Journal of Bioenergetics and Biomembranes, Vol. 14, Nos 5/6, 1982

Tryptic Digestion of the (Na + K)-ATPase Is Both Sensitive to and Modifies K^+ Interactions with the Enzyme

Joseph D. Robinson[1]

Received March 4, 1982; revised April 22, 1982

Abstract

Tryptic digestion of the (Na + K)-ATPase in the presence of choline chloride or NaCl ("Na-type") and in the presence of KCl ("K-type") produced distinct patterns of peptide fragments and losses of catalytic activity. The $K_{0.5}$ for K^+ to shift digestion from the Na-type, and its sensitivity to dimethyl sulfoxide and Triton X-100, were consistent with K^+ acting at sites on the cytoplasmic face of the enzyme through which the K-phosphatase reaction also is activated. Reagents favoring the E_1 conformational states, oligomycin, Triton, and ATP, shifted the pattern toward the Na-type, whereas those favoring E_2 states, dimethyl sulfoxide, $MgCl_2$, and $MnCl_2$, shifted the pattern toward the K-type. Na-type digestion caused a greater loss of K-phosphatase than (Na + K)-ATPase activity, and the residual K-phosphatase activity was more sensitive to inhibition by Triton and ATP but stimulated more by dimethyl sulfoxide and inhibited less by P_i and $MnCl_2$; all these effects are consistent with such digestion shifting equilibria toward E_1 enzyme states. Accordingly, the $K_{0.5}$ for K^+ to activate the (Na + K)-ATPase was increased. However, the $K_{0.5}$ for the K-phosphatase was unchanged; this observation requires revision of previous formulations, and bears on additional aspects of enzyme activity as well.

Key Words: (Na + K)-ATPase; enzyme conformational states; oligomycin; dimethyl sulfoxide; tryptic proteolysis.

Introduction

Since conformational changes in the (Na + K)-ATPase may be pertinent to the physiological role of this enzyme as the Na,K-pump, such changes have been widely investigated in terms of activation kinetics for monovalent cations and nucleotide substrate (Robinson, 1967), cardiac glycoside inhibition and binding (Schwartz *et al.*, 1968; Albers *et al.*, 1968), and the properties of

[1]Department of Pharmacology, State University of New York, Upstate Medical Center, Syracuse, New York 13210.

0145-479X/82/1200-0319$03.00/0

modifiers such as oligomycin (Fahn *et al.*, 1966). The early formulations in terms of two major families of conformational states, E_1 and E_2, continue to be salient features of current reaction schemes portraying cyclical interconversions of the phosphorylated and dephosphorylated states:

$$E_1 \rightarrow E_1 - P \rightarrow E_2 - P \rightarrow E_2 \rightarrow E_1$$

(for recent reviews see Robinson and Flashner, 1979a; Cantley, 1981, Schuurmans Stekhoven and Bonting, 1981).

The individuality of such conformational states was recently emphasized by studies on the sensitivity of tryptic digestion of the enzyme to the presence of Na^+ or K^+ (Jorgensen, 1975). Thus, Na^+ and K^+ not only favor the alternate conformational states, E_1 and E_2, but also select two different patterns of tryptic digestion, the Na-type and the K-type. The experiments described here were designed to explore two aspects of such tryptic digestion: (a) to identify those ligands that can influence transitions between the Na-type and the K-type and to quantitate the K sensitivity in order to permit comparison with previously characterized K^+ sites of the enzyme; and (b) to examine the enzymatic properties remaining after tryptic digestion in terms of attributes of the E_1 and E_2 states, following the proposal that the Na-type digestion alters the equilibrium toward the E_1 states (Jorgensen, 1975; Jorgensen and Karlish, 1980), and to define the previously unexamined kinetic parameters of the modified enzyme. These experiments thus bear on relationships between availability and occupancy of distinct K^+ sites on the enzyme and the conformational states of the enzyme, and demonstrate the necessity for modifying previous formulations. The revised scheme is, moreover, pertinent not only to issues of tryptic digestion but also to unresolved problems of ligand sensitivity.

Methods and Materials

The enzyme preparation was obtained from medullae of frozen canine kidneys, following the procedure of Jorgensen (1974). The specific activity of the (Na + K)-ATPase activity ranged from 15 to 20 μmol P_i liberated/mg protein · min at 37°C.

Tryptic digestion of the enzyme was at 37°C for 10 min unless otherwise specified. The medium contained enzyme (about 15 μg/ml medium), 30 mM histidine · HCl/Tris (pH 7.8), 100 mM monovalent cation chloride, approximately 5 μg trypsin/ml medium, and other additions where noted. Digestion was halted by addition of a 4-fold excess of trypsin inhibitor (weight to weight of trypsin), followed by dilution with 0.25 M sucrose at 0°C, centrifugation for 40 min at 140,000 × *g*, and resuspension of the sedimented enzyme in 0.25 M

sucrose. Before use the trypsin was assayed using *Nα-p*-tosyl arginine methyl ester, and the concentration adjusted empirically so that tryptic digestion for 10 min in the presence of 100 mM NaCl reduced (Na + K)-ATPase activity to 45–50% of control activity. In all cases the residual activity after tryptic digestion is compared to activity of a concurrent control treated identically except that trypsin was added after trypsin inhibitor.

