Biochimica et Biophysica Acta, 485 (1977) 434—445

BBA 68290

SUBSTRATE INHIBITION OF THE (Na^+, K^+)-ATPase IN THE PRESENCE OF EXCESS Mg^{2+}

GILAD RIMON

Biophysics Section, Institute of Life Science, Hebrew University, Jerusalem (Israel)

(Received April 22nd, 1977)

Summary

1. High concentrations of ATP inhibit completely the activity of (Na^+, K^+)-ATPase (ATP phosphohydrolase, EC 3.6.1.3) prepared from sheep brain.

2. The inhibition depends on the concentration of total ATP, i.e. complexed ATP + free ATP.

3. The inhibition by high ATP concentrations persists in the absence of K^+, and is then independent of the Na^+ concentration between 2 and 140 mM Na^+.

4. Raising the K^+ concentration at 20 mM Na^+ increases the ATP concentration required for the maximal hydrolysis rate.

5. The Hill number for the inhibition process is about three.

6. The inhibition by ATP is temperature-dependent, in that as the temperature is increased, higher ATP concentrations are required for inhibition.

Introduction

Since Skou in 1957 [1] suggested that the ATPase activity of crab nerves correlated with the Na^+ and K^+ active transport system, an enormous amount of data has been accumulated (see last reviews [2—6]) on the (Na^+, K^+)-ATPase enzyme. But the detailed molecular mechanism of the sodium-potassium pump is still not well understood, partly because the large number of variables that participate or are present in the enzyme cycle (Na, K, Mg, ATP, Mg · ADP, Mg · ATP) cause difficulties in analysing the role of every variable through the enzyme cycle.

For reasons which are not relevant to the purpose of the present paper, it became necessary to measure the apparent affinity of the enzyme for ATP at various temperatures. Preliminary experiments performed at optimal pH and cation concentrations (140 mM Na^+, 14 mM K^+, 5 mM Mg^{2+}, pH 7.4) revealed, however, that below 17°C the (Na^+, K^+)-ATPase activity was inhibited completely at 5 mM ATP, while the optimal activity at this temperature was at

0.75 mM ATP. At higher temperatures above 25°C, the optimal (Na^+, K^+)-ATPase activity was around 3 mM ATP and much higher ATP concentrations were needed in order to get inhibition. The purpose of the present paper is to test whether this phenomenon of inhibition by ATP at excess of Mg^{2+}, which was clearly revealed at low temperatures and does not seem to have been studied previously, could be explained by competition between free ATP and Mg · ATP at the same catalytic site. It is suggested that the inhibition indeed occurs by ATP occupying low affinity sites that at physiological temperature and ATP concentrations are empty. The effect of Na^+ and K^+ on the shape of the inhibition curve is explored and discussed in relation to the suggested mechanism for inhibition.

It is important to differentiate the present study from that of Hexum et al. [7], in which the concentration of Mg^{2+} was limiting as the ATP concentration was itself varied and, on the other hand, that of Skou [8] in which the concentration of Mg^{2+} was itself varied at the Mg · ATP concentration was changed. The results of these earlier studies will be considered together with the present results in the Discussion.

Methods and Materials

Enzyme preparation

Microsomes were prepared from sheep brain by the method of Jorgensen et al. [9]. The microsomes were terated with NaI, according to the method of Nakao et al. [10].

Protein was measured according to the method of Lowry [11]. The specific activity at 37°C in the presence of Tris · HCl was measured at pH 7.4, 3 mM ATP, 5 mM $MgCl_2$, 14 mM KCl, 140 mM NaCl and under these conditions, 98% of the total activity was inhibited by 1.25 mM ouabain. The specific activity of the preparations were 40—60 μmol of P_i liberated per h/mg of protein. At 15°C, under the same conditions of pH, cation concentrations and optimal ATP concentration, about 85—90% of the total activity was inhibited by ouabain.

Measurement of activity

The activity at various concentrations of cations and ATP was tested by measuring the amount of ^{32}P released from ATP labelled with ^{32}P in the γ position. The hydrolysis reaction was started by addition of 0.1 ml of enzyme suspension of about 1 mg/ml protein to a stirred volume of 0.9 ml containing 50 mM Tris buffer adjusted with HCl to pH 7.4 as measured at the reaction temperature, NaCl, KCl, $MgCl_2$ and ATP (the latter possessing a specific activity of 10^6—10^7 counts per min/μmol) as mentioned in the legends to the figures.

