Biochimica et Biophysica Acta, 403 (1975) 538—543

BBA 67622

DISULFIDE OF THIOINOSINE TRIPHOSPHATE, AN ATP-ANALOG INACTIVATING ($Na^+ + K^+$)-ATPase

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(Received April 28th, 1975)

Summary

($Na^+ + K^+$)-activated ATPase in beef brain microsomes is inactivated by the disulfide of thioinosine tri[γ-^{32}P]phosphate, an ATP analog. The inactivation of the enzyme, which is accompanied by an incorporation of radioactivity into the membrane protein, is abolished by ATP or dithiothreitol. Since dithiothreitol restores the activity of ($Na^+ + K^+$)-ATPase, which had previously been inactivated by this ATP analog, it is concluded that thioinosine triphosphate disulfide reacts with a sulfhydryl group in the ATP binding site of ($Na^+ + K^+$)-activated ATPase.

Introduction

($Na^+ + K^+$)-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3), which presumably catalyzes the active transport of Na^+ and K^+ through cellular membranes [1], contains 2—6 sulfhydryl groups per active center [2]. Recently, some evidence has been presented that an SH group in the ATP binding site may interact with the 6-amino group of ATP [3]. This conclusion is based on studies with the ATP analogues thioinosine triphosphate and dinitrophenylthioinosine triphosphate, which inactivate ($Na^+ + K^+$)-activated ATPase relatively slowly [3]. In order to obtain additional evidence for the participation of a sulfhydryl group in the ATP binding site, we felt it worthwhile to look for an ATP analog which reacts more rapidly with sulfhydryl groups. This paper reports on the action of the disulfide of thioinosine triphosphate on ($Na^+ + K^+$)-activated ATPase.

Abbreviations: $(sITP)_2$, thioinosine triphosphate disulfide; sITP, 6-mercaptopurine riboside-5′-triphosphate; sIMP, 6-mercaptopurine riboside-5′-monophosphate; $s(IMP)_2$, thioinosine monophosphate disulfide.

Material and Methods

Chemicals

[^{32}P]Orthophosphate was obtained from Amersham-Buchler, Braunschweig. Biochemicals were obtained from Boehringer Mannheim GmbH, Mannheim. Dithiothreitol was obtained through Serva, Heidelberg. 6-Mercaptopurine riboside was from Pharma-Waldhof GmbH, Düsseldorf. All other chemicals were of analytical grade and obtained from E. Merck, Darmstadt and Merck-Schuchard, München.

Preparations of 6-mercaptopurine riboside-5'-tri[γ-^{32}P]phosphate

6-Mercaptopurine riboside-5'-triphosphate (sITP) which had been synthesized from the monophosphate with method 2 of Murphy et al. [4] was a substrate of 3-phosphoglycerate kinase. Consequently the method of Glynn and Chappell [5] was used to exchange [^{32}P] orthophosphate with the terminal phosphate of thioinosine triphosphate.

The disulfide of 6-mercaptopurine riboside-5'-triphosphate ((sITP)$_2$)

This was prepared by oxidation of the SH-groups of thioinosine triphosphate with I_2 according to Doerr et al. [6]: 63.5 μmol thioinosine triphosphate were dissolved in 3.52 ml 0.2 M potassium phosphate buffer pH 7.5 or 0.2 M borate buffer pH 7.6. To this solution 0.06 ml 1 M iodine was added dropwise. After some minutes of reaction the mixture was applied to a Sephadex G 10 column (1.5 × 20 cm) which had been equilibrated with 50 mM potassium phosphate buffer pH 7.5 or 50 mM borate buffer pH 7.6. The disulfide of thioinosine triphosphate was eluted near the void volume and well separated from any remaining thioinosine triphosphate and I_2. It appeared homogeneous by chromatographic analysis on a Sephadex G 10-column of 1.4 m length (Fig. 1). The molecular weight of the disulfide of thioinosine triphosphate is 1194; this value was also found by gel chromatography (Fig. 1). The product was analyzed for purine content, ribose, acid labile phosphate and total phosphorus content. The ratio of the amount of purine moiety:ribose:total phosphorus was 1 : 1 : 3. The spectrum of the disulfide of thioinosine triphosphate

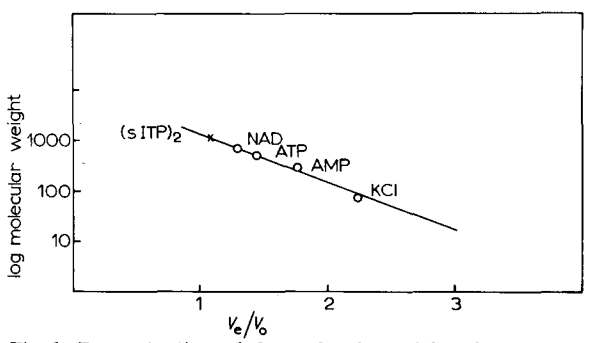

Fig. 1. Determination of the molecular weight of (sITP)$_2$ by gel filtration. 10 μmol of NAD, ATP, AMP and KCl (each) were applied to a 1400 × 1 cm column of Sephadex G 10 equilibrated with 50 mM potassium phosphate buffer pH 7.5.

