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The locus of nucleotide specificity in the reaction mechanism of $(Na^+ + K^+)$-ATPase determined with ATP and GTP as substrates *

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(1) ATP and GTP have been compared as substrates for $(Na^+ + K^+)$-ATPase in Na^+-activated hydrolysis, Na^+-activated phosphorylation, and the $E_2K \rightarrow E_1K$ transition. (2) Without added K^+ the optimal Na^+-activated hydrolysis rates in imidazole-HCl (pH 7.2) are equal, but are reached at different Na^+ concentrations: 80 mM Na^+ for GTP, 300 mM Na^+ for ATP. The affinities of the substrates for the enzyme are widely different: K_m for ATP 0.6 μM, for GTP 147 μM. The Mg-complexed nucleotides antagonize activation as well as inhibition by Na^+, depending on the affinity and concentration of the substrate. (3) The optimal 3-s phosphorylation levels in imidazole-HCl (pH 7.0) are equally high for the two substrates (3.6 nmol/mg protein). The K_m value for ATP is 0.1–0.2 μM and for GTP it ranges from 50 to 170 μM, depending on the Na^+ concentration. The affinity of Na^+ for the enzyme in phosphorylation is lower with the lower affinity substrate: $K_m(Na^+)$ is 1.1 mM with ATP and 3.6 mM with GTP. The GTP-phosphorylated intermediate exists, like the ATP-phosphorylated intermediate, in the E_2P conformation. (4) Addition of K^+ increases the optimal hydrolytic activity 30-fold for ATP (at 100 mM Na^+ + 10 mM K^+) and 2-fold for GTP (at 100 mM Na^+ + 0.16 mM K^+). K^+ greatly increases the K_m values for both substrates (to 430 μM for ATP and 320 μM for GTP). Above 0.16 mM K^+ inhibits GTP hydrolysis. (5) GTP does not reverse the quenching effect of K^+ on the fluorescence of the 5-iodoacetamidofluorescein-labeled enzyme. ATP fully reverses this effect, which represents the transition from E_1K to E_2K. Hence GTP is unable to drive the $E_2K \rightarrow E_1K$ transition.

Introduction

Any enzyme has a certain degree of substrate specificity, some like cytochrome *c* oxidase very narrow, others very broad. $(Na^+ + K^+)$-ATPase is an intermediate example, which can hydrolyse ATP, CTP, ITP, GTP, UTP and TTP with decreasing activity in that order, down to 0.5% for TTP as compared to ATP [1–3]. A relation has been sought between the increase in the dissociation constant of nucleotide binding to the native enzyme (from 0.2 to 3000 μM) and structural features of the substrate [3]. If this were the only cause, a sufficiently high concentration of nucleotide should give the same maximal activity, provided that the reaction steps following the binding

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Abbreviations: 5-IAF, 5-iodoacetamidofluorescein; CDTA, *trans*-1,2-diaminocyclohexane tetraacetic acid.

step would display the same activity for all substrates. This is evidently not the case [4].

The reaction mechanism of $(Na^+ + K^+)$-ATPase comprises five partial reactions: (1) binding of the nucleotide to the high-affinity phosphorylating site, (2) $(Mg^{2+} + Na^+)$-dependent phosphorylation to an ADP-sensitive, K^+-insensitive phosphoenzyme E_1P, (3) conformational change to a K^+-sensitive, ADP-insensitive phosphoenzyme E_2P, (4) K^+-stimulated dephosphorylation of the phosphoenzyme resulting in the formation of E_2K, (5) conformational change of E_2K to E_1K, accelerated by low-affinity non-phosphorylating nucleotide binding to the enzyme, and exchange of K^+ for Na^+ [5].

We decided to determine which step is responsible for the substrate specificity of the enzyme and whether it involves a change in K_m only or also a change in V_{max}. In first instance we have determined the $(Na^+ + K^+)$-ATPase activity, which covers all five steps, and the Na^+-ATPase activity which covers steps 1–4 (step 4 being the spontaneous or Na^+-activated dephosphorylation of E_2P). Subsequently we have narrowed down the localization of the responsible step by more specific assays: Na^+-activated phosphorylation (step 2), K^+-activated dephosphorylation (step 4), and monitoring the change in fluorescence accompanying the nucleotide induced $E_2K \rightarrow E_1K$ transition (step 5), using covalently bound fluorescent probes that do not affect overall activity [6,7].

In the present study we have compared GTP with ATP. GTP, which differs from ATP in having the amino group in the 2-position of the purine nucleus rather than in the 6-position, has previously been found to be hydrolyzed at 0.6–8% of the rate for ATP, depending on the enzyme source [1–3].

Materials and Methods

Preparation of the enzyme. $(Na^+ + K^+)$-ATPase is prepared from rabbit kidney outer medulla using zonal centrifugation of the sodium dodecylsulfate treated microsomal fraction as described by Jørgensen [8]. Removal of contaminating ATP, subsequent washing and storage of the resulting preparation follows the method of Schoot et al. [9], except for the composition of the storage medium, which contains 50 mM imidazole-HCl (pH 7.0), 0.25 M sucrose and no CDTA. The specific activity of the enzyme used in these experiments is 1000–1200 μmol P_i/mg protein per h. Protein is determined by means of the trichloroacetic acid precipitation modification of the Lowry method against bovine serum albumin as standard [8].