About 5—7 samples of 0.1 ml were taken during the course of the reaction. The samples were delivered into 0.3 ml of 0.2% sodium dodecyl sulfate (SDS) solution to stop the hydrolysis. The ATP was separated from the released ^{32}P by absorption to activated charcoal [12] as follows: 5 ml of 100 mM KH_2PO_4 solution at 0°C, pH 1, containing 50 mg per ml activated charcoal was added to the 0.4 ml containing SDS and sample suspension. The resulting mixture was stirred on a vortex mixer and then centrifuged at 2000 × *g* for 15 min. 3 ml of

the clear supernatant were taken directly for counting, using the Cerenkov effect. The counting of radioactivity was performed using a Packard Tri Carb Liquid scintillation counter.

The sampling times were chosen so that no more than 10—15% of the total ATP was hydrolysed. Under these conditions, the hydrolysis was a linear function of time. The reaction temperature was kept constant within ±0.1°C using a Hetofrig thermostatically temperature-controlled bath.

All the hydrolysis rates were obtained by subtraction of the activity in the presence of 1.25 mM ouabain at the various ATP concentrations, from the total activity under the same conditions.

The hydrolysis rates in all the figures are presented as percentages of the optimal hydrolysis rate at 37°C, for the particular preparation studied, (relative activity). The relative activities are presented as function of the total ATP concentration. Every point in every figure is the result of an individual experiment.

Materials

All the salts used were of analytical grade. $Na_2 \cdot ATP$, Tris · ATP, sodium dodecyl sulfate, activated charcoal No. C-5510, were obtained from Sigma. [γ^{32}P]ATP was prepared according to the method of Glynn and Chappell [13]. ^{32}P was obtained from the Radiochemistry Department, Beersheba, Nuclear Research Centre-Negev.

Calculation of $ATP_{free}/Mg \cdot ATP$

It was necessary to calculate the ratio of ATP_{free} to Mg · ATP for a realistic assessment of the present data. The concentrations of Mg · ATP and ATP_{free} were calculated according to the solution of the following equations:

$$K_1 = \frac{[Mg \cdot ATP]}{([ATP_{total}] - ([Mg \cdot ATP] + [Na_2 \cdot ATP] + [K_2 \cdot ATP]))([Mg_{total}] - [Mg \cdot ATP])} \quad (1)$$

$$K_2 = \frac{[Na_2 \cdot ATP] + [K_2 \cdot ATP]}{([Na] + [K])^2[ATP_{total}] - ([Mg \cdot ATP] + [Na_2 \cdot ATP] + [K_2 \cdot ATP])} \quad (2)$$

The reported value of K_1, the formation constant for Mg · ATP, ranges between 10 000 and 20 000 M^{-1} [14—16]. The reported value for K_2, the formation constant for $Na_2 \cdot ATP$ and $K_2 \cdot ATP$ is 15 M^{-1} [14]. For the present calculations the lower value for the Mg · ATP formation constant was chosen in order to avoid conclusions based on over-estimate of the formation constant. As will be shown later in this paper a very accurate value of the formation constant is not essential to the conclusions to be derived. The formation constant at 15°C was estimated as 7000 M^{-1} by extrapolation of Burton's data measured at 25°C and 65°C [16].

Calculations show that at the experimental range of temperature, Mg^{2+}, K^+ and Na^+ concentration, the concentrations of $Na_2 \cdot ATP$ and $K_2 \cdot ATP$ can be neglected with respect to the Mg · ATP concentration. In addition, the concentrations of $H \cdot ATP^{2-}$ and $H_2 \cdot ATP^-$ have been shown to be negligibly small under the conditions used in the present study [17].

Results

The phenomena of excess substrate inhibition and its dependence on the ratio $ATP_{free}/Mg \cdot ATP$ and the free Mg^{2+} concentration

The aim of this section was to investigate how the (Na^+, K^+)-ATPase activity varied as a function of the ATP concentrations at various concentrations of free and total Mg^{2+} at 15°C (Fig. 1). The procedure used was as follows:

1. The total Mg^{2+} concentration was held constant at 5 mM. At this Mg^{2+} concentration, the ratio $ATP_{free}/Mg \cdot ATP$ rises from 0.039 to 0.24, on raising the ATP concentration from 0.25 mM to 5 mM.

2. The concentration of free Mg^{2+} was held constant at 5 mM. This was done simply by adding equimolar concentrations of ATP and Mg^{2+} and then adding a further concentration of 5 mM Mg^{2+}. Calculation of the ratio ATP_{free}/Mg ATP, shows that the ratio remains 0.0365 through all ATP concentrations.

