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Characterization of an Inorganic Phosphate Binding Site on the Isolated, Reconstitutively Active β Subunit of $F_0 \cdot F_1$ ATP Synthase†

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ABSTRACT: One binding site for phosphate (P_i) has been demonstrated on the reconstitutively active β subunit that has been removed from the *Rhodospirillum rubrum* membrane-bound ATP synthase ($RrF_0 \cdot F_1$). Under optimal conditions 1 mol of P_i is bound per mole of β subunit with a K_d of 270 ± 30 μM and a half-time of 15 min. P_i binding to β is absolutely dependent on $MgCl_2$, and for its stable binding, $MgCl_2$ must be present not only during the binding step but also during the elution–centrifugation step used to separate the bound and free [^{32}P]P_i. The binding of P_i is inhibited by the presence of ATP or ADP. When present at low concentrations (5–50 μM) both nucleotides inhibit P_i binding to β in a noncompetitive manner with a K_i of 10 μM. At higher concentrations (0.1–10 mM) the inhibition becomes competitive with ATP being a much more effective inhibitor (K_i = 350 μM) than ADP (K_i = 10 mM). These results indicate that P_i binds to the $MgCl_2$-dependent low-affinity nucleotide binding site that has been demonstrated on the isolated *R. rubrum* β subunit [Gromet-Elhanan, Z., & Khananshvili, D. (1984) *Biochemistry 23*, 1022–1028] probably at the site occupied by the γ-phosphoryl group of ATP. The observation that estimated K_d values for binding of P_i, ADP, and ATP to this $MgCl_2$-dependent low-affinity binding site on β are very similar to the reported K_m for ATP hydrolysis and for P_i and ADP during photophosphorylation indicates that this site might be the catalytic site of the $RrF_0 \cdot F_1$ ATP synthase.

The molecular mechanism of ATP synthesis and hydrolysis by the proton-translocating reversible $F_0 \cdot F_1$ ATP synthase is still unknown, although a number of mechanisms have been proposed (Boyer et al., 1977; Cross, 1981). One possible approach to the elucidation of this problem is to identify and characterize the substrate binding sites on this enzyme complex. Previous studies have demonstrated the presence of several nucleotide binding sites that reside in the two larger subunits of the $F_0 \cdot F_1$ ATP synthase, α and β (Harris, 1978; Shavit, 1980; Cross, 1981; Senior & Wise, 1983). They seem to include both catalytic and regulatory sites, but the exact subunit location of each category is not clear. A number of F_1-ATPases have also been reported to bind P_i[1] (Penefsky, 1977; Kasahara & Penefsky, 1978) or its analogue 4-azido-2-nitrophenyl phosphate (Lauquin et al., 1980; Pougeois et al., 1983a,b). One (Penefsky, 1977) or possibly two (Kasahara & Penefsky, 1978) P_i binding sites have been found on MF_1. The binding site of the phosphate analogue has been located on the β subunit (Pougeois et al., 1983a,b).

A detailed characterization of individual substrate binding sites on the $F_0 \cdot F_1$ enzyme complex is, however, very difficult because (a) it contains two or three copies of $\alpha\beta$ pairs that could be in different conformational states in the catalytically active complex (Grubmeyer et al., 1982; O'Neal & Boyer, 1984) and (b) its catalytic activity leads to interconversion of the substrates. These problems could be circumvented by direct examination of substrate binding sites on isolated, purified, reconstitutively active α and β subunits. Such subunits have been obtained up to now only from three different bacterial sources, i.e., from a thermophilic bacterium (Yoshida et al., 1977), from *Escherichia coli* (Futai, 1977), and from *Rhodospirillum rubrum* (Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982a). The isolated subunits provide

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[1] Abbreviations: $RrF_0 \cdot F_1$, proton-translocating ATP synthase–ATPase complex of *R. rubrum*; RrF_1, soluble *R. rubrum* ATPase; CF_1, soluble chloroplast ATPase; MF_1, soluble mitochondrial ATPase; P_i, inorganic phosphate; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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a much simpler system for studying substrate binding sites, as they show no subunit–subunit interactions and no catalytic activity by themselves, although they retain the capacity to restore ATP synthesis and/or hydrolysis after their reconstitution (Futai, 1977; Philosoph et al., 1977; Yoshida et al., 1977).

Direct binding studies with labeled ATP and ADP have been carried out until now only on the α subunit of *E. coli* (Dunn & Futai, 1980) and on the β subunit of *R. rubrum* (Gromet-Elhanan & Khananshvili, 1984a; Khananshvili & Gromet-Elhanan, 1984). These studies have identified one high-affinity nucleotide binding site on the α subunit, whereas on the β subunit two binding sites for both ATP (Gromet-Elhanan & Khananshvili, 1984a) and ADP (Khananshvili, & Gromet-Elhanan, 1984) have been found. One of them, which is very similar to the binding site located on the α subunit, is a $MgCl_2$-independent high-affinity site, whereas the second is a $MgCl_2$-dependent low-affinity site.