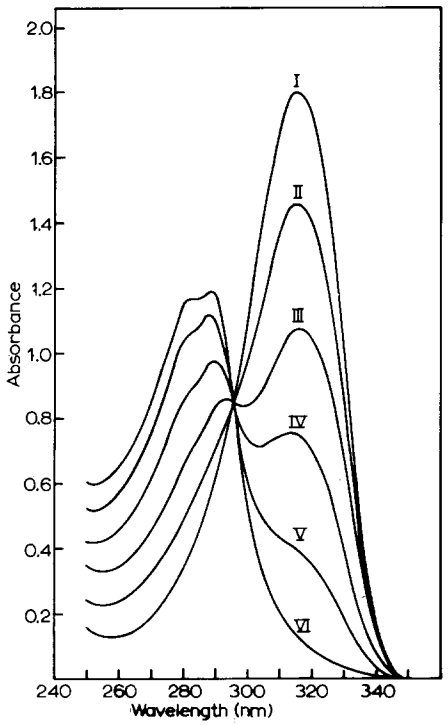

Fig. 2. Change of the ultraviolet spectrum by oxidation of 6-mercaptopurine riboside-5'-triphosphate (sITP) to $(sITP)_2$ by I_2. 16.02 μmol sITP were solubilized in 2 ml 0.2 M potassium phosphate buffer pH 7.5 and treated with 0.1 ml of the following I_2 solutions: I = no I_2; II = $3.8 \cdot 10^{-4}$ M I_2; III = $7.7 \cdot 10^{-4}$ M I_2; IV = $11.6 \cdot 10^{-4}$ M I_2; V = $15.5 \cdot 10^{-4}$ M I_2; VI = $20.8 \cdot 10^{-4}$ M I_2. The spectra were measured in a 1 cm cuvette.

(Fig. 2) showed a maximum at 290 nm as does the 6-mercaptopurine disulfide [6]. The concentration of the triphosphate was calculated from $E_{290}^{1\,cm} = 30.4 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ at pH 7.5. Terminally labelled [γ-^{32}P] $(sITP)_2$ was prepared from [γ-^{32}P]sITP by the same procedure as well as $(sIMP)_2$ from sIMP.

Enzyme and assays

$(Na^+ + K^+)$-activated ATPase from beef brain with a specific activity of 1—4 units/mg was prepared as described previously [7]. The enzymatic activity was measured with the coupled optical assay [7]. Contaminations by an Mg^{2+} activated ATPase amounted to about 1% of $(Na^+ + K^+)$-ATPase. One enzyme unit is defined as the amount of enzyme hydrolyzing 1 μmol ATP per min at 37° C. Protein was estimated by the procedure of Lowry et al. [8].

Inactivation of $(Na^+ + K^+)$-ATPase by the disulfide of thioinosine monophosphate and triphosphate

0.29 mg of $(Na^+ + K^+)$-ATPase from beef brain was incubated in a total volume of 0.5 ml at 37° C with 100 mM imidazole-HCl pH 7.25 and the reagents indicated in the figures. Because of the lability of $(sITP)_2$ [11] only freshly prepared solutions of this compound were used. Aliquots of 0.04 ml were withdrawn at the times indicated and used in the coupled optical assay.

Results

Incubation of $(Na^+ + K^+)$-ATPase with $(sITP)_2$ results in a time- and concentration-dependent decrease of enzymatic activity (Figs 3, 4). The inactivation half life at infinitely high concentrations reaches a maximal value of 0.6 min^{-1} (Fig. 4). This saturation effect indicates that the inactivation by $(sITP)_2$ is preceded by a reversible binding of the inhibitor to the enzyme and that the inactivation is not simply random bimolecular [9,10]. From these data an apparent first order rate constant k_2 for inactivation of $1.9 \cdot 10^{-2}$ s^{-1} and apparent dissociation constant of the $(sITP)_2$ enzyme complex of 0.56 mM can be calculated at 37°C and pH 7.25 (Eqn 1). This value agrees with the K_m for ATP which was found to be between 0.1 and 0.8 mM [12—14]. (For the derivations of the equations used to calculate these constants see references [9,10]). The rate of inactivation increases with increasing pH until pH 8.2 and decreases at more alkaline pH. This pH-optimum of inactivation is more alkaline than the pH-optimum of the enzymatic activity. Apparently the formation of a covalent bond is favoured at alkaline pH-values.

