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Vanadate Binding to the (Na + K)-ATPase

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Abstract

A particulate (Na + K)-ATPase preparation from dog kidney bound [^{48}V]-ortho-vanadate rapidly at 37°C through a divalent cation-dependent process. In the presence of 3 mM $MgCl_2$ the K_d was 96 nM; substituting $MnCl_2$ decreased the K_d to 12 nM but the maximal binding remained the same, 2.8 nmol per mg protein, consistent with 1 mol vanadate per functional enzyme complex. Adding KCl in the presence of $MgCl_2$ increased binding, with a $K_{0.5}$ for KCl near 0.5 mM; the increased binding was associated with a drop in K_d for vanadate to 11 nM but with no change in maximal binding. Adding NaCl in the presence of $MgCl_2$ decreased binding markedly, with an I_{50} for NaCl of 7 mM. However, in the presence of $MnCl_2$ neither KCl nor NaCl affected vanadate binding appreciably. Both the nonhydrolyzable, β,γ-imido analog of ATP and nitrophenyl phosphate, a substrate for the K-phosphatase reaction that this enzyme also catalyzes, decreased vanadate binding at concentrations consistent with their acting at the low-affinity substrate site of the enzyme; the presence of KCl increased the concentration of each required to decrease vanadate binding. Oligomycin decreased vanadate binding in the presence of $MgCl_2$, whereas dimethyl sulfoxide and ouabain increased it. With inside-out membrane vesicles from red blood cells vanadate inhibited both the K-phosphatase and (Na + K)-ATPase reactions; however, with the K-phosphatase reaction extravesicular K^+ (corresponding to intracellular K^+) both stimulated catalysis and augmented vanadate inhibition, whereas with the (Na + K)-ATPase reaction intravesicular K^+ (corresponding to extracellular K^+) both stimulated catalysis and augmented vanadate binding.

Key Words: (Na + K)-ATPase; vanadate binding; ligand effects; inside-out membrane vesicles.

Introduction

Kinetic studies directed toward deciphering the complex reaction process of the (Na + K)-ATPase, the enzymatic representation of the plasma

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membrane sodium pump, have revealed a multitude of interacting classes of sites for the physiological ligands, Na, K, Mg, and ATP, and some of these classes of sites have also been demonstrated in binding experiments (Robinson and Flashner, 1979; Beauge' and Glynn, 1979; Yamaguchi and Tonomura, 1980a, 1980b). Studies using specific inhibitors have also assisted efforts to depict the reaction process, and a recently discovered inhibitor, orthovanadate, has proved to be a particularly interesting and mechanistically revealing reagent (Cantley *et al.*, 1978; Beauge', 1979; Bond and Hudgins, 1979; Beauge' *et al.*, 1980; Smith *et al.*, 1980). But despite these detailed kinetic studies of enzyme inhibition by vanadate, a number of uncertainties remain, including whether or not K^+ is required for vanadate to react with the enzyme (Bond and Hudgins, 1979; Beauge' *et al.*, 1980), the nature of the sites through which Na^+ antagonizes vanadate inhibition and their relation to the K^+-sites (Bond and Hudgins, 1979; Beauge' *et al.*, 1980; Smith *et al.*, 1980), and the interrelationship between these sites, enzyme conformations, and substrate binding (Cantley *et al.*, 1978; Bond and Hudgins, 1979; Beauge' *et al.*, 1980).

The experiments described here were designed to extend the initial brief account of vanadate binding (Cantley *et al.*, 1978) to approach these issues. Measurement of vanadate binding to the purified enzyme permits determination of ligand effects in the absence of the full complement of ligands required for kinetic studies of enzymatic activity and thus allows direct examination of the effects of these ligands individually as well as in combination. These data indicate roles for the ligands in vanadate binding corresponding to particular aspects of their roles in the reaction process of the (Na + K)-ATPase.

Materials and Methods

The enzyme preparation was obtained from the medullae of frozen canine kidneys by a modification (Liang and Winter, 1976) of the procedure of Jorgensen (1974). The specific activity of the (Na + K)-ATPase reaction ranged from 13 to 19 μmol P_i liberated/mg protein/min. Vanadate binding was routinely assayed by incubation for 15 min at 37°C in the standard medium, which contained 30 mM histidine · HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 50 nM [^{48}V]-ortho vanadate, and 0.5–0.8 μg enzyme protein/ml of medium. Vanadate was added as the ammonium salt; there was no added Na^+ or K^+ in the standard incubation medium. The incubation was terminated by filtration of the mixture through Gelman Metricel membrane filters (0.45 μm pore size) and each filter washed twice with 1.0 ml of ice-cold 0.3 mM histidine · HCl/Tris (pH 7.8); filtration and washing were completed within 15 sec. The filters were air dried and the radioactivity remaining on them measured by liquid scintillation counting. Specific binding was taken as

the difference in ^{48}V content between that in incubations under conditions analogous to those described above and that in corresponding incubations to which was added 1 mM unlabeled vanadate.

