Biochimica et Biophysica Acta 813 (1985) 161–166
Elsevier

BBA 72472

Affinity labeling of the ($Na^+ + Mg^{2+}$)-ATPase from *Acholeplasma laidlawii* B membranes by the 2′,3′-dialdehyde derivative of adenosine 5′-triphosphate

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(Received September 3rd, 1984)

Key words: ($Na^+ + Mg^{2+}$)-ATPase; ATP dialdehyde; Affinity labeling; Enzyme inactivation; (*A. laidlawii*)

The ($Na^+ + Mg^{2+}$)-ATPase of the *Acholeplasma laidlawii* B plasma membrane was inactivated by the 2′,3′-dialdehyde derivative of ATP (oATP). oATP behaved as a reversible competitive inhibitor of this ATPase and was slowly hydrolyzed by the enzyme. In addition, oATP induced an irreversible inactivation of the enzyme. A 62% inactivation of the enzyme correlated with the binding of 16 moles of oATP per mole of the enzyme. In the presence of 5′-adenylyl imidodiphosphate, a non-hydrolyzable substrate analogue, the stoichiometry was 8 moles oATP per mole of ATPase. By SDS-polyacrylamide gel electrophoresis, [U-^{14}C]oATP was found to bind covalently to four of the five subunits of the enzyme, but specific labeling was highest for the γ-subunit of the ATPase.

Introduction

The Na^+-stimulated, Mg^{2+}-dependent ATPase of *Acholeplasma laidlawii* B is an integral membrane protein [1] which is involved in the transmembrane ion transport required to regulate the osmotic balance of the organism [2]. Recently, this enzyme has been purified to homogeneity and its subunit composition defined [3]. The purified ATPase could be resolved into five subunits (α,β,γ,δ,ε) on SDS-polyacrylamide gel [3]. Although the subunit composition of *A. laidlawii* B plasma membrane ATPase resembles that of the F_1 part of some bacterial F_0F_1-ATPases, its kinetic properties and insensitivity to many of the inhibitors of bacterial and mitochondrial F_0F_1-ATPases suggest that this enzyme is dissimilar to other known bacterial ATPases. In addition, the *A. laidlawii* B ATPase is tightly bound to the plasma membrane by hydrophobic interactions and its activity is sensitive to the phase state and possibly to the fluidity of the membrane lipids [4–7].

The 2′,3′-dialdehyde derivatives of adenine nucleotides have been successfully used as affinity labels for a variety of enzymes [8–10], including several ATPases [11–14]. In order to gain information about the nature of the substrate binding sites of the membrane-bound ATPase of *A. laidlawii*, we have studied the interaction of oATP with the enzyme. The results presented in this report demonstrate that oATP covalently modifies the enzyme and that at least one lysine residue is involved in the binding of substrate.

Materials and Methods

Materials

[U-^{14}C]ATP (593 mCi/mmol) was purchased from NEN, Boston, MA. [8-^{14}C]ATP (1 mCi/mmol) and ACS were purchased from Amersham,

Abbreviations: oATP, 2′,3′-dialdehyde derivative of ATP; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; SDS, sodium dodecyl sulphate; Ado*PP*[NH]*P*, 5′-adenylyl imidodiphosphate; DMPC, L-α-dimyristoylphosphatidylcholine.

Ontario, Canada. Sigma Chemical Co. supplied Na_2ATP, Ado*PP*[NH]*P*, Hepes and DMPC. Cellulose-polyethyleneimine sheets were purchased from J.T. Baker Chemical Co., Phillipsburg, NJ.

Methods

Purification of ATPase and reconstitution with DMPC. ATPase was purified from *Acholeplasma laidlawii* B membranes as described by Lewis and McElhaney [3]. Purified enzyme was reconstituted with DMPC using sodium cholate solubilization and removal of the detergent by extensive dialysis against reconstitution buffer (10 mM Hepes (pH 7.6)/10 mM NaCl/1 mM EDTA/1 mM NaN_3).

