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Evidence for the Presence and Role of Tightly Bound Adenine Nucleotides in Phospholipid-Free Purified *Micrococcus lysodeikticus* Adenosine Triphosphatase

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Abstract

[^{32}P]-labeled ATPase was isolated in a highly purified state from *Micrococcus lysodeikticus* strain PNB grown in medium supplemented with [^{32}P]orthophosphate. Selective extraction procedures allowed us to determine that at least 25% of the firmly bound label belonged to adenine nucleotides, ATP and ADP being present in equimolar amounts. However, no ^{32}P label was found to be part of phospholipids. This was confirmed by purification of the ATPase from cells fed with [2—^{3}H]glycerol. Using the luciferin–luciferase assay we estimated that ATPase freshly isolated by Sephadex chromatography (specific activity 10–14 μmole substrate transformed · min^{-1} · mg protein^{-1}) contained 2 moles ATP/mole of enzyme. The ratio fell with the age of enzyme and its purification by gel electrophoresis and this was paralleled by a loss of ATPase activity. The endogenous nucleotides were readily exchanged by added ADP or ATP. This result suggests that the sites for tight binding of adenine nucleotides are equivalent, although ADP seems to have a higher affinity for them. The last properties represent a peculiar characteristic of this bacterial ATPase as compared with other bacterial and organelle energy-transducing proteins.

Introduction

Soluble, purified bacterial adenosine triphosphatases (ATPases or BF_1 factors) resemble the coupling factors from mitochondria (F_1) and

chloroplasts (CF_1) [1–4] and, therefore, seem to be suitable models to study the molecular mechanisms underlying the process of energy transduction. These complex molecules may contain firmly associated nonprotein components such as nucleotides [5–8], lipids [9], and carbohydrates [10]. The soluble, purified mitochondrial and chloroplast coupling factors did not contain associated phospholipids [3, 4]. In the case of bacterial ATPases there is some controversy over this aspect. While the ATPases or BF_1 factors purified from *Streptococcus faecalis* [1, 11] and *Micrococcus lysodeikticus* [12, 13] did not show any apparent requirement of lipid for their activity, Peter and Ahlers [9] have recently presented evidence to support the claim that *Escherichia coli* ATPase is a lipoprotein requiring about 50 molecules of phospholipid per mole of enzyme for full activity.

F_1 and CF_1 factors contain tightly bound adenine nucleotides [1, 8, 14–16] whose significance in the mechanisms of oxidative and photosynthetic phosphorylation has been the subject of recent formulations [17–19]. On the other hand, very few studies have been carried out on the existence and role of tightly bound nucleotides in bacterial membrane ATPases. In 1972, Abrams and Nolan reported on the incorporation of [^{32}P]orthophosphate into the ATPase of *S. faecalis* [20], and subsequently Abrams et al. [5] found that ATP and ADP formed very stable complexes with that enzyme. Maeda et al. [21] studied the firmly bound nucleotides in highly purified ATPase from *E. coli*. In more recent reports, Harris et al. [21a] found ATP and ADP tightly bound to the coupling ATPase from *Paracoccus denitrificans* and Lee et al. [21b] studied the binding of exogenous nucleotides to the purified latent ATPase of *Mycobacterium phlei*.

In the present work we studied the possible cellular incorporation of ^{32}P into the plasma membrane ATPase of *M. lysodeikticus* as a sensitive means to detect phospholipids and/or nucleotides intimately associated with the protein. By this, we identified in highly purified BF_1 preparations the presence of ATP and ADP, but were unable to detect reasonable amounts of phospholipids in them. This last was confirmed by purifying ATPase from *M. lysodeikticus* grown in [2—^{3}H]glycerol. We determined by luciferin–luciferase assay the amount of firmly bound ATP and attempted to correlate its level with the ATPase activity of different catalytic forms of *M. lysodeikticus* enzyme.

Materials and Methods

Microorganism and Isolation of Membranes

The growth characteristics and conditions of culture in peptone–water–yeast extract medium of *Micrococcus lysodeikticus* strain PNB have

been described [22]. Membranes were isolated as already reported [13, 22]. For radioactive labeling experiments, the medium was supplemented with 0.5 mCi/liter [^{32}P]orthophosphate (Junta Energía Nuclear, Madrid, carrier free) or with 0.85 mCi/liter [2—^{3}H]glycerol (Radiochemical Centre, Amersham, England, specific activity 143 Ci/mole).