Inside-out red blood cell vesicles were prepared by a modification (Mercer and Dunham, 1981) of the method of Steck (1974). Fresh, washed human cells were lysed by suspending in 40 volumes of 5 mM Tris · HCl (pH 8.0)/1 mM EGTA for 10 min at 0°C; the mixture was centrifuged at 30,000 *g* and the resulting pellet then suspended in 10 volumes of water for 10 min at 0°C, when 20 volumes of 5 mM Tris · HCl (pH 8.0)/1 mM EGTA was added and the mixture centrifuged again. This suspension and centrifugation was repeated, and the pellet then suspended in 0.5 mM Tris · HCl (pH 8.0)/1 mM EGTA for 1 hr at 0°C before centrifugation again. The pellet was next homogenized by passing five times through a 1-inch 27 gauge needle, and 2 ml of the homogenate layered on 3 ml of Dextran T-70 (1.115 g in 25 ml of 0.5 mM Tris · HCl (pH 7.8)/1 mM EGTA), which was then centrifuged for 40 min at 30,000 *g*. The vesicles at the interface were washed by suspending and centrifuging in 10, 20, and 40 mM Tris · glycylglycine (pH 7.4)/0.1 mM $MgCl_2$, and then stored, for up to 4 days at 0–4°C, in 3 mM Tris · glycylglycine/25 mM choline chloride. To adjust the intravesicular contents, the vesicles were incubated for 24–48 hr at 0–4°C, in one-ninth volume of either (i) 0.1 M choline chloride, (ii) 0.1 M KCl, (iii) 0.09 M choline chloride/0.01 M KCl, or (iv) 0.1 M NaCl, all containing in addition 3 mM Tris · glycylglycine (pH 7.4); these vesicles are thus considered to contain, respectively, (i) choline chloride, (ii) 10 mM KCl, (iii) 1 mM KCl, and (iv) 10 mM NaCl.

To assay K-phosphatase activity the vesicles were first incubated for 10 min at 37°C in 19 volumes of media (final concentration of vesicle protein: 0.6 mg/ml) containing 10 mM histidine · HCl/Tris (pH 7.4), 3 mM $MgCl_2$, and 10 mM chloride salt of choline, K^+, and/or Na^+, both in the absence and presence of 10 nM vanadate; the assay incubation was initiated by adding nitrophenyl phosphate (as the Tris salt) to a final concentration of 3 mM and the incubation continued for 20 min at 37°C before termination by trichloroacetic acid. Phosphatase activity was determined spectrophotometrically in terms of nitrophenol production (Robinson, 1969). K-phosphatase activity is considered as the difference between corresponding incubations in the absence and presence of 20 μM strophanthidin; the specific activity averaged 1.3 nmol nitrophenol liberated/mg protein/min. (Na + K)-ATPase activity was assayed similarly, in the absence and presence of vanadate and of strophanthidin, except that 3 mM ATP (as the Tris salt) was added to initiate the assay incubation, and the reaction was measured in terms of P_i production (Robinson, 1967); the specific activity averaged 3.7 nmol P_i liberated/mg protein/min.

Values presented are means of four or more experiments, each

performed in duplicate or triplicate, with the standard error of the mean where appropriate.

Frozen canine kidneys were obtained from Pel Freeze; ATP (vanadium-free), nitrophenyl phosphate, oligomycin, ouabain, and strophanthidin from Sigma; AMP-PNP from ICN; and ammonium ortho-vanadate from Fisher. [^{48}V]-Vanadyl chloride was obtained from Amersham and converted to ortho-vanadate as described by Cantley *et al.* (1978).