Determination of ATPase activity. The enzyme was incubated at 37°C with 1 mM Na_2ATP, 15 mM $MgCl_2$, 50 mM NaCl and 50 mM Tris at pH 7.5 in a total volume of 0.5 ml. The reaction was terminated by the addition of 50 μl of 5% SDS. Phosphate was estimated according to the method of Atkinson et al. [15]. Protein was estimated by the method of Hartree [16] after precipitation according to Bensadoun and Weinstein [17].

Modification of ATPase by oATP. oATP was synthesized from [U-^{14}C]ATP by the method of Lowe and Beechey [18]. The purity of synthesized oATP was confirmed by chromatography on cellulose-polyethyleneimine sheets, using 0.8 M NH_4HCO_3 as the developing solvent [19]. oATP migrated with an R_F value of 0.02 and no ATP (R_F 0.24) was detectable under ultraviolet light. The concentration of oATP was estimated by measuring the absorbance at 258 nm, using 14900 $cm^{-1} \cdot M^{-1}$ for the absorption coefficient. The specific radioactivity of oATP was 2.98 mCi/mmol.

Reconstituted ATPase (300 μg protein) was incubated with 5 μCi of oATP at 37°C for 30 min in reconstitution buffer. At the end of the incubation, sodium borohydride solution was added to a final concentration of 2.5 mM. The mixture was incubated at 37°C for a further 30 min, transferred to a Sepharose 4B column (1.6 × 30 cm) and eluted with reconstitution buffer. ATPase-containing fractions were pooled, concentrated and dialyzed against reconstitution buffer.

SDS-polyacrylamide gel electrophoresis. Acrylamide gels (7%) were used to separate the [^{14}C]oATP-labeled subunits of the ATPase [3]. The gels were stained with Coomassie blue G, frozen in sold CO_2/acetone bath and sliced in 2-mm slices. Slices were digested by overnight incubation in 0.5 ml of 30% H_2O_2 at 60°C. The radioactivity was estimated in a Beckman LS 7800 scintillation counter using ACS scintillant.

Results

Kinetics of inactivation of the ATPase by oATP

As shown in Fig. 1, preincubation of ATPase with oATP resulted in a concentration-dependent inhibition of the ATPase activity. oATP also served as a substrate for the enzyme, but was hydrolyzed at only 10% of the rate of equimolar amounts of ATP. Further proof that oATP functioned as a slowly hydrolyzable substrate came from the time-course of the ATPase in the presence and absence of ATP. The enzyme was incubated either with oATP, alone or in combination with equimolar concentration of ATP, and ATPase activity was determined at various time intervals. Results in Fig. 2 suggest that oATP functions as a substrate of the enzyme, but is hydrolyzed at a much lower rate compared to ATP. The synthesized oATP was free of contaminant ATP, as judged by polyethyleneimine chromatography (see Methods). Therefore, contaminating ATP could

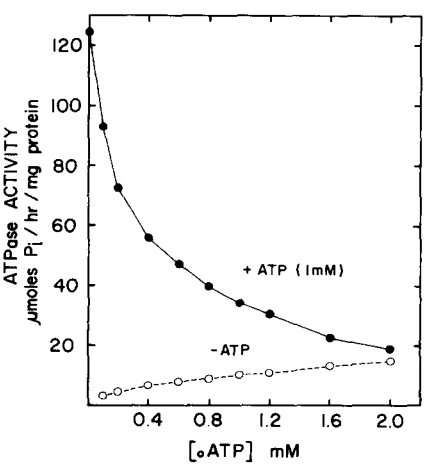

Fig. 1. Effect of oATP concentration on ATPase activity. Reconstituted ATPase (7.9 μg protein) was preincubated at 37°C for 10 min with varying concentrations of oATP. ATPase activity was estimated after the addition of 1 mM ATP.