(Na + K)-ATPase activity was measured in terms of the production of P_i, as described previously (Robinson, 1967). The standard incubation medium contained 30 mM histidine · HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM ATP, 90 mM NaCl, and 10 mM KCl. K-phosphatase activity was measured in terms of nitrophenol production from incubation with nitrophenyl phosphate as substrate (Robinson, 1969). The standard incubation medium contained 30 mM histidine · HCl/Tris (pH 7.8), 3 mM $MgCl_2$, 3 mM nitrophenyl phosphate, and 10 mM KCl.

SDS-gel electrophoresis[2] on 7.5% polyacrylamide gels was performed as previously described (Robinson and Flashner, 1979b).

Incubations for assaying enzymatic activity were performed in duplicate or triplicate, and the data presented are averages of four or more such experiments (except for Fig. 1, which shows a single experiment with assays in triplicate). Where appropriate, data are presented with S.E.M.

Frozen canine kidneys were obtained from Pel Freeze; 7.5% polyacrylamide gels from BioRad; and ATP (vanadium free), *p*-nitrophenyl phosphate, oligomycin, Triton X-100, trypsin (type III, salt free), soybean trypsin inhibitor (type I-S), and *Nα-p*-tosyl arginine methyl ester from Sigma.

Results

Factors Favoring the Na-Type or K-Type Tryptic Digestion

Incubation of the (Na + K)-ATPase enzyme preparation with trypsin produced both a time- and ligand-dependent loss of enzymatic activity (Fig. 1). Tryptic digestion in the presence of 100 mM NaCl caused a slightly lesser loss of (Na + K)-ATPase activity but a far greater loss of K-phosphatase activity than digestion in the presence of 100 mM KCl. Moreover, when tryptic digestion was continued further in the presence of NaCl, then a second, slower loss of (Na + K)-ATPase activity occurred, but such a biphasic loss did not result from digestion in the presence of KCl (data not presented). Substituting 100 mM choline chloride for NaCl resulted in losses of both

[2]Abbreviations used: SDS, sodium dodecylsulfate.

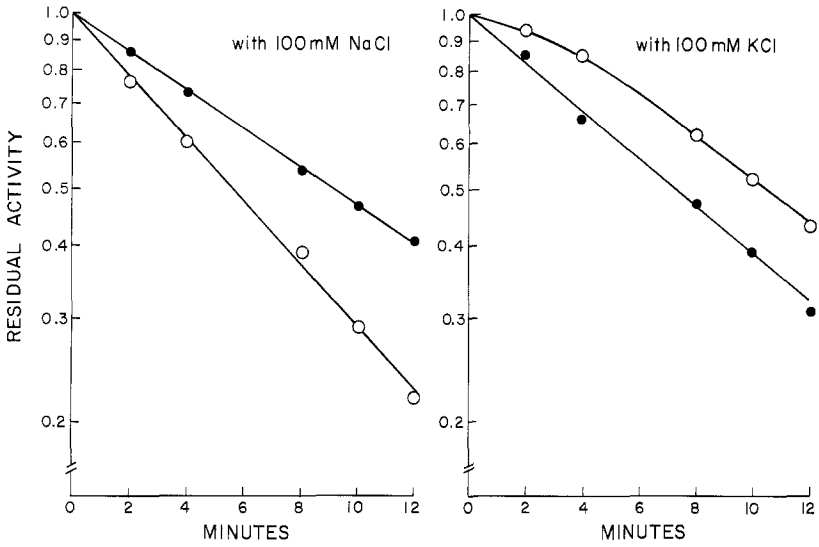

Fig. 1. Loss of enzymatic activity following tryptic digestion. The (Na + K)-ATPase preparation was incubated at 37°C in the presence of 30 mM histidine. HCl/Tris (pH 7.8), approximately 3 μg trypsin/ml medium, and either 100 mM NaCl (left-hand panel) or 100 mM KCl (right-hand panel). After the times indicated, digestion was halted by adding an excess of trypsin inhibitor, followed by dilution and centrifugation, as described under Methods and Materials. Residual (Na + K)-ATPase activity (●) and K-phosphatase activity (○) are presented relative to that of concurrent controls treated identically except that trypsin was added after trypsin inhibitor; data are presented from a single experiment with assays for activity performed in triplicate.

(Na + K)-ATPase and K-phosphatase activities quantitatively similar to those with NaCl; on the other hand, substituting 100 mM RbCl, 100 mM CsCl, or 1 mM TlCl plus 99 mM choline chloride for KCl resulted in a pattern of loss activities similar to those with KCl (data not presented). All these observations are in accord with earlier reports by Jorgensen (1975, 1977).

As Jorgensen (1975) also showed, tryptic digestion in the presence of 100 mM NaCl, during the initial monophasic loss of ATPase activity, did not alter the catalytic subunit of the enzyme detectably by SDS-gel electrophoresis, whereas digestion in the presence of 100 mM KCl produced two fragments on SDS-gel electrophoresis, of roughly 60,000 and 40,000 daltons (data not presented). To evaluate the extent of tryptic modification and to characterize the effects of various ligands on such digestion, measuring the loss of enzymatic activity rather than the appearance of fragments on electrophoretic patterns was thus chosen, since (a) no such fragments occurred during digestion with NaCl, and (b) those occurring during digestion with KCl were difficult to quantitate because they were of comparable magnitude to, and partially overlapped on SDS-gel electrophoresis, the glycoprotein subunit of the enzyme.

