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POTASSIUM BINDING TO THE (Na^+ + K^+)-ATPase

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Summary

The number of K^+ bound to the (Na^+ + K^+)-ATPase has been measured under equilibrium conditions by a differential-titration technique (Hastings, D.F. (1977) Anal. Biochem. 83, 416—432). 5.1 K^+ were bound per ^{32}P-labelling site. The K'_D for K^+ was dependent on the concentration of choline, which was included to give ionic strength. K'_D was 59 ± 2.5 μM with 97 mM choline, 26 ± 1.9 μM with 30 mM choline. The K^+ : choline selectivity was 2564 : 1 and the calculated K'_D for K^+ with zero choline was 11 μM and for choline with zero K^+ was 28 mM. 20 μM ATP in the presence of 97 mM choline increased the K'_D for potassium 3-fold to 177 ± 14 μM. The K'_D for K^+ with 3 mM Na^+ in the presence of 27 mM choline was 81 ± 10 μM and with 30 mM Na^+ without choline 700 ± 250 μM. The calculated K'_D for Na^+ at zero K^+ and zero choline was 0.6 ± 0.2 mM. The K^+ : Na^+ selectivity was 54 : 1.

Introduction

In a previous paper, a differential potentiometric titration method has been described which allows a titration of high-affinity K^+ sites on the (Na^+ + K^+)-ATPase [1]. In the present paper, the method has been used to titrate the number of K^+ bound to the (Na^+ + K^+)-ATPase under equilibrium conditions.

Methods

The enzyme source was salt glands from the spiny dogfish, *Squalas acanthias*. The microsomes were prepared as described previously [2] with the following modifications. The supernatant after the 5900 × *g* centrifugation was centri-

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fuged at 150 000 × *g* for 45 min at 0°C. The pellet was washed three times in K^+-free titration buffer: 22% glycerol (w/v), 97 mM choline chloride, 30 mM histidine, 0.5 mM EDTA, pH 7.3, measured at 20°C, and twice in titration buffer containing 10 μM KCl by repeated centrifugation: 150 000 × *g*, 45 min, 15°C. Final K^+ content was determined by flame photometry.

K^+ binding was measured by differential titration [1] using K^+-selective valinomycin electrodes (Radiometer, Copenhagen). The reference cell contained an identical suspension of enzyme which had been inactivated by incubation at 65°C for 5 min. K^+ binding to the membrane phospholipids and protein which was not heat-labile occurred in both the sample and reference vessels to the same degree and did not influence the titration. The precision of the technique is best at moderate K^+ concentrations and decreases in a predictable manner at high and low concentrations of K^+. The titrations were performed at 22°C, pH 7.3.

A weighted nonlinear-regression routine was used to determine the binding constants [3]. For further information about errors and calculations see Ref. 1.

Protein was measured by using the method of Lowry et al. [4].

($Na^+ + K^+$)-ATPase activity was determined by measuring the inorganic phosphate released by the enzyme during a 5 min incubation period in the presence and absence of 1 mM ouabain. The reaction medium contained: 130 mM NaCl, 20 mM KCl, 4 mM $MgCl_2$, 3 mM ATP, 0.2 mM EGTA, 1 mM phospho*enol*pyruvate, 30 mM histidine, 330 μg/ml bovine serum albumin, 50 μg/ml pyruvate kinase, pH 7.4, at 37°C.

Enzyme phosphorylation using [γ-^{32}P]ATP was measured as described by Skou and Hilberg [5].

Results

The specific activity of the preparation used was 1330 ± 80 μmol P_i/mg protein per h (Table I). The ATPase : *p*-nitrophenylphosphatase ratio measured under the optimum conditions was 6.9. The site number measured by ^{32}P incorporation from [^{32}P]ATP under conditions specific for the ($Na^+ + K^+$)-ATPase reaction was 1.7 ± 0.1 nmol/mg protein.

Fig. 1 shows a Scatchard plot of the K^+ binding at two ionic strengths and choline chloride concentrations (I = 0.1 and 0.33; choline concentration 97 and 30 mM). In Table I the results of five different experiments with each of the two choline concentrations are given. There are 7.9 ± 0.2 nmol K^+ sites per mg protein, and with an apparent affinity of 17 ± 1 mM^{-1} (K'_D 59 μM) at the high choline concentration. The 2.1-fold increase in affinity when the choline concentration is decreased is larger than would be expected from the 1.06-fold increase in the K^+ activity coefficient calculated by Debye-Hückel theory.

