AFFINITY OF THE ($Na^+ + K^+$)-DEPENDENT ATPase FOR Na^+ MEASURED BY Na^+-MODIFIED ENZYME INACTIVATION

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1. Introduction

Since the ($Na^+ + K^+$)-dependent ATPase represents an essential part of, if not the entire, mechanism for transporting Na^+ and K^+ across cell membranes [1–3], the nature of the activation of the enzyme by these same ions seems particularly crucial. Recently, the affinity of the ATPase for K^+ was investigated by measuring the K^+-dependent inactivation of the enzyme by Be^{2+} [4]. Schoner has described [2] a Na^+-inhibited inactivation of the ATPase by dicyclohexylcarbodiimide (DCCD), which suggests a similar approach to evaluating the Na^+-affinity of the enzyme.

This report describes affinities for Na^+, measured in terms of alterations in the rate of inactivation by DCCD, as a function of various ligand states of the ATPase. With a rat brain enzyme preparation the dissociation constant for Na^+, K_D, was 2.3 mM in the absence of other ligands. Phlorizin decreased the affinity for Na^+, as it decreased the kinetically determined apparent affinity of the ATPase reaction [5]. ATP alone halved the K_D, but ATP plus $MgCl_2$, which with Na^+ markedly decrease the K_D for K^+ [4], had little effect. Thus, unlike the K^+-sites that greatly increase in affinity under conditions producing enzyme phosphorylation, the demonstrated affinity for Na^+ changed relatively little in the presence of ligand representing different functional states of the ATPase.

2. Methods

The ($Na^+ + K^+$)-dependent ATPase was obtained from a rat brain microsomal preparation by treatment with deoxycholate and NaI, as previously described [6].

Inactivation by DCCD was accomplished during brief incubations ('preincubations') at 37°C of the enzyme preparation (0.5 mg protein/ml final volume) in 30 mM histidine–Tris (pH 7.8), together with other modifiers when indicated, plus DCCD added last (final concentration: 0.2 mM). Inactivation was terminated and residual activity measured by adding 4 vol of media to bring the concentrations of reactants to their standard assay concentrations: 30 mM histidine HCl (adjusted to pH 7.8 with Tris), 3 mM ATP, 3 mM $MgCl_2$, 90 mM NaCl, and 10 mM KCl. ATPase activity was measured during this incubation, for 4–8 min at 37°C., in terms of the production of P_i, as previously described [6]. Inactivation was calculated relative to control activity, measured concurrently, representing corresponding preincubation and incubation conditions but without DCCD. The low level of Na^+- and K^+-independent ATPase activity was corrected for, as previously described [4].

3. Results and discussion

After brief initial incubations with DCCD, in the presence and absence of NaCl, the resulting inactivation could be measured by short assay incubations in the standard medium: the extent of inactivation did

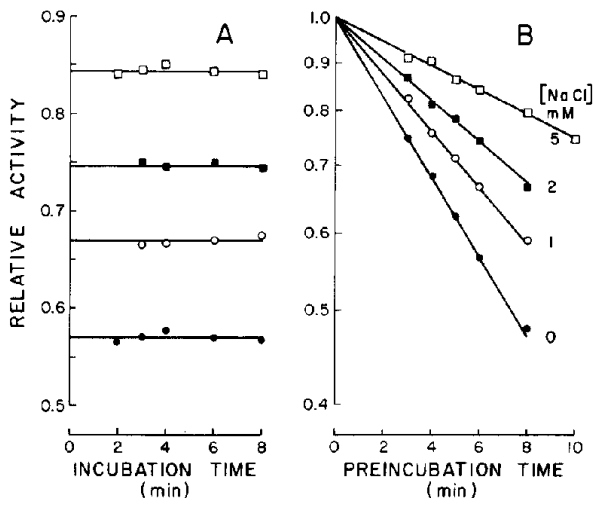

Fig. 1. Effects of DCCD on enzyme activity. In panel A experiments are presented in which the enzyme was first incubated for 6 min at 37°C in media containing 30 mM histidine HCl–Tris (pH 7.8) and 0.2 mM DCCD alone (•) or with 1 (○), 2 (■), or 5 (□) mM NaCl. These inactivating incubations were terminated by adding 4 vol of media to bring the final concentrations of reactants to their standard values for assay (see Methods), and the incubations continued for the times indicated. In panel B experiments are presented in which the enzyme was first incubated ('preincubation') at 37°C for the times shown, in the media described above, and these preincubations then terminated at the times shown by adding the assay media as cited above; in this instance the assay incubations were for 6 min. In both panels values are presented relative to corresponding controls without DCCD, representing averages of 5 or more experiments performed in duplicate.

not change measurably during the assay incubation (fig. 1A). This lack of change is due both to the essentially irreversible inactivation (activity was not restored by three washes) and to the apparent cessation of further inactivation upon adding the assay medium, probably as a result of dilution by DCCD and the high NaCl of the medium.

