Modification of the Conformational Equilibria in the Sodium and Potassium Dependent Adenosinetriphosphatase with Glutaraldehyde†

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ABSTRACT: Glutaraldehyde treatment of electroplax membrane preparations of Na,K-ATPase leads to irreversible changes in the enzymic behavior of the protein, which are not due to modification of the active site. When the glutaraldehyde treatment is carried out in a medium containing K^+ and without Na^+, the "K^+-modified enzyme" so produced shows the following changes in enzymic properties: The steady-state phosphorylation by ATP and the rate of ATP–ADP exchange are decreased to ~40% of control, while Na,K-ATPase activity decreases to ~15% of control. Phosphatase activity is decreased very little, but the potassium activation parameters of the reaction are changed, from $K_{0.5} \approx 5$ mM and $n_H = 1.9$ in control to $K_{0.5} \approx 0.5$ mM and $n_H = 1$ in K^+-modified enzyme. K_I(app) for nucleotide inhibition of phosphatase activity is increased significantly. Changes in the cation dependence of the ATPase reaction are also observed. All of these effects can be explained by assuming that the cross-linking of surface groups in protein subunits when they are in conformation E_2 shifts the intrinsic conformational equilibrium of the enzyme toward E_2. We considered the simplest mathematical model for the coupling between K^+ binding and the conformational equilibrium, with equivalent potassium sites that must be simultaneously in the same state. If one assumes that the potassium activation of phosphatase activity in the K^+-modified enzyme reflects the affinity for K^+ of E_2, the behavior of the phosphatase activity in the native enzyme can be fit if there are only two potassium sites, whose affinity is 80-fold higher in E_2 than in E_1, and the equilibrium constant for $E_2 \rightleftharpoons E_1$ is about 250. The same sites can explain the activation of dephosphorylation during ATP hydrolysis. Independent of the model chosen, potassium ions must be required for the catalytic action of form E_2 and cannot be merely "allosteric activators". The enzyme modified with glutaraldehyde in a medium containing Na^+ also has interesting properties, but their rationalization is less straightforward. The Na,K-ATPase activity is inhibited more than the "partial reactions", as in the K^+-modified enzyme. We suggest that this is a generally expected result of modifications of the enzyme.

The sodium and potassium dependent adenosinetriphosphatase (Na,K-ATPase)[1] is a complex membrane enzyme with multiple, interacting sites for monovalent cations and substrates (Glynn & Karlish, 1975; Cantley, 1981). The ligand concentration dependences of various processes catalyzed by the enzyme have often been used to infer the properties of the ligand sites and the nature of the interactions among them [e.g., Gache et al. (1977) and Robinson (1977)]. According to the generally accepted mechanism for the action of the enzyme, however, the protein exists in two conformational states, E_1 and E_2, which differ in their affinities for various ligands (Glynn & Karlish, 1975; Cantley, 1981). It is obvious that transitions between these states, which may be at equilibrium under one set of conditions but not under another, will strongly influence the observed concentration dependence of any reaction of the enzyme [e.g., Moczydlowski & Fortes (1981)]. If it were possible to freeze the protein in a particular conformational state or to shift its conformational equilibrium strongly toward one of the states, it would then be possible to directly study the properties of the ligand sites in that state. Such studies could in turn be useful for the clarification of the number and mutual interaction of ion sites and other properties of the Na,K-ATPase.

The conformations of the Na,K-ATPase apparently differ in the arrangement and relative positions of exposed peripheral groups (Jorgensen, 1977), as well as in the structure of the ligand binding sites. The cross-linking of groups at the enzyme surface with a bifunctional reagent, when the enzyme is in a medium favoring a specific conformation, should thus stabilize that conformation. We have studied the effect of glutaraldehyde treatment on Na,K-ATPase from electroplax (Chipman et al., 1983) and have been able to produce modified enzyme preparations in which the equilibrium between E_1 and E_2 is apparently significantly altered. In this paper, we report further details of the properties of such preparations and some insights that they provide into the behavior of the native Na,K-ATPase.

Materials and Methods

Materials. ADP, ATP, AMP-PNP, PNPP, strophanthidin, ouabain, β-galactosidase, and the mixture of molecular weight marker proteins were purchased from Sigma. [2-^{3}H]ADP was obtained from the Radiochemical Centre, Amersham, and [^{32}P]phosphoric acid from the Nuclear Research Center, Dimona. [γ-^{32}P]ATP was prepared by the method of Avron (1961). 2,4-Dinitrophenyl phosphate monolutidine salt (DNPP) was prepared according to Kirby & Varvoglis (1966). The imidazolium salts of ADP, ATP, and PNPP were prepared from the commercial sodium salts by ion exchange on a Dowex W50 column and stored in frozen solution. Concentrations of these solutions were determined spectrophotometrically.

Enzyme. Torpedo ocellata was obtained from Dr. D. Michaelson of Tel Aviv University. *Electrophorus electricus* was supplied by World Wide Scientific Animals (Ardsley, NY). The electroplax tissue was kept at −70 °C from the time of death of the animals. Plasma membrane preparations were prepared from the electroplax of either species as described by Perrone et al. (1975). The pellets were suspended for use by homogenization with a Teflon homogenizer in 0.32 M

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[1] Abbreviations: Na,K-ATPase, sodium and potassium dependent adenosinetriphosphatase (EC 3.6.1.3); AMP-PNP, 5′-adenylyl imidodiphosphate; EDTA, ethylenediaminetetraacetic acid; PNPP, *p*-nitrophenyl phosphate; DNPP, 2,4-dinitrophenyl phosphate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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sucrose–1 mM EDTA brought to pH 7.0 with imidazole ("sucrose–EDTA solution") at a protein concentration of 10–25 mg/mL. Such suspensions could be stored frozen at −15 °C for several months with little loss of activity. Purified *Electrophorus* enzyme (Dixon & Hokin, 1978) was a gift of Dr. L. Hokin.

Glutaraldehyde Treatment. The standard modified enzyme was prepared by treatment with 0.035% glutaraldehyde as follows: A 20-μL aliquot of glutaraldehyde solution (2.6%, prepared from Sigma grade II 25% glutaraldehyde) was added to 1.5 mL of a *Torpedo* membrane fragment enzyme suspension (5 mg/mL) in 100 mM imidazole buffer, pH 7.0, containing 1 mM EDTA and either (a) 30 mM KCl and 10 mM KH_2PO_4 ("K^+ medium") or (b) 100 mM NaCl and 3 mM ATP ("Na^+ medium") and the mixture incubated at 25 °C. After 20 min, unreacted glutaraldehyde was reduced by addition of 10 μL of fresh 0.65 M $NaBH_4$ (a 2.4-fold stoichiometric excess) and the sample transferred to an ice bath. Control samples were treated similarly, except that $NaBH_4$ was added immediately before the glutaraldehyde instead of at the end of the incubation. The reaction mixture was diluted with an equal volume of 100 mM imidazole buffer and centrifuged at 17000*g* for 30 min at 4 °C. The pellet was resuspended in 2 mL of sucrose–EDTA and centrifuged once more. The final pellet was resuspended in the same solution and stored at −15 °C. The protein concentration of samples prepared in this way [method of Lowry et al. (1975) with BSA as standard] was 2–2.5 mg/mL. The potassium content of samples prepared in K^+ medium was determined by flame emission spectrophotometry and was about 2 μmol of K/mg of enzyme. This could be reduced to less than 0.1 μmol of K/mg by washing the preparation again in sucrose–EDTA containing 100 mM NaCl and finally washing once more in the solution without NaCl.