Enzyme assays. Na^+-stimulated ATPase activity is measured at 37°C in terms of the liberation of $^{32}P_i$ from [γ-^{32}P]ATP, present in micromolar or millimolar concentrations in a medium containing 0.01–500 mM NaCl, 50 mM imidazole-HCl or Tris-HCl (pH 7.2) and 0.15 mM Mg^{2+} in excess of the ATP concentration in a total volume of 0.3 ml. Na^+-stimulated hydrolytic activity with [γ-^{32}P]GTP (1 mM) as substrate is assayed in identical fashion. The P_i production is measured by stopping the reaction at a given time through addition of 0.3 ml 10% (w/v) trichloroacetic acid, followed by mixing with 0.4 ml of a 20% (w/v) aqueous charcoal suspension. The charcoal adsorbs adenosine phosphates from the reaction medium but not inorganic phosphate [10]. The charcoal is sedimented by centrifugation for 10 min at $2000 \times g$ and 0°C, and a 0.2 ml aliquot of the supernatant is mixed with 4 ml liquid scintillation fluid (Aqualuma Plus). Radioactivity is measured with a liquid scintillation counter. ATPase activity is taken as the difference in the activity in the presence of NaCl and that in the absence of NaCl but with 1 mM ouabain plus 5 mM $MgCl_2$. The latter activity is considered to represent contaminating Mg^{2+}-ATPase activity. In order to minimize background ATPase activity, the control is incubated with ouabain and $MgCl_2$ for 30 min at 22°C prior to assay [11]. $(Na^+ + K^+)$-dependent hydrolytic activity is determined in identical fashion but with K^+ present in the indicated concentrations. Assays are performed in duplicate and each experiment is repeated at least once. Conversion of ATP or GTP by hydrolysis is never allowed to exceed 15%.

Phosphorylation by ATP or GTP and dephosphorylation, both at 22°C, follow the methods earlier described by us [12,13]. The $E_2K \rightarrow E_1K$ conversion is assayed by determining fluorescence changes upon addition of K^+ and ATP or GTP to 5-iodoacetamidofluorescein-labeled $(Na^+ + K^+)$-ATPase at 22°C and pH 7.0 [7]. All kinetic data

are plotted according to the Henri-Michaelis-Menten method; V_{max} and K_m values are determined from Scatchard plots.

Reagents. All chemicals are of analytical grade. Ouabain is purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). ATP and GTP are obtained from Boehringer Mannheim (F.R.G.) and are converted to the imidazole- or Tris-salt by cation exchange over a Dowex 50W X4 column in the H^+-form, followed by neutralization with imidazole or Tris. [γ-^{32}P]ATP and [γ-^{32}P]GTP are supplied by Amersham International (U.K.) and 5-iodoacetamidofluorescein by Molecular Probes, Inc. (Junction City, OR, U.S.A.). Scintillation fluid (Aqualama Plus) is from Lumac/3M, Schaesberg (The Netherlands).

Results

Na^+-activated substrate hydrolysis in imidazole buffer

The Na^+-nucleosidetriphosphatase activity is measured as the Na^+-dependent hydrolysis of ATP and GTP in the absence of added K^+ and at varying Na^+ concentration in 50 mM imidazole-HCl buffer (pH 7.2). The concentrations for each substate have been chosen such as to saturate the relevant binding site: 1 mM GTP (higher concentations are inhibitory), 0.02 mM ATP (saturating the high-affinity binding site only), 3 mM ATP (saturating both high- and low-affinity binding sites). Different profiles are obtained for the two substrates (Fig. 1).

For ATP a relatively high activity is obtained in the absence of added Na^+, about half of the optimal activity at 300 mM Na^+. This represents the imidazole-activated phosphorylation, which we have recently reported [14]. Addition of 0.1–1 mM Na^+ lowers the activity by 30% at low ATP concentration (0.02 mM). This inhibition corresponds to that of the Na^+ pump in erythrocytes by extracellular Na^+, observed by Glynn et al. [15,16] and ascribed to inhibition of the spontaneous hydrolysis of E_2P. In line with their results, further increase of the Na^+ concentration changes this inhibition into a stimulation, in which Na^+ displays a K^+-like effect. The optimum is reached at 300 mM Na^+. Higher concentrations again give rise to inhibition, which may be due to either an