Results

[^{48}V]-Ortho vanadate bound tightly to the particulate (Na + K)-ATPase preparation during incubation at 37°C in the presence of 3 mM $MgCl_2$, such that after filtration of the reaction mixture and two washes with ice-cold buffer (filtration and washing accomplished within 15 sec) two subsequent washes failed to remove additional radioactivity. This stability is thus in accord with a half-life of the enzyme–vanadate complex of 210 sec at 37°C (Cantley *et al.*, 1978) and of 15 h at 4°C (Smith *et al.*, 1980). In the absence of added divalent cation, the binding of radioactivity was far less,

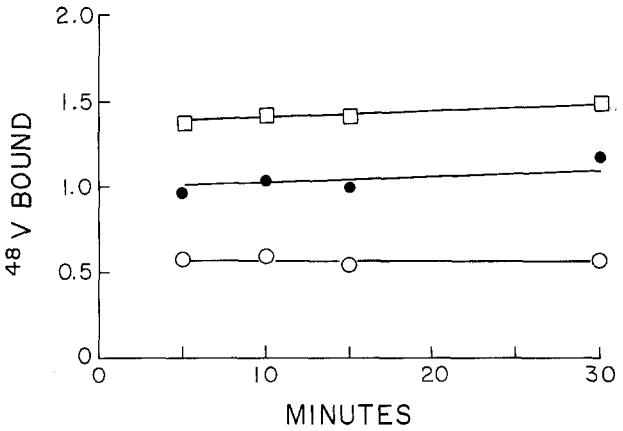

Fig. 1. Time course of [^{48}V]-vanadate binding to the (Na + K)-ATPase. The enzyme was incubated with 50 nM [^{48}V]-ortho-vanadate for the times indicated, either in the standard medium containing 30 mM histidine · HCl/Tris (pH 7.8) and 3 mM $MgCl_2$ (●), or that medium containing in addition 10% (v/v) dimethyl sulfoxide (□) or 10 μg/ml oligomycin (○). The incubation was terminated by filtration and the radioactivity retained with the enzyme counted, as described under Methods. Specific binding of ^{48}V is presented relative to that bound in the standard medium after incubation for 15 min, defined as 1.0.

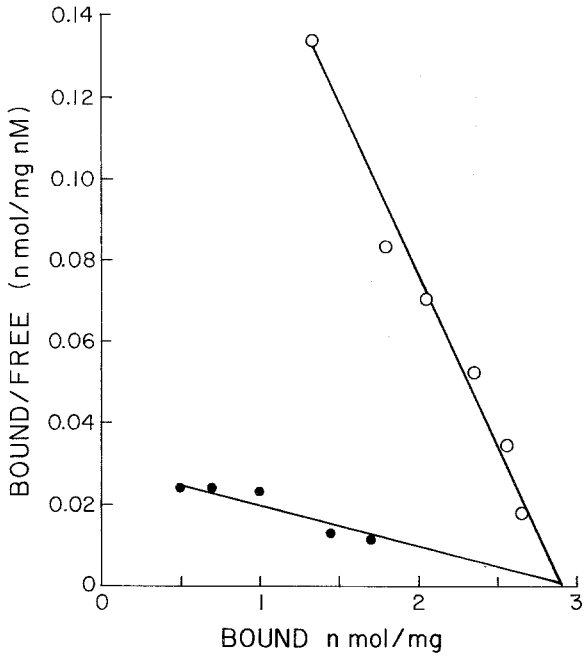

Fig. 2. Binding of [^{48}V]-vanadate as a function of concentration. The enzyme was incubated with a range of [^{48}V]-vanadate concentrations for 15 min at 37°C in either the standard medium containing 3 mM $MgCl_2$ (●) or in that medium in which 3 mM $MnCl_2$ (○) was substituted for $MgCl_2$. Data are presented in the form of a Scatchard plot.

about 10% of that in the standard medium; an equivalent amount of radioactivity was retained when the enzyme was incubated in the standard medium (containing 3 mM $MgCl_2$) to which was added 1 mM unlabeled vanadate. This low level of ^{48}V retention, in the absence of divalent cation or the presence of high concentrations of unlabeled vanadate, was thus considered to represent nonspecific binding, with specific binding defined here as the difference between that in the absence and presence of 1 mM unlabeled vanadate, measured concurrently in otherwise identical media.

With this experimental system, specific binding of [^{48}V]-vanadate to the enzyme in the standard medium occurred rapidly at 37° with little change during the period 5 to 30 min (Fig. 1); in subsequent studies incubations were for 15 min. The K_d for vanadate binding, in the presence of 3 mM $MgCl_2$, was 96 nM with a maximal binding of 2.9 nmol vanadate/mg protein (Fig. 2). This latter value is consistent with a molar ratio of 1 vanadate per functional enzyme complex, present in the preparation at 75–90% purity (Robinson, 1981).