3. The concentration of free Mg^{2+} was held constant at 10 mM using the same strategy as in 2 above. At this concentration of free Mg^{2+}, the ratio $ATP_{free}/Mg \cdot ATP$ remains 0.0184 at all ATP levels.

As is demonstrated in Table 1, the ratio $ATP_{free}/Mg \cdot ATP$ depends on the value of the formation constant chosen. But all the chosen values of the formation constant the ratio $ATP_{free}/Mg \cdot ATP$ remains constant in procedures 2 and 3 at the ATP concentration range studied. Since the following conclusions are based only on the constancy of the ratio $ATP_{free}/Mg \cdot ATP$, through all ATP concentrations, the exact value of the formation constant is not important for derivation of these conclusions.

The results (Fig. 1) show that inhibition of the (Na^+, K^+)-ATPase activity by excess ATP is complete by 5 mM ATP. The inhibition pattern in all three experimental procedures is the same. This means that the degree of inhibition is not affected by the free Mg^{2+} concentration, nor by the ratio $ATP_{free}/Mg \cdot ATP$. The fact that the inhibition occurs at high ATP concentrations, even though the ratio $ATP_{free}/Mg \cdot ATP$ is held constant, excludes the possibility that the inhibition is due to simple competition between free ATP and MgATP

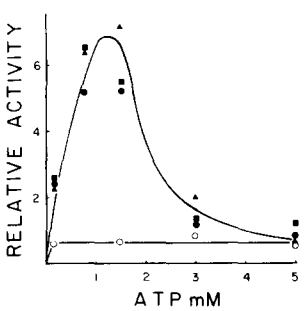

Fig. 1. Substrate inhibition of the (Na^+, K^+)-ATPase hydrolytic activity at 15°C at varied $MgCl_2$ concentration. The incubation media contained 140 mM NaCl 15 mM KCl and 50 mM Tris buffer (pH 7.4). The total hydrolytic activity was measured as follows: ■———■ constant concentration of 5 mM Mg^{2+} total; ●———●, constant concentration of 5 mM Mg^{2+} free; ▲———▲, constant concentration of 10 mM Mg^{2+} free; ○———○, hydrolytic activity in the presence of 1.25 mM ouabain at 10 mM Mg^{2+} free. The hydrolytic activity in the presence of ouabain is independent of the Mg^{2+} concentration, at the studied range of Mg^{2+} concentration.

TABLE I

CALCULATION OF THE RATIO $ATP_{free}/Mg \cdot ATP$ USING VARIOUS FORMATION CONSTANTS OF $Mg \cdot ATP$, MAINTAINING A TOTAL Mg^{2+} CONCENTRATION IN CONSTANT EXCESS OF THE TOTAL ATP CONCENTRATION

Mg ATP formation constant (M^{-1})	ATP_{free}/Mg ATP at constant excess of 5 mM Mg^{2+}	ATP_{free}/Mg ATP at constant excess of 10 mM Mg^{2+}
3 000	0.086 —0.081	0.043 —0.0424
7 000	0.0398 —0.036	0.0185—0.01835
10 000	0.0258 —0.0254	0.013 —0.01297
15 000	0.0173 —0.0172	0.0093—0.0087

The left-hand values in the second and third columns are the values of $ATP_{free}/Mg \cdot ATP$ at 0.25 mM total ATP concentration, and the right-hand values in the second and third columns are the ratio of $ATP_{free}/Mg \cdot ATP$ at 5 mM total ATP concentration.

for the same catalytic site. The relation between the hydrolytic velocity of the (Na^+, K^+)-ATPase with $Mg \cdot ATP$ as a substrate, in a case of simple competition by ATP_{free} can be written as:

$$v = V/\left(1 + \frac{[K]}{[Mg \cdot ATP]} + \frac{[ATP_{free}]}{[Mg \cdot ATP]} \cdot \frac{K}{K_i}\right). \quad (3)$$

V is the maximal hydrolytic velocity at infinite substrate concentration, K is the Michaelis constant for $Mg \cdot ATP$ and K_i is the dissociation constant for free ATP.

