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Binding of Adenine Nucleotides to the F_1-Inhibitor Protein Complex of Bovine Heart Submitochondrial Particles†

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ABSTRACT: The binding of ATP radiolabeled in the adenine ring or in the γ- or α-phosphate to F_1-ATPase in complex with the endogenous inhibitor protein was measured in bovine heart submitochondrial particles by filtration in Sephadex centrifuge columns or by Millipore filtration techniques. These particles had 0.44 ± 0.05 nmol of F_1 mg^{-1} as determined by the method of Ferguson et al. [(1976) *Biochem. J. 153*, 347]. By incubation of the particles with 50 μM ATP, and low magnesium concentrations (<0.1 μM MgATP), it was possible to observe that 3.5 mol of [γ-^{32}P]ATP was tightly bound per mole of F_1 before the completion of one catalytic cycle. With [γ-^{32}P]ITP, only one tight binding site was detected. Half-maximal binding of adenine nucleotides took place with about 10 μM. All the bound radioactive nucleotides were released from the enzyme after a chase with cold ATP or ADP; 1.5 sites exchanged with a rate constant of 2.8 s^{-1} and 2 with a rate constant of 0.45 s^{-1}. Only one of the tightly bound adenine nucleotides was released by 1 mM ITP; the rate constant was 3.2 s^{-1}. It was also observed that two of the bound [γ-^{32}P]ATP were slowly hydrolyzed after removal of medium ATP; when the same experiment was repeated with [α-^{32}P]ATP, all the label remained bound to F_1, suggesting that ADP remained bound after completion of ATP hydrolysis. Particles in which the natural ATPase inhibitor protein had been released bound tightly only one adenine nucleotide per enzyme. The results indicate that one of the first events that occurs during ATP hydrolysis by the F_1-inhibitor protein complex is the binding of two to three adenine nucleotides to sites that apparently are not hydrolytic. In addition, it was found that in the complex, the affinity of two to three of its adenine nucleotide binding sites is higher than in particulate enzymes devoid of the inhibitor protein.

The membranes of mitochondria, chloroplasts, and bacteria have an enzyme known as H^+-ATPase synthase or H^+-ATPase. This enzyme utilizes the energy of electrochemical H^+ gradients to catalyze the synthesis of ATP; under some conditions, the enzyme hydrolyzes ATP, and this results in the formation of electrochemical H^+ gradients (Mitchell, 1961). The enzymes have a hydrophobic portion (F_o) that transports H^+ across the membrane to a hydrophilic moiety known as F_1. The latter catalyzes the synthesis and hydrolysis of ATP [for reviews, see Cross (1981), Senior and Wise (1983); Hatefi (1985a), Futai et al. (1989), and Senior (1990)] and is formed by five different subunits with a stoichiometry α_3, β_3, γ, δ, and ϵ. In addition, the mitochondrial F_1 has a detachable low molecular weight protein, known as the natural ATPase inhibitor (Pullman & Monroy, 1963), that inhibits ATP hydrolysis, the ATP-dependent reactions of the inner mitochondrial membrane (Asami et al., 1970; Ernster et al., 1973), and the initial events of oxidative phosphorylation (Gomez-Puyou et al., 1979; Harris et al., 1979; Schwerzman et al., 1981) and favors the accumulation of ATP as driven by electron transport (Beltran et al., 1986).

Mitochondrial F_1-ATPase has six adenine nucleotide binding sites, three of which are considered catalytic; the other are

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considered regulatory in both mitochondrial F_1 (Slater et al., 1979; Kironde & Cross, 1986; Rosing et al., 1975; Fitin et al., 1979; di Pietro et al., 1980; Vasilyeva et al., 1980; Cross & Nalin, 1982; Drobinskaya et al., 1985; Martins et al., 1988) and CF_1 (Xue & Boyer, 1989; Guerrero et al., 1990; Milgrom et al., 1990, 1991; Milgrom, 1991). As the mechanisms involved in the synthesis and hydrolysis of ATP by the mitochondrial enzyme differ from those of other translocating ATPases (Webb et al., 1980; Pedersen & Carafoli, 1987a,b), it is possible that the tight binding of adenine nucleotides during a catalytic cycle is part of the intermediate states of such event. Thus, measurements of the occupancy of the binding sites before the completion of a single hydrolytic cycle, as well as at steady-state hydrolysis, may shed light on the sequence of steps that take place during the reaction.

Even though it is probable that synthesis and hydrolysis of ATP take place with F_1–inhibitor protein complexes (Schwerzman & Pedersen, 1981; Beltran et al., 1986), few studies have been done on the binding of adenine nucleotides to these complexes. These have dealt with the binding of nucleotides to soluble F_1 reconstituted with inhibitor protein (Klein et al., 1981; di Pietro et al., 1988), to particles that contained endogenous inhibitor protein (Harris et al., 1977; Myers & Boyer, 1983), and to soluble F_1 during its reconstitution with the inhibitor protein (Di Pietro et al., 1988; Penin et al., 1988). Here the characteristics of adenine nucleotide binding to F_1–inhibitor protein complexes have been further studied in submitochondrial particles. The results show that the enzyme binds tightly three to four adenine nucleotides before a single catalytic cycle takes place; at the steady-state of hydrolysis, these nucleotides undergo continuous exchange with medium nucleotides. The data are in apparent contrast with results in soluble F_1 reconstituted with inhibitor protein, in which it was shown that exchange of bound nucleotides was largely impaired (Klein et al., 1981; di Pietro et al., 1988), but in agreement with Penin et al. (1988), who showed that the soluble reconstituted F_1–inhibitor protein complex could release some of its bound nucleotides when attached to submitochondrial particles.

