosphate by native F_1 depends on the concentration of Mg^{2+} (Gómez-Puyou et al., 1986) with a maximum at 4–10 mM. Figure 4 shows the pattern of ATP and PP_i formation at various concentrations of Mg^{2+} by native and adenine nucleotide depleted F_1. With the latter, phosphate uptake was not saturated at 10 mM Mg^{2+} (Figure 4A); higher concentrations were not assayed due to precipitation. At all Mg^{2+} concentrations, the amount of ATP formed was of a similar extent with the native and the adenine nucleotide depleted enzyme (Figure 4B). Regarding PP_i formation, it was found that in both preparations PP_i synthesis was hardly detectable at Mg^{2+} concentrations below 0.4 mM, but as Mg^{2+} was gradually raised, PP_i formation increased sharply (Figure 4C). The Mg^{2+} effect was more important in the depleted enzyme (Figure 4C).

The inclusion of PP_i in the reaction mixtures inhibited [^{32}P]-phosphate uptake (Figure 5). With 500 μM PP_i, the diminution in uptake occurred mainly at the expense of PP_i; PP_i and ATP formation were inhibited by about 80% and 60%, respectively. The detrimental effect of added PP_i on both ATP and PP_i synthesis could be related to the inhibiting effect of PP_i on phosphate binding by F_1 (Issartel et al., 1987). However, for the case of ATP formation, it is possible that added PP_i released some of the enzyme-bound ADP, thus decreasing synthesis of ATP.

Hydrolysis of Pyrophosphate by Soluble F_1. Issartel et al. (1987), using a colorimetric method for assay of inorganic phosphate, found that F_1 did not hydrolyze PP_i. In confirmation, we found that in all water media in a wide range of Mg^{2+} concentrations, native F_1 hardly hydrolyzed [^{32}P]PP_i (Figure 1). On the other hand, with F_1 depleted of adenine nucleotides, a low but significant Mg^{2+}-dependent PP_i hydrolysis was observed (Figure 6). In various experiments carried out in all water mixtures, the adenine nucleotide depleted F_1 hydrolyzed about 0.2–0.3 nmol of PP_i min^{-1} mg^{-1} in a Mg^{2+}-dependent process (Figure 6A); active hydrolysis could be observed for at least 2 h (Figure 6B). In the presence of 30% Me_2SO, no hydrolysis of [^{32}P]PP_i was detected with either the native or the adenine nucleotide depleted enzyme.

The effect of various agents that affect ATP hydrolysis by F_1 and PP_i hydrolysis by inorganic pyrophosphatase was assayed on PP_i hydrolysis by adenine nucleotide depleted F_1 (Table II). AMPPNP (Penefsky, 1974) inhibited the activity by about 50%. NaF, an inhibitor of inorganic pyrophosphatase (Baykov et al., 1979), had no effect. Surprisingly, NaN_3, an inhibitor of ATP hydrolysis (Ebel & Lardy, 1975; Moyle & Mitchell, 1975; Vasilyeva et al., 1982; Muratalieve et al., 1991) brought about an enhancement of activity. Methanol at concentrations lower than those that stimulate ATP hydrolysis in F_1 (Penefsky et al., 1965) brought about an increase in activity. The highly specific F_1 inhibitor aurovertin

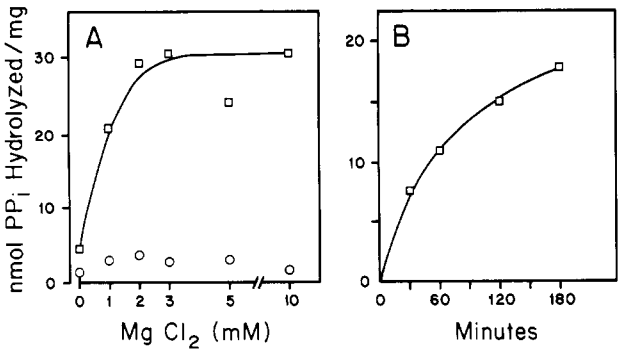

FIGURE 6: Pyrophosphate hydrolysis by native (○) and adenine nucleotide depleted (□) F_1. (A) Native (400 μg) and adenine nucleotide depleted F_1 (40 μg) were incubated in 100 mM MOPS–KOH, pH 6.5, the indicated concentrations of $MgCl_2$, and 500 μM [^{32}P]PP_i for 120 min. (B) F_1 depleted of adenine nucleotides (40 μg) was incubated for the indicated times in the aforementioned reaction mixture, except that the concentration of $MgCl_2$ was 0.9 mM.