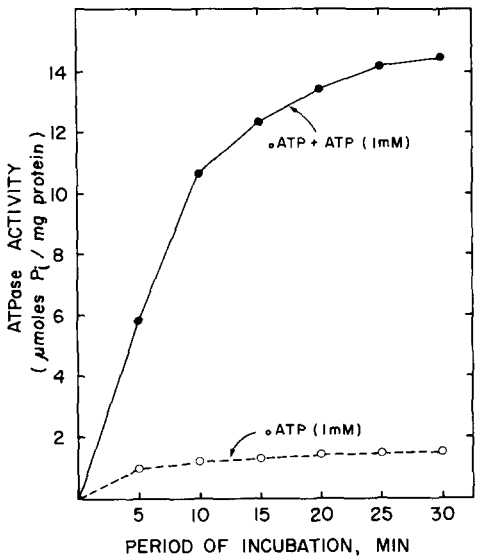

Fig. 2. Time-course of ATPase with oATP and ATP as substrates. Reconstituted ATPase (7.9 μg protein) was incubated at 37°C with either oATP (1 mM) or oATP+ATP (1 mM each) and the ATPase assay carried out for the specified periods. The specific activity of ATPase with 1 mM ATP alone as substrate was 104 μmol P_i/h per mg protein.

not have contributed to the ATPase activity detected in the presence of oATP. Since ATP dialdehyde is known to decompose to adenine-containing compounds and tripolyphosphates [11], the possibility of the tripolyphosphates breaking down to yield P_i could not be ruled out.

Nature of the inactivation of the ATPase by oATP

As oATP functioned as a less effective substrate of the enzyme, it was of interest to determine the nature of the inhibition of the ATPase by oATP. The enzyme was preincubated for 10 min at 37°C with specified concentrations of oATP, followed by the assay for ATPase activity in the presence of ATP. The results are shown as Lineweaver-Burk plots in Fig. 3. It is clear that oATP acts as a competitive inhibitor of the enzyme. The K_i value for oATP calculated from the double reciprocal plot was 0.13 mM.

Since oATP acts as a poor substrate and as a covalent modifier of the enzyme, there should be two types of inhibition, reversible competitive inhibition (Fig. 3) and irreversible inactivation due to covalent modification of the enzyme. Competi-

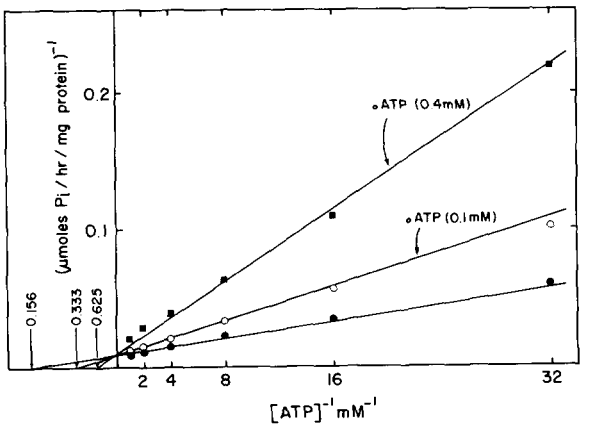

Fig. 3. Nature of inactivation of ATPase by oATP. Reconstituted ATPase (9.26 μg protein) was preincubated with (0.1 or 0.4 mM) and without oATP (●) for 10 min at 37°C and ATPase activity was determined after addition of different concentrations of ATP. Results are represented as Lineweaver-Burk plots. K_m values (mM) are shown with arrows at each concentration of oATP.

tive reversible inhibition of the ATPase is apparent at lower concentrations of oATP (Fig. 3). The irreversible inactivation becomes more pronounced at higher concentrations of the inhibitor (Fig. 3) and with longer periods of incubation. Up to 62% inactivation of the enzyme could be achieved by incubating the enzyme with oATP for 30 min (Table I). Since oATP competes for the

TABLE I

INHIBITION OF ATPase BY [U-^{14}C]oATP

ATPase (300 μg protein) was incubated with 0.692 mM [U-^{14}C]oATP at 37°C. 20–50-μl aliquots were withdrawn at specified time intervals and ATPase activity determined. At the end of 30 min incubation, $NaBH_4$ (final concentration 2.5 mM) was added and the mixture was incubated for another 30 min, following which the unbound label was removed by gel filtration and dialysis. ATPase activity was estimated thereafter.