The change in affinity could be due to an ionic-strength effect on the enzyme or to a competition between K^+ and choline. Assuming that is due to a simple competition, an estimate of the selectivity of the K^+ sites can be obtained by solving the binding function (Eqn. 1), at two choline concentrations, for K_a, the association constant for K^+, and the selectivity S (Eqn. 2). The selectivity is defined as the ratio of the choline affinity to the K^+ affinity.

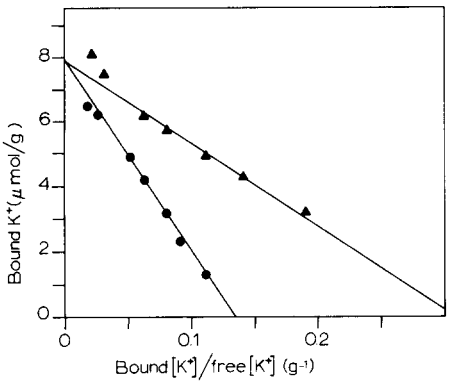

Fig. 1. The effect of the organic cation choline on K^+ binding. K^+ binding was measured by a differential-titration technique with 97 mM choline (●———●) or 30 mM choline (▲———▲) as the ionic strength agent. The protein concentrations were 2.52 and 2.58 g/l, respectively. The abscissa shows bound K^+/free K^+ and the ordinate bound K^+, a Scatchard plot. For the high choline concentration, maximum bound K^+ was 7.9 ± 0.2 μmol/g (n = 5). For the low choline concentration, the maximum bound K^+ was 7.8 ± 0.2 μmol/g (n = 5) and the dissociation constant was 25.5 ± 1.9 μM (n = 5), 22°C, pH 7.3.

Choline, the competing ion, is represented by the symbol M^+.

$$\frac{1}{\text{Apparent } K^+ \text{ affinity}} = \frac{1}{K_a} + S(M^+) \quad (1)$$

$$S = \Delta(\text{Apparent affinity})^{-1}/\Delta(M^+) \quad (2)$$

The calculation gives a K^+ affinity of 93 mM^{-1} (K'_D = 11 μM) at zero choline and a choline : K^+ selectivity ratio of $3.9 \cdot 10^{-4}$ (Table I). Even though the selectivity ratio is very small, millimolar concentrations of the organic cation would decrease the apparent affinity of the sites for K^+.

The effect of Na^+ on the binding was measured in a similar series of experiments where a portion of the 0.03 M choline chloride was replaced by NaCl.

TABLE I

Enzyme activity (μmol P_i/mg protein per h 1330 ± 80) (S.E., n = 13). Number of experiments (n) is given in parentheses.

	K^+ binding (nmol/mg protein)	$K^+/^{32}P$ site	K'_D (μM)
Choline (97 mM)	7.9 ± 0.2 (5)	4.6	59 ± 2.5 (5)
Choline (30 mM)	7.8 ± 0.2 (5)	4.6	26 ± 1.9 (5)
Choline * (0 mM)			11
Na^+ (3 mM), choline (27 mM)	9.6 ± 1.7 (3)	5.2	81 ± 10 (7)
Na^+ (30 mM), choline (0 mM)	9.6 ± 1.7 (3)	5.6	700 ± 250 (3)
Choline (97 mM), Tris (100 μM)	8.7 ± 0.1 (5)	5.1	46 ± 2 (5)
Choline (97 mM), Tris-ATP (20 μM)	9.2 ± 0.6 (6)	5.4	177 ± 14 (6)
	mean	5.1	
Calculated K^+:choline selectivity	2564:1		
Calculated K^+:Na^+ selectivity	54:1		
Calculated K'_D for Na^+ 0.6 ± 0.2 mM			

* Calculated from a K^+:choline selectivity of 2564:1.