Solubilization and Purification of ATPase

The ATPase was solubilized by suspending the membranes in low ionic strength (3 mM) tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.5) as described elsewhere [23] and purified by Sephadex G-200 filtration [13] but using 30 mM Tris-HCl (pH 8.0) as effluent [24]. This yielded form B_A of the ATPase, nonstimulated by trypsin, whose specific activity ranged from 10–15 μmoles $P_i \cdot min^{-1} \cdot mg\ protein^{-1}$. In some instances, the enzyme was purified by preparative gel electrophoresis which yielded enzyme forms of lower specific activity [22] (specific activity range 2–4 μmoles $P_i \cdot min^{-1} \cdot mg\ protein^{-1}$). For specific purposes, the Sephadex G-200 preparations were repurified by semipreparative gel electrophoresis. The purified enzymes were stored at −20°C (2–3 mg protein/ml 30 mM Tris-HCl, pH 8.0) and their ATPase activity and ATP content measured at dinferent intervals to follow the time-related change of these parameters.

Radioactively labeled ATPase was isolated by the same procedure from cells grown up to the stationary phase in medium supplemented with [^{32}P]orthophosphate. Under these experimental conditions about 50% of the radioactivity was incorporated into the cells.

Determination of ATPase and Protein Content

ATPase activity was determined by incubating the enzyme (5–10 μg) with 8 mM ATP : CA^{2+} at 37°C and the P_i liberated measured colorimetrically [13, 23]. Where stated, trypsin was added to the ATPase assay at a weight ratio of 1 : 5 [24] in order to estimate trypsin stimulability of ATPase. One unit of enzyme activity is defined as the amount of enzyme able to liberate 1 μmole P_i in 1 min and specific activity as the number of units per milligram of protein. Protein content was estimated by the method of Lowry et al. [25] using bovine serum albumin (Sigma Chemical Company, St. Louis, Missouri) as standard.

Extraction and Identification of Phospholipids

The phospholipids eventually associated with the purified ATPase and/or the peak eluting in the void volume of the Sephadex column (i.e., with a $V_e/V_o = 1$) were extracted from radioactively labeled fractions as

follows. Aliquots of each fraction were shaken with 3 vol of chloroform–methanol (2:1, vol/vol) at room temperature and then centrifuged (5000 × *g*), 15 min) to facilitate the separation into two phases. The organic phase was drawn off, and the aqueous phase reextracted twice with the same volume of chloroform–methanol. The organic phases were pooled, taken to dryness with a rotary evaporator, and dissolved in a small volume of chloroform–methanol for subsequent identification by thin-layer chromatography. Phospholipids were identified by chromatography on silica gel thin-layer plates (Merck, Darmstadt, Germany) using the following solvent systems: (i) chloroform–acetone–acetic acid–water, 5:2:1:0.5 (vol/vol) for one dimension, or (ii) chloroform–methanol–water, 65:25:4 (vol/vol) in a first dimension and, after drying, solvent (i) in a second dimension. Lipids were visualized by exposure to iodine vapour [26]. For specific identification of phosphatidylglycerol, the plates were sprayed with 1% periodate and then with Schiff's reagent [27]. Radioactive samples were identified from their R_f values as compared with those of known standards (phosphatidylethanolamine, phosphatidylglycerol, phosphatidylchloline, and cardiolipin, all from Sigma Chemical Company) and, after scratching from the plates counted in Bray's Mixture [28], on a Nuclear Chicago Mark II liquid scintillation spectrophotometer.

