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STUDIES ON ($Na^+ + K^+$)-ACTIVATED ATPase

XLV. MAGNESIUM INDUCES TWO LOW-AFFINITY NON-PHOSPHORYLATING NUCLEOTIDE BINDING SITES PER MOLECULE *

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ATP and adenylylimidodiphosphate (Ado*PP*[NH]*P*) bind to ($Na^+ + K^+$)-ATPase in the absence of Mg^{2+} (EDTA present) with a homogeneous but 15-fold different affinity, the K_d values being 0.13 μM and 1.9 μM, respectively. The binding capacities of the two nucleotides are nearly equal and amount to 3.9 and 4 nmol/mg protein or 1.7 and 1.8 mol/mol ($Na^+ + K^+$)-ATPase, respectively. The K_d value for ATP is equal to the K_m for phosphorylation by ATP (0.05–0.25 μM) and the binding capacity is equivalent to the phosphorylation capacity of 1.8 mol/mol ($Na^+ + K^+$)-ATPase. Hence, the enzyme contains two high-affinity nucleotide binding and phosphorylating sites per molecule, or one per α-subunit. Additional low-affinity nucleotide binding sites are elicited in the presence of Mg^{2+}, as shown by binding studies with the non-phosphorylating (Ado*PP*[NH]*P*). The K_d and binding capacity for Ado*PP*[NH]*P* at these sites is dependent on the Mg^{2+} concentration. The K_d increases from 0.06 mM at 0.5 mM Mg^{2+} to a maximum of 0.26 mM at 2 mM Mg^{2+} and the binding capacity from 1.5 nmol/mg protein at 0.5 mM Mg^{2+} to 3.3 nmol/mg protein at 4 mM Mg^{2+}. Extrapolation of a double reciprocal plot of binding capacity vs. total Mg^{2+} concentration yields a maximal binding capacity at infinite Mg^{2+} concentration of 3.8 nmol/mg protein or 1.7 mol/mol ($Na^+ + K^+$)-ATPase. The K_d for Mg^{2+} at the sites, where it exerts this effect, is 0.8 mM. The K_d for the high-affinity sites increases from 1.5–1.9 μM in the absence of Mg^{2+} to a maximum of 4.2 μM at 2 mM Mg^{2+} concentration. The binding capacity of these sites (1.8 mol/mol enzyme) is independent of the Mg^{2+} concentration. Hence, Mg^{2+} induces two low-affinity non-phosphorylating nucleotide binding sites per molecule ($Na^+ + K^+$)-ATPase in addition to the two high-affinity, phosphorylating nucleotide binding sites.

Introduction

The ($Na^+ + K^+$)-activated ATPase contains two different types of subunits, which are designated α and β. Their protein molecular weight, determined by sedimentation equilibrium analysis of the isolated, detergent-free subunits from a rabbit kidney outer medulla preparation is 120 600 ± 4 600 for the α subunit and 42 800 ± 1 000 for the β subunit [1]. For enzyme preparations from dog kidney and shark rectal gland values of 106 000–121 000 for the α-subunit and 36 600–56 000 for the β-subunit, obtained in the presence of detergent via sedimentation equilibrium or gel filtration analysis, have been reported [2–4]. From the protein mass ratio ($\alpha/\beta = 3.04$), the 280 nm absorbance ratio ($\alpha/\beta = 2.02$; $\epsilon_\alpha = 143\,000$, $\epsilon_\beta = 78\,000\ l \cdot mol^{-1} \cdot cm^{-1}$) and comparison of their amino acid composition with that of the native enzyme the α/β molar ratio has been shown to be 1 : 1, and the $\alpha\beta$ molecular weight 163 400 [1]. This value agrees with the protein molecular weight of

* For Part XLIV see Ref. 11.
Abbreviation: TNP, trinitrocyclohexadienylidene.

170 000 ± 9 000, determined by sedimentation velocity and gel filtration analysis of the detergent solubilized $\alpha\beta$ dimer from a pig kidney outer medulla preparation [5]. From the reported protein molecular weights of total enzyme (280 000–380 000, Refs. 2, 6, 7) it follows that the most likely subunit composition is $\alpha_2\beta_2$, corresponding to a protein molecular weight of 326 800 for the $(Na^+ + K^+)$-ATPase preparation from rabbit kidney outer medulla here investigated.