So far there has been no report on the capacity of the isolated α and β subunits to bind P_i. In this paper the optimal conditions required for demonstrating P_i binding to β are established and the properties of P_i binding in the absence and presence of ATP and ADP are defined.

Experimental Procedures

R. rubrum cells were grown as outlined by Philosoph et al. (1977). Chromatophores were prepared by the Yeda press (Gromet-Elhanan, 1970, 1974) with the modifications described by Gromet-Elhanan & Khananshvili (1984a). The reconstitutively active β subunit of RrF_0F_1 was extracted from *R. rubrum* chromatophores by LiCl (Philosoph et al., 1977), purified, and stored according to Khananshvili & Gromet-Elhanan (1982a). In all the experiments reported here, three different preparations of β were used. All of them were electrophoretically pure, showing no contamination even when 50 μg of protein was loaded on SDS–polyacrylamide gels, and all of them restored 92%–97% of the photophosphorylation of Mg^{2+}-ATPase activities of β-less chromatophores.

Before incubation of the β subunit with [^{32}P]P_i, concentrated EDTA was added to β at 2–3 mg/mL in the storage buffer (50 mM Tricine–NaOH, pH 8.0, 4 mM $MgCl_2$, 4 mM ATP, and 10% glycerol) to give a final EDTA concentration of 2 mM. After 20 min at 23 °C, the β subunit was freed from the storage buffer by elution–centrifugation (Penefsky, 1977) at 4 °C on Sephadex G-50 columns as described by Khananshvili & Gromet-Elhanan (1983a). In some cases, larger columns of 3, 5, or 10 mL were used. In these columns no more than 10% of the column volume was loaded with suspensions of β, and the centrifugation time was increased to 15 min to ensure effective removal of all the buffer in the void volume. The columns were preequilibrated either with TGEN buffer containing 50 mM Tricine–NaOH, pH 8.0, 20% glycerol, 2 mM EDTA, and 50 mM NaCl or with MGEN buffer containing 50 mM Mes–NaOH, pH 6.5, instead of Tricine–NaOH. For complete removal of all the ATP previously bound to the β subunit, the elution–centrifugation step was repeated at least twice (see Table I). Such ATP-free β preparations retained their reconstitutive activity and P_i or nucleotide binding capacity when kept in TGEN or MGEN buffer for several hours at 23 °C or for at least 2 weeks in liquid nitrogen.

P_i binding studies were carried out by incubating [^{32}P]P_i with the β subunit at 10 μM (Gromet-Elhanan & Khananshvili, 1984a) either in MGM buffer containing 50 mM Mes-NaOH, pH 6.5, 20% glycerol, and 1 mM $MgCl_2$ or in TGM buffer containing 50 mM Tricine-NaOH, pH 8.0, 20% glycerol, and 5 mM $MgCl_2$, at 23 °C under the conditions described in the figure legends. Incubation was initiated by addition of the β subunit, and at the specified time intervals, the unbound P_i was removed by elution–centrifugation on Sephadex columns preequilibrated with the incubation buffer unless otherwise stated. The samples were placed in Eppendorf tips, which were inserted about 3–5 mm above the top of each column as described by Gromet-Elhanan & Khananshvili (1984a). The effluent from each column was diluted with 1 mL of water, and appropriate aliquots were assayed for ^{32}P radioactivity and protein content.

Protein was determined according to Lowry et al. (1951). ^{3}H or ^{32}P radioactivity was measured by liquid scintillation counting (Penefsky, 1977). Binding data were calculated by using a M_r of 50 000 for the β subunit (Bengis-Garber & Gromet-Elhanan, 1979).

[2,8-^{3}H]ATP (24–29 Ci/mmol) was purchased from New England Nuclear and was diluted with chromatographically purified nonradioactive ATP to give a specific radioactivity of 1.4×10^5 cpm/nmol. [^{32}P]P_i was obtained from The Nuclear Research Center, Negev, and purified as follows: Concentrated HCl was added to 100–200 μL of [^{32}P]P_i (about 3–5 mCi) to a final concentration of 1 N. After 2 h at 35 °C, the sample was diluted to 50 mL with H_2O and applied to a Dowex AG 1-X4 column (0.7 × 2cm). The column was washed with H_2O (10 mL) and 5 mM HCl (10 mL), and the [^{32}P]P_i was eluted with 60 mM HCl (4 mL). The recovery of radioactivity was approximately 80%, and it contained less than 0.0005% impurities measured by 2-methyl-1-propanol–benzene extraction (Avron, 1960).

