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The molecular size required varies according to the reaction step round the sodium pump cycle

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Progress along the path of the sodium pump cycle requires a stepwise recruitment of additional subunits for maximal activity. These results show that whereas a particle the size of the $\alpha\beta$ protomer presents Na^+,K^+-ATPase activity at 10 μM ATP, an additional subunit, perhaps a second α-chain, is required to obtain the much greater Na^+,K^+-ATPase activity resulting from the occupation of low-affinity ATP sites at physiological ATP concentrations. A non-phosphorylating ATP analogue, however, will modestly stimulate the Na^+,K^+-ATPase activity acting at an alternative low-affinity site or step on the $\alpha\beta$ protomer.

Na^+,K^+-ATPase; Na^+ pump; Subunit; Radiation inactivation; ATP-binding site; ATP analog

1. INTRODUCTION

Kinetic studies of the sodium pump have shown that the system behaves at times with features of a subunit enzyme [1,2]. Examples of this are the apparent need for simultaneous loading of internal Na^+ and external K^+ at high ATP [3], the complex dependence of the ATPase activity on the ATP concentration [4] and the heterogeneity of ATP- and ouabain-binding sites [5,6]. Purified preparations of Na^+,K^+-ATPase consist of α and β polypeptide chains, present in a 1:1 or 1:2 ratio [7,8]. The catalytic, or α-chain, has a molecular mass of 112.5 kDa [9]; the β-chain, of unknown function, is a glycoprotein of 41–55 kDa [10,11]. From radiation inactivation studies, there is evidence that the molecular size required to support Na^+,K^+-ATPase activity in the membrane-bound enzyme is greater than the sum of α and β polypeptide chains [12–14], except when irradiating at very low temperatures [15]. However, solubilized preparations can be made which contain a protomeric ($\alpha\beta$) sodium pump and have Na^+,K^+-ATPase activity [16]; also, the need for concomitant internal and external cation binding does not arise at low ATP concentrations [17]. The latter observations favour the possibility of a self-sufficient protomer as the active species in the membrane.

The experiments presented here were aimed at exploring the possibility that at least some of these contradictions were more apparent than real. The radiation inactivation technique, which allows one to measure the minimal size of the enzymically active particle [18], was used to estimate molecular size. The method is particularly well suited to the study of membrane-bound enzymes, in which case testing for a subunit structure is logistically difficult. With hormone-stimulated adenylate cyclase, for instance, it yields for the hormone receptor and the cyclase-regulator complex sizes similar to those obtained by hydrodynamic analysis of the solubilized components [19], whereas in the case of acetylcholinesterase [20], radiation inactivation exposes a purification-induced aggregation of the solubilized enzyme. For

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Na^+,K^+- [12–14], Ca^{2+},Mg^{2+}- [21,22], H^+- [12] and H^+,K^+- [14] transport ATPases, the radiation inactivation technique consistently returns molecular sizes which point at multimeric structures.

In an earlier study on the sodium pump [23] the irradiated samples were used to measure ATP-ADP exchange and Na^+-ATPase activity simultaneously in the same tubes, at high Na^+ concentrations: the average target sizes found were 118 and 201 kDa, respectively. This suggested that isolated α-chains could reversibly perform the Na^+-dependent partial reactions from E_1 to E_2P (steps a–b in fig.4). The larger particle size needed to obtain ATP hydrolysis in the absence of K^+ (path a-b-c in fig.4) implied that one or two β-chains would be needed in addition to the catalytic polypeptide.

The design adopted for the present study was to utilize the same irradiated samples to measure Na^+,K^+-ATPase activity under two different conditions.