Consequently, the rate of inactivation during the initial incubation could be assessed by measuring the residual activity in a subsequent assay. The loss of activity followed a first-order time course (fig. 1B); NaCl slowed the rate of inactivation, and pseudo first-order rate constants could thus be measured in the absence of NaCl, k_{in}, and in its presence k_{ob}.

These data suggest that Na^+ slows inactivation by forming an enzyme–Na complex resistant to inactivation:

$$E \cdot Na \rightleftharpoons E_f \longrightarrow E_{in}$$

where E_f is free enzyme, E_{in} inactivated enzyme, and $E \cdot Na$ the resistant complex; for convenience the sum of these may be set equal to unity: $E \cdot Na + E_f + E_{in} = 1.0$. Since inactivation is slow (fig. 1B) relative to the probable rate of Na^+-binding, E_f and $E \cdot Na$ are essentially in equilibrium. Therefore, the dissociation constant for Na^+, K_D, is given by:

$$K_D = \frac{[E_f]\,[Na]}{[E \cdot Na]}$$

Initially, in the absence of inactivated enzyme, $E_f + E \cdot Na = 1.0$;

$$\text{thus } [E \cdot Na] = \frac{1}{1 + \frac{K_D}{[Na]}}$$

The rate of inactivation, v_{in}, equals $k_{in}[E_f]$, where k_{in} is the pseudo first-order rate constant measured in the absence of Na^+. Initially, and in the absence of Na^+, $v_{in} = k_{in}(1.0)$. In the presence of Na the observed rate of inactivation, v_{ob}, equals $k_{ob}(1.0 - [E_{in}])$, and initially $v_{ob} = k_{ob}(1.0)$, where k_{ob} is the pseudo first-order rate constant measured under these conditions. Since the initial rate of inactivation in the presence of Na may also be written as $v_{ob} = k_{in}(1.0 - [E \cdot Na])$, then $k_{ob} = k_{in}(1.0 - [E \cdot Na])$. Thus:

$$[E \cdot Na] = 1 - \frac{k_{ob}}{k_{in}} = \frac{1}{1 + \frac{K_D}{[Na]}}$$

This is formally equivalent to the Michaelis–Menten equation, so that plots of $(1 - k_{ob}/k_{in})^{-1}$ against $[Na]^{-1}$ will give $-K_D$ at the intercept with the $[Na]^{-1}$ axis.

When the pseudo first-order rate constants from experiments such as that in fig. 1 are plotted against [Na] in this fashion, a K_D for Na^+ of 2.3 mM was found (fig. 2; table 1). This value is close to the concentration for half-maximal activation of the ATPase at low K^+-concentrations [7] and of the Na^+ plus nucleotide stimulation of K^+-dependent phosphatase activity of the ATPase [8]. Unlike conventional kinetic studies, where product formation is measured, this approach can be used under various ligand states including absence of substrate.

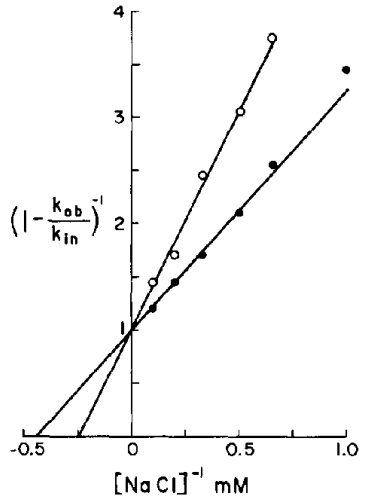

Fig. 2. Effects of NaCl on inactivation by DCCD. Experiments were performed as in fig. 1, with the pseudo first-order rate constants calculated from plots such as fig. 1B. The inactivating incubations ('preincubations') were in 30 mM histidine HCl–Tris (pH 7.8), 0.2 mM DCCD and the concentrations of NaCl indicated, in the absence (•) or presence (○) of 1 mM $MgCl_2$. Data are plotted as described in the text.