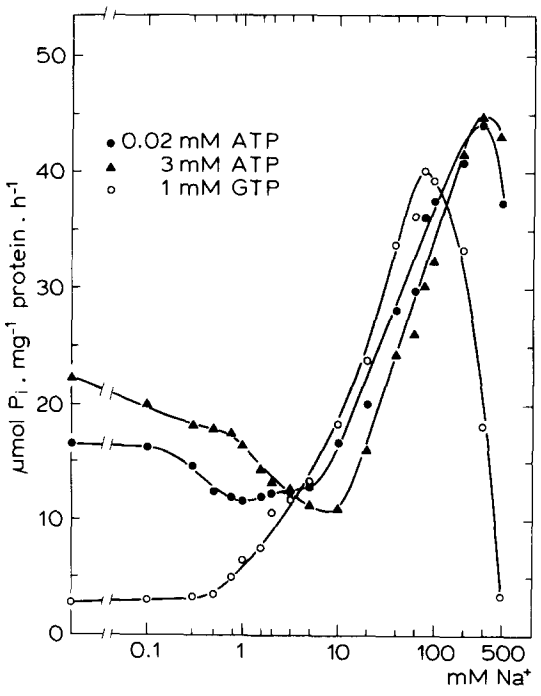

Fig. 1. Enzymatic hydrolysis of ATP or GTP in imidazole-HCl (pH 7.2), without added K^+, as a function of the Na^+ concentration. Na^+ and substrate concentrations as indicated, enzyme 3.3 µg/ml for ATP and 16.7 µg/ml for GTP, incubation 3 min (0.02 mM ATP), 120 min (3 mM ATP) and 30 min (1 mM GTP). Optimal activity for GTP is at 80 mM Na^+ ($Na^+_{0.5}$ = 14.4 mM), and for ATP at 300 mM Na^+ ($Na^+_{0.5}$ = 53 mM at 0.02 mM ATP and 100 mM at 3 mM ATP).

ionic strength effect, a slowdown of the E_1-P → E_2P transition [17,18] or a return of the inhibition of E_2P hydrolysis. At a high ATP concentration (3 mM) the curve is shifted to the right ($Na^+_{0.5}$ increasing from 53 to 100 mM), but the optimum remains at 300 mM and reaches the same height. An antagonism between MgATP and Na^+, as observed here, has previously been described by Robinson [19].

For GTP there is nearly no hydrolysis below 1 mM Na^+ and hence no inhibition at low Na^+ concentrations. The Na^+-activation curve has shifted to the left ($Na^+_{0.5}$ = 14 mM, Na^+_{max} = 80 mM). This indicates that there is in this case less antagonism between Mg-nucleotide and Na^+, which is probably due to a lower affinity of the enzyme for MgGTP than for MgATP. This is reflected in the increasing $Na^+_{0.5}$ ratio of 1 : 3.4 : 6.4

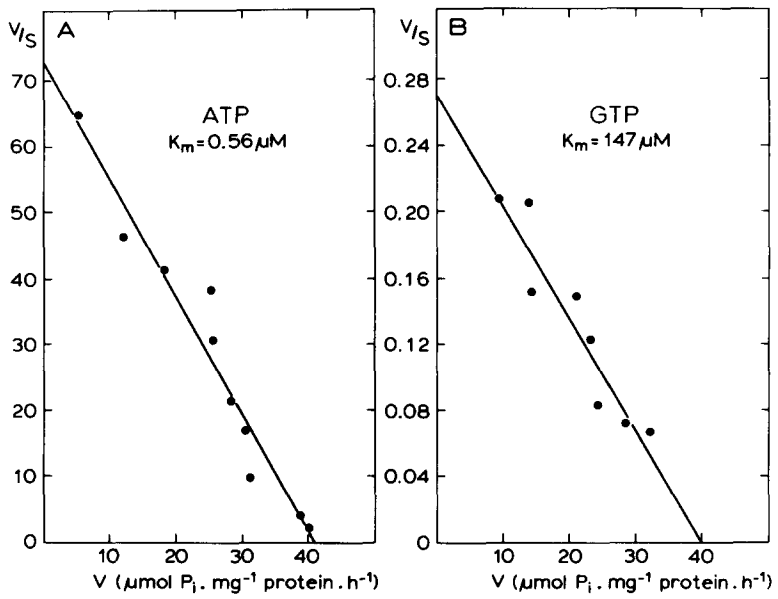

Fig. 2. Scatchard plots for hydrolysis of ATP (A) and GTP (B) in imidazole-HCl (pH 7.2) in the absence of added K^+. ATP hydrolysis is followed for 0.25–5 min in the presence of 0.1–20 μM ATP (initial concentrations), 1.7 μg/ml protein and 300 mM Na^+. GTP hydrolysis is followed for 15 min in the presence of 50–500 μM GTP (initial concentrations), 5 μg/ml enzyme and 80 mM Na^+. The substrate concentrations used for calculating v/S on the ordinate scale are averages of the initial and final concentrations. Lines have been calculated by linear regression analysis.

in the presence of 1 mM GTP, 0.02 mM ATP and 3 mM ATP, respectively.

The most remarkable observation to be made from Fig. 1 is that the peak activities for GTP and ATP are almost the same: 40 vs. 44 μmol/mg protein per h. This is confirmed in Scatchard plots of the activity, determined at the optimal Na^+ concentration for each substrate (Fig. 2A and B). While the V_{max} is nearly the same for the two substrates (approx. 40 μmol/mg protein per h), the K_m values differ by a factor 260 (147 μM for GTP vs. 0.56 μM for ATP).