Treatment	ATPase activity (μmol P_i/h per mg protein)	Percent inhibition
none	241	0
oATP, zero time	224	7
oATP, 30 min	91	62
oATP, 30 min, followed by gel filtration, dialysis	88	63.5

substrate binding site, it can be assumed that some specific labeling occurs at the catalytic site of the ATPase, which in turn brings about the irreversible inhibition of the enzyme.

[U-^{14}C]oATP binding to ATPase

Incubation of ATPase with [U-^{14}C]oATP resulted in a time-dependent inhibition of ATPase activity. Removal of unbound oATP by gel filtration and dialysis did not result in any change in the degree of inhibition of ATPase (Table I), suggesting that the label was covalently bound to the enzyme protein. Under the conditions of incubation used here, a 62% inactivation of the enzyme corresponded to the binding of 16 moles of oATP per mole of enzyme. When labeled in the presence of 0.5 mM Ado*PP*[NH]*P*, the stoichiometry was 8 moles oATP per mole of enzyme.

The ATPase labeled with oATP was subjected to SDS gel electrophoresis and the labeling pattern is shown in Fig. 4. The label was found to bind to all subunits of the enzyme except ϵ. In order to determine the relative amounts of specific labeling, small amounts of the reconstituted ATPase were subjected to SDS-gel electrophoresis and the gel was stained with Coomassie blue. The area under each of the subunit peaks was quantitated by densitometric scanning of the gel at 595 m. The radioactively labelled gels were not used for this purpose, as the gels had to be overloaded with protein so as to get detectable amounts of label in the protein bands. This fact accounts for the fair amount of radioactivity associated with undissociated enzyme remaining at the origin (Fig. 4). The proportion of undissociated enzyme is larger when reconstituted enzyme is used for electrophoresis. Since it is reasonable to assume that the intensity of each band corresponds to the relative quantity of the individual subunits, the area under each peak was used to calculate the radioactivity bound per unit area. The results are shown in Table II. Although it would appear from Fig. 4. that maximum incorporation of label occurs with the α-subunit, when the degree of specific labeling was calculated based on the relative proportion of each of the subunits, the γ-subunit was found to bind 45% of the label and the other subunits 20% or less. Assuming that all of the subunits were equally accessible to the label, the γ-subunit shows some

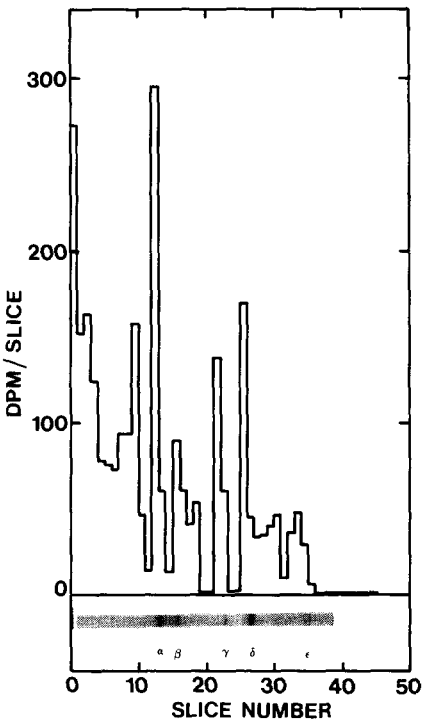

Fig. 4. SDS-polyacrylamide gel electrophoresis of ATPase inactivated by [U-^{14}C]ATP. ATPase was incubated at 37°C with 0.692 mM [U-^{14}C]ATP, 50 mM Hepes (pH 7.5) 15 mM $MgCl_2$ and 50 mM NaCl for 30 min. $NaBH_4$ (2.5 mM final concentration) was added and incubated for 30 min. Unreacted oATP was removed, ATPase subunits separated and the radioactivity estimated as described in Materials and Methods. The photograph of an analytical gel is shown to indicate the position of the individual subunits.