Materials and Methods

Bovine heart mitochondria were prepared according to Low and Vallin (1963). Mn-ATP particles were prepared in the presence of succinate and $MnCl_2$ as described by Hatefi (1985b). Except in the experiment of Table VI, these particles were used in all experiments. In some cases, these particles were also prepared from mitochondria preincubated with 100 μM atractyloside (at least 10 min) before addition of ATP and sonication. MgATP particles were prepared (without succinate and $MnCl_2$) according to Beltran et al. (1986). Both types of particles had their ATPases largely controlled by the inhibitor protein (Beltran et al., 1986) and had an ATPase activity of around 0.4 μmol min^{-1} mg^{-1} as measured by an ATP-regenerating system.

The F_1 content of the particles was determined following the methodology of Ferguson et al. (1976). Briefly, 15 mg of particles was incubated for 1 h at room temperature in 200 mM sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA),[1] 4 mM ATP, and 10 mM triethanolamine chloride, pH 7.5; the solution also contained 10 μmol/mL iodoacetate, *N*-ethylmaleimide, and iodoacetamide. The particles were thereafter centrifuged and washed once in sucrose/EDTA/ATP media without the thiol-blocking agents. Afterward, the particles were suspended in the same media and incubated with 50 μM NBD-Cl until the ATPase activity was inhibited by more than 70%. The particles were either washed 5 times with sucrose/EDTA/ATP or dialyzed against 20 volumes of a suspension of Mg-ATP particles (20 mg/mL) in 250 mM sucrose for 24 h. At this time, 150 μM freshly prepared cysteine was added. After a 30-min incubation, the suspension was centrifuged. The supernatant was withdrawn, and from its absorbance at 475 nm, the amount of cysteine–NBD was calculated. The value obtained was considered to correspond to the amount of F_1 in the particles after correction for the percentage of inhibition of ATPase activity.

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

[γ-^{32}P]Phosphate was obtained from the Atomic Energy Commission (Brazil) or from ICN. [γ-^{32}P]ATP and [γ-^{32}P]ITP were prepared according to Glynn and Chappel (1964), [α-^{32}P]ATP was prepared as described by Maia et al. (1984), and [2,8,3-^{3}H]ATP and [2,8,3-^{3}H]ADP were obtained from ICN.

Measurements of ATPase and ITPase Activities. ATPase activity was measured spectrophotometrically in the presence of an ATP-regenerating system (Pullman et al., 1960). In some experiments, the amount of inorganic [^{32}P]phosphate formed from [γ-^{32}P]ATP or [γ-^{32}P]ITP was used to assay their hydrolysis. The reaction was arrested with 5% trichloroacetic acid (final concentration). After centrifugation, [^{32}P]phosphate was extracted into water-saturated isobutyl alcohol/benzene (1:1) as a phosphomolybdate complex; the radioactivity in the latter was used to estimate the amount of ATP or ITP hydrolyzed (Tuena de Gomez-Puyou et al., 1984).

Binding of Nucleotides to Particulate F_1. The standard conditions for the binding of radioactive nucleotides were as follows. Unless otherwise indicated, submitochondrial particles (0.1–0.2 mg of protein) were incubated in 50 μL of 50 mM Tris–acetate, pH 7.5, 20 mM KCl, 1 mM EDTA, 200 mM sucrose, and 50 μM labeled nucleotides. At the times indicated under Results, the mixture was filtered through Sephadex G-50 (medium) columns by the centrifugation technique (Penefsky, 1977); this procedure was followed by Myers and Boyer (1983) to estimate binding of adenine nucleotides to particulate F_1. The columns were previously equilibrated with 50 mM Tris-HCl, pH 7.5, 20 mM KCl, and 200 mM sucrose. The filtrate was used to measure radioactivity and protein. The amount of protein recovered after filtration was about 70%; the radioactivity of the mixtures filtered without particles was less than 0.001%. The specific activity of [^{3}H]AdN was about 2×10^5 cpm/nmol, whereas that of the [^{32}P]AdN was between 2×10^5 and 10×10^5 cpm/nmol. The variation in the binding values was about 10%.

In some experiments, 50 μM Ap5A, 1 μM FCCP, and 100 μM carboxyatractyloside or atractyloside were included in the reaction mixture; they did not affect the level of radioactivity found in the particles (not shown).

The kinetics of adenine nucleotide exchange were determined with the rapid filtration system of Biologic Instruments, RFS-04. Submitochondrial particles were incubated as described above, and 50 μL was applied to 0.45-μm Millipore filters. The filter was washed (using a pipet) with 1 mL of the same solution used to equilibrate the Sephadex columns. Subsequently, the solution of the syringe containing cold nucleotide was passed through the filter. The flux changed between 4 mL/s (time of 100 ms) to 0.1 mL/s (time of 5 s);

[1] Abbreviations: AMPPNP, adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; NBD-Cl, 7-chloro-4-nitro-2,1,3-benzoxadiazole; Tris, tris(hydroxymethyl)aminomethane.