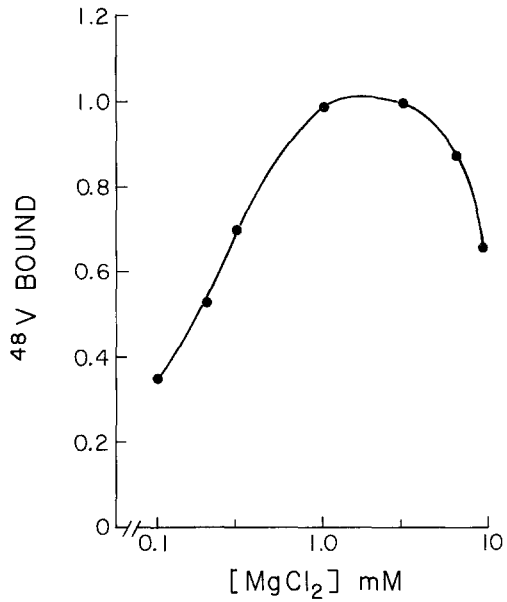

Fig. 3. Effect on $MgCl_2$ concentration on [^{48}V]-vanadate binding. The enzyme was incubated for 15 min at 37°C in the standard medium but with the concentrations of $MgCl_2$ indicated. Specific binding of [^{48}V]-vanadate is presented, as in Fig. 1, relative to that bound with 3 mM $MgCl_2$, defined as 1.0.

The optimal $MgCl_2$ concentration was in the range 1–3 mM (Fig. 3), with a $K_{0.5}$ of 0.25 mM (estimated from a replot of these data).

Substituting 3 mM $MnCl_2$ for 3 mM $MgCl_2$ increased vanadate binding (Table I). The magnitude of this increase, however, varied with the vanadate concentration, reflecting a decrease in K_d to 12 nM (Fig. 2). The maximal

Table I. [^{48}V]-Vanadate Binding to the (Na + K)-ATPase[a]

Ligands added	[^{48}V]-Vanadate binding		
	With no divalent cation	With 3 mM $MgCl_2$	With 3 mM $MnCl_2$
None	0.01 ± 0.01	1.00	2.49 ± 0.13
10 mM KCl	0.04 ± 0.01	2.40 ± 0.10	2.56 ± 0.14
10 mM NaCl	0.02 ± 0.02	0.43 ± 0.05	2.54 ± 0.11
10% Dimethylsulfoxide	0.00 ± 0.02	1.41 ± 0.08	2.51 ± 0.14
0.1 mM ouabain	0.04 ± 0.02	2.92 ± 0.16	2.62 ± 0.14
10 μg/ml oligomycin	—	0.56 ± 0.06	—

[a]Specific binding of [^{48}V]-vanadate was measured after incubation with the enzyme for 15 min at 37°C, as described under Methods. The results are presented relative to that with 3 mM $MgCl_2$ defined as 1.00.

binding was the same with either 3 mM $MgCl_2$ or $MnCl_2$ (Fig. 2). On the other hand, $CaCl_2$ was less effective, so that when 3 mM $CaCl_2$ was substituted the specific uptake was only one-fifth that with 3 mM $MgCl_2$.

Adding KCl to the incubation medium in the absence of $MgCl_2$ had no effect on vanadate binding but in the presence of 3 mM $MgCl_2$ markedly increased the specific binding (Table I), as expected from previous studies on inhibition of the enzyme by vanadate (Cantley *et al.*, 1978; Beauge', 1979; Bond and Hudgins, 1979). The optimal KCl concentration was in the range 3–10 mM (Fig. 4), with a $K_{0.5}$ of 0.5 mM (estimated from a replot of the data). This value is near the $K_{0.5}$ for K^+ as activator of the K-phosphatase reaction (Robinson, 1969; 1975a; 1981) that this enzyme also catalyzes. A replot in double-reciprocal form of the data from 0.1 to 3.0 mM KCl was concave upward, consistent with a positive cooperative interaction between K^+ sites, as also seen in the K-phosphatase reaction (Robinson, 1969).