Table II: Effect of Inhibitors on Pyrophosphate Hydrolysis by F_1 Depleted of Adenine Nucleotides[a]

expt	additions	nmol of [^{32}P]pyrophosphate hydrolyzed/mg of F_1
1	none	21
	200 μM AMPPNP	10
2	none	20
	5 mM NaF	23
	200 μM NaN_3	38
3	none	15
	2.0% methanol	25
	20 μM aurovertin in 2.0% methanol	7

[a] The incubating medium was detailed in Materials and Methods. The concentration of [^{32}P]PP_i was 500 μM. The reaction was initiated by the addition of 50 or 100 μg of adenine nucleotide depleted F_1 in 10 μL of 50% glycerol medium. The reactions were allowed to proceed for 120 min (experiments 1 and 2) or 60 min (experiment 3) at 30 °C. The reaction was arrested with 1 mL of 6.0% trichloroacetic acid that contained 1 mM phosphate. The suspension was extracted with 2-butanol–benzene, and radioactivity of the organic phase was determined. The indicated agents were assayed in different experiments as indicated.

(Lardy et al., 1964; Chang & Penefsky, 1973) dissolved in methanol produced substantial but partial inhibition.

DISCUSSION

Pyrophosphatase activity has been observed in mitochondria and in submitochondrial particles (Mansurova et al., 1977; Kondrashin et al., 1980; Irie et al., 1970). Also, it has been reported that the equilibrium constant of PP_i hydrolysis is drastically changed by cosolvents (such as Me_2SO) that perturb the organization of water molecules (de Meis, 1989); indeed, in Me_2SO, inorganic pyrophosphatase can catalyze net synthesis of medium PP_i from inorganic phosphate (de Meis, 1984). Therefore, the possibility that the described synthesis and hydrolysis of pyrophosphate took place by a contaminating pyrophosphatase was considered; however, the following observations indicate that this is not the case:

(1) Adenine nucleotide depleted F_1 was obtained by filtration of native F_1 through a Sephadex column as described by Penefsky (1977). PP_i synthesis was of a similar extent with both types of F_1, but PP_i hydrolysis was much higher by the adenine nucleotide depleted enzyme. These observations would not be consistent with the existence of a contaminating pyrophosphatase. Rather, the data suggest that PP_i hydrolysis is affected by the content of adenine nucleotides in F_1.

(2) PP_i hydrolysis by F_1 depleted of adenine nucleotide was inhibited or stimulated by agents that produce such effects on the ATPase activity of F_1 (AMPPNP, aurovertin, and methanol).

(3) On average, the amount of PP_i and protein that filtered from Sephadex centrifuge columns was in a ratio of 0.05 mol of PP_i/mol of F_1. On a molar basis, if this PP_i was bound to all pyrophosphatases, the amount of the contaminating pyrophosphatase in F_1 would have been about 5%. This amount of pyrophosphatase should have yielded important PP_i hydrolysis, i.e., the activity of soluble inorganic pyrophosphatase is around 500 μmol min^{-1} mg^{-1}, whereas that of purified membrane pyrophosphatase is around 30 μmol min^{-1} mg^{-1} (Volk et al., 1982). Neither we nor Issartel et al. (1987) observed significant hydrolysis of PP_i by native F_1, although we did detect hydrolysis by the adenine nucleotide depleted enzyme, but on the order of 0.3 nmol min^{-1} (mg of depleted F_1)$^{-1}$. Also, it is noted that solubilization of membrane-bound pyrophosphatases requires drastic detergent treatments (Efremovich et al., 1980; Nyren et al., 1991), whereas our F_1 preparations were obtained by sonication of submitochondrial particles.