Table I: Binding of Various Nucleotides to Particulate F_1[a]

expt	nucleotide tested	binding (mol of AdN/mol of F_1)
1	[^{3}H]ATP	3.5 ± 0.45 (3)
2	[α-^{32}P]ATP	3.8 ± 0.42 (3)
3	[γ-^{32}P]ATP	3.5 ± 0.11 (12)
4	[^{3}H]ADP	3.6 ± 0.13 (7)
5	[^{3}H]ADP-Mg	3.4 ± 0.38 (3)
6	[γ-^{32}P]ITP	0.9 ± 0.31 (3)

[a] The binding of the indicated nucleotides was measured in 50 μL of an incubation mixture that contained 50 mM Tris-HCl, pH 7.5, 200 mM sucrose, 1 mM EDTA, and 0.2 mg of particle protein; the indicated radioactive nucleotides were added at a concentration of 50 μM. In experiment 5, 2 mM $MgCl_2$ was included. After 15 s of incubation at room temperature, the mixtures were filtered once through Sephadex columns equilibrated with 200 mM sucrose and 50 mM Tris-HCl, pH 7.5. The filtrates were used for determination of radioactivity and protein. The numbers in parentheses indicate the number of experiments.

regardless of the time, all filters were washed with a total of 0.5 mL of cold nucleotide solutions. In the procedure, all the protein that was applied to the filter was recovered.

Determination of the Adenine Nucleotide Content of Submitochondrial Particles. Adenine nucleotides were extracted from the particles with 1 N perchloric acid. The supernatant was neutralized with 5 M K_2CO_3 and perchlorate removed. Nucleotides were separated by HPLC (Waters) in a radial PAK SAX P 3082AOI column that was eluted either isocratically with 0.25 M KH_2PO_4 and 0.5 M KCl, pH 5.0, or by 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 0.5 M KCl, pH 5.0. The amount of ADP and ATP was calculated with external standards. The content of ADP was also quantitated enzymatically as described by Bergmeyer (1985). Essentially the same results were obtained.

Results

Binding of Nucleotides to F_1 of Submitochondrial Particles before the Completion of One Hydrolytic Cycle. Relatively high concentrations of particles are required for adenine nucleotide binding assays. Thus, in measurements of the binding of ATP to particulate F_1, important decreases in ATP concentration may take place unless the rates of ATP hydrolysis are substantially diminished. Here we diminished the rate of ATP hydrolysis by including EDTA in the reaction mixture. In the time of the experiment shown in Table I, less than 10% of the ATP added was hydrolyzed. In these conditions, 3–4 mol of nucleotides was bound per mole of F_1 with either [^{3}H]ATP, [α-^{32}P]ATP, or [γ-^{32}P]ATP (Table I, experiments 1–3). The particles also bound 3–4 ADP per F_1, either at low or at high Mg^{2+} concentrations (Table I, experiments 4 and 5). With [γ-^{32}P]ITP, only 1 mol was bound per mole of F_1. This suggests that most of the binding sites detected with adenine nucleotides were specific for such nucleotides.

In the presence of EDTA buffer which poised the concentration of ATP-Mg below 0.1 μM (Fabiato & Fabiato, 1979), it was possible to follow the time course of adenine nucleotide binding before the completion of a single catalytic cycle. In the experiments outlined in Figure 1, hydrolysis of [γ-^{32}P]ATP took place at rates of 0.3 nmol min^{-1} mg^{-1}; this corresponded to a value of less than 1 enzyme turnover/min. Also as shown in Figure 1, the particles bound 3.5 mol of [γ-^{32}P]ATP/mol of F_1 in less than 30 s. The amount of [γ-^{32}P]ATP bound remained constant during the course of steady-state hydrolysis; it is noted that for the length of the experiment, the level of [γ-^{32}P]ATP in the reaction media did not decrease to a significant extent.

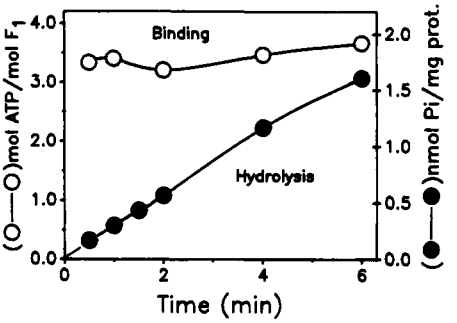

FIGURE 1: Hydrolysis and binding of [γ-^{32}P]ATP to submitochondrial particles before completion of one catalytic cycle. Submitochondrial particles (2 mg/mL) were incubated at room temperature in 50 μL of media that contained 50 mM Tris–acetate, pH 7.5, 200 mM sucrose, 20 mM KCl, 1 mM EDTA, and 50 μM [γ-^{32}P]ATP. At the indicated times, aliquots of 10 μL were transferred to 240 μL of 5.2% trichloroacetic acid, and inorganic [^{32}P]phosphate was extracted and determined as described under Materials and Methods. The binding of [γ-^{32}P]ATP was determined by filtering aliquots of 50 μL through Sephadex columns as described under Materials and Methods. The filtrates were used for the determination of radioactivity and protein.