Extraction and Identification of Nucleotides

Nucleotides associated with the ATPase were extracted as follows. (A) Fractionation after protein precipitation with trichloroacetic acid. Delipidated protein was precipitated with the same volume of 10% (wt/vol) trichloroacetic acid. After centrifugation (4000 × *g*, 30 min), the supernatant was withdrawn to estimate its nucleotide content and the pellet extracted first with 70% (vol/vol) ethanol–0.1% (vol/vol) perchloric acid and then with 70% ethanol. (B) Adsorption into charcoal following protein denaturation. The protein was denaturated by treatment with 8 M urea (Merck, Darmstadt) and 1 mM dithiothreitol (Calbiochem, San Diego, California) at room temperature. To the denaturated protein an equal volume of activated charcoal (Merck) (9% wt/vol) in 0.2 M KCl (hydrochloride, pH 2.0) was added and the mixture was magnetically stirred for 10 min. After centrifuging at 400 × *g* for 10 min, the supernatant was poured off and the pellet containing the nucleotides was extracted twice with 50% (vol/vol) ethanol in 0.5 M NH_4OH. The extracted nucleotides were recovered in the supernatants resulting after centrifugation at 4000 × *g* for 30 min. In order to identify nucleotides, the different extracts were electrophoresed, after reducing the volume by rotary evaporator, on Whatman 3 MM paper as reported [29]. Electrophoreses

were run at 400 V for 5 hr and the nucleotides visualized by ultraviolet absorption. The radioactive spots were localized on the paper with a Packard radiochromatogram scanner. ATP, ADP, and AMP (PL Biochemicals, Milwaukee, Wisconsin) were run as standards.

Polyacrylamide Gel Electrophoresis

This technique was carried out using the Tris-glycine system at alkaline pH (8.5 ± 0.2) as previously described [2]. Gels of 11×0.6 cm were used. Electrophoresis was run at a constant current of 2.5 mA/gel until the tracking dye (bromphenol blue) entered the gel and then the current rose to 5 mA/gel. Proteins were stained according to the procedure described by Fairbanks et al. [30].

To locate [^{32}P]-labeled ATPase, unstained gels were sliced into 1-mm pieces with a Mickle Gel Slicer (The Mickle Laboratory Engineering Company, Gomshall, Surrey, England). To each slice, 0.3 ml of 0.2% (wt/mol) sodium dodecyl sulfate (BDH Chemicals Ltd., Poole, England) in water was added. The slices were frozen and thawed three times, then shaken overnight at 30°C, and after suspension in Bray's solution counted in a Packard Tri-Carb liquid scintillation spectrometer. In some instances, gels were sliced longitudinally into two halves, one was stained for protein and the other, dried under vacuum, was used to locate radioactivity with a Packard Radiochromatogram Scanner.

Determination of ATP Content

ATP content was measured in samples of purified ATPase (350 μg protein equivalent to 1 nmole/150 μl) using the firefly lantern luciferin–luciferase assay (Sigma Chemical Company) [31]. ATP content was measured on ATPase samples without any treatment (run as controls to estimate the level of free or labile-bound ATP) or on ATPase denaturated with 8 M urea to facilitate release of tightly bound nucleotides. In other instances, the nucleotides were extracted with the two procedures outlined previously. ATPase denaturation which gave the best quantitative results (see Results) was used in most of the experiments.

Experiments designed to study the displacement of endogenous bound ATP with exogenous ADP were carried out as follows: purified ATPase (350 μg protein in 150–200 μl 30 mM Tris-HCl, pH 7.5) was incubated for 20 min at 40 or 20°C with concentrations of ADP ranging from 0.1 to 1 mM. To eliminate the excess of ADP which would interfere with the luciferin reaction, the incubation mixtures were filtered on a small column ($V_o = 1.8$ ml; $V_i = 5$ ml) of Sephadex G-100 and the ATP content of the ATPase peak eluting in the void volume was measured as already

described. The displacement of endogenous bound nucleotides by external ATP was studied with [^{32}P]-labeled ATPase. The protein (50 μg in 100 μl 30 mM Tris-HCl, pH 7.5) was incubated at 40°C for 20 min with ATP or ATP : Ca at concentrations ranging from 0.1 to 8 mM. After incubation, the samples were electrophoresed in polyacrylamide gels and the extent of exchangeable radioactivity was measured by comparison with a control incubated without added nucleotides.

