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TRYPTOPHAN FLUORESCENCE OF ($Na^+ + K^+$)-ATPase AS A TOOL FOR STUDY OF THE ENZYME MECHANISM

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Summary

1. The protein fluorescence intensity of ($Na^+ + K^+$)-ATPase is enhanced following binding of K^+ at low concentrations. The properties of the response suggest that one or a few tryptophan residues are affected by a conformational transition between the K-bound form $E_2 \cdot (K)$ and a Na-bound form $E_1 \cdot Na$.

2. The rate of the conformational transition $E_2 \cdot (K) \rightarrow E \cdot Na$ has been measured with a stopped-flow fluorimeter by exploiting the difference in fluorescence of the two states. In the absence of ATP the rate is very slow, but it is greatly accelerated by binding of ATP to a low affinity site.

3. Transient changes in tryptophan fluorescence accompany hydrolysis of ATP at low concentrations, in media containing Mg^{2+}, Na^+ and K^+. The fluorescence response reflects interconversion between the initial enzyme conformation, $E_1 \cdot Na$ and the steady-state turnover intermediate $E_2 \cdot (K)$.

4. The phosphorylated intermediate, E_2P can be detected by a fluorescence increase accompanying hydrolysis of ATP in media containing Mg^{2+} and Na^+ but no K^+.

5. The conformational states and reaction mechanism of the ($Na^+ + K^+$)-ATPase are discussed in the light of this work. The results permit a comparison of the behaviour of the enzyme at both low and high nucleotide concentrations.

Introduction

In order to understand the mechanism of a complex enzymatic mechanism such as that of the active ion-transporting ($Na^+ + K^+$)-ATPase it is necessary to

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identify the reaction intermediates and investigate their properties. Much information has been obtained from observations on phosphoryl enzymes [1,2] but studies of this sort do not of course throw direct light on the nature of non-phosphorylated forms. Therefore, we have preferred on alternative approach and made a search for fluorescent probes of both non-phosphorylated and phosphorylated intermediates in order to study their properties by kinetic techniques such as stopped flow fluorimetry.

In recent experiments with a purified membranous preparation of (Na^+ + K^+)-ATPase we reported [3] that formycin triphosphate (FTP), and formycin diphosphate, (FDP), which are fluorescent analogues of the adenine nucleotides [4], bind tightly to the enzyme with enhancement of the formycin nucleotide fluorescence. These features enabled us to identify intermediates and observe elementary steps of the enzymatic process, previously innaccessible to experimentation [5,6]. This work led to important conclusions (see refs. 5 and 6) but one can point out two limitations to the use of the extrinsic probes. Firstly, measurements were restricted to a low range of formycin nucleotide concentration in which the small signal changes, due to binding of FTP and FDP, could be detected against the fluorescence background. Also, enzymic intermediates which do not bind FTP and FDP could not be identified positively, but their properties were inferred from their kinetic relationship with states in which the nucleotides are bound tightly. Thus, alternative probes which are not subject to these limitations were very desirable.

The present experiments show that the intrinsic tryptophan fluorescence of (Na^+ + K^+)-ATPase is enhanced upon transition from the Na-form to the K-form or to the phosphorylated state. The Na-form of the enzyme is thought to predominate in Na^+- and K^+-free media (E_1) or in Na^+-rich media containing little or no K^+ ($E_1 \cdot Na$), and the alternate conformational state, the K-form ($E_2 \cdot (K)$) is the major species present in K^+-rich media (see refs. 7 and 8). We have exploited the tryptophan fluorescence change as a tool to study signals accompanying hydrolysis of ATP and Na^+- or K^+-induced conformational transitions. The experiments are similar in type to those with the formycin nucleotides [3,5,6], but there are two advantages in the direct detection of the K-form of the enzyme. Firstly the identity of the intermediate one is studying, is in no doubt, and it has also been possible to observe the behavior of the

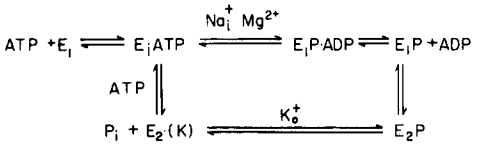


Fig. 1. A simplified scheme for the mechanism of (Na^+ + K^+)-ATPase (see refs. 1, 2 and 9—11). E_1 is a conformation of the enzyme which predominates in Na^+- and K^+-free or Na^+-rich media. It has a high affinity for ATP. In the presence of ATP, Mg^{2+}, and Na^+ ions bound to internal loading sites, the enzyme is phosphorylated. The primary phosphoenzyme is $E_1P \cdot ADP$ and dissociation of ADP leads to E_1P, a phosphoenzyme which can transfer the phosphoryl group back to ADP. A spontaneous conformational change is thought to produce the phosphoenzyme E_2P. This phosphoenzyme can be dephosphorylated slowly in the absence of K^+ ions, but very rapidly with K^+ ions bound to external loading sites. K^+-activated hydrolysis of E_2P gives rise to the dephosphoenzyme $E_2 \cdot (K)$ containing occluded K^+ ions. Completion of the cycle by conformational transition from $E_2 \cdot (K)$ back to the original state $E_1 \cdot ATP$ is slow at low nucleotide concentrations, but is accelerated by binding of ATP to low affinity sites. For a more detailed version which defines explicitly the Na^+ and K^+ transport steps (see ref. 6).

enzyme at much higher nucleotide concentrations (ATP) than hitherto.