When $ATP_{free}/Mg \cdot ATP$ is held constant, it is seen immediately that no inhibition should occur at all, as the concentration of total ATP is varied. So, it can be concluded that the inhibition by ATP at excess Mg^{2+} concentration is not a consequence of competition between free ATP and $Mg \cdot ATP$ at the same catalytic site. A remaining possibility is that $Mg \cdot ATP$ or ATP inhibits at high substrate concentrations by occupying low affinity sites for ATP. In order to see if the phenomenon of excess substrate inhibition was a more general one, the inhibition of the (Na^+, K^+)-ATPase activity was measured at 35°C as func-

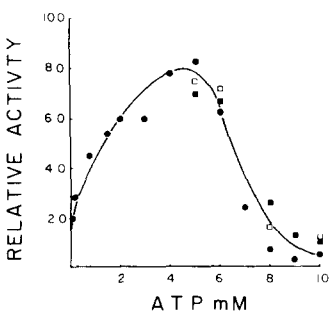

Fig. 2. Substrate inhibition of the (Na^+, K^+)-ATPase hydrolytic activity at 35°C at varied $MgCl_2$ concentrations. The incubation media contained 140 mM NaCl, 14 mM KCl and 50 mM Tris buffer (pH 7.4). The ouabain inhibitable hydrolytic activity (at 35°C 98% of the total activity is ouabain inhibitable (see the text)) was measured at three different $MgCl_2$ concentrations as follows: ■———■, 5 mM Mg^{2+} total; ●———●, 10 mM Mg^{2+} total; □———□, 15 mM Mg^{2+} total. NaCl is 140 mM and KCl 15 mM.

tion of ATP concentration at three constant total Mg^{2+} concentrations: 5, 10, 15 mM (Fig. 2). At 35°C, the inhibition appeared above 5 mM ATP, and at a concentration of 10 mM ATP, 85—95% of the optimal activity was inhibited. The shape of the inhibition, at all three Mg^{2+} concentrations, was the same although the ratio $ATP_{free}/Mg \cdot ATP$, at 10 mM ATP and 5 mM total Mg^{2+} is about 1, while at 15 mM total Mg^{2+}, the ratio is about 0.014, about 70 times less. This shows that, at high ATP concentrations, the inhibition depends on the total ATP concentration. The dependence of the inhibition on the total ATP concentration indicates that the inhibitory sites for ATP have the same affinity for free ATP as for complexed ATP.

The inhibition of the enzyme activity by excess of ATP concentrations at 35°C seems to contradict the results of Hexum et al. [7] who did not find any hint of substrate inhibition at a total ATP concentration of 10 mM at 37°C. But since Hexum et al. did not measure the enzyme activity at ATP concentrations intermediate between 1 and 10 mM ATP, it seems possible that they missed the rise of the enzyme activity and the following inhibition of this activity which occurs between 1 ATP and 10 mM ATP.

Effect of Na^+ on excess substrate inhibition

The phenomenon of excess substrate inhibition occurs also in the absence of external K^+ (Fig. 3). One can therefore study the effect of Na^+ concentration on the inhibition of ATP (Fig. 4). Decreasing the Na^+ concentration in the absence of K^+, from 140 mM Na^+ to 2 mM, does not change the optimal concentration of ATP that is required for maximal activity. At 1 mM Na^+, there was almost no ATPase activity, and it was thus not possible to test whether

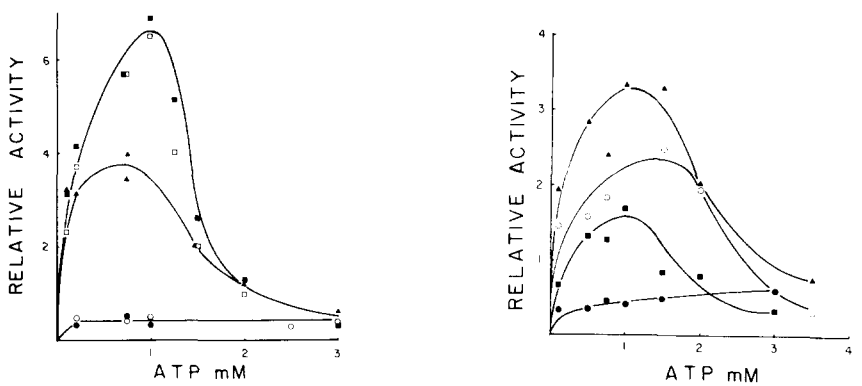

Fig. 3. Substrate inhibition of the (Na^+, K^+)-ATPase hydrolytic activity at 15°C in the presence and absence of K^+. The incubation media contained 140 mM NaCl 15 mM KCl, 50 mM Tris buffer (pH 7.4) and 10 mM $MgCl_2$. The total hydrolytic activity was measured as follows: In the absence of external KCl, ▲——▲; with 15 mM KCl, ■——■; with 125 mM K^+, □——□; with 15 mM KCl and 1.25 mM ouabain, ●——●; with 125 mM KCl and 1.25 mM ouabain, ○——○.