With this approach, the concentration dependence for KCl altering the Na-type tryptic digestion to the K-type could be measured by substituting equimolar amounts of KCl for choline chloride during tryptic digestion and then measuring the residual K-phosphatase activity (Fig. 2). As the KCl concentration was increased, less K-phosphatase activity was destroyed during the tryptic digestion, with a $K_{0.5}$ for sparing K-phosphatase activity of 1.3 mM. By contrast, substituting equimolar amounts of NaCl (from 1 to 100 mM) for choline chloride during tryptic digestion did not affect the amount of residual K-phosphatase activity (data not presented), confirming the identity of the Na- and choline-types of tryptic digestion.

Dimethyl sulfoxide and Triton X-100 affect differently the apparent

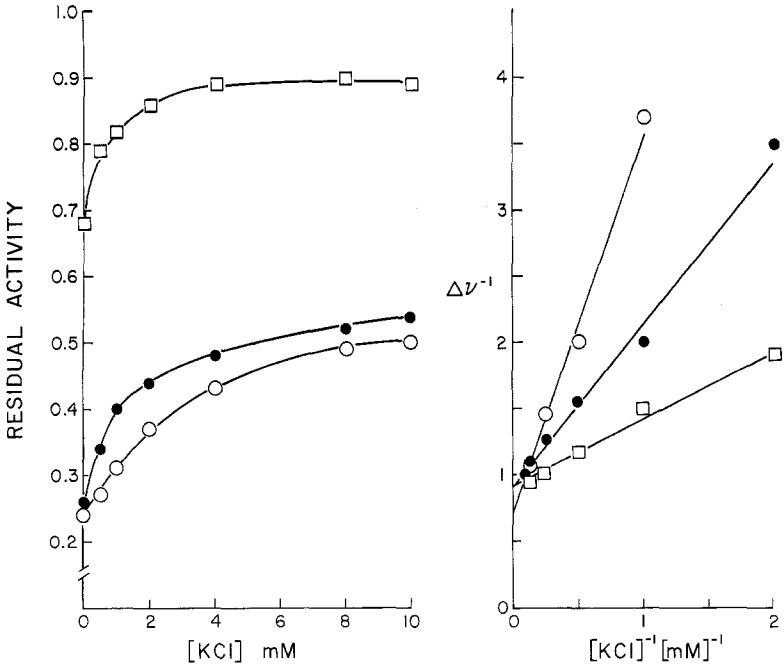

Fig. 2. Effect of KCl, present during tryptic digestion, on residual K-phosphatase activity. The (Na + K)-ATPase preparation was incubated with trypsin for 10 min at 37°C as described under Methods and Materials, but with either 100 mM choline chloride, or with the concentration of KCl indicated substituted for an equimolar concentration of choline chloride. In the left-hand panel the residual K-phosphatase activity (relative to concurrent controls treated identically except for trypsin being added after trypsin inhibitor) is plotted against the KCl concentration substituted for choline chloride, for experiments with no other additions during tryptic digestion (●), or for those with 0.008% (v/v) Triton X-100 (○) or with 10% (v/v) dimethyl sulfoxide (□) present during tryptic digestion. In the right-hand panel these data are replotted in double reciprocal form, in terms of the increment in K-phosphatase activity (Δv) due to KCl, with the increment at 10 mM KCl normalized to 1.0 in all cases.

Table I. Residual K-Phosphatase Activity After Tryptic Digestion[a]

Additions to tryptic digestion incubation	Residual K-phosphatase activity (% control) after tryptic digestion		
	100 mM choline chloride	1 mM KCl plus 99 mM choline chloride	100 mM KCl
None	26 ± 1	40 ± 2	54 ± 2
ATP, 1 mM	26 ± 2	29 ± 2	36 ± 3
Oligomycin, 10 μg/ml	25 ± 2	32 ± 3	49 ± 3
Triton X-100, 0.008%	24 ± 2	32 ± 3	50 ± 3
$MgCl_2$, 3 mM	42 ± 3	—	56 ± 4
$MnCl_2$, 3 mM	51 ± 2	—	60 ± 4
Dimethyl sulfoxide, 10%	68 ± 3	82 ± 4	89 ± 4

[a]Residual K-phosphatase activity, expressed as a percent of control activity treated identically except for the absence of trypsin, is presented for (Na + K)-ATPase enzyme preparations digested in media containing 100 mM choline chloride, 1 mM KCl and 99 mM choline chloride, or 100 mM KCl, plus the additions noted.

affinity of the two classes of sites through which K^+ activates the (Na + K)-ATPase and K-phosphatase reactions (Robinson, 1972, 1980). Correspondingly, these reagents affected the $K_{0.5}$ for KCl sparing the K-phosphatase activity during tryptic digestion (Fig. 2); dimethyl sulfoxide decreased the $K_{0.5}$ whereas Triton X-100 increased it. In the absence of KCl, Triton X-100 had little effect on the loss of K-phosphatase activity, but dimethyl sulfoxide markedly reduced such loss (Fig. 2; Table I). Dimethyl sulfoxide, at the concentration used here, had no effect on tryptic digestion of its artificial substrate, *Nα-p*-tosyl arginine methyl ester (data not presented).