Recently, we have demonstrated that the phosphorylation capacity for ATP is 5.6 nmol P/mg protein (based on amino acid analysis), corresponding to 0.92 molecule per $\alpha\beta$ dimer or 1.8 molecule per enzyme molecule [8]. Since the molar binding value of ATP equals the phosphorylation level [9] with a K_d or K_m in the range 0.05–0.25 μM [10,11], it can be concluded that the enzyme contains two high-affinity phosphorylating ATP binding sites.

Kinetic evidence has been presented, which can be interpreted to support the presence of separate high-affinity (K_m or K_d = 1–5 μM) and low-affinity (K_m or K_d = 0.1–0.5 mM) ATP binding sites [12–15]. Studies on the inactivation by the disulfide of thioinosine triphosphate in relation to its binding seem to agree with this interpretation [16]. In this case the ratio of high-affinity (phosphorylating) and low-affinity (non-phosphorylating) ATP binding sites was 1 : 3 and their sum amounted to 4 mol/mol enzyme, assuming a protein molecular weight of 280 000 for the $\alpha_2\beta_2$ tetramer. On the other hand, studies with a trinitrocyclohexadienylidene analogue of ATP suggest that at any time only one type of binding site exists (one per 175 000 g protein) with an affinity for ATP, which is either high or low, depending on the presence of ligands: K_d = 1 μM without ligands; K_d = 70–120 μM with 20 mM K^+ + 3.9 mM Mg^{2+} [17].

In view of these conflicting results and the scarcity of actual binding studies, we have investigated the binding of the non-phosphorylating ATP analogue adenylyl imidodiphosphate (Ado*PP*[NH]*P*) to a highly purified $(Na^+ + K^+)$-ATPase preparation as a function of the Mg^{2+} concentration. Mg^{2+} in millimolar concentrations has various effects on the partial reactions of $(Na^+ + K^+)$-ATPase, including nucleotide binding. It has been suggested that Mg^{2+} exerts these effects via binding to the low-affinity substrate sites [18], quite distinghuishable in their affinity to Mg^{2+} from the high-affinity ATP binding and phosphorylation sites: K_d = 0.5–1 mM vs. K_d = 5 μM [19,20]. We find that Mg^{2+} in millimolar concentrations elicites two low-affinity non-phosphorylating nucleotide binding sites per $\alpha_2\beta_2$ tetramer of 327 000 protein molecular weight, in addition to the two high-affinity binding sites involved in phosphorylation.

Materials and Methods

Preparation of $(Na^+ + K^+)$-ATPase

A highly purified $(Na^+ + K^+)$-ATPase preparation is obtained from rabbit kidney outer medulla according to Jørgensen [22]. Removal of ATP, subsequent washing and storage of this preparation have been described elsewhere [23]. Protein is determined by means of the trichloroacetic acid precipitation modification of the Lowry method against bovine serum albumin as standard [22]. $(Na^+ + K^+)$-ATPase activity is determined at 37°C via the non-radioactive assay method described by Schoot et al. [23]. Enzyme preparations with a specific activity of at least 1.5 mmol ATP hydrolyzed per h per mg Lowry protein have been used (range 1.5–1.6 mmol/h per mg protein).

*Binding of Ado*PP*[NH]P*

Radioactive [2,8-^{3}H]Ado*PP*[NH]*P* (tetrasodium salt, ICN Chemical and Radioisotope Division, Irvine, CA, U.S.A.) is mixed with non-radioactive Ado*PP*-[NH]*P* (tetralithium salt, Boehringer Mannheim GmbH, Biochemica, F.R.G.) to obtain a specific radioactivity of 130–200 Ci/mol. The imidazole salt is prepared by passing the aqueous solution over a Dowex 50 WX4 cation exchange resin (200–400 mesh, Fluka AG, Buchs, Switzerland), the final nucleotide concentration being determined by reading the 260 nm absorbance. [^{14}C]Sucrose (480 Ci/mol, The Radiochemical Centre, Amersham, U.K.) is added to the Ado*PP*[NH]*P* solution to give a $^3H/^{14}C$ ratio of 1.8–4.6 to correct for remaining free Ado*PP*-[NH]*P* (see below).