In various experiments, different enzyme, glutaraldehyde, and ligand concentrations were used. The time course of the enzyme modification was followed by preparing an enzyme suspension in the treatment medium, adding glutaraldehyde, and transferring aliquots from the reaction mixture to tubes containing cold $NaBH_4$ solution at appropriate times. Aliquots of the quenched solutions were then assayed for enzymic activities without washing, and the activities were compared to controls in which $NaBH_4$ was added immediately before glutaraldehyde. In control experiments (Table I), we showed that $NaBH_4$ itself has no significant effect on the activities of the enzyme and reduces glutaraldehyde rapidly enough so that "zero time of treatment" samples ($NaBH_4$ 1–2 s before glutaraldehyde) behave like native enzyme. In addition, the controls (Table I) demonstrate that $NaBH_4$ does not effect the behavior of enzyme that has already reacted with glutaraldehyde.

Assays of Activity. Na^+ and K^+ dependent ATPase activity was routinely assayed by colorimetric determination of the inorganic phosphate released (Taussky & Shorr, 1953), in a solution containing 3 mM ATP, 3 mM $MgCl_2$, 100 mM NaCl, and 20 mM KCl in 60 mM imidazole buffer, pH 7.0. The ATPase activity at low substrate concentrations was determined by using [γ-^{32}P]ATP and analysis by a modification of the phosphate extraction method of Martin & Doty (1949; Avron, 1960). After the reaction was stopped with TCA, an aliquot of the mixture (usually 1 mL) was diluted to 2.5 mL with water saturated with 2-methyl-1-propanol and xylene, and to this was added 0.8 mL of 5% ammonium molybdate in 4 N HCl, 50 μL of 20 mM phosphate as carrier, and 4 mL of 2-methyl-1-propanol–xylene (1:1 v/v) saturated with water. After vortex agitation and separation of phases, 2 mL of the (upper) organic phase was taken for scintillation counting.

ADP–ATP exchange was assayed by following the appearance of label in ATP in a reaction mixture containing 5 mM ATP, 1.25 mM [2-^{3}H]ADP, 0.15 mM $MgCl_2$, and 100 mM NaCl in a total volume of 50 μL. The reaction was stopped by addition of 10 μL of 25 mM Na_2EDTA, and an aliquot was spotted on a poly(ethylenimine)–cellulose thin-layer chromatography sheet (Macherey-Nagel). The chromatogram was developed with 0.2 N NH_4CO_3, and the UV-absorbing spots corresponding to ADP and ATP were cut out and placed in scintillation vials. One milliliter of 0.7 M $MgCl_2$–0.02 M Tris buffer, pH 7, was added to each vial to extract the nucleotides, and scintillation fluid was added after 1 h.

Phosphatase activity was routinely assayed by spectrophotometric measurement (400 nm) of the *p*-nitrophenylate released from *p*-nitrophenyl phosphate (PNPP) after the enzymic reaction was stopped with NaOH. The reaction mixture contained 5 mM PNPP, 10 mM $MgCl_2$, and 30 mM KCl in 100 mM imidazole buffer, pH 7.0. The dinitrophenylphosphatase activity was measured by continuous spectrophotometric assay of dinitrophenylate release ($\epsilon = 1.1 \times 10^4$ at 395 nm) from 2,4-dinitrophenyl phosphate (DNPP) in magnetically stirred cells in a Cary 219 spectrophotometer.

All enzyme activities were determined at 25 °C under conditions where the reaction was linear with time, with appropriate blank controls. All activities quoted are measured activity minus the activity in the presence of 1 mM strophanthidin, unless otherwise stated. For the native enzyme under optimal conditions, the ATPase and phosphatase activities were over 98% strophanthidin sensitive.

Kinetic parameters were calculated by fitting the experimental data (e.g., strophanthidin-sensitive velocity vs. potassium concentration) to the appropriate equation with a Grid Search nonlinear least-squares program (Bevington, 1969) written in Basic for an Apple II+ microcomputer.

The phosphorylation of the enzyme with ATP was determined by a variation of the method of Schuurmans Stekhoven et al. (1980). [α-^{32}P]ATP (20 μM) was added to 100 μg of the enzyme in 0.2 mL of a solution containing 5 mM $MgCl_2$, 60 mM NaCl, and 100 mM imidazole, pH 7.5, at 0 °C. After 10 s, 0.1 mL of 10% TCA was added. The precipitated protein was collected on a Millipore filter (0.45 μm) and washed 5 times with 15 mL of 0.5 M TCA containing 20 mM phosphate and 0.1 mM ATP. The filter was dried and dissolved in Triton–toluene-based scintillation fluid and counted. Nonspecific trapping of label by the protein and filter was determined by adding TCA before the labeled ATP.

Gel Electrophoresis. Polyacrylamide slab gel electrophoresis in the presence of SDS was carried out by the procedure of Laemmli (1970) with some modifications: The denaturing buffer contained 1% mercaptoethanol. The acrylamide concentration was 3.75% in the stacking gel and 6% in the separating gel. A mixture of six proteins of known molecular weights from 29000 to 240000 was used as a molecular weight marker run parallel to samples. In order to determine the relative amount of a given peptide in different samples, a known quantity of a standard, β-galactosidase, which did not overlap with other polypeptide bands, was added to each aliquot. The gels were stained in 0.05% Coomassie Blue R in 25% 2-propanol–10% acetic acid, destained, and photographed, and a positive reproduction on film was scanned with the "gel-scanning accessory" of the Cary 219 spectrophotometer. The relative areas of the standard peak and the peak due to

Table I: Influence of $NaBH_4$ on Glutaraldehyde Treatment of Enzyme[a]

medium	treatment[b]	activity[c] ATPase[d]	activity[c] *p*-nitrophenylphosphatase[e] 30 mM KCl	activity[c] *p*-nitrophenylphosphatase[e] 0.5 mM KCl
100 mM NaCl + ATP	no additions	93	92	
	$NaBH_4$, 30 min	102	99	
	$NaBH_4$, glutrald, 30 min	106	99	
	glutrald, 30 min	15	28	
	glutrald, 30 min, $NaBH_4$	15	25	
30 mM KCl	no additions	99	105	1
	$NaBH_4$, 30 min	110	112	0.5
	$NaBH_4$, glutrald, 30 min	90	94	0.5
	glutrald, 30 min	16	64	31
	glutrald, 30 min, $NaBH_4$	12	57	29

[a] 3 mg/mL of microsomal enzyme from *Torpedo* electroplax in 100 mM imidazole-HCl buffer at pH 7.0, containing either 30 mM KCl or 100 mM NaCl + 1 mM ATP, was treated with $NaBH_4$ or glutaraldehyde (glutrald) or both and its activity assayed. [b] Samples incubated 30 min total at 25 °C, with various combinations of additions of glutaraldehyde (to 0.016%) and $NaBH_4$ (to 4 mM final, a 5-fold stoichiometric excess over glutaraldehyde). After the last addition and/or incubation, samples were cooled to 0 °C, diluted 2-fold in sucrose-EDTA, and centrifuged. The enzyme pellet was resuspended in sucrose-EDTA and assayed for protein concentration. Each treatment was repeated in duplicate. [c] Activities are given as percent of control (untreated) enzyme and were determined in duplicate for each treated sample. [d] ATPase assayed colorimetrically, routine conditions. [e] *p*-Nitrophenylphosphatase activity assayed under routine conditions (30 mM KCl) or at 0.5 mM KCl. (For latter conditions, activities as percent of control *at 30 mM*. See Figure 2 and text for significance.)

