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PHYSICAL AND ENZYMATIC PROPERTIES OF NUCLEOTIDE-DEPLETED BEEF HEART MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE

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Tightly bound adenine nucleotides are removed from multiple binding sites on beef heart mitochondrial ATPase (F_1) by chromatography on columns of Sephadex equilibrated with 50% glycerol. Release of nucleotides from the enzyme is associated with large decreases in sedimentation velocity (from 11.9 S to 8.4 S) which may be observed in concentrated solutions of polyols. Polyol-induced conformational changes are reversed when the enzyme is returned to dilute buffers. The nucleotide-depleted enzyme restores oxidative phosphorylation in F_1-deficient submitochondrial particles. Reconstitution of nucleotide-depleted F_1 with the ATP analog (adenylyl-imidodiphosphate (AMP-PNP), almost 5 moles of AMP-PNP per mole of enzyme, results in preparations with substantially inhibited ATPase activity which nevertheless restores oxidative phosphorylation and the ^{32}Pi-ATP exchange reaction in F_1-deficient submitochondrial particles. Incubation of the analog-labeled enzyme with ATP and Mg^{++} results in partial displacement of the analog and a time-dependent recovery of ATPase activity.

INTRODUCTION

Previous studies have shown that beef heart mitochondrial ATPase prepared in this laboratory contained 2 moles of ADP and 1 of ATP which were firmly bound to the protein and were not removed by repeated precipitation with ammonium sulfate or by chromatography on columns of Sephadex or Dowex-2-chloride but were released following acid denaturation (1). These results were similar to those of Harris et al. (2) except that the latter workers reported the presence of 5 moles of adenine nucleotide per mole of enzyme. Further support for the conclusion that the nucleotides were not covalently linked to the enzyme was provided by evidence that high concentrations of polyhydric alcohols, such as sucrose or glycerol, facilitated release of the nucleotides. Chromatography on columns of Sephadex equilibrated with 50% glycerol led to virtually complete removal of adenine nucleotides from the protein (1). This paper describes some of the physical and enzymatic properties of samples of F_1[1] which were virtually completely free of adenine nucleotides.

[1] Abbreviations: F_1, soluble mitochondrial ATPase; OSCP, oligomycin sensitivity-conferring protein; SU-particles, submitochondrial particles deficient in coupling factors; AMP-PNP, adenylylimidodiphosphate.

[2] N. E. Garrett and H. S. Penefsky, unpublished experiments.

METHODS

The preparation of F_1 (3), OSCP (4), and SU-particles (5) and the measurement of oxidative phosphorylation catalyzed by SU-particles reconstituted with coupling factors (4) was carried out as described. Adenine nucleotides were removed from F_1 by chromatography in the presence of 50% glycerol (1). A sample of the ammonium sulfate suspension of F_1 (3) containing 10–20 mg was centrifuged and the pellet redissolved with 1 ml of a buffer containing 100 mM Tris sulfate, pH 8, and 4 mM EDTA. An equal volume of saturated ammonium sulfate was added and after 10 min at 0°C, the enzyme was centrifuged and the pellet dissolved by adding 0.2 ml of a buffer containing 50% glycerol, 100 mM Tris-sulfate, pH 8, and 4 mM EDTA. The solution was chromatographed on a column of Sephadex G-50 medium (0.9 × 11.5 cm) equilibrated with the same buffer. Twenty fractions (0.3 ml per fraction) were collected from the column at a flow rate of about 0.03 ml per min. The enzyme usually appeared in fractions 9–14.

Binding of radioactive adenine nucleotides was measured in a pressure dialysis cell constructed according to the description of Paulus (6) by procedures described earlier (1). Protein was measured by refractometry (7) or by a spectrophotometric method (8). The latter values were corrected to dry weight when multiplied by 1.87. Protein concentration was also measured by the method of Lowry, et al. (9), and the values were divided by 1.18 to convert to dry weight. A unit of ATPase activity is defined as the amount of protein which catalyzes the hydrolysis of 1 μmole of ATP per min. Specific activity is defined as units per milligram of protein.