The increased binding in these experiments with KCl did not represent a

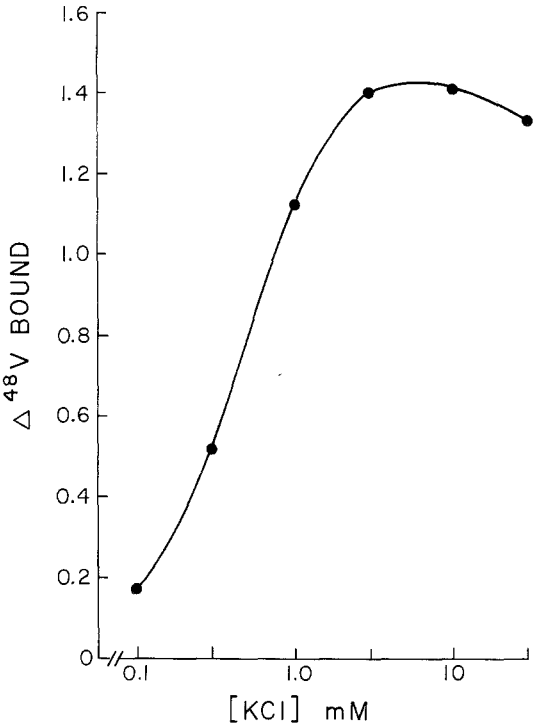

Fig. 4. Increase in [^{48}V]-vanadate binding with KCl. The enzyme was incubated for 15 min at 37°C in the standard medium to which was added the KCl concentrations indicated. The increments in ^{48}V-binding, Δ^{48}V, are presented relative to that bound in the standard medium (without KCl), defined as 1.0.

greater maximal binding, but rather a change in K_d for vandate binding, which in the presence of 10 mM KCl decreased to 11 nM (data not presented). On the other hand, in the presence of 3 mM $MnCl_2$ such addition of KCl had little effect on the vanadate binding (Table I), in accord with the already low K_d in the presence of this divalent cation (Fig. 2). Even with 10 nM vanadate, however, the addition of 10 mM KCl did not increase vanadate binding in the presence of 3 mM $MnCl_2$ (Robinson, 1981).

Substituting NaCl for KCl, in the presence of 3 mM $MgCl_2$, decreased vanadate binding (Table I), again as expected from its antagonism to enzyme inhibition by vanadate (Cantley *et al.*, 1978; Beauge', 1979; Bond and Hudgins, 1979). The concentration of NaCl required for 50% inhibition was independent of the vanadate concentration and averaged 7 mM (data not presented), near the K_i for NaCl as an antagonist to KCl activating the K-phosphatase reaction (Robinson, 1969). In the presence of 3 mM $MnCl_2$, however, the addition of NaCl had little effect on vanadate binding (Table I). This was also true with 10 nM vanadate (Robinson, 1981).

LiCl resembled KCl more than NaCl in this system, increasing specific binding in the presence of 3 mM $MgCl_2$ by 16% at 10 mM and by 36% at 50 mM. Neither choline chloride nor Tris hydrochloride, in the range 10–50 mM, increased vanadate binding.

To examine the effects of nucleotides on vanadate binding the nonhydrolyzable β,γ-imido analog of ATP, AMP-PNP, was used to avoid possible problems due to hydrolysis, even in the presence of vanadate inhibition, of the low concentrations of nucleotide used. As in pilot experiments using ATP, the analog AMP-PNP decreased vanadate binding in the presence of 3 mM $MgCl_2$, with 50% inhibition occurring near 0.25 mM AMP-PNP (Fig. 5), a concentration near the K_i for AMP-PNP as a competitor toward ATP at the low-affinity substrate sites of the enzyme (Robinson, 1976a). When 10 mM KCl was present as well, the concentration of AMP-PNP required to inhibit vanadate binding by 50% was increased markedly, to 2 mM (data not presented); this observation is in accord with the effects of K^+ on the apparent affinity for nucleotides at the low-affinity substrate sites (Robinson, 1967; Robinson, 1975b). The AMP-PNP was used without further purification, and thus effects of possible contaminants in the material cannot be ruled out.

The substrate for the K-phosphatase reaction, nitrophenyl phosphate, also inhibited vanadate binding in the presence of 3 mM $MgCl_2$, with 50% inhibition occurring near 0.8 mM (Fig. 5). Again, addition of 10 mM KCl increased the concentration required for 50% inhibition, to 3 mM nitrophenyl phosphate (data not presented), in agreement with the K_m as substrate for the K-phosphatase reaction in the presence of 3 mM $MgCl_2$ and 10 mM KCl (Robinson, 1969).

Current formulations of the reaction sequence (Karlish *et al.*, 1978; Robinson and Flashner, 1979; Smith *et al.*, 1980) include transitions between