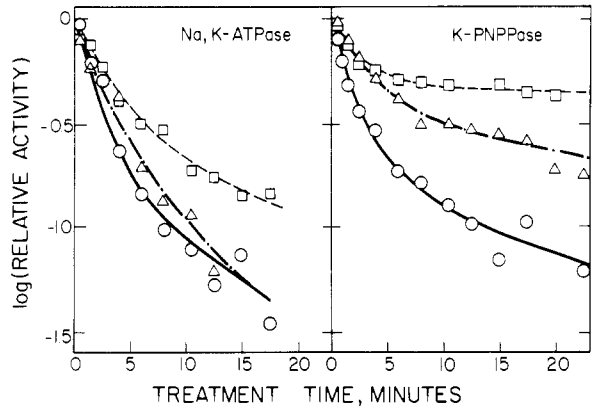


FIGURE 1: Time course of loss of Na^+ and K^+ dependent ATPase activity (left panel) and K^+-dependent *p*-nitrophenylphosphatase activity (right panel) upon glutaraldehyde treatment of *Torpedo* enzyme. Microsomal enzyme (2.0 mg/mL) was treated with 0.02% glutaraldehyde in 100 mM imidazole–1 mM EDTA buffer, pH 7.0, containing 30 mM KCl (△), 100 mM NaCl (○), or 100 mM NaCl + 1 mM ATP (□). At various times, 100-μL aliquots were removed, and the reaction was stopped with $NaBH_4$. Activities were measured under standard conditions as described under Materials and Methods.

a given peptide were used to determine the amount of the peptide remaining free in each sample after treatment.

Results

Selective Inactivation by Glutaraldehyde. Glutaraldehyde treatment of a membrane fragment preparation of *T. ocellata* electroplax Na,K-ATPase leads to a progressive, irreversible loss or alteration of its various enzymic activities (Figure 1). Although $NaBH_4$ has been used in most of the experiments reported here (to destroy excess glutaraldehyde and thus allow convenient control of the time of reaction), the observed changes in the enzyme do not depend on the addition of $NaBH_4$ (Table I). The kinetics of the inactivation process depend on the ligands present during the treatment (Figure 1). For example, the K^+-dependent phosphatase activity is lost more than twice as fast in a buffer containing NaCl (100 mM) as in a buffer containing KCl (30 mM). The substrates and products of the enzyme provide protection against inactivation (e.g., ATP in NaCl in the presence of EDTA to ensure sequestering of endogenous Mg^{2+}, Figure 1). However, such protection is only partial, even at saturation with ATP. Under

Table II: Effect of Glutaraldehyde Treatment in Na^+ and K^+ Media on Activities of the Na,K-ATPase[a]

reaction	% residual activity after glutaraldehyde treatment[b] treatment in Na^+ medium	treatment in K^+ medium
Na^+,K^+-ATPase	14 ± 4 (6)	13 ± 6
ADP-ATP exchange	60 ± 15 (3)	38 ± 3
phosphorylation by ATP	70 ± 10 (5)	35 ± 19
K^+-*p*-nitrophenylphosphatase	33 ± 7 (3)	72 ± 14
K^+-2,4-dinitrophenylphosphatase	21 (1)	73

[a] Microsomal enzyme from *Torpedo* electroplax (5 mg/mL) was treated with 0.035% glutaraldehyde for 20 min in a medium containing 100 mM imidazole–1 mM EDTA buffer, pH 7.0, and either 100 mM NaCl + 3 mM ATP (treatment in Na^+ medium) or 30 mM KCl + 10 mM KH_2PO_4 (treatment in K^+ medium). After 20 min, the reaction was stopped with $NaBH_4$ and the enzyme washed by centrifugation as described under Materials and Methods. [b] Activities were measured under standard conditions (see Materials and Methods) and are given as percent of activity of control enzyme treated in parallel in the same medium, with $NaBH_4$ added before glutaraldehyde. Errors are standard deviations of determinations on a number of separate treatment experiments (number in parentheses). In each case, Na^+ and K^+ treatments were carried out in parallel, and differences in residual activities in the two treatments are more significant than suggested by errors.

all conditions we have examined, the Na^+ and K^+ dependent ATPase activity is more susceptible to inhibition by glutaraldehyde than is the K^+-dependent phosphatase activity; this is a result frequently observed with modifications of Na,K-ATPases [e.g., Cantley et al. (1978) and Henderson et al. (1979)]. This point will be discussed below.

The kinetics of inactivation clearly cannot be fit by a single exponential decay of activity (Figure 1). We believe that the process is a complex one, involving multiple reactions of the protein molecule with glutaraldehyde and consuming a significant fraction of the reagent, and is therefore not expected to be pseudo first order.

In order to further clarify the effect of the glutaraldehyde modification on the catalytic properties of the enzyme, we have compared a number of activities of modified enzymes to those of the controls. Table II summarizes the activities, relative

Table III: Kinetic Properties of Microsomal Na,K-ATPase after Glutaraldehyde Modification in K^+ Medium[a]

reaction[b]	parameter	control	modified enzyme
ATP hydrolysis	activity [nmol (mg of protein)$^{-1}$ min^{-1}]	500	75 (15%)
	$K_{0.5}(K^+)$ (mM)[c]	1.0	0.6
	$n_H(K^+)$[c]	1	0.7
	rel act. at 50 mM Na^+–100 mM K^+[d]	0.61	0.47
ADP–ATP exchange	activity [nmol (mg of protein)$^{-1}$ min^{-1}]	26	10 (38%)
^{32}P incorporation from ATP	steady-state level (nmol/mg of protein)[e]	0.26	0.09 (35%)
PNPP hydrolysis	activity [nmol (mg of protein)$^{-1}$ min^{-1}]	180	130 (75%)
	$K_{0.5}(K^+)$ (mM)[f]	5.5 ± 1.6	0.62 ± 0.07
	$n_H(K^+)$[f]	1.9 ± 0.2	0.93 ± 0.10
	K_I(app) for ADP (mM)[g]	0.74 ± 0.09	2.6 ± 0.3
DNPP hydrolysis	activity [nmol (mg of protein)$^{-1}$ min^{-1}]	850	620 (73%)
	$K_{0.5}(K^+)$ (mM)[h]	6.2	1.1
	$n_H(K^+)$[h]	1.5	0.9

[a] Modified enzyme prepared by treatment with 0.4–0.7 μmol of glutaraldehyde/mg of enzyme in 30 mM KCl + 10 mM KH_2PO_4 at pH 7.0 for 20 min. [b] Assays as described under Materials and Methods, with modifications as noted. [c] Determined under standard conditions, except with varying KCl and using [^{32}P]ATP. [d] Activity at 50 mM Na^+ + 100 mM K^+ relative to that at 120 mM Na^+ + 30 mM K^+, using 0.25 mM [^{32}P]ATP. [e] In the absence of added K^+. [f] Determined under standard conditions, except with varying KCl. Figures given are mean ± standard deviation for six repetitions of experiment. [g] At 30 mM K^+ and 0.4 mM substrate. See Figure 6 for conditions. [h] Determined at standard conditions, except varying KCl.

to controls, of enzyme treated with glutaraldehyde in K^+ medium or in Na^+ medium under identical standard conditions. We have repeated such preparations many times, and the properties of the resulting modified enzymes are quite reproducible. The overall Na^+- and K^+-dependent ATPase activity is depressed more than any of the "partial activities" of the enzyme, whether the protein is treated in Na^+ or K^+ media. The phosphatase activities are retained to a greater extent if the enzyme is treated in the K^+ medium, while the partial activities involving nucleotides are retained somewhat more if the enzyme is treated in the Na^+ medium. Under either set of modification conditions, however, the enzyme retains to a considerable extent its ability to undergo phosphorylation by ATP in the presence of sodium ions, as reflected both in the ATP–ADP exchange reaction (Fahn et al., 1966) and in the incorporation of ^{32}P from [γ-^{32}P]ATP. This implies that the observed loss of the Na^+- and K^+-dependent ATPase activity cannot be due *primarily* to the blocking of the nucleotide binding sites or the inactivation of the catalytic apparatus required for the transfer of the phosphoryl group between a nucleotide and the enzyme.