RESULTS

Removal of virtually all of the adenine nucleotides from F_1 was not accompanied by a change in the state of aggregation of the molecule. It may be seen in Fig. 1 that electrophoresis of F_1 on acrylamide gels containing 50% glycerol (left gel) or 1.5 M sucrose (right gel) did not alter the single protein disc pattern usually observed with the highly purified native enzyme (3). The somewhat slower migration of the enzyme in glycerol may reflect the higher viscosity of the gel but may also reflect glycerol-induced changes in the shape of the protein discussed below.

In order to investigate the possibility that promotion of the release of nucleotides was due to a polyol-induced conformational change in the protein, in a manner analogous to that reported by Meyers and Jakoby for a variety of enzymes (10, 11), a study was made of the effects of high concentrations of sucrose on the sedimentation velocity of native F_1. As shown in Fig. 2, inset, a considerable decrease in $S_{20,w}$ occurred, from a value of 11.9 S, the value expected at the protein concentration of the experiment (7) to an S value of 8.4 in the presence of 1.5 M sucrose. The solvent-induced conformational changes were reversible. If the glycerol-treated enzyme was transferred from, for example, a glycerol-containing buffer to a buffer containing 0.25 M sucrose, the sedimentation coefficient of the sample was indistinguishable from that of the native enzyme. It may be seen that the slope of the lines obtained from a plot of the radial position of the sedimenting boundaries vs. time of centrifugation was the same within experimental error for the two samples, Fig. 2.

Fig. 1. Acrylamide gel electrophoresis of native F_1 in the presence of 50% glycerol or 1.5 M sucrose. Gels containing glycerol or sucrose were prepared exactly as described earlier (3) except that urea was replaced by the polyol indicated. Other conditions of electrophoresis were as described (3). Each gel contained 20 μg of native F_1 Left gel, 50% glycerol. Right gel, 1.5 M sucrose.

The UV absorption spectrum of nucleotide-depleted F_1 is shown in Fig. 3. The A_{280}/A_{260} ratio of the preparation was 1.80, indicative of the low adenine nucleotide content of the preparation (1). The fine structure in the spectrum reflects contributions from tyrosine and phenylalanine. F_1 contains no tryptophane (12, 13).

The ATPase activity of nucleotide-depleted F_1 was unchanged from that of the native enzyme (1). The enzyme preparation also was capable of restoring oxidative phosphorylation in F_1-deficient submitochondrial particles. A large stimulation in the P/O ratio was observed when SU-particles were reconstituted with either native F_1 or glycerol-treated F_1, Table I. F_1 chemically modified with dicyclohexylcarbodiimide (14) did not restore oxidative phosphorylation in these particles.[2] Thus, it is unlikely that restoration of oxidative phosphorylation in the experiment occurred via important contributions to membrane structure (14, 15, 5).

It was reported earlier that samples of F_1 prepared in this laboratory contained 3 moles of tightly bound adenine nucleotide per mole of protein and, in addition two sites which engaged in rapidly reversible binding of AMP-PNP (1). Hilborn and Hammes (16) also reported the presence of two binding sites on F_1 which engaged in reversible binding of ADP. It would appear that there are five nucleotide-binding sites on the protein, three

[2] N. E. Garrett and H. S. Penefsky, unpublished experiments.