Na^+-activated substrate hydrolysis in Tris buffer

Similar determinations of the Na^+-nucleoside triphosphatase activity have been made in Tris buffer (50 mM, pH 7.2). The results, shown in Fig. 3, have been obtained at optimal conditions for each substrate (1 mM GTP, 60 mM Na^+; 0.02 mM ATP, 150 mM Na^+; 3 mM ATP, 300 mM Na^+). The triphosphatase activity for GTP (1 mM) is slightly lower than for 0.02 mM ATP (33 vs. 40 μmol/mg protein per h), but higher than for 3 mM ATP (33 vs. 25 μmol/mg protein per h). The $Na^+_{0.5}$ value for GTP is, like in imidazole buffer, far lower than that for ATP: 8 mM Na^+ for GTP, 25 mM for 0.02 mM ATP and 50 mM for 3 mM ATP. However, all three $Na^+_{0.5}$ values are about

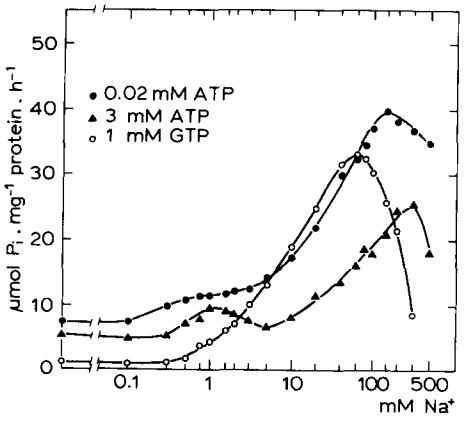

Fig. 3. Enzymatic hydrolysis of ATP and GTP in Tris-HCl (pH 7.2), without added K^+ as a function of the Na^+ concentration. Conditions are the same as in Fig. 1. Optimal activity for 1 mM GTP is at 60 mM Na^+ ($Na^+_{0.5}$ = 7.9 mM), for 0.02 mM ATP at 150 mM Na^+ ($Na^+_{0.5}$ = 25 mM) and for 3 mM ATP at 300 mM Na^+ ($Na^+_{0.5}$ = 50 mM).

half of those in imidazole buffer, which is another example of an interaction between imidazole and Na^+ previously noticed for the phosphorylation step [14]. The equal ratio's of the $Na^+_{0.5}$ values in the two buffers, as well as the near equality of the K_m values for the two substrates in Tris and imidazole (100 vs. 147 μM for GTP; 0.56 vs. 0.56 μM for ATP, each at optimal Na^+ concentration), indicate a similar antagonism between Mg-nucleotide and Na^+ in these buffers.

The most striking differences between the activity profiles in imidazole and Tris are the lower buffer-stimulated ATPase activities and the strong MgATP inhibition revealed by the profile with 3 mM ATP present. The first phenomenon is due to the lower phosphorylation capability in Tris than in imidazole, which we have recently reported [14]. The second phenomenon is reminiscent of the substrate inhibition described by Robinson et al. [20] for Na^+-ATPase activity in Tris-histidine buffer, particularly at high Na^+ concentration. In addition, inhibition at low Na^+ concentrations is absent and is replaced by stimulation, as in previous reports on Na^+-ATPase activity in imidazole-glycylglycine [21] or Tris-HCl [16,22,23] or is shifted to higher Na^+ concentrations (1–5 mM) at elevated MgATP level (3 mM), as also occurs in imidazole (Fig. 1).

Na^+-dependent phosphorylation by GTP

Schoner et al. [4] first reported that GTP is a phosphorylating substrate for $(Na^+ + K^+)$-ATPase. Fig. 4 confirms this for our preparation and shows that in 3 seconds phosphorylation levels as high as 3.6 nmol/mg protein can be reached. This is the same level as obtained with ATP for the same preparation, but at a greatly increased K_m value (50–170 μM for GTP, 0.1–0.2 μM for ATP). The K_m value for GTP strongly depends on the Na^+ concentration (ionic strength being kept constant at 140 mM by addition of choline chloride). It decreases from about 170 μM at 1–3 mM Na^+ to a minimum value of 50 μM at 8–12 mM Na^+, and increases again to 75 μM at 100 mM Na^+ The K_m value for ATP is fairly constant at 0.1–0.2 μM in this range of Na^+ concentrations. The K_m values for Na^+ also differ: 3.6 mM for GTP vs. 1.1 mM for ATP, accompanied by a similar trend in the Hill coefficients for Na^+ activation: nH is 2.4 for GTP vs. 1.4 for ATP. The GTP-phosphorylated intermediate is predominantly in the E_2P form, since it is ADP-insensitive and K^+-sensitive. Its K^+-sensitivity appears to be equally high as that of the ATP-generated phosphoenzyme: 97% K^+ (0.1 mM) induced hydrolysis within 3 s (cf. Ref. 13).