Fig. 4. Substrate inhibition of the (Na^+, K^+)-ATPase activity at 15°C at various NaCl concentrations in the absence of KCl. The incubation media contained 10 mM $MgCl_2$, 50 mM Tris buffer (pH 7.4). Every point was obtained by subtraction of the activity in the presence of 1.25 mM ouabain, from the total activity under the same condition. The ouabain inhibitable hydrolytic activity was measured as follows: ●——●, 1 mM NaCl; ■——■, 1.9 mM NaCl; ○——○, 7 mM NaCl; ▲——▲, 140 mM NaCl.

high ATP concentrations inhibit the enzyme when the sodium concentration is low.

The concentration of ATP which produces the optimal activity is likely to be a function both of the affinity of the catalytic sites and the postulated inhibitory sites for ATP [18]. The lack of dependence of the optimal ATP concentration on the Na^+ concentration suggests that Na^+ has no effect on the affinity of either class of sites.

Effect of K^+ on the phenomenon of excess substrate inhibition at a constant Na^+ concentration

Raising the K^+ concentration, at a constant Na^+ concentration of 20 mM, from 0 up to 15 mM, appears to increase the optimal concentration of ATP required for maximal activity (Fig. 5), from 3 to 6 mM ATP. This effect may be explained by a decrease in the affinity for ATP at the catalytic site, or at the inhibitory site, or both. Raising the K^+ concentration from 2 up to 30 mM, at constant concentration of 140 mM Na^+, fails to change the optimal ATP concentration of 3 mM. High concentrations of Na^+ thus seems to abolish the effect of K^+ on the optimal concentration that is required for maximal activity.

The effect of temperature on the ATP concentration required for maximal activity

The concentration of ATP found to yield a maximal activity is highly temperature dependent, in that increasing the temperature increases the concentration of ATP required for maximal activity (Table II).

Different preparations show some differences in the optimal ATP concentrations required for maximal activity at any given temperature, but the effect of temperature for any given preparation is a systematic one (Table II).

The fact that the inhibition by ATP is dependent on the total ATP concentration indicates that the temperature dependence of the optimal ATP concen-

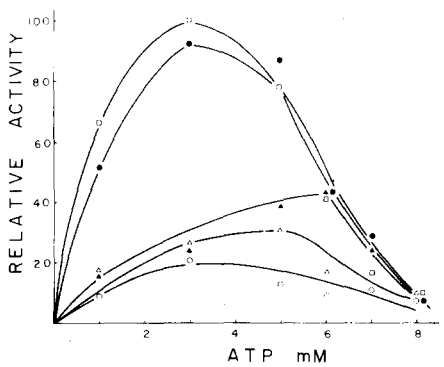

Fig. 5. Substrate inhibition of the (Na^+, K^+)-ATPase hydrolytic activity at 35°C when KCl concentration is changed at low and at high NaCl concentration. The incubation media contained 10 mM $MgCl_2$ 50 mM Tris buffer (pH 7.4) and the indicated KCl and NaCl concentrations. The ouabain inhibitable hydrolytic activity was measured as follows: ○———○, 20 mM NaCl in the absence of KCl; △———△, 20 mM NaCl and 7.5 mM KCl; ▲———▲, 20 mM NaCl and 15 mM KCl; ●———●, 140 mM NaCl and 2 mM KCl; □———□, 140 mM NaCl and 30 mM K^+.

TABLE II

THE ATP CONCENTRATIONS REQUIRED FOR OPTIMAL ACTIVITY AT VARIOUS TEMPERATURES FOR THREE DIFFERENT PREPARATIONS

All the experiments were carried out at concentrations of 14 mM KCl and 140 mM NaCl, 50 mM Tris buffer, pH 7.4, at the indicated $MgCl_2$ concentrations. All values are mM.

Preparation No.	Temperature			Mg^{2+} concentration
	15°C	25°C	35°C	
1	1 —2	3.5—4.5	5—8	10
2	0.5—1	—	2—4	10
3	1 —2	3.5—5	5—7	15

trations required for maximal activity reflects temperature dependent changes in the enzyme, rather than an effect of temperature on the formation constant for Mg · ATP.

