Na,K-dependent adenosine triphosphate phosphohydrolase: activation of the phosphatase reaction by ATP analogs

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The effect of N_1-substituted analogs of ATP on the hydrolysis of umbelliferone phosphate by Na,K-ATPase has demonstrated: (i) analogs having a negatively charged substituent (N_1-oxy- or N_1-carbo-methoxy-ATP) and capable of accepting H^+ induce an activation similar to that of ATP; ii) N_1-methoxy-ATP, containing an uncharged substituent, does not affect the phosphatase reaction at low concentration and inhibits it at higher concentration. It has been assumed that ATP binding to Na,K-ATPase induces formation of a hydrogen bond between the nitrogen atom at the first position of the purine base and appropriate amino acid of active centre, with a subsequent attachment of H^+ to ATP, thus facilitating the transition of Na,K-ATPase from the K^+- to the Na^+-form.

Na,K-ATPase *ATP analog* *Phosphatase reaction* *Duck salt gland*

1. INTRODUCTION

Besides ATP, Na,K-activated, Mg-dependent adenosine triphosphate phosphohydrolase (Na,K-ATPase) uses other nucleoside triphosphates (CTP, ITP, GTP, UTP) as substrates [1,2]. The hydrolysis of ATP and CTP by Na,K-ATPase occurs via non-Michaelis kinetics [2–4]. The hydrolysis of ITP, GTP and UTP by this enzyme can be described in terms of a simple hyperbolic dependence on substrate concentration [2]. All of the nucleotides can eliminate the Na^+-induced inhibition of K^+-dependent hydrolysis of *p*-nitrophenyl phosphate or umbelliferone phosphate, which are also substrates for Na,K-ATPase [5–8]. The activating effect of nucleotides decreases in the following order: ATP CTP ITP UTP GTP [7] and thus correlates at first approximation with the affinity of them to ATP-binding site of Na,K-ATPase [9,10] as well as with the enzyme turnover number for these substrates [2].

These data indicate that among all substrates investigated CTP bears the highest resemblance with ATP. Although CTP is a pyrimidine nucleotide, its structural properties are similar to those of ATP. At physiological pH both nucleotides exist in the amino form. The substituent at the sixth position of the purine base (fourth position of the pyrimidine base) is an amino group; the N_1 nitrogen of purine (N_3 nitrogen of pyrimidine) is in the non-protonated form (fig.1). It is known that

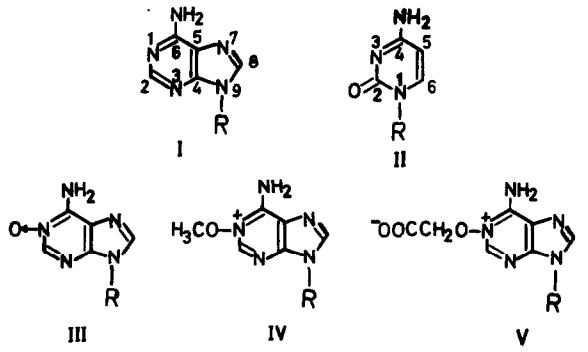

Fig.1. Structural formulas of ATP (I), CTP (II), N_1-oxy-ATP (III), N_1-methoxy-ATP (IV) and N_1-carboxymethoxy-ATP (V). R, ribose-5′-triphosphate tail of the nucleotide molecule.

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this nitrogen atom can bind H^+ and participate in the formation of a hydrogen bond, e.g., with the NH_2 group of the appropriate base in a double helix of a nucleic acid, or with the tyrosine residue of the active centre of lactate dehydrogenase during formation of the NAD^+-enzyme complex [11].

In order to evaluate the role of the proton-accepting ability of the N_1-nitrogen of purine base during ATP recognition by Na,K-ATPase, we studied the activating effect of N_1-substituted analogs of ATP on the phosphatase reaction. In these experiments two analogs, containing negatively charged substituents (N_1-oxy-ATP and N_1-carboxymethoxy-ATP) and an analog containing an uncharged substituent (N_1-methoxy-ATP) were used (fig.1).