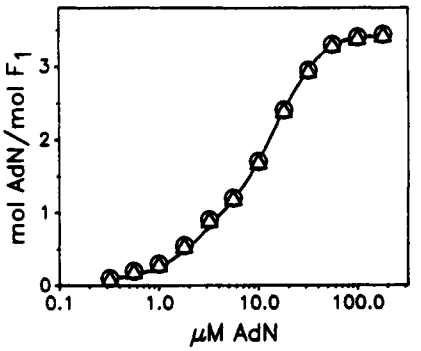

FIGURE 2: Binding of [^{3}H]adenine nucleotides to particulate F_1 at various concentrations of adenine nucleotides. Submitochondrial particles (0.2 mg of protein) were incubated as in Figure 1 with the indicated concentrations of [^{3}H]ADP (△) or [^{3}H]ATP (○). After 15 s of incubation, the particles were filtered once through Sephadex columns. The filtrates were used for the determination of protein and radioactivity.

Table II: Exchange of Nucleotides Bound to Particulate F_1[a]

expt	additions	mol of [^{32}P]Adn/mol of F_1
1	[α-^{32}P]ATP‖buffer‖	3.3
2	[α-^{32}P]ATP‖1 mM ATP‖	0.2
3	1 mM ATP‖50 μM [α-^{32}P]ATP‖	3.1

[a] The reaction mixture was as in Table I, except that the incubation mixture contained the indicated concentrations of radioactive or nonradioactive ATP. Filtration through Sephadex columns is indicated by ‖. Where indicated, the first filtrate was incubated for 15 s with the specified concentrations of radioactive or nonradioactive ATP and filtered for a second time. The final filtrates were used for the determination of radioactivity and protein.

Characteristics of the Tight Binding Sites. With adenine nucleotides, near-saturation of the binding sites was achieved with a concentration of 50 μM [^{3}H]ATP or [^{3}H]ADP; half-maximal binding was attained with 10 μM (Figure 2). In the binding curve, no cooperative behavior was observed.

The binding of adenine nucleotides to particulate F_1 could be due to the occupancy of empty sites or to a exchange reaction with previously bound nucleotides. Accordingly, particles were incubated with [α-^{32}P]ATP and subsequently filtered. The resulting particles were then exposed to 50 μM nonradioactive ATP, and filtered again. The particles of the second filtration had lost most of the bound radioactive nucleotides (Table II). Also it was found that particles that had

Table III: Total Nucleotide Content of Submitochondrial Particles after Hydrolysis of ATP[a]

condition	mol/mol of F_1		
	ADP	ATP	total
particles‖	1.2	1.9	3.1
particles + MgATP‖	1.5	2.2	3.7

[a] For the determination of adenine nucleotides in the particles of the first row, the particles were filtered through Sephadex columns. Those shown in the second row were allowed to hydrolyze 50 μM MgATP for 15 s and then filtered through Sephadex columns. The nucleotides were extracted after the addition of 1 mM EGTA and 1 N perchloric acid. After incubation for 30 min in the cold, the mixture was centrifuged and the supernatant neutralized with K_2CO_3. The amount of nucleotides in the supernatant was determined enzymatically or by HPLC (see details under Materials and Methods).

Table IV: Exchange of Bound ATP with Different Compounds[a]

expt	additions	mol of [γ-^{32}P]ATP/mol of F_1
1		3.8
2	1 mM ATP	0.3
3	1 mM ADP	0.3
4	1 mM AMPPNP	0.4
5	1 mM ITP	2.9
6	25 mM P_i	3.7

[a] The reaction mixture was as in Table I. After 5 s of incubation with [γ-^{32}P]ATP, the indicated compounds were added in a volume of 5 μL at the final concentration shown. One minute later, the mixture was filtered through a Sephadex column. The later filtrates were used for the determination of radioactivity and protein.

been incubated with nonradioactive ATP and filtered could bind [α-^{32}P]ATP to an extent similar to that which had been released. The content of adenine nucleotides (ATP plus ADP) in submitochondrial particles was about 3 mol/mol of F_1; this value was increased, but slightly, in particles that had been allowed to carry out hydrolysis (Table III). This indicates that the total content of adenine nucleotides of the particles did not change significantly upon exposure to ATP. Therefore, the overall results of Tables II and III indicate that binding was due to an exchange reaction with previously bound adenine nucleotides, and not to the filling of empty sites.

To determine the specificity of the adenine nucleotide tight binding sites, particulate F_1 was incubated with [γ-^{32}P]ATP for 5 s; at this time, a cold chase was made with different nonradioactive compounds (Table IV). ATP and ADP brought about extensive removal of [γ-^{32}P]ATP. A similar effect was observed with the nonhydrolyzable analog AMPPNP. Inorganic phosphate did not affect the level of bound nucleotides.

ITP produced the exchange of only one of the three to four bound [γ-^{32}P]ATP (Table IV). Since ITP is effectively hydrolyzed by particulate F_1, and since apparently its binding only occurs at catalytic sites (Cross, 1981; Hatefi, 1985a; Futai et al., 1989; Senior, 1990), the experiment of Table IV suggests that at least one of the sites that bound [γ-^{32}P]ATP had the capacity to carry out hydrolysis. This would seem to agree with Kironde and Cross (1986), who showed that in soluble F_1 only one adenine nucleotide was tightly bound to a catalytic site.