Other reagents also thought to influence the equilibrium between E_1 and E_2 states of the enzyme correspondingly affected the loss of K-phosphatase activity during tryptic digestion (Table I). Thus, ATP, oligomycin, and Triton X-100 had little effect during tryptic digestion in 100 mM choline, but promoted the loss of K-phosphatase activity during tryptic digestion in the presence of KCl, particularly at the lower KCl concentration. Such changes support the conclusions from different experimental approaches that ATP (Post *et al.*, 1972), oligomycin (Fahn *et al.*, 1966), and Triton X-100 (Robinson, 1980) favor the E_1 states whereas K^+ favors the E_2 states (Post *et al.*, 1972). Conversely, $MgCl_2$, $MnCl_2$, and dimethyl sulfoxide, which other approaches indicate as favoring E_2 states (Robinson 1980, 1981), decreased the loss of K-phosphatase activity during tryptic digestion in 100 mM choline chloride (the concentrations of $MgCl_2$ and $MnCl_2$ listed were optimal for sparing K-phosphatase activity).

Enzymatic Properties After Na-Type Digestion

On the basis of increased Na-ADP/ATP exchange and decreased K-phosphatase activities after Na-type tryptic digestion, Jorgensen (1977)

Table II. Sensitivity of K-phosphatase Activity to Modifiers[a]

Additions to assay medium	Change in residual K-phosphatase acitivity	
	Control enzyme preparations	Enzyme digested with trypsin
None	(100)	(100)
Triton X-100, 0.008%	52 ± 3	41 ± 3
ATP, 0.1 mM	68 ± 3	59 ± 2
P_i, 2 mM	55 ± 2	67 ± 2
$MnCl_2$, 1 mM	56 ± 1	83 ± 4
Dimethyl sulfoxide, 10%	156 ± 9	197 ± 14

[a]The effects of various modifiers of the K-phosphatase reaction, when added to the standard assay medium, are presented relative to the activity in the absence of the modifier, for both the control enzyme preparation and that digested for 10 min in the presence of 100 mM choline chloride.

proposed that such treatment shifted the equilibria between the conformational states toward the E_1 family. To examine this proposal further, the sensitivity of the modified enzyme to a diverse group of reagents favoring the E_1 or E_2 states was compared to the response of the undigested enzyme. For the K-phosphatase reaction, probably catalyzed entirely by E_2 states, dimethyl sulfoxide, which selects such states, stimulated more after tryptic digestion (Table II). Similarly, inhibitors that react with E_2 states, P_i and Mn^{2+}, inhibited the modified enzyme less, whereas those that react with E_1 states, ATP and Triton X-100, inhibited more (Table II). The (Na + K)-ATPase reaction is catalyzed by both families of conformational states, cyclically, and thus effects are less readily predictable: dimethyl sulfoxide inhibited the modified enzyme more and oligomycin and Triton X-100 inhibited less, but Mn^{2+} also inhibited less (Table III).

Also in accord with this interpretation of a shift toward E_1 states is the increased $K_{0.5}$ for KCl as activator of the (Na + K)-ATPase reaction (Fig. 3), since the pertinent sites become available only after E_2-P formation (Robinson, 1973). Loss of (Na + K)-ATPase activity could not, however, be

Table III. Sensitivity of (Na + K)-ATPase Activity to Modifiers[a]

Additions to assay medium	Change in residual (Na + K)-ATPase acitivity	
	Control enzyme preparations	Enzyme digested with trypsin
None	(100)	(100)
Oligomycin, 10 μg/ml	34 ± 3	71 ± 6
Triton X-100, 0.008%	69 ± 6	74 ± 5
$MnCl_2$, 1 mM	70 ± 2	87 ± 3
Dimethyl sulfoxide, 10%	73 ± 3	62 ± 4

[a]The effects of various modifiers of the (Na + K)-ATPase reaction, when added to the standard assay medium, are presented relative to the activity in the absence of the modifier, for both the control enzyme preparation and that digested for 10 min in the presence of 100 mM choline chloride.

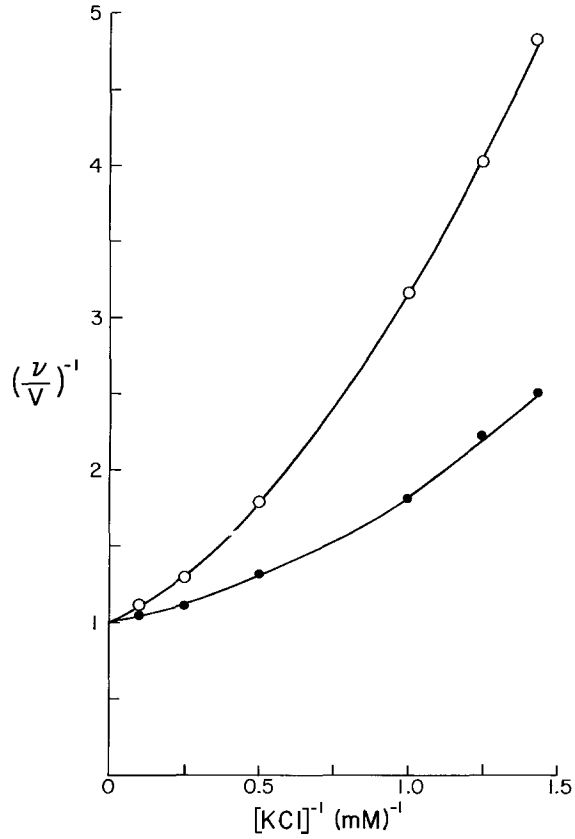

Fig. 3. Effect of tryptic digestion on K-activation of the (Na + K)-ATPase reaction. The enzyme preparation was incubated for 10 min in 100 mM choline chloride in the absence (●) or presence (○) of trypsin, as described under Methods and Materials. The residual (Na + K)-ATPase activity was then assayed in the standard medium modified to contain the concentration of KCl indicated. Data are presented in double-reciprocal form, with the velocity divided by the maximal velocity to permit easier comparison. $K_{0.5}$ values for KCl were 0.8 mM for control and 1.7 mM for trypsin-digested enzyme.

overcome merely by raising the KCl concentration further. The $K_{0.5}$ for Na^+ was not detectably altered (Fig. 4).