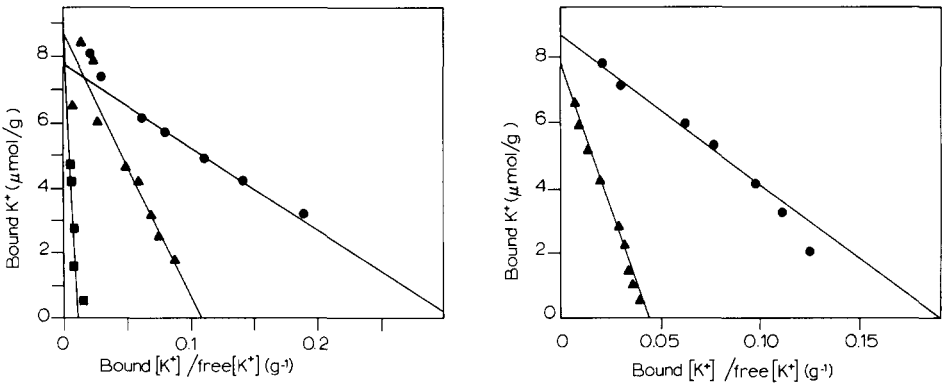

Fig. 2. The effect of Na^+ on K^+ binding. K^+ binding was determined in the presence of 0, 3, or 30 mM NaCl which replaced the same concentration of the 30 mM choline chloride in the medium. The abscissa shows bound K^+/free K^+ and the ordinate bound K^+, a Scatchard plot. With zero Na^+ (●———●) the dissociation constant was 26 ± 1.9 μM (n = 5) and the site concentration was 7.5 ± 0.2 μmol/g (n = 5). With 3 mM Na^+ (▲———▲) the dissociation constant increased to 81 ± 10 μM (n = 7). With 30 mM Na^+ (■———■) the dissociation constant increased to 700 ± 250 μM (n = 3) and the site concentration to 9.6 ± 1.7 μmol/g (n = 3), 22°C, pH 7.3.

Fig. 3. The effect of 20 μM ATP on K^+ binding. K^+ binding was determined in the presence of 100 μM Tris chloride (●———●) or in the presence of 20 μM Tris-ATP (▲———▲), pH 7.4. The concentration represents final values, the standard 97 mM choline chloride titration medium was diluted 3%. The abscissa shows bound K^+/free K^+ and the ordinate bound K^+, a Scatchard plot. In the absence of nucleotide the dissociation constant was 46 ± 2 μM (n = 5) and the site concentration was 8.7 ± 0.1 μmol/g (n = 5). In the presence of nucleotide the dissociation constant was 177 ± 14 μM and the site concentration was 9.2 ± 0.6 μmol/g (n = 6), 22°C, pH 7.3.

The ionic strength was unchanged. Fig. 2 and Table I show the results. The presence of Na^+ decreased the apparent affinity of the sites for K^+ from 38.5 ± 1.5 mM^{-1} (K'_D 26 μM) in the absence of Na^+ to 12.3 ± 1.5 mM^{-1} (K'_D 81 μM) in the presence of 3 mM Na^+, and to 1.4 ± 0.4 mM^{-1} (K'_D 700 μM) in the presence of 30 mM Na^+. There was no significant change in the specific-site concentration. Assuming simple competition between K^+, Na^+ and choline and using the calculated choline and K^+ affinities, the K'_D value for Na^+ was calculated to be 0.6 ± 0.2 mM. The K^+ : Na^+ selectivity was 54 : 1.

To verify that the high-affinity K^+ sites are associated with the (Na^+ + K^+)-ATPase, the titration was repeated in the presence of 20 μM ATP (Tris salt). Nørby and Jensen [6] observed that 7.5 mM K^+ decreases the affinity of the enzyme for ATP 5.8-fold. A similar decrease in the K^+ affinity would be observed if the two binding phenomena were related. Upon addition of ATP (Fig. 3), the K^+ affinity decreases from 21.8 ± 1.0 mM^{-1} (K'_D 46 μM) to 5.7 ± 0.5 mM^{-1} (K'_D 177 μM), a 3.8-fold decrease. The specific-site concentration remained unchanged.

The mean of the experiments given in Table I was 5.1 K^+ bound per ^{32}P-labelling site.

Discussion

The K^+-binding sites are measured from a difference in binding of K^+ to an active and to a heat-denatured inactivated system, i.e., the K^+ sites which are

measured are connected to the activity of the enzyme system and require an organized structure.

Furthermore, the high affinity for K^+, the decrease in affinity when ATP is added and a $K^+ : Na^+$ selectivity of 54 support the observation that it is a specific binding of K^+ to the $(Na^+ + K^+)$-ATPase.

Using a centrifugation technique, Matsui et al. [7] found that with 0.2 mM K^+ there were 16.4 nmol K^+ bound per mg $(Na^+ + K^+)$-ATPase with 2.5 nmol ouabain-binding sites, i.e., 6.6 K^+ bound per ouabain-binding site. In these experiments, there was no correction for K^+ bound to inactive enzyme or for the K^+ in the water phase of the pellet. Half of the 16.4 nmol K^+ bound disappeared when 5 mM Mg^{2+} was added and another 4 nmol when, besides Mg^{2+}, ouabain was added. There was thus an ouabain-sensitive K^+ binding of 1.7 K^+ per ouabain site. In another set of experiments, it was found that the binding of the two ouabain-sensitive K^+ gave non linearity in a Scatchard plot; the apparent K'_D value was approx. 50 μM.

In experiments with a forced-dialysis procedure, Cantley et al. [8] found two Rb^+ bound per ouabain-binding site with an affinity of 770 μM.