Prior to the nucleotide binding experiment we remove EDTA, present during storage [23], from the enzyme by washing in cold (4–5°C) 0.25 M sucrose, 100 mM imidazole-HCl (pH 7.0). The enzyme suspension is centrifuged at 240 000 × *g* for 15 min, and is resuspended in the same medium to a protein concentration of 2.5 mg/ml.

An aqueous solution of the ligands, containing [^{3}H]Ado*PP*[NH]*P* + [^{14}C]sucrose and $MgCl_2$ or EDTA, is mixed with the enzyme suspension in a volume ratio of 3 : 2 (total volume 100 μl). Final concentrations are for [^{3}H]Ado*PP*[NH]*P*: 1–300 μM, $MgCl_2$: 0.5–5 mM, EDTA: 2 mM, enzyme protein: 1 mg/ml, and imidazole-HCl (pH 7.0): 40 mM. After ample equilibration (5 min) at room temperature (22°C), two 40 μl aliquots of the suspension are filtered in duplicate by suction via 0.45 μm pore width Type HA filters (Millipore Corporation, Bedford, MA, U.S.A.). The filters are extracted for 30 min in 1 ml 10% (w/v) SDS at room temperature, and the resulting solution is mixed with 10 ml PicoFluor 15 (Packard Instr. Co., Inc., Downers Grove, IL, U.S.A.). The solutions are counted for ^{3}H and ^{14}C in a liquid scintillation counter programmed for double label analysis, and the radioactivity of each isotope is calculated by means of the external standard ratio method.

Counts of [^{14}C]sucrose serve, through the ^{3}H/^{14}C ratio, to correct for non-protein-bound [^{3}H]-Ado*PP*[NH]*P* remaining in the variable amount (about 7%) of water adhering to the filter, on the assumption that the neutral sugar does not bind to ($Na^+ + K^+$)-ATPase [24]. ^{14}C-labeled sucrose is particularly suitable for this purpose, since a large excess (0.1 M) of non-radioactive sucrose is present in the medium. Binding of [^{3}H]Ado*PP*[NH]*P* to the protein reduces the ^{3}H/^{14}C ratio in the free solution and with this lower ratio the correction for free [^{3}H]Ado*PP*-[NH]*P* on the filter is repeated, leading to a higher value of protein-bound Ado*PP*[NH]*P* and a new and still lower ^{3}H/^{14}C ratio in solution. This procedure of mutual correction between bound and free [^{3}H]Ado-*PP*[NH]*P*, with ^{14}C on the filter as absolute standard for the free nucleotide, was repeated 5 times, when an asymptotically approached maximal value for protein-bound Ado*PP*[NH]*P* (and a minimum for the free ^{3}H/^{14}C ratio) is obtained.

This correction method is illustrated with an example. In the presence of 5 μM Ado*PP*[NH]*P*, the medium (40 μl) contains 200 pmol nucleotide. Let the initial ^{3}H/^{14}C counting ratio be x and the ^{14}C counts retained on the filter be y. Then the non-corrected value for free Ado*PP*[NH]*P* (*F*) on the filter corresponds to xy counts, which are converted by comparison with [^{3}H]Ado*PP*[NH]*P* standards of known concentration to pmol. In an actual experiment this led to 20.4 pmol for *F*. The total amount of protein-bound (*B*) + free (*F*) nucleotide on the filter was 86 pmol. Hence $B = 86-20.4 = 65.6$ pmol Ado*PP*[NH]*P* and the medium (*M*) contained 200–65.6 = 134.4 pmoles. This means that the initial ^{3}H/^{14}C ratio in the medium was reduced to (134.4/200) x = 0.672x and the correction for free Ado*PP*-[NH]*P* on the filter to $0.672 \times 20.4 = 13.7$ pmol. Since $B + F$ remains 86 pmol, *B* now becomes 86–13.7 = 72.3 and *M* 200–72.3 = 127.7 pmol. This leads to a new ^{3}H/^{14}C ratio of (127.7/200) x = 0.638x and a new correction for *F* of $0.638 \times 20.4 = 13.0$ pmol. After these three corrections *F*, *B*, *M* and the ^{3}H/^{14}C ratio did not change anymore and yielded values of 13.0, 73.0, 127.0 pmol and 0.635x, respectively. Substracted are blanks, which are treated by the same procedure, but contain an additional 10 mM non-radioactive Ado*PP*[NH]*P*. In the above example the non-specific retention of Ado*PP*[NH]*P* on the filter was $(13/86) \times 100 = 15\%$. Over the entire range of Ado*PP*[NH]*P* concentrations the retention of non-protein-bound Ado*PP*[NH]*P* rose from 10 to 90%. The accuracy of the method corresponds to a relative standard error for specifically bound nucleotide of 1–5%.