If $(sITP)_2$ binds to the substrate-binding site of the enzyme, then it is to be expected that ATP may protect the enzyme against the inactivation. As can be seen from Fig. 3, already low concentrations of ATP reduce the rate of inactivation considerably. High concentrations of ATP (30 mM) completely abolish the inactivation. The amount of ATP giving half maximal protection against the inactivation (ATP protection constant) is found at about 0.1 mM ATP. In order to ascertain that $(sITP)_2$ is really bound at the ATP-binding site of $(Na^+ + K^+)$-ATPase, we compared the action of $(sIMP)_2$ with that of $(sITP)_2$. In agreement with previous results of Hegyvary and Post [15] demonstrating a weak affinity of AMP for the ATP binding site, we find under the experimental

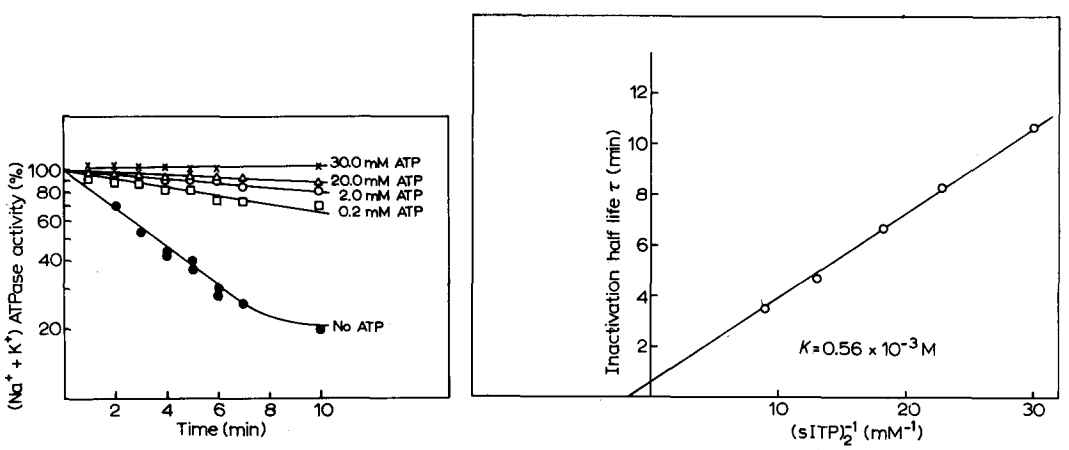

Fig. 3. Effect of increasing ATP concentrations on the inactivation of $(Na^+ + K^+)$-ATPase by 110 μM $(sITP)_2$. (For details see Fig. 4)

Fig. 4. Inactivation half life as a function of the reciprocal concentration of $(sITP)_2$. 0.29 mg $(Na^+ + K^+)$-ATPase (spec. activity 1.6 units/mg) was incubated at 37°C in 100 mM imidazole-HCl, pH 7.25, and increasing concentrations of $(sITP)_2$ (total volume 0.5 ml). Aliquots of 0.04 ml were tested for activity at the times indicated in the coupled optical assay.

conditions of Fig. 3 a small rate of inactivation (5% decrease of activity within 6 min of incubation) by a 15-fold higher $(sIMP)_2$ concentration than $(sITP)_2$. In order to get hints for the kind of interaction of $(Na^+ + K^+)$-activated ATPase with $(sITP)_2$, we studied the effect of dithiothreitol on an enzyme, which had previously been inactivated by $(sITP)_2$: Dithiothreitol re-activated $(Na^+ + K^+)$-ATPase activity (not shown). This finding suggests that membrane-bound $(Na^+ + K^+)$-ATPase from beef brain is inactivated by the formation of a mixed disulfide with 6-mercaptopurine riboside-5′-triphosphate in the active site of the enzyme.

If this conclusion were right, radioactive $(sITP)_2$ should label the membrane protein during the inactivation procedure. As is demonstrated in Fig. 5, incubation of beef brain microsomes with $([\gamma\text{-}^{32}P]sITP)_2$ results in a simultaneous inactivation of $(Na^+ + K^+)$-ATPase and an incorporation of radioactivity into the acid-precipitable membrane protein. The radioactivity incorporated is most likely the result of a transfer of $[\gamma\text{-}^{32}P]sITP$. A transfer of the terminal phosphate group, which seems to be possible, because sITP is a substrate of $(Na^+ + K^+)$-activated ATPase [3], can be excluded, since the inactivation procedure is performed in the presence of 20 mM potassium, and it is well known that $(Na^+ + K^+)$-ATPase is phosphorylated in the presence of Na^+, but not in the presence of K^+ [1]. Moreover, presence of dithiothreitol abolishes the inactivation and the incorporation of radioactivity (Fig. 5). Since the presence of dithiothreitol does not affect the procedure of the Na^+-dependent formation of a phospho-intermediate with $[\gamma\text{-}^{32}P]ATP$, it appears that the incorporation of radioactivity under the conditions of Fig. 5 is due to the formation of a mixed disulfide with a sulfhydryl group.

