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DEFECTIVE CONFORMATIONAL RESPONSE IN A SELECTIVELY TRYPSINIZED ($Na^+ + K^+$)-ATPase STUDIED WITH TRYPTOPHAN FLUORESCENCE

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Summary

1. Monitoring protein conformations of purified ($Na^+ + K^+$)-ATPase with intrinsic fluorescence we have examined if altered conformational responses accompany the defective catalytic and transport processes in selectively modified 'invalid' ($Na^+ + K^+$)-ATPase which is obtained by graded tryptic digestion of the Na^+ form of the protein.

2. The protein fluorescence intensity of the K^+ form (E_2K) of both control and invalid ($Na^+ + K^+$)-ATPase is 2—3% higher than that of the Na^+ form (E_1Na). By varying the NaCl concentration we found evidence for different fluorescence intensities of the two phosphoenzymes; E_2P has the same fluorescence intensity as E_2K and the intensity of E_1P is similar to that of E_1Na. The fraction of phosphoenzyme present as E_2P can therefore be determined as the amplitude of the fluorescence change accompanying phosphorylation in the absence of K^+ divided by the amplitude of the full response to K^+.

3. Titration of the fluorescence responses of the invalid ($Na^+ + K^+$)-ATPase shows that the tryptic split alters the poise of the equilibria between the cation-bound conformations, E_1Na and E_2K, and between the phosphoforms, E_1P and E_2P, in the direction of the E_1 forms.

4. Vanadate binds to the Mg^{2+}-bound form of E_2K and prevents further changes in fluorescence intensity of the protein. The conformative responses of invalid ($Na^+ + K^+$)-ATPase are insensitive to vanadate in agreement with the reduced vanadate binding affinity of this enzyme.

5. The defective conformative response of the invalid ($Na^+ + K^+$)-ATPase in

Abbreviation: SDS, sodium dodecyl sulfate.

relation to its catalytic defects, reduced Na^+ transport, and insensitivity to vanadate suggest that the transitions between Na^+ forms (E_1) and K^+ forms (E_2) of the protein are coupled to the catalytic and transport reactions of the ($Na^+ + K^+$)-pump.

Introduction

Selective protein modification is a useful way of establishing structure-function relationships in several enzymes and transport systems. A selectively modified ($Na^+ + K^+$)-ATPase is produced by graded tryptic digestion of the Na^+ form of the purified enzyme from outer renal medulla membranes [1,2]. The controlled cleavage results in a stable, membrane-bound preparation termed 'invalid ($Na^+ + K^+$)-ATPase (TENa)' which has an intact number of binding sites for ATP and phosphorylation, but a defective K^+-stimulated dephosphorylation and K^+-phosphatase activity [1,2]. The trypsinized ($Na^+ + K^+$)-ATPase has become resistant to vanadate [3]. It reconstitutes well into phospholipid vesicles and catalyzes active coupled ($Na^+ + K^+$)-transport, but the transport ratio is reduced to 1—2 Na^+/2 K^+ as compared with the ratio of 3 Na^+/2 K^+ for control ($Na^+ + K^+$)-ATPase [4]. In the present work we have examined if the defective catalytic and transport processes in the trypsinized ($Na^+ + K^+$)-ATPase are attributable to altered conformational responses of the protein.

Two conformational states, a Na^+ form (E_1) and a K^+ form (E_2) of the α-subunit with molecular weight close to 100 000 were detected in the purified renal ($Na^+ + K^+$)-ATPase with trypsin as tool [1,5]. Transitions between the conformations can now be conveniently studied with the aid of extrinsic and intrinsic fluorescent probes [6—9]. Intrinsic protein fluorescence turns out to be a particularly useful tool for comparing native [8] and invalid ($Na^+ + K^+$)-ATPase. The protein fluorescence intensity of the K^+ form of unmodified ($Na^+ + K^+$)-ATPase is 2—3% higher than that of the Na^+ form and phosphorylation of the catalytic protein from ATP is also accompanied by an increase in fluorescence intensity [8]. In the trypsinized ($Na^+ + K^+$)-ATPase the fraction of ADP-sensitive phosphoenzyme E_1P is increased and K^+-stimulated dephosphorylation is impaired [2]. This could reflect an altered equilibrium between the E_1P and the K^+-sensitive E_2P and so we decided to characterize further the phosphoenzyme conformations in normal and invalid ($Na^+ + K^+$)-ATPase by means of the tryptophan fluorescence signal. The proportions of E_1P and E_2P can be manipulated by varying the NaCl concentration [10,11] and we have exploited this observation in order to look for differences in the protein fluorescence intensities of the two phosphoenzyme conformations. Vanadate ion has recently been reported to inhibit ($Na^+ + K^+$)-ATPase [12,13] and binds [14] to the enzyme with a very high affinity in the presence of Mg^{2+} and K^+. With invalid enzyme the binding affinity for VO_3^- is reduced [4] and the ATPase [3] and residual active Na^+ transport [4] are resistant to vanadate. Since the effects of vanadate on unmodified enzyme could imply that it affects the conformational transitions between the Na^+ form and K^+ form we have also looked at its actions on the protein fluorescence changes in both control and invalid ($Na^+ + K^+$)-ATPase.

Comparison of the conformational changes in the protein of control and trypsinized ($Na^+ + K^+$)-ATPase following binding of cations or nucleotides and phosphorylation from ATP shows that the tryptic split alters the poise of the equilibria between both dephosphoforms E_1Na and E_2K, and between phosphoforms E_1P and E_2P in the direction of the E_1 state.

Methods

($Na^+ + K^+$)-ATPase was purified from the outer medulla of pig kidney by isopycnic centrifugation in the Ti-14 Beckman zonal rotor of a microsomal fraction after incubation for 30 min at 20°C with SDS in presence of ATP as before [15]. The preparations had specific activities of 28—36 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ protein and the binding capacities for [^{3}H]ouabain, [^{14}C]ATP and [^{48}V]-vanadate were 2.9—3.6 $nmol \cdot mg^{-1}$ protein. Preparation of invalid ($Na^+ + K^+$)-ATPase by digestion with trypsin in presence of 150 mM NaCl to 40% of control ($Na^+ + K^+$)-ATPase activity and wash of the membranes by centrifugation was done as before [1,2]. Prior to fluorescence assays the membranes were washed twice in 0.1 mM EDTA, 25 mM Tris, pH 7.2, by centrifugation for 10 min at 100 000 rev./min in the Beckman Airfuge.

Fluorescence measurements were made as described in detail by Karlish and Yates [8] using a Perkin Elmer MPF 44A spectrofluorimeter, fitted with a continuously stirred 1 cm cuvette, maintained at 25°C. Aliquots of the ($Na^+ + K^+$)-ATPase containing 20—26 μg protein were diluted to about 30 nM with 2.5 ml 0.1 mM EDTA, 25 mM Tris-HCl, pH 7.2. Impurities were removed from buffer and electrolyte solutions by filtration through 0.2 μm Millipore filters mounted on syringes. 0.1 mM EDTA was added to the 25 mM Tris-HCl buffer in order to avoid interference with the changes in fluorescence signals by contaminant heavy metals.

Results

Transitions between E_1 and E_2

Recordings of the steady-state levels of fluorescence intensity in Fig. 1 illustrate the antagonism between K^+ and ADP or Na^+ (cf. Refs. 5 and 7). The 3% increase in fluorescence intensity after addition of K^+ accompanied the transition from the Na^+ form (E_1) to the K^+ form (E_2) of the purified ($Na^+ + K^+$)-ATPase. Reversal of the fluorescence signal to the original level accompanied transition from the K^+ form (E_2) to the Na^+ form (E_1) after addition of low concentrations of ADP, ATP (see also Fig. 5), or NaCl.

At equal protein concentrations, the amplitude of the increase in fluorescence intensity after addition of excess K^+ was very similar for control and invalid ($Na^+ + K^+$)-ATPase (Figs. 2 and 6). Titration of the responses to K^+ in the absence of Na^+ or with 10 mM Na^+ showed, however, that the invalid ($Na^+ + K^+$)-ATPase was more resistant to K^+ than the control (Figs. 2 and 3). The $K_{0.5}$ for K^+ was about 2-fold lower for the control than for the invalid ($Na^+ + K^+$)-ATPase (Fig. 3). Conversely, titration of the response to ADP (Fig. 1) in presence of a low concentration of K^+ showed that the invalid ($Na^+ + K^+$)-ATPase was 2—3-fold more sensitive to ADP than the control

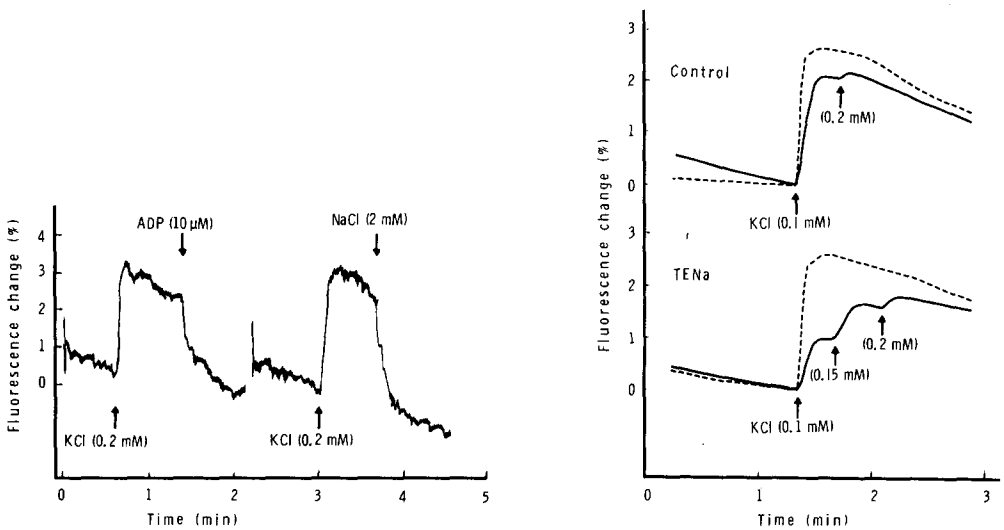


Fig. 1. Record of the increase in fluorescence intensity accompanying transition from the Na^+ form (E_1) to the K^+ form (E_2) after addition of KCl and of the decrease in fluorescence intensity accompanying transition from the K^+ form (E_2) to the Na^+ form (E_1) after addition of ADP (left) or NaCl (right). Conditions given in Methods. After recording the emission spectrum with excitation wavelength 295 nm using slit widths of 10 nm on both monocromators, the fluorescence intensity at 328 nm was monitored with 10-fold amplification of the total signal and a time constant of 1.5 s.

Fig. 2. Averaged records showing titration of the fluorescence response to K^+ of control (above) and trypsinized (below) ($Na^+ + K^+$)-ATPase. ------, the response to 1.2 mM KCl, which yields a maximal increase in fluorescence intensity.

(Fig. 4). The apparent affinity of the invalid ($Na^+ + K^+$)-ATPase to K^+ is reduced, and the apparent affinity to ADP appears to be increased, as compared with the control enzyme. Thus the equilibrium between the Na^+ form

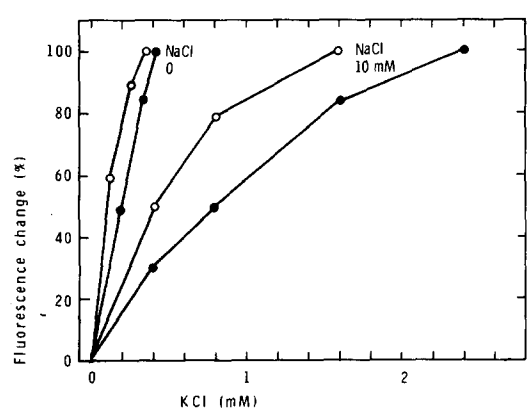
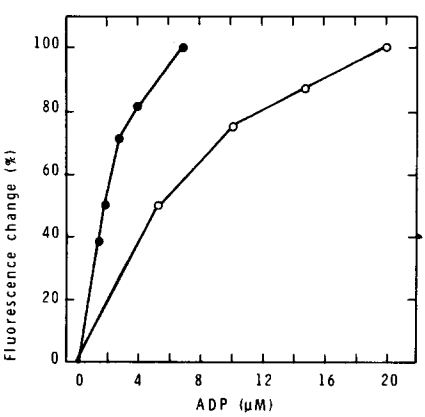


Fig. 3. Concentration dependence of the increase in fluorescence intensity following addition of K^+ to control (○) and trypsinized (●) ($Na^+ + K^+$)-ATPase without NaCl (left) and with 10 mM NaCl (right). Titrations were performed as in Fig. 2. The data are mean values of two determinations. The amplitude of the increase in fluorescence intensity following addition of KCl is given in percent of the maximum response to KCl.



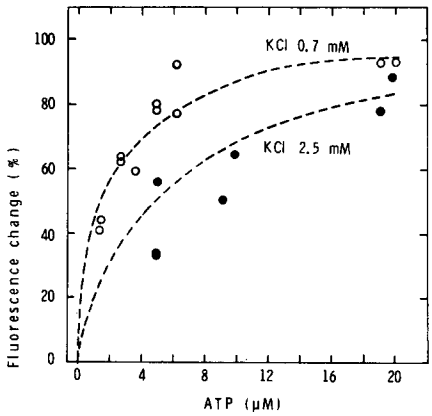


Fig. 4. Concentration dependence of the decrease in fluorescence intensity following addition of ADP to control (○) and trypsinized (●) $(Na^+ + K^+)$-ATPase in presence of 0.3 mM KCl. Procedure as in Fig. 1. The data are mean values of two determinations.

Fig. 5. Concentration dependence of the decrease in fluorescence intensity following addition of ATP to control $(Na^+ + K^+)$-ATPase in the presence of 0.7 mM KCl (○) and 2.5 mM KCl (●). Procedure as in Fig. 4. The data are the results of a series of consecutive titrations. In each experiment were added three 0.5 μl portions of ATP solutions with concentrations from 10 mM to 30 mM and the data were calculated from steady levels of fluorescence attained after each addition. ------, the ATP titration curves calculated for the two concentrations of KCl on basis of the model in Appendix.

(E_1) and the K^+ form (E_2) of the protein in the invalid $(Na^+ + K^+)$-ATPase appears to be shifted in direction of E_1.

The K^+-nucleotide antagonism was investigated further with control enzyme as shown in Fig. 5. Low concentrations of ATP reversed the K^+-dependent fluorescence change, and the apparent affinity for the nucleotide was clearly reduced as the concentration of K^+ was raised. The dotted lines represent the same ATP titration curves calculated on the basis of the model (Appendix) which is discussed below.

Transition from E_1P to E_2P

In Fig. 6 the responses to addition of K^+ (a) are compared to the increases in fluorescence intensity following addition of ATP to media containing Mg^{2+} and Na^+ (b), or Mg^{2+}, Na^+, and K^+ (c). In the invalid $(Na^+ + K^+)$-ATPase the magnitude of the increase in fluorescence intensity following phosphorylation from ATP (Fig. 6b) was about the same as in control, but the further increase in fluorescence intensity after subsequent addition of K^+ was abolished in the trypsinized preparation. Furthermore, the amplitude of the response of the invalid $(Na^+ + K^+)$-ATPase to addition of ATP in the presence of Mg^{2+}, Na^+, and K^+ was diminished (Fig. 6c).

The increase in fluorescence intensity following addition of ATP to media containing Mg^{2+} and Na^+ was about 2/3 of the maximum response to K^+ (Fig. 6). In other experiments somewhat variable and smaller responses (cf. Ref. 8) were observed. In order to examine whether this reflects differences in the fluorescence intensities of E_1P and E_2P, we have varied the NaCl concentration as discussed in Introduction. The responses of the control (Na^+ +

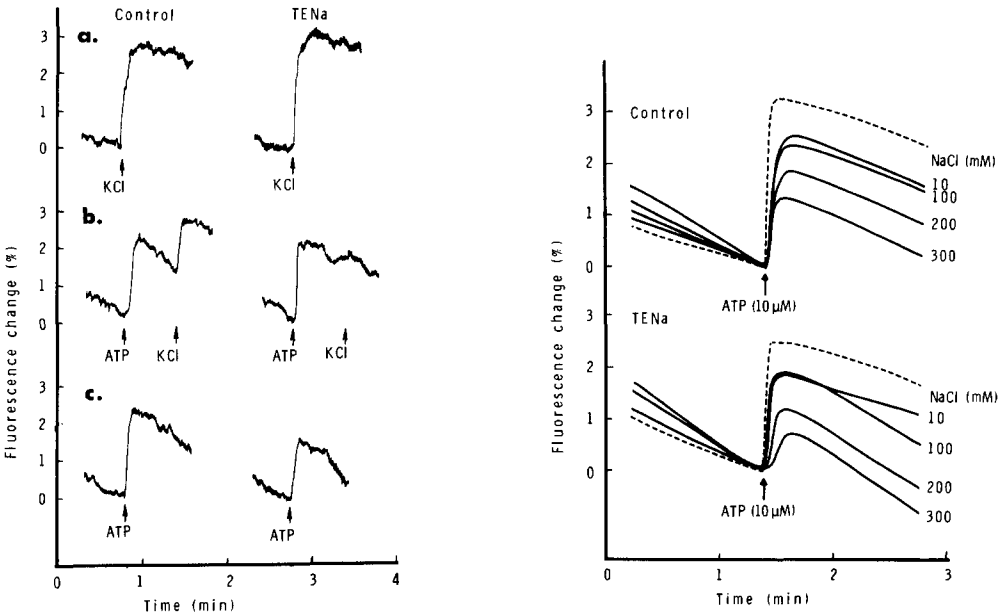


Fig. 6. Record of the changes in fluorescence intensities of control (left) and trypsinized (right) ($Na^+ + K^+$)-ATPase following transition from the Na^+ form (E_1) to the K^+ form (E_2) by binding of K^+ (a) or by phosphorylation from ATP (b and c). The conditions were: (a) 0.1 mM EDTA, 25 Tris, pH 7.2. (b) 100 mM NaCl and 2 mM $MgCl_2$ were added. (c) 1.5 mM KCl, 97.5 mM NaCl and 2 mM $MgCl_2$ were added.

Fig. 7. Averaged records showing the effect of NaCl on the amplitude of the increase in fluorescence intensity accompanying phosphorylation from ATP of control (above) or trypsinized (below) ($Na^+ + K^+$)-ATPase. ———, tracings of the responses to phosphorylation from ATP at varying concentrations of NaCl in the presence of 2 mM $MgCl_2$. The ionic strength was kept constant by adding Tris-HCl. - - - - - -, tracings of the responses to ATP in the presence of 2 mM $MgCl_2$, 100 mM NaCl, 2 mM KCl.

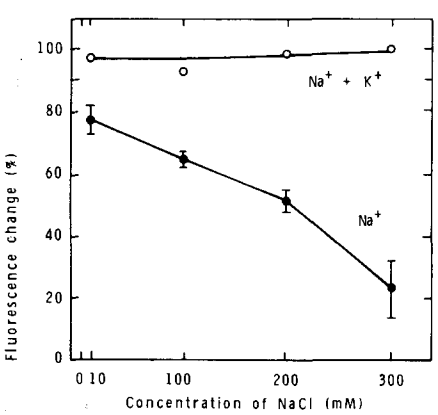


Fig. 8. Effect of NaCl on the amplitude of the increase in fluorescence intensity accompanying addition of ATP to a NaCl or a NaCl plus KCl medium. Procedure as in Fig. 7. Tris-HCl was added to keep the ionic strength constant. To obtain the Na^+ plus K^+ data, 0.2—6 mM KCl was added to maintain a $Na^+ : K^+$ ratio of 100 : 2. Data with Na^+ alone are mean values ± S.E. of three or four measurements.

TABLE I

THE DEPENDENCE OF [^{14}C]ADP-ATP EXCHANGE ACTIVITY ON THE CONCENTRATION OF NaCl

Mean values ± S.E. of three determinations on control ($Na^+ + K^+$)-ATPase are given. For assay of exchange activity, aliquots each containing 5 μg protein were incubated for 15 min at 26°C in a total volume of 100 μl with 5 mM ATP, 1.25 mM [^{14}C]ADP, 5 mM $MgCl_2$ and 10—300 mM NaCl. The ionic strength was kept constant by exchanging NaCl with Tris-HCl. The nucleotides were separated by thin-layer chromatography on 20 cm polygram gel 300 poly(ethyleneimine)-impregnated plastic sheets in 1.2 M LiCl. The spots were cut out with scissors and were eluted with 0.7 mM $MgCl_2$, 25 mM Tris [11] and the radioactivity in ADP and ATP was determined in a Packard scintillation counter.

NaCl concentration (mM)	[^{14}C]ADP-ATP exchange activity (nmol/min per mg protein)
10	67.5 ± 1.5
100	163 ± 3.0
200	201 ± 17
300	313 ± 5.0

K^+)-ATPase are shown in Figs. 7 and 8. In this experiment the amplitude of the fluorescence change accompanying phosphorylation at a low concentration of NaCl, was about 80% of that of the full response to K^+. With increasing concentrations of NaCl without KCl the amplitudes became smaller. The increase in fluorescence intensity following addition of ATP with both NaCl and KCl in the medium is due to the formation of E_2P and in particular E_2K in conditions of turnover [8]. The amplitude of this turnover-associated change remained identical to that following addition of K^+ alone, even at high NaCl concentrations (as in Fig. 6a).

Comparison of the data in Fig. 8 with the data in Table I shows that the fluorescence response to addition of ATP in presence of Mg^{2+} and Na^+ varied inversely with the activity of the Na^+-dependent ADP-ATP exchange. In agreement with previous observations [10,11] this reflects the increase in proportion of E_1P to E_2P with increasing concentrations of NaCl.

In the invalid ($Na^+ + K^+$)-ATPase, the amplitude of the fluorescence change accompanying addition of ATP to a medium containing Mg^{2+}, Na^+, and K^+ was smaller than in the control (Fig. 6c and Fig. 7). The amplitude of the signal accompanying phosphorylation from ATP in presence of Mg^{2+} and Na^+ was depressed to a larger extent than in control by increasing the NaCl concentration (Fig. 7). Previously, we found that the Na^+-dependent ADP-ATP exchange activity of the invalid ($Na^+ + K^+$)-ATPase was higher than in control and that the relative amount of E_1P was increased from 14% of the total phosphoenzyme in control to 40% in the invalid ($Na^+ + K^+$)-ATPase [1,2]. The defective fluorescence responses of the protein in the invalid ($Na^+ + K^+$)-ATPase are therefore in agreement with a shift in the equilibrium between E_1P and E_2P forms of the protein of the invalid ($Na^+ + K^+$)-ATPase in direction of the E_1P form.

Effects of vanadate on the conformational response in control and invalid ($Na^+ + K^+$)-ATPase

In the absence of Mg^{2+}, 10 μM vanadate did not affect the fluorescence

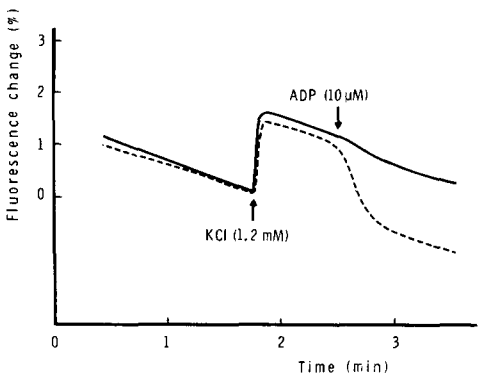


Fig. 9. Effect of 1 μM vanadate in the presence of 2 mM $MgCl_2$ on the fluorescence response of control (———) and trypsinized (- - - - -) $(Na^+ + K^+)$-ATPase to sequential addition of KCl and ADP.

response to addition of K^+ or ADP (as in Fig. 1) to control or invalid $(Na^+ + K^+)$-ATPase. Similarly, in the absence of K^+, the increase in fluorescence intensity accompanying phosphorylation from ATP in the presence of Mg^{2+} and Na^+ was unaffected by 10 μM vanadate (not shown). This is in agreement with the observation that Mg^{2+} and K^+ are required for high-affinity binding of vanadate [13]. In a medium containing Mg^{2+}, $NaVO_3$ and KVO_3 at 10 μM prevented the increase in fluorescence intensity of control $(Na^+ + K^+)$-ATPase after addition of ATP. In invalid $(Na^+ + K^+)$-ATPase the same VO_3^- caused only a small reduction in the amplitude of this change in fluorescence (not shown). This resistance to vanadate is in agreement with the reduced binding affinity (K_{diss} = 32 nM) of the invalid $(Na^+ + K^+)$-ATPase as compared with the control enzyme (K_{diss} = 4.5 nM) [4] and with the insensitivity to vanadate of the ATPase activity [3] and of the residual active transport in vesicles reconstituted with invalid $(Na^+ + K^+)$-ATPase [4].

The mechanism of action of vanadate was further examined in the following experiment (Fig. 9). Addition of Mg^{2+} increased the fluorescence intensity of control and invalid $(Na^+ + K^+)$-ATPase to a level about midway between the levels of E_1Na and E_2K. The increase in fluorescence after addition of K^+ was therefore smaller than usual (Fig. 9). This response to K^+ was not prevented by vanadate, but after the addition of K^+, vanadate prevented the reversal of the fluorescence change following addition of ADP to the control, whereas the decrease in fluorescence intensity after addition of ADP to the invalid was unaffected. Thus, the control $(Na^+ + K^+)$-ATPase must complete the transition to the Mg^{2+}-bound E_2K form of the protein before vanadate can bind and prevent further motion within the protein. In the invalid $(Na^+ + K^+)$-ATPase, the binding affinity of the E_2K form to vanadate is reduced [4]. The low concentration of the inhibitor does therefore not interfere with the conformational transition.

Discussion

The agreement between the changes in fluorescence intensity levels and the previous enzymatic [1,2] and active transport data [4] show that the fluo-

rescence technique allows comparison of the conformational state of the protein of invalid ($Na^+ + K^+$)-ATPase with that of control enzyme, both in the absence of ATP and during turnover. The analysis of the transitions between the cation-bound conformations E_1Na and E_2K and between the phosphoforms, E_1P and E_2P of the protein of invalid and control ($Na^+ + K^+$)-ATPase shows that the tryptic split alters the poise of the equilibria between these conformations in the direction of the E_1 forms.

Non-phosphorylated conformations

The K^+- or Na^+-induced conformational transition between the E_1 and E_2 forms of invalid and control ($Na^+ + K^+$)-ATPase can be described conveniently by the following scheme proposed in previous publications [7,9]:

$$E_1 \cdot Na \underset{K_s}{\rightleftarrows} E_1 \underset{K_p}{\rightleftarrows} E_1 \cdot K \underset{K_c}{\rightleftarrows} E_2 \cdot (K)$$

K_s and K_p are intrinsic cation binding constants. K_c is the conformational equilibrium constant. E_1 is the predominant native conformation to which Na^+ or K^+ may bind in competition (see Ref. 7). A spontaneous conformational change occurs between E_1K and $E_2 \cdot (K)$ or the K^+ form which contains occluded K^+. The K^+ concentration for a half-maximal change between the E_1 and E_2 forms, say in the absence of Na^+, is given by the expression:

$$K_{0.5} = \frac{K_p}{K_c - 1}$$

As discussed previously (Ref. 7) the conformational equilibrium is poised for in the direction of $E_2 \cdot (K)$ and even though K^+ binds weakly to E_1 the overall result in titrations such as in Fig. 3 is a high apparent K^+ affinity. The difference in $K_{0.5}$ for K^+ between the control and invalid of 2—3-fold at either zero or 10 mM Na^+ in Fig. 3, is consistent with a 2—3-fold shift in the K_c of the invalid protein towards E_1K.

This concept of ion binding and conformational changes in the normal and invalid protein is strongly supported by the data on the K^+-nucleotide antagonism (Figs. 1, 4 and 5). Consider the scheme shown in the Appendix. Nucleotide and K^+ are assumed not to compete at the same binding sites. ATP is assumed to be able to bind to either conformation E_1 or $E_2(K)$ but the affinity for E_1 is high (K_a = 0.15 μM, Refs. 16 and 17) and it is unaffected by the tryptic split [1], while that for $E_2(K)$ is very low (Refs. 8 and 18). ATP and K^+ binding are necessarily antagonistic at non-saturating concentrations because each ligand stabilizes an alternative protein conformation. The calculated ATP titration curves in Fig. 5 are based on this scheme as developed in the Appendix. Given the uncertainty of the values of the various equilibrium constants and the inevitable scatter in the data points, the agreement between the predicted and observed curves is encouraging. Thus the scheme serves as a good working hypothesis. The apparent affinity for ADP of the invalid enzyme (Fig. 4) is raised 2—3-fold as compared to control and the apparent K^+ affinity is reduced by about the same factor (Fig. 3). This is rather convincing evidence for a shift in the conformational equilibrium in the invalid towards the E_1 state. The alternative possibility that the intrinsic binding constants for K^+ and ATP (K_p and K_a in the scheme) should be altered

is quite unlikely because this would require the binding of structurally unrelated ligands to be affected by tryptic modification to the same extent but in opposite directions.

Phosphorylated conformations

In the control ($Na^+ + K^+$)-ATPase the amplitude of the signal accompanying phosphorylation from ATP with 10 mM NaCl, is about 75% of the maximal fluorescence change ($+K^+$) and it is reduced with increasing concentrations of NaCl in parallel with the change in ratio of E_1P to E_2P. In the trypsinized ($Na^+ + K^+$)-ATPase the level of E_1P at 100 mM NaCl is 3-fold higher than in control [2], the amplitude of the fluorescence signal accompanying phosphorylation is smaller than in control and is more sensitive to increasing NaCl concentrations. Taken together, these observations imply that the fluorescence change accompanying phosphorylation from ATP in the absence of K^+ reflects the formation of E_2P, which has the same fluorescence intensity as $E_2(K)$, and conversely the fluorescence intensity of E_1P is similar to that of the dephosphoform E_1. Therefore the fraction of the total phosphoenzyme present as E_2P may be determined as the amplitude of the fluorescence change accompanying phosphorylation in the absence of K^+ divided by the amplitude of the full response to K^+. For example, the experiment in Fig. 7 with 10 mM NaCl yields a steady-state ratio of E_2P to E_1P about 3 : 1 in the control and about 1.7 : 1 in the invalid ($Na^+ + K^+$)-ATPase. This result is in good agreement with the ratios obtained in direct measurements of E_1P and E_2P [2]. The tryptophan fluorescence technique thus provides a simple and rapid assay of the steady-state proportions of the two phosphoenzymes which have been isolated kinetically [18].

Vanadate

Vanadate clearly inhibited the conformational change $E_2K \rightarrow E_1 \cdot ADP$ in the unmodified enzyme presumably by forming the stable complex E-K^+-Mg^{2+}-vanadate (Ref. 13). This finding parallels recent observations on the effects of vanadate on a fluorescein-labelled ($Na^+ + K^+$)-ATPase [19].

The insensitivity to vanadate of the conformative response of the protein of invalid ($Na^+ + K^+$)-ATPase is in agreement with the reduced vanadate binding affinity of this enzyme [4] and with the insensitivity of the residual active Na^+ transport [4] and of the ($Na^+ + K^+$)-ATPase activity [3] of the trypsinized ($Na^+ + K^+$)-ATPase. Vanadate is assumed to bind to the site from which phosphate is released during ATP hydrolysis [14]. The lower affinity for vanadate is thus in agreement with the shift in equilibrium of the cation-bound form of the protein in direction of E_1Na and of the phosphoprotein in direction of E_1P. The weak effect of K^+ on the rate constant for dephosphorylation [2] and the low K^+-phosphatase activity [1] of the invalid ($Na^+ + K^+$)-ATPase also reflects the abnormal transformation from E_1P to E_2P of the protein. There is an unchanged number of vanadate binding sites in the invalid ($Na^+ + K^+$)-ATPase, but the dissociation constant is much higher than in control [4]. The altered equilibrium between the conformations of the cation-bound forms and the phosphoforms of the protein in the invalid ($Na^+ + K^+$)-ATPase may therefore result in inadequate exposure of the site for binding of vanadate.

Invalid enzyme, conformational transitions and cation movements

Together with previous data the present results show that the defective conformational response, the catalytic defects [1,2], the reduced active Na^+ transport [4] and the insensitivity to vanadate [3,4] of the invalid ($Na^+ + K^+$)-ATPase are caused by the same tryptic split in the purified enzyme. The split does not alter the conformation of the ATP binding area or of the site in the α-subunit that receives the γ-phosphate from ATP [1]. Kinetic evidence for defective binding of Na^+ to invalid ($Na^+ + K^+$)-ATPase could not be obtained [4]. The cleavage may therefore affect protein domains of more general importance for the conformational transition between E_1 and E_2 states of ($Na^+ + K^+$)-ATPase.

The changes in fluorescence intensity in native ($Na^+ + K^+$)-ATPase can be assumed to arise from changes in structure of the α-subunit because they are elicited by the same ligands and take the same direction as the transitions between the Na^+ form and the K^+ form of the α-subunit that can be detected with trypsin [5]. The structure of α-subunit in E_1P has not been examined with trypsin, but the present observations suggest that its fluorescence intensity is similar to that of the dephosphoforms of E_1. The major structural change in the α-subunit seems therefore to occur when E_1P is transformed into E_2P and not when γ-phosphate is transferred from ATP to the protein. The pattern of tryptic cleavage of the α-subunit in E_2P is identical to that in E_2K [5] and the two forms appear to have the same fluorescence intensity. The K^+-stimulated dephosphorylation reaction seems therefore not to be accompanied by significant changes in protein structure. The data on the conformational changes and considerations on the nature of the transport reaction lead one to expect that the major conformational transitions between Na^+ forms and K^+ forms of the α-subunits are coupled to the translocation steps for Na^+ and K^+ across the membrane. Our observations on the invalid ($Na^+ + K^+$)-ATPase are also consistent with the notion that the conformational changes constitute the cation translocation steps, Na^+ movements involving the E_1P to E_2P stage and K^+ movements requiring the $E_2 \cdot (K)$ to E_1ATP transition (see also Ref. 7). In addition Na^+-dependent phosphorylation precedes the E_1P to E_2P conformational change, and K^+-dependent hydrolysis of E_2P precedes the $E_2 \cdot (K)$ to E_1ATP transition. The phosphoryl transfer reactions themselves seem to be accompanied by only minor changes in structure of the α-subunit. This finding adds substantially to the kinetic evidence suggesting that the phosphoryl transfers are coupled to cation-exchange reactions on the cytoplasmic and extracellular surfaces rather than to the movements as such of cations across the membrane.

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Appendix

The K^+-nucleotide antagonism

Consider the scheme:

$$\begin{array}{ccccc} E_1 & \xleftarrow{K_p} & E_1 \cdot K & \xrightarrow{K_c} & E_2 \cdot (K) \\ {\scriptstyle K_a}\uparrow\downarrow{\scriptstyle ATP} & & {\scriptstyle ATP}\uparrow\downarrow{\scriptstyle K_a} & & {\scriptstyle ATP}\uparrow\downarrow{\scriptstyle K_{ap}} \\ E_1 \cdot ATP & \underset{K_p}{\overset{K^+}{\rightleftharpoons}} & E_1 K\ ATP & \xrightarrow[K_{ca}]{} & E_2 \cdot (K) \cdot ATP \end{array}$$

By hypothesis: ATP binds to available E_1 forms with affinity K_a; K^+ binds to available E_1 forms with affinity K_p, and ATP binds to the occluded form $E_2 \cdot (K)$ with affinity K_{ap}. The conformational equilibrium constant between E_1K and $E_2 \cdot (K)$ is K_c. The conformational equilibrium constant between $E_1 \cdot K \cdot ATP$ and $E_2 \cdot (K)$ is K_{ca}.

$$E_{total} = E_2 \cdot (K) + E_2(K)ATP + E_1 \cdot K + E_1 \cdot K \cdot ATP + E_1 \cdot ATP + E_1$$

The equilibria lead to the relationships:

$$\frac{E_2 \cdot (K)}{E_{total}} = \frac{1}{a + b\ ATP} \quad . \tag{1}$$

$$\frac{E_2 \cdot (K)ATP}{E_{total}} = \frac{ATP}{c + d\ ATP} \tag{2}$$

Assuming that all E_1 forms have 'low' and all E_2 forms have 'high' intrinsic protein fluorescence, then in titrations as in Fig. 5 we have

$$\frac{\Sigma E_2\ forms}{E_{total}} = \frac{C + (a + d)\ ATP + b\ ATP^2}{ac + (ad + bc)\ ATP + bd\ ATP^2} \tag{3}$$

$$a = 1 + \frac{1}{K_{ca}}\left[1 + \frac{K_p}{K^+}\right] \quad ; \qquad b = \frac{1}{K_{ap}} + \frac{1}{K_c K_a}\left[1 + \frac{K_p}{K^+}\right]$$

$$c = K_{ap} + \frac{K_a}{K_{ca}}\left[1 + \frac{K_p}{K^+}\right] \quad ; \quad d = 1 + \frac{1}{K_{ca}}\left[1 + \frac{K_p}{K^+}\right]$$

The major problem in calculating ΣE_2 forms/E_{total} and the shape of the ATP titration curves is the lack of accurate values of the individual equilibrium constants. From the previous studies of the conformational transitions using formycin nucleotides [7] it was inferred that K^+ binds weakly to E_1 and the conformational equilibrium constant K_c is poised for in the direction of $E_2 \cdot (K)$. But values for K_p and K_c could not be obtained due to the restricted range of K^+ concentrations over which the rates of the transition $E_1 \rightarrow E_2 \cdot (K)$ could be monitored. More recently the kinetic measurements have been greatly improved with the aid of a fluorescein-labelled ($Na^+ + K^+$)-ATPase and these experiments lead to the following values of $K_p \approx 75$ mM and $K_c \approx 1000$ (Karlish, S.J.D., unpublished results).

The binding constant K_a of ATP to the native conformation E_1 has been measured directly by several workers, and has values in the range 0.12—0.22 μM [16,17]. In making our calculations we have chosen a value of $K_a = 0.15$

μM obtained from fluorescence titrations performed at room temperature [7].

Binding of ATP to $E_2 \cdot (K)$ is weak [8,18] and difficult to estimate. A figure of $K_{ap} \approx 450$ μM was inferred previously [8] on the basis of the stimulatory effect of ATP on the rate of the transition $E_2 \cdot (K) \rightarrow E_1 \cdot Na$. K_{ca} can be calculated from the relationship $K_{ca} = K_a \cdot K_c/K_{ap}$. Fortunately binding of ATP to the form $E_2 \cdot (K)$ is virtually negligible in the concentration range 1—20 μM used in the titrations and so the ratio E_2/E_{total} in Fig. 5 is almost completely insensitive to the value of K_{ap} and K_{ca}. At saturating ATP and K^+ concentrations (several orders of magnitude higher than in these experiments) the equilibrium between E_1 and E_2 will be governed by K_{ca}.

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