Results

Isolation of ATPase From M. lysodeikticus *Grown in [32P]Orthophosphate*

Figure 1 illustrates the elution profile of ATPase activity and [^{32}P] radioactivity following Sephadex G-200 filtration of crude BF_1 isolated from

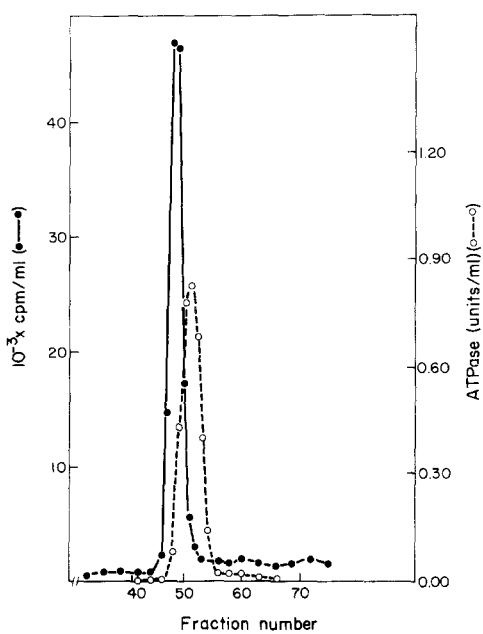

Figure 1. Purification of ^{32}P-labeled ATPase from *M. lysodeikticus*. Crude ATPase (0.5–0.6 mg protein/ml; 700,000 cpm/mg protein) was isolated from *M. lysodeikticus* grown in the presence of [^{32}P]orthophosphate and concentrated to 5–6 mg/ml by Amicon ultrafiltration with an XM 50 Diaflo membrane. After concentration, the protein was centrifuged at 100,000 × *g* for 1 hr to remove high-molecular-weight aggregates and 7–8 mg protein/1.5 ml of this preparation was charged into a column of Sephadex G-200 (total volume 450 ml; V_0 = 145 ml). The column was eluted with 30 mM Tris-HCl (pH 8.0) at a flow rate of 18 ml/hr. Fractions of 4 ml were collected and aliquots of 200 and 100 μl used to determine ATPase activity and radioactivity, respectively. Fractions 48–54 were pooled and concentrated to give purified ATPase. For other details see the text.

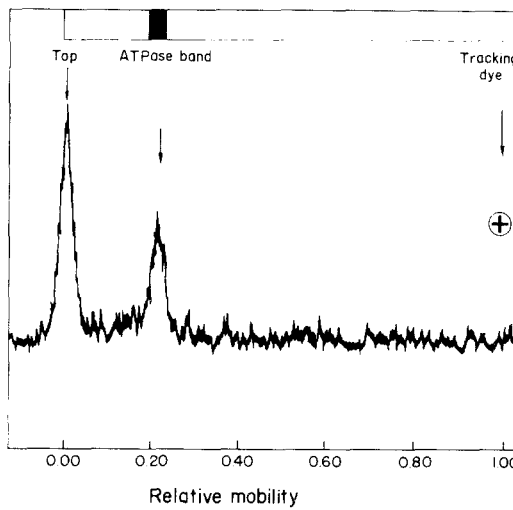

Figure 2. Radiochromatogram scan and ATPase identification after gel electrophoresis of purified ^{32}P-labeled ATPase. The ATPase was purified as described in Fig. 1 and an aliquot (40 μg protein) electrophoresed in Tris-glycine (pH 8.5). The gel was sliced into two halves to detect the ATPase band and radioactivity. For other experimental details see Materials and Methods.

cells grown in [^{32}P]-labeled medium. The bulk of the radioactivity was eluted in the void volume in a peak corresponding to that containing most of the carotenoid material present in the crude enzyme and revealed by absorbance at 475 nm (see Abrams et al. [5] and Maeda et al. [21]). It was difficult to tell from this result whether some ^{32}P remained associated specifically with the ATPase. The association of ^{32}P label firmly fixed to the active enzyme was demonstrated by analytical gel electrophoresis of the Sephadex ATPase fraction as shown in Fig. 2. The scan shows the existence of two peaks of radioactivity, one of them possessing the same relative mobility as the ATPase band and the other remaining at the top of the gel. This last behaved like a lipid, as it ran ahead to the position of the tracking dye if sodium deoxychlolate was added to the sample [32]. From the radioactivity scan it was calculated that 40% of the ^{32}P label of the Sephadex ATPase fraction remained bound to the ATPase molecule. This was confirmed by repurification of BF_1 by either gel chromatography or semipreparative gel electrophoresis. Table I outlines the distribution of radioactivity and ATPase activity along different stages of purification of the ATPase solubilized from *M. lysodeikticus* membranes.

