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ON THE SPECIFICITY OF THE ATP-BINDING SITE OF ($Na^+ + K^+$)-ACTIVATED ATPase FROM BRAIN MICROSOMES

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SUMMARY

In order to obtain information on the specificity of the ATP-binding site of ATPase from brain microsomes, the influence of pyrophosphate, adenine, adenosine, AMP, ADP, CTP, GTP and ITP on ATP binding was investigated by a rapid dialysis rate technique previously described.

1. Pyrophosphate, adenine, adenosine and AMP in concentrations up to about 3 orders of magnitude higher than that of free ATP did not affect the binding of ATP.

2. Less ATP was bound in the presence than in the absence of ADP. Based on a model for competitive binding the affinity of the ATP-binding site for ADP was found to be 5 times smaller than for ATP.

3. CTP, GTP and ITP were only able to displace ATP from the ATP-binding site in concentrations very high relative to that of free ATP. The affinity for these triphosphates was at least 250–850 times lower than for ATP, CTP having the highest affinity of the three compounds examined.

4. The fact that the nucleotide specificity of the ATP-binding site is similar to the substrate specificity of ($Na^+ + K^+$)-activated ATPase strongly suggests that this binding site is identical with the substrate site of ($Na^+ + K^+$)-activated ATPase.

5. It is concluded that the 6-amino group of the purine and pyrimidine ring as well as the β-phosphate group are essential for binding to the substrate site of ($Na^+ + K^+$)-activated ATPase, and that binding of ATP also involves a link between the γ-phosphate group and the enzyme.

INTRODUCTION

In a previous paper[1] we have reported on the binding of ATP to ($Mg^{2+} + Na^+ + K^+$)-activated ATPase from ox brain microsomes. This report indicated that the ATP molecule as such will form a complex with the enzyme, and that the ATP-binding capacity is proportional to the ($Mg^{2+} + Na^+ + K^+$)-ATPase and the ($Na^+ + K^+$)-ATPase activity, suggesting that the enzyme–ATP complex formation is part of the reaction scheme for ATP hydrolysis.

In order to gain information about the specificity of the ATP-binding site we have investigated the influence of pyrophosphate, adenine, adenosine, AMP, ADP, ITP, GTP and CTP on [^{32}P]ATP binding. The results of this investigation are given in

the present paper and are discussed in relation to earlier kinetic studies concerning the interaction of these substances with the ($Na^+ + K^+$)-activated ATPase.

MATERIALS AND METHODS

Reagents

The reagents were the purest obtainable and were purchased from Merck (imidazole, HCl, EDTA and $Na_4P_2O_7$), Sigma (Trizma base), Analar (sucrose), and Boehringer (adenine, adenosine, AMP, trisodium salts of ADP, ITP, GTP and CTP and disodium salt of ATP). ATP labelled with ^{32}P in the γ-position ([^{32}P]ATP) was obtained from The Radiochemical Centre, Amersham, England.

[^{32}P]ATP, ATP and ADP were purified and converted to their Tris salts as described previously[1]. ATPase from ox brain was prepared by the method of SKOU AND HILBERG[2] except that the final suspension was in 12.5 mM imidazole–HCl buffer (pH 7.1 at 37°) containing 250 mM sucrose[1]. The concentration of protein, K^+, Na^+ and ATP-binding sites in the four enzyme preparations used in this investigation as well as the specific activities were determined according to NØRBY AND JENSEN[1] and are given in Table I.

TABLE I

CHARACTERISTICS OF THE ENZYME PREPARATIONS USED IN THE PRESENT INVESTIGATION

The parameters shown were measured as described by NØRBY AND JENSEN[1].

Enzyme prep.	*Protein (mg/ml)*	*K^+ (μM)*	*Na^+ (μM)*	*Specific activity (μmoles P_i/mg protein per h)*			*ATP-binding capacity, [E_t] (μM)*
				($Mg^{2+} + Na^+ + K^+$)-ATPase	*(Mg^{2+})-ATPase*	*($Na^+ + K^+$)-ATPase*	
I	2.04	133	314	148	21	127	0.6*
II	2.46	55	127	169	31	138	0.95
III	3.36	179	448	171	22	149	1.20
IV	2.83	170	366	220	36	184	1.20

* Calculated from the ($Na^+ + K^+$)-activated ATPase activity according to NØRBY AND JENSEN[1].