Fig. 1 shows a recent version of the mechanism of ($Na^+ + K^+$)-ATPase proposed by Albers [9,2] and Post [10,11]. The scheme is useful as an aid in understanding the experimental design and the terminology is used throughout this text. The brackets around the K^+ ion in the K-form $E_2 \cdot (K)$ indicate that the cation binding sites are occluded and K^+ is not free to exchange with the medium [11,8,6]. The model is somewhat oversimplified, for the Na^+ and K^+ transport steps are not defined. The relations between phosphoryl transfer, conformational changes and the transporting steps are postulated explicitly in a similar but more complete kinetic model described in our previous publication [6].

Methods

($Na^+ + K^+$)-ATPase was purified from the red outer medulla of sheep, pig or rabbit kidney by the simpler procedure of Jorgensen [12]. In order to remove traces of ATP the enzyme preparations were washed at 0°C with a medium containing imidazole, 25 mM, pH 7.5, EDTA, 1 mM, and they were stored in this medium at 0°C. Specific activities of the preparations were in the range 12—20 units/mg protein (i.e. 35—65% pure on a protein basis, see ref. 13). ATPase and Lowry protein assays were performed as described in ref. 13. Oubain-insensitive ATPase activity was usually not detectable.

Fluorescence measurements

Experiments were conducted at room temperature, 20°C ± 2°C. All fluorescence measurements, other than the stopped-flow experiments, were performed on a Perkin-Elmer MPF 44A Fluorescence Spectrophotometer. The fluorescence cell was fitted with a home-made mixing device which enables one to add solutions during constant stirring of the enzyme suspension. Hamilton syringes were used for addition of known small volumes of solutions. Unless otherwise stated the protein fluorescence of the ($Na^+ + K^+$)-ATPase was excited at 295 nm and emission was detected at 325 nm. Slit widths on the excitation and emission monochromators were chosen to give the best compromise between opposing requirements. On the one hand the measurement of small ligand-induced changes in fluorescence (1—3%) is optimized by high excitation and emission light intensities, since this minimizes the signal noise. But widening the slits to achieve this end reduces the magnitude of the fluorescence change which is optimal at the chosen excitation and emission wavelengths, i.e. at 295 and 325 nm respectively. Furthermore at very high excitation intensities there is a rapid and constant fall in the fluorescence of the membranes which interferes with the measurements. This process is excited optimally at 287 nm and reflects presumably photoinactivation of the protein by the ultraviolet light. Taking all these factors into consideration, the slit widths found to give the best results were 10 nm on both monochromators. If very small signal changes were to be recorded, the instrument time constant was set to 1.5 or 3 s (see for example Fig. 8), but usually the response time was 0.3 s (e.g. Fig. 9).

Stopped-flow fluorescence measurements

Experiments were done at room temperature (20°C ± 2°C) with the instrument described by Bagshaw et al. [14]. The protein fluorescence was excited at 295 nm and the emitted light was detected after passage through a U.G. 11 and a W.G. 335 cut off filter which exclude most light below 335 nm. Signals were recorded on a Tektronix type 564 storage oscilloscope, and photographed.

Results

The K-form of ($Na^+ + K^+$)-ATPase

The intrinsic protein fluorescence of the ($Na^+ + K^+$)-ATPase preparation shows characteristics typical of many soluble and membrane bound proteins, including an excitation maximum at about 287 nm and emission maximum at about 337 nm (see also Fig. 10). A small, but distinct, increase in the intensity of the tryptophan fluorescence was observed when low concentrations of K^+ ions (1 mM) were added to the membranes suspended in a medium free of Na^+ ions (Fig. 2a). A second addition of KCl produced no further response. In view of the small size of the signal it is worth mentioning our efforts to increase its amplitude and exclude the possibility of optical artefacts. Using kidney microsomal preparations which serve as the starting material for purification of the ($Na^+ + K^+$)-ATPase (see ref. 12) the K^+-dependent fluorescence change was barely detectable (amplitude <0.3%). This suggests that enrichment of the membranes with ($Na^+ + K^+$)-ATPase at the expense of irrelevant proteins improves the signal to background ratio, and makes unlikely the possibility of a non-specific effect of K^+ ions on the protein fluorescence or light-scattering of the membranes. It was also found that a sheep enzyme preparation of specific activity 18 U/mg gave a decidedly better signal change (approx. 2%) compared to a less purified sample (spec. act. 9 U/mg with a response of about 1%). The K^+ response (and the turnover-associated signals see Fig. 6) were clearly observable with enzyme preparations from sheep, pig or rabbit, but there seemed to be species differences. A highly purified pig enzyme (spec. act. 32 U/mg,