Results

Determination of Conditions Required for Demonstration of Binding of P_i to the β Subunit. The purified *R. rubrum* β subunit is stored in a buffer containing 4 mM $MgCl_2$ and ATP since previous studies have shown that in order to preserve its reconstitutive activity $MgCl_2$ and ATP must be present during the isolation, purification, storage, and reconstitution steps (Binder & Gromet-Elhanan, 1974; Philosoph et al., 1977). These concentrations of $MgCl_2$ and ATP provide, however, optimal conditions for ATP binding to the two nucleotide binding sites that have been identified on this isolated β subunit (Gromet-Elhanan & Khananshvili, 1984a). We have, therefore, examined whether the presence of bound ATP does affect the ability of β to bind medium P_i (Table I). For this examination, β was first precipitated by ammonium sulfate, to remove all bound unlabeled ATP, and then incubated in storage buffer containing [^{3}H]ATP. Any remaining ammonium sulfate was removed by elution–centrifugation on a Sephadex column preequilibrated with the same buffer. An aliquot of the effluent from this step (Table I, step 0) was freed from unbound ATP (Gromet-Elhanan & Khananshvili, 1984a) and was assayed for ^{3}H radioactivity and protein content, as well as for its capacity to bind [^{32}P]P_i by incubation with 2 mM [^{32}P]P_i in MGM buffer for 30 min. As is illustrated in Table I, step 0, 1.82 mol of ATP was bound per mole of β during the incubation in storage buffer, and this β could not bind any P_i. The bound ATP could, however, be removed by successive elution–centrifugation steps on Sephadex columns preequilibrated with EDTA containing buffer (Table I, steps 1–3). As the binding stoichiometry of ATP is gradually decreased, the capacity of β to bind P_i increases, so that after three successive elution–centrifugation steps in the presence of EDTA (Table I, step 3) the binding stoichiometry of ATP decreased from 1.82 to 0.03 mol/mol of β and that of P_i increased from 0.01 to 0.63 mol/mol of β. These results

Table I: Effect of Bound ATP on Binding of [^{32}P]P_i to the β Subunit[a]

no. of elution–centrifugation steps with EDTA	bound [^{3}H]ATP (mol/mol of β)	bound [^{32}P]P_i (mol/mol of β)	recovery of treated β (%)
0	1.82 (100)	0.01	100
1	0.63 (34)	0.22	82
2	0.12 (6)	0.62	75
3	0.03 (2)	0.63	68

[a] A total of 250 μL of β at 3 mg/mL was precipitated by ammonium sulfate, and the precipitate was resuspended in 250 μL of the storage buffer (see Experimental Procedures) containing [^{3}H]ATP and incubated at 23 °C for 2 h. After removal of any remaining ammonium sulfate by elution–centrifugation, an aliquot of the effluent (step 0) containing the β subunit was assayed for bound [^{3}H]ATP and for its capacity to bind [^{32}P]P_i as described in the text. The bulk of the effluent from step 0 was subjected to successive elution–centrifugation steps on Sephadex columns equilibrated with TGEN buffer (steps 1–3). Aliquots of the effluent from each of these steps were assayed for remaining bound [^{3}H]ATP and for [^{32}P]P_i binding. The numbers in parentheses represent the percent of the remaining bound [^{3}H]ATP in each step.

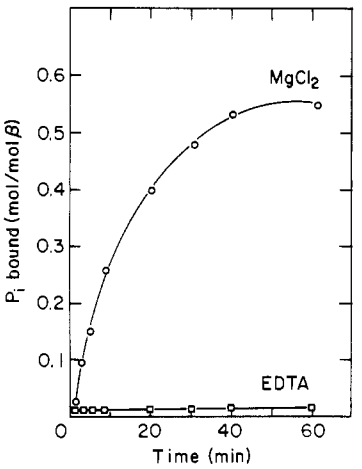

FIGURE 1: Time dependence of P_i binding to the β subunit. β was incubated at 10 μM with 2 mM [^{32}P]P_i in MGM buffer containing 1 mM $MgCl_2$ (O) or 2 mM EDTA and no $MgCl_2$ (□). At the time indicated, 100-μL aliquots were freed from unbound [^{32}P]P_i and assayed for bound [^{32}P]P_i as described under Experimental Procedures.

indicate that bound ATP inhibits the binding of medium P_i to β. Therefore, in all further experiments β free of bound ATP was used (see Experimental Procedures).