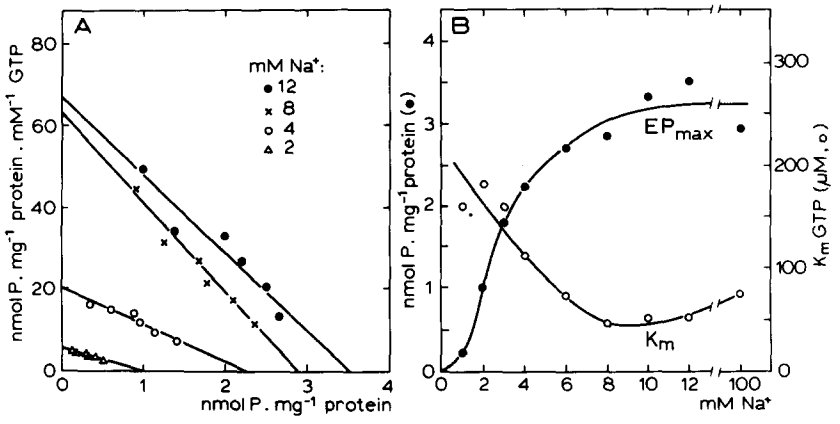

Fig. 4. Phosphorylation by GTP as a function of the Na^+ concentration in 50 mM imidazole-HCl (pH 7.0). (A) Scatchard plots for 3 s phosphorylation at 22°C in the presence of 5 mM $MgCl_2$, 0.33 mg/ml enzyme, 20–200 μM GTP and the indicated Na^+ concentrations. Ionic strength (140 mM) is kept constant by addition of choline chloride. Lines have been calculated by linear regression analysis. (B) Michaelis-Menten plot of abscissa intercepts in (A), indicating EP_{max}, as a function of the Na^+ concentration. Also shown is the K_m for GTP, determined from the slopes in A, as a function of the Na^+ concentration.

The effects of K^+ on the hydrolysis of ATP and GTP

Fig. 5 shows the effects of K^+ on Na^+-activated hydrolysis of ATP and GTP at varying Na^+ concentration. Again some striking differences are noticed. In the case of ATP (5 mM), in the absence of added K^+ (residual K^+ concentration 6 μM) there is a peak activity at 300 mM Na^+ (Fig. 5A), as previously shown in Fig. 1. Addition of K^+ to the assay medium results in the appearance of a second peak at 2 mM Na^+. At 80 μM K^+ the two peaks are of the same magnitude. Further increase of the K^+ concentration raises the height of both peaks and shifts them together. At 1.8 mM K^+ the two peaks merge at 40 mM Na^+. The appearance of the left hand peak can be explained by K^+-stimulated hydrolysis of E_2P accompanied by a change of the rate-limiting step from the hydrolysis of E_2P to the ATP-assisted $E_2K \rightarrow E_1Na$ transition (see Discussion). In this transition K^+ and Na^+ compete at the intracellular sites [24], leading to a shift of this peak to higher Na^+ concentrations when K^+ is increased. The Na^+/K^+ concentration ratio for the left hand peak remains rather constant at 22–25.

A very different behaviour is observed in the Na^+-activated hydrolysis of GTP upon addition of K^+ (Fig. 5B). The position of the peak, present at 80 mM Na^+ in the absence of added K^+ (6 μM residual K^+), is not changed. Its height is slightly increased by 0.2 mM K^+, and then decreases at higher K^+ concentrations (1–5 mM). The half maximally stimulating Na^+ concentration changes little. The low activity level between zero and 0.5 mM Na^+ is almost doubled upon addition of 0.2–1 mM K^+, but is decreased with 5 mM K^+.

The near absence of stimulation by K^+ of the GTP hydrolysis is in marked contrast to the large stimulation of the ATP hydrolysis. The absence of this effect for GTP could be due to an increased K_m value for this nucleotide at increasing K^+ level. Thus we have determined the K_m and V_{max} values for GTP at constant (100 mM) Na^+ concentration and variable K^+ concentrations (Fig. 6).