Binding studies

Binding of ATP to the enzyme preparations was measured by the dialysis rate technique of COLOWICK AND WOMACK[3]. The equipment, the experimental conditions and the counting procedure were as described previously[1]. The influence of adenine, adenosine, AMP, PP_i, ADP, GTP, ITP and CTP on the binding of ATP was investigated by comparing ATP binding in the absence and presence of these compounds at constant ATP concentration. Experimental details will appear in RESULTS.

THEORY

The results of these experiments were evaluated according to the model described below in which it is assumed that the substance in question competes with ATP

for the same binding site

$$E + ATP \rightleftharpoons EATP$$
$$E + X \rightleftharpoons EX$$

in which X symbolizes one of the above compounds, and E = free ATP-binding sites.

At equilibrium

$$\frac{[E]\cdot[ATP]}{[EATP]} = K'_A \quad (1)$$

$$\frac{[E]\cdot[X]}{[EX]} = K'_X \quad (2)$$

$$[E_t] = [E] + [EATP] + [EX] \quad (3)$$

$$[X_t] = [X] + [EX] \quad (4)$$

Measuring [EATP] and [ATP] in the absence of X (called B_0 and F_0, respectively) and in the presence of X (B_X and F_X) we are able to calculate [EX].

Combination of Eqn. (1) and (3) gives

$$K_A' = \frac{([E_t] - B_X - [EX])\cdot F_X}{B_X} = \frac{([E_t] - B_0)\cdot F_0}{B_0} \quad (5)$$

from which

$$[EX] = [E_t] - B_X - \frac{B_X\cdot F_0}{F_X\cdot B_0}([E_t] - B_0) \quad (6)$$

Furthermore,

$$[X] = [X_t] - [EX] \quad (7)$$
$$[E] = [E_t] - B_X - [EX] \quad (8)$$

Eqns. 6, 7 and 8 are now substituted into Eqn. 2

$$K_X' = \frac{([E_t] - B_X - [EX])\,([X_t] - [EX])}{[EX]} \quad (9)$$

K'_X can now be calculated from Eqns. 9 and 6 since $[E_t]$ is known (Table I).

RESULTS

Adenine, adenosine, and AMP

It appears from Fig. 1 that an increase in the concentration of adenosine or AMP from 0 to 3.2 μM and further to 51 μM does not change counts/min per sample of the effluent from the lower chamber*. This means that the ATP binding is unaffected by adenine, adenosine and AMP in concentrations which are about 500 times that of ATP_{total} (1500 times that of free ATP). In this connection it should be recalled that

* The same held true for adenine at a concentration of 50 μM (experiment not shown).

liberation of [^{32}P]ATP from the EATP complex is mirrored by an increase in counts/min per sample[1]. The steady, although slow, increase in counts/min per sample with time has previously been shown to be due to a slow hydrolysis of [^{32}P]ATP in the upper chamber[1].

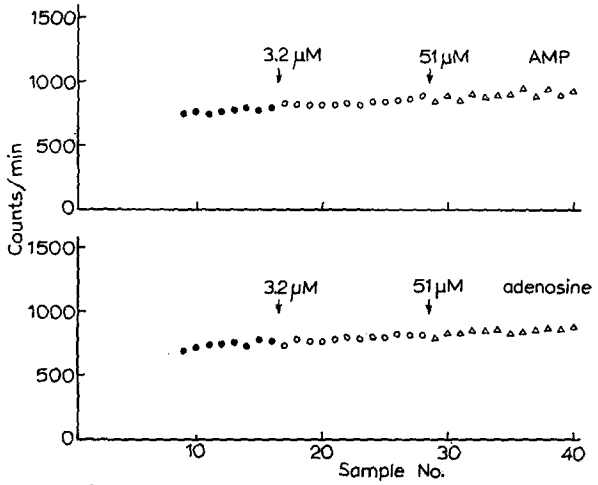

Fig. 1. [^{32}P]ATP binding in the absence and presence of adenosine (lower curve) or AMP (upper curve). Adenosine and AMP dissolved in 300 mM Tris (pH 6.3) (37°) were added to the upper chamber solution (1.56 ml) in 5-μl aliquots. Points of addition (indicated by arrows) and concentrations achieved are shown in the figure. Enzyme preparation I (Table I) was used. ATP concentration in both experiments 0.115 μM of which about 2/3 were bound to the enzyme. Temperature 2°, ionic strength 0.073 M, pH (37°) 7.05.