TABLE I. Distribution of ^{32}P Label in *M. lysodeikticus* ATPase at Different Stages of Purification [a]

Enzyme preparation	ATPase activity (μmoles · min^{-1} · mg $protein^{-1}$) —Trypsin	ATPase activity (μmoles · min^{-1} · mg $protein^{-1}$) +Trypsin	^{32}P radioactivity (cpm/mg protein)
Crude	0.4	1.4	700,000
Sephadex G-200	13.0	13.0	90,000
Repurified	13.0	12.5	35,000

[a] For experimental details see Materials and Methods and Fig. 1. The radioactivity of all the protein preparations was normalized to 0 day by correcting for decay.

Identification of ^{32}P-Labeled Nucleotides Associated with M. lysodeikticus *ATPase*

When the ATPase of *M. lysodeikticus* purified by Sephadex filtration (95,000 cpm/mg protein, Table I) was extracted with chloroform–methanol, about 60% of the radioactivity went into the organic phase. This was a confirmation of the results illustrated in Fig. 2. However, if a repurified protein (35,000 cpm/mg protein, Table I) was subjected to the same extraction procedure, virtually all the radioactivity remained associated with the protein in the aqueous phase. When the lipid-depleted protein was extracted specifically for nucleotides, the results presented in Table II were obtained. About 25% of the ^{32}P radioactivity behaved as belonging to nucleotides although the 25% remaining in the charcoal may also probably correspond to this class of compounds. The 50% radioactivity nonadsorbed onto charcoal may represent inorganic

TABLE II. Distribution of ^{32}P Label Following Extraction of Nucleotides from Highly Purified ATPase of *M. lysodeikticus* [a]

	^{32}P radioactivity Total cpm	^{32}P radioactivity %	[^{32}P] compounds Identified
Denatured ATPase	17,632	100	ATP, ADP, P_i
Supernatant from charcoal-treated ATPase	8,700	49.3	P_i
Ethanol extract from charcoal	4,110	23.3	ATP, ADP
Ethanol-extracted charcoal	4,800	27.2	

[a] Repurified ^{32}P-labeled ATPase (500 μg/300 μl 30 mM Tris-HCl pH 7.5) was extracted with chloroform–methanol and then treated to extract nucleotides following procedure B (Materials and Methods).

phosphate associated with the protein as suggested by a radioactive spot running similarly in an electrophoresis of this fraction. Its final identification would require further work. Electrophoretic analyses of the nucleotide extracts allowed us to identify ATP and ADP in approximately equimolar amounts. Similar results were obtained if nucleotide extraction was carried out according to procedure A (see Materials and Methods).

Experiments with Cells of M. lysodeikticus *Grown in [2-^{3}H] Glycerol. Confirmation of the Absence of Tightly Bound Phospholipid in the ATPase*

Phospholipids were selectively labeled by feeding *M. lysodeikticus* with [2-^{3}H]glycerol. Table III illustrates the distribution of radioactive glycerol and phospholipid content of the fractions obtained in a typical experiment with stationary phase cells leading to "shock wash" release of *M. lysodeikticus* ATPase. As might be expected, membranes showed the highest specific incorporation of [2-^{3}H]glycerol, although the shock washes, (i.e. see wash 6) also had relatively high proportions. Similar results were obtained with exponentially growing cells.

However, the elution profile of radioactivity and ATPase activity following Sephadex G-200 filtration strongly suggested (Fig. 3) that the

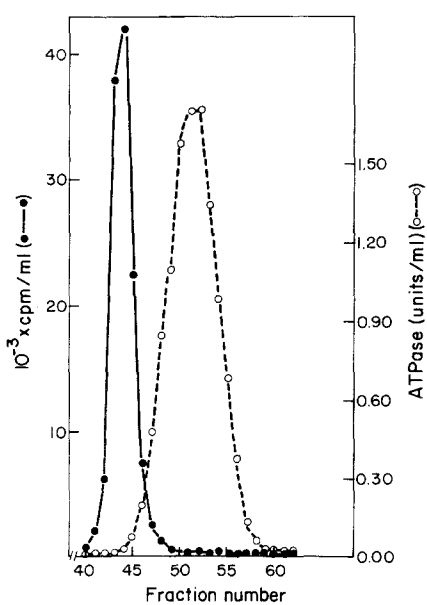

Figure 3. Purification of ATPase from *M. lysodeikticus* cells grown in [2-^{3}H]glycerol. Crude ATPase (0.5 mg protein/ml) was obtained by pooling shock washed 5 and 6 described in Table III and was subsequently processed as detailed in Fig. 1. Fractions 48–55 were pooled and concentrated in this case to give purified ATPase.