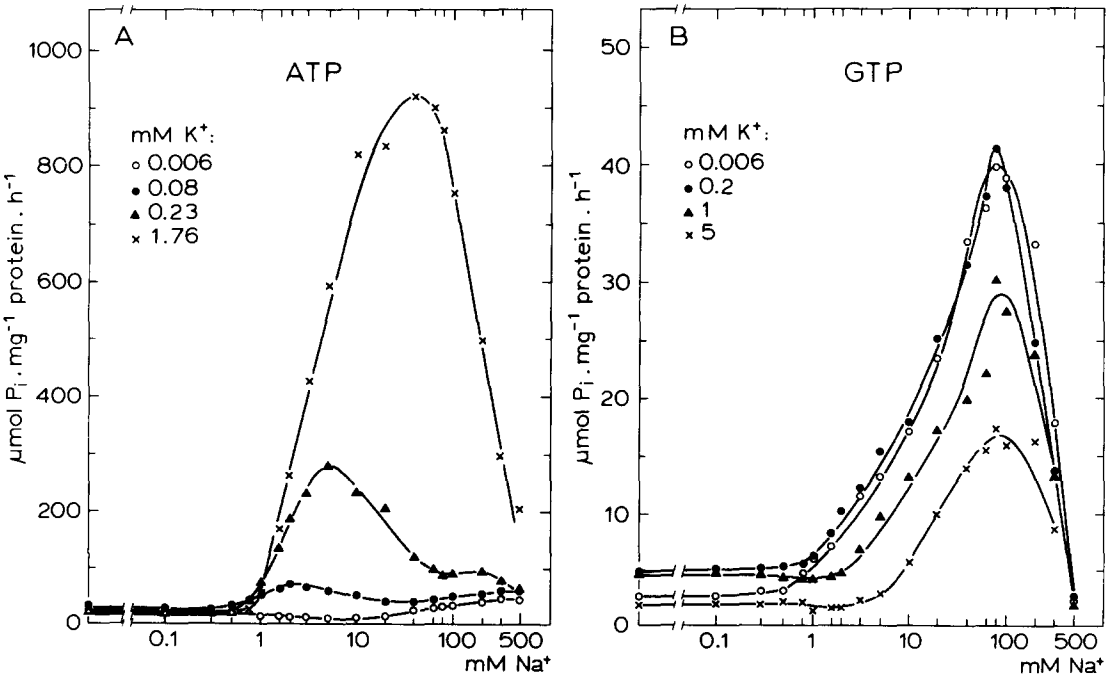

Fig. 5. Effect of $Na^+ + K^+$ on the hydrolysis of ATP and GTP. The hydrolysis is assayed in 50 mM imidazole-HCl (pH 7.2) in the presence of the indicated Na^+ and K^+ concentrations. (A) Hydrolysis of ATP: ATP 5 mM, enzyme 16.7 μg/ml, incubation for 5, 10 and 30 min at decreasing K^+ concentrations. (B) Hydrolysis of GTP: GTP 1 mM, enzyme 16.7 μg/ml and incubation for 30 min. Maximal activity at all K^+ concentrations is at 80 mM Na^+ ($Na^+_{0.5} = 17$–21 mM).

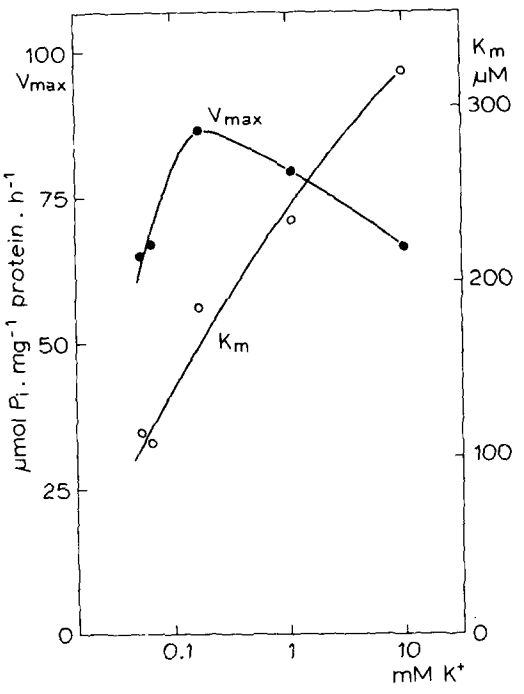

Fig. 6. Plots of V_{max} and K_m for GTP hydrolysis as a function of the K^+ concentration. V_{max} and K_m have been determined from Scatchard plots at 0.06–1 mM GTP (inhibition above 1 mM GTP). Na^+ is 100 mM, K^+ as indicated, enzyme 16.7 μg/ml, incubation 1–15 min, depending on the GTP concentration.

Up to 0.16 mM K^+, or about twice the K_m value for stimulation of E_2P hydrolysis [13], the V_{max} value is twice that in the absence of K^+ (Fig. 2B). At 10 mM K^+ there is a 23% inhibition of V_{max}, hence the stimulation is reduced to 1.6-fold. This phenomenon is accompanied by a continuous increase in the K_m value for GTP, going from 110 μM at 0.06 mM K^+ to 320 μM at 10 mM K^+. Under these conditions the hydrolysis of ATP is optimal (1200 μmol/mg protein per h) and 30-times higher than that in the absence of K^+ (Fig. 2A). The K_m value for ATP increases even more than that for GTP: 0.6 μM without K^+ (Fig. 2A), 430 μM at 10 mM K^+ and 100 mM Na^+ [25].

The GTP-phosphorylated intermediate is sensitive to K^+, like the ATP-phosphorylated intermediate. Hence, the inhibitory effect of K^+ above 0.2 mM on the overall activity (Figs. 5B and 6) must occur in a step subsequent to the dephosphorylation. A likely candidate is the $E_2K \rightarrow E_1K$ transition, which is examined in the following section.

The $E_2K \rightarrow E_1K$ transition

Several fluorescent probes can monitor the conformational change from E_2 to E_1, but most of these are unsuitable for following the ATP-driven transition as they interfere with nucleotide binding, e.g. fluorescein isothiocyanate [26], eosin and eosin maleimide [6,27]. A covalently binding fluorescent label, which does not inhibit $(Na^+ + K^+)$-ATPase activity and is able to record the ATP-driven $E_2K \rightarrow E_1K$ transition, is 5-iodoacetamidofluorescein (5-IAF, Ref. 7). Fig. 7 demonstrates the fluorescence changes upon sequential addition of K^+ and ATP or GTP to the IAF-labeled enzyme. Addition of K^+ up to 50 μM quenches the fluorescence and induces the E_2K conformation. Subsequent addition of ATP (up to 0.75 mM) reverses this effect and restores the fluorescence to the original level (Fig. 7A). However, addition of GTP (up to 1 mM) is virtually without effect (Fig. 7B). Subsequent addition of ATP again gives an increase in fluorescence, but now the restoration is incomplete, probably due to inhibition exerted by