Changes in Potassium Activation Parameters. The dependence of enzyme activities on the concentrations of monovalent cations provides an insight into the nature of the modification caused by glutaraldehyde. In Figure 2, the [K^+] dependence of the catalyzed hydrolysis of *p*-nitrophenyl phosphate (PNPP) is compared for native or control-treated enzymes and enzymes treated with glutaraldehyde. The native and control-treated enzymes show highly cooperative potassium activation ($K_{0.5}$ = 5 mM, $n_H \simeq 2$) as previously reported (Gache et al., 1976). The enzyme treated with glutaraldehyde in K^+ medium has an apparent affinity for K^+ nearly 1 order of magnitude greater ($K_{0.5}$ = 0.7 mM in this experiment) and shows hyperbolic activation (n_H = 1.0). In the experiment illustrated, with a relatively low glutaraldehyde to protein ratio, the activity of the treated enzyme at optimal [K^+] is nearly equal to that of the controls. The enzyme treated in Na^+ medium, on the other hand, has lowered phosphatase activity at all K^+ concentrations. Its activation parameters cannot be determined very accurately because of its low activity, but $K_{0.5}$ seems essentially unchanged, while n_H may be somewhat lowered (1.7 ± 0.3).

The potassium activation parameters for the hydrolysis of 2,4-dinitrophenyl phosphate (DNPP), a much more active pseudosubstrate of the enzyme, are altered by the glutaraldehyde treatment in the same way as are those for the hydrolysis of PNPP. Kinetic parameters for these and other reactions of control enzyme and enzyme treated with glutaraldehyde in KCl plus KH_2PO_4 are collected in Table III.

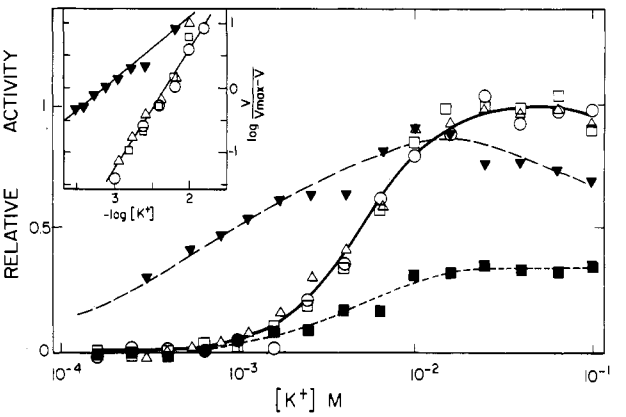


FIGURE 2: Potassium ion dependence of hydrolysis of PNPP by native Na,K-ATPase (○) and by enzyme treated with glutaraldehyde. Enzyme (9 mg/mL) and 0.03% glutaraldehyde were incubated for 20 min in the buffer as in Figure 1, containing either 30 mM KCl (▼) or 100 mM NaCl + 3 mM ATP (■). Control enzyme incubated in either K^+ medium (△) or Na^+ medium (□), but with $NaBH_4$ added before glutaraldehyde, is also shown. Enzyme samples were washed twice as described under Materials and Methods, and *p*-nitrophenylphosphatase activities were determined under standard conditions, except with varying KCl concentration. (Solid line) (controls) n_H = 1.9, $K_{0.5}$ = 4.8 mM; (long dashed line) (treated in K^+ medium) n_H = 0.97, $K_{0.5}$ = 0.75 mM; (short dashed line) (treated in Na^+ medium) n_H = 1.7, $K_{0.5}$ = 5 mM. Activity at high [KCl] was fit by eye only. (Insert) Hill plots for control and K^+-modified enzyme.

Changes in the potassium activation parameters for phosphatase activities similar to those shown in Figure 2 have been reproducibly observed as a result of treatment of *Torpedo* enzyme in K^+ medium at various glutaraldehyde to protein ratios. A similar effect is observed with a membrane fragment preparation from *E. electricus* electroplax: $K_{0.5}$ for potassium activation of PNPP hydrolysis is reduced 10-fold and n_H reduced from 2 to 1 after cross-linking in the presence of KCl (not shown). Preliminary experiments with purified *Electrophorus* enzyme and various phosphate ester pseudosubstrates give similar results.

We considered the possibility that potassium retained in the enzyme preparation after the glutaraldehyde treatment could be partly responsible for the observed change in the response

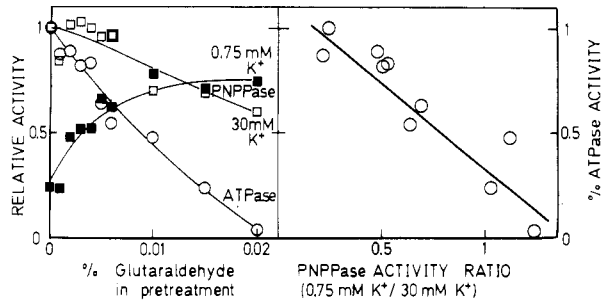


FIGURE 3: Glutaraldehyde dependence of loss of Na^+ and K^+ dependent ATPase activity and changes in phosphatase activity of the Na,K-ATPase. Enzyme (2 mg/mL) was treated for 20 min in 100 mM imidazole, 1 mM EDTA, 30 mM KCl, and 10 mM KH_2PO_4 at pH 7.0 with varying amounts of glutaraldehyde. (Left panel) ATPase activity (○) and PNPP hydrolysis activity (□) under standard conditions and PNPPase activity at 0.75 mM KCl (■), relative to activity of control enzyme at 30 mM KCl; (right panel) ATPase activity plotted against the ratio of phosphatase activities at low and high [KCl].

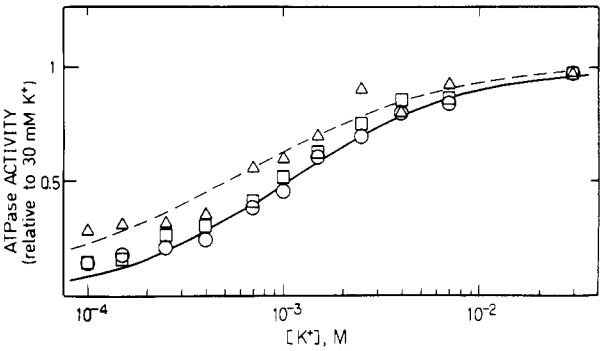


FIGURE 4: Potassium dependence of hydrolysis of ATP by control and glutaraldehyde-treated Na,K-ATPase. Enzyme treated under standard conditions (see Table II) in K^+ medium (△) or Na^+ medium (□) and native enzyme (○) were assayed for ATPase activity with [γ-^{32}P]ATP under standard conditions, except for varying KCl concentration. All activities are given relative to that of the same sample at 30 mM KCl. The absolute activities of the treated enzymes are very low (~15% of native). (Solid line) (control) n_H = 1.0, $K_{0.5}$ = 1.0 mM; (dashed line) (KCl treated) n_H = 0.7, $K_{0.5}$ = 0.6 mM.