TABLE III. Distribution of Protein and [2-^{3}H]Glycerol-Labeled Phospholipids in Subcellular Fractions Isolated from *M. lysodeikticus* [a]

Fraction	Protein (mg/ml)	[2-^{3}H]Glycerol		Incorporated (cpm/mg protein)	Phospholipid content (nmoles/mg protein)
		cpm/ml	%		
Cytoplasm	13.2	190,490	10.8	14,431	0.12
Wash	1.52	56,080	3.2	36,894	0.30
Wash 2	0.36	46,510	2.6	129,194	1.07
Wash 3	0.26	57,910	3.3	222,130	1.85
Wash 4	0.21	52,110	3.0	248,142	2.06
Shock wash 5	0.70	114,480	6.5	204,971	1.70
Shock wash 6	0.23	91,900	5.2	400,565	3.33
Residual membranes	1.24	1149,000	65.4	926,613	7.72

[a] Membranes were isolated and washed to release membrane ATPase from *M. lysodeikticus* cells grown in [2-^{3}H]glycerol following standard procedures [13, 22, 23]. For other experimental details see Materials and Methods.

active enzyme had very few, if any, associated phospholipid molecules. This was confirmed by quantitative analysis which showed that less than 0.02 mole of phospholipid was associated with 1 mole of ATPase as well as by analytical polyacrylamide gel electrophoresis which in contrast with Fig. 2 showed the complete absence of tritium label in the ATPase band. From these results, it seems reasonable to conclude that there are no tightly bound phospholipids in the purified BF_1 of *M. lysodeikticus*.

Enzymatic Determination of Tightly Bound ATP in Purified ATPase Preparations

Once the presence of adenine nucleotides in purified BF_1 had been demonstrated (see earlier), we considered it to be interesting to extend this study and to determine the ATP content in different preparations. We first attempted to find the best method to measure the ATP content by the luciferin-luciferase assay. Denaturation by 8 M urea yielded the best results (i.e. 1.50 nmoles ATP/mole ATPase) as compared with conventional procedures of nucleotide extraction. These methods gave values 10–20% lower than the denaturation by urea. However, the differences were too small to account for the low yield in the extraction of ^{32}P label from the ATPase as found previously (see Table II). On the other hand, the low value estimated in native ATPase confirmed that ATP is tightly bound to the ATPase molecule and that this binding is dependent on the substructure of the protein.

Taking advantage of the inactivation of *M. lysodeikticus* ATPase depending on its storage and purification procedure [22], we attempted to find a correlation between the level of bound nucleotide and that of ATPase activity.

Table IV summarizes the results on the variation in the level of total ATP in the preparation (denatured ATPase), released or free ATP (native ATPase), and ATPase activity. Figures for bound ATP can be calculated from the difference between the denatured and the native columns. Results show a correlation between the level of total and bound ATP with activity but also point out that the decrease in ATPase activity, i.e., its inactivation, is not simply dependent on or accompanied by a release of nucleotide into the medium. As a matter of fact, the low ATP content estimated under native conditions demonstrates the absence of free ATP in the medium. A concomitant degradation of ATP should also occur in order to account for the decrease in total amount of ATP. An intrinsic ATPase activity of the purified enzyme from *M. lysodeikticus* (i.e., an activity independent of externally added divalent cations) which was able to hydrolyze ATP into ADP and P_i slowly has been demonstrated in this laboratory (J. Carreira and E. Muñoz, unpublished observations). This intrinsic ATPase or

TABLE IV. Variations in the Level of ATPase Activity and Tightly Bound ATP of *M. lysodeikticus* ATPase Preparations Purified and Stored under Different Conditions[a]