Kinetics of the Exchange Reaction. The kinetics of the exchange of tightly bound nucleotides in particulate F_1 were followed by the rapid filtration technique (see Materials and Methods). Particles were first loaded with radioactive ATP; this was followed by a cold chase of ATP or ITP, and the amount of [γ-^{32}P]ATP that remained tightly bound to the enzyme was determined at different times. With ATP, all the

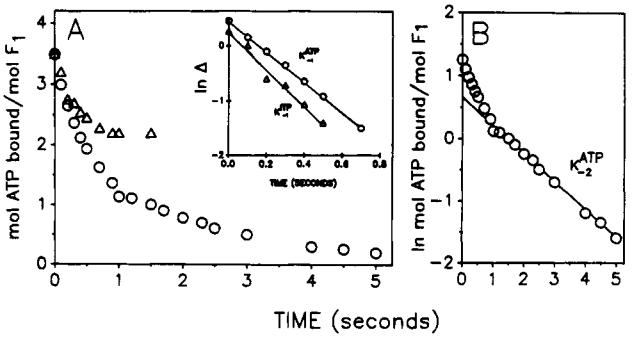

FIGURE 3: (A) Exchange kinetics with ATP or ITP. Submitochondrial particles (2.4 mg of protein/mL) were incubated with 50 μM [γ-^{32}P]ATP, in the medium described in Figure 1. Aliquots of 50 μL (0.12 mg of protein) were placed on the Millipore filter and washed under vacuum with 1 mL of the same reaction medium, but without ATP; in this step, the unbound nucleotides were removed. After that, the filter was washed, for the times shown on the abscissa, with the same reaction medium that contained 1 mM ATP (○) or 1 mM ITP (△). (B) Logarithmic plot of the data obtained after the addition of cold ATP [results of (A)]. The inset of (A) shows a plot of the ln of the amount of ATP bound against time (see text for details).

Table V: Rate Constants and Number of Tight Binding Sites[a]

nucleotide	k^{-1} (s^{-1})	n^1	k^{-2} (s^{-1})	n^2
ATP	2.79	1.57	0.45	1.92
ITP	3.20	1.30		

[a] The experimental conditions were as in the Figure 3. k^{-1} and k^{-2} are the rate constants for release of tightly bound ATP. n^1 and n^2 are the number of sites that exchange at faster or slower rates, respectively. These values were obtained from the intercept at the ordinate as obtained from the data in the inset of Figure 3A and from Figure 3B.

tightly bound nucleotides exchange within 5 s (Figure 3A). With ITP only one site exchanged; in this case, the level of the remaining bound nucleotides (2.2 mol of ATP/mol of F_1) was not modified in a 10-min incubation time (not shown).

The velocity of exchange with ATP did not fit a single exponential (Figure 3A); rather, the exchange took place with two clearly distinguishable rates (Figure 3B). A value of 0.45 s^{-1} for the slower rate was obtained from the slope of Figure 3B. The inset of Figure 3A shows the plot from which the rate constant of the fast component was calculated without the interference of the slow component (Ferst, 1977); in this, the ln of the difference between the experimental values at a particular time, as extrapolated from the linear portion of the graph, was plotted against time. Values of 2.79 and 3.20 s^{-1} were obtained for ATP and ITP, respectively (Table V). The data therefore suggest that the hydrolytic site exchanged tightly bound ATP with medium ATP at a rate 6 times faster than that of the nonhydrolytic site.

To gain further insight into the nature and properties of the ATP binding sites of particulate F_1, particles were incubated with [γ-^{32}P]ATP. At 15 s of reaction time, less than 2% of the added ATP had been hydrolyzed (curve 2, Figure 4), and 3.5 mol of ATP was bound per mole of F_1 (curve 1 of Figure 4); this latter value corresponded to 12% of the total [γ-^{32}P]ATP in the medium. At this stage, a chase of 1 mM nonradioactive ATP was applied, and the amount of radioactivity remaining in the particles and the amount of radioactive phosphate formed were determined (curves 3 and 4, Figure 4). The amount of radioactivity in the particles decreased upon the addition of cold ATP. If all or part of the bound radioactive ATP had been committed to hydrolysis, a burst in inorganic [^{32}P]phosphate would have taken place. Since the release of radioactive ATP was not accompanied by a proportional increase in radioactive inorganic phosphate