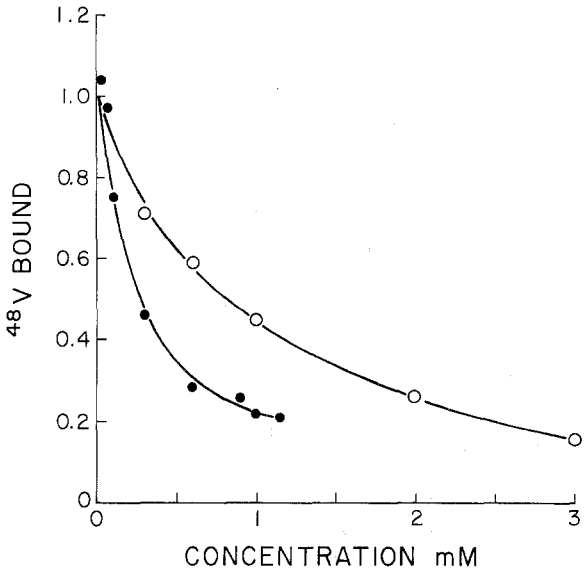

Fig. 5. Effect of AMP-PNP and nitrophenyl phosphate on [^{48}V]-vanadate binding. The enzyme preparation was incubated for 15 min at 37°C in the standard medium containing, in addition, the concentrations indicated of the β,γ-imido analog of ATP, AMP-PNP (●), or nitrophenyl phosphate (○). Specific binding of ^{48}V-vanadate is presented relative to that in the absence of these substances, defined as 1.0.

two major conformational states of the enzyme, E_1 and E_2, with an equilibrium between these states sensitive to the substrates and activators of the enzyme: ATP and Na^+ favoring the E_1 state and Mg^{2+} and K^+ favoring the E_2 state. Moreover, inhibition by vanadate may be attributed to its binding tightly to the E_2 state, mimicking phosphate as a leaving group from the enzyme aspartyl residue in the hydrolytic step of the reaction sequence (Cantley *et al.*, 1978). Consequently, agents that affect transition between the E_1 and E_2 states should then alter vanadate binding. Two such agents are oligomycin, which appears to favor the E_1 conformation, and dimethy sulfoxide, which appears to favor the E_2 conformation (Fahn *et al.*, 1966; Robinson, 1980a). In accord with these considerations, oligomycin decreased vanadate binding whereas dimethyl sulfoxide increased it (Table I); in both cases the effect was constant over the incubation time 5–30 min (Fig. 1). Ouabain binds to and stabilizes the E_2 state (Glynn and Karlish, 1975), and it too increased vanadate binding (Table I).

$MnCl_2$ appears to favor the E_2 conformational state even more strongly than does $MgCl_2$ (Robinson, 1981), and when oligomycin and dimethyl sulfoxide were tested in the presence of $MnCl_2$ neither altered the specific uptake of vanadate appreciably (Table I).

To determine on which face of the membrane-bound enzyme K^+ acted to stimulate inhibition of the (Na + K)-ATPase by vandate, initial experiments using red blood cell ghosts demonstrated that raising extracellular K^+ from 5 to 20 mM increased inhibition, whereas raising intracellular K^+ from 5 to 40 mM did not increase inhibition (Beauge' and Glynn, 1978). Nevertheless, the properties of the K^+ sites potentiating vandate binding resemble those of the K^+ sites activating the K-phosphatase reaction in apparent affinity for K^+ and Na^+ and in sensitivity to dimethyl sulfoxide (Robinson, 1975a), and these K^+ sites have recently been localized to the intracellular face of the membrane-bound enzyme (Blostein *et al.*, 1979). To re-examine the issue of sidedness, inside-out vesicles from red blood cell membranes were used because of the easier access of substrates to the active site (*in vivo* on the intracellular face). With the inside-out vesicles the K-phosphatase reaction was stimulated by extravesicular (i.e., intracellular) K^+ and not by intravesicular (i.e., extracellular) K^+ (Table II), confirming the report of Blostein *et al.* (1979). But in contrast to the experiments measuring (Na + K)-ATPase

Table II. Effect of Vanadate on K-Phosphatase Activity of Inside-Out Red Blood Cell Vessicles[a]

Intravesicular medium	Extravesicular medium	K-Phosphatase activity		Percent inhibition by vanadate
		Without vanadate	With 10 μM vanadate	
Choline Chloride without KCl or NaCl	10 mM KCl	100	37 ± 3	65
	2 mM KCl	52 ± 2	26 ± 2	50
	1 mM KCl	24 ± 3	16 ± 1	33
	no KCl	2 ± 1	—	—
With 10 mM KCl	10 mM KCl	102 ± 4	36 ± 2	65
	1 mM KCl	26 ± 2	17 ± 1	35
	no KCl	8 ± 2	—	—
With 10 mM NaCl	10 mM KCl	96 ± 3	35 ± 2	64

[a] K-Phosphatase activity of inside-out red blood cell vesicles was measured in the extravesicular medium indicated during incubations for 20 min at 37°C, after preincubation for 10 min in the absence or presence of 10 μM vanadate, as described under Methods. Three groups of vesicles were used: those equilibrated so that the intravesicular medium contained choline chloride without KCl or NaCl, those equilibrated with 10 mM KCl in place of an equivalent amount of choline chloride, and those equilibrated with 10 mM NaCl in place of an equivalent amount of choline chloride. Strophanthidin-sensitive K-phosphatase activity is presented relative to that of incubations in the presence of 10 mM KCl of vesicles equilibrated in the presence of choline chloride only, defined as 100.