The time dependence of P_i binding to the β subunit was tested (Figure 1) with a saturating concentration of P_i (see Figure 5). The binding of P_i reached a maximal level of 0.6 mol/mol of β at about 40 min with a $t_{1/2}$ of 15 min. This binding is absolutely dependent on the presence of $MgCl_2$ during the incubation, since when $MgCl_2$ was replaced by EDTA no binding of P_i could be observed (Figure 1). This effect was not due to any interference of EDTA with P_i binding to β, because when P_i was incubated with β in the absence of both $MgCl_2$ and EDTA (see Figure 3), less than 0.03 mol of P_i was bound per mol of β. The amount of bound P_i increased linearly with increasing β concentrations (not shown). This led to a constant binding stoichiometry of P_i to β of 0.63 mol/mol at a range of β concentrations between 2 and 40 μM. In all further experiments, β at 10 μM (=0.5 mg/mL) was therefore used since, as we have earlier reported (Gromet-Elhanan & Khananshvili, 1984a), this is the minimal protein concentration that is fully recovered after elution centrifugation.

As is illustrated in Figure 2, the optimal pH for binding of P_i to β is dependent on the concentration of $MgCl_2$ in the

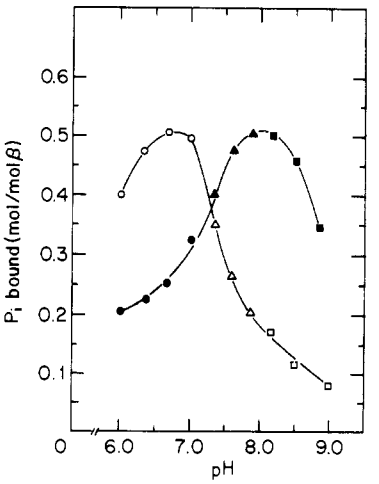

FIGURE 2: Effect of pH on binding stoichiometry of P_i to β at two different $MgCl_2$ concentrations in the incubation mixture. β was incubated at 10 μM with 2 mM [^{32}P]P_i, 20% glycerol, and 50 mM of Mes–NaOH (O, ●), Hepes–NaOH (△, ▲), or Tricine–NaOH (□, ■) at the indicated pH in the presence of either 1 (O, △, □) or 5 mM (●, ▲, ■) $MgCl_2$. After 1 h, aliquots of 100 μL were freed from unbound [^{32}P]P_i and assayed as described in Figure 1.

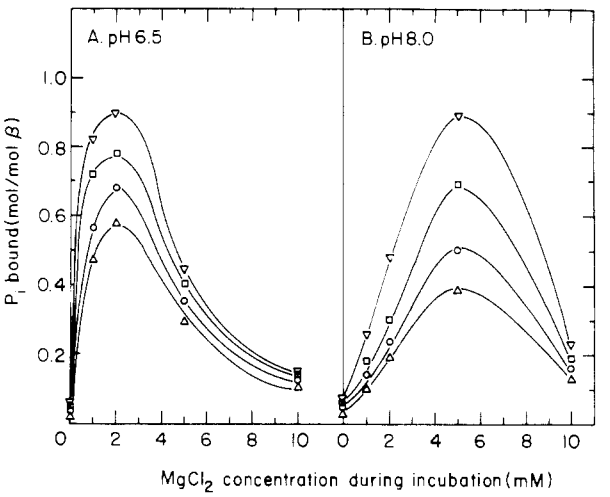

FIGURE 3: Dependence of binding stoichiometry of P_i to β on the concentration of $MgCl_2$ during the incubation and in the elution–centrifugation steps. β was incubated at 10 μM with 2 mM [^{32}P]P_i and 20% glycerol either in 50 mM Mes–NaOH, pH 6.5 (A), or in 50 mM Tricine–NaOH, pH 8.0 (B), with the indicated concentrations of $MgCl_2$. After 1 h, aliquots were loaded on Sephadex G-50 columns preequilibrated with the buffer used during the incubation in each series of experiments, except that the $MgCl_2$ concentrations were as follows: 2 (△), 5 (O), 10 (□), and 20 mM (▽). The effluents were assayed for bound [^{32}P]P_i as described under Experimental Procedures.

incubation mixture. In the presence of 1 mM $MgCl_2$, P_i binding to β was optimal at pH 6.6, whereas with 5 mM $MgCl_2$ the optimal pH for P_i binding was shifted to pH 8.0. The different pH optimum recorded in Figure 2 seems to be due to a shift in the optimum concentration of $MgCl_2$ in the incubation mixture from about 2 mM at pH 6.5 (Figure 3A) to about 5 mM at pH 8.0 (Figure 3B). Higher $MgCl_2$ concentrations during incubation decrease the binding of P_i to β (Figure 3).