Preparation	Method purification	Age and storage conditions	ATPase activity (μmoles · min^{-1} · mg^{-1})	ATP/ATPase (mole/mole)	
				Native	Denatured
I	Sephadex G-200	Fresh	12–14	0.18	2.07
		2 weeks, −20°C	9–10	0.10	1.33
		2 months, frozen and thawed several times	2.5–3	–	0.30
II	Sephadex G-200	1 week, −20°C	8–9	0.20	1.50
		6 months, −20°C	6	–	0.83
III	Gel electrophoresis, pH 7.2	Fresh	7	0.10	1.30
		6 months, −20°C	4	–	0.72
IV	Gel electrophoresis, pH 8.5	Fresh	2.5–3	–	0.55

[a] For experimental details see Materials and Methods and the text.

another activity not yet defined might be able to act on the ATP when bound or after its release into solution.

Exchange of the Tightly Bound ATP of M. lysodeikticus *ATPase by External Nucleotides*

In order to determine whether the endogenous bound ATP occupied readily exchanging binding sites, we incubated BF_1 with exogenous ADP and measured its residual ATP content after filtration on Sephadex G-100. We also examined the effect of this displacement on the level of ATPase activity to test whether the binding of ADP could responsible for ATPase inactivation. The results are summarized in Table V. As can be seen, ADP was able to displace the tightly bound ATP at concentrations as low as 0.1 mM, and this displacement seemed to be independent of the temperature and the type of ATPase preparation. ADP (about 1–1.5 moles/mole ATPase) remained firmly bound to the ATPase as estimated after these experiments. This exchange of ATP by ADP did not apparently affect the ATP hydrolytic activity of the enzyme, strongly suggesting that activity is independent of ATP or ADP occupying the tight-binding sites. It is also worth noting that by simple dilution and/or incubation of the ATPase, some of the tightly bound ATP was released which was then measured without protein denaturation (cf. the values obtained for native proteins in the experiments summarized in Table V). Then the subsequent filtration on Sephadex of a protein so treated resulted in a decrease of level of bound ATP but without noticeable change in its ATPase activity. This result clearly shows that nucleotides can be released from the ATPase without inactivation and may therefore suggest that nucleotides are not essential for the activity (see Discussion).

Exchange of endogenous nucleotides with exogenous ATP was carried out with [^{32}P]-labeled ATPase. About 50% of the label was displaced by 1 mM ATP but 8 mM ATP did not exceed this percentage whereas 0.1mM ATP exchanged only 10% of the label. The results were independent of the presence of Ca in the incubation mixture. The complexity of these results, probably involving a combination of ATP hydrolysis and binding, prevented further analysis.

Discussion

In this work we have presented conclusive evidence to establish that phospholipids are not integral parts of the molecule of *M. lysodeikticus* ATPase. This is a confirmation of previous conclusions reached by different methods [12, 13]. In a recent report, Peter and Ahlers [9]

TABLE V. Exchange of Tightly Bound ATP of *M. lysodeikticus* ATPase by External ADP[a]

Preparation	Experimental conditions	ATPase activity (μmoles · min^{-1} · mg^{-1})	ATP/ATPase (mole/mole)	
			Native	Denatured
I, 2 weeks, −20°C	—	9.50	0.10	1.33
	Incubated 20 min, 40°C	9.20	0.60	1.30
	Incubated, Sephadex filtered	9.00	0.10	0.80
	Incubated ADP (1 mM), Sephadex filtered	9.10	0.01	0.10
	Incubated ADP (0.1 mM), Sephadex filtered	9.20	0.01	0.10
IV, fresh	—	3.10	–	0.55
	Incubated 20 min, 20°C	2.86	0.39	0.25
	Incubated, Sephadex filtered	2.70	–	0.25
	Incubated ADP (0.1 mM), Sephadex filtered	2.80	0.01	0.01

[a] For identification of the preparations see Table IV. Other experimental details are given under Materials and Methods and Table IV.

provided evidence suggesting that the ATPase from *Escherichia coli* is a lipoprotein. Moreover, these authors criticized our previous work on *M. lysodeikticus* ATPase on the basis that we used methods of low sensitivity. In their work, Peter and Ahlers [9] showed that 80 μg of phospholipids (phosphatidylethanolamine) was required for maximum activity of 1 mg ATPase, i.e., 40 nmoles phospholipid/nmole enzyme. The method used in the present work allowed us to detect 1 nmole phospholipid/100 nmoles BF_1. Therefore, our earlier conclusion now seems to be substantiated by significant experimental evidence.