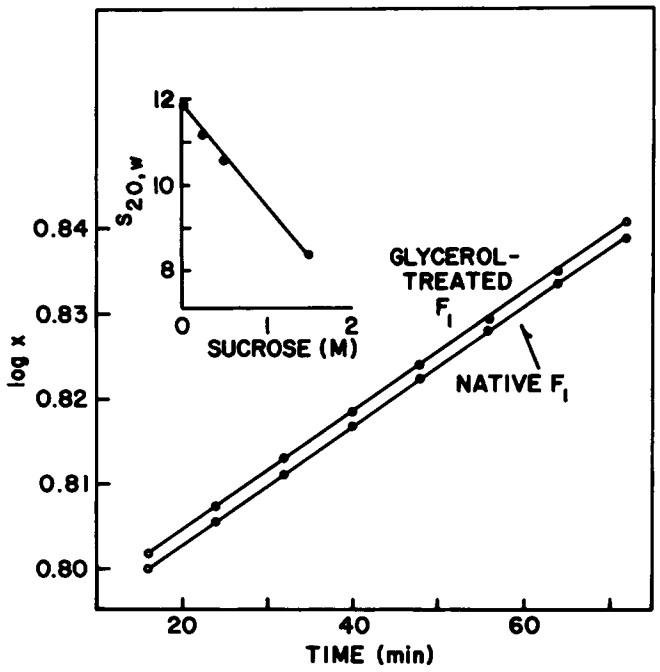

Fig. 2. Sedimentation velocity of native and nucleotide-depleted F_1. Glycerol-treated F_1 (A_{280}/A_{260} = 1.95) was prepared as described in Methods. Both the glycerol-treated enzyme and the native enzyme were chromatographed on columns of Sephadex G-50 equilibrated with 0.25 M sucrose, 50 mM Tris-sulfate, pH 8.0, 2 mM EDTA. The protein concentration was 4.1 mg/ml. Sedimentation velocity was measured in a Spinco Model E ultracentrifuge as described earlier (7). The sedimentation coefficient, $S_{20,w}$, for both the native enzyme and the glycerol-treated enzyme was 11.2. Inset: $S_{20,w}$ for native F_1. The buffer was the same as that given above except for the concentration of sucrose. Progress of the sedimenting boundaries in 0.5 and 1.5 M sucrose was monitored at 280 nm with the UV scanner attachment to the ultracentrifuge. All of the observed s values were corrected for the effects of solvent viscosity and density (22). No corrections were made for possible effects of solvents on the partial specific volume of the protein.

of which bind nucleotides which are not readily released into solution, and two which participate in freely reversible binding. It may be expected, however, that variation will be observed among individual preparations of enzyme giving rise to heterogeneity in the number of sites occupied by tightly bound nucleotides. Binding of [^{3}H] ADP to such a preparation is described in Fig. 4. The A_{280}/A_{260} of the preparation was 1.52, which, according to a relationship developed between the absorbance ratio and nucleotide content of native F_1 (1), suggests a nucleotide to protein ratio of about 2. It might be expected that the preparation would exhibit about three binding sites for ADP. The total amount of ADP bound by the protein sample was in fact three (Fig. 4B). A Scatchard-type (17) plot of the data indicated approximately two sites characterized by a K_D of 1.8 μM (Fig. 4A). Additional binding of ADP was weaker.

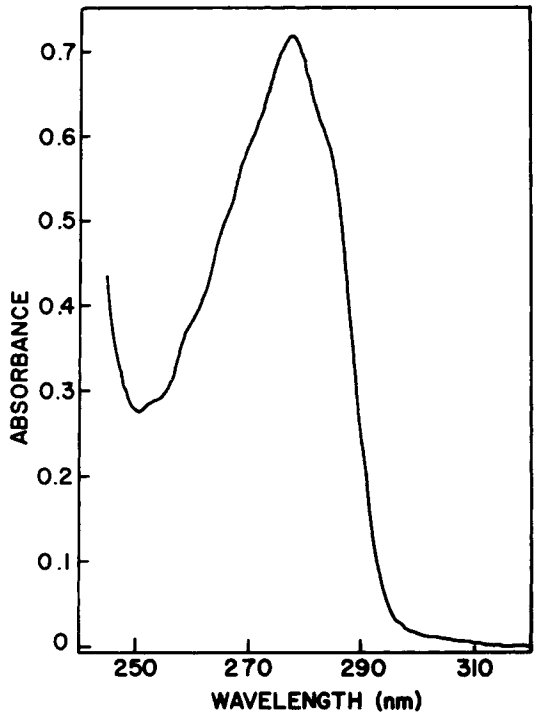

Fig. 3. UV absorption spectrum of glycerol-treated F_1. The preparation of glycerol-treated F_1 is described in Methods. A sample of the enzyme, 0.05 ml, was added to 0.95 ml of 0.25 M sucrose, 50 mM Tris-sulfate, pH 8.0, 2 mM EDTA. The final protein concentration was 1.4 mg/ml. UV absorbance was measured on a Cary Model 14 spectrophotometer equipped with the 0–1.0 absorbance units slidewire.