2. EXPERIMENTAL

Na^+,K^+-ATPase was purified from pig kidneys [24] and was initially at 11 μmol·min^{-1}·mg protein^{-1} and 99.6% ouabain-sensitive. Enzyme samples (in 250 mM sucrose, 25 mM histidine, pH 7.4, 1 mM EDTA) were freeze-dried, and kept as controls or irradiated with 16 MeV electrons at 2 Mrad/min in the linear electron accelerator of Addenbrooke's Hospital (Cambridge). This was done in sealed evacuated ampoules at room temperature, with chilled air circulation. Thereafter, the samples were reconstituted with cold distilled water and diluted. These samples, or aliquots of the original purified enzyme, were assayed at 10 μM or both 10 μM and 1 mM [γ-^{32}P]ATP, in triplicate initial and final tubes, as described earlier [22]. The basic reaction medium contained 150 mM NaCl, 15 mM KCl, 25 mM histidine (pH 7.4) and 0.1 mM EGTA, in 40 μl. Other additions and specific experimental conditions are described in the figure legends. Target sizes were calculated according to Kepner and Macey [12]. ATP (disodium salt) was purchased from Boehringer and AMP-PNP from Sigma. [γ-^{32}P]ATP was prepared by a modification of the method of Glynn and Chappell [25]. $^{32}P_i$ was obtained from New England Nuclear.

3. RESULTS AND DISCUSSION

In the presence of K^+, a description of the ATP concentration dependence of the ATPase activity requires at least two apparent affinities for ATP binding, with $K_{0.5}$ values of less than 1 μM and 100–200 μM [4]. Whereas the high-affinity ATP effect is related to the enzyme phosphorylation requirement (fig.4, step a), the low-affinity effect, of much greater extent, should result from an acceleration of release of the K^+ occluded in the

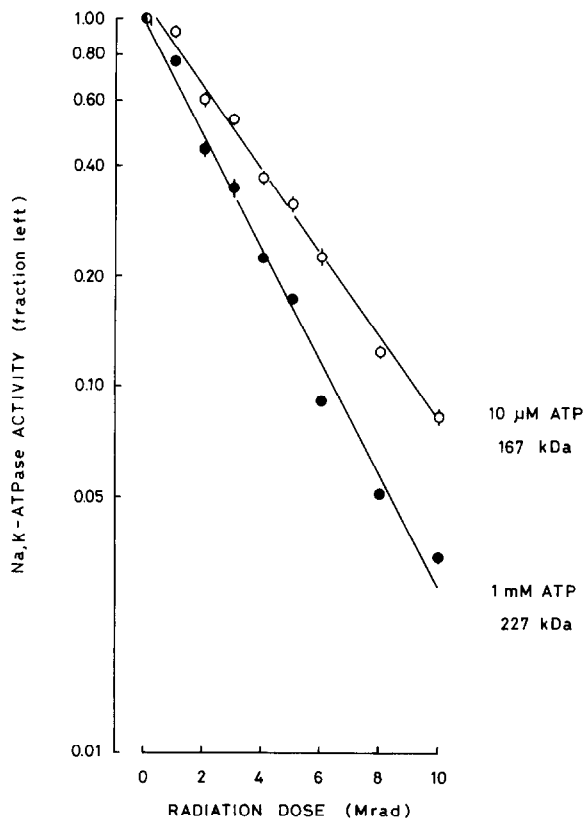
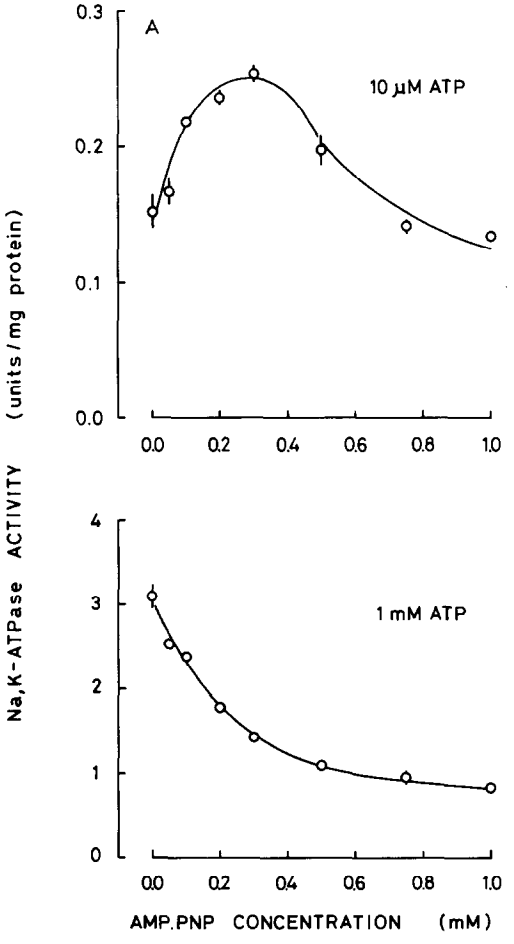