As indicators that the Na^+-site modifying inactivation was related to the Na^+-site activating the ATPase the effects of phlorizin and dimethylsulfoxide (DMSO) were examined. Phlorizin decreases apparent Na^+-affinity while increasing K^+-affinity [4, 5], whereas DMSO has little effect on Na^+-affinity while increasing K^+-affinity [4, 9]. Here phlorizin significantly increased the K_D for Na^+, while DMSO had little effect (table 1).

Adding 1 mM $MgCl_2$ nearly doubled the K_D for Na^+ (fig. 2; table 1); this may simply reflect competition between Mg^{2+} and Na^+ for the Na^+-site [8, 10]. However, Mg^{2+} is a major determinant of alternative conformational states of the ATPase [3, 10], and it has been proposed that the enzyme state selected by Mg^{2+}, E_2, has a lower affinity for Na^+ [11]. Oligomycin, also a major selector of conformational states, favoring E_1 [3, 12], had little effect on the K_D in the absence of Mg^{2+} (table 1).

Free ATP nearly halved the K_D for Na^+ (table 1); this was not simply due to removing endogenous Mg^{2+} since EDTA was ineffective. In the presence of both Mg^{2+} and Na^+, ATP phosphorylates the enzyme

Table 1
Dissociation constants for Na^+ in the presence of various reactants.

Addition	Concentration	K_D for Na^+ (mM)[a]
None	–	2.3 ± 0.3 (8)
Phlorizin	0.3	3.7 ± 0.3[b] (6)
Dimethylsulfoxide	(10%, v/v)	2.0 ± 0.3 (5)
$MgCl_2$	1.0	3.9 ± 0.4[b] (6)
Oligomycin	(2 μg/ml)	1.9 ± 0.2 (5)
ATP	1.0	1.4 ± 0.2[b] (6)
EDTA	0.4	2.2 ± 0.3 (5)
ATP + $MgCl_2$	1.0 and 1.0	2.0 ± 0.3 (5)
ATP + EDTA	1.0 and 0.4	2.1 ± 0.3 (6)

[a] Data are presented ± standard deviation for the number of experiments indicated in parenthesis.
[b] Significantly different from control ($P < 0.05$).

[3], but under these conditions the K_D for Na^+ was not significantly changed from its value in the absence of these ligands. With 1 mM ATP and $MgCl_2$ the concentration of free Mg^{2+} is only 0.1 mM.

By contrast, in the presence of a phosphorylating nucleotide, Mg^{2+}, and Na^+ the apparent affinity for K^+ increases 20-fold, measured either by the Be^{2+}-inactivation technique [4] or by the phosphatase activity [8]. Thus while the affinity for K^+ increases markedly during the reaction cycle the affinity for Na^+ is little changed. This is perhaps reasonable in light of the usual formulation of the reaction sequence [3] whereby the enzyme is first phosphorylated in a Na^+- and Mg^{2+}-dependent step, followed by a K^+-dependent dephosphorylation (with high K^+-affinity following phosphorylation). In contrast to schemes proposing cyclical conversion of Na^+- and K^+-sites with the reaction sequence [1–3] the continued existence of Na^+- and K^+-sites throughout the ligand states supports formulations of separate sites for these ions [1, 4, 7, 8, 13].

If the observed sites reflect the transport sites for Na^+ it is clear that the dissociation constant, under any of the ligand conditions, is inadequate to discharge Na^+ into the extracellular fluid (approx. 135 mM Na^+ and 5 mM K^+). As with the K^+-sites demonstrated in Be^{2+} inactivation experiments there was no evidence of a major decrease in affinity: either the decrease is so transient that it cannot be approximated by the ligand states used, or, as previously

proposed, the continuing existence of the moderate affinity sites may represent merely the affinity at one face of a transport channel or pore (thus obscuring efforts to demonstrate a low affinity site). Transport may occur by a gate mechanism such that throughout the cycle sites of moderate to high affinity exist at one face, while low affinity discharge sites exist across the diffusion barrier at the opposite face.

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