Table III. Effect of Vanadate on (Na + K)-ATPase Activity of Inside-Out Red Blood Cell Vesicles[a]

Intravesicular medium	Extravesicular medium	(Na + K)-ATPase activity		Percent inhibition by vanadate
		Without vanadate	With 10 μM vanadate	
Choline chloride with 10 mM KCl	10 mM NaCl	100	39 ± 3	61
Choline chloride with 1 mM KCl	10 mM NaCl	84 ± 4	45 ± 2	46
Choline chloride without KCl	10 mM NaCl	1 ± 1	—	

[a](Na + K)-ATPase activity of inside-out red blood cell vesicles was measured in the presence of 10 mM extravesicular NaCl during incubations for 20 min at 37°C, after preincubation for 10 min in the absence or presence of 10 μM vanadate, as described under Methods. Three groups of vesicles were used: those equilibrated so that the intravesicular medium contained choline chloride without KCl, those equilibrated with 10 mM KCl in place of an equivalent amount of choline chloride, or those equilibrated with 1 mM KCl in place of an equivalent amount of choline chloride. Strophanthidin-sensitive (Na + K)-ATPase activity is presented relative to that of vesicles equilibrated with 10 mM KCl, defined as 100.

activity in red blood cell ghosts, extravesicular K^+ increased inhibition of the K-phosphatase reaction by vanadate (Table II). Intravesicular Na^+ did not measurably affect inhibition. On the other hand, when the sensitivity of the (Na + K)-ATPase reaction was measured using thse vesicles, then intravesicular K^+ increased inhibition by vanadate (Table III). Thus, when the (Na + K)-ATPase reaction was assayed, extracellular K^+ increased inhibition by vanadate, just as in experiments using red blood cell ghosts, whereas when the K-phosphatase reaction was assayed, intracellular K^+ increased inhibition. Attempts to measure [^{48}V]-vanadate binding to the (Na + K)-ATPase of the inside-out vesicles were frustrated by the much larger binding to other sites (Mercer, R. W., Dunham, P. B., and Robinson, J. D., unpublished observations).

Discussion

Five major points may be derived from these experiments on vanadate binding to the (Na + K)-ATPase in the context of the reaction scheme

$$E_1 \cdot ATP \xrightarrow[-ADP]{Na,Mg} E_1\text{-}P \rightarrow E_2\text{-}P \xrightarrow[-P_i]{K} E_2 \xrightarrow{ATP} E_2 \cdot ATP \rightarrow E_1 \cdot ATP$$

(i) Divalent cations are an absolute requirement for binding. The site at which Mg acts resembles in apparent affinity that at which the K-

phosphatase reaction is activated (Swann and Albers, 1978), nucleotide binding to the high-affinity substrate site is decreased (Robinson, 1980b), and the ADP/ATP exchange inhibited (Robinson, 1976b); studies on antagonism to Mn^{2+} binding, measured by electron paramagnetic resonance, indicate one such Mg^{2+} site per functional enzyme complex (Grisham and Mildvan, 1974). Mn^{2+} is more effective than Mg^{2+} in terms of a decreased K_d for vanadate, althouth the maximal binding is the same with each; Ca^{2+} is far less effective than Mg^{2+}. Smith *et al.* (1980) showed that Mn^{2+} is tightly bound with vanadate in a ternary complex, but a second aspect of the role of the divalent cation may be selecting the appropriate enzyme conformation: Mn^{2+} is more effective than Mg^{2+} in selecting the E_2 conformational state of the enzyme (Robinson, 1981), whereas Ca^{2+} favors the E_1 state (Tobin *et al.*, 1973).