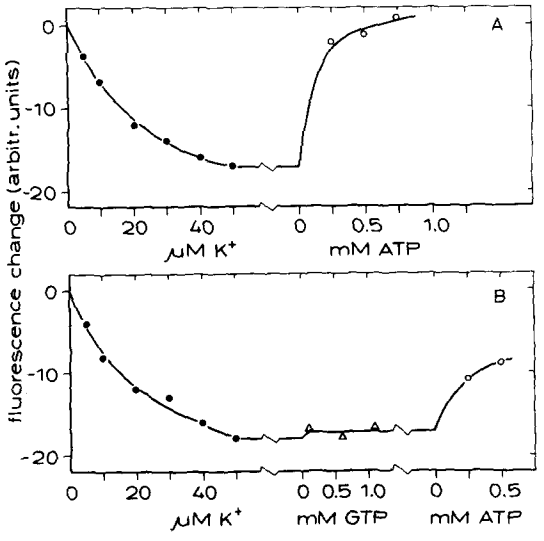

Fig. 7. Fluorescence response of 5-IAF-labeled enzyme to K^+ and substrate. Fluorescence change (arbitrary units) in 50 mM imidazole-HCl (pH 7.0, 22°C), upon addition of K^+ and ATP (A) or GTP (B) in concentrations as indicated. Enzyme concentration is 27 μg/ml. Excitation at 490 nm, emission at 520 nm.

the GTP. These data indicate that GTP in millimolar concentration, unlike ATP, is unable to drive the $E_2K \rightarrow E_1K$ transition and even inhibits the ATP-driven transition.

Discussion

The $E_2K \rightarrow E_1K$ transition as rate-limiting step

In this study we have provided evidence that in the case of GTP the $E_2K \rightarrow E_1K$ transition is the major locus of substrate specificity in the overall reaction mechanism in the presence of $Na^+ + K^+$. The reduced activity with GTP as compared to ATP is not a matter of a reduced affinity for the substrate, since the K_m values for ATP (0.43 mM, Ref. 25) and GTP (0.32 mM) are not appreciably different. The K_d values for the low-affinity substrate site, which is thought to be involved in the $E_2K \rightarrow E_1K$ transition, do not appreciably differ either [25]. Hence the reduction of the hydrolytic activity with GTP as substrate upon addition of K^+ is due to a reduction of the maximal rate of the $E_2K \rightarrow E_1K$ transition. This conclusion is supported by the 5-IAF fluorescence experiments.

Further support for this conclusion is provided by our finding that in imidazole buffer the Na^+-dependent activities in the absence of K^+, viz. the steady-state phosphorylation level and the Na^+-activated dephosphorylation, are equally high for the two substrates. This implies that steps 1–3, i.e. substrate binding, phosphorylation and $E_1P \rightarrow E_2P$ transition, are not rate-limiting for the Na^+-activated hydrolysis of E_2P for either substrate. Neither does the K^+-stimulated dephosphorylation appear to be rate-limiting for GTP. The phosphoenzyme generated by GTP is, like that generated by ATP [13], ADP-insensitive and K^+-sensitive, indicating that the $E_1P \rightleftharpoons E_2P$ equilibrium is poised far to the right. This explains also the two-foldl increase in V_{max} of GTP hydrolysis upon addition of about twice the half-maximally stimulating concentration of K^+. This means that the large difference in GTP and ATP hydrolysis in the presence of K^+ cannot be ascribed to a reduced K^+-sensitivity of the GTP-phosphorylated intermediate. Rather step 5, the $E_2K \rightarrow E_1K$ transition following the K^+-stimulated dephosphorylation of E_2P (step 4), must be responsible. GTP is unable to drive this rate-limiting step in the overall activity.

Still another phenomenon may be explained by this insufficiency of GTP. In the hydrolysis of ATP K^+ induces an additional peak of activity in the 2–40 mM Na^+ concentration range ($Na^+_{0.5}$ is 1–4 mM), which increases with increasing K^+ concentration. The $Na^+_{0.5}$ value for the $E_2 \rightarrow E_1$ transition in the absence of added K^+ (about 2 mM, Ref. 28) falls in this range of competition between Na^+ and K^+ ions (K_m $Na^+ = 1.3$ mM, K_i $K^+ = 0.23$ mM, Ref. 26). ATP apparently compensates for the greater part of the inhibition by K^+. When no such compensation by the nucleotide occurs, as in the case of GTP, Na^+ must exert this action and the $Na^+_{0.5}$ value remains high. In the absence of K^+ there is also no activation but rather inhibition in the low Na^+ concentration range, even though the $E_2 \rightarrow E_1$ transition is then not hampered by K^+. However, under these conditions the dephosphorylation step is rate-limiting but not the $E_2 \rightarrow E_1$ transition.