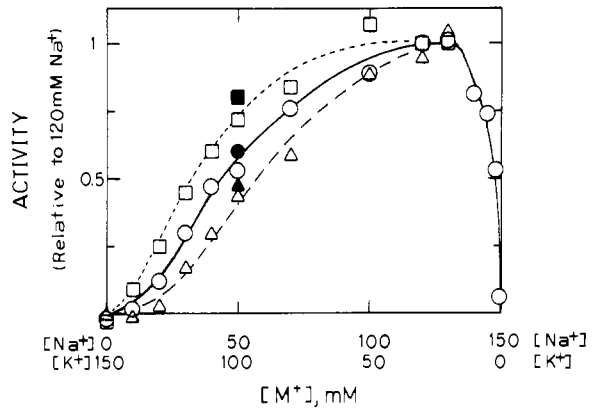


FIGURE 5: Monovalent cation dependence of hydrolysis of ATP at constant $[Na^+] + [K^+]$ = 150 mM, by native (○, ●) and glutaraldehyde-treated Na,K-ATPase. Enzyme was treated under standard conditions (see Table III) in K^+ medium (△, ▲) or Na^+ medium (□, ■). ATPase activity was determined with 0.25 mM [γ-^{32}P]ATP and 0.5 mM $MgCl_2$ in 30 mM imidazole buffer, pH 7.0. Activities are given relative to average activity of same sample at 120 mM NaCl + 30 mM KCl. Open symbols are averages from three separate experiments over the concentration range, and full symbols are from two experiments in triplicate comparing activity at 50 mM NaCl + 100 mM KCl to that at 120 mM NaCl + 30 mM KCl.

to added potassium. A series of washings of the *Torpedo* preparation after treatment in K^+ medium (including a wash with 100 mM NaCl as described under Materials and Methods) reduced the potassium content to less than 0.1 μmol/mg of protein but had no effect on the apparent potassium activation parameters. This amount of potassium would contribute less than 5×10^{-6} M to the total potassium concentration in a typical assay solution with 50 μg of protein/mL. (The phosphatase activity of the washed modified preparation in the absence of added potassium is 18% of its activity at optimal $[K^+]$.) Potassium entrained in closed vesicles cannot be responsible for the observed effects, either, since an enzyme molecule in a sealed membrane vesicle would either be inactive (active site inward) or have its ouabain sites inaccessible (Perrone & Blostein, 1973). The ouabain-insensitive phosphatase activity of the modified enzyme under optimal conditions is higher than that of the native enzyme, but is still only 12% of the total. We can tentatively assume, then, that the glutaraldehyde treatment in KCl stabilizes the enzyme in a conformation with a high affinity for K^+ (E_2?).

Relationship between Effects. It can be seen from the potassium activation curves of Figure 2 that at low potassium concentration (say, <2 mM) the phosphatase activity is *increased* by glutaraldehyde treatment in the K^+ medium. We could thus test the relationship between the changes in the behavior of the phosphatase reaction and the inhibition of the ATPase reaction, by examining the activity after treatment with increasing amounts of glutaraldehyde (Figure 3). When the relative ATPase activity is plotted against the ratio of phosphatase activities at low and optimal potassium concentrations (right panel in Figure 3), the data fall on a straight line. The same relationship is found when the *kinetics* of the changes are followed at a single glutaraldehyde concentration (Chipman et al., 1983), and a similar correlation is found with the potassium activation parameters for DNPP hydrolysis. The changes in the enzyme that lead to modification of the parameters for the phosphatase activity therefore occur simultaneously with those leading to inhibition of the ATPase activity.

Other Changes in Kinetic Parameters. We have found several other changes in the kinetic parameters of reactions catalyzed by the Na,K-ATPase after modification of the enzyme with glutaraldehyde. Figure 4 shows the potassium dependence of the ATPase activity of the enzyme in the presence of saturating NaCl (120 mM) and substrate (3 mM) concentrations. The potassium dependence of this activity in the *native* enzyme shows little or no cooperativity (n_H = 0.9–1.3). The apparent $K_{0.5}$ for K^+ is dependent on the sodium concentration (0.2–1 mM at $[Na^+]$ = 10–100 mM, not shown) and considerably lower than that of the phosphatase reaction. It should be noted that these properties of the ATPase reaction in the native enzyme, which are similar to those reported for several other Na,K-ATPases [e.g., Gache et al. (1976)], are close to those of the phosphatase reaction of the enzyme *modified in K^+ medium.* The residual ATPase activities of the modified enzymes are quite low (10–15% of control) and are therefore presented in Figure 4 relative to optimal activities for these enzymes. Despite the greater experimental uncertainty that results from this low activity, it can be seen that the apparent potassium affinity for this reaction of the enzyme is increased slightly by glutaraldehyde treatment in K^+ medium. The residual activity of the enzyme modified in the Na^+ medium is altered much less, if at all.

The monovalent cation dependence of the Na,K-ATPase is often studied at a constant total $[Na^+]$ plus $[K^+]$. Figure 5 shows the ion dependence of the reaction at 0.25 mM ATP and $[Na^+]$ plus $[K^+]$ = 150 mM. The behavior of the glu-

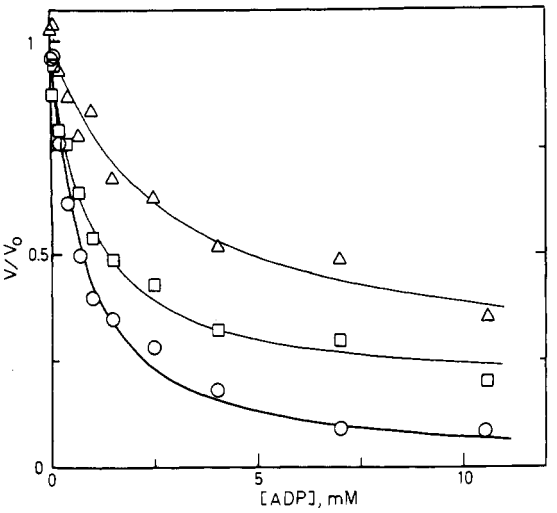


FIGURE 6: Inhibition of PNPP hydrolysis by ADP. Activity was measured at 0.4 mM PNPP, 10 mM $MgCl_2$, and 30 mM KCl in 100 mM imidazole buffer, pH 7.0, for control enzyme (○) and enzyme modified in K^+ medium (△) or Na^+ medium (□). The solid lines are calculated fits to the data: control enzyme, K_I(app) = 0.74 mM; K^+-modified enzyme, K_I(app) = 2.64 mM, leading to 78% inhibition; Na^+-modified enzyme, K_I = 0.90 mM, leading to 82% inhibition (see text).

taraldehyde-modified enzymes was compared to that of the native enzyme in the high range of potassium concentrations. In this range, the activity relative to optimal activity is higher for the enzyme treated in Na^+ medium than for the native enzyme and lower for the enzyme treated in K^+ medium (although the latter difference is on the borderline of statistical significance).

Since the intrinsic affinity of enzyme conformation E_1 for nucleotides is assumed to be several orders of magnitude greater than that of E_2 (Smith et al., 1980; Moczydlowski & Fortes, 1981), we have also looked for changes in the apparent nucleotide affinities of the modified enzymes. Figure 6 shows the inhibition by ADP of PNPP hydrolysis by native and modified enzymes at 30 mM KCl. A similar result is observed in the AMP-PNP inhibition of DNPP hydrolysis. The enzyme modified in K^+ medium has a considerably lower *apparent* affinity for the nucleotide than the native enzyme, although it shows slightly heterogeneous behavior (i.e., a better fit to the data is obtained by assuming two inhibition constants, only the lower of which is well determined, then by assuming a single constant). The enzyme modified in Na^+ medium has an affinity for ADP closer to that of the native enzyme under these conditions.

Relation between Kinetic Changes and Subunit Cross-Linking. Since the Na,K-ATPase is a multisubunit protein, it is relevant to examine the relationship between intersubunit cross-links and the observed modifications in the enzymic behavior of the protein [although the intersubunit cross-links formed with many different reagents have been studied recently by a number of groups (Giotta, 1976; Sweadner, 1977; De Pont et al., 1979; Askari et al., 1980; Askari & Huang, 1980; Craig & Kyte, 1980; Harris & Stahl, 1980; Craig, 1982), such studies were not concerned with the relationship between the cross-links and enzymic behavior of the products]. Samples of the protein modified with increasing amounts of glutaraldehyde (as in Figure 3) in Na^+ or in K^+ media were analyzed by SDS gel electrophoresis on 6% acrylamide gel slabs. The intensity of the bands due to subunits α (105 000) and β (apparent M_r 46 000) decreased, and large aggregates that did not enter the gel were formed at their expense. Bands due to small oligomers of the polypeptides could not be detected (not shown). This phenomenon has previously been observed by Craig (1982). The disappearance of free subunit α upon modification in K^+ medium was followed quantitatively by analyzing the treated protein with an added internal marker (see Materials and Methods). The changes in enzyme activities are compared to the extent of cross-linking of α subunits in Figure 7.