(ii) K^+ is not required for vanadate binding, contrary to some proposals (Bond and Hudgins, 1979; Beauge' *et al.*, 1980), but it does decrease the apparent K_d without affecting maximal binding in the presence of Mg^{2+}. The inability of K^+ to increase vanadate binding appreciably in the presence of Mn^{2+} suggests that a major factor in the action of K^+ is selection of the E_2 conformational state: if 3 mM Mn^{2+} already selects the E_2 state optimally, then further addition of K^+ would not lead to additional conversion to the E_2 state and additional binding. In accord with this assignment of the role of K^+ in augmenting vanadate binding through favoring the E_2 conformational state are the characteristics of the K^+ site affecting binding: it resembles in apparent affinity for K^+ that at which the K^+-phosphate reaction is activated (Robinson, 1975a; 1981) and ATP binding is diminished (Robinson, 1975), but not in apparent affinity that at which the (Na + K)-ATPase reaction is activated (Robinson, 1975a; Bond and Hudgins, 1979; Beauge', 1979). This assignment is consistent with the sidedness of the K^+ effects on vanadate inhibition, for those sites at which the K-phosphatase reaction are activated are accessible most readily from the intracellular face except in the presence of Na^+ and ATP when they are readily accessible from the extracellular face (Blostein *et al.*, 1979). Thus when the vanadate sensitivity of the K-phosphatase reaction is tested, intracellular K^+ modifies vanadate inhibition (Table II), whereas when sensitivity of the (Na + K)-ATPase reaction is tested, extracellular K^+ modifies vanadate inhibition (Table III; Beauge' and Glynn, 1978). In either case the "occluded K^+ site" (Post *et al.*, 1972; Beauge' and Glynn 1979), an intermediate step in the ATPase reaction cycle and perhaps representing a stage in the normal transport sequence from extracellular to intracellular media, may be the specific site.

(iii) Inhibition of vanadate binding by Na^+ occurs even in the absence of K^+, in contrast to proposals that Na^+ acts by displacing K^+ (Bond and

Hudgins, 1979) or that K^+ acts by displacing Na^+ (Beauge' *et al.*, 1980). The inability of intravesicular Na^+ (i.e., extracellular Na^+) to diminish vanadate inhibition of the K-phosphatase reaction of inside-out red blood cell vesicles makes it unlikely that the pertinent Na^+ sites are located, at least in this system, on the extracellular face of the enzyme, as proposed by Beauge' *et al.* (1980). The K_i for Na^+, about 7 mM, is the same as the K_i for Na^+ as an inhibitor of the K-phosphatase reaction (Robinson, 1969), strengthening the identification of K^+ sites modifying vanadate inhibition with those activating the phosphatase reaction. Moreover, the insensitivity to both Na^+ and K^+ of vanadate binding in the presence of Mn^{2+} (Table I) is in accord with the relative insensitivity to these cations of the K-phosphatase reaction, but not of the (Na + K)-ATPase reaction, in the presence of Mn^{2+} (Robinson, 1981).

(iv) As deduced from kinetic studies on enzyme inhibition (Cantley *et al.*, 1978), the nucleotide concentration antagonizing vanadate binding is in the range of the low-affinity substrate site, with an apparent K_i on the order of 0.1–1 mM. The apparent affinity for the nucleotide also is diminished by KCl, just as the apparent affinity for ATP at the low-affinity substrate sites in the (Na + K)-ATPase reaction is diminished (Robinson, 1967; 1975b). Nitrophenyl phosphate, the substrate for the phosphate reaction, acts as a competitor to ATP at the low- but not the high-affinity substrate sites (Robinson, 1976a; 1980b), and here hinders vanadate binding. Finally, in current schemes of the reaction sequence (Karlish *et al.*, 1978; Robinson and Flashner, 1979; Smith *et al.*, 1980) it is ATP binding to these low-affinity substrate sites that favors conversion of the enzyme from the E_2 to the E_1 conformational state; it should be emphasized that the high- and low-affinity substrate sites demonstrable kinetically may represent a single site that changes affinity with enzyme conformational state (Smith *et al.*, 1980).

(v) Ligands and reagents that favor the E_1 state, such as ATP, Na^+, and oligomycin, decrease vanadate binding, whereas those that favor the E_2 state, such as Mn^{2+}, K^+, dimethyl sulfoxide, and ouabain, increase binding. Aside from the divalent cation, it is not obvious that any of these agents affects binding through any other means than selecting enzyme conformation.

Overall, these experiments favor a model in which vanadate can bind only to the E_2 conformational state, selected by divalent cations, K^+, ouabain, and/or dimethyl sulfoxide in a ternary complex including a trapped divalent cation (Smith *et al.*, 1980) and by virtue of its resemblance to the transition state of phosphate during hydrolysis (Cantley, 1978). K^+ increases binding by favoring the E_2 state, approaching its controlling site from the intracellular face, or in the presence of ATP and Na^+ from the extracellular face by means of the cation transport pathway. Na^+, on the other hand, decreases binding through favoring the E_1 state, as do ATP and oligomycin.

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