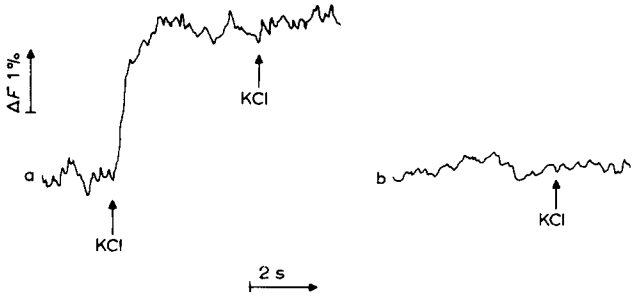


Fig. 2. Enhancement of tryptophan fluorescence of ($Na^+ + K^+$)-ATPase caused by binding of K^+ ions. 100 μg of rabbit enzyme protein (specific activity approx. 15 U/mg) was suspended in a medium of volume 2.5 ml which contained: A. Tris-HCl, 100 mM, pH 7.0; EDTA (Tris) 1 mM. B. NaCl, 80 mM; Tris Cl, 20 mM pH 7.0; EDTA (Tris) 1 mM). 2-μl aliquots of KCl, 1.25 M were added as indicated to give a concentration in the medium of 1 mM per addition. The signal change was recorded at an amplification 10-fold higher than the total signal and the instrument time constant was 0.3 s. Other conditions were as described in Methods.

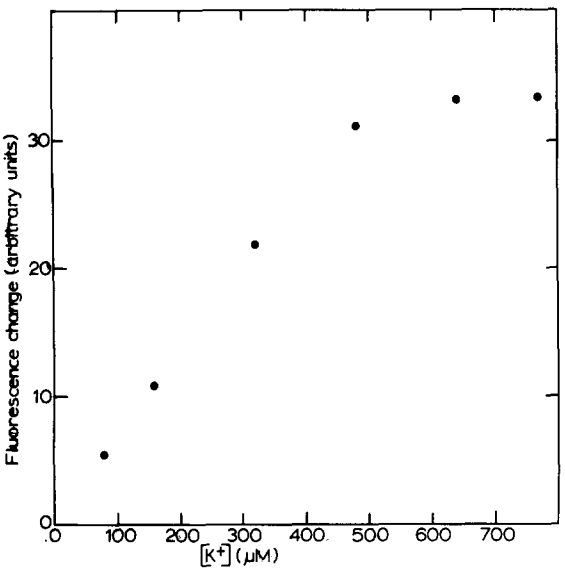


Fig. 3. Dependence on K^+ concentration of the tryptophan fluorescence enhancement. 50 μg of rabbit ($Na^+ + K^+$)-ATPase (spec. act., 20 U/ml) was suspended in 2.5 ml of a medium containing: Tris-HCl, 100 mM, pH 7.0, EDTA, 1 mM and NaCl, 0.5 mM. The fluorescence changes were recorded at a 30-fold amplification over the total fluorescence signal, and the large background noise was therefore minimized by raising the instrument time constant to 3.0 s. Successive aliquots of KCl, 100 mM, were then added as accurately as possible, to give concentration increments of 100—200 μM KCl, and the fluorescence change after each addition was measured. The amplitude of the fluorescence enhancement at saturating levels of KCl was about 2.7% of the total signal, in this experiment. Points are given ±1 S.E.M.

kindly supplied by P.L. Jørgensen) showed about the same amplitude of the K^+ response (approx. 2%) and the turnover signal as the less purified sheep enzyme (18 U/mg). The best signals observed todate (approx. 3%, see Fig. 3) were observed with a rabbit enzyme of specific activity 20 U/mg and this has now become the preparation of choice. It has also been found that somewhat better results were obtained in media of pH 6.8—7.0 than at the more customary pH 7.5—7.7.

In the experiment of Fig. 2 the enzyme is present initially in the conformation E_1 and the fluorescence change is presumably associated with conversion to the K-form $E_2 \cdot (K)$. The lack of response upon addition of 1 mM KCl to enzyme in a high-Na medium Fig. 2b illustrates antagonism between Na^+ and K^+ ions. This experiment is an essential control for the ATP turnover experiment described in Fig. 6a. A titration of the K^+ dependent effect in a medium containing 0.5 mM Na^+ ions showed that a half-maximal response was produced by addition of about 200 μM KCl (Fig. 3), and in the Na-free medium of Fig. 2a or in media containing 80 mM NaCl half maximal responses required addition of approx. 50 μM and 6 mM KCl, respectively (not shown).

Rate of the transition $E_2 \cdot (K) \rightarrow E_1 \cdot Na$

When enzyme preincubated with low concentrations of K^+ ions was suddenly exposed to high concentrations of Na^+ ions, by mixing in the stopped-flow