Fig.1. Radiation inactivation of the Na^+,K^+-ATPase activity at two ATP concentrations. The slopes of the regression lines (as mean ± SE) are (Mrad^{-1}) −0.2601 ± 0.0027 (10 μM) and −0.3540 ± 0.0050 (1 mM). For assays at 1 mM ATP, the medium contained besides 1.1 mM $MgCl_2$ and enzyme ranging from (μg protein/ml) 2.5 (controls) to 25 (10 Mrad). For 10 μM ATP, $MgCl_2$ was at 0.2 mM and the enzyme was between 0.75 and 7.5 μg protein/ml. Incubation times at 37°C varied between 20 and 60 min, according to expected hydrolysis, which did not exceed 20%. Vertical bars represent ± 1 SE.

dephosphoenzyme [26,27] (step f). Since only one ATP-binding site has been identified so far in the α-chain and none in the β-chain [10], it was decided to determine whether low-affinity ATP effects required the presence of a second α-chain.

A set of irradiated Na^+ pump samples was then assayed at both 10 μM ATP and 1 mM ATP. At 10 μM, in practice, only the high-affinity ATP site should be operational; at 1 mM ATP, the large stimulation swamps any high-affinity contribution. The result of one of three such experiments is presented in fig.1, and shows a clear-cut difference in molecular size. At 10 μM ATP, the target size appears as 173 ± 6 kDa (mean ± SD) and at 1 mM, as 241 ± 15 kDa. Thus, the size necessary for high-affinity ATP hydrolysis seems to be the sum of α- and β-chains and is closely similar to that observed for the solubilized and active protomeric pump [16] (in which case all ATP-binding heterogeneity has disappeared [28]). Instead, when the assay is performed at physiological ATP levels, something the size of α_2, or perhaps $\alpha_2\beta$, is the active species. The implication is that for ATP to bind at a low-affinity site to catalyse the release of the K^+-occluded form [27] (fig.4, path a-b-d-f), another α-chain is required, but not if the release occurs spontaneously at a low rate (at catalytic ATP concentrations, path a-b-d-e). A target size of 117 kDa has been reported [15] for an ATP-independent Rb^+-Rb^+ exchange catalysed by the Na^+ pump, which seemingly shares steps d-e of the same path. However, from table II of the same article it is clear that, for irradiations at −135°C, the estimates for Na^+,K^+-ATPase (high ATP) and