Surprisingly, the $K_{0.5}$ for KCl as activator of the K-phosphatase reaction was not increased (Fig. 5), despite the evidence in the preceding section indicating that occupancy of such sites is a major selector for the E_2 states. Inhibition of K-phosphatase activity by NaCl also was not affected by Na-type tryptic digestion (Fig. 6). The K_m for nitrophenyl phosphate as substrate for the K-phosphatase reaction was not altered either (data not presented).

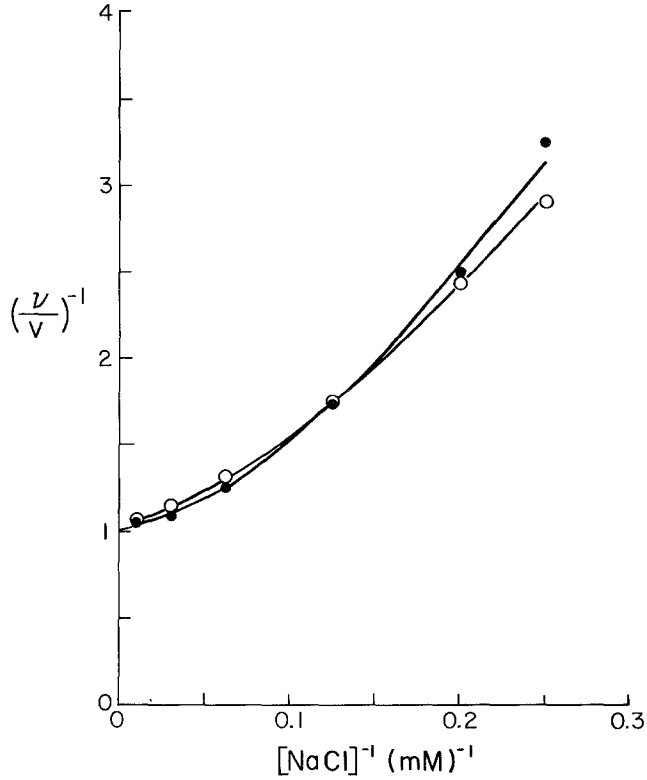

Fig. 4. Effect of tryptic digestion on Na-activation of the (Na + K)-ATPase reaction. The enzyme preparation was treated as in Fig. 3, and the assay of residual (Na + K)-ATPase activity measured identically, except that in this case the NaCl concentration was modified as indicated. Data are presented as in Fig. 3, for control (●) and digested (○) enzyme; the $K_{0.5}$ values for NaCl were 7 mM in both cases.

Just as the loss of (Na + K)-ATPase activity after Na-type digestion could not be overcome by infinite KCl concentration (Fig. 3), so the loss of K-phosphatase activity could not be overcome by raising the KCl concentration (Fig. 5), nor, as deduced by extrapolation, with infinite concentrations of reagents favoring E_2 states, such as dimethyl sulfoxide or $MgCl_2$ (data not presented).

Discussion

Early studies on the catalytic properties of the (Na + K)-ATPase revealed two forms of the phosphorylated enzyme, one susceptible to dephosphorylation by ADP, termed E_1-P, and the other by water in the presence of

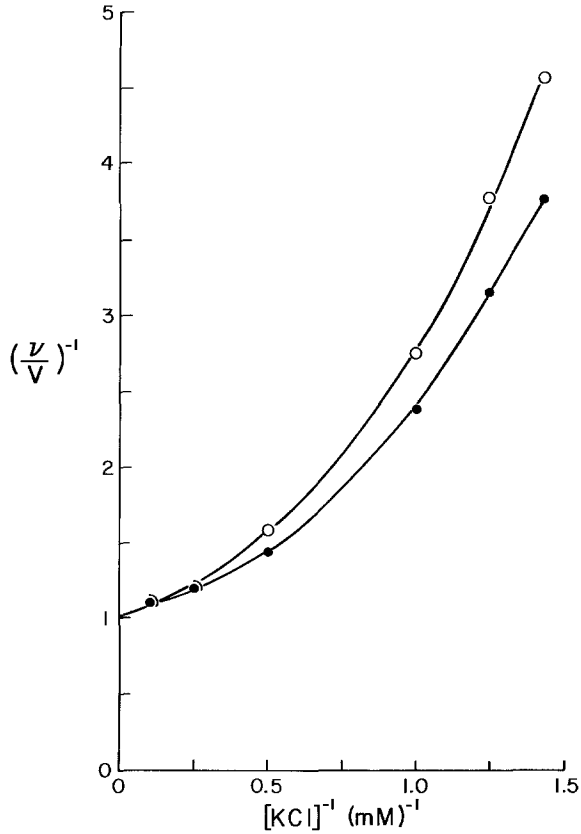

Fig. 5. Effect of tryptic digestion on K-activation of the K-phosphatase reaction. The enzyme preparation was treated as in Fig. 3, and the residual K-phosphatase activity was then assayed in the standard medium modified to contain the concentration of KCl indicated. Data are presented as in Fig. 3, for control (●) and digested (O) enzyme; the $K_{0.5}$ values for KCl were 1.2 and 1.4 mM, respectively.