TABLE II

RELATIVE LABELING OF ATPase SUBUNITS BY [U-^{14}C]oATP

The ATPase subunits were separated by analytical scale SDS-gel electrophoresis. The intensity of the stain was quantitated by densitometric scanning of the Coomassie blue-stained gels. The area under each of the subunits was used to calculate the relative radioactive labeling of each of the subunits from Fig. 4.

Subunit	Area	Radio-activity (dpm)	dpm/unit area	Relative labeling % total
α	59	355	6.02	19.0
β	34	150	4.41	13.9
γ	13	186	14.31	45.2
δ	31	215	6.94	21.9
ϵ	6	–	–	–

preferential incorporation of the label compared to the other subunits. This points to the possibility that the lysine residue(s) presumably involved in the binding of substrate might be associated with the γ-subunit of the enzyme.

Discussion

The results presented here show that oATP can be used as an affinity label for the *A. laidlawii* ($Na^+ + Mg^{2+}$)-ATPase, as for other ATPases [12,13]. Evidence that oATP interacts with a catalytically active site is that oATP acts as a slowly hydrolyzable substrate (Figs. 1 and 2) and as a competitive inhibitor of ATP hydrolysis (Fig. 3). In the presence of Ado*PP*[NH]*P*, a nonhydrolyzable analogue of ATP, there was a 50% decrease in the binding of oATP, again suggesting that oATP interacts with the catalytic site. Since the removal of non-covalently bound oATP by gel filtration and dialysis did not change the extent of inhibition, the observed inhibition of enzyme activity should be due to the covalent binding of oATP to the enzyme. The stoichiometry of labeling is rather high compared to other ATPases. However, considering the probable subunit stoichiometry of $\alpha_3\beta_3\gamma\delta_2\epsilon_3$ as estimated from the relative staining intensities of the subunits on SDS-polyacrylamide gels [3], and the fact that some non-specific binding occurs, it is probable that the number of catalytic sites per mole of enzyme is less than eight. Since oATP binds to an amino group, there should be at least one amino group essential for the activity of the enzyme.

Despite the finding that oATP can act as a reversible competitive inhibitor and induce irreversible inactivation of the ATPase, the large unspecific labeling of the enzyme is a drawback in the use of oATP as an affinity label. The identification of the lysine residue involved in substrate binding and the quantitation of the number of binding sites must await methods for purification of the enzyme in large amounts and for the determination of the amino acid sequence of the individual subunits.

Although dialdehyde derivatives of adenine nucleotides have been successfully used as affinity labels for different ezymes, these compounds are known to decompose into adenine-containing compounds and the tripolyphosphate [11]. When we used [8-^{14}C]oATP instead of [U-^{14}C]oATP for labeling, under the acidic conditions of staining and destaining the gels, the radioactivity was completely removed from the protein band. But when [U-^{14}C]oATP was used, the radioactive label could be detected in the protein band after the staining-destaining procedure. The open sugar ring probably becomes unstable to acidic conditions, thus the adenine moiety of the oATP is cleaved off during the staining-destaining of gels. This explains the lower amounts of radioactivity detected on the gels after staining.

Unlike the modification on the sugar ring of the nucleotide, the enzyme was extremely sensitive to chemical modifications of the adenine moiety. We attempted the use of 8-azidoATP, a photoaffinity label widely used for affinity labeling of ATPases [20]. 8-azidoATP was not hydrolyzed by the enzyme, and did not affect the enzyme activity, when ATP was used as the substrate. Similar results were obtained with 8-bromoATP. It is noteworthy that Formycin A-triphosphate functioned at 40% efficiency as a substrate for the enzyme [3]. These findings suggest that position 8 on the nucleotide molecule is involved in its binding at the active site of the enzyme, since slight modifications at this position made the molecule unrecognizable by the enzyme.

Acknowledgements

Financial support from the Alberta Heritage Foundation for Medical Research and the Medical Research Council of Canada are gratefully acknowledged. We thank Dr. Ruthven N.A.H. Lewis for many helpful discussions.

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