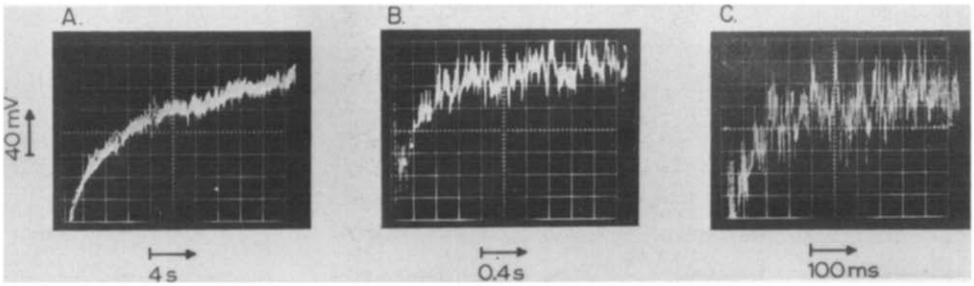


Fig. 4. Stopped-flow records of the $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ transition monitored by the tryptophan fluorescence change. An upward deflection indicates a fall in fluorescence. Syringe 1 contained in a volume of 5 ml: 400 μg of pig-enzyme (specific activity 18.5 units/mg); Tris-HCl 150 mM, pH 7.0; EDTA (Tris) 1 mM; KCl, 1 mM. Syringe II contained in the same volume: NaCl, 130 mM; Tris-HCl, 20 mM pH 7.0; EDTA, 1 mM and ATP as follows:Scan A-none; B-40 μM and C-200 μM. The final concentration after mixing, of any component not present in both syringes is half that given above. Time constants on the stopped flow instrument were set at: A, 100 ms; B, 20 ms and C, 2 ms. The temperature was 22°C. The negatives of the photographs were enlarged and projected onto graph paper. The rate constants of the initial phase was obtained graphically from semi-log plots of the trace obtained after subtractions of the linear portion. These plots were satisfactorily linear as expected for an exponential process. The correction for photoinactivation was appreciable only for the slowest scans (eg. Fig. 2A). Values of the apparent first-order rate constants from the traces in Fig. 4 are included in the data of Fig. 5.

fluorimeter, the conversion from the initial state $E_2 \cdot (K)$ to the final state $E_1 \cdot Na$ could be followed by the associated fall in fluorescence, Fig. 4A. The relevant signal change which is the initial portion of the trace in Fig. 4A is slightly complicated by superimposition of a constant fall in the total fluorescence due to photoinactivation of the protein by the exciting light. Subtraction of this linear portion from the total signal change reveals an initial exponential phase. The rate of this phase is very slow in the absence of ATP. If ATP was added to the Na^+-containing medium, and any Mg^{2+} ions present were chelated to prevent hydrolysis, the rates of transition were very much accelerated (Fig. 4B and C). The photoinactivation process was barely detectable at the higher sweep-speeds used to monitor these faster signals. The relation between the rate constant of the conformational change and the ATP concentration is shown in Fig. 5. The figure includes data from 2 large experiments with pig and rabbit enzyme, and it is clear that the enzyme from the two species shows essentially identical rates of the transition. It is important to note that the very low rate constant observed in the absence of ATP, 0.26 s^{-1}, is not significantly different from that measured with the formycin nucleotides (approx. 0.2 s^{-1}) [6]. The other striking features of Fig. 5 are the very large acceleration by ATP and the lack of saturation up to the highest ATP concentrations at which measurements were possible. If 1 mM K^+ was mixed with enzyme suspended in a medium containing 0.5 mM NaCl, a small but rapid rise in fluorescence intensity was observed, due presumably to the reverse conformational transition $E_1 \cdot Na \rightarrow E_2 \cdot (K)$. We have not been able to characterize this process satisfactorily since the rates involves is much higher than for $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ transition [6].

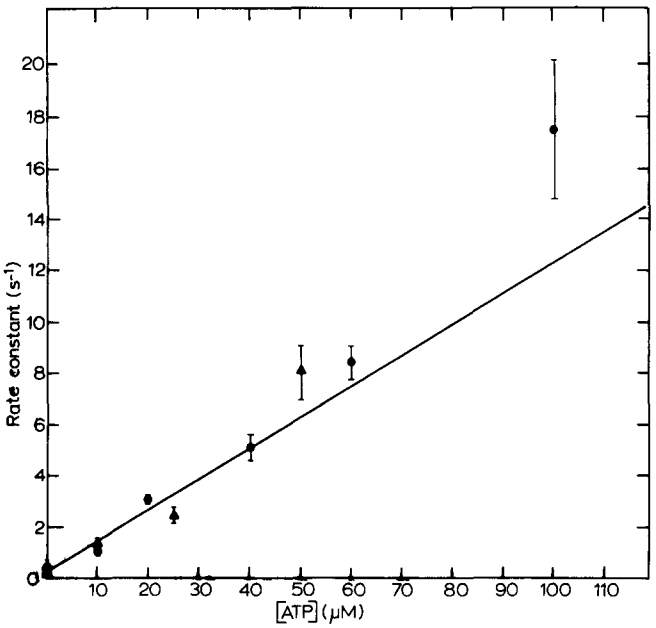


Fig. 5. Dependence of the rate of the transition $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ on the concentration of ATP. Points marked -●- are from the pig enzyme experiment, details of which are given in the legend to Fig. 4. Points marked -▲- are from an experiment with a rabbit enzyme (specific activity 19 U/min) performed in the same conditions as in Fig. 4 except that EDTA was replaced with CDTA, 2 mM. Each point represents the average value (±S.E.M.) of the rate constants estimated from at least five traces. The rate constant at zero ATP in the pig enzyme experiment 0.29 s^{-1} ± 0.017 s^{-1} is the average figure from 17 separate records. The straight line drawn through the points represents the equation $k = 0.120$ [ATP] + 0.256. It has been calculated by the method of least squares using values of the rate constants weighted according to the inverse of their variance (see ref. 15).