It was found that nucleotide-depleted F_1 could bind up to 5 moles of AMP-PNP per mole of protein and that the bound nucleotide resisted removal by ammonium sulfate precipitation or Sephadex chromatography (1). However, some of the bound analog could be displaced by ATP in the presence of Mg^{++}. As shown in Fig. 5, the initial analog to enzyme ratio in three different experiments ranged from 4.1 to 4.8. Following addition of 4 mM ATP (curves 1 and 2) or 5.8 mM ATP (curve 3), a time-dependent release of [^{3}H]-AMP-PNP from the protein occurred. The limiting value of analog bound to protein which was reached after about 1 hr ranged from 1.5 to 2 moles of AMP-PNP per mole F_1. The ATPase activity of F_1 labeled with 4 or more moles of AMP-PNP was about 10 μmoles of ATP hydrolyzed per min per mg protein (Fig. 5). However, release of the analog appeared to precede recovery of enzymatic activity (compare curves 1 and 4).

Although the ATPase activity of F_1 was thus substantially inhibited when the enzyme contained more than 2 moles of AMP-PNP per mole of protein, the analog-labeled enzyme exhibited an undiminished capacity to restore oxidative phosphorylation (Table II) and the ^{32}Pi-ATP exchange reaction (Table III) in SU-particles. The slightly higher rates of ATP synthesis and incorporation of ^{32}Pi into ATP observed with the analog-labeled enzyme

TABLE I. Restoration of Oxidative Phosphorylation by Nucleotide-Depleted F_1

Additions	Oxygen consumed (μatoms)	Pi uptake (μmoles)	P/O
0.5 mg Su-particles	3.93	0.019	0.005
+233 μg native F_1	4.20	1.14	0.271
+467 μg native F_1	4.64	1.42	0.306
+700 μg native F_1	4.52	1.56	0.345
+124 μg nucleotide-depleted F_1	4.98	0.906	0.182
+248 μg nucleotide-depleted F_1	4.84	1.07	0.221
+496 μg nucleotide-depleted F_1	3.38	1.06	0.314
+467 μg native F_1 + 0.5 mM 2, 4-dinitrophenol	2.68	0.025	0.009

Oxidative phosphorylation catalyzed by SU-particles reconstituted with F_1 and OSCP was measured as described previously (4). F_1 was depleted in adenine nucleotides by chromatography on a column of Sephadex G-50 equilibrated with 50% glycerol as described in Methods. The $A_{280}/_{260}$ ratio of the protein was 1.80. Each vessel contained 0.5 mg Su-particles, 50 μg OSCP, and F_1 as shown.

may be a reflection of the decreased ATPase activity in particles reconstituted with the inhibited enzyme. The ATPase activity of particles reconstituted with native F_1 was about two times higher than that observed with AMP-PNP-F_1 (Table III).

DISCUSSION

The physical properties of F_1 in concentrated solutions of polyols has provided insights into the mechanism of the release of tightly bound adenine nucleotides from the enzyme. It is clear from the acrylamide gel electrophoresis experiments (Fig. 1) that dissociation of the enzyme did not occur under these conditions. Rather, the large decreases in sedimentation velocity which were observed (Fig. 2) support the conclusion that high concentrations of sucrose caused large changes in the conformation of the molecule. These changes in S value were reminiscent of the reversible alterations in the structure of F_1 which occurred when the enzyme was incubated at 5°C, giving rise to an equilibrium mixture of 11.9 S, 9.1 S, and 3.5 S components (7). The 11.9 S value was that expected for the native enzyme at the protein concentration of the experiment while 9.1 S component was viewed as a possible intermediate in the formation of subunits (the 3.5 S component). Rewarming of the solution led, under the appropriate conditions, to reappearance of a single sedimenting boundary with a value of 11.9 S (7).