If soluble F_1 from heart mitochondria has the capacity to synthetize PP_i the question arises as to why we and other authors (Sakamoto & Tonomura, 1983; Sakamoto, 1984a,b; Kandpal et al., 1987; Beharry & Bragg, 1991a,b, 1992; Gómez-Puyou et al., 1986) failed to observe synthesis of PP_i when this F_1 was examined for synthesis of ATP in the presence of Me_2SO. Sakamoto and Tonomura (1983) determined synthesis of ATP by measuring the formation of ATP from radioactive ADP previously bound to the enzyme; with this methodology, it would not have been possible to detect PP_i. However, the latter authors, as well as Kendpal et al. (1987) and Beharry and Bragg (1991a), used [^{32}P]phosphate uptake to estimate ATP synthesis. Sakamoto and Tonomura (1983) and Kendpal et al. (1978), after arrest of the reaction, passed the supernatant through a charcoal column and subsequently eluted radioactivity; the latter was considered to correspond to the synthetized ATP. Thus, it is possible that PP_i that may have been formed would not have adsorbed to the charcoal. In fact, Crane and Lipmann (1953) reported that PP_i does not adsorb to charcoal. In the protocols of Beharry and Bragg (1991a), after arrest of the reaction, the extracts were applied and run in poly(ethylenimine)–cellulose thin-layer plates; apparently only the radioactivity of the spot that corresponded to ATP as identified by its UV absorption and R_f was determined. Again, with this methodology the existence of PP_i would have been overlooked. However, perhaps the principal reason for not detecting PP_i synthesis was that the researchers did not explore the possibility.

In our previous experiments (Gómez-Puyou et al., 1986), we used chromatography in poly(ethylenimine) columns (Magnusson et al., 1976) to separate [γ-^{32}P] ATP formed from [^{32}]phosphate. A key step in the identification of the radioactive components was the use of yeast hexokinase to determine if indeed the label was in γ phosphate of ATP. In those studies we observed that after treatment with hexokinase all the radioactivity in the ATP region moved to the region in which inorganic phosphate (and glucose 6-phosphate) eluted from the column. In light of the present data, we reexamined our protocols and found that yeast hexokinase (Sigma Type IV) brought about hydrolysis of PP_i. This was on the order of 25 nmol of phosphate formed mg^{-1} min^{-1} with 3.0 mM pyrophosphate, 3.0 mM $MgCl_2$, and 20 mM Tris-HCl, pH 7.4. This is an activity that easily could have hydrolyzed PP_i that most likely was present in our previous experiments. In fact, we have now seen that treatment of the extracts with hexokinase releases radioactivity from the ATP region in poly-

(ethylenimine) columns, even though the samples applied contained pyrophosphatase-sensitive radioactivity.

Hence, the overall findings indicate that for our case the previously reported amount of ATP formed by F_1 was overestimated. For the case of other authors, adsorption of the extracts to charcoal and chromatography in thin-layer plates produced a more specific assay for ATP formed, but uptake of [^{32}P]phosphate into PP_i could have been overlooked.

The finding that soluble mitochondrial F_1 has the capacity to synthesize and hydrolyze PP_i does not necessarily imply that PP_i transformations by the mitochondrial F_oF_1 complex are of physiological significance. However, the findings do indicate that pyrophosphate may be added to the list of molecules (such as GTP and ITP) that are transformed at the catalytic site(s) of F_1. In addition, the present results indicate that in F_1 at least one of the binding sites for PP_i has catalytic properties. This would agree with previous conclusions (Kironde & Cross, 1986; Issartel et al., 1987).

The present results also show that ATP is formed at the expense of the ADP that remains in F_1 after adenine nucleotide depletion. Thus, this ADP binding site has catalytic properties. This site would correspond to that detected by Beharry and Bragg (1991a), who showed that after loading F_1 with adenine nucleotides only endogenous bound ADP was phosphorylated by medium phosphate.

Regarding the mechanisms for pyrophosphate synthesis by F_1, it is relevant that its formation requires Me_2SO and that the synthesized PP_i remains in the enzyme after filtration. This suggests that the same energetic considerations proposed for synthesis of a tightly bound ATP [see reviews by de Meis (1989) and Al-Shawi and Senior (1992)] also apply to synthesize of PP_i by mitochondrial F_1. It is also of interest that the energy-transducing membranes of *Rhodospirillum rubrum* possess a membrane-bound pyrophosphatase that forms PP_i with the energy of electrochemical H^+ gradients (Baltscheffsky et al., 1966). Moreover, Baltscheffsky et al. (1987) suggested that there may be functional similarities between this pyrophosphatase and the α and β subunits of ATP synthases; the present findings on the catalytic synthesis and hydrolysis of PP_i by soluble F_1 lend support to this idea.

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