Binding of ATP

Radioactive [2,8-^{3}H]ATP (tetrasodium salt, ICN Chemical and Radioisotope Division, Irvine, CA, U.S.A.) is mixed with non-radioactive ATP (disodium salt, Boehringer Mannheim GmbH, Biochemica, FRG) so as to obtain a specific radioactivity of 100 Ci/mol. Further processing and procedures are the same as described for Ado*PP*[NH]*P*, except that no Mg^{2+} is used in the binding studies. At low [^{3}H]ATP concentrations (0.4–2 μM), the final protein concentration is reduced to 0.25 mg/ml vs. 1 mg/ml at high nucleotide concentrations (10–300 μM). This reduction in protein concentration is not required in the Ado*PP*-[NH]*P* binding studies, since Ado*PP*[NH]*P* has a lower affinity for the enzyme than ATP. The initial radioactivity ratio of [^{3}H]ATP to [^{14}C]sucrose in the free solution is 2 : 1 in these experiments.

Plotting of data and determination of binding parameters

The binding data are graphically presented in Scat-

chard plots as nmol nucleotide bound per mg protein per μmol/l nucleotide on the ordinate versus nmol nucleotide bound per mg protein or mol per mol enzyme on the abscissa. Conversion of nmol nucleotide bound per mg protein to mol nucleotide per mol enzyme is carried out by multiplication with the factor 0.44 (1.35 X 0.327, i.e. the ratio of protein weight determined by the Lowry method over protein weight determined via amino acid analysis [8], multiplied by the enzyme's micromolar weight [1]). The equilibrium concentration of free nucleotide is taken as initial minus enzyme-bound nucleotide. When the enzyme displays two types of nucleotide binding sites, K_d values and binding capacities for the two types of sites have been calculated using a 3-fold iterative process [25]. Straight lines through experimental points have been calculated by means of linear regression analysis.

Results

Fig. 1 demonstrates the binding of ATP (0.4–300 μM) to $(Na^+ + K^+)$-ATPase in Mg^{2+}-free solution. From the Scatchard plot a uniform K_d of 0.13 μM is derived, which matches the 0.1–0.2 μM values reported by Hegyvary and Post [26] and Nørby and Jensen [27]. The maximal binding capacity, derived from the intercept at the abscissa, equals 3.9 nmol/mg protein or 1.7 mol ATP per mol enzyme. This is in good agreement with our recently reported phosphorylation capacity of 1.8 mol/mol $(Na^+ + K^+)$-ATPase in the presence of excess (1–2.5 mM) [γ-^{32}P]ATP [8]. The K_m value for phosphorylation by ATP (0.05–0.25 μM [11]) agrees with the K_d value of 0.13 μM for ATP binding demonstrated in Fig. 1. These data indicate that the enzyme molecule has two high-affinity sites, both for ATP binding and for phosphorylation by ATP, one each per α-subunit.

Fig. 2 demonstrates that these sites can also be occupied by Ado*PP*[NH]*P* with a K_d value of 1.9 μM, about 15-times as high as for ATP. The binding capacity is 4 nmol/mg protein or 1.8 mol/mol enzyme, equal to that for ATP. The K_d value for Ado*PP*[NH]*P* agrees with the K_i value of 3 μM for Ado*PP*[NH]*P*, determined from the kinetics of $(Na^+ + K^+)$-ATPase at low substrate concentrations [13].

However, in the presence of 5 mM $MgCl_2$, a concentration commonly used in the assay of $(Na^+ + K^+)$-ATPase activity [23], the binding plot for Ado*PP*-[NH]*P* becomes biphasic and reaches nearly twice the binding capacity encountered in the absence of Mg^{2+} (Fig. 3). We have interpreted this biphasic behaviour as representing the simultaneous occurrence of two sets of different binding sites, one set with a low and