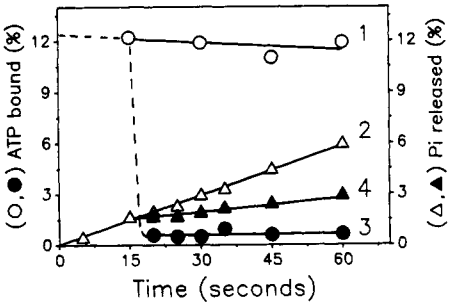

FIGURE 4: Inorganic phosphate released after a cold chase with 1 mM ATP. Submitochondrial particles (4 mg/mL) were incubated at room temperature in a medium containing 50 mM Tris–acetate, pH 7.5, 200 mM sucrose, 20 mM KCl, 0.1 mM $MgCl_2$, 1 mM EDTA, and 50 μM [γ-^{32}P]ATP. After 15 s of reaction, an aliquot was transferred to a tube that contained 1 mM ATP, 0.1 mM $MgCl_2$, and 5 mM EDTA (final concentrations). In this case, the ATP–Mg complex increased from 0.1 μM (curves 1 and 2) to 0.38 μM (curves 3 and 4). At the indicated times, two aliquots of each tube were withdrawn, and both [γ-^{32}P]ATP binding (O, ●) and inorganic [^{32}P]phosphate formed (Δ, ▲) were determined as described in Figure 1. The specific hydrolytic activity obtained from the data in curves 2 and 4 was 0.75 and 3.3 nmol of P_i mg^{-1} min^{-1}, respectively.

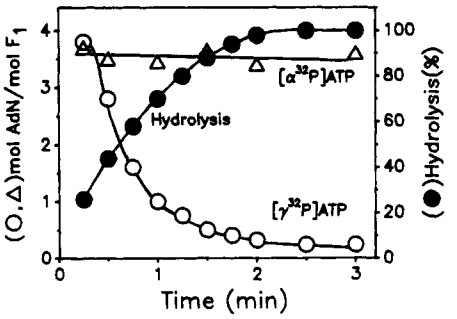

FIGURE 5: Binding of [α-^{32}P]ATP and [γ-^{32}P]ATP to particulate F_1 at different times of incubation. Submitochondrial particles (5 mg of protein/mL) were incubated in the medium of Figure 1 that in addition contained 0.5 mM $MgCl_2$ and 50 μM [α-^{32}P]ATP (Δ) or [γ-^{32}P]ATP (O). At the indicated times, aliquots of 50 μL were filtered once through Sephadex columns to determine adenine nucleotide binding. Aliquot were also taken from the tubes that contained [γ-^{32}P]ATP in order to determine ATP hydrolysis (●).

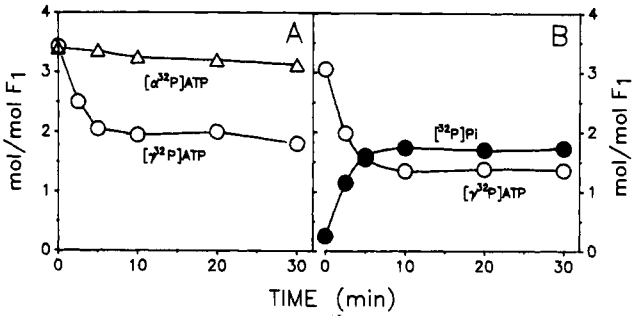

FIGURE 6: Hydrolysis of bound ATP by particulate F_1 in media free of ATP. (A) Particles were incubated as in Figure 1 with 50 μM [α-^{32}P]ATP (Δ) or [γ-^{32}P]ATP (O). After 15 s, the mixtures were filtered. At the times indicated, aliquots of the filtrates were filtered for a second time through Sephadex columns (time before second filtration in the figure). The amounts of radioactivity and protein were determined in the filtrates. The sample at zero time did not receive a second filtration. (B) Particles were incubated as in (A) with [γ-^{32}P]ATP and filtered after 15 s. At the indicated times, aliquots of the filtrate were withdrawn and added to 5% trichloroacetic acid (time before TCA addition in the figure), and the amount of inorganic [^{32}P]phosphate formed was assayed (●). In the sample at zero time, the first filtrates were received in trichloroacetic acid.

Table VI: Binding of Adenine Nucleotides to F_1 of Different Types of Submitochondrial Particle Preparations[a]

expt	particles	nmol of AdN/mg of protein	nmol of F_1/mg of protein	mol of AdN/mol of F_1
1	MgATP	1.2	0.4	2.8
2	MgATP/succ-Mn^{2+}	1.7	0.4	3.9
3	noncontrolled[b]	0.4	0.4	1.0
4	ammonium–Sephadex	0.4	0.4	0.9
5	Klein's particles[c]	0.4	0.4	1.1

[a] The reaction mixture was as in Table I, with 50 μM [γ-^{32}P]ATP. The F_1 content of these particles was determined as described under Materials and Methods. In all cases, the amount of ATP hydrolyzed was less than 10%. [b] Particles as obtained in experiment 2 were incubated for 3 h at 38 °C in a solution containing 50 mM Tris–acetate, pH 7.5, 200 mM sucrose, and 150 mM KCl, before the binding determination. This treatment promotes the release of the natural inhibitor protein (Beltran et al., 1986). [c] Klein's particles refer to particles prepared as described by Klein et al. (1981).

(curve 4 of Figure 4), the released radioactivity corresponded to [γ-^{32}P]ATP. In addition, the results also indicate that radioactive ATP already bound to the hydrolytic site could exchange with medium ATP. Therefore, the data indicate that in the conditions of the experiment, the splitting of bound ATP was the rate-limiting step of the overall reaction.