Fluorescence changes accompanying hydrolysis of ATP

(a) The K-form $E_2 \cdot (K)$. A transient change in tryptophan fluorescence was observed (Fig. 6a) when low concentrations of ATP were added to enzyme suspended in a medium containing Mg^{2+}, 80 mM NaCl and 1 mM KCl, conditions which permit hydrolysis of the ATP. The response could be elicited several times by further additions of ATP. Eventually photoinactivation of the protein or accumulation of ADP and P_i brought about a reduction in the magnitude of the change. The amplitude of the hydrolysis associated signal was the same as that produced by binding of K^+ to the enzyme in a Na^+-free medium (Fig. 6b). The rise in fluorescence following addition of ATP is explained conversion of the enzyme from the "low" fluorescence initial conformation $E_1 \cdot Na$ (see the control experiment in Fig. 2B) to the "high" fluorescence steady-state intermediate $E_2 \cdot (K)$, presumably via the rapid phosphorylation and dephosphorylation steps. The duration of the high fluorescence steady-state should reflect the time required to hydrolyse the ATP and when the substrate becomes exhausted the initial conformation $E_1 \cdot Na$ is regained and the fluorescence falls to its original level. The duration is estimated from the interval between the half-rise and half-fall time of the signal. This value was roughly

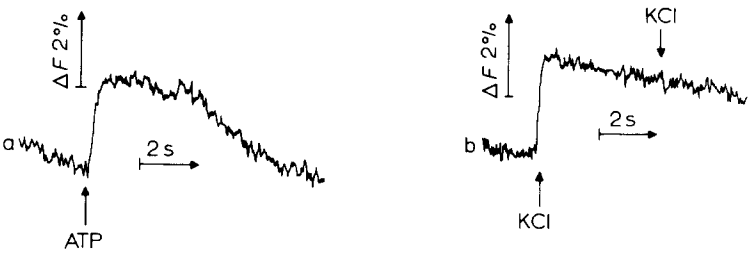


Fig. 6. Transient fluorescence change showing the appearance of the K-form of $(Na^+ + K^+)$-ATPase during hydrolysis of ATP. In Fig. 6a the fluorescence cell obtained in a volume of 2.5 ml the following reaction constituents: 100 μg of rabbit enzyme protein (spec. act. $\approx$ 19 units/mg^{-1}); NaCl, 80 mM; KCl, 1 mM; $MgCl_2$, 1 mM and Tris-HCl, 70 mM, pH 7.0. 2.5 μl of ATP, 1 mM was addedm where indicated, to give a final concentration of 1 μM. In Fig. 6b the reaction mixture obtained in a volume of 2.5 ml: 100 μg of rabbit enzyme protein; Tris-HCl, 150 mM, pH 7.0. 2.5 μl of KCl 1 M was added as indicated to give a final concentration of 1 mM KCl per addition. The fluorescence changes were recorded with instrumental conditions as in Fig. 2. Notice the relatively slow photoinactivation of the protein (in Fig. 6a and b) which causes a drift in the fluorescence baseline. The destruction of the protein decreases with time, and in some experiments e.g. Fig. 2a and b, it is barely detectable.

proportional to the amount of ATP added in the concentration range 0.5—3 μM (not shown).

We have not yet been able to monitor the rate of appearance of $E_2 \cdot (K)$ in the stopped-flow fluorimeter but useful information from turnover experiments can still be obtained by observations on the amplitude of the signal and the duration of the steady state. The amplitude depends on the difference in fluorescence between the initial enzyme conformation and that in the steady state. For example, it was possible to titrate the K^+ requirement for conversion of $E_1 \cdot Na$ to $E_2 \cdot (K)$, in the presence of Mg^{2+} ions, by adding ATP at a fixed concentration, to the medium containing different ratios of Na^+ and K^+ ions. The amplitude of the turnover association signal was depressed at K^+ concentrations in which the initial state of the enzyme had been shifted in the direction of $E_2 \cdot (K)$ (see Fig. 7). The K^+ concentration for a half maximal effect at 80 mM NaCl (5—6 mM KCl) was not significantly different from that observed in the direct titration of the tryptophan response. The duration of the steady state, at fixed concentrations of ATP is proportional to the turnover rate of the enzyme. In an experiment in which the effect of a low concentration of Tl^+ was compared with K^+, signals of the same amplitude were observed but the steady state with Tl^+ lasted 2—3 times longer than with K^+ (not shown). This observation implies clearly that with Tl^+ the turnover rate is lower than with K^+, due probably to a lower rate of the transition $E_2 \cdot (Tl) - E_1 \cdot Na$ (see ref. 6), but a detailed study was not carried out.

(b) Phosphorylated intermediate E_2P. In media containing Mg^{2+} and Na^+ ions but no added K^+ ions, addition of ATP produced a transient fluorescence change with an amplitude approximately half of that observed in the presence of K^+ ions (Fig. 8). The nature of the change in absence of K^+ can be inferred from a comparison of the response with 5 or 80 mM NaCl, respectively (Fig. 8). The striking difference in the length of the steady state period reflects a large increase in the rate of Na dependent ATP hydrolysis, in the absence of K^+, which is known to occur in this range of Na^+ concentration [11,16]. The phe-