K^+, termed E_2-P (Fahn *et al.*, 1966; Post *et al.*, 1969). Furthermore, E_1-P bears high-affinity sites for Na^+ accessible to the cytoplasm, whereas E_2-P bears high-affinity sites for K^+ accessible extracellulary; corresponding dephosphorylated forms, E_1 and E_2, complete a reaction cycle allowing sequential transport of Na^+ out of the cell, and K^+ into the cell. The major goal of the experiments described here is an understanding of the relationship between the ligand-selected states of the enzyme manifested by the two patterns of tryptic digestion, the Na- and K-types, and, reciprocally, the properties of the modified enzyme with particular regard to ligand-selected states and the E_1 and E_2 conformational families.

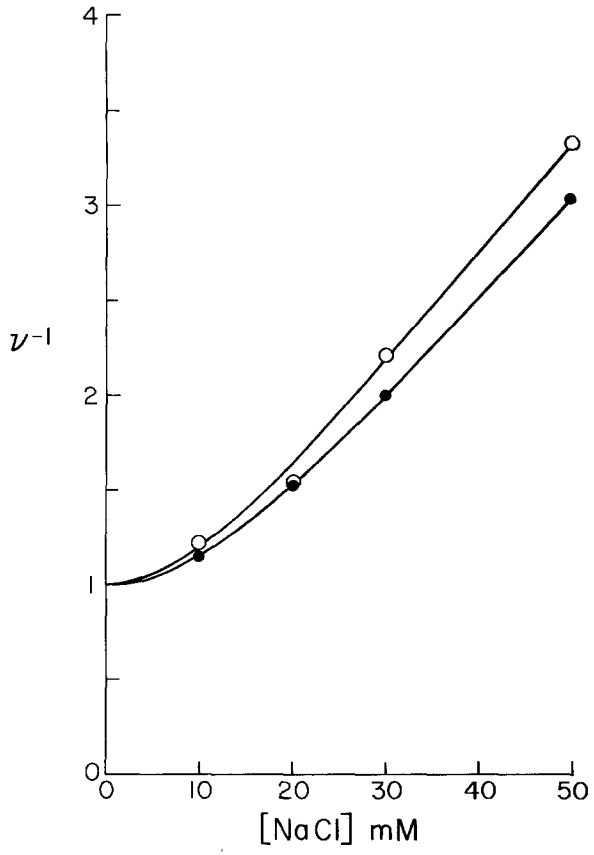

Fig. 6. Effect of tryptic digestion on inhibition of the K-phosphatase reaction by NaCl. The enzyme preparation was treated as in Fig. 3, and the residual K-phosphatase activity was then assayed in the standard medium modified to contain the concentration of NaCl indicated. Data are presented in the form of a Dixon plot, for control (●) and digested (○) enzyme.

Interpretations of Na^+ and K^+ as selectors of E_1 and E_2 states, respectively, are supported by Na^+ and K^+ selecting alternate patterns of tryptic digestion (Jorgensen, 1975) and influencing intrinsic protein fluorescence oppositely (Karlish and Yates, 1978). The ability of Na^+, at high-affinity sites accessible intracellulary, to activate enzyme phosphorylation by ATP and thus form E_1-P fits these formulations readily; that Na^+ is not required to select the Na-type of tryptic digestion (i.e., choline chloride will substitute, although not for enzyme phosphorylation) may be attributed to the enzyme in the absence of both Na^+ and K^+ existing largely in the E_1 state. More complicated are relationships with K^+: dephosphorylation of E_2-P is activated

by K^+ at high-affinity sites accessible extracellularly, termed β-sites (Robinson, 1975), whereas in the absence of enzyme phosphorylation moderate-affinity sites, termed α-sites, are available cytoplasmically to activate reactions considered to represent E_2 functions, such as the K-phosphatase reaction (Drapeau and Blostein, 1980) and vanadate binding (Robinson and Mercer, 1981). Thus, the E_2 conformational state, as so defined, is selected by K^+ from the cytoplasmic but not the extracellular face. In these cases the actual locus at which K^+ acts may correspond to the "occluded K^+ sites," accessible, as is activation of the phosphatase reaction and vanadate binding, from the extracellular face only in conjunction with E_2-P formation (Post *et al.*, 1972; Beaugé and Glynn, 1979; Drapeau and Blostein, 1980; Robinson and Mercer, 1981).

In this context, the Na-type of digestion corresponds to tryptic hydrolysis of the (Na + K)-ATPase in the E_1 conformational state. Thus, not only does Na^+ select the Na-type digestion, but in the presence of K^+ the Na-type digestion is favored by oligomycin, Triton X-100, and ATP (Table I): oligomycin blocks conversion of E_1-P to E_2-P (Fahn *et al.*, 1966) and also selects E_1 over E_2 states (Robinson, 1980); Triton X-100 alters the equilibrium toward E_1 states (Robinson, 1980); and ATP binds to high-affinity sites on E_1 thereby favoring conversion to E_1 states (Post *et al.*, 1972).

Conversely, the K-type of tryptic digestion corresponds to hydrolysis of the (Na + K)-ATPase in the E_2 conformational state, favored by K^+ acting through the cytoplasmic moderate-affinity α-sites. Thus, the $K_{0.5}$ for K^+, 1.3 mM (Fig. 2), corresponds to that for K^+ activating the K-phosphatase reaction (Fig. 5), not to K^+ at the high-affinity β-sites where in the absence of competing Na^+ the $K_{0.5}$ is on the order of 0.1 mM (Robinson, 1975). Also in accord with this assignment are the effects of dimethyl sulfoxide and Triton X-100 on the $K_{0.5}$ for K^+ (Fig. 2): dimethyl sulfoxide decreases the $K_{0.5}$ for K^+ in the K-phosphatase reaction but increases it in the (Na + K)-ATPase reaction utilizing the extracellularly available β-sites (Robinson, 1972), whereas Triton X-100 increases the $K_{0.5}$ in the K-phosphatase reaction but has little effect on it in the (Na + K)-ATPase reaction (Robinson, 1980). A further link with the α-sites was provided in a paper published by Karlish and Pick (1981) after these studies were completed, showing that K^+ selects the K-type digestion through sites accessible cytoplasmically.

Identification of the K-type digestion with the E_2 state is also supported by the effects of dimethyl sulfoxide, $MgCl_2$, and $MnCl_2$ in the absence of K^+ (Table I), since these agents have been considered to favor the E_2 state (Robinson, 1980, 1981). The relative efficacy for producing the K-type digestion was $K^+ > Mn^{2+} > Mg^{2+}$ (Table I), whereas for activating the K-phosphatase reaction the sequence is $K^+ + Mg^{2+} > K^+ + Mn^{2+} > Mn^{2+} > Mg^{2+} > K^+$ (Robinson, 1981), further indicating different classes within the E_2 family of conformational states.

Karlish (1980), on the other hand, found that Mg^{2+} produced an E_1-type of fluorescence with fluorescein isothiocyanate-labeled enzyme, and Jorgensen (1975) found no effect of Mg^{2+} on the Na-type pattern of digestion. Nevertheless, Castro and Farley (1979), in agreement with the results presented here, reported that Mg^{2+} favored the K-type pattern of tryptic fragments. The reason for these discrepancies is unknown.

The properties of the (Na + K)-ATPase modified by Na-type digestion appear to reflect a shift toward E_1 states of the enzyme, as originally proposed by Jorgensen (1975) on the basis of increased Na-ADP/ATP exchange and decreased K-phosphatase activities after such treatment. Such changes were particularly clear-cut in the K-phosphatase reaction, probably catalyzed entirely by E_2 states. After Na-type digestion dimethyl sulfoxide, which shifts equilibria toward E_2 states (Robinson, 1980), stimulated even more (Table II). Inorganic phosphate, a potent inhibitor of this but not the (Na + K)-ATPase reaction (Robinson *et al.*, 1978), inhibited less, as did Mn^{2+}, which may inhibit by hindering dissociation of P_i from the hydrolytic site of the E_2 state (Robinson, 1982). Conversely, ATP, which binds with low affinity to E_2 but with high affinity to E_1 states (Post *et al.*, 1972), inhibited the modified enzyme more, as did Triton X-100, which also favors E_1 states (Robinson, 1980).

Correspondingly, the $K_{0.5}$ for K^+ as activator of the (Na + K)-ATPase reaction is increased by such digestion (Fig. 3), in accord with its binding to β-sites on E_2P. That K^+ could not overcome the inactivation implies, however, that tryptic digestion inactivates by other processes in addition to merely shifting equilibria toward E_1 states.

By contrast, the $K_{0.5}$ for K^+ as activator of the K-phosphatase reaction was little changed (Fig. 5).[3] This observation is especially surprising in light of the evidence (above) that the same sites for K^+, the α-sites, mediate activation of this reaction and the shifts in equilibria toward E_2 states. In addition, Jorgensen and Karlish (1980) showed that after Na-type digestion the $K_{0.5}$ for K^+ to shift the equilibrium from E_1 to E_2 states, measured in terms of intrinsic protein fluorescence, is increased twofold. Assuming that $E_2 \cdot K$ is the active species for catalysis, their model,

$$E_1 + K^+ \overset{K_p}{\rightleftharpoons} E_1 \cdot K \overset{K_c}{\rightleftharpoons} E_2 \cdot K \qquad (1)$$

gives the same $K_{0.5}$ for both intrinsic protein fluorescence and the K-

[3] A trivial explanation, that Na-type digestion destroys K-phosphatase activity, and thus any residual activity represents only undigested enzyme with its unchanged $K_{0.5}$, is contradicted by experiments showing altered sensitivity of the residual K-phosphatase activity to modifiers (Table II). Nevertheless, the modified enzyme preparation most probably is heterogeneous, containing both digested and undigested enzyme molecules.

phosphatase reaction:

$$K_{0.5} = \frac{K'_p}{K_c - 1} \tag{2}$$

(In this model K_c is the equilibrium constant for the isomerization, K_p is the association constant for K-binding, and K'_p is the dissociation constant; rapid equilibrium is assumed, and the possibility of cooperative binding is ignored.)