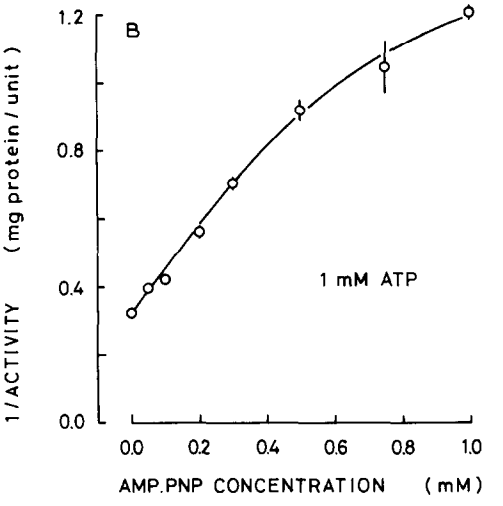


Fig.2. Effects of a non-phosphorylating analogue on the Na^+,K^+-ATPase activity of non-irradiated enzyme. (A) Effect of increasing AMP-PNP concentrations on Na^+,K^+-ATPase activity at 10 μM (upper panel) and 1 mM [γ-^{32}P]ATP (lower panel). Equimolar AMP-PNP and $MgCl_2$ were added to the medium, to attain the final concentrations shown. The enzyme was at 0.072 (top) and 1.43 μg protein/ml (bottom). The ATPase activities and their errors were calculated from the fitted slopes of linear time courses (5 data points over 20 min at 37°C). 'Units' refer to μmol P_i/min. (B) Dixon plot of the Na^+,K^+-ATPase activities in the lower panel of part A. A second experiment under the same conditions gave a closely similar curve. The errors on the reciprocals have been calculated according to Wilkinson [35].

K^+-phosphatase activities are too low by 33% with respect to the average of all quoted studies. If the Rb^+-Rb^+ exchange target size is corrected accordingly, one obtains 174 kDa. This is identical with the size reported here for Na^+,K^+-ATPase activity in the absence of regulatory ATP effects, and is consistent with an $\alpha\beta$ configuration.

The low-affinity ATP stimulation of hydrolysis was further explored by the use of the non-phosphorylating $\beta\gamma$-imido analogue of ATP, adenylyl imidodiphosphate (AMP-PNP). This nucleotide can replace ATP in stimulating K^+-K^+ exchange by the sodium pump [29], but not in supporting Na^+-Na^+ exchange in a catalytic capacity [30]. Fig.2A shows that at 10 μM [γ-^{32}P]ATP, low concentrations of AMP-PNP stimulate the Na^+,K^+-ATPase activity (cf. [31]), up to about 65%, whereas it is only inhibitory at 1 mM [γ-^{32}P]ATP. This behaviour implies that the substrate must add more than once during the enzymic cycle [32], the analogue behaving as a 'better-than-nothing' replacement for the substrate in its non-catalytic binding. Fig.2B shows a Dixon plot of the data in the lower panel of fig.2A. The downward curvature indicates that complete inhibition will not be attained at saturating AMP-PNP concentrations. Thus, substrate and inhibitor should be binding at different sites or steps [33]. The easiest interpretation is that at 1 mM [γ-^{32}P]ATP, the analogue inhibits by competing at the low-affinity site (or step), rather than during phosphorylation, and that it is much less effective than ATP at accelerating this step (f in fig.4). Since at 1 mM [γ-^{32}P]ATP, the effect of 1 mM AMP-PNP is nearly saturated, at 10 μM [γ-^{32}P]ATP and 1 mM AMP-PNP one should be observing the hydrolysis of radioactive ATP following its binding at the catalytic step, with the analogue entering at the low-affinity step every cycle. This occurs irrespective of some inhibition at the catalytic step caused by high concentrations of AMP-PNP under this extreme condition [34] (fig.2A, upper panel).

The Na^+,K^+-ATPase activity of a set of irradiated samples was then assayed at 10 μM ATP with and without 1 mM AMP-PNP. Contrary to expectations, the result (fig.3) shows no shift in target size. This is in sharp contrast with the observation in fig.1 and did not result from an anomaly with this enzyme batch: as a control experiment

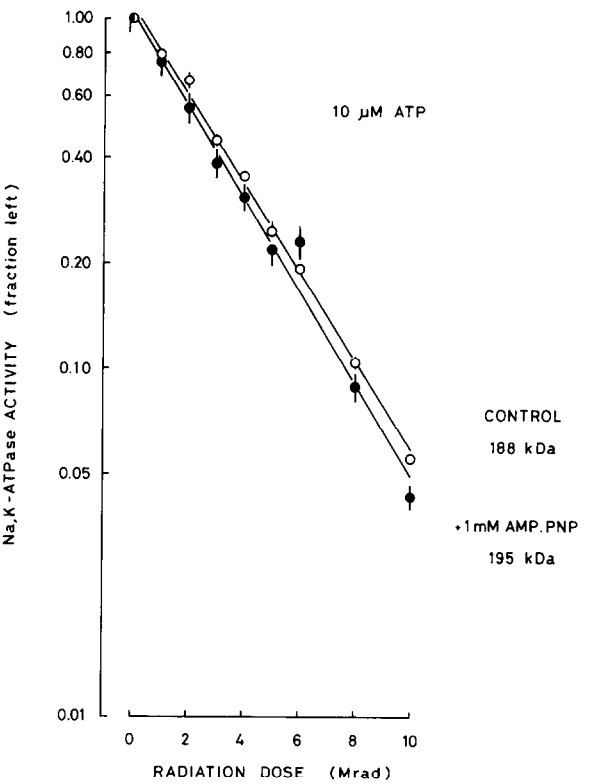


Fig.3. Radiation inactivation of the Na^+,K^+-ATPase activity measured at 10 μM [γ-^{32}P]ATP in the presence and absence of 1 mM AMP-PNP + 1 mM $MgCl_2$. The enzyme concentration varied between 0.75 and 7.5 μg/ml and the incubation at 37°C between 20 and 60 min. The slopes (in $Mrad^{-1}$) are -0.3046 ± 0.0049 and -0.2934 ± 0.0021, with and without AMP-PNP, respectively.

(not shown), the same irradiated samples, which had been kept frozen, were assayed at 10 μM and 1 mM ATP (no AMP-PNP) with the protocol of fig.1. The target sizes were 179 kDa (10 μM ATP) and 257 kDa (1 mM ATP). One then must conclude that the $\alpha\beta$ protomer can go through steps of high and low ATP affinity or have more than one ATP site. However, this low-affinity step (site) in the protomer (the only one that seems to be available for the analogue) should be regarded as 'less effective', since it only leads to a modest increase in Na^+,K^+-ATPase activity. The more than 20-fold increase obtained with high ATP should operate through the 'more effective' low-affinity site, which involves an additional α-subunit.

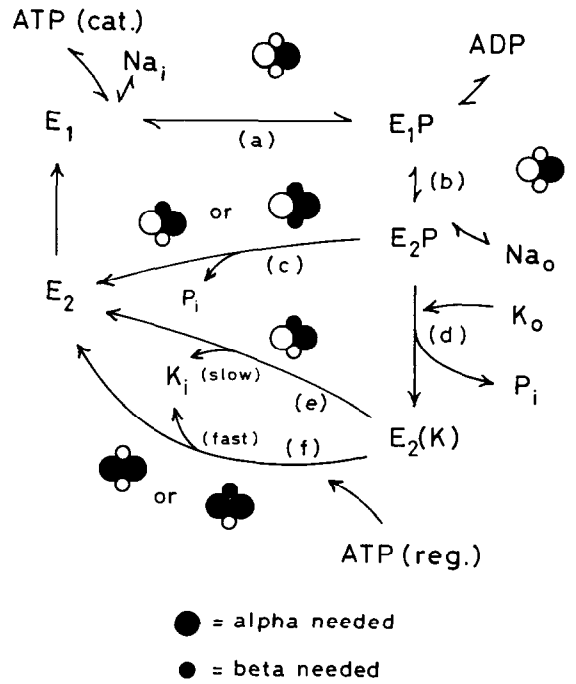


Fig.4. Simplified scheme of the reaction steps of the Na^+ pump, depicting the subunit arrangement required at each stage, as it emerges from the present and an earlier study [23]. The low-affinity AMP-PNP effect should be along step e. The outline represents the diprotomer.

It is possible that this more effective low-affinity ATP site is in fact located in an interacting α-chain and is not accessible to AMP-PNP. However, it is also possible that ATP binds again to the protagonist or 'occluded' α-chain with a low affinity and that an association of the ATP-liganded protagonist with a second α-chain is necessary to accelerate K^+ release. In this case, the docking would be rather ineffectual if the $\beta\gamma$ analogue replaces ATP in the protagonist α-chain. These possibilities need not be mutually exclusive, since it is conceivable that the overall behaviour represents the sum of both and perhaps other contributions.

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