ADP

Preliminary experiments of the kind shown in Fig. 1 revealed that addition of ADP did cause an increase in free [^{32}P]ATP in the upper chamber. In order to evaluate the effect of ADP quantitatively, two separate experiments were performed, one of which is shown in Fig. 2.

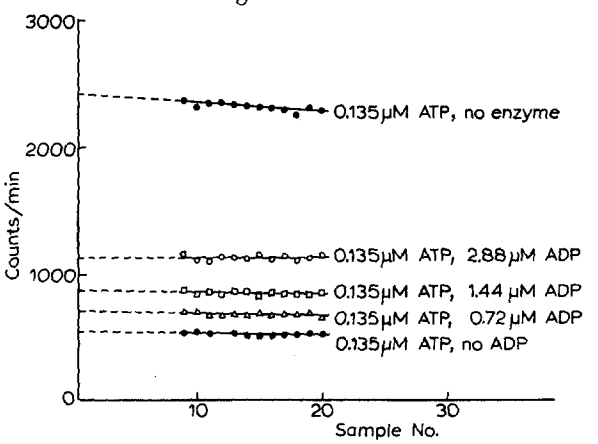

Fig. 2. The effect of ADP on ATP binding by enzyme preparation II (Table I). The composition of the upper chamber solution (1.56 ml) with regard to ATP and ADP is given for each curve. Apart from the ADP concentration, the upper chamber solutions were identical in the five experiments[1], except that in the standard experiment (upper curve) no enzyme was present. Temperature 2°, ionic strength 0.073 M, pH (37°) 7.05.

Fig. 2 shows that increasing concentrations of ADP lead to increasing concentrations of free ATP. The concentration of free (F) and bound ATP (B) in each experiment was calculated from the total ATP concentration and the extrapolated value for counts/min per sample of effluent at zero time[1] obtained from the experimental curve and the standard curve (no enzyme).

The enzyme–ADP dissociation constant, K'_{ADP}, was then calculated from Eqns. 9 and 6 in the THEORY section by inserting B_0, F_0, $[E_t]$ (Table I) and corresponding values of B_X, F_X and $[ADP]_{total}$. The results of this and a similar experiment with enzyme preparation III ($[ATP]_{total} = 0.195$ μM) are given in Table II together with the K^+ concentration in the upper chamber and the apparent dissociation constant of EATP determined separately under identical experimental conditions.

It appears from Table II that K'_{ADP} seems to be independent of the concentration of ADP_{total}, and that K'_{ADP} is 5 times K'_{ATP} in both experiments.

TABLE II

APPARENT DISSOCIATION CONSTANT OF ENZYME–ADP COMPLEX, K'_{ATP}, DETERMINED AS DESCRIBED IN THE TEXT

The data obtained with enzyme preparation II originate from the experiments illustrated in Fig. 2. K'_{ATP} is given for comparison.

Enzyme prep.	$[K^+]$ *in the upper chamber* (μM)	K'_{ATP} (μM)	$[ADP_t]$ (μM)	K'_{ADP}	
				Individual (μM)	*Mean ± S.E.* (μM)
II	49	0.20	0.72	1.06	
			1.44	1.04	1.06 ± 0.01
			2.88	1.08	
III	161	0.26	0.29	1.58	
			0.89	0.82	1.20 ± 0.22
			2.93	1.21	

GTP, ITP and CTP

In experiments like those shown in Fig. 1 with 0.135 or 0.547 μM ATP in the upper chamber, GTP, ITP or CTP in concentrations up to 5 μM were without effect on the ATP binding.

However, when these triphosphates were present in concentrations 200 times that of ATP an effect on ATP binding was observed, less ATP being bound than in the absence of GTP, ITP or CTP. This is apparent from Fig. 3 which gives the results of binding experiments with 0.25 μM ATP without and with 50 μM GTP, ITP or CTP. For comparison an experiment without these triphosphates but with 1.12 μM ATP is included.

Table III gives for each curve the value of the intercept and the slope. It is seen that inclusion of GTP, ITP or CTP leads to a significant increase in the intercept-value which means that less ATP is bound. Furthermore, GTP and ITP significantly decrease the slope suggesting that these two nucleotides decrease the rate of hydrolysis of ATP in the upper chamber.

The concentrations of free ATP (F) and bound ATP (B) corresponding to each curve in Fig. 3 were calculated as described in the section dealing with ADP (see also

TABLE III

THE EFFECT OF GTP, ITP AND CTP ON ATP BINDING AND HYDROLYSIS, ILLUSTRATED BY THE VALUES FOR INTERCEPT AND SLOPE OF THE CURVES SHOWN IN FIG. 3

For further explanation see text.

	0.25 μM ATP	*50 μM GTP 0.25 μM ATP*	*50 μM ITP 0.25 μM ATP*	*50 μM CTP 0.25 μM ATP*	*1.12 μM ATP*
Intercept, counts/min per sample	316 ± 8	379 ± 5	359 ± 3	448 ± 7	500 ± 7
Slope, counts/min per min	15.9 ± 1.0	6.6 ± 0.6	7.2 ± 0.4	17.2 ± 0.8	15.3 ± 0.8

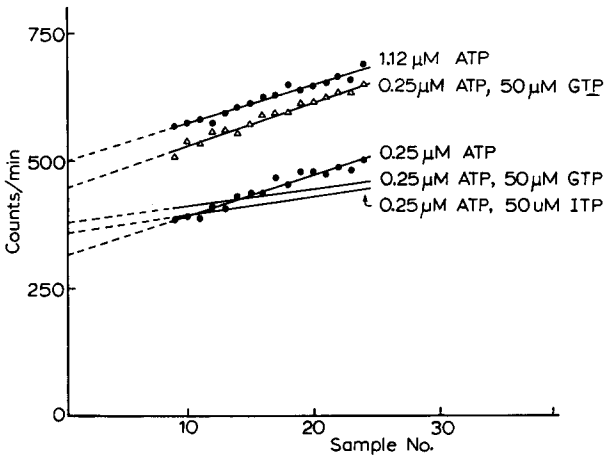

Fig. 3. The effect of GTP, ITP and CTP on [^{32}P]ATP binding by enzyme preparation IV. The composition of the upper chamber solution (1.56 ml) with regard to ATP and the nucleotides in question is given for each curve. For clarity, the points corresponding to the GTP and ITP regression lines shown, are omitted from the figure. The extrapolated intercept with the ordinate axis of the standard curve (no enzyme, 0.25 μM ATP), which is not shown, was 1230 counts/min per sample. Temperature 2°, ionic strength 0.073 M, pH (37°) 7.05.

ref. 1). Based on the model for competitive binding (THEORY section), GTP–, ITP– and CTP–enzyme dissociation constants (K'_{GTP}, K'_{ITP} and K'_{CTP}) were now calculated from Eqn. 9. The results are shown in Table IV.

TABLE IV

APPARENT DISSOCIATION CONSTANTS (μM) OF THE GTP–, ITP– AND CTP–ENZYME COMPLEXES

Calculated from the data given in Fig. 3 and Table III as described in the text. K'_{ATP} is given for comparison.

Enzyme prep.	[K^+] *in the upper chamber* (μM)	K'_{ATP}	K'_{GTP}	K'_{ITP}	K'_{CTP}
IV	153	0.28	161	242	70

Pyrophosphate

The influence of PP_i on ATP binding was investigated as described for GTP, ITP and CTP, but no effect was observed even in the experiment where the concentration of PP_i was 50 μM and that of ATP 0.25 μM. Neither the intercept, nor the slope was changed by PP_i.

DISCUSSION

It is obvious from the above results that adenine, adenosine, AMP, and pyrophosphate even in concentrations very high relative to that of ATP are without effect on ATP binding. Thus, if binding of these compounds to the ATP-binding site can take place at all, the dissociation constants of the formed complexes must be several orders of magnitude higher than that of the enzyme–ATP complex. The observation that AMP and PP_i are not hydrolyzed by $(Na^+ + K^+)$-stimulated ATPases from a variety of tissues[4–10], and that AMP does not inhibit ATP hydrolysis by such preparations[11,12], is in agreement with our present results and our previous suggestion[1] that the binding of ATP studied is confined to the $(Na^+ + K^+)$-activated ATPase.

ADP, on the other hand, was able to displace ATP from the binding site and it is proposed that this observation reflects competition between ADP and ATP for the site. This assumption is supported by the results in Table II which indicates that the dissociation constant, K'_{ADP}, calculated as described in the THEORY section is independent of the ADP concentration (and the $[ADP_t]/[ATP_t]$ ratio). In both experiments the affinity of the enzyme for ADP was found to be about 5 times lower than for ATP.

The inhibition of ATP binding by ADP is probably the mechanism for the ADP-inhibition of ATP hydrolysis in the presence of $(Na^+ + K^+)$-activated ATPase[8,11–16] (and J. C. SKOU, personal communication). ADP inhibition of hydrolysis is, however, also found for other ATPases (for refs. see NØRBY[17]).

GTP, ITP and CTP in concentrations 10–40 times that of ATP_{total} did not influence ATP binding significantly. Only when these triphosphates were present in concentrations 200 times that of ATP a slight inhibition of ATP binding by GTP and ITP was observed, CTP having a somewhat greater effect. The experiments revealed another difference between the effect of GTP and ITP on one hand and CTP and ATP on the other, namely that GTP and ITP both, and to the same extent, reduced the rate of hydrolysis of $[^{32}P]$ATP (decrease in slope, Table III), an effect not obtained with CTP or ATP (Fig. 3).

The lesser binding of ATP in the presence of GTP, ITP or CTP might arouse the suspicion that the commercial triphosphate preparations used were contaminated with ATP. A content of 0.5 % ATP in the GTP and ITP preparations and about 1.5 % ATP in CTP can exclusively explain the observed decrease in binding of $[^{32}P]$ATP. For these reasons and for reasons discussed below the dissociation constants for the GTP–, ITP– and CTP–enzyme complexes listed in Table IV must be considered as minimum values.

The lower rate of hydrolysis of $[^{32}P]$ATP in the presence of GTP or ITP cannot be explained by a displacement of ATP from the site which is responsible for practically all the ATP binding observed in our studies[1]. A likely explanation for this observation is that GTP and ITP as such compete with ATP for a site which, under the experimental conditions used, binds only a small fraction of total ATP bound and has

a relatively high turnover rate. A possible candidate for such a site is the unspecific, Mg-activated (ouabain-insensitive) purine nucleoside triphosphatase known to be present in microsomal preparations [5,7–9,11,13,18–24].

The above results disclose a pronounced specificity of the ATP-binding site, ATP and ADP being bound with a much higher affinity than the other compounds tested.

If the GTP, ITP and CTP preparations are free of ATP, our results furthermore indicate the following order as to the affinity of the site for these compounds: CTP > GTP > ITP.

As far as we are aware, nucleotide–ATPase dissociation constants are not available in the literature. Several investigators have, however, under various conditions, studied the nucleotide preference of ($Na^+ + K^+$)-activated ATPase in experiments on nucleosidetriphosphate hydrolysis[2,4–13,15,18,20,21,23–25], inhibition of ATP hydrolysis by nucleotides[12,13,15] (for refs. concerning AMP and ADP, see above), phosphorylation of the enzyme[2,12,15] and on the di–triphosphonucleotide exchange reaction[26,27]. The general picture emerging from the just mentioned literature is that ATP and ADP are the nucleotides greatly preferred by the substrate site of the ($Na^+ + K^+$)-activated ATPase. Judged from the hydrolysis experiments, the ($Na^+ + K^+$)-activated ATPase seems to have a higher affinity for CTP than for ITP, GTP or UTP[5,7,8,10,13,20,24,25].

In this connection the studies of ROBINSON[28], who showed that only ATP and CTP but not GTP, ITP or UTP were able to induce structural changes in brain microsomes, are of interest. Furthermore, it seems important to note that the Na^+-transport system of human red cell ghosts only uses ATP as substrate, the ouabain-sensitive outflux in the presence of ITP, GTP, UTP or CTP being very small or absent[29,30].

It appears from the above discussion that the nucleotide specificity of the binding site with which we are primarily concerned in the present study is similar to the substrate specificity of ($Na^+ + K^+$)-activated ATPase. This, together with the previously demonstrated proportionality between ATP-binding capacity and ($Na^+ + K^+$)-ATPase activity[1], strongly suggest that this binding site is identical with the substrate site of ($Na^+ + K^+$)-ATPase.

Moreover, we feel justified to conclude that the 6-amino group on the purine or pyrimidine ring as well as the β-phosphate group is essential for binding. The fact that the dissociation constant of the enzyme–ADP complex is five times that of the enzyme–ATP complex, presumably indicates that binding of ATP also involves a link between the γ-phosphate group and the enzyme. Similar conclusions can be drawn from studies on the interaction of nucleotides with myosin ATPase[31] and heavy meromyosin[32].

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