The exchange reaction was also studied during the steady-state of [γ-^{32}P]ATP hydrolysis poised at relatively high rates by high Mg^{2+} concentrations (Figure 5). In these conditions, a progressive decrease in the radioactivity bound to the particles was observed as nonradioactive ADP accumulated in the media. No such decrease was observed when [α-^{32}P]ATP was used (Figure 5); in this case, after all the ATP was hydrolyzed, the enzyme remained with three to four tightly bound radioactive ADP. These observations indicate that during steady-state hydrolysis, bound and medium nucleotides were in continuous exchange.

Stability of [γ-^{32}P]ATP Bound to Particulate F_1. Particles were incubated with [γ-^{32}P]ATP or [α-^{32}P]ATP and subsequently filtered (Figure 6A). At various times, the loaded particles were again filtered, and the amount of radioactivity that remained in the particles was determined. The results showed that with [γ-^{32}P]ATP, the radioactivity in the particles obtained in the second filtrate decreased progressively from 3.5 to 1.8 mol/mol of F_1 as the time between the first and second filtrates increased (Figure 6A). This was accompanied by a corresponding increase of inorganic [^{32}P]phosphate (Figure 6B). Therefore, in the absence of medium nucleotides, two hydrolytic sites became clearly evident.

In contrast to the experiments with [γ-^{32}P]ATP, particles that were incubated with [α-^{32}P]ATP retained a constant level of radioactivity after the second filtration (Figure 6A). The results obtained with the two types of radioactive ATP showed that although ATP was hydrolyzed, the resulting ADP remained bound to the enzyme. This will be in accordance with the data on the adenine nucleotide content of submitochondrial particles (Table III) that showed that the total content of adenine nucleotides was nearly the same in the stock particles and in those that had been allowed to carry out hydrolysis.

Binding of Adenine Nucleotides by F_1 in Different Types of Particles. The content of F_1 of the particles used in the described experiments, as well as that of other types of submitochondrial particles, was determined. All types of particles had nearly the same content of F_1 per milligram of protein (Table VI); however, there were important variations in the binding capacity of the different preparations. From the data (Table VI), it was apparent that particles that exhibited high rates of ATP hydrolysis, bound tightly only one adenine nucleotide per enzyme (experiments 3 and 4) under conditions in which less than 10% of the added ATP was hydrolyzed. In

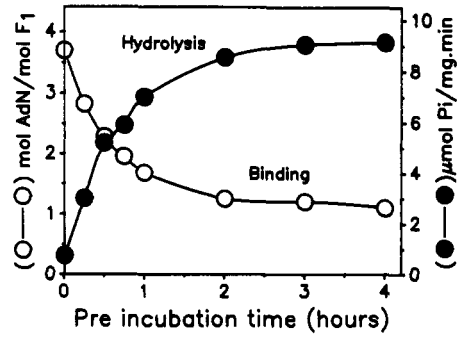

FIGURE 7: Correlation between the hydrolytic activity and the capacity to exchange tightly bound nucleotides. Submitochondrial particles (15 mg of protein/mL) were incubated at 38 °C in a medium containing 50 mM Tris–acetate, pH 7.5, 200 mM sucrose, and 150 mM KCl. At the times indicated, two aliquots of 10 μL were withdrawn. One was transferred to 40 μL of a medium containing 50 mM Tris–acetate, 200 mM sucrose, 1.25 mM EDTA, and 62.5 μM [α-^{32}P]ATP. After 15 s, the amount of radioactive adenine nucleotide bound was determined after filtration through Sephadex columns (○). The second aliquot was used to determine the ATP hydrolysis (●) spectrophotometrically as described under Materials and Methods.

contrast, in particles in which the hydrolytic activity was largely controlled, three to four nucleotides bound tightly per F_1 (experiments 1 and 2).

The correlation between the level of ATPase activity and the binding capacity of submitochondrial particles is also shown in Figure 7. A progressive increase in the specific activity of the ATPase of controlled particles was accompanied by a decrease in the capacity to bind nucleotides.

Discussion

The data of this work show that submitochondrial particles that have their F_1 controlled by the inhibitor protein could exchange three to four adenine nucleotides per enzyme. That the exchange took place at the level of F_1, and not at the level of the adenine nucleotide translocator or through its activity, is evidenced by the following observations: (a) The exchange was not affected by the presence of atractyloside or carboxyatractyloside in the incubation media. (b) The exchange was not diminished in particles that had been prepared by adding atractyloside (100 μM) in the sonication step (data not shown); according to Klingenberg (1977), in these conditions atractyloside drastically diminished the activity of the translocator. (c) AMPPNP is poorly transported by the translocator in submitochondrial particles (Klingenberg, 1977), yet we observed that AMPPNP caused rapid release of previously bound radioactive nucleotides (Table IV). In soluble F_1-ATPase reconstituted with the inhibitor protein, Klein et al. (1981), Di Pietro et al. (1988), and Milgrom (1991) showed that the complex had a low capacity to exchange adenine nucleotides. We have observed the same behavior in preparations of native soluble F_1 in complex with the inhibitor protein as isolated from MgATP particles (unpublished data). Apparently, and in agreement with the data of Valdez and Dreyfus (1987) and Okada et al. (1986), there are important differences between the soluble and particulate enzymes.

