J Bioenerg Biomembr (2008) 40:561–568
DOI 10.1007/s10863-008-9192-4

Catalysis by isolated β-subunits of the ATP Synthase/ATPase from Thermophilic bacillus PS3. Hydrolysis of Pyrophosphate

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Received: 10 December 2008 / Accepted: 12 December 2008 / Published online: 13 January 2009

Abstract Although the capacity of isolated β-subunits of the ATP synthase/ATPase to perform catalysis has been extensively studied, the results have not conclusively shown that the subunits are catalytically active. Since soluble F_1 of mitochondrial H^+-ATPase can bind inorganic pyrophosphate (PP_i) and synthesize PP_i from medium phosphate, we examined if purified His-tagged β-subunits from Thermophilic bacillus PS3 can hydrolyze PP_i. The difference spectra in the near UV CD of β-subunits with and without PP_i show that PP_i binds to the subunits. Other studies show that β-subunits hydrolyze [^{32}P] PP_i through a Mg^{2+}-dependent process with an optimal pH of 8.3. Free Mg^{2+} is required for maximal hydrolytic rates. The Km for PP_i is 75 μM and the Vmax is 800 pmol/min/mg. ATP is a weak inhibitor of the reaction, it diminishes the Vmax and increases the Km for PP_i. Thus, isolated β-subunits are catalytically competent with PP_i as substrate; apparently, the assembly of β-subunits into the ATPase complex changes substrate specificity, and leads to an increase in catalytic rates.

Keywords ATP synthase · Pyrophosphate · Pyrophosphatase activity · Beta subunits of the ATP synthase · Soluble F_1 of the ATP synthase

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Introduction

The transport of electrons through photosynthetic or respiratory chains generates electrochemical proton gradients that can be used for the formation of high energy phosphate bonds of ATP through the catalytic action of ATP synthases. The action of the enzyme is fully reversible, since it can hydrolyze ATP and generate electrochemical proton gradients. The ATP synthases are multisubunit complexes formed by two principal components, Fo and F_1. Fo channels H^+ to the F_1 moiety which has the catalytic machinery for ATP synthesis and hydrolysis. F_1 may be isolated as a soluble protein; it is formed by five different subunits α_3, β_3, γ, δ, and ε in order of decreasing molecular weight. F_1 has three catalytic sites that are at the interface of α and β subunits, albeit the vast majority of the residues that form the active site are on the β-subunit (Abrahams et al. 1994; Stock et al. 1999).

Studies on the isolated subunits of F_1 have been made in order to gain insight into their particular contributions to ATP synthesis and hydrolysis. For example, it has been extensively studied if the isolated β-subunits from several sources have the capacity to catalyze hydrolysis of ATP. The results are somewhat controversial (Avital and Gromet-

Elhanan 1991; Frasch et al. 1989; Futai 1977; Hsu et al. 1984; Kagawa et al. 1989; Markan et al. 1990; Harris et al. 1985; Miwa and Yoshida 1989; Philosoph et al. 1977; Richter et al. 1986; Roux-Fromy et al. 1987; Yoshida et al. 1977; Kasamo et al. 1989) in the sense that some authors have observed low rates of ATP hydrolysis, whereas other groups have failed to detect catalytic function of the isolated subunits. Along this line, it has been reported that the β-subunit of thermophilic bacillus PS3 can bind adenine nucleotides, and that their binding induces conformational changes (Pérez-Hernández et al. 2002; Yagi et al. 2004). Collectively, the data suggest that β-subunits can bind ATP, but lack the capacity to perform catalytic turnover. In regard to the catalytic properties of F_1, it is relevant that the enzyme from bovine heart can synthesize PP_i from medium P_i in the presence of dimethyl sulfoxide (Tuena de Gómez-Puyou et al. 1993), and that PP_i induces the release of adenine nucleotides from F_1 (Issartel et al. 1987). In the latter work, it was also reported that PP_i binds to the residues of F_1 that interact with the α and β phosphates of ADP. It is also noted that Zancani et al (2003) described a soluble protein of the matrix of pea stem mitochondria that exhibits high pyrophosphatase activity and that according to its amino acid sequence corresponds to the β subunit of the ATP synthase. However as discussed below, there are structural differences between the latter protein and β subunits from other sources. Therefore, to explore if β subunits have catalytic properties, we examined if the isolated recombinant β subunits of F_1 from thermophilic bacillus PS3 have the capacity to hydrolyze PP_i. The results show that indeed, isolated β-subunits can bind and hydrolyze PP_i.

Material and methods

Preparation of the β-subunits The vector pUC-β, derived from pUC18 and encoding the protein sequence of the thermophilic β subunit was kindly donated by Dr. Masasuke Yoshida (Tokyo Institute of Technology, Japan). This vector was modified in order to introduce a six-residue Histag sequence at the N-terminus using a two stage PCR procedure based on the QuickChange site directed mutagenesis method (Novagen) as described by Wang and Malcolm, 2002. The plasmids were transformed into *Escherichia coli* β-minus strain DK8. Cells grown in LB media containing ampicillin were induced for 3 h with 1 mM isopropyl beta-D-1-thiogalactopyronoside at an OD at 600 nm of 0.6. The pellet of cells from a 3 L culture was suspended in 20 ml of buffer A (50 mM sodium phosphate buffer pH 8.0, 300 mM NaCl and 10 mM imidazole). Cells were lysed by sonication in the presence of complete protease inhibitor cocktail (Roche Diagnostics) and centrifuged at 20,000 g for 20 min. The resulting supernatant was loaded into a column that contained 10 ml of NiNTA agarose resin (Qiagen). The protein was eluted with a linear gradient of buffer A plus 500 mM imidazole and precipitated with ammonium sulfate at 70% saturation. Hydrophobic interaction chromatography was then carried out (Butyl-Toyopearl resin) in the presence of ammonium sulfate. Finally, the protein was loaded into a gel filtration column (Superdex 200 10/300 GL) in the presence of buffer containing 50 mM Tris/HCl pH 7.4 and 250 mM NaCl. The fractions that contained β-subunits were concentrated by centrifugation/filtration through Centricon filters. The solution of concentrated protein was centrifuged at 12,000 rpm for 20 min in order to eliminate any aggregates that may have been formed. The resulting β-subunits were more than 99% pure and homogenous as judged by denaturing and native gel electrophoresis. Aliquots that contained β-subunits were stored at 4°C for not more than 2 weeks.

Gel electrophoresis The preparations of β-subunits were analyzed in denaturing SDS gels formed with 8% acrylamide (Laemmli 1970), as well as in native gels with 12% acrylamide. Both types of gels were stained with either Coomassie blue or silver (Bollag et al. 1996; Hevkeshoven and Dernick 1985). Pyrophosphatase activity of the protein in the native gels was assessed by incubating the non-stained gel with a mixture of 50 mM Tris-HCl, 3 mM PP_i and 5 mM $MgCl_2$ for 30 min. at 37°C. After washing, the gel was soaked in 50 ml of a solution that contained 5% ammonium molybdate, 0.5 M H_2SO_4 and 0.75 g ascorbic acid. After incubation for 5 or 30 min at 45°, the gel was washed with water. Pyrophosphatase activity was visualized as a blue band.

Pyrophosphate hydrolysis Unless otherwise indicated all experiments were made at 46°C. The basic medium for the assay of PP_i hydrolysis was 40 mM MOPS-Tris, 2 mM KH_2PO_4, 10 mM $MgCl_2$, 250 μM [^{32}P] PP_i (approximately 5000 cpm/nmol), and 50 μg of the β-subunit in a final volume of 100 μL. Except when PP_i hydrolysis was measured as function of pH the reaction was carried out at pH 8.3. Variations of the incubation media are detailed in the"Results" section. The reaction was arrested with 6% trichloroacetic acid (final concentration). The mixture was supplemented with 0.7 ml of 3.3% ammonium molybdate in 3.7 N H_2SO_4 and 0.2 ml acetone. After stirring and mild centrifugation, 0.5 ml of the organic phase was applied to filter papers and radioactivity in the filter papers was determined by liquid scintillation. In all experiments blanks were included; these comprised identical mixtures in which pyrophosphatase activity was determined, except for the presence of the enzyme.

Circular dichroism The CD spectra of the β-subunit were recorded in Jasco J-750 spectropolarimeter. For the recording of far UV CD spectra, the concentration of protein was 0.45 mg per ml. For the near UV CD spectra at 25°C, β-subunit was at a concentration of 1.9 mg/ml of buffer that contained 40 mM tricine (pH 8) in a cell with a light path of 1 cm; the spectra were also recorded in presence of 1 mM PP_i and 2 mM $MgCl_2$. The CD at 222 nm of β-subunits as a function of temperature was also recorded; in this case the temperature of the cell with a light path of 0.1 cm was raised at a rate of 1°C per minute from 25° to 85°C.

Mass spectrometry analysis. Tandem Mass Spectrometry (LC/ESI-MS/MS) The protein bands were excised from a Coomassie stained SDS gel, distained, reduced, carbamidomethylated, washed, digested with modified porcine trypsin, and extracted as previously described (Xolalpa et al. 2007). After concentration, the samples were analyzed by mass spectrometric on a 3,200 Q TRAP hybrid tandem mass spectrometer (Applied Biosystems/MDS Sciex), equipped with a nano electrospray ion source and a MicroIonSpray II head. Protein identification was performed from the MS/MS spectra datasets using MASCOT (Version 1.6b9, Matrix Science, UK available at http://www.matrixscience.com). Searches were conducted using the Bacteria subset of the National Center for Biotechnology Information non-redundant database (NCBInr, http://www.ncbi.nih.gov). Trypsin was used as specific protease; one missed cleavage was allowed with an MS/MS tolerance of 0.5 Da. A protein "hit" was accepted as valid when at least one matched MS/MS spectrum had a confidence level of 95% ($p<0.05$).

Results

The ß-subunit of thermophilic Bacillus PS3 hydrolyzes pyrophosphate In the evaluation of the PPi hydrolytic activity by the β-subunit, it is necessary to ascertain if the activity is indeed carried out by the β-subunit or by a contaminant pyrophosphatase. In this regard, we think it is appropriate to describe our attempts to purify β-subunits that lacked the His-tag. After rupture of the cells, the supernatant that contained β-subunit (as evidenced by SDS gels) were passed successively through the following columns: DEA-Macroprep (Biorad), Mono-Q HR 10/5 (Pharmacia Biotech), and Superdex S75 by FPLC. When the samples from the last column were analyzed in non-denaturing gels, we observed that it contained two proteins that were clearly distinguishable by their different migration (Fig. 1a, lane 1). Surprisingly when pyrophosphatase activity was assessed in the native gels, it was found that the two bands exhibited activity (Fig. 1a). To identify the proteins, the two bands were excised from the gels and analyzed by MS/MS spectrometry (See Methods). The protein with the faster mobility was found to correspond to subunits β of the ATP synthase of bacillus PS3, *Geobacillus kautophilus* and other related bacteria. The MASCOT score was 1,432 with 32 matching peptides that covered 61% of the sequence. The protein had a nominal

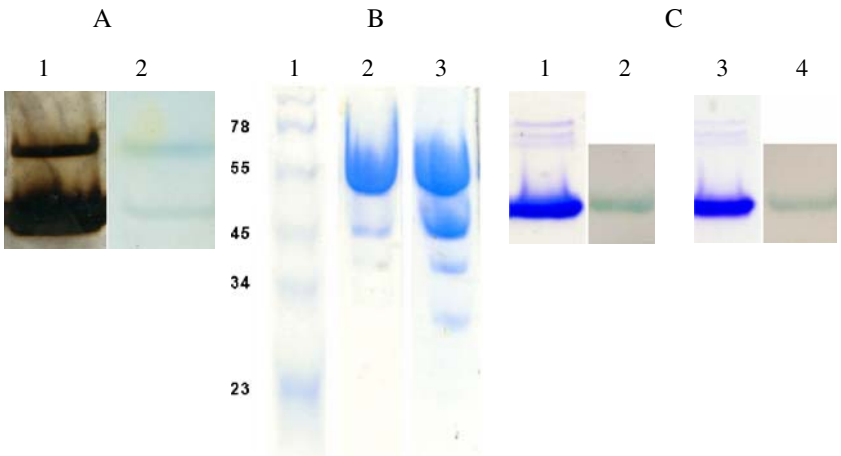

Fig. 1 Gel electrophoresis of β-subunits and His-tagged β-subunits under native and denaturing conditions and in gel pyrophosphatase activity. In **A**, lane 1 shows a silver stained native gel that was loaded with the proteins obtained after "purification" of β-subunits (for details see text). The upper band corresponds to the elongation factor and the lower band to β-subunits; these were identified by mass spectrometry as described in the text. Lane 2 shows the results after the gel was incubated under conditions for assay of pyrophosphatase activity. **B** shows the SDS gel of standard molecular weight proteins (lane 1), the protein band of His-tagged β-subunits with an activity of 582 pmol/min/mg (lane 2), and His-tagged β-subunits with an activity of 246 pmol/min/mg (lane 3). **C** shows the electrophoretic profile of the latter proteins under non-denaturing conditions; lanes 1 and 3 show respectively, the high and low activity His-tagged β-subunits stained with Coomassie blue, and lanes 2 and 4 when the proteins were assessed for pyrophosphatase activity

mass of 51,821 and a calculated pI of 5.03. The other protein band was identified as the elongation factor G of *Escherichia coli* (gi/168738924). The MASCOT score was 1,313; 33 different peptides were found with a sequence coverage of 43%. This protein displayed a nominal mass of 75,732 and a calculated pI of 5.13. Further attempts to purify the two proteins were carried out, but we were unable to obtain clear separation. To our knowledge, pyrophosphatase activity of the elongation factor has not been previously observed; however, this issue was not explored further.

Because the latter preparations of β-subunits consistently had the elongation factor that exhibited pyrophosphatase activity, we opted for purification of β-subunits that had been His-tagged at the N-terminus. In this case, we obtained (see "Methods" section) a preparation that exhibited a single protein band in native gels: in SDS gels; the protein exhibited a molecular weight of about 52,000 kDa. In native gels, the protein band exhibited pyrophosphatase activity; the activity band coincided with the protein band that stained with Coomassie blue. We have made five independent preparations of His-tagged β-subunits, when freshly prepared they exhibited an average activity of 595±20 pmol/min/mg. However, we had a preparation with an activity of 246 pmol/min/mg.

In this connection, it is noted that when the high and low activity His-tagged β-subunits were analyzed in SDS gels, it was found that the protein with high activity exhibited a single protein band. In contrast, the His-tagged β-subunits with low activity exhibited His-tagged β-subunits plus a protein band of approximate M_w of 45 kDa, and other minor protein bands (see B in Fig. 1). In *native* gel electrophoresis, the two preparations of His-tagged β-subunits exhibited a single protein band with pyrophosphatase activity, albeit it was lower in the low activity preparation (see C in Fig. 1). Thus, the existence of a single protein in native gels and more than two proteins in SDS gels indicated that the preparation of low activity corresponded to nicked His-tagged β-subunits. It is important to point out that the storage of His-tagged β-subunits at 4°C for more than 2 weeks was accompanied by a decrease of pyrophosphatase activity through a process that was accompanied by nicking of the protein. Thus, the experiments reported here were performed with His-tagged β-subunits that had been stored for less than 2 weeks.

Hydrolysis of pyrophosphate by the purified His-tagged β-subunit The incubation of the purified His-tagged β-subunit with [^{32}P] PP_i brought about the progressive appearance of substantial amounts of $^{32}P_i$. Some of the characteristics of the hydrolytic reaction were determined. In media with 10 mM $MgCl_2$, and 250 μM [^{32}P] PP_i, at pH 8.3, hydrolysis was directly proportional to the concentration of β-subunits up to 100 μg per ml (Fig. 2a). It is pointed out that up to 100 μg per ml, the specific activity of the β-subunit remained constant, indicating that the subunits were catalytically active in their monomeric state. Hydrolysis of [^{32}P] PP_i was linear with time for at least 2 h (Fig. 2b). [^{32}P] PP_i hydrolysis by

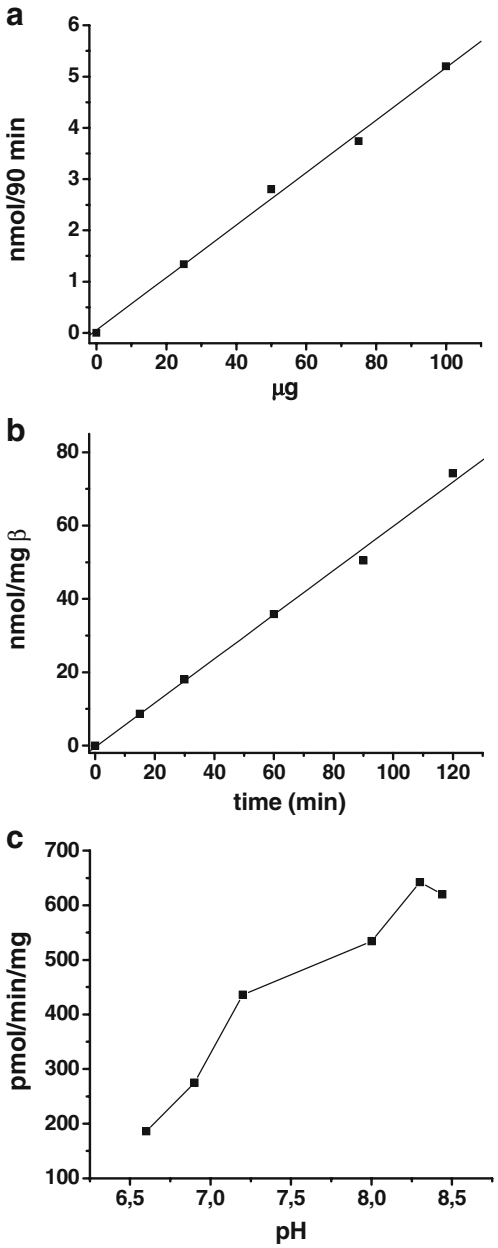

Fig. 2 Hydrolysis of [^{32}P] PP_i by His-tagged β-subunits. In **a**, the indicated amounts of β-subunits were incubated in 40 mM MOPS-Tris, 10 mM $MgCl_2$, and 250 μM [^{32}P] PP_i, 40 mM MOPS-Tris, 2 mM KH_2PO_4, and 10 mM $MgCl_2$ (pH 8.3) in a final volume of 0.1 ml. After 90 min, the reaction was arrested and the amount of $^{32}P_i$ was determined. **b** shows hydrolysis of [^{32}P] PP_i as function of time with 50 μg of β-subunit protein. In **c**, the activity was measured at different pH with 50 μg of protein and 90 min of incubation

the β-subunit was also measured at different pH; the optimal pH of the reaction was 8.3 (Fig. 2c). In eight independent experiments performed under identical conditions (50 μg per ml, 250 μM [^{32}P] PP_i, 10 mM $MgCl_2$, 2 mM KH_2PO_4 at pH 8.3 and 46°C), the average rate of hydrolysis was 577±20.6 pmol/min/mg.

Since the His-tagged β-subunits we used for the present experiments were derived from thermophilic bacillus PS3, we examined their capacity to hydrolyze [^{32}P] PP_i at different temperatures. At 22°, 37°, and 46°C, hydrolysis was linear with time and increased as the incubation temperature was raised (not shown). At 55°C, an initial phase of high activity was followed by a progressive decrease in the rate of hydrolysis, indicating that at this temperature, the subunit underwent thermal denaturation. In the light of these data all the experiments of this work were performed at 46°C.

PP_i hydrolysis is Mg^{2+} dependent Although there are preferences by distinct pyrophosphatases for a particular divalent cation, the pyrophosphate activities of all pyrophosphatases, soluble or membranal, are Mg^{2+} dependent (Halonen et al. 2005). Likewise, hydrolysis of ATP by soluble and particulate F_1 requires Mg^{2+}, although some preparations of F_1 hydrolyze ATP in presence of Ca^{2+} (Pullman et al. 1960). Accordingly, we examined the dependence of the [^{32}P] PP_i hydrolytic activity of β-subunits on divalent cations (Table 1). We found that PP_i hydrolysis by the β-subunit is strictly dependent on Mg^{2+}. Ca^{2+} and Mn^{2+} did not support the catalytic breakdown of [^{32}P] PP_i. K^+ and Na^+ did not affect to a significant extent PP_i hydrolysis supported by Mg^{2+} (Table 1). Similarly to its effect on other pyrophosphatase activities (Baykov 1979), fluoride induced a partial inhibition of hydrolysis (Table 1),

The effect of different concentrations of Mg^{2+} on the hydrolytic activity of β-subunit was studied. The experiments were performed with 250 μM [^{32}P] PP_i. In the light of the association constant of PP_i with Mg^{2+}, it may be calculated that with 250 μM Mg^{2+}, essentially all the substrate would be in complex with Mg^{2+}. At the latter concentration of $MgPP_i$, the enzyme exhibited substantial activity; however, the data showed that optimal activity was reached at Mg^{2+} concentrations higher than 250 μM (Fig. 3), indicating that maximal activity requires free Mg^{2+}.

Table 1 Effect of different ions on the Pyrophosphatase activity of His-tagged β-subunits

	pmol/min/mg
1 mM $MgCl_2$	582
500 μM NaF	177
1 mM $CaCl_2$	6
1 mM $MnCl_2$	18
1 mM $MgCl_2$ + 100 mM KCl	460
1 mM $MgCl_2$ + 100 mM NaCl	626

Activity was measured in the standard conditions except that the indicated salts were included in the incubation mixture

Kinetics of [^{32}P] PP_i hydrolysis by β-subunits and the effect of ATP Hydrolysis of PP_i by β-subunits incubated with different [^{32}P] PP_i concentrations was determined (Fig. 4a). The enzyme exhibited classical Michaelis-Menten behavior; the Km and Vmax of the hydrolytic reaction were 75 μM and 800 pmol/min/mg, respectively.

Because it has been reported that ATP binds to isolated β-subunits (Nájera-Peña et al. 1999; Khananshvili and Gromet-Elhanan 1985; Kironde and Cross 1986), we examined if ATP affects PP_i hydrolysis. ATP at mM concentrations induced a rather modest inhibition of [^{32}P] PP_i hydrolysis, albeit its effect was concentration dependent (Fig. 4b); for example with 250 μM [^{32}P] PPi, 7 mM ATP inhibited the activity by about 20%. It is noted that the inhibition was not due to chelation of free Mg^{2+}, since in the experiments, the concentration of free Mg^{2+} was maintained at a concentration of 1 mM. Lineweaver-Burk plots of [^{32}P] PP_i hydrolysis at various concentrations of [^{32}P] PP_i with and without ATP were linear. Although in presence of ATP the Km increased to 121 μM, its effect was not strictly competitive, since it also decreased the Vmax of [^{32}P] PP_i to 676 pmol/min/mg (inset Fig. 4a).

Near and far CD spectra of β-subunits with and without PP_i The binding of ATP to isolated β-subunits has been successfully determined by recording the CD spectra of the protein with and without ligands (Ohta et al 1980). Thus, to gain insight into the characteristics of the binding of PP_i to the isolated β-subunits, we determined the CD spectra of

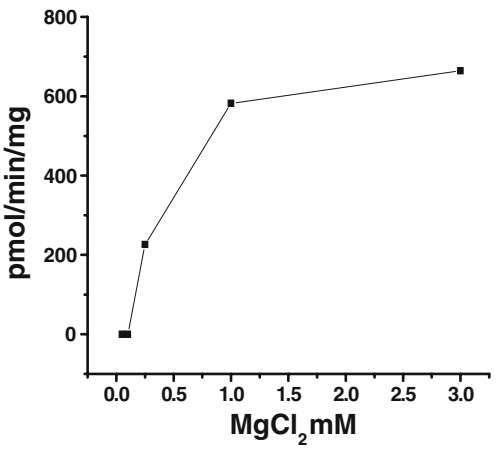

Fig. 3 Effect of Mg^{2+} on [^{32}P] PP_i hydrolysis by His-tagged β-subunits. Hydrolysis of [^{32}P] PP_i was measured in the conditions of Fig. 2 b, in 90 min of incubation, except that the concentration of Mg^{2+} was varied as indicated

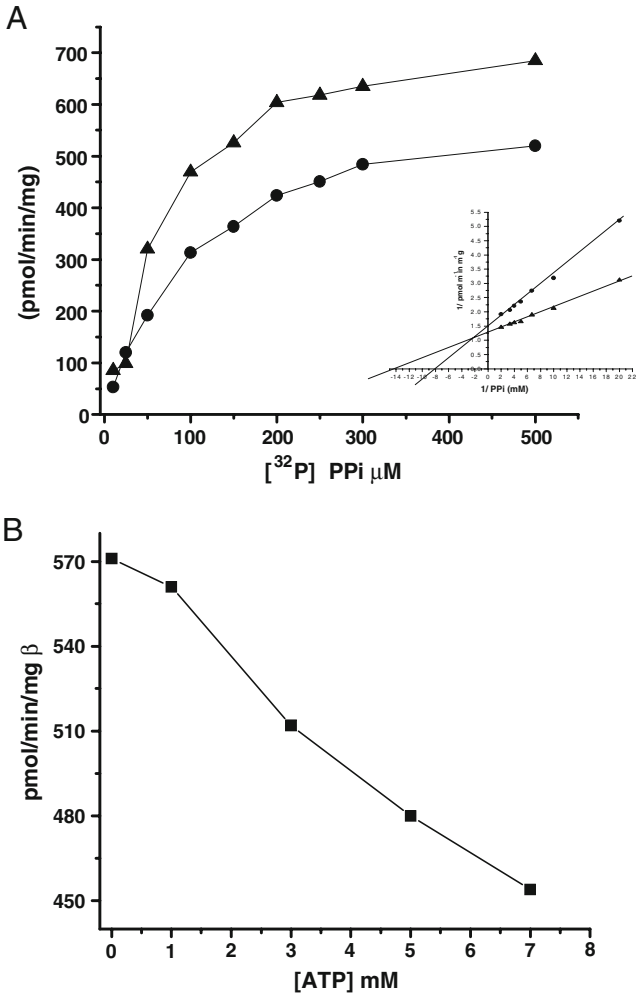

Fig. 4 Hydrolysis of [^{32}P] PP_i by His-tagged β-subunits at different concentrations of [^{32}P] PP_i. Effect of ATP. In **A**, The conditions were as in Fig. 2 **B**, except that the concentration of substrate was varied as indicated. The filled triangles indicate [^{32}P] PP_i hydrolysis at the indicated concentrations; the closed circles indicate mixtures that contained 7 mM ATP. The inset shows a Lineweaver plot of the data with and without ATP. In **B**, ATP at the indicated concentrations was included in the standard incubation media for [^{32}P] PP_i hydrolysis

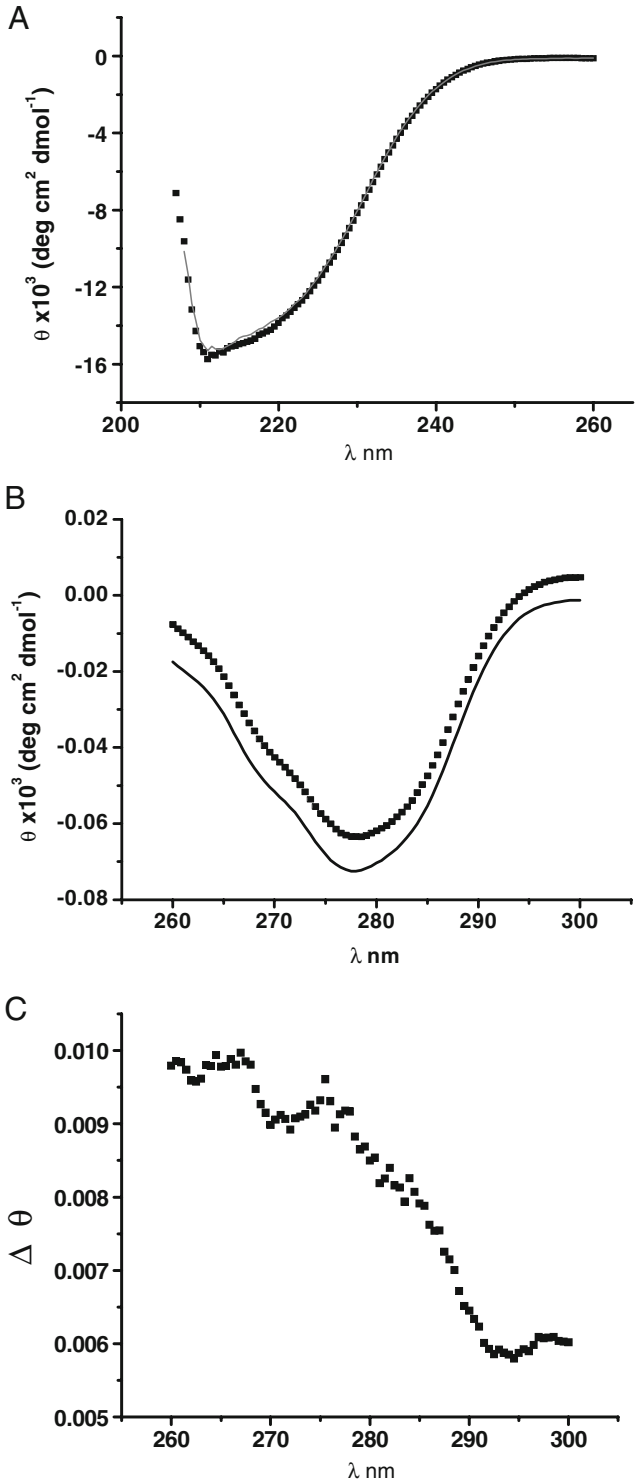

Fig. 5 Far and near CD spectra of β-subunits with and without Mg-PP_i. **A** shows the far UV CD spectra of the subunits with and without 1 mM PP_i and 1 mM $MgCl_2$. **B** depicts the change induced by Mg-PP_i on the near UV CD spectra. **C** shows the difference spectra of the near UV CD data with and without PP_i. For experimental details, see Methods sectiona

the protein with and without PP_i. The far UV CD spectra of β-subunit with and without PP_i showed no significant differences in the two conditions (Fig. 5A). On the other hand, the difference CD spectra of β-subunits with and without PP_i in the near UV showed that PP_i modifies the environment of aromatic residues (Fig. 5B and C). Taken together, the data illustrate that PP_i does not affect to a large extent the secondary structure of the β-subunit, although it changes the environment of its aromatic residues.

We also considered of interest to perform a thermal scanning of the β-subunit at a wavelength of 222 nm. The protein exhibited an unusual transition. As the temperature of the cell was increased in the range of 50° to 70°C, there was a progressive loss of secondary structure. However, as the temperature was further increased to 85°C, the protein

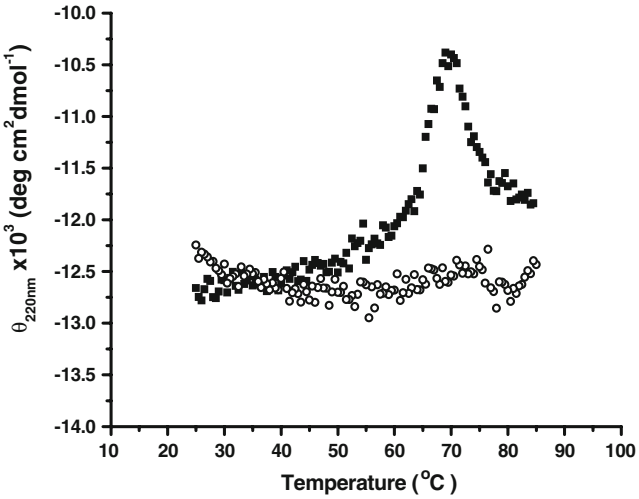

Fig. 6 Thermal scanning of β-subunits as monitored by changes in CD absorption at 220 nm. The temperature of the 0.1 cm light path cell was raised at a rate of 1°C per minute (closed squares) from 25°C to 85°C. Afterwards the temperature of the cell was brought down to 25 (open circles) at the same rate

acquired some of the lost secondary structure (Fig. 6). The temperature of the same sample was then lowered to 25°C; after cooling, the protein had a secondary structure similar to that observed at the beginning of the experiment. It is possible that this phenomenon is related to the thermophilic nature of the protein, since it has been shown by CD thermal scanning that the unfolding that a thermophilic protein undergoes at low temperatures is reversed by relatively high temperatures (Chandrayan and Guptasarma 2008).

Discussion

The H^+- PP_i synthase of *R. rubrum* is an integral membrane protein that catalyzes the synthesis of PP_i from medium P_i with the energy of electrochemical H^+ gradients (Baltscheffsky 1993). The reaction is fully reversible, as shown by its ability to induce the formation of H^+ gradients during PP_i hydrolysis (Baltscheffsky 1967; Baltscheffsky et al. 1999; Sosa and Celis 1995). These functions are also expressed by ATP synthases, except that ATP is the product and substrate of the reactions, and thus, it has been suggested that PP_i synthases represent evolutionary precursors of ATP synthases (Baltscheffsky 1967; Baltscheffsky 1993). However, the analysis of amino acid sequences and crystallographic data show no clear evidence of structural similarities between the catalytic sites of ATP and PP_i synthases (Heikinheimo et al. 1996).

Although the two enzymes do not seem to be structurally related, it has been reported that soluble F_1 from bovine heart mitochondria has the capacity to bind PP_i (Issartel et al. 1987) and to catalyze its synthesis from medium phosphate in presence of dimethyl sulfoxide (Tuena de Gómez-Puyou et al. 1993). Moreover, a peptide of the β-subunit that comprises residues 141–190 binds PP_i and ATP with relatively good affinity (Garboczi et al. 1988). Therefore, and because the various reports on ATP hydrolysis by isolated β-subunits are rather ambiguous, we examined if the subunits have the capacity to bind and hydrolyze pyrophosphate. With respect to PP_i binding, the difference of the near UV CD spectra of β-subunits with and without PP_i was strongly suggestive that the subunits have the capacity to bind PP_i. In fact, we also observed that the subunits catalyze hydrolysis of PP_i. In this connection, it is relevant that the crystal structure of F_1 (Stock et al. 1999) shows that the catalytic sites of F_1 are formed by residues that belong to both α and β subunits, although the majority of the residues belong to the β-subunit, suggesting that the proper docking of the β and γ phosphates for catalytic action requires the participation of the α subunit. On the other hand, the structural data also show that the β and γ phosphates of ATP interact with residues of the β-subunit. Apparently this is sufficient to support PP_i hydrolysis.

Of interest to the present work is that Zancani et al. (2003) isolated a soluble protein from pea stem mitochondria that according to the mass spectrometry analysis corresponds to the β-subunit of the mitochondrial ATP synthase. A report on the proteome of pea mitochondria also revealed the presence of soluble β-subunits (Bardel et al. 2002) in the mitochondrial matrix. Although it cannot be excluded that the soluble subunits correspond to unassembled subunits or subunits that are detached from the complex or failed to assemble, the data of Zancani et al. (2003) show that their protein hydrolyzes PP_i at rates that are many-fold higher than the activity we detected in the isolated β-subunits of thermophilic bacillus PS3.

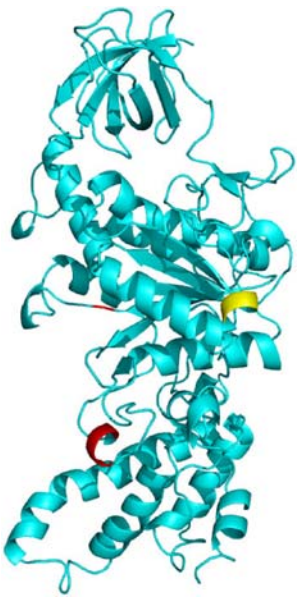

Fig. 7 Some sequence differences located on the nucleotide binding domain between the β-subunit of thermophilic bacillus PS3 and the β-subunit of pea mitochondria. Two regions that differ (residues 306 and 336–338 shown in red) are in the vicinity of the region of the nucleotide binding site. The pea protein has an eight residue fragment that is absent in the β-subunit of the thermophile; it is between residues 208 and 209 of the thermophilic protein (depicted in yellow). The residues were mapped on the β-subunit from the crystal structure of the α3β3 complex of F_1 ATPase from the Thermophilic Bacillus PS3 (PDB code 1SKY)

Thus, the question that stems from the current observations and those of Zancani et al. (2003) concerns the structural features that make the pea protein a more effective catalyst of PP_i hydrolysis. To address this issue, we compared the amino acid sequence of the pea protein and the β-subunit of thermophilic bacillus PS3. The two proteins have an identity of 63% and a similarity of 75%. However, it is noteworthy that the pea protein has an eight-residue insertion between residues 208 and 209 of the thermophilic protein that protrudes into the solvent (Fig. 7). However, other differences were detected in the vicinity of the nucleotide binding site. Therefore, at the moment, the cause of the different pyrophosphatase activity of the two proteins remains to be established. It is noted that the pea protein does not catalyze hydrolysis of ATP or other triphosphates to a significant extent (Zancani et al. 2003). This would be in consonance with previous reports on the lack or very low ATPase activity of isolated β-subunits; as well as with the present data that show that the pyrophosphatase activity of the β-subunit of thermophilic bacillus SP3 is very weakly inhibited by ATP.

Although β-subunits are instrumental in the expression of ATP synthesis and hydrolysis by the whole ATPase complex, there has been a long standing question of whether the isolated subunits have the capacity to perform catalysis. The data that have accumulated throughout the years show that they are essentially catalytic inert with ATP as substrate However, our results show that the isolated subunits are indeed catalytically active albeit with a much simpler substrate.

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