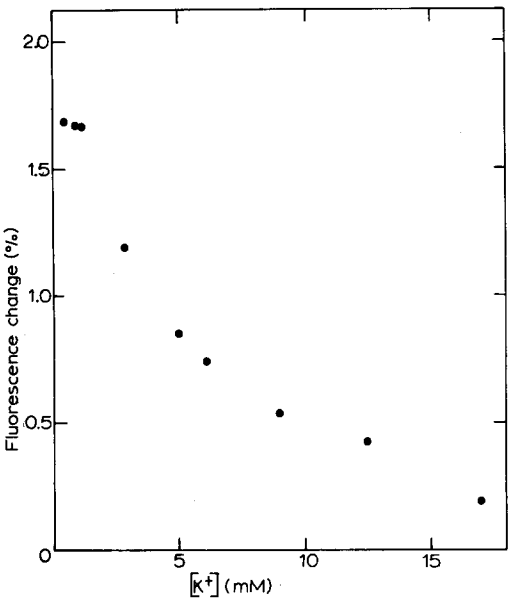


Fig. 7. Stabilization of the enzyme in the form $E_2 \cdot (K)$ prior to hydrolysis at ATP. 50 μg of sheep enzyme (spec. act. ≈14 units/min) was suspended in 2.5 ml of medium containing: NaCl, 80 mM, $MgCl_2$, 2 mM; EDTA 1 mM, histidine, 26 mM, pH 6.9 and KCl was varied as indicated. Each point in the figure represents the average value of the signal amplitudes from three successive fluorescence responses following addition of ATP, 0.5 μM (±1 S.E.M.). In this experiment the slit-width on the emission monochromator was set at 20 nmetre. Other recording conditions were as in Fig. 2 and as described in the Methods.

nomenon implies clearly that this fluorescence response is associated with conversion of the initial conformation $E_1 \cdot Na$ to the phosphoenzyme E_2P, which is the major turnover intermediate in these conditions [11]. One might suspect that the smaller signal in media containing 80 mM NaCl and no added KCl was in fact due to the presence of contaminating K^+ ions at a concentration sufficient approximately to half-saturate the full K^+-dependent change. But the similar amplitude of the responses and differences in the duration of the steady-state with 5 or 80 mM NaCl, makes this unlikely because one would expect the higher Na concentration to compete much more effectively with

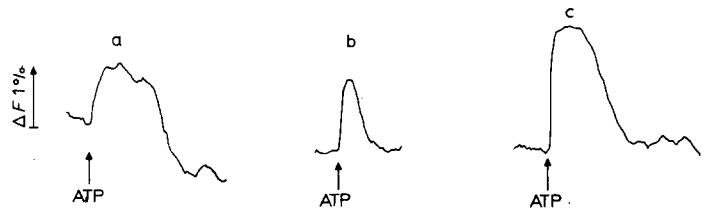


Fig. 8. Phosphoenzyme detected by a tryptophan fluorescence change accompanying hydrolysis of ATP in the absence of K^+ ions. 100 μg of sheep enzyme (specific activity 14 units/min) was suspended in 2.5 ml of reaction medium containing respectively: a. NaCl, 5 mM, Tris-HCl, 75 mM; histidine 25 mM; pH 6.9, $MgCl_2$ 2 mM; b. NaCl, 80 mM; histidine, 25 mM, pH 6.9, $MgCl_2$ 1 mM; c. NaCl 80 mM; KCl, 1 mM; histidine, 25 mM, pH 6.9, $MgCl_2$ 1 mM. 2.5 μl of ATP 3 mM was added as indicated to give a final concentration of 3 μM. The time constant of the instrument was set to 3 s in order to minimize noise and enable clear visualization of the small fluorescence changes of traces a and b.

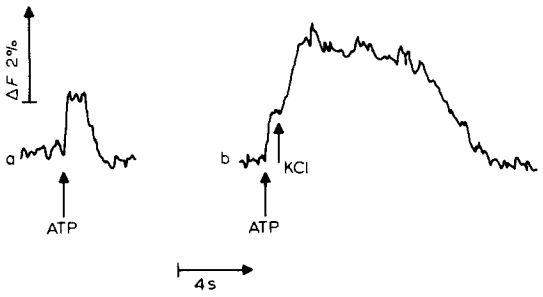


Fig. 9. Sequential additions of ATP and K^+ ions to $(Na^+ + K^+)$-ATPase suspended in medium containing Na^+ and Mg^{2+} ions. Initial conditions were as in Fig. 8b except that 40 μg of the sheep enzyme was used. ATP was added where indicated to a final concentration of 2.5 μM, and KCl was added in the experiment of 9b to a concentration of 1 mM. The time constant on the fluorimeter was set to 0.3 sec. Note the similarity to trace 8b excepting the noisiness of the signal.

very low concentrations of contaminating K^+ ions. In the experiments of Fig. 9 ATP was first added to enzyme in a medium containing Mg^{2+} and 80 mM NaCl (Fig. 9a). If after the initial rise in fluorescence, KCl was added quickly (Fig. 9b), this produced a further increase in the fluorescence to the steady-state level observed when K^+ ions were present initially. The duration of the steady-state with K^+ ions and 80 mM NaCl is greatly extended by comparison with turnover in the absence of K^+ (compare Fig. 8b and c, and Fig. 9a and b). This reflects the well-known inhibition of hydrolysis by K^+ ions at low concentrations of ATP [11,16—18] and is attributed to the very slow step $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ discussed above [6,11].