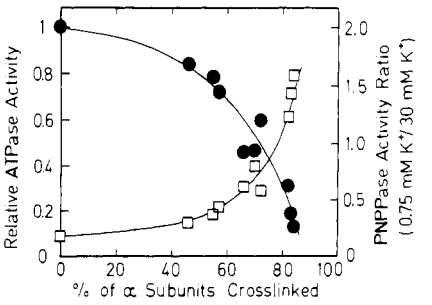


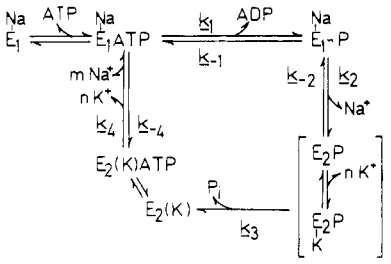
FIGURE 7: Comparison of changes in enzymic properties of enzyme with extent of cross-linking of peptides. Enzyme was treated with varying amounts of glutaraldehyde as in Figure 3. Aliquots were analyzed by SDS–acrylamide gel electrophoresis with an internal marker of β-galactosidase, as described under Materials and Methods, to determine the fraction of α remaining electrophoretically unchanged. Aliquots of treated enzyme were assayed for ATPase activity relative to controls (●) and for the ratio of phosphatase activities at 0.75 and 30 mM PNPP (□). Data are the average of four to six experiments.

Discussion

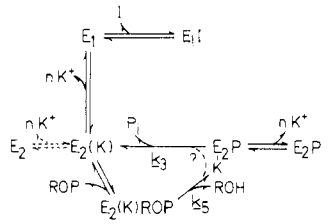
The treatment of the Na,K-ATPase with glutaraldehyde leads to significant changes in the enzymatic activities of the protein, summarized in Tables II and III. Glutaraldehyde tends to exist as an oligomer in solution and probably reacts with proteins as a complex multivalent reagent that can form several stable α,β-unsaturated imine bonds at once with primary amine groups on the protein surface (Monsan et al., 1975). The formation of cross-linked aggregates of the ATPase polypeptides is apparently not in itself sufficient to cause changes in enzymatic behavior, since there can be a significant extent of cross-linking with little effect on activities (Figure 7), at low glutaraldehyde concentrations. While a single cross-link would be sufficient to incorporate a polypeptide into an aggregate, the observed changes in activity apparently require more extensive, multiple modification (note that in experiments such as those of Figures 3 and 7 the glutaraldehyde to polypeptide ratio ranges from about 5 to over 100 mol/mol). An analysis of the kinetics, stoichiometry, and sites of attack of this reagent would be a formidable problem. However, an important negative conclusion can be drawn from our data: The changes in the catalytic parameters of the protein are not primarily due to the reaction of groups at or around the catalytic sites. Saturating concentrations of ATP in the presence of NaCl or of phosphate in the presence of KCl do not prevent the loss of Na^+ and K^+ dependent ATPase activity upon glutaraldehyde treatment. In addition, both modified enzymes retain a significant fraction of their ability to interact with nucleotides (Table II).

Jorgensen (1977) has shown that the tryptic digestion patterns of native Na,K-ATPase are completely different in NaCl and KCl solutions, which suggests that the exposure and/or configuration of some lysine and arginine residues at the enzyme surface are fundamentally changed on going from the conformation stabilized by sodium (E_1) to that stabilized by potassium (E_2). In addition, the quaternary structure of the protein is apparently different in the two states (Askari, 1982). The pattern of glutaraldehyde cross-linking of surface groups is therefore expected to be different in the two con-

Scheme I



Scheme II



formations. This prediction is born out by the rather different properties of the enzymes modified in Na^+ and K^+ media. A corollary prediction is that the cross-linking of surface groups in the arrangement they occupy in one conformation should cause the alternative conformation to be relatively unfavorable; that is, the cross-linking should shift the intrinsic conformational equilibrium of the protein subunit toward the state in which it was modified. Such conformational shifts could explain many of the observations reported above, particularly those involving the enzyme modified by glutaraldehyde in 30 mM KCl, with or without added KH_2PO_4 (K^+-modified enzyme).

Properties of K^+-Modified Enzyme. In order to see how changes in the conformational equilibrium would affect various properties of the Na,K-ATPase, let us consider the proposed kinetic mechanisms for the reactions that it catalyzes. Scheme I is a version of the Post–Albers mechanism for the Na,K-ATPase (Fahn et al., 1968; Post et al., 1969) that includes those steps relevant to ATP hydrolysis under the conditions we have used here, to ATP–ADP exchange, and to phosphorylation (Glynn & Karlish, 1975; Cantley, 1981). Scheme II includes the reaction steps we believe to be relevant to the phosphatase activity in the absence of sodium and ATP (I represents a nucleotide inhibitor).

We will consider the phosphatase activity first. Several lines of evidence suggest that if the phosphatase reaction does proceed via an enzyme phosphate (Robinson, 1971), the phosphorylation of $E_2(K)$ (rate k_5) must be slower than the dephosphorylation (k_3) (Odom et al., 1981), so that only a small fraction of the enzyme will be phosphorylated at steady state. The rate of the reaction will therefore depend on k_5 and on the fraction of the enzyme in $E_2(K)$ forms, as opposed to E_1 forms. Conformation E_2 has a higher affinity for potassium than does E_1, so the binding of potassium will produce a shift in the conformational equilibrium toward E_2 (Karlish et al., 1978b; Karlish, 1980; Beaugé & Glynn, 1980; Hobbs et al., 1980; Skou & Esmann, 1980). If the number of potassium ions involved (n) is greater than 1 and the intrinsic conformational equilibrium of the enzyme favors E_1, the potassium activation will show positive cooperativity and an observed potassium concentration for 50% of maximal activity ($K_{0.5}$) higher than that expected from the affinity of E_2 for potassium (Levitzki, 1978). A shift in the intrinsic equilibrium toward E_2, due to modification of the protein, would then be expected to decrease the cooperativity for potassium activation and decrease $K_{0.5}$ toward that characteristic of E_2. The rate of phosphate ester hydrolysis at optimal potassium concentration should not be effected by such a shift, if it were the only change in the enzyme.

The binding of a nucleotide inhibitor (ADP or AMP-PNP) will also be coupled to the conformational equilibrium, since E_2 and E_1 differ in their affinities for nucleotides by several orders of magnitude (Smith et al., 1980; Moczydlowski & Fortes, 1981). Under the conditions of our inhibition experiments (Figure 6), the conformation of the enzyme is pushed strongly toward E_2 by the saturating potassium concentration, and the apparent inhibition constant for ADP (K_I) is quite high [compare, e.g., K_D = 5 μM for ADP–Mg (Karlish et al., 1978a) or K_D = 3–6 μM for AMP-PNP–Mg (Robinson, 1980; Schuurmans Stekhoven et al., 1981) in the absence of potassium]. A shift in the intrinsic conformational equilibrium toward E_2 due to modification of the protein should make ADP an even poorer inhibitor. The K^+-modified enzyme appears to fulfill all of the above predictions concerning the phosphatase activity, except that its maximal activity is decreased by about 30% compared to native enzyme.