Of relevance to the action of the inhibitor protein, it is of interest to compare the exchange of adenine nucleotides in particulate F_1 with and without control by the inhibitor protein. Penin et al. (1988) showed that progressive abolition of the inhibitory action of the protein on the ATPase activity of particulate F_1 was accompanied by release of adenine nucleotides. In confirmation, we observed that as particulate F_1 gradually lost control by the inhibitor protein, the amount of exchangeable adenine nucleotides diminished (Figure 7). Hence, the results indicate that the release of hydrolysis in particulate F_1 is accompanied by a loss in the affinity for adenine nucleotides. However, in conditions of steady-state ATP hydrolysis, we observed a rapid exchange of tightly bound adenine nucleotides; this was not observed in the experiments of Penin et al. (1988) with particulate F_1–inhibitory protein complexes that had been previously formed with the soluble enzyme.

Nature of the Adenine Nucleotide Binding Sites. With respect to the nature of the tight binding sites, several authors (Cross, 1981; Futai et al., 1989; Senior, 1990) proposed that the ITP binding site(s) are catalytic. Particles with F_1 in complex with the inhibitor protein bound three to four nucleotides per enzyme; of these, only one exchanged with ITP. This suggests the existence of only one hydrolytic site. However, in the absence of medium nucleotides, two ATP tight binding sites capable of carrying out hydrolysis were detected. This set of observations thus suggests that in particulate F_1 in complex with the inhibitor protein, there is heterogeneity of the hydrolytic sites. In CF_1, similar conclusions were reached by Fromme and Graber (1989).

The distinct nature of two classes of adenine nucleotide binding sites in particulate F_1 in complex with the inhibitor protein was also evidenced by the results of the experiments on the kinetics of the exchange. The data showed that exchange took place with two clearly distinguishable rate constants, i.e., 2.8 and 0.45 s^{-1}. The more rapid probably corresponds to the site that catalyzed hydrolysis, since ITP exchanged with a rate constant of 3.2 s^{-1}. Therefore, in the particles with F_1 in complex with the inhibitor protein, the hydrolytic site exchanged with medium ATP at a rate 300 times faster than the hydrolytic event. Therefore, it would appear that the rate-limiting step in hydrolysis would be the splitting of ATP. Moreover, by adjusting the rate of hydrolysis to less than one catalytic cycle per minute, it was observed that each of the enzymes bound three to four adenine nucleotides before the completion of one cycle of hydrolysis. As a consequence, the exchange of medium ATP at three to four sites in the enzyme seems to be one of the first events in the hydrolysis of ATP.

It is worthwhile to point out that although the exchange of three to four ATP to the enzyme was the first event detected during hydrolysis of ATP, the finding does not necessarily imply that the binding of ATP at nonhydrolytic sites was necessary for hydrolysis. In fact, particles effectively hydrolyzed ITP, even though only one binding site was detected.

Occupancy of Binding Sites during Hydrolysis. It is known that during hydrolysis F_1 can bind one or two ADP at sites that are inhibitory for ATP hydrolysis (Fitin et al., 1979; Vasilyeva et al., 1980; Drobinskaya et al., 1985; Martins et al., 1988; di Pietro et al., 1988) and that the bound ADP remains in the enzyme after many catalytic cycles. Moreover, studies at the pre-steady-state of hydrolysis by particles whose ATPase had been activated showed that the enzyme bound one ADP that arose from ATP hydrolysis and that this bound ADP was inhibitory for hydrolysis (Martins et al., 1988). Here, it was observed that the enzyme bound three to four ATP before a catalytic cycle was completed. Therefore, in particles with both F_1 free or F_1 in complex with the inhibitor protein, one of the earliest events in ATP hydrolysis is the occupancy of adenine nucleotide binding sites. In free F_1, the sites would seem to be filled by ADP derived from ATP hydrolysis, whereas during ATP hydrolysis by the F_1–inhibitor protein complex, the sites would be occupied by ATP. In addition, it is interesting that in particulate F_1 in complex with

the inhibitor protein, two of the bound ATP were hydrolyzed after removal of medium ATP and that the resulting ADP remained bound to the enzyme (Figure 6A,B). As noted, in F_1 free of inhibitor protein, a similar process was observed except that the rate at which the bound ATP was hydrolyzed was much faster (Martins et al., 1988).

The adenine nucleotide binding sites of F_1 have been extensively explored [see Cross (1981), Hatefi (1985a), Futai et al. (1989), and Senior (1990)]. It is generally accepted that the catalytic sites exchange rapidly, require Mg^{2+}, and are nonspecific. The noncatalytic sites are assumed to have a low rate of exchange, are independent of Mg^{2+}, and have a high specificity for adenine nucleotides. Here we found that particulate F_1–inhibitor protein complexes bound three to four adenine nucleotides in less than 5 s, suggesting catalytic site behavior. The exchange in all sites was independent of Mg^{2+}, but only one bound ITP, even though we detected two hydrolytic sites. Therefore, it seems that particulate F_1 when complexed with the inhibitor protein has tight binding sites that have properties different from those of soluble F_1, and in agreement with Fromme and Graber (1989), it appears that sites with catalytic properties may be heterogeneous.

Registry No. ATP, 56-65-5; ADP, 58-64-0; ADP-Mg, 7384-99-8; ITP, 132-06-9.

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