Figure 3 also demonstrates that for stable binding of P_i to the β subunit $MgCl_2$ must be present not only during the binding step but also during the elution–centrifugation step used to separate the β subunit bound [^{32}P]P_i from the remaining free ligand. In the experiments described in Table I and Figures 1 and 2, β was freed from unbound [^{32}P]P_i by elution–centrifugation on Sephadex columns, which were

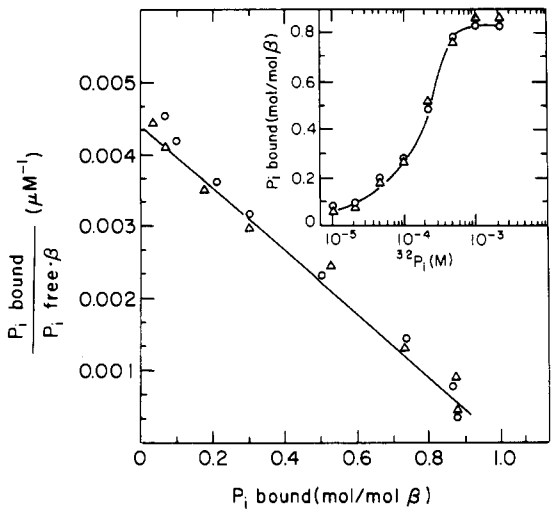

FIGURE 4: Scatchard plot analysis of P_i binding to the β subunit. β was incubated at 10 μM with the indicated concentrations of [^{32}P]P_i in MGM (○) or TGM (△) buffer. After 1 h, aliquots were freed from unbound [^{32}P]P_i by elution–centrifugation on Sephadex columns preequilibrated with the same buffer, but containing 20 mM $MgCl_2$, and the effluents were assayed for bound [^{32}P]P_i.

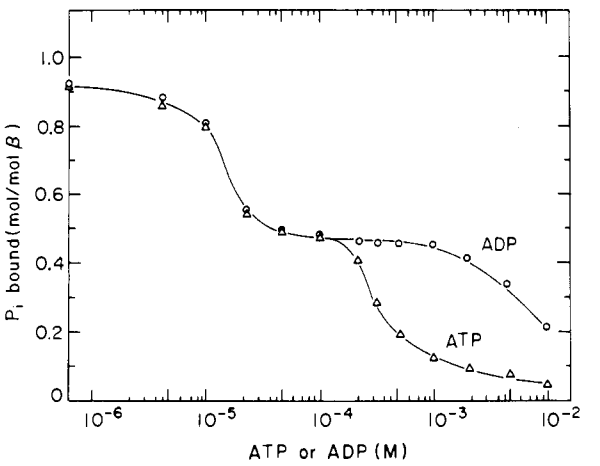

FIGURE 5: Inhibition of P_i binding to the β subunit on addition of ATP and ADP together with [^{32}P]P_i to the incubation mixture. β was incubated at 10 μM with 2 mM [^{32}P]P_i in TGM buffer containing the indicated concentrations of ATP (△) or ADP (○). After 1 h, aliquots of 80 μL were freed from unbound [^{32}P]P_i and nucleotides by elution–centrifugation on Sephadex columns preequilibrated with TGM buffer containing 20 mM $MgCl_2$, and the effluents were assayed for bound [^{32}P]P_i.

preequilibrated with the same buffer that was used for the incubation of β with [^{32}P]P_i, as described under Experimental Procedures. Under these conditions, the maximal binding stoichiometry of P_i to β was 0.6 mol/mol. A higher binding stoichiometry approaching 1 mol of P_i bound per mole of β was, however, obtained when the concentration of $MgCl_2$ in the preequilibration buffer was increased to 20 mM (Figure 3), although the optimal $MgCl_2$ concentrations for the binding step were much lower, ranging between 2 and 5 mM. In light of these results, the binding of P_i to β was assayed in all further experiments after removal of unbound P_i by elution–centrifugation on Sephadex columns preequilibrated with the incubation buffer, but containing 20 mM $MgCl_2$.

We have recently observed that the binding of ADP to the $MgCl_2$-dependent low-affinity nucleotide binding site on β shows a similar requirement for higher $MgCl_2$ concentrations during the elution–centrifugation step than during the incubation step (Khananshvili & Gromet-Elhanan, 1984). On the other hand, the binding of ATP to this site shows no such requirement, since this site was fully occupied by ATP when $MgCl_2$ was present during the incubation but removed together with the unbound ATP during the elution–centrifugation step (Gromet-Elhanan & Khananshvili, 1984a).

The affinity of the β subunit for P_i and the number of binding sites on β for P_i were investigated by using the optimal conditions defined above for P_i binding to β, in the presence of a wide range of P_i concentrations (Figure 4, inset). About 0.9 mol of P_i binding site/mol of β was titrated at both pH 6.5 and pH 8.0, showing a monophasic binding curve that saturates at 1 mM P_i. A Scatchard plot of the binding data reveals one binding site with a K_d of 270 ± 30 μM. This K_d is similar to the K_m for P_i incorporation into ATP measured by Selman & Selman-Reimer (1981) in spinach thylakoids, so this site might be a catalytic one.