Under the standard conditions for the ATPase reaction, in contrast to the phosphatase reaction, $E_2(K)$ and E_1 (or their ATP complexes) are not at equilibrium; E_1ATP will go on more rapidly to $E_1\sim P$ (in a step irreversible in the absence of ADP) than it will revert to $E_2(K)$ (Mårdh & Lindahl, 1977; Smith et al., 1980; Moczydlowski & Fortes, 1981). A steady-state analysis of the mechanism under these conditions (Appendix) suggests that $K_{0.5}$ for potassium activation will be determined by the potassium affinity of E_2P and by the ratio k_{cat}/k_3'. If there is no step on the forward path much slower than k_3' (Froehlich et al., 1976; Mårdh & Lindahl, 1977), then $K_{0.5}$ for activity will be only slightly lower than that for conversion of E_2P to E_2P–K. A shift in the conformational equilibrium toward E_2 would be expected to lead to inhibition of the ATPase activity (see eq A3), and the inhibited enzyme should have a lower $K_{0.5}$ for potassium than the native enzyme (eq A6 and A7). The K^+-modified enzyme seems to behave according to this prediction as well (Figure 4).

At very high potassium, relatively low sodium, and moderately low ATP concentrations (e.g., at 50 mM NaCl and 100 mM KCl, in Figure 5), it may be that the conversion of $E_2(K)$ to Na–E_1ATP is sufficiently reversible for competition between potassium and sodium to become significant (Skou & Esmann, 1981). A shift in the conformational equilibrium toward E_2 should make this effect more important, as observed in Figure 5 for the K^+-modified enzyme.

The phosphorylation of the enzyme by [γ-^{32}P]ATP and the ATP–ADP exchange reaction presumably involve E_1 forms of the enzyme (Scheme I). A modified enzyme whose intrinsic conformational equilibrium is shifted toward E_2 should have activities comparable to the native enzyme, so long as the conformational balance can be shifted to E_1 at the high sodium concentrations involved in these reactions (note that the exchange reaction is carried out at high nucleotide concentrations as well). For the K^+-modified enzyme, the activities in question are somewhat lower than those of the native enzyme (35–40%), suggesting that some of the enzyme may be "frozen" in conformation E_2. However, this may also be due to dephosphorylation activated by residual potassium in the preparation. An additional possibility is that the catalytic competence of the "sodium form" of the enzyme is somewhat poorer (i.e., k_1 and k_{-1} are lowered) because the cross-linking constrains it and prevents it from attaining the "normal" E_1 conformation.

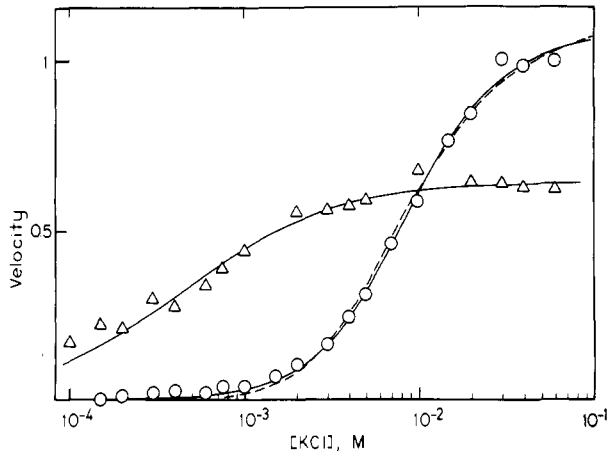


FIGURE 8: MWC fit to data for potassium activation of PNPP hydrolysis by native and K^+-modified *Torpedo* Na,K-ATPase. Activity was assayed (in duplicate) as described under Materials and Methods for native enzyme (○) and for enzyme treated with glutaraldehyde (△) in 30 mM KCl + 10 mM KH_2PO_4 and washed 4 times as described to remove potassium. Data for K^+-modified enzyme were fit arbitrarily assuming L (MWC conformational equilibrium) = 0. Solid line is best fit; K_R = 0.45 mM. Data for native enzyme (○) were fit by *assuming* K_R = 0.45 mM, with arbitrary values of n. Solid line is best fit for n = 2; dotted line is best fit for n = 4 (see Table IV).

Table IV: Estimated MWC Parameters for the K^+ Activation Data of Figure 8[a]

n	2	3	4
K_R (mM)	0.45	0.45	0.45
L	264	1190	2650
c	0.0125	0.060	0.125
V_{max}[b]	1.15	1.41	1.88
E_2/E_{total} (at saturation)[c]	0.96	0.80	0.61
ΣD^2[d]	7.1×10^{-4}	1.06×10^{-3}	1.21×10^{-3}

[a] The data for the potassium dependence of PNPP hydrolysis by native *Torpedo* Na,K-ATPase (Figure 8, circles) were fit by nonlinear least-squares computation (see Materials and Methods) to the equation $v/V_{max} = \alpha(1+\alpha)^{n-1}/[(1+\alpha)^n + L(1+c\alpha)^n]$, where $\alpha = [K^+]/K_R$, assuming that K_R = 0.45 mM, for arbitrary values of n. We believe that n = 2 is most reasonable for this model (see text). [b] V_{max} relative to the observed rate at 60 mM K^+ defined as 1. [c] Calculated conformational ratio at infinite $[K^+]$. [d] Sum of the squares of deviations from the theoretical line.

Implications of Behavior of K^+-Modified Enzyme. The kinetic properties of the protein modified with glutaraldehyde in K^+ medium, then, are as predicted for an enzyme preparation with an intrinsic conformational equilibrium shifted toward E_2 (which has perhaps also suffered a certain amount of additional nonspecific inactivation). Several groups have previously shown that the conformational equilibria in other enzymes showing allosteric or hysteretic behavior can be shifted or frozen by treatment with cross-linking reagents (Nucci et al., 1978; Enns & Chan, 1978; Klemes & Citri, 1979; Monneron & d'Alayer, 1980). In addition to supporting generally accepted notions about the mechanism of the Na,K-ATPase, however, the K^+-modified enzyme also provides a number of new qualitative and quantitative insights into the properties of the enzyme. The intrinsic potassium affinity of the sites activating phosphate ester hydrolysis ($K_{0.5} \approx 0.5$ mM) is seen to be very similar to that of the sites activating [dephosphorylation in] ATP hydrolysis [and other effects as well; cf., e.g., Smith et al. (1980)], and there is therefore no reason to propose two different classes of catalytically significant potassium sites [as, e.g., Robinson (1975)]. Our results show that the apparent low affinity for potassium in the phosphatase reaction and the cooperativity for potassium activation are due to coupling of potassium binding to the conformational equilibrium $E_1 \rightleftharpoons E_2$.

We have analyzed the potassium activation of PNPP hydrolysis by the enzyme using the simplest possible model for coupling between cation binding and the conformational equilibrium: n equivalent cation sites that are all simultaneously in either one state or the other (Figure 8). This is mathematically essentially a Monod–Wyman–Changeux (MWC) model and is characterized by five parameters (Levitzki, 1978): K_R, the dissociation constant for the "relaxed" state; c, the ratio of dissociation constants for the relaxed and "taut" states; L, the conformational equilibrium in the direction of the taut state (E_1 here); n, the number of sites; and V_{max}, the velocity of the reaction when the enzyme is saturated with potassium (if it were completely) in the relaxed state. Without an independent estimate of any of the parameters, it is not possible to fit a single activation curve uniquely with such a model. In this case, however, we have used the noncooperative potassium activation curve for a thoroughly washed preparation of K^+-modified enzyme (Figure 8) to obtain an upper estimate for K_R, 0.45 mM. With this value of K_R and the arbitrary assumption that the number of sites, n, is 2, the data for the native enzyme could be fit well and unambiguously by L = 264 and c = 0.0125 (Figure 8 and Table IV). They could be fit almost as well for n = 3 or n = 4, but the parameters L and c required for a reasonable fit in these cases imply that even at infinite potassium concentration, a significant portion of the enzyme remains in conformation E_1 [E_2/E_{total} (at saturation), Table IV]. This is inconsistent with observations by other methods with other Na,K-ATPases [e.g., Jorgensen (1977) and Karlish et al. (1978b)], with the high apparent K_I for ADP found here (Table III) and with the potassium dependence of nucleotide inhibition in electroplax enzymes (D. M. Chipman, unpublished results).