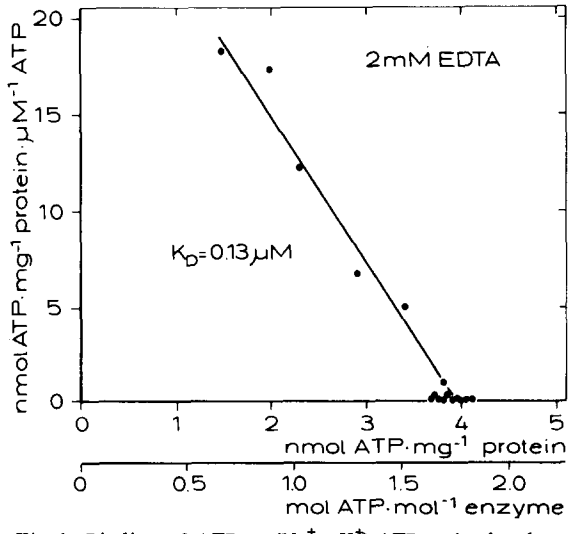

Fig. 1. Binding of ATP to $(Na^+ + K^+)$-ATPase in the absence of Mg^{2+}. EDTA (2 mM) is present in the incubation medium. The K_d value derived from the slope is 0.13 μM, and the binding capacity derived from the intercept on the abscissa is 1.7 mol/mol enzyme.

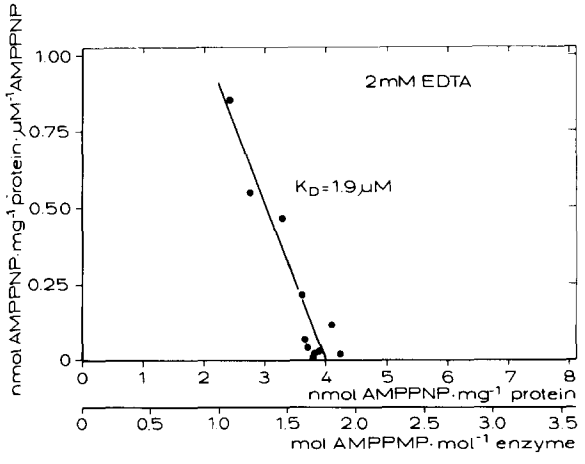

Fig. 2. Binding of Ado*PP*[NH]*P* (AMPPNP) to $(Na^+ + K^+)$-ATPase in the absence of Mg^{2+}. EDTA (2 mM) is present in the incubation medium. The K_d value is 1.9 μM and the binding capacity 1.8 mol/mol enzyme.

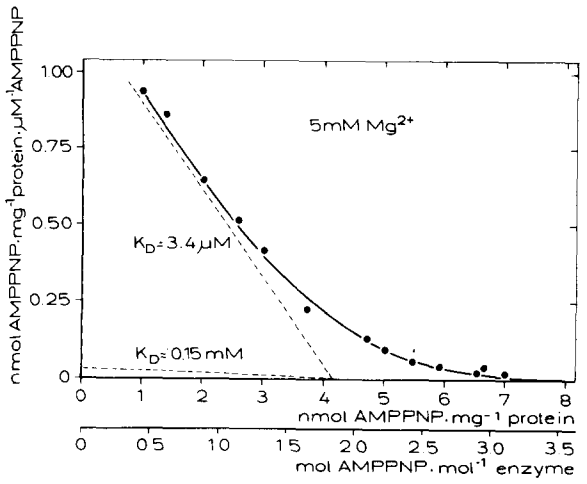

Fig. 3. Binding of Ado*PP*[NH]*P* (AMPPNP) to $(Na^+ + K^+)$-ATPase in the presence of 5 mM Mg^{2+}. The curved plot has been resolved into two linear plots (dashed lines) by 3-fold mutual correction. The high-affinity plot yields a K_d value of 3.4 μM and a binding capacity of 1.81 mol/mol enzyme, the low-affinity plot a K_d of 0.15 mM and a binding capacity of 1.85 mol/mol enzyme.

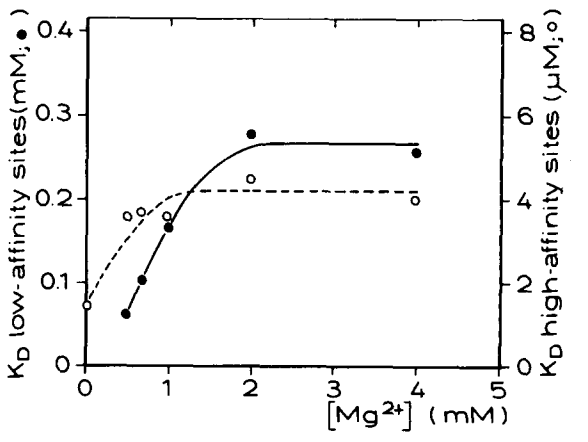

Fig. 4. Effect of Mg^{2+} on the K_d value of high-affinity and low-affinity Ado*PP*[NH]*P* (AMPPNP) binding to $(Na^+ + K^+)$-ATPase. A series of binding plots like the one shown in Fig. 3 has been determined at different Mg^{2+} concentrations. These have been analyzed for high-affinity and low-affinity binding as explained in the legend of Fig. 3. On the abscissa the total magnesium concentration (free plus Ado*PP*[NH]*P* complexed) is shown, on the left ordinate the K_d for low-affinity Ado*PP*[NH]*P* binding, and on the right ordinate the K_d for high-affinity Ado*PP*[NH]*P* binding.

one with a high K_d value. Hence, we have resolved the composite graph into two partial linear plots (dashed lines in Fig. 3) by an iterative mutual correction procedure [25]. From the partial plots K_d values of 3.4 and 150 μM and binding capacities of 1.81 and 1.85 mol/mol enzyme have been derived. This means that in the presence of Mg^{2+} the enzyme acquires two low-affinity ATP binding sites in addition to the two high-affinity sites it already has in the absence of Mg^{2+}.

In subsequent experiments we have investigated the dependence of the K_d values and the binding capacities on the Mg^{2+} concentration. Fig. 4 demonstrates that the K_d values for the high-affinity, as well as for the low-affinity binding sites increases with the Mg^{2+} concentration to maximal levels of about 4.2 and 260 μM, respectively. The binding capacity of the high-affinity sites remains constant at 1.8 mol/mol enzyme, while that of the low-affinity sites increases with the Mg^{2+} concentration, leading to a maximal binding capacity of 1.7 mol/mol $(Na^+ + K^+)$-ATPase for this preparation (Fig. 5). The apparent K_d value of Mg^{2+} for this effect is 0.77 mM, matching the value of 0.77 mM determined by Robinson [28] by means of a Be^{2+}-inactivation procedure.

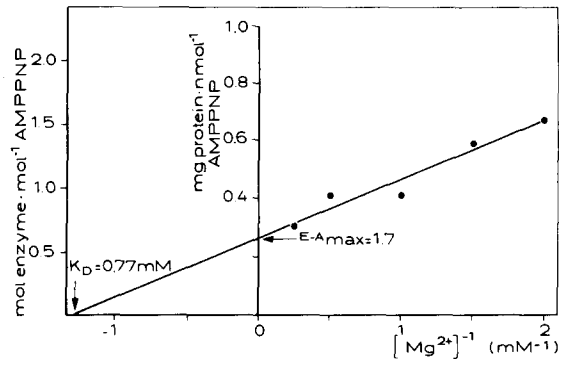

Fig. 5. Effect of Mg^{2+} on the low-affinity Ado*PP*[NH]*P* (AMPPNP) binding capacity. The series of binding plots obtained in the experiment of Fig. 4 has been analyzed for high-affinity and low-affinity binding as explained in the legend of Fig. 3. The data are presented in a double reciprocal plot. The maximal binding capacity of the low-affinity sites ($E\text{-}A_{max}$), derived from the intercept on the ordinate, is 1.7 mol/mol enzyme. The K_d value for Mg^{2+}, derived from the intercept on the abscissa is 0.8 mM. The abscissa shows the reciprocal total magnesium concentration (free plus Ado*PP*-[NH]*P* complexed).

Comparison of Fig. 3 with Figs. 4 and 5, obtained with two different enzyme preparations, shows that the dependence of the K_d values and the binding capacities on the Mg^{2+} concentration may vary with the enzyme preparation, but that the divalent cation invariably tends to double the number of binding sites by uncovering two low-affinity binding sites per enzyme molecule.

Discussion

Kinetic experiments had already provided evidence that in the overall $(Na^+ + K^+)$-ATPase reaction steps occur with low (1 μM) and high (100–500 μM) K_m values for ATP [12,13]. The high-affinity sites were interpreted to be involved in phosphorylation, and the low-affinity sites in driving a conformational transition $E_2 \cdot K \rightarrow E_1 \cdot K$ following K^+-stimulated hydrolysis of the phosphointermediate to $E_2 \cdot K$ [29]. Evidence that the latter action of ATP does not involve phosphorylation comes from an observation made when the enzyme is forced into the unphysiological K^+/K^+ exchange transport mode. In this transport mode, which is considered to operate via the $E_2 \cdot K \rightarrow E_1 \cdot K$ transition, ATP can be replaced by non-phosphorylating analogues such as Ado*PP*[NH]*P* [30].

Yet, it was not clear whether or not the low-affinity nucleotide binding sites in the $E_2 \cdot K$ state are separate from the high-affinity sites or represent the same sites in a different conformation of the enzyme ($E_2 \cdot K$ versus $E_1 \cdot Na$), which would not only have a different substrate affinity, but also a reduced phosphorylation ability. Upwardly convex double reciprocal plots of ATPase activity vs. substrate concentration can indeed be obtained, when the high and low affinities are assumed to be expressions of the same site in different enzyme conformations [31].

However, in the present study we show that two non-phosphorylating, low-affinity binding sites per enzyme molecule can be elicited by Mg^{2+} in addition to the two high-affinity binding and phosphorylating sites already present. A relatively high Mg^{2+} concentration is also required for maximal velocity of the overall $(Na^+ + K^+)$-ATPase reaction [21]. This could point to a relevance of the Mg^{2+}-induced low-affinity binding sites to the enzyme mechanism.

The calculated stoichiometries of 1.7–1.8 mol/mol enzyme for high- and low-affinity binding sites are obviously dependent on the enzyme molecular weight (see under 'plotting of data and determination of binding parameters' in Methods). We have used the molecular weight of 327 000 recently determined in our laboratory from the α- and β-subunit molecular weights of the rabbit kidney outer medulla $(Na^+ + K^+)$-ATPase complex used in the present study, the determined α/β molar ratio of 1 and an assumed $\alpha_2\beta_2$ stoichiometry in this complex. Values of subunit molecular weights deviating from those used in this study relate to $(Na^+ + K^+)$-ATPase complexes derived from other tissues or species (see Introduction) and hence cannot properly be applied in this study.

Another matter is the relatively high nucleotide binding capacity (4 nmol/mg protein) obtained for $(Na^+ + K^+)$-ATPase preparations with suboptimal specific activities (1.5–1.6 mmol/h per mg protein) as compared to preparations of higher specific activity (2.2 mmol/h per mg protein) with an equal (high-affinity) nucleotide binding capacity [10]. Removal of ATP from the preparation (see under Materials and Methods) leads to a partial loss of activity [8], apparently without loss of nucleotide binding capacity. Such a non-parallel decrease of $(Na^+ + K^+)$-ATPase activity and ATP binding capacity has previously been described by Nørby and Jensen for ox brain preparations [32].

A matter of mechanistic importance is that the enzyme apparently contains two different sets of Mg^{2+} binding sites. One of these (the 'affinity sites') increases the K_d for the high-affinity as well as for the low-affinity nucleotide binding sites and is saturated at 2 mM Mg^{2+} (Fig. 4), but may not be involved in the induction of extra nucleotide binding sites. The other set of Mg^{2+} binding sites (the 'capacity sites') is involved in the induction of the extra nucleotide binding sites and is saturated at (Fig. 3) or beyond 5 mM Mg^{2+} (Fig. 5). Thus, although the affinity of Ado*PP*[NH]*P* for its low-affinity binding sites is appreciably higher at 0.5 mM Mg^{2+} than at 5 mM Mg^{2+} (Fig. 4), the maximal nucleotide binding capacity ($E\text{-}A_{max}$, Fig. 5) is not reached since at 0.5 mM Mg^{2+} the 'capacity sites' are not saturated by Mg^{2+}.

With regard to the existence of high-affinity and low-affinity nucleotide binding sites on the enzyme, studies with the fluorescent non-phosphorylating substrate analogue trinitrocyclohexadienylidene-ATP

(TNP-ATP) would seem to conflict with the concept of separate regulatory ATP binding sites. This ATP analogue competitively inhibits the ATPase activity at low ATP concentrations in the absence of K^+ and at high ATP concentrations in the presence of K^+ [31]. This would suggest that only one binding site per $\alpha\beta$ dimer is involved in the reaction, the affinity of which depends on the absence or presence of K^+ in the medium. However, the properties of the binding sites for TNP-ATP strikingly resemble those of the low-affinity ATP- or Ado*PP*[NH]*P*-binding sites derived from kinetic and binding studies. (1) They are non-phosphorylating [31]. (2) The dissociation constant for TNP-ATP has a relatively high temperature coefficient [17] like the low-affinity K_m for ATP in kinetic experiments [13]. (3) The substrate specificity for TNP-adenosine derivatives is low [31] like the low substrate specificity at the kinetically determined low-affinity sites for ATP [13,14]. (4) TNP-ATP competitively inhibits the *p*-nitrophenylphosphatase reaction [31], which is competitively inhibited by ATP via the low-affinity ATP sites [13]. (5) TNP-ATP binding is relatively ouabain-insensitive (i.e. 1 mM ouabain merely increases its K_d value from 0.4 [17] to 1.2 μM [31]), like binding of Ado*PP*[NH]*P* to the Mg^{2+}-induced low-affinity sites undergoes only a relatively small increase in K_d by 1 mM ouabain (Schuurmans-Stekhoven et al., unpublished data). It may be that TNP-ATP, because of its lipophylic nature [17], has access and binds preferentially to the sites which for less lipophylic nucleotides are elicited by Mg^{2+}. If this were true, it would follow from the increase in K_d for TNP-ATP upon high-affinity ATP binding [17], that high-affinity and low-affinity nucleotide binding sites interact by negative cooperativity as suggested by Grosse et al. [14,15].

The SH-group reactive disulfide of thioinosine triphosphate appears to sense simultaneously the high-affinity and low-affinity nucleotide binding sites in the absence of Mg^{2+}, but the reaction rates differ by an order of magnitude [16]. The rapidly reacting sites, equivalent to the high-affinity sites, amount to 0.97–1.12 mol/mol enzyme, based on Lowry protein determination and an assumed protein molecular weight of 280 000. Taking into account that this enzyme from pig kidney outer medulla has an $\alpha_2\beta_2$ molar weight of 340 000 [5] and the finding that the Lowry protein values are 35% too high [8], we arrive at a binding capacity at the high-affinity sites of 1.7 mol/mol enzyme, matching the value of 1.7–1.8 in the present study. The proportion of slowly reacting binding sites (protected by ATP with low affinity) is three to one high-affinity binding site [16], greatly exceeding our 1 : 1 ratio for the two types of sites. However, their ratio suffers from the uncertainty that the protection by ATP may extend beyond its binding centre, so that the number of protected inhibitor sites may exceed the number of nucleotide binding sites.

It is still uncertain at which step in the reaction cycle of $(Na^+ + K^+)$-ATPase the additional Mg^{2+} and ATP are bound. It is tempting to assume that low-affinity binding of ATP is required to drive the $E_2 \cdot K \rightarrow E_1 \cdot K$ transition. Against this assumption would plead that Karlish et al. [33] do not find an effect of Mg^{2+} on this transition. However, their Mg^{2+} (1 mM) and nucleotide (4 μM) concentrations were low, so that only high-affinity substrate sites would be occupied. Hegyvary and Jørgensen [34], however, recently described an interaction of Mg^{2+} on this transition when it was driven by Na^+ alone. Under those conditions Mg^{2+} inhibited (half-maximally at 1.2 mM). Unfortunately, their enzyme preparation, labeled with fluorescein isothiocyanate in order to follow conformational transitions fluorimetrically, had lost the ability to bind ATP due to the labeling process. They concluded, however, that $E_2 \cdot K \cdot Mg$ is the occluded K^+ complexing form, rather than $E_2 \cdot K$ as originally proposed by Post et al. [29], and they suggested that this complex is converted by low-affinity ATP binding into a non-occluded form, able to release K^+. Grosse et al. [15], on the other hand, place the low-affinity binding of ATP at the start of the reaction cycle, from which it might enhance K^+-dependent dephosphorylation and transformation of the liberated site into a high-affinity ATP phosphorylating site, while the companion site would decrease its affinity and thus release its product ADP in an anti-cooperative 'flip-flop' mechanism [14]. From a mechanistic point of view, it will thus be of interest to know whether the Mg^{2+}-induced low-affinity binding sites are permanently exposed, independent of other ligands or of partial reactions like phosphorylation.

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