Estimate of the stoichiometry for the effect of ATP molecules on the inhibition process

In order to estimate the number of ATP molecules required for the inhibition process, the Hill numbers [19] for the descending part of eleven separate experimental curves (including the curves in Figs. 1 and 3) were calculated. All these curves were derived from experiments that were performed at 15°C at 140 mM NaCl, at various KCl concentrations (Table III). All such experimental curves consist of a steep rise in the activity followed by a steep fall in activity as the ATP concentration is raised without any intermediate plateau between the activation and the inhibition process. The lack of intermediate plateau indicates that the inhibitory process begins before all the catalytic sites are

TABLE III

THE CALCULATED HILL NUMBER FOR THE DESCENDING PART OF ELEVEN EXPERIMENTAL CURVES. ALL THE EXPERIMENTS WERE DONE AT 15°C, AT 140 mM NaCl, 10 mM $MgCl_2$, KCl AS INDICATED, 50 mM TRIS BUFFER, pH 7.4.

KCl concentration (mM)	Hill number ± S.E.
14	2.2 ± 0.9
14	3.67 ± 0.05
14	4.07 ± 0.75
14	2.76 ± 0.2
14	1.24 ± 0.46
14	4.01 ± 0.55
30	1.5 ± 0.64
60	2.9 ± 0.5
90	3.26 ± 1.5
0	4.2 ± 0.85
0	4.3 ± 1
	The mean Hill number is 3.1 ± 0.67

occupied. Therefore, the maximal determined rate of hydrolysis is lower than the true maximal hypothetical hydrolysis rate, which would be the activity at infinite substrate concentration, when the inhibition does not occur. This hypothetical maximal hydrolysis rate was estimated by simulation of theoretical curves, with the same shape and apparent maximal activity as the experimental curves. The change in the calculated Hill number when different values of maximal velocity was used was negligible with respect to the experimental error. The standard error for every experiment was calculated according to the deviation of the experimental points from the linear regression of $\ln(s)$ against $\ln(V/v - 1)$, where s is the substrate concentration, V is the hypothetical maximal activity and v is the activity at s. The calculated mean Hill number is about 3 (Table III). This suggests that at least three molecules of ATP bind to one enzyme unit to induce inhibition in addition to the ATP molecule that is bound at the catalytic site.

Discussion

Inhibition of the (Na^+, K^+)-ATPase activity at high ATP concentrations, at constant low Mg^{2+} concentration, has been explained by Hexum et al. [7] as due to competition between Mg · ATP and free ATP on the catalytic site.

Such an inhibition mechanism predicts that the degree of inhibition would be a function of ATP_{free}/Mg · ATP ratio. The ratio ATP_{free}/Mg · ATP can be controlled easily by changing the Mg^{2+} concentration. One would expect that reducing the level of free ATP by increasing the concentration of Mg^{2+} at high ATP concentration would reduce the degree of inhibition. The present results show that the degree of inhibition at high ATP concentrations and high Mg^{2+} concentrations is not dependent on the ATP_{free}/Mg · ATP ratio.

The finding that 3 ATP molecules are needed for the inhibition process, at excess Mg^{2+} concentration, is also not readily compatible with an inhibition mechanism of competition between free ATP and Mg · ATP at the catalytic site.

The present results suggest, in contrast to the above view, that the inhibition at high ATP concentrations, at excess Mg^{2+} concentration, occurs by the occupation of low affinity sites for ATP.

Skou [8] found that, for any given total ATP concentration, there is an optimal Mg · ATP concentration above which the hydrolysis rate is inhibited to a certain degree. Skou suggested that the presence of free ATP is associated with activation of the hydrolytic activity. Raising the Mg^{2+} concentration at a fixed total ATP concentration brings about a certain degree of inhibition because the activating free ATP becomes complexed. According to Skou's results, raising the Mg · ATP concentration increased the hydrolytic activity when free ATP was present or not.

The present experiments, carried out at low concentration of ATP free but at higher Mg · ATP concentration than those studied by Skou, show that raising the Mg · ATP concentration causes a decrease in the hydrolytic activity. The present results, covering thus a different substrate concentration range, do not contradict the results of Skou.