Effect of ATP and ADP on Binding of P_i to the β Subunit. The occupation of both high-affinity and low-affinity nucleotide binding sites on the β subunit with ATP was found to inhibit completely the binding of medium P_i to β (Table I). It was therefore very interesting to test whether the addition of ATP (or ADP) together with P_i to the incubation mixture does also inhibit the binding of P_i to β. As is illustrated in Figure 5, P_i binding to β is inhibited by the presence of either

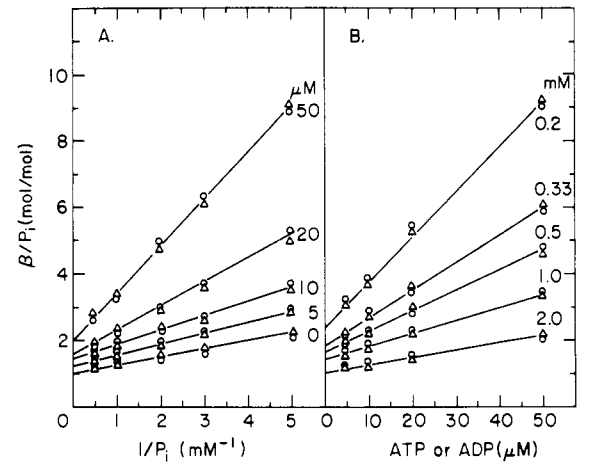

FIGURE 6: Noncompetitive inhibition of P_i binding to the β subunit by 5–50 μM ATP or ADP. β was incubated at 10 μM in TGM buffer with 0.2–2.0 mM [^{32}P]P_i in the presence of the indicated concentrations of ATP (△) or ADP (○). After 1 h, aliquots were freed from unbound [^{32}P]P_i and assayed as described in Figure 5. In (A) the reciprocal of the molar binding ratio $(P_i/\beta)^{-1}$ is plotted vs. the reciprocal of P_i concentrations. In (B), the reciprocal of the molar binding ratio is plotted vs. the concentration of ATP or ADP.

ATP or ADP together with P_i during incubation of β under conditions that have been shown to be optimal for binding of both nucleotides (Gromet-Elhanan & Khananshvili, 1984a; Khananshvili & Gromet-Elhanan, 1984) and of P_i (Figures 1–4) to β. This inhibition is dependent on the nucleotide concentration, exhibiting a biphasic inhibition curve. In the presence of 5–50 μM concentrations of either ATP or ADP, the binding stoichiometry of P_i to β decreases from 0.9 to 0.5 mol of P_i/mol of β. A further decrease is observed with increasing nucleotide concentrations, but under these conditions ATP becomes a much more effective inhibitor than ADP. Thus, in the presence of 2 mM ATP or ADP, the binding stoichiometry of P_i decreases to 0.1 and 0.4 mol of P_i/mol of β, respectively (Figure 5).

A detailed analysis of the kinetic parameters of the inhibition of P_i binding by ATP and ADP was carried out by varying the concentration of P_i and of the nucleotides during the incubation. In the experiment summarized in Figure 6, P_i binding to β was measured with P_i concentrations of 0.2–2.0

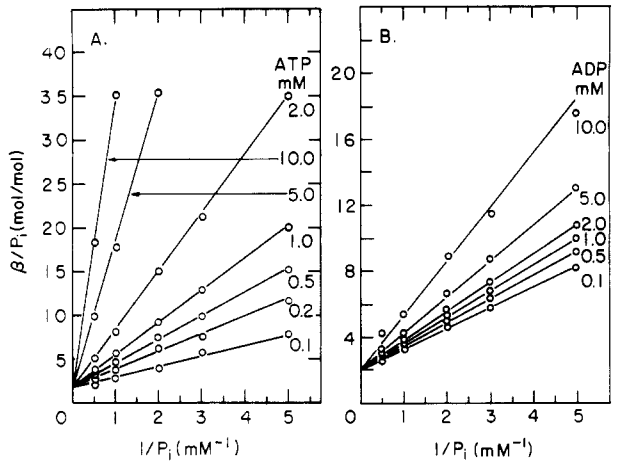

FIGURE 7: Competitive inhibition of P_i binding to β by 0.1–10 mM concentrations of ATP and ADP. Incubation and assay conditions were as described in Figure 6, except that in (A) the incubation was carried out with the indicated concentrations of ATP and in (B) with the indicated concentrations of ADP.

mM and with ATP or ADP concentrations of 5–50 μM. The Lineweaver and Burk plot of the data (Figure 6A) indicates that at 5–50 μM both nucleotides inhibit the binding of P_i to β in a very similar noncompetitive manner, decreasing the affinity of P_i binding by about 4-fold, as the K_d of P_i binding increases from 250 to 970 μM. The K_i of this noncompetitive inhibition, calculated from the Dixon plot (Figure 6B), is 10 μM. This K_i value is similar to the K_d values reported for the binding of ATP or ADP to the $MgCl_2$-independent high-affinity binding site on β (Gromet-Elhanan & Khananshvili, 1984a), suggesting that the noncompetitive inhibition of P_i binding to β by micromolar concentrations of both nucleotides could result from the occupation of the high-affinity nucleotide binding site on β.