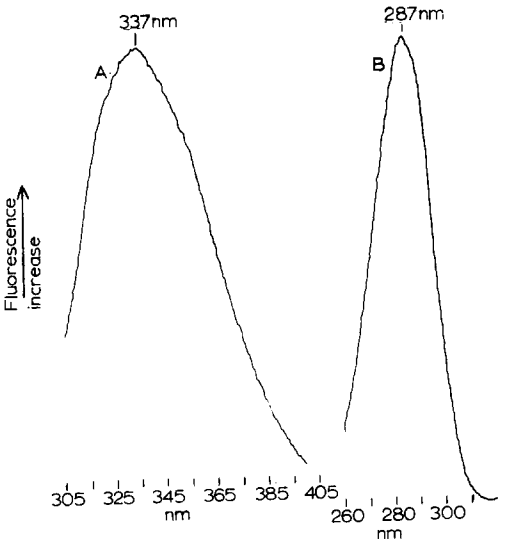


Fig. 10. Emission and excitation spectra of the $(Na^+ + K^+)$-ATPase protein fluorescence. 60 μg of sheep $(Na^+ + K^+)$-ATPase (approx. 14 units/min) was suspended in the 2.5 ml of the following medium: NaCl, 80 mM KCl, 1 mM $MgCl_2$ 2 mM Tris-HCl, 40 mM, pH 7.0. The emission spectrum was recorded with a fixed excitation wavelength of 295 nmetre and the excitation spectrum was recorded with a fixed emission wavelength of 325 nmetres. Spectra were not corrected for the wavelength sensitivity of the fluorimeter detection systems in order to allow a comparison in identical recording conditions with the difference spectra shown in Fig. 11. Slit-widths on both emission and excitation monochromators were fixed at 10 nm.

Protein fluorescence spectra

Emission and excitation spectra of the intrinsic fluorescence of $(Na^+ + K^+)$-ATPase are shown in Fig. 10. The amplitudes of signals accompanying ATP hydrolysis by this enzyme in conditions of Fig. 6a, are also plotted point by point in Fig. 11 (a and b) as a function of emission and excitation wavelengths. Difference spectra obtained in this way cannot of course be as accurate as continuous recordings, but it seems clear that the maxima of the emission (320—325) and excitation (295—300) curves are significantly shifted to the blue or red side respectively of the total fluorescence peaks. The excitation maximum in Fig. 11b implies clearly the involvement of a tryptophan residue(s). The K^+-dependent enhancement of tryptophan fluorescence in Na^+-free media (Fig. 2a) showed similar spectral shifts (not shown) but here it was not possible to average several signals on the same protein sample and so the accuracy of individual points was less satisfactory. The sheep enzyme used in these experiments is about 40% pure $(Na^+ + K^+)$-ATPase, on a protein basis. Since one is comparing in Figs. 10 and 11 characteristics of a specific response of the $(Na^+ + K^+)$-ATPase with the fluorescence of all the proteins in the preparation, it is important to exclude the possibility that the protein impurities disguise the true fluorescence characteristics of the $(Na^+ + K^+)$-ATPase protein. A very highly purified pig enzyme (32 units/min) showed essentially the same features as the spectra in Fig. 10. This makes one confident of the legitimacy of comparing spectra, using the less purified sheep enzyme. Although the spectral shifts seem

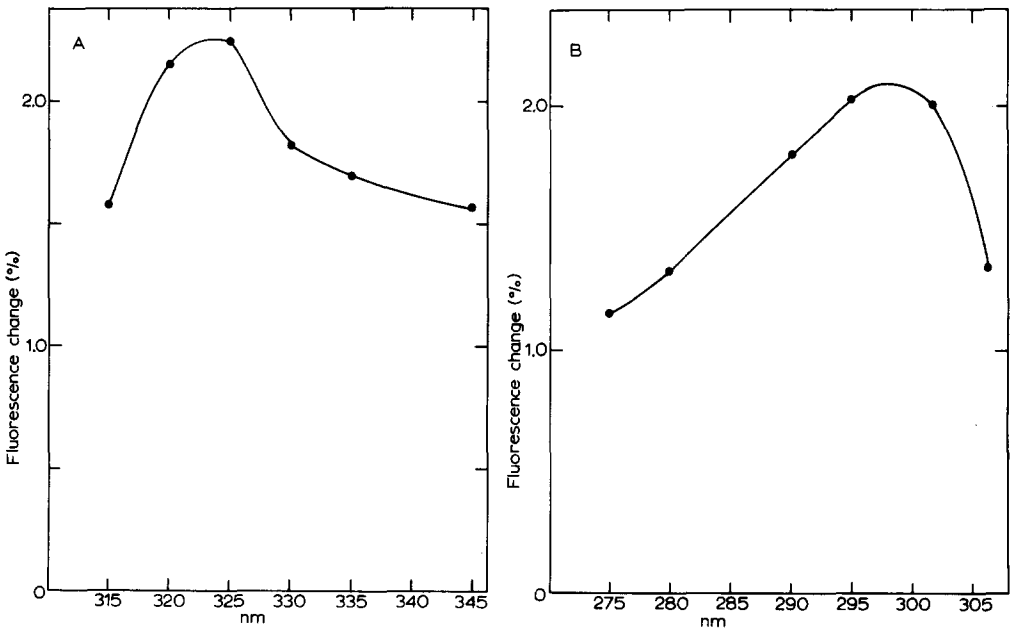


Fig. 11. Difference spectra of the tryptophan fluorescence change. Experimental conditions were as in Fig. 10. At each wavelength indicated in the figures, the amplitude of the fluorescence change accompanying hydrolysis of 0.5 μM ATP was measured. Each point represents the average amplitude of 2 or 3 signals recorded from the same protein sample. With variable emission wavelengths, the excitation wavelength was 295 nm, and for variable excitation wavelengths, the emission wavelength was 325 nm, chosen as described in Methods to optimize the signal amplitudes.