2. MATERIALS AND METHODS

Na,K-ATPase was purified from supraorbital glands of domestic ducks after 10 days of a high salt and water diet [12,13]. The specific activity of Na,K-ATPase was about 25 μmol ATP split/mg protein per min (37°C). The rate of umbelliferone phosphate hydrolysis was determined from the changes in the fluorescence of umbelliferone as described previously [14]. The measurements were performed on a Hitachi 512 spectrofluorimeter (exciting light wavelength 365 nm, fluorescence 440 nm, spectral slit widths of exciting and emitting light paths 3 and 20 nm, respectively).

The incubation medium (final volume 3 ml) contained: 1 mM KCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.4 mM umbelliferone phosphate and 30 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonate (HEPES), pH 7.8, at 37°C. The reaction was initiated by adding 6–8 μg protein.

The nucleotides used were purchased from Serva and Reanal. HEPES was obtained from Merck and umbelliferone phosphate from IsoLab. The purity of nucleotides was checked by thin layer chromatography, nucleotide concentration was determined spectrophotometrically.

N_1-Oxy-ATP was synthesized according to [15]. N_1-Methoxy-ATP and N_1-carboxymethoxy-ATP were prepared according to a procedure developed in our laboratory [16]. All synthetic preparations were characterized by an elementary analysis, electrophoresis and UV and IR spectroscopy [16].

3. RESULTS AND DISCUSSION

As shown previously [7,8] ATP addition to Na,K-ATPase in the presence of a low concentration of K^+ (1 mM) and a moderate concentration of Na^+ (10 mM) results in activation of umbelliferone phosphate hydrolysis, the degree and duration of this activating effect being dependent on ATP concentration (fig.2). This effect of ATP on the phosphatase reaction is a result of ATP hydrolysis and can be eliminated by complete breakdown of ATP to ADP and P_i [7]. The degree of activation also depends on the amount of ATP added, i.e., as the ATP concentration rises (from 3 to 10 μM), the activating effect increases. With a further increase in the nucleotide concentration the activating effect is diminished, eventually resulting in inhibition of the phosphatase reaction at ATP concentrations exceeding 250 μM (fig.3).

The activating effect of CTP and ITP on the phosphatase reaction is less pronounced than that of ATP and is observed at higher nucleotide concentrations (fig.3). This is consistent with the data reported previously [6,7]. Inhibition of the phosphatase reaction by CTP and ITP is attained only at concentrations above 400 μM. The activation by CTP and ITP is more prolonged than that of ATP, presumably due to the lower hydrolytic activity of the enzyme towards these substrates.

Our study on the effects of some ATP analogs has demonstrated that N_1-oxy-ATP and N_1-carboxymethoxy-ATP activate the phosphatase reaction almost to the same extent as ATP (figs 2 and 3). The maximal activating effect is observed in the presence of 20 μM N_1-carboxymethoxy-ATP and 30 μM N_1-oxy-ATP (fig.3). The diminution of

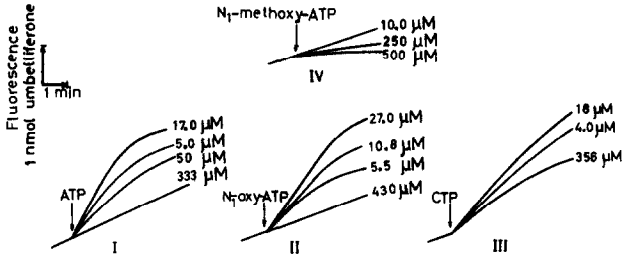

Fig.2. Activation of enzymatic hydrolysis of umbelliferone phosphate by ATP (I), N_1-oxy-ATP (II), CTP (III) and N_1-methoxy-ATP (IV). Concentrations are given in brackets.