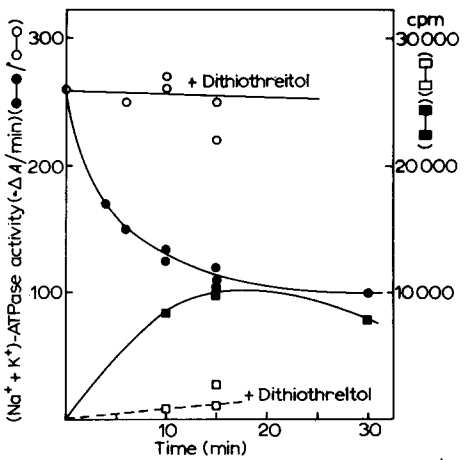

Fig. 5. Comparison of the inactivation of $(Na^+ + K^+)$-ATPase and the $[\gamma\text{-}^{32}P]sITP$ incorporation into the membrane protein during incubation with radioactive $(sITP)_2$. 1.7 mg $(Na^+ + K^+)$-ATPase (spec. activity 2.7 units/mg) was incubated at 37° C in a total volume of 1.05 ml in 100 mM imidazole-HCl (pH 7.25) 20 mM potassium phosphate (pH 7.6) and 2 mM $[\gamma\text{-}^{32}P](sITP)_2$ (spec. activity 42.9 · 10^6 cpm/μmol). Control experiments contained in addition 1 mM dithiothreitol. Aliquots of 0.03 ml of the reaction mixture were used in the optical assay [7] for determinations of the enzymatic activity. The labelling procedure was terminated by the addition of 5 ml cold 5% trichloroacetic acid containing 1 mM P_i and ATP. The precipitate was washed 3 times with 5 ml cold 5% trichloroacetic acid. The last sediment was dissolved in 0.5 ml 1 M NaOH and counted in the medium of Nørby and Jensen [16]. Open symbols (○,□) represent experiments in the presence of 1 mM dithiothreitol, closed symbols (●,■) represent experiments in the absence of dithiothreitol.

Discussion

From these studies with $(sITP)_2$ it appears that this compound acts as an affinity label of the ATP-binding site of $(Na^+ + K^+)$-activated ATPase. This conclusion is based on the findings that:

(a) $(sITP)_2$ but not $(sIMP)_2$ inactivates $(Na^+ + K^+)$-activated ATPase;

(b) ATP protects the enzyme completely against the inactivation by $(sITP)_2$ with the low ATP protective constant of 0.1 mM (Fig. 3). The K_m-value of the enzyme for ATP is in the same concentration range [11—13];

(c) $(sITP)_2$ inactivates $(Na^+ + K^+)$-activated ATPase at an infinitely high concentration with a limited inactivation velocity (Fig. 4). This saturation effect [9,10] confirms that the inactivation of the enzyme by $(sITP)_2$ is preceded by a reversible enzyme-inhibitor complex prior to the formation of a covalent bond (Eqn 1):

$$\text{E-SH} + (\text{sITP})_2 \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{E-SH} \cdot (\text{sITP})_2 \xrightarrow{k_2} \text{E-s-s-ITP} + \text{sITP} \qquad (1)$$

The finding that dithiothreitol protects the enzyme against the inactivation (Fig. 5) or re-activates the already inactivated enzyme, suggests that $(sITP)_2$ forms a mixed disulfide with a sulfhydryl group in the ATP binding site. Similar conclusions have been drawn by Yount et al. [11] in their studies on the action of $(sITP)_2$ on heavy meromyosin. The action of $(sITP)_2$ on $(Na^+ + K^+)$-ATPase as an affinity label supports the earlier suggestions [3] that the 6-amino group of ATP may interact with a sulfhydryl group in the substrate binding site. The ATP analogues 2,4-dinitrophenyl thioinosine triphosphate, which has been shown previously [3] to react at the ATP binding site, and the disulfide of thioinosine triphosphate are voluminous molecules compared with the normal substrate ATP. It therefore appears that the ATP binding site of $(Na^+ + K^+)$-ATPase is more probably a shallow groove than a deep cleft.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft (Scho 139/7) and the Fond der Chemischen Industrie.

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