There are a number of studies in the literature that deal with other subjects

but, incidentally, demonstrate substrate inhibition at excess or equimolar Mg^{2+} concentrations. The inhibition in these cases cannot be explained by the inhibitory effect of free Mg^{2+}, which has a low K_i, about 20—40 mM [20—22]. Inhibition of the activity by ATP has been found in (Na^+, K^+)-ATPase extracted from human erythrocytes [23], rabbit kidney outer medulla [24], beef brain [24], ox brain [25], and electroplax microsomes [26]. In the last case the substrate was UTP and the reduction of the activity appeared above 5 mM UTP at 26°C. In all the other cases the reduction in activity appeared clearly above 3—5 mM ATP at 37°C. The same phenomenon was found when ITP and GTP were used as substrates [25]. It was found that high Mg · ATP concentrations and probably also high Mg · ADP inhibit the Na^+-dependent ADP/ATP exchange [27].

It is worthwhile to note that high concentrations of Mg · ATP inhibit the uptake rate of Ca^{2+} by the Ca^{2+} pump [28], the catalytic unit of which is similar to the catalytic unit of the (Na^+, K^+)-ATPase [29]. The fact that the substrate inhibition appears in preparations which were extracted from a variety of species and tissues suggests that the substrate inhibition is an intrinsic property of the (Na^+, K^+)-ATPase enzyme.

The reason that the substrate inhibition appears in so few kinetic studies is that, at the usual range of temperatures used for kinetic studies (25—37°C), the inhibition appears only at an ATP concentration above 3—5 mM. These concentrations are at the upper limit of those generally used in kinetic studies. The fact that complete inhibition by ATP at 35°C requires a concentration of more than 10 mM indicates that only a small fraction of inhibitory sites that bind ATP are occupied at physiological temperature, ATP and cation concentrations.

The properties of the inhibitory sites, such as are revealed by studying the dependence of the substrate inhibition on the Na^+, K^+ and Mg^{2+} concentrations are as follows:

1. The affinity of the inhibitory sites for ATP is independent of the Na^+ concentration in the absence of K^+.

2. The affinity for ATP of the catalytic sites, or of both the catalytic and the inhibitory sites, is decreased by raising the K^+ concentration at low Na^+ concentration, but this effect is abolished at high Na^+ concentrations.

3. The affinity of the inhibitory sites for free ATP is the same as that for ATP complexed to Mg^{2+}.

The first two properties have been shown already for the catalytic sites in many kinetic [30,31] and binding studies [32,33]. The third property resembles Skou's finding that the optimal Na^+/K^+ ratio for half maximal activation of the hydrolytic activity is controlled by an ATP site which has the same affinity for complexed ATP as for free ATP [8]. The resemblance between the inhibitory class of sites and the catalytic class of sites might suggest that those sites which inhibit while they are occupied are related to the hydrolytic mechanism. These sites might perhaps be empty ADP sites, which coexist with the ATP binding sites. Coexistence of occupied ATP sites and empty ADP sites was postulated originally by Stein et al. [34] and Repke and Schon [35] in their flip-flop models. It is possible that at abnormal conditions of temperature and ATP concentrations, ATP mimics the action of ADP as a product inhibitor, by

binding to such empty ADP sites. The justification for the assumption that an ATP molecule might mimic the action of an ADP molecule at the ADP site is based upon the various analogous interactions of the ATP and ADP molecules with the enzyme. Binding [36] and kinetic [31] experiments show that ATP and ADP compete at the same sites and their affinity is equally affected by various conditions of Na^+ and K^+. Both molecules activate ouabain binding [26] and PNPase activity at the appropriate Na^+ and K^+ concentration [37]. Experiments with *N*-ethylmaleimide show that the protection of either ADP or ATP against inactivation of the hydrolysis activity by *N*-ethylmaleimide is affected equally by various concentrations of Na^+ and K^+ [38].

The Hill number of 3 which was found above for the inhibition process is not compatible with the Hill number that has been found for protection of the hydrolytic activity by ATP against inactivation by Chlorpromazine ($h = 1.3$) [39], or with the Hill number for the variation of the rate with ATP concentration ($h = 0.3—1$) at the low substrate range [31] of ATP concentrations. But it is not to be expected that the number of molecules participating in these latter processes will be necessarily equal to the number of molecules participating in the inhibition process at higher substrate concentrations. A Hill number of about three for the inhibition process suggests that the minimal number of inhibitory sites and catalytic sites taken together per enzyme unit is four.

There is also a possibility that the inhibition process occurs bot by binding of three ATP molecules at three separate binding sites, but by the binding of more than one ATP molecule per binding site. It seems unlikely that the inhibition occurs through binding of an additional three molecules of ATP to a single catalytic site which binds already one ATP molecule. It is possible, however, that two binding sites exist per unit enzyme, as was suggested by Skou [8], Robinson [40] and Lazdunski et al. [31], and inhibition may occur when two ATP molecules bind at each binding site.

Acknowledgement

I want to thank Professor W.D. Stein and Dr. A. Tolkowsky for encouragement, helpful discussions and criticism of the manuscript.

References

1 Skou, J.C. (1957) Biochem. Biophys. Acta 23, 394—401
2 Dahl, J.L. and Hokin, L.E. (1974) Annu. Rev. Biochem. 43, 327—356
3 Skou, J.C. (1975) Q. Rev. Biophys. 7, 401—434
4 Glynn, I.M. and Karlish, S.J.D. (1975) Annu. Rev. Physiol. 37, 13—55
5 Askari, E. (1974) Ann. N.Y. Acad. Sci. 242,
6 Whittam, R. and Chipperfield, A.R. (1975) Biochim. Biophys. Acta 415, 149—171
7 Hexum, T., Samson, Jr., F.E. and Himes, R.H. (1970) Biochim. Biophys. Acta 212, 322—331
8 Skou, J.C. (1974) Biochim. Biophys. Acta. 339, 246—257
9 Jorgensen, P.L. and Skou, J.C. (1971) Biochim. Biophys. Acta, 233, 366—380
10 Nakao, T., Tashima, Y., Hagano, K. and Nakao, N. (1965) Biochem. Biophys. Res. Commun. 19, 755—758
11 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
12 Crane, R.K. and Lipmann, F. (1953) J. Biol. Chem. 201, 235—243
13 Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147—149
14 O'Sullivan, W.J. and Perrin, D.D. (1964) Biochemistry 3, 18—26
15 Norby, J.G. (1970) Acta Chem. Scand. 24, 3274—3286

16 Burton, K. (1959) Biochem. J. 71, 388—395
17 Storer, A.C. and Cornish-Bowden, A. (1976) Biochem. J. 159, 1—5
18 Laidler, K.J. and Bunting, P.S. (1973) The Chemical Kinetics of Enzyme Action, p. 84—88, Clarendon Press, Oxford
19 Hill, A.V. (1910) J. Physiol. (Lond.) 40, IV—VII
20 Robinson, J.D. (1974) Biochim. Biophys. Acta. 341, 232—247
21 Atkinson, A., Hunt, S. and Lowe, A.G. (1968) Biochim. Biophys. Acta, 167, 469—472
22 Gibbs, R., Roddy, R.R. and Titus, E. (1965) J. Biol. Chem. 240, 2181—2187
23 Dunham, E.T. and Glynn, I.M. (1968) J. Physiol. (Lond.) 156, 174—293
24 Baskin, L.S. and Leslie, R.B. (1968) Biochem. Biophys. Acta. 159, 509—513
25 Schoner, W., Benuch, R. and Kramer, R. (1968) Eur. J. Biochem. 7, 102—110
26 Siegel, G.J. and Goodwin, B. (1972) J. Biol. Chem. 247, 3630—3637
27 Robinson, J.D. (1976) Biochim. Biophys. Acta 440, 711—722
28 Moore, L., Fitzpatrick, D.F., Chen, T.S. and Landon, E.J. (1974) Biochim. Biophys. Acta. 345, 405—418
29 Bastide, F., Meissner, G., Fleischer, S. and Post, R.L. (1973) J. Biol. Chem. 248, 8385—8391
30 Robinson, J.D. (1967) Biochemistry 6, 3250—3258
31 Gache, C., Rossi, B. and Lazdunski, M. (1976) Eur. J. Biochem. 65, 293—306
32 Jensen, J. and Norby, J.G. (1971) Biochim. Biophys. Acta. 233, 395—403
33 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5234—5240
34 Stein, W.D., Lieb, W.R., Karlish, S.J.D. and Eilam, Y. (1973) Proc. Natl. Acad. Sci. U.S. 70, 275—278
35 Repke, K.R.H. and Scon, R. (1973) Acta Biol. Med. Germ. 31, K 19—30
36 Norby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta. 233, 104—116
37 Swann, A.C. and Albers, R.W. (1975) Biochim. Biophys. Acta. 382, 437—456
38 Skou, J.C. (1974) Biochim. Biophys. Acta. 339, 234—245
39 Squires, R.F. (1965) Biophys. Res. Commun. 19, 27—32
40 Robinson, J.D. (1976) Biochim. Biophys. Acta. 429, 1006—1019