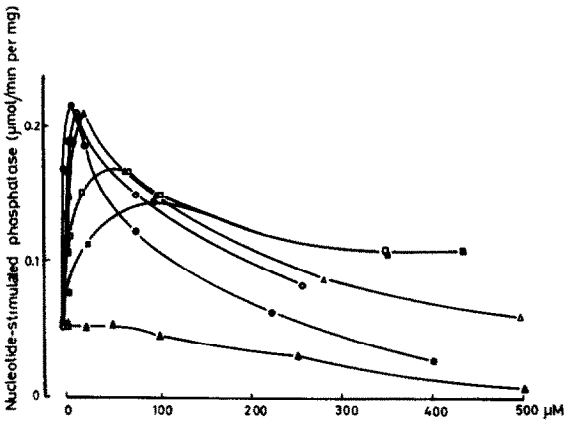

Fig.3. Dependence of the rate of umbelliferone phosphate hydrolysis on nucleotide concentration; ATP (●), N_1-carboxymethoxy-ATP (○), N_1-oxy-ATP (△), CTP (□), ITP (■), N_1-methoxy-ATP (▲).

the activating effects of these analogs with a rise in their concentrations occurs more gradually than with ATP.

No inhibition of the phosphatase reaction is observed, until N_1-oxy-ATP concentration becomes as high as 500 μM.

Unlike the analogs containing a negatively charged substituent in the first position of the purine base, N_1-methoxy-ATP, bearing an uncharged substituent and thus being incapable of binding H^+ does not activate the phosphatase reaction in the range of 5–50 μM but inhibits it at concentrations exceeding 100–500 μM.

A comparison of structural properties of N_1-methoxy-ATP and ATP suggests that the lack of activation can be due to three possible reasons, namely: (i) the presence of a substituent in the N_1-position changes the electron structure of the purine base and prevents stacking interaction of the analog with the corresponding site of the active centre; (ii) the lack of the proton-accepting ability of this analog makes it impossible to form the hydrogen bond with the corresponding group in the active centre of Na,K-ATPase and, finally, (iii) the presence of this substituent creates steric hindrance to the analog interaction with the enzyme.

Since two other ATP analogs with the substituents in the same N_1-position of the purine base do activate the phosphatase reaction, the first possibility can be excluded. The presence of a bulky substituent at the same position in the N_1-carboxymethoxy-ATP molecule does not prevent the activating effect either; therefore we may also reject the third assumption.

Hence, the only reason for the elimination of the activating effect of N_1-methoxy-ATP on the phosphatase reaction is the loss of the proton-accepting ability by this nucleotide.

The necessity of 'deprotonation' of Na,K-ATPase for its conversion from the K^+- to the Na^+-form has been demonstrated by authors in [17]. These authors have shown that the steady-state effect of alkalinization from pH 7.4 to 8.4 on $K_{0.5}$ for Na^+ is comparable with the increase in ATP concentration from 0.1 μM to 4 μM at constant pH (7.4). The increase in pH has a so-called 'ATP effect' not only on $K_{0.5}$ for Na-activation, but also on the rate of transformation of the enzyme from K^+-form to Na^+-form [17]. At this point, the alleviation of the conversion of the enzyme from one conformation to the other induced by ATP may be interpreted in terms of the ability of enzyme-bound nucleotide to accept a proton. This property of ATP provides for the formation of a hydrogen bond between the appropriate donor (e.g., tyrosyl or lysyl residue, see [17,18]) in the Na,K-ATPase active centre and the N_1-atom of the base. It was noted in [17] that there is a difference between alkalinization of medium on one hand and the effect of ATP (at fixed pH) on the other. The ATP effect is more complex and does not only relate to the ability to facilitate the conversion from the potassium to the sodium form of the enzyme. However, the ability of ATP to facilitate this conversion is actually determined by the proton-accepting properties of this molecule [17].