The discrepancy between Fig. 5 and the fluorescent data may, however, be reconciled merely by altering the model to include an E_2 form in the absence of K^+ that is in equilibrium with E_1 (like the above model, binding of K^+ to E_2 is assumed to be extremely tight):

$$E_2 \overset{K_1}{\rightleftharpoons} E_1 + K^+ \overset{K_2}{\rightleftharpoons} E_1 \cdot K \overset{K_3}{\rightleftharpoons} E_2 \cdot K \tag{3}$$

In this case, the $K_{0.5}$ for intrinsic protein fluorescence changes, when $E_1 + E_1(K) = E_2 + E_2(K)$, is given by

$$K_{0.5} = \frac{K'_2(K_1 - 1)}{K_1(K_3 - 1)} \tag{4}$$

whereas the $K_{0.5}$ for the K-phosphatase reaction, when $E_2(K) = E_2 + E_1 + E_1(K)$, is given by

$$K_{0.5} = \frac{K'_2(K_1 + 1)}{K_1(K_3 - 1)} \tag{5}$$

(in both these formulae K'_2 is the reciprocal of K_2, to express the relationship as a dissociation constant). With these relationships it is then possible for the $K_{0.5}$ for fluorescence changes [Eq. (4)] to double while the $K_{0.5}$ for the K-phosphatase reaction [Eq. (5)] is unchanged (for example, with $K_1 = 2$, $K'_2 = 8$ mM, and $K_3 = 10$ the respective $K_{0.5}$ values are 0.44 and 1.33 mM; modifications causing a shift toward E_1 states, as represented by $K_1 = 6$ and $K_3 = 8$ but with K'_2 unchanged, more than double the $K_{0.5}$ for fluorescence changes to 0.95 mM, while the $K_{0.5}$ for the K-phosphatase is unchanged at 1.33 mM). Moreover, this formulation can also account (J. D. Robinson, in preparation) for such heretofore puzzling observations as the insensitivity of the K-phosphatase reaction to oligomycin despite oligomycin favoring E_1 states, and the induction of sensitivity by Na^+ (Robinson, 1970) and by Triton X-100 (Robinson, 1980).

Acknowledgments

The careful technical assistance of Mrs. Nancy Martin is gratefully acknowledged. This work was supported by U.S. Public Health Service research grant NS-05430.

References

Albers, R. W., Koval, G. J., and Siegel, G. J. (1968). *Mol. Pharmacol.* **4,** 324–336.
Beauge, L. A., and Glynn, I. M. (1979). *Nature (London)* **280,** 510–512.
Cantley, L. C. (1981). *Curr. Top. Bioenerg.* **11,** 201–237.
Castro, J., and Farley, R. A. (1979). *J. Biol. Chem.* **254,** 2221–2228.
Drapeau, P., and Blostein, R. (1980). *J. Biol. Chem.* **255,** 7827–7834.
Fahn, S., Koval, G. J., and Albers, R. W. (1966). *J. Biol. Chem.* **241,** 1882–1889.
Jorgensen, P. L. (1974). *Biochim. Biophys. Acta* **356,** 36–52.
Jorgensen, P. L. (1975). *Biochim. Biophys. Acta* **401,** 399–415.
Jorgensen, P. L. (1977). *Biochim. Biophys. Acta* **466,** 97–108.
Jorgensen, P. L., and Karlish, S. J. D. (1980). *Biochim. Biophys. Acta* **597,** 305–317.
Karlish, S. J. D. (1980). *J. Bioenerg. Biomembr.* **12,** 111–136.
Karlish, S. J. D., and Yates, D. W. (1978). *Biochim. Biophys. Acta* **527,** 115–130.
Karlish, S. J. D., and Pick, U. (1981). *J. Physiol.* **312,** 505–529.
Post, R. L., Kume, S., Tobin, T., Orcutt, B., and Sen, A. K. (1969). *J. Gen. Physiol.* **54,** 306S–326S.
Post, R. L., Hegyvary, C., and Kume, S. (1972). *J. Biol. Chem.* **247,** 6530–6540.
Robinson, J. D. (1967). *Biochemistry* **6,** 3250–3258.
Robinson, J. D. (1969). *Biochemistry* **8,** 3348–3355.
Robinson, J. D. (1970). *Arch. Biochem. Biophys.* **139,** 164–171.
Robinson, J. D. (1972). *Biochim. Biophys. Acta* **274,** 542–550.
Robinson, J. D. (1973). *Arch. Biochem. Biophys.* **156,** 232–243.
Robinson, J. D. (1975). *Biochim. Biophys. Acta* **384,** 250–264.
Robinson, J. D. (1980). *Biochim. Biophys. Acta* **598,** 543–553.
Robinson, J. D. (1981). *Biochim. Biophys. Acta* **642,** 405–417.
Robinson, J. D. (1982). *Curr. Top. Membr. Transport,* in press.
Robinson, J. D., Flashner, M. S., and Marin, G. K. (1978). *Biochim. Biophys. Acta* **509,** 419–428.
Robinson, J. D., and Flashner, M. S. (1979a). *Biochim. Biophys. Acta* **549,** 145–176.
Robinson, J. D., and Flashner, M. S. (1979b). *Arch. Biochem. Biophys.* **196,** 350–362.
Robinson, J. D., and Mercer, R. W. (1981). *J. Bioenerg. Biomembr.* **13,** 205–218.
Schuurmans Stekhoven, F., and Bonting, S. L. (1981). *Physiol. Rev.* **61,** 1–76.
Schwartz, A., Matsui, H., and Laughter, A. H. (1968). *Science* **160,** 323–325.