The results presented in this paper indicate therefore that high concentrations of polyols caused a change in the conformation of F_1 which decreased the affinity of the nucleotide binding sites. Since slow chromatography was more effective in removing nucleotides from the enzyme than more rapid chromatography (1), it would appear that the rate of dissociation of nucleotides from the enzyme as well was slow. In addition, it should be mentioned that the stabilization of F_1 which occurred in concentrated solutions of polyols (7) also may be an important factor in the ability to remove nucleotides. These experiments further indicate that the hydrodynamic profile of native F_1 was not directly

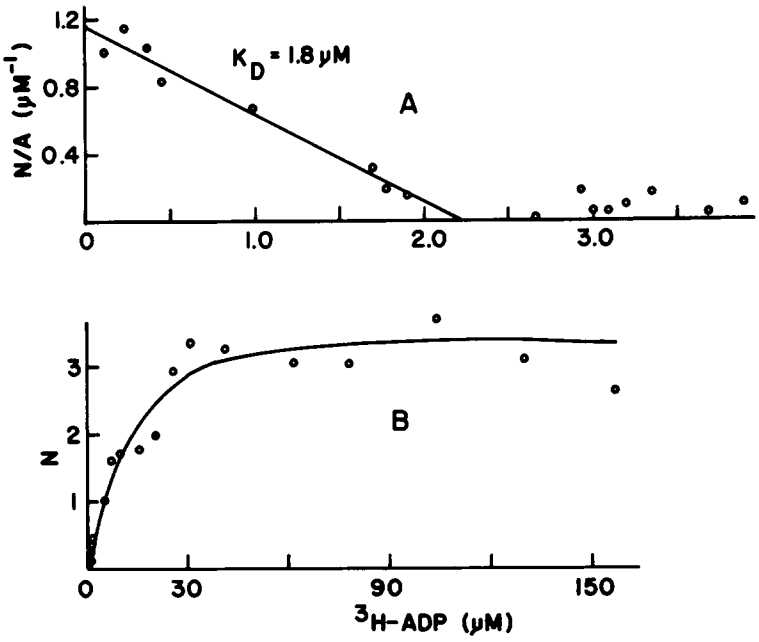

Fig. 4. Binding of [^{3}H]ADP to native F_1. A sample of native F_1 (3), 2.9 mg, was dissolved in 0.5 ml of a buffer which contained 0.25 M sucrose, 50 mM Tris-sulfate, pH 8.0, and 2 mM EDTA. An equal volume of saturated ammonium sulfate was added and the mixture allowed to stand 10 min at 0°C. The precipitate was collected by centrifugation, dissolved in 0.1 ml of 50 mM Tris-sulfate, pH 8.0, 2 mM $MgSO_4$, and the enzyme was applied to a Sephadex G-50 column (0.6 cm × 10 cm) equilibrated with the same buffer. The concentration of enzyme collected from the column was 5.5 mg/ml. The reaction mixture for binding contained in a final volume of 0.3 ml, 1.0 nmole of F_1, 50 mM Tris-sulfate, pH 8.0, 2 mM $MgSO_4$, and [^{3}H]ADP as shown (6.5 × 10^4 cpm/nmole). Binding of ADP to F_1 was measured in the pressure dialysis cell as described (1). A. Scatchard-type (18) plot of the binding data. B. Titration of F_1 with increasing concentrations of [^{3}H]ADP. N is the average number of moles of ligand bound to protein. A is the concentration of free ligand.

dependent on the presence of bound nucleotides since the sedimentation coefficient of the nucleotide-depleted enzyme was indistinguishable from the native enzyme when both were analyzed in dilute buffers, Fig. 2. The longer term stability of the enzyme in dilute buffers was, however, dependent on adenine nucleotides, since glycerol-treated enzyme solutions became turbid and formed precipitates after continued standing in 0.25 M sucrose.[2]

Rosing et al. concluded from studies of cold treatment of F_1 that transformation to the 9.1 S component was not accompanied by release of adenine nucleotides (18). Release was said to accompany formation of the 3.5 S component (18). According to the current study, however, it appears that alterations in protein conformation which result in considerable reduction in the sedimentation velocity of F_1 are important contributing factors in the release of nucleotides and that dissociation of the protein is not a necessary condition for release.