Conversely, in the present work we have demonstrated that the purified ATPase contains tightly bound nucleotides. This result represents a new example of this property shared by all the energy-transducing proteins so far studied. Nevertheless, differences seem to exist with regard to the number and type of nucleotides bound. Two laboratories found differences in the ATP content of beef heart mitochondrial F_1 which varied between 1 and 3 moles/mole enzyme [7, 8]. However, 2 moles of firmly bound ADP was found in the two preparations [7, 8]. Pedersen estimated that F_1 from rat liver mitochondria had a simple site for high affinity binding of ADP [3]. All these authors concurred in asserting that these F_1 factors possessed multiple nucleotide binding sites [7, 8, 15, 16]. Abrams and Nolan [20], studying the ATPase (BF_1 factor) from *Streptococcus faecalis*, reported that the enzyme purified from cells grown in ^{32}P contained 5 to 10 atoms of P_i but these workers did not analyze their quantitative and qualitative distribution into nucleotides. The same group [5] reported that, in *in vivo* studies, 1 mole of enzyme was able to bind 1 mole ATP 1 mole ADP, and 0.1 mole P_i. In a very recent study Maeda et al. [21] reported that a homogeneous ATPase from *E. coli* contained 2.2 moles ATP, 0.4 mole ADP, and 0.1 mole P_i. From the present studies we can conclude that an active purified form of *M. lysodeikticus* ATPase contains 2 moles of tightly bound ATP per mole of enzyme. From an extrapolation of the results obtained with cells grown in [^{32}P]orthophosphate, it seems reasonable to conclude that ADP is bound to *M. lysodeikticus* ATPase in approximately the same amount as ATP. AMP and P_i may also be part of *M. lysodeikticus* BF_1 but their exact identification and quantitation must await further experimentation.

The rapid exchange of the endogenous bound nucleotides of *M. lysodeikticus* ATPase is a unique property of this enzyme as compared with other energy-transducing ATPases (e.g., beef heart mitochondrial F_1 [8], *S. faecalis* and *E. coli* ATPase [6, 21]). Also interesting is the fact that the sites appeared to be equivalent for ATP and ADP, although ADP showed a higher affinity for them.

Of particular interest in these studies was the attempt to find a

correlation between the level of ATPase activity and the amount of nucleotide bound. The analysis of ATP content in ATPase preparations with different levels of ATPase activity showed that a correlation seems to exist, at least, between ATP bound and activity. These results also proved that ATPase inactivation did not correlate with the release of ATP into the medium. The tightly bound ATP was likely transformed before or after its release. It is therefore tempting to think that this transformation should precede inactivation of the ATPase and be somewhat related to it perhaps by inducing a conformational change in the protein. On the other hand, the saturation of the binding sites with ADP did not appear to be responsible for enzyme inactivation.

Some experiments described in Table V which suggested that nucleotides may not be essential for activity were in contradiction with the idea just discussed. However, it is possible that the short period of time ATPase remained with unbound nucleotide in these experiments (see Table V) was insufficient to allow the transformation of the nucleotides and/or to induce an irreversible conformational change of the protein. This may account for the discrepancy.

To accommodate all the evidence we would like to propose a mechanism to explain the loss of ATPase activity: (i) the slow release of a part of the ATP bound by an equilibrium process with the medium; (ii) the hydrolysis of the free ATP inducing conformational changes in the enzyme and/or a change in its ability to bind nucleotides; (iii) the irreversibility of the process because of the lack of concentration of ATP and/or ADP in the medium sufficient to load the site; (iv) protein storage under these conditions which would lead to ATPase inactivation. There is as yet no sufficient experimental evidence to support this mechanism although conformational differences between catalytically different forms of the enzyme [22, 23] and the induction of molecular changes by substrate hydrolysis have been demonstrated [34]. The specific properties (compare this work with Refs. 7, 8, 35) of the tightly bound nucleotides of *M. lysodeikticus* ATPase constitute a new example that in spite of a general pattern of similarities between energy-transducing proteins, peculiarities also seem to exist between them. They may help to understand the physiological role of each one of these proteins.

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