The simple model implies, therefore, that the phosphatase activity involves only two equivalent interacting potassium sites. This result has interesting implications for questions such as the molecular basis of the observed transport stoichiometry [two K^+ per ATP hydrolyzed (Glynn & Karlish, 1975), two occluded potassium ions per phosphorylation site (Beaugé & Glynn, 1979)] and the nature of subunit–subunit interactions in the Na,K-ATPase, but we must remember that there is no reason a priori to accept the simple model, with its assumption of equivalent cation sites. In fact, we cannot rule out completely such complex possibilities as the presence of nonequivalent sites involved in the activation, in one of which potassium is irreversibly trapped in the K^+-modified enzyme.

Whatever model is correct, however, an additional inference can be drawn from the behavior of the K^+-modified enzyme. This modified enzyme still requires potassium activation, so the cation is not merely an "allosteric effector" but rather must also be required for the operation of the catalytic apparatus in the E_2 conformation.

A wide variety of modifications of Na,K-ATPases have been reported to lead to preferential inhibition of the Na^+ and K^+ dependent ATPase activity of the preparation in question, while its K^+ phosphatase activity is retained [e.g., Henderson et al. (1979), Cantley et al. (1978), and Ottolenghi, (1979)]. It is often suggested that such effects imply that the ATPase reaction involves a site or functional groups that are not involved in the phosphatase activity of the enzyme. In our discussion of the properties of the enzyme modified with glutaraldehyde

in K^+ medium, we have shown that such suggestions are not necessarily correct. Any modification of the protein that changes its intrinsic conformational equilibrium or slows the interconversion between conformations would be expected to inhibit the Na^+ and K^+ dependent ATPase activity, which requires cycling between the two states. As long as conformation E_2 remains accessible at the standard potassium concentration used in assays, on the other hand, the phosphatase activity will be preserved. Similar expectations apply to any partial reaction involving only a single conformation of the protein.

Nature of Na^+-Modified Enzyme. The properties of the Na^+-modified enzyme are not as easily explained as those of the K^+-modified enzyme and are not simply as expected if the intrinsic conformational equilibrium were shifted toward E_1. One must consider, in addition, other possibilities. Some of the enzyme molecules may be completely frozen or have their catalytic sites modified. The normal conformations of E_2 and E_2P may not be accessible, and slightly altered states of lowered catalytic competence may take their places. An appropriate combination of such assumptions could explain the results reported here, but this modified enzyme obviously requires further experimental clarification.

Conclusions

The modification of the Na,K-ATPase with glutaraldehyde has yielded two preparations with interesting properties. The K^+-modified enzyme corroborates accepted notions about the mode of action of the enzyme, especially those concerning the key role of conformational changes in the protein. In addition, this modified enzyme has led to some new insights into the kinetics of the Na,K-ATPase. A simple model for the potassium activation of the phosphatase activity of the enzyme can be based on it: the enzyme has two equivalent potassium sites with $K_D \approx 0.5$ mM in conformation E_2 but an affinity some 80-fold lower in conformation E_1. The intrinsic conformational equilibrium constant for $E_2 \rightleftharpoons E_1$ implied by this model is about 260, and the coupling between potassium binding and this unfavorable equilibrium leads to the low apparent potassium affinity of the phosphatase reaction. It would be useful to modify the enzyme with other cross-linking agents that would allow a more clear-cut definition of the modified products, particularly a Na^+-modified enzyme. Pre-steady-state kinetic analysis of the ATPase reaction should provide an important test of the explanations proposed above for the changes in the steady-state kinetic parameters of the enzyme upon modification. We are continuing our work with these goals in mind. We are also expanding our studies of the monovalent cation dependence of the steady-state kinetics of native *Torpedo* and *Electrophorus* electroplax enzymes, exploiting some of the information our current work has provided (to be published).

Acknowledgments

This work could not have been carried out without the careful and enthusiastic technical assistance of Edith Elhanany and Bathsheva Damri. We are also very grateful to Prof. Dan Michaelson (Department of Biochemistry, Tel Aviv University) and his assistants for generous supplies of *Torpedo ocellata*, which made our work possible. Prof. M. Klapper of The Ohio State University furnished us with programs that were incorporated into our least-squares computation routines. Some of our early experiments were carried out in the laboratory of Prof. S. A. Bernhard at the University of Oregon, and we also thank him and his co-workers for useful discussions. We thank Academic Press for permission to reproduce Figure 2 of this paper, which appeared in a preliminary report (Chipman et al., 1983).

Appendix

Steady-State Analysis of ATPase Reaction. We have several assumptions in our analysis of the steady-state kinetics of the mechanism of Scheme I: (a) Steps 1 and 3 are irreversible (ADP and phosphate are absent). (b) The concentration of ATP is such that E_1 and E_2 are saturated. (c) ATP binding to $E_2(K)$ is in rapid equilibrium. (d) Potassium binding to E_2P is in rapid equilibrium. The fraction of E_2P in the form activated for phosphorylation by binding of the required number of potassium ions is a monotonic increasing function of the concentration of potassium ions, $f([K^+])$ (with a value between 0 and 1), and the fraction in the form that can revert to E_1P is a monotonic decreasing function $g([K^+])$.

The steady-state expression for this scheme will be given by

$$\frac{v}{E_T} = \frac{k_1k_2k_3k_4}{k_2k_3k_4 + k_1k_{-2}k_4 + k_1k_3k_4 + k_1k_2k_4 + k_2k_3k_{-4} + k_1k_2k_3} \quad \text{(A1)}$$

Inserting $k_3 = k_3'f$ and $k_{-2} = k_{-2}'g$, we obtain

$$\frac{v}{E_T} = \frac{k_1k_2k_3'k_4f}{k_1k_2k_4 + k_1k_{-2}'k_4g + (k_2k_4 + k_1k_4 + k_2k_{-4} + k_1k_2)k_3'f} \quad \text{(A2)}$$

The value of k_{cat} is v/E_T when f goes to 1 and g goes to 0 (saturation with potassium):

$$k_{cat} = \frac{k_1k_2k_3'k_4}{k_4(k_1k_2 + k_1k_3' + k_2k_3') + k_2k_3'(k_1 + k_{-4})} \quad \text{(A3)}$$

At the potassium concentration for half of maximal activity, f and g will have values $f_{0.5}$ and $g_{0.5}$ such that

$$v/E_T = k_{cat}/2 \quad \text{(A4)}$$

By combining eq A2–A4 and solving for $f_{0.5}$, we obtain

$$f_{0.5} = \frac{k_{cat}(k_2 + k_{-2}'g_{0.5})}{(k_{cat} + k_3')k_2} \quad \text{(A5)}$$

Under conditions where $k_{-2}'g_{0.5} \ll k_2$ (e.g., the equilibrium between E_1P and E_2P lies toward E_2P)

$$f_{0.5} = \frac{k_{cat}}{k_{cat} + k_3'} \quad \text{(A6)}$$

Since k_{cat} must be less than or equal to k_3', $f_{0.5}$ must be less than 0.5; that is, the ATPase reaction will reach half of maximal velocity at a potassium concentration less than that required for half-saturation of E_2P. If potassium binding to E_2P is a simple hyperbolic function [i.e., $f_{0.5} = [K^+]/(K_d + [K^+])$], then it can be shown that

$$K_M(app) = \frac{k_{cat}}{k_3'}K_d \quad \text{(A7)}$$

Registry No. ATPase, 9000-83-3; Na, 7440-23-5; K, 7440-09-7; glutaraldehyde, 111-30-8; ATP, 56-65-5; PNPP, 330-13-2; DNPP, 2566-26-9.

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