Yamaguchi and Tonomura [9], using a membrane-filtration technique, found two kinds of cation-binding sites on the $(Na^+ + K^+)$-ATPase: about three sites with high affinity for Na^+ as well as K^+ (K'_D for Na^+ about 0.2 mM); about three sites with a high affinity for K^+ (K'_D less than 0.2 mM), one of which had a high affinity for Na^+ and was apparently one of the above three Na^+ sites, the other two had a low affinity for Na^+ (the K : Na selectivity was 62 : 1). Besides this, there were a number (ten or more, see Figs. 12 and 18 in Ref. 9) with a moderate affinity for K^+ which saturates with 1.5—2 mM K^+. From this they concluded that there were five specific cation-binding sites on the system, three Na^+ sites and two K^+ sites.

The apparent affinity for K^+ observed in the present experiments was 59 μM with 97 mM choline and 26 μM with 30 mM choline and was calculated to be 11 μM without choline. It is of the same order as that of 50 μM found by Matsui et al. [7] and less than the value of 0.2 mM found by Yamaguchi and Tonomura [9] and it agrees with the value of 87 μM calculated by Nørby and Jensen [6] from the effect of K^+ on binding of ATP.

The apparent affinities for K^+ observed in the binding experiments also agree with values obtained by titration with potassium using changes in fluorescence as a tool: 0.05 mM in the presence of 100 mM Tris at pH 7.0 [10] and 0.12 mM in the presence of 150 mM choline, pH 7.2 [11], using intrinsic fluorescence, and at pH 7.2 with 30 mM histidine a $K_{0.5}$ for K^+ of 0.15 mM in the presence of 150 mM choline, 0.08 mM with 50 mM choline and 0.024 mM without choline using the change in fluorescence of eosin maleimide-labelled enzyme [11].

The number of high-affinity sites for K^+ (about 5) is, however, higher than the two observed by Matsui et al. [7] and the three observed by Yamaguchi and Tonomura [9], two of which had a $K^+ : Na^+$ selectivity of 62 : 1 equal to the 54 : 1 found in the present paper.

Previous experiments [2] showed that the enzyme preparation contains about 2.5 α-chains per ^{32}P-labelling site and not 2 as suggested from molecular weight determination [12,13]. The disrepancy may be due to polypeptide

impurities. But, if it is due to underestimation of the number of enzyme molecules from the ^{32}P-labelling procedure and if the 'extra' molecules are active from the point of view of K^+ binding, the number of K^+ bound per enzyme molecule is about 20% lower than 5.1, i.e., about 4.

There is a certain deviation from the linearity in the upper part of Scatchard plots shown in Figs. 1 and 2. This is in the K^+ concentration range where the method becomes uncertain, and it is not possible to tell whether this is due to another class of K^+-binding sites, the K^+ sites with the moderate affinity observed by Yamaguchi and Tonomura [9]. But, if it does, the resolution of the Scatchard plot will give a line for the high-affinity binding sites which intercepts with the ordinate at a lower value than that shown in the figures.

They may thus be two errors in the present experiments, the correction of which may decrease the number of high-affinity binding sites for K^+ and bring them closer to the results obtained by Matsui et al. [7] and by Yamaguchi and Tonomura [9].

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References

1 Hastings, D.F. (1977) Anal. Biochem. 83, 416—432
2 Skou, J.C. and Esmann, M. (1979) Biochim. Biophys. Acta 567, 436—444
3 Chandler, J.P. (1971) STEPIT, Program 66, Quantum Chemistry Program Exchange, Indiana University Chemistry Dept., Bloomington, IN 47401, U.S.A.
4 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
5 Skou, J.C. and Hilberg, C. (1969) Biochim. Biophys. Acta 195, 198—219
6 Nørby, J.G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104—116
7 Matsui, H., Hayashi, Y., Homareda, H. and Kimimura, M. (1977) Biochem. Biophys. Res. Commun. 75, 373—380
8 Cantley, L.C., Jr., Cantley, L.C. and Josephson, L. (1978) J. Biol. Chem. 253, 7361—7368
9 Yamaguchi, M. and Tonomura, Y. (1979) J. Biochem. 86, 509—523
10 Karlish, S.J.D. and Yates, D.W. (1978) Biochim. Biophys. Acta 525, 115—130
11 Skou, J.C. and Esmann, M. (1980) Biochim. Biophys. Acta 601, 386—402
12 Hastings, D.F. and Reynolds, J.A. (1979) Biochemistry 18, 817—821
13 Esmann, M., Skou, J.C. and Christiansen, C. (1979) Biochim. Biophys. Acta 567, 410—420