to be real phenomena, their extent cannot be estimated accurately by the methods employed. This follows from the necessity of optimizing measurements of the small fluorescence changes which leads inevitably to a reduction in the spectral discrimination of the fluorimeter (see Methods).

Discussion

The Na-form and K-form

The tryptophan fluorescence change observed upon binding of K^+ ions to ($Na^+ + K^+$)-ATPase provides further evidence for differences between the Na form and K-form of the enzyme. The small size of the signal, even in a highly purified ATPase preparation, implies perhaps that a single or small fraction of the total tryptophan residues are involved in the conformational change. The increase in fluorescence emission and spectral shifts described in Fig. 11 probably reflect an increase in the quantum yield of the relevant tryptophan residue(s). This could be due to a change from a relatively hydrophilic microenvironment in the Na form to a more hydrophobic state in the K^+ media (see ref. 19). The sarcoplasmic reticulum Ca^{2+}-ATPase has recently also been reported to show a small Ca-dependent change in protein fluorescence [20].

Opposing effects of Na^+ or K^+ on the ($Na^+ + K^+$)-ATPase have been observed in studies of nucleotide binding [6,21,22], the response to tryptic digestion [7], phosphorylation by inorganic phosphate [8,24] and modification of irreversible inhibition of activity by Be^{2+} and F^- ions [25]. Nucleotide binding is antagonized by the presence of K^+ or its congeners with the exception of Na^+ [21,22,3]. Published data indicate that displacement of ATP or FTP by K^+ ions at fixed low concentrations of nucleotide and very low Na concentrations (<130 μM) requires about 100—200 μM KCl for a half-maximal response [6,22, 21]. With 80 mM NaCl, we observed a $K_{0.5}$ of about 10 mM KCl for displacement of FTP and FDP [6]. These values correspond rather closely with the K^+ concentrations for a half maximal tryptophan fluorescence change shown in Fig. 3, and given in the text. Unfortunately, the accuracy of these K^+ titrations is such that one cannot distinguish interesting features such as sigmoidicity at the foot of the curves. Therefore it is not possible to conclude whether one or more K^+ ions must bind to the enzyme in order to provoke the tryptophan response. Nevertheless, it is reasonable to attribute the effects of K^+ ions on nucleotide binding and the tryptophan fluorescence to occupancy of the same cation-loading sites. Our previous observations of the effects of K^+ ions on equilibrium binding and the kinetics of displacement of FTP and FDP, lead us to propose that these effects are induced by binding of the K^+ to internal cation-loading sites (see ref. 6 for detailed reasoning).

Reaction mechanism

(a) Behaviour at low nucleotide concentrations. The tryptophan fluorescence changes associated with hydrolysis of ATP can be interpreted simply in terms of mechanisms like that shown in Fig. 1. These experiments have been useful in defining the kinetic relationships between the enzyme conformation $E_1 \cdot Na$, $E_2 \cdot (K)$ and E_2P which in the past could be inferred only from indirect observations.

(1) The experiments in Figs. 2 and 6 identify directly the K-form of the enzyme and show that it can be formed either by binding K^+ ions to the non-liganded enzyme or during hydrolysis of ATP in the presence of K^+. Prior to addition of ATP the enzyme is almost entirely in the state $E_1 \cdot Na$. Upon addition of ATP at low concentrations, the $E_2 \cdot (K)$ species appears, and in these conditions it must be the only hydrolysis intermediate present in appreciable amounts.

(2) In the absence of K^+ ions the phosphoenzyme E_2P is the major turnover intermediate [11]. The different amplitudes of the tryptophan fluorescence change in the absence or presence of K^+ ions presumably distinguishes between the phosphoenzyme and the K-form. The experiment in which ATP and K^+ were added sequentially (Fig. 9) is explained most economically on the assumption that the K^+-sensitive E_2P precedes $E_2 \cdot (K)$ during hydrolysis of ATP. Inhibition of ATP hydrolysis by K^+ ions indicated by the fluorescence responses in Figs. 8b and c, or Figs. 9a and b, implies of course that reconversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$ at low nucleotide concentrations is appreciably slower than hydrolysis of E_2P in the absence of K^+ ions.

(3) The influence of the Na^+ ion concentration on the duration of the tryptophan fluorescence response, without K^+ (see in Figs. 8a and b) is strikingly similar to effects we reported on the formycin fluorescence signals accompanying FTP hydrolysis [5]. One can infer from observation of this kind that the large rise in hydrolysis of ATP in the range 5—80 mM Na^+ which occurs in the absence of K^+ [11,16] is due to an increase in the rate of dephosphorylation of E_2P. Beaugé and Glynn have recently confirmed this conclusion in direct measurement of the rates of dephