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# HYDROLYSIS OF ADENYLYL IMIDODIPHOSPHATE IN THE PRESENCE OF $Na^+ + Mg^{2+}$ BY $(Na^+ + K^+)$-ACTIVATED ATPase *

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**(1) Contrary to what has usually been assumed, $(Na^+ + K^+)$-ATPase slowly hydrolyses Ado*PP*[NH]*P* in the presence of $Na^+ + Mg^{2+}$ to ADP-$NH_2$ and $P_i$. The activity is ouabain-sensitive and is not detected in the absence of either $Mg^{2+}$ or $Na^{2+}$. The specific activity of the $Na^+ + Mg^{2+}$ dependent Ado*PP*[NH]*P* hydrolysis at 37°C and pH 7.0 is 4% of that for ATP under identical conditions and only 0.07% of that for ATP in the presence of $K^+$. The activity is not stimulated by $K^+$, nor can $K^+$ replace $Na^+$ in its stimulatory action. This suggests that phosphorylation is rate-limiting. Stimulation by $Na^+$ is positively cooperative with a Hill coefficient of 2.4; half-maximal stimulation occurs at 5–9 mM. The $K_m$ value for Ado*PP*[NH]*P* is 17 μM. At 0°C and 21°C the specific activity is 2 and 14%, respectively, of that at 37°C. AMP, ADP and Ado*PP*[$CH_2$]*P* are not detectably hydrolysed by $(Na^+ + K^+)$-ATPase in the presence of $Na^+ + Mg^{2+}$. (2) In addition, Ado*PP*[NH]*P* undergoes spontaneous, non-enzymatic hydrolysis at pH 7.0 with rate constants at 0, 21 and 37°C of 0.0006, 0.006 and 0.07 $h^{-1}$, respectively. This effect is small compared to the effect of enzymatic hydrolysis under comparable conditions. $Mg^{2+}$ present in excess of Ado*PP*[NH]*P* reduces the rate constant of the spontaneous hydrolysis to 0.005 $h^{-1}$ at 37°C, indicating that the MgAdo*PP*[NH]*P* complex is virtually stable to spontaneous hydrolysis, as is also the case for its enzymatic hydrolysis. (3) A practical consequence of these findings is that Ado*PP*[NH]*P* binding studies in the presence of $Na^+ + Mg^{2+}$ with enzyme concentrations in the mg/ml range are not possible at temperatures above 0°C. On the other hand, determination of affinity in the $(Na^+ + K^+)$-ATPase reaction by competition with ATP at low protein concentrations (μg/ml range) remains possible without significant hydrolysis of Ado*PP*[NH]*P* even at 37°C.**

## Introduction

The ATP analogue Ado*PP*[NH]*P*, with an imino group replacing the oxygen linking the β- and γ-phosphate group, has been successfully used as a non-hydrolyzed, non-phosphorylating substrate analogue in studies on ATP phosphohydrolases [1,2], including $(Na^+ + K^+)$-activated ATPase [3–5]. Simons [3] found some hydrolysis of the analogue by red cell membranes in the presence of $Na^+ + K^+ + Mg^{2+}$, but this was not inhibited by ouabain, suggesting that $(Na^+ + K^+)$-ATPase activity was not involved.

We have used Ado*PP*[NH]*P* in studies on nucleotide binding to $(Na^+ + K^+)$-ATPase from rabbit kidney outer medulla, proving the uncovering of a non-phosphorylating low-affinity nucleotide binding site in the presence of $Mg^{2+}$ in addition to the phosphorylating high-affinity site [6].

* This is article No. 51 in the series Studies on $(Na^+ + K^+)$-activated ATPase. Article No. 50 is Ref. 19.

Abbreviations: Ado*PP*[NH]*P*, adenylyl imidodiphosphate; CDTA, *trans*-1,2-diaminocyclohexane tetraacetic acid.

Ado*PP*[NH]*P* has also been used in studies of the effect of $Na^+$ or $K^+$, in the presence or absence of $Mg^{2+}$, on high-affinity nucleotide binding to ($Na^+ + K^+$)-ATPase from canine kidney medulla [7] and pig kidney outer medulla [8]. In these experiments $Na^+$ increased the $K_d$ for the nucleotide at mmolar $Mg^{2+}$.

In the context of further studies on the function of the low-affinity nucleotide binding site, we began to study the effects of $Na^+$ and $K^+$ on the affinity and capacity of Ado*PP*[NH]*P* binding to this $Mg^{2+}$-induced site. We find that $Na^+$ in the presence of millimolar $Mg^{2+}$ reduces Ado*PP*[NH]*P* binding in a slow process, taking hours for completion at 22°C and resulting in an apparent increase of the $K_d$. Prior binding of the nucleotide is essential, but preincubation of the enzyme with $Na^+ + Mg^{2+}$ has no additional effect. $Na^+$ exerts a specific effect in the 1–5 mmolar range, while only $10^{-6}$ M $Mg^{2+}$ is required for near-maximal inhibition of binding. Since these conditions resemble those required for phosphorylation by ATP, it occurred to us that Ado*PP*[NH]*P* in the presence of $Mg^{2+}$ and $Na^+$ may slowly phosphorylate ($Na^+ + K^+$)-ATPase and thus be slowly hydrolyzed. This has prompted us to investigate the hydrolysis of Ado*PP*[NH]*P* by ($Na^+ + K^+$)-ATPase.

## Materials and Methods

*Enzyme preparation and hydrolytic assays*

($Na^+ + K^+$)-ATPase is purified from rabbit kidney outer medulla as described by Jørgensen [9]. Removal of contaminating ATP, subsequent washing and storage of the preparation follows the method of Schoot et al. [10], except that the storage medium contains 50 mM imidazole-HCl (pH 7.0), 0.25 M sucrose and no CDTA.

($Na^+ + K^+$)-ATPase activity with ATP as the substrate is determined essentially according to the non-radioactive assay method of Schoot et al. [10], following 10–15 min incubations at enzyme concentrations of 1.2–3 μg/ml. The specific ATPase activities of the preparations used in this study are 1330–1540 $\mu mol \cdot (mg\ protein)^{-1} \cdot h^{-1}$, while their enzymatic hydrolysis rates for Ado*PP*[NH]*P* are 0.06–0.07% of that for ATP.

Enzymatic as well as non-enzymatic (spontaneous) hydrolysis of 1 mM Ado*PP*[NH]*P* is assayed in 50 mM imidazole-HCl (pH 7.0) with and without various additions as indicated in Table I. ($Na^+ + K^+$)-ATPase, when present, is used in a concentration of 0.1–0.15 mg/ml. After 1–4 h, 0.3 ml aliquots of the incubation medium are added to 0.45 ml 11.3% trichloroacetic acid. When enzyme is present, the mixture is then centrifuged for 15 min at $2000 \times g$ at 0°C. To 0.6 ml of the supernatant 0.475 ml of colour reagent [10] is added and after 30 min at room temperature the absorbance is read at 700 nm. In the assays of enzymatic hydrolysis blanks, containing 1 mM ouabain, are subtracted.

Other nucleotides (ATP, ADP, AMP and Ado*PP*[$CH_2$]*P*), have been compared with Ado*PP*[NH]*P* for enzymatic hydrolysis in the presence of $Na^+ + Mg^{2+}$. The nucleotides, obtained from Boehringer Mannheim, GmbH, Biochemica, F.R.G., are first converted to their imidazole salts, either by neutralization of the acids (ADP, AMP) or Dowex 50 WX4 (imidazole form) column chromatography (ATP, Ado*PP*[NH]*P*, Ado*PP*[$CH_2$]*P*) as previously described [6]. They are incubated in 1 mM concentration with the enzyme at 37°C for 8 min in the case of ATP and for 3 h in the case of the others. The reaction is stopped by addition of trichloroacetic acid.

The $K_m$ value for Ado*PP*[NH]*P* in the $Na^+ + Mg^{2+}$ stimulated hydrolysis has been determined using the very sensitive though rather unstable malachite green staining method for $P_i$ [11]. Ado*PP*[NH]*P* (20–100 μM) is incubated with 22 μg protein/ml for 35 min at 37°C in the presence of 100 mM $Na^+$ and 5 mM $Mg^{2+}$. Hydrolysis is stopped by cooling in ice and 0.23 ml aliquots are stained. The 625 nm absorbance is read within 1 min after colour development. As protein affects the 625 nm absorbance [12], the $P_i$ standards contain the same concentration of ($Na^+ + K^+$)-ATPase as the incubation medium for Ado*PP*[NH]*P* hydrolysis. The $K_m$ is determined from a Lineweaver-Burk plot, taking as substrate concentrations the mean of the Ado*PP*[NH]*P* concentrations at 0 and 35 min.

*Nucleotide analysis*

The hydrolysis product of Ado*PP*[NH]*P* has been identified by high pressure liquid chromatog-

raphy (HPLC) as described by Salceda et al. [13] with minor modifications. A 50-min gradient elution time, a 15-min elution with the high concentration buffer at a pump speed of 1.5 ml/min and a full scale recorder setting of 0.2 at 254 nm absorbance are used. Ado*PP*[NH]*P* (1 mM) is incubated for 1 h at 37°C with ($Na^+ + K^+$)-ATPase (0.86 mg/ml) in 50 mM imidazole-HCl (pH 7.0) in the presence of 5 mM $Mg^{2+}$ with or without $Na^+$ (100 mM). The reaction is stopped by addition of 50 μl 2.4 M $HClO_4$ to 0.25 ml of the incubation medium. After standing for 10 min at 0°C, the mixture is centrifuged for 15 s at 10000 × *g*. To 0.2 ml of the supernatant 50 μl 1.6 M KOH is added and the $KClO_4$ precipitate removed by centrifugation. A 25-μl aliquot, containing 17 nmol nucleotide, is injected into the HPLC column. A mixture of nucleotide standards (AMP, ADP, ATP, Ado*PP*[NH]*P*, 1 mM each) is processed in the same way (from addition of $HClO_4$). ADP-$NH_2$ is prepared by incubating 1 mM Ado*PP*[NH]*P* for 1 h at 37°C with alkaline phosphatase from *Escherichia coli* (Sigma Chemical Co., St. Louis, MO), 15 units in 0.25 ml 25 mM imidazole-HCl (pH 8.0) in the presence of 5 mM $Mg^{2+}$, further processing as described above for the Ado*PP*[NH]*P* hydrolysis by ($Na^+ + K^+$)-ATPase (from $HClO_4$ addition).

## Results

Fig. 1 clearly demonstrates that ($Na^+ + K^+$)-ATPase purified from rabbit kidney outer medulla hydrolyzes Ado*PP*[NH]*P* in the presence of $Mg^{2+} + Na^+$. The hydrolysis rate at 37°C is constant during the first two hours and then declines to 64% of the initial rate in the next two hours. The average specific activity with Ado*PP*[NH]*P* as substrate at 37°C is 1.02 (S.E. 0.07) μmol · (mg protein)$^{-1} \cdot h^{-1}$ for the four enzyme preparations. This is 4% of the specific $Na^+$-ATPase activity ($K^+$ absent) under identical conditions but with ATP as substrate, and only 0.07% of the specific ($Na^+ + K^+$)-ATPase activity in the presence of ATP + $Mg^{2+} + Na^+ + K^+$. Addition of $K^+$ does not increase the phosphohydrolase activity with Ado*PP*[NH]*P* as substrate, neither can $K^+$ replace $Na^+$ (Table I). Other additions than $Na^+ + Mg^{2+}$ or $Na^+ + K^+ + Mg^{2+}$ (EDTA with or without $Na^+$ and/or $K^+$; $Na^+$ and/or $K^+$ alone; $Mg^{2+}$ alone or with $K^+$) do not give rise to any significant ouabain-sensitive hydrolysis of Ado*PP*[NH]*P*. In all those cases the ouabain-insensitive hydrolysis of Ado*PP*[NH]*P*. In all those cases the ouabain-insensitive hydrolysis in the presence of enzyme is equal to the non-enzymatic hydrolysis discussed in the next paragraph.