If the activating effect of nucleotides on the phosphatase reaction is really related to this property, the order of efficiency should correlate with the pK_a values for the $N_1(N_3)$-atom of the corresponding base. Comparison of these (adenine, 3.5; cytidine, 4.15; inosine, 8.8; uridine and guanosine, 9.2, see [19]) shows that the activating effect does decrease with increase in pK_a values. This fact supports the view that the ability of substrate to form a complex with the enzyme, providing for its 'deprotonation', facilitates transformation of the K^+-form to Na^+-form.

From this point of view the different dependence of Na,K-ATPase on the Na/K-ratio during hydrolysis of different nucleotides could for the most

part be explained by different abilities of the latter to modify the affinity of the enzyme for Na^+ and K^+ in the course of operation. Since this modification should be directly connected with the substitution of one kind of ion for another on ionic sites of the enzyme with their subsequent translocation, the H^+-accepting properties of nucleotides have to correlate with their ability to provide for active ion transport by the Na-pump. It is interesting to note that the ability for transport of sodium and potassium by Na,K-ATPase may be provided for by only ATP or CTP hydrolysis (see [2]). Hydrolysis of other nucleotides by Na,K-ATPase is performed in an uncoupled mode. This fact gives strong evidence for the idea of a direct connection between 'protonation–deprotonation' of the enzyme and ion translocating ability [20].

REFERENCES

[1] Skou, J. (1974) Biochim. Biophys. Acta 339, 246–257.
[2] Boldyrev, A.A. and Svinukhova, I.A. (1982) Biochim. Biophys. Acta 707, 167–170.
[3] Robinson, J. and Flashner, M. (1979) Biochim. Biophys. Acta 549, 145–176.
[4] Schuurmans Stekhoven, F. and Bonting, S. (1981) Physiol. Rev. 61, 1–76.
[5] Yoshida, H.K., Nagai, K., Ohashi, T. and Nakagava, Y. (1969) Biochim. Biophys. Acta 171, 178–185.
[6] Skou, J. (1974) Biochim. Biophys. Acta 339, 258–273.
[7] Svinukhova, I.A. (1983) Ukr. Biochim. J. (USSR) 55, 286–291.
[8] Pitts, B.J.R. (1974) Annu. NY Acad. Sci. (USA) 242, 293–304.
[9] Hegivary, C. and Post, R. (1971) J. Biol. Chem. 246, 5334–5340.
[10] Jensen, J. and Norby, J. (1971) Biochim. Biophys. Acta 233, 295–303.
[11] Holbrook, J., Liljas, A., Steindel, S. and Rossmann, M.G. (1975) in: The Enzymes (Boyer, P.D. ed.) vol.xi, pp.240–248.
[12] Hopkins, B., Wagner, H. and Smith, T. (1976) Biochemistry 14, 5593–5601.
[13] Boldyrev, A.A., Lopina, O.D. and Svinukhova, I.A. (1981) Biokhimija (USSR) 46, 1519–1525.
[14] Pitts, B.J.R. and Askari, A. (1971) Biochim. Biophys. Acta 227, 453–459.
[15] Mantsch, H.H., Goia, J., Kezdi, M., Barzi, O., Dansoreani, M., Jebeieani, G. and Ty, N.G. (1976) Biochemistry 14, 5593–5601.
[16] Baranova, L.A., Grivennikov, I.A. and Gulyaev, N.N. (1982) Biokhimija (USSR) 47, 1806–1813.
[17] Skou, J. and Esmann, M. (1980) Biochim. Biophys. Acta 601, 386–402.
[18] Cantley, L., Gelles, J. and Josephson, L. (1978) Biochemistry 17, 418–423.
[19] Sober, H.A. ed. (1973) Handbook of Biochemistry, 2nd edn, pp.G8–G95, CRC Press, Ohio.
[20] Skou, J. (1982) Biochim. Biophys. Acta 688, 369–380.