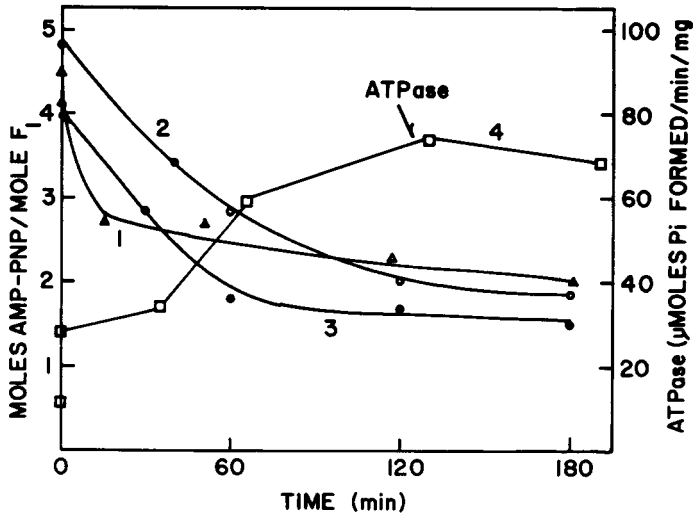

Fig. 5. Relationship between ATPase activity and AMP-PNP content of glycerol-treated F_1. Glycerol-treated F_1 (A_{280}/A_{260} = 1.92–1.95) was prepared as described in Methods. [^{3}H]AMP-PNP labeled F_1 was prepared as described in the legend to Table II. The ratio of moles of analog per mole of F_1 at the beginning of each of the three experiments is indicated by points on the ordinate. The reaction mixture contained in a final volume of 1.14 ml, 2 μM analog-labeled F_1, 4 mM (curves 1 and 2) or 5.8 mM (curve 3) ATP, 50 mM Tris-sulfate, pH 8, and 0.5 mM $MgSO_4$. Aliquots of the reaction mixture (0.15 ml) were transferred to the pressure dialysis cell, and, at the times indicated, binding was measured as described (1). The ATPase activity (curve 4) was measured in the experiment corresponding to curve 1. ATPase activity was measured as described in reference 23.

The enzymatic properties of glycerol-treated F_1 were remarkably similar to those of the native enzyme. Thus, the ATPase activity of the two preparations was identical (specific activity = 100 U/mg and there was no apparent difference in the ability of the two preparations to restore oxidative phosphorylation in F_1-deficient submitochondrial particles. These experiments do not, however, rule out the possibility that a rapid binding of nucleotides to the glycerol-treated enzyme preceded initiation of ATPase activity (1) or of the reactions of oxidative phosphorylation.

Studies on the properties of glycerol-treated F_1 reconstituted with almost 5 moles of AMP-PNP are relevant to the role of the multiple adenine nucleotide-binding sites in the mechanism of action of the enzyme. The observation that the ATPase activity of the enzyme was substantially inhibited when more than about two sites were occupied by the analog (Fig. 5) suggests that these sites are different from the hydrolytic site but may exert allosteric control on ATPase activity since AMP-PNP was readily displaced from the hydrolytic site by 4 mM ATP (19). The amount of the analog carried over into the reaction mixture for the measurement of ATPase activity in Fig. 5, about 0.05 nmoles, would not have inhibited ATPase activity if added directly.

The experiments of Fig. 5 suggest that the presence of AMP-PNP on the binding sites of F_1 perturbs the hydrolytic activity of the enzyme via an additional mechanism. Since

TABLE II. Coupling Factor Activity of F_1 Reconstituted with AMP-PNP

Additions	Oxygen consumed (μatoms)	Pi Uptake (μmoles)	P/O
I. 0.5 mg SU-particles	4.1	0.002	0.001
+ 52 μg AMP-PNP-F_1	3.3	0.199	0.061
+ 104 μg AMP-PNP-F_1	3.3	0.243	0.073
+ 53 μg native F_1	3.5	0.147	0.042
+ 106 μg native F_1	3.7	0.170	0.047
II. 0.5 mg SU-particles	2.15	0.006	0.003
+ 127 μg native F_1	2.4	0.243	0.102
+ 135 μg AMP-PNP-F_1	2.2	0.282	0.128