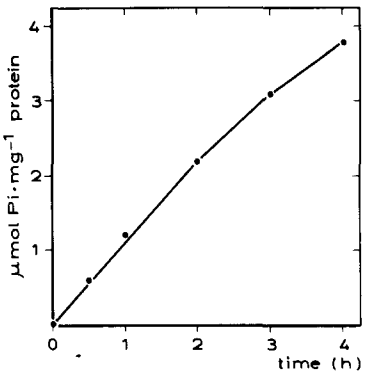


Fig. 1. ($Na^+ + Mg^{2+}$)-dependent enzymatic hydrolysis of Ado*PP*[NH]*P*. The ouabain-sensitive hydrolysis of Ado*PP*[NH]*P* (1 mM) by ($Na^+ + K^+$)-ATPase (0.15 mg/ml; spec. act. 1500 μmol·(mg protein)$^{-1} \cdot h^{-1}$) in the presence of 5 mM $Mg^{2+}$ and 100 mM $Na^+$ is followed at 37°C and pH 7.0 for the indicated lengths of time.

In addition to the enzymatic ouabain-sensitive hydrolysis of Ado*PP*[NH]*P*, there is non-enzymatic hydrolysis with a rate constant which is only dependent on the presence or absence of $Mg^{2+}$. In the absence of $Mg^{2+}$ the rate constant amounts to 0.066 (S.E. 0.003; $n = 9$) $h^{-1}$ at pH 7.0 and 37°C, which is equivalent to only 7% of that of the maximal enzymatic hydrolysis per mg protein. Addition of excess $Mg^{2+}$ in the presence or absence of enzyme, $Na^+$ being absent in the former case, reduces the hydrolysis rate constant to 0.005 (S.E. 0.001; $n = 5$) $h^{-1}$ or less than one tenth the rate present in the absence of $Mg^{2+}$, both in the presence of enzyme ($Na^+$ omitted) or its absence. This indicates that the $Mg^{2+}$-nucleotide complex is virtually stable to spontaneous hydrolysis, as well as to enzymatic hydrolysis in the absence of $Na^+$.

Other nucleotides tested, viz. ADP, AMP and Ado*PP*[$CH_2$]*P*, do not show ouabain-sensitive, ($Na^+ + Mg^{2+}$)-stimulated, enzymatic hydrolysis. Hence, the β-γ methylene phosphate linkage, the

TABLE I

HYDROLYSIS OF Ado*PP*[NH]*P*

The enzymatic hydrolysis of Ado*PP*[NH]*P* (1 mM) is assayed for 4 h at the indicated temperatures with 0.15 mg/ml ($Na^+ + K^+$)-ATPase (specific activity 1430 $\mu$mol·(mg protein)$^{-1}$·h$^{-1}$) present. Cations added as chlorides. The results are expressed in $\mu$mol·(mg protein)$^{-1}$·h$^{-1}$. The non-enzymatic hydrolysis in 4 h is assayed with 1 mM Ado*PP*[NH]*P* in the same media but without enzyme and $Mg^{2+}$. The results are expressed as rate constants in h$^{-1}$. Fractional increment per °C in specific activity and rate constant of non-enzymatic hydrolysis have been calculated for the 0–21°C and 21–37°C temperature range separately and the range is indicated.

| Enzymatic hydrolysis<br>Additions (concn. in mM) | Specific activity ($\mu$mol·(mg protein)$^{-1}$·h$^{-1}$) | | | Fractional increments per °C |
|---|---|---|---|---|
| Temperature: | 0°C | 21°C | 37°C | |
| $Mg^{2+}$(5) | < 0.01 | 0.02 | < 0.01 | – |
| $Mg^{2+}$(5)+$Na^+$(100) | 0.02 | 0.14 | 1.01 | 0.29–0.39 |
| $Mg^{2+}$(5)+$K^+$(10) | 0.01 | < 0.01 | < 0.01 | – |
| $Mg^{2+}$(5)+$Na^+$(100)+$K^+$(10) | 0.01 | 0.15 | 0.97 | 0.34–0.67 |
| Non-enzymatic hydrolysis | Rate constant (h$^{-1}$) | | | |
| Temperature: | 0°C | 21°C | 37°C | |
| Various $Na^+$ and $K^+$ additions as above, with and without 2 mM EDTA | 0.00057 | 0.0063 | 0.066 | 0.48–0.59 |

$\alpha$-phosphoester and the $\alpha$-$\beta$ pyrophosphate linkage are not susceptible to hydrolysis by ($Na^+ + K^+$)-ATPase.

The ($Na^+ + Mg^{2+}$)-dependent enzymatic hydrolysis of Ado*PP*[NH]*P*, as well as its non-enzymatic hydrolysis, are strongly temperature dependent, as is the case for the enzymatic hydrolysis of ATP by ($Na^+ + K^+$)-ATPase. Fractional increments in specific activities and in the rate constant of non-enzymatic hydrolysis range between 0.3 and 0.67/C deg. in the temperature range of 0–37°C (Table I).

The rate of hydrolysis is dependent on the $Na^+$ concentration in sigmoidal fashion, with half-maximal stimulation occurring at 5–9 mM $Na^+$ (Fig. 2A). A biphasic stimulation, as often observed with ATP as substrate in the absence of $K^+$ [14–16], is not seen here. The $K_m$ value of 5–9 mM for $Na^+$ agrees with its value (5–8 mM) in the $Na^+ + Mg^{2+}$-dependent phosphorylation by ATP [14,17]. The Hill coefficient of 2.4 (Fig. 2B) agrees with that of 2.5–3 for the binding of $Na^+$ to the enzyme [18].

The $K_m$ for Ado*PP*[NH]*P* has been determined as described under Materials and Methods. No concentrations below 20 $\mu$M have been used as the phosphate production then sinks below the detection limit of the malachite green assay. The $K_m$ value of 17 $\mu$M thus obtained is close to the $K_d$ for high-affinity binding. The $K_d$ value, determined in the presence of 5 mM $Mg^{2+}$ but absence of $Na^+$ at 37°C and pH 7.0, is 6 $\mu$M [19], but in the presence of $Mg^{2+}$ and $Na^+$ it increases to 12 $\mu$M, even at 0°C [7] where no hydrolysis is to be expected according to our findings (Table I).

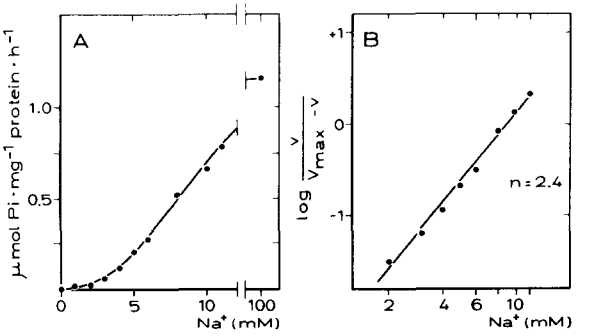


Fig. 2. ($Na^+ + Mg^{2+}$)-dependent enzymatic hydrolysis of Ado*PP*[NH]*P* as a function of the $Na^+$ concentration. (A) The ouabain-sensitive hydrolysis of Ado*PP*[NH]*P* (1 mM) by ($Na^+ + K^+$)-ATPase (0.15 mg/ml, spec. act. 1540 $\mu$mol·(mg protein)$^{-1}$·h$^{-1}$) in the presence of 5 mM $Mg^{2+}$ and the concentrations of $Na^+$ as indicated is assayed after 2 h incubation at 37°C and pH 7.0. (B) Same data presented in a Hill plot. The Hill coefficient or cooperativity index $n$, equal to the slope, is indicated. The line through the experimental points is determined by linear regression analysis.

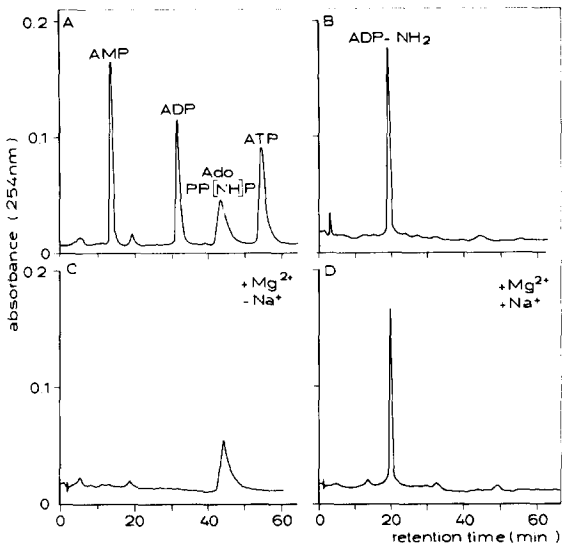


Fig. 3. HPLC elution pattern of Ado*PP*[NH]*P* and its hydrolysis product. (A) Elution pattern of AMP, ADP, Ado*PP*[NH]*P* and ATP. (B) Elution profile of ADP-$NH_2$, prepared by enzymatic hydrolysis of Ado*PP*[NH]*P* with *E. coli* alkaline phosphatase. (C) Elution profile of Ado*PP*[NH]*P* after 1 h incubation of the nucleotide (1 mM) with ($Na^+ + K^+$)-ATPase (0.86 mg/ml) in the absence of $Na^+$ and the presence of 5 mM $Mg^{2+}$ at 37°C and pH 7.0, showing no hydrolysis products. (D) Elution profile of Ado*PP*[NH]*P* after 1 h incubation under the conditions of (C) but with 5 mM $Mg^{2+}$ + 100 mM $Na^+$ present, showing its hydrolysis product.

The hydrolysis product, which in analogy to the hydrolysis product of ATP would be expected to be ADP-$NH_2$, has been determined by HPLC. Fig. 3A presents the HPLC elution pattern of a number of adenine nucleotides. The retention times are 13.5 min for AMP, 31.5 min for ADP, 43.3 min for Ado*PP*[NH]*P*, and 54.8 min for ATP. ADP-$NH_2$, generated from Ado*PP*[NH]*P* with *E. coli* alkaline phosphatase, has a retention time of 18.3–19.5 min (Fig. 3B). Incubation of Ado*PP*[NH]*P* with ($Na^+ + K^+$)-ATPase for 1 h in the presence of $Mg^{2+}$ without $Na^+$ gives no detectable break-down of the nucleotide (retention time 43.3 min, Fig. 3C), but incubation under the same conditions in the presence of $Mg^{2+} + Na^+$ gives a product with the same retention time (19.5 min, Fig. 3D) as the product of hydrolysis by alkaline phosphatase. This identifies the ($Na^+ + K^+$)-ATPase hydrolysis product of Ado*PP*[NH]*P* as ADP-$NH_2$.

## Discussion

Contrary to what is commonly assumed, we find that Ado*PP*[NH]*P* is hydrolysed by ($Na^+ + K^+$)-ATPase in the presence of $Na^+ + Mg^{2+}$, although at an extremely low rate, i.e. 0.07% of the rate of ATP hydrolysis in the presence of $Na^+ + K^+ + Mg^{2+}$. $K^+$ does not stimulate Ado*PP*[NH]*P* hydrolysis, which indicates that $Na^+$-dependent phosphorylation is the rate-limiting step, rather than $K^+$-stimulated dephosphorylation. This conclusion is supported by the absence of a biphasic $Na^+$ stimulation curve, which is commonly observed with ATP as substrate in the absence of $K^+$ [14–16], and where the second phase is attributed to $Na^+$-stimulated dephosphorylation. It is also supported by the agreement between the $K_m$ values for $Na^+$ in Ado*PP*[NH]*P* hydrolysis (5–9 mM) and in ($Na^+ + Mg^{2+}$)-dependent phosphorylation by ATP (5–8 mM) and between the $K_m$ value for Ado*PP*[NH]*P* in its enzymatic hydrolysis and the $K_d$ value for its binding to the high-affinity phosphorylating site (17 and 6 $\mu$M, respectively).