When P_i binding to β was measured with P_i concentrations of 0.2–2.0 mM and with ATP or ADP concentrations of 0.1–10 mM, which were responsible for the second phase of inhibition of P_i binding by the nucleotides (see Figure 5), a different picture was obtained (Figure 7). The inhibition becomes competitive, with ATP being a much more effective inhibitor than ADP. Thus, with 0.1–10 mM ATP, the K_d of P_i binding to β increased from 0.67 to 10 mM (Figure 7A), whereas with 0.1–10 mM ADP, this K_d increased only to 1.3 mM (Figure 7B). The estimated K_i for the competitive inhibition of P_i binding to β by ATP is 350 μM, and it is very similar to the K_d value of 200 μM reported for the binding of ATP to the $MgCl_2$-dependent low-affinity nucleotide binding site on β (Gromet-Elhanan & Khananshvili, 1984a). On the other hand, the estimated K_i for the competitive inhibition of P_i binding by ADP is 10 mM, and it is more than a 100-fold greater than the K_d value of 80 μM reported for the binding of ADP to the $MgCl_2$-dependent low-affinity nucleotide binding site on β (Khananshvili & Gromet-Elhanan, 1984).

The above-described competitive inhibition of P_i binding to β by ATP and ADP suggests that P_i binds to the $MgCl_2$-dependent low-affinity nucleotide binding site on the β subunit. The much more effective inhibition of this binding by ATP could indicate that P_i binds at the site occupied by the γ-phosphoryl group of ATP, which is not occupied in the presence of ADP. Further proof for the identification of the P_i binding site on the β subunit with the γ-phosphoryl site in the $MgCl_2$-dependent low-affinity ATP binding site has recently been obtained from studies with a chemically modified β subunit (Khananshvili & Gromet-Elhanan, 1985).

Discussion

This study provides evidence that under optimal conditions 1 mol of P_i is bound per mole of β subunit, with a K_d of 270 $\pm$ 30 μM and a $t_{1/2}$ of 15 min (Figures 1 and 4). The conditions required for obtaining the maximal binding stoichiometry include (a) the use of β-subunit preparations that were freed from all bound ATP (Table I), (b) the presence of $MgCl_2$ in the incubation mixture as well as during the separation of bound and free P_i (Figures 1 and 3), and (c) a proper adjustment of pH and $MgCl_2$ concentration in the incubation mixture (Figures 2 and 3).

An ATP-free β subunit is required because ATP and ADP inhibit P_i binding to β. At micromolar nucleotide concentrations a noncompetitive inhibition is obtained that decreases the P_i binding stoichiometry to β by about 50% whereas at millimolar concentrations the nucleotides become competitive inhibitors that eliminate completely P_i binding to β. Partial inhibition of P_i binding to MF_1 by micromolar concentrations of both ADP and ATP has been reported by Penefsky (1977). Tightly bound ATP has been shown to decrease the affinity of P_i binding to CF_1 when assayed by an anilinonaphthalenesulfonate fluorescence change (Pick & Finel, 1983), and a partial protection by ADP and ATP of F_1 photoinactivation by a photoreactive P_i analogue has been observed by Pougeois et al. (1983a,b). But, no detailed kinetic investigation has been performed in any of these studies.

The detailed analysis presented in Figures 5–7 has identified two phases of inhibition of P_i by ADP and ATP. The kinetic parameters of these phases correspond to those reported for the two nucleotide binding sites that we have demonstrated on the isolated β subunit (Gromet-Elhanan & Khananshvili, 1984a; Khananshvili & Gromet-Elhanan, 1984), indicating that the binding of ADP and ATP to the high-affinity as well as the low-affinity nucleotide binding sites on β affects the binding of P_i to β. Occupation of the high-affinity site causes noncompetitive inhibition, whereas occupation of the low-affinity nucleotide binding site leads to a competitive inhibition of P_i binding to β, thus suggesting that P_i binds to this $MgCl_2$-dependent low-affinity nucleotide binding site. The observation that the K_i for the competitive inhibition of P_i binding by ATP is very similar to the K_d of its binding to this low-affinity site, whereas the K_i for the competitive inhibition by ADP is about 100-fold greater than the K_d of its binding to this site, suggests that P_i binds to the low-affinity nucleotide binding site on the β subunit at the place occupied by the γ-phosphoryl group of ATP, which remains empty in the presence of ADP.

A requirement for the presence of $MgCl_2$ in the incubation mixture as well as during the elution–centrifugation step separating the bound and free ligand has been observed also for the binding of ADP (Khananshvili & Gromet-Elhanan, 1984), but not of ATP (Gromet-Elhanan & Khananshvili, 1984a), to the $MgCl_2$-dependent low-affinity nucleotide binding site on β. Moreover, with ADP as well as with P_i the concentration of $MgCl_2$ required for retaining the bound ligand during the elution–centrifugation step is higher than that required for the binding itself. Our experiments with ADP led us to conclude that the Mg·ADP·β complex is unstable and dissociates very rapidly when $MgCl_2$ is removed together with the free ADP, while the Mg·ATP·β complex is stable and does not dissociate upon removal of $MgCl_2$. In light of the above results, the Mg·P_i·β complex seems to be as unstable as the ADP complex. The remarkable stability of the Mg·ATP·β complex, as compared to the P_i or ADP complexes, may be due to the fact that ATP can bind with both the adenine ring

and the γ-phosphoryl group, as compared to ADP and P_i each of which has less binding groups than ATP. These results are in accord with the suggestion that P_i binds to the site occupied by the γ-phosphoryl group of ATP.

The binding of P_i to β was found to be optimal at pH 6.5, when assayed in the presence of 1 mM $MgCl_2$ during the incubation (Figure 2). Using the same concentration of $MgCl_2$, Penefsky (1977) and Kasahara & Penefsky (1978) have observed a similar pH optimum of 6.5 for binding of P_i to MF_1. They have interpreted the observed pH-dependence curve as indicating that the monovalent anion is the charged form of P_i, which binds to the enzyme. Our observation that the presence of higher $MgCl_2$ concentrations during the incubation of P_i with the β subunit shifts the pH optimum to pH 8.0 indicates that the observed pH optimum might be due to a more complicated interplay of different effects, which could involve also the β subunit itself. One such possibility is an anion-induced base shift in the protein (Dixon & Webb, 1966; Cornisch-Bowden, 1979) that could shift the pH optimum for P_i binding to the β subunit. For a full explanation of the intricate adjustment of the pH and the $MgCl_2$ concentration, more experiments will be required.

The apparent K_d of phosphate binding to β (270 ± 30 μM), which was identical when measured at pH 6.5 with 1 mM $MgCl_2$ and at pH 8.0 with 5 mM $MgCl_2$ (Figure 4), is very similar to the K_m for P_i incorporation into ATP that has been measured during steady-state photophosphorylation with spinach thylakoid membranes (Selman & Selman-Reimer, 1981). Moreover, the K_d values measured for ADP and ATP binding to the $MgCl_2$-dependent low-affinity binding site on β (Khananshvili & Gromet-Elhanan, 1984; Gromet-Elhanan & Khananshvili, 1984a) are also very similar to the reported K_m values for ADP phosphorylation (Selman & Selman-Reimer, 1981) and for ATP hydrolysis by *R. rubrum* chromatophores (Horio et al., 1971). This similarity suggests that the $MgCl_2$-dependent low-affinity binding site for ATP, ADP, and P_i observed on the *R. rubrum* β subunit might be the catalytic site of the $RrF_0{\cdot}F_1$ ATP synthase. This suggestion does not rule out the possibility that the low-affinity catalytic site could be shifted into a high-affinity state upon reconstitution of the isolated β subunit into the multisubunit $F_0{\cdot}F_1$ complex. Ligand binding to catalytic and regulatory sites in the fully assembled $F_0{\cdot}F_1$ complex that leads to negative cooperative interactions between these sites could decrease this high-affinity state of the catalytic site, causing an apparent similarity between the K_d values and the steady-state K_m values (Grubmeyer et al., 1982; O'Neal & Boyer, 1984).

We have earlier modified the *R. rubrum* β subunit with a number of reagents known to interact with specific amino acid residues (Khananshvili & Gromet-Elhanan, 1982b; Khananshvili & Gromet-Elhanan, 1983a,b; Gromet-Elhanan & Khananshvili, 1984b). Recently, we have compared the capacity of such modified β preparations to bind P_i, ADP, and ATP with their capacity to rebind to β-less chromatophores and to restore their photophosphorylation activity (Khananshvili & Gromet-Elhanan, 1985). The results provide experimental evidence for the identification of the P_i binding site on β with the γ-phosphoryl site of ATP binding to the $MgCl_2$-dependent low-affinity nucleotide binding site on β. They also provide evidence for the direct involvement of this site in the catalytic activity of the $RrF_0{\cdot}F_1$ ATP synthase.

Registry No. P_i, 14265-44-2; Mg, 7439-95-4; ATP, 56-65-5; ADP, 58-64-0; ATP synthase, 37205-63-3.

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