Oxidative phosphorylation catalyzed by reconstituted SU-particles was measured as described previously (4). Each vessel contained 0.5 mg SU-particles, 50 μg OSCP, and F_1 as shown. Glycerol-treated F_1 [A_{280}/A_{260}] = 1.95) was prepared as described in Methods and a sample was labeled with [^{3}H] AMP-PNP by passing the protein through a Sephadex G-50 column equilibrated with 0.25 M sucrose, 50 mM Tris-sulfate, pH 8.0, 2 mM $MgSO_4$, and 50 μM [^{3}H] AMP-PNP (3.2×10^4 cpm/nmole). The analog-labeled enzyme was then chromatographed on a second column of Sephadex containing 0.25 M sucrose, 50 mM Tris-sulfate, pH 8.0, 0.5 mM $MgSO_4$ in order to remove loosely bound and unbound radioactivity. The enzyme eluted from the second column contained 4.1 moles of AMP-PNP per mole protein (expt. I) and 4.7 moles per mole F_1 (expt. II). Native F_1 was freed of ammonium sulfate as described (3).

TABLE III. Restoration of the ^{32}Pi-ATP Exchange Reaction in SU-Particles with F_1-Containing AMP-PNP.

Additions	ATP[a] (μmoles)	(^{32}P)ATP (cpm/μmole)	ATPase (μmoles/min/mg)
2 mg SU-particles	9.09	0.000	0.02
+ native F_1	0.87	1.51×10^5	1.22
+ AMP-PNP-F_1	2.30	2.04×10^5	0.64

A sample of glycerol-treated enzyme (A_{280}/A_{260} = 1.95) was prepared as described in Methods and labeled with [^{3}H] AMP-PNP as described in the legend to Table II. The enzyme was allowed to stand overnight before chromatography on the second column to remove unbound and loosely bound nucleotides. The enzyme eluted from the second column contained 4.8 moles of [^{3}H] AMP-PNP per mole F_1. SU-particles were reconstituted with coupling factors by incubating in a total volume of 4.9 ml, 7.3 mg SU-particles, 0.6 mg OSCP, 0.15 M sucrose, 4.9 mM ATP, 12.3 mM Pi, 7.4 mM $MgCl_2$, and 1.1 mg of F_1 as shown. After incubation for 10 min at room temperature, the particles were collected by centrifugation after washing the top of the pellet and the walls of the centrifuge tube with 5 ml of 0.25 M sucrose, 10 mM Tris-Tes, pH 7.5. The particles were homogenized in 0.6 ml of the same buffer. The particles reconstituted with AMP-PNP-F_1 contained 0.52 nmoles of AMP-PNP per mg protein. The ^{32}Pi-ATP exchange reaction was measured as described previously (20). The reaction mixture contained 2 mg reconstituted SU-particles and ^{32}Pi (4.7×10^6 cpm/μmole). An aliquot of the deproteinized reaction mixture was neutralized with KOH and the ATP remaining was measured as described (21). ATPase activity of the reconstituted particles was measured by a spectrophotometric method as described (19).

[a]ATP remaining at the end of the reaction.

displacement of the analog from F_1 by ATP largely preceded recovery of ATPase activity, (curves 1 and 4), it seems possible that a time-dependent rearrangement of the structure of the molecule accompanies reactivation. It should be noted, however, that no lag was detected in the catalysis of oxidative phosphorylation by SU-particles reconstituted with AMP-PNP-labeled F_1.[2] The efficiency of such particles was at least equal to that of particles reconstituted with native F_1 (Tables II and III).

The tenacious binding of AMP-PNP to F_1 which is observed in solution was preserved when the enzyme was recombined with SU-particles. Neither uncouplers of oxidative phosphorylation nor the presence of an oxidizable substrate influenced binding. However, partial release was observed in the presence of ADP or of ADP and Pi.[2] These results suggest that there may be important differences in the manner of release of AMP-PNP from F_1 when the enzyme is in solution and when it is bound to the mitochondrial membrane.

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