From the agreement between the Hill coefficients of 2.4 for the $Na^+$ effect on Ado*PP*[NH]*P* hydrolysis and of 2.5–3 for $Na^+$-binding to the enzyme [18] and the Na/ATP transport ratio of nearly 3 [20], we conclude that three $Na^+$ sites display positive cooperative behaviour in the hydrolytic process. This can also be seen when phosphorylation by ATP is assayed in the presence of $K^+$ [21], which slows down phosphorylation by shifting the $E_1K \rightleftharpoons E_2K$ equilibrium towards $E_2K$ [22]. On the other hand, no cooperativity between $Na^+$ sites is apparent, either in the $Na^+$-ATPase reaction, where dephosphorylation is rate limiting, or in the phosphorylation reaction by ATP, both of which are characterized by non-sigmoid $Na^+$-stimulation curves [14–17,23]. The cooperative interaction between the $Na^+$ sites may either occur in the phosphorylation step or in the preceding reaction step, i.e. the $E_2 \rightarrow E_1$ transition.

It appears that ($Na^+ + K^+$)-ATPase is not the only cation transport ATPase, which can split Ado*PP*[NH]*P*. ($Ca^{2+} + Mg^{2+}$)-ATPase in sarcoplasmic reticulum also hydrolyzes Ado*PP*[NH]*P*, but at 0.2% the rate for ATP, and yielding the same products as ($Na^+ + K^+$)-ATPase, viz. ADP-

$NH_2$ and $P_i$ [24]. Ado*PP*[$CH_2$]*P* is not hydrolyzed by either enzyme, possibly because of its deviating P-C-P bond angle and stretches [1]. Unfortunately, at present it cannot very well replace Ado*PP*[NH]*P* in binding studies, since a radioactive compound is not commercially available.

An important question is whether Ado*PP*[NH]*P* can still be useful in binding studies. There appears to be no problem when the reaction is carried out in the presence of excess $Mg^{2+}$ but in the absence of $Na^+$ as in our earlier studies [6], since then there is no enzymatic hydrolysis. The non-enzymatic hydrolysis at 37°C in the presence of excess $Mg^{2+}$ is less than 1% per h, while in the absence of $Mg^{2+}$ it is still only 6.4% per h at 37°C and less than 1% per h at 21°C.

However, in the presence of $Na^+$ (and $Mg^{2+}$) our values for $V_{max}$ (Table I) and $K_m$ (17 $\mu$M) predict hydrolysis rates of 0.06–1.0 $\mu mol \cdot mg^{-1} \cdot h^{-1}$ at 37°C in the 1–300 $\mu$M range of Ado*PP*[NH]*P* concentrations used in our binding studies [6]. This means that such appreciable amounts of the nucleotide will be hydrolyzed (45–90%/min per mg protein in the concentration range, 1–20 $\mu$M, of high-affinity binding) that reliable binding studies in the presence of $Mg^{2+} + Na^+$ cannot be executed. At 21°C we calculate a breakdown of 6–13%/min per mg protein (1–20 $\mu$M nucleotide). Only at 0°C, hydrolysis becomes negligible, approx. 0.5–2% per min per mg protein (1–20 $\mu$M nucleotide).

We have also used Ado*PP*[NH]*P* (100–400 $\mu$M) as a competitor for ATP (200–2000 $\mu$M) in the $(Na^+ + K^+)$-ATPase reaction during 10 min assays at 11–37°C [19]. Calculation by means of Michaelis-Menten kinetics and the known kinetic parameters shows that the hydrolysis of Ado*PP*[NH]*P* would be less than 0.01% under those conditions (0.2 mM ATP, 0.5–23 $\mu$g/ml enzyme protein). This means that in studies of high-affinity competition by ATP at low protein concentrations hydrolysis of Ado*PP*[NH]*P* via the high-affinity sites is negligible.

In conclusion, Ado*PP*[NH]*P* cannot be used to analyse high-affinity binding at relatively high protein concentrations ($\geq$ 1 mg/ml) in the presence of $Na^+ + Mg^{2+}$ and at temperatures above 0°C, whereas it can be used to analyse its affinity in the ATPase reaction by competition with ATP at relatively low protein concentrations at all feasible temperatures.

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