Antagonism between Na^+ and substrate or imidazole

Some other interesting mechanistic effects appear from our study. In the Na^+-activated hydrolysis of GTP and ATP the activity curve strongly depends on the affinity and concentration of the Mg-complexed nucleotide. The curve for GTP, which has a 260-fold lower affinity for the enzyme in this process, is shifted to lower Na^+ concentrations than the curve for ATP. The latter curve shifts even further to the right at higher ATP concentrations (3 mM). This represents an antagonism between Mg-nucleotide and Na^+. This phenomenon was previously detected by Robinson [19] for the $(Na^+ + K^+)$-ATPase activity, where the $Na^+_{0.5}$ value, after extrapolation to zero $[K^+]$, showed an increase with the Mg-ATP concentration.

Comparison of the ratio of half-maximally activating Na^+ concentrations at 1 mM GTP, 0.02 mM ATP or 3 mM ATP in either imidazole or Tris medium shows that the Mg-nucleotide-Na^+ antagonism is not affected by the buffer. This indicates that in the intact cell there must be an interaction between the substrate at the intracellular side and Na^+ at the extracellular side of the membrane, since Na^+ in that situation stimulates the hydrolysis process from the extracellular side

[15,16]. This antagonism is opposite to the synergism that appears to occur between the nucleotide and Na^+ at the same (intracellular) side of the membrane. Here the ratio K_m values for intracellular Na^+ in the formation of E_2P from the low-affinity substrate GTP instead of the high-affinity substrate ATP is 3.3 : 1, which is opposite to the 1 : 3.3 ratio for the stimulation of E_2P hydrolysis by extracellular Na^+.

For the Na^+-activated hydrolysis the $Na^+_{0.5}$ values in Tris are about half of those in imidazole buffer. At high Na^+ concentrations the substrate inhibition in Tris medium is stronger than in the imidazole medium. The latter phenomenon has earlier been detected by Robinson et al. [20], who found the inhibition to increase with the Na^+ concentration in Tris-histidine buffer. The different effects of imidazole and Tris medium may be due to the stronger Na^+-like properties of imidazole-H^+ as compared to Tris-H^+, as has also been observed for phosphorylation [14]. However, lacking the K^+-like properties of Na^+, which stimulates E_2P hydrolysis, imidazole buffer inhibits this Na^+ effect more than Tris buffer does. Imidazole-H^+ increases the $Na^+_{0.5}$ values for the hydrolysis, while Tris-H^+ increases the extent of the Na^+-dependent substrate inhibition. Here also, the antagonism between imidazole-H^+ and Na^+ at the low-affinity extracellular hydrolytic sites is replaced by a synergism lat the high-affinity intracellular phosphorylating sites, in which imidazole increases instead of decreases the apparent affinity for Na^+ [14]. The increase in the cooperativity index n_H for Na^+ in phosphorylation from 1.4 for ATP to 2.4 for GTP indicates that the Na^+ sites interact more strongly when the substrate has a lower affinity [29].

Substrate structure and the locus of specifity

Nucleotides can be modified in the purine ring, the ribose group or the phosphate chain, with different effects on the location of the rate-limiting step in the reaction mechanism. Our data on the partial reactions with ATP and GTP, where only the purine ring is modified, suggest that the $E_2K \rightarrow E_1K$ transition is the main locus of substrate specificity, since the other partial reactions are not markedly affected. Similar conclusions can be reached for other substrates with a modified purine ring, viz. CTP [30] and UTP [31]. The conclusion is supported by the low effectiveness of ITP, GTP, UTP and CTP in supporting the K^+-K^+ exchange, which involves the $E_2K \rightleftharpoons E_2K$ transition [32]. This may be due to the relatively high pK value of the N_1 nitrogen of the purine ring (N_3 of the pyrimidine group) in these substances, precluding them from taking up a proton from the enzyme in this transition [33].

Modification of the phosphate chain shifts the rate-limiting step to other partial reactions. Adenylyl imidodiphosphate (Ado*PP*[NH]*P*), modified in the β-γ phosphate link, is hydrolyzed by the enzyme in the presence of $Mg^{2+} + Na^+$, yielding ADP-NH_2 and P_i [34]. K^+ neither stimulates nor inhibits the activity. Our interpretation is that the phosphorylation step and not the $E_2K \rightarrow E_1K$ transition is here the rate-limiting step. This is in agreement with the finding of Simons [35] that Ado*PP*[NH]*P* can effectively (55%) substitute for ATP in K^+-K^+ exchange transport.

When the γ-phosphate group is modified as in [γ-S]ATP, there is a general reduction in the rate constants of the partial reactions, but the $E_1P \rightarrow E_2P$ transition (and the phosphorylation step) appears to be most retarded as reflected in the size of the ADP-sensitive phosphoenzyme fraction and its reduced K^+-sensitivity [13]. Hence, there is no constant locus of specificity for the various substrates in the reaction mechanism of ($Na^+ + K^+$)-ATPase. The general picture emerging is: (1) modification of the phosphate chain predominantly affects reactions involving the phosphoenzyme (formation of E_1P and its transition to E_2P), (2) modification of the purine ring mainly affects the $E_2K \rightarrow E_1K$ transition of the dephosphorylated enzyme, in addition to a general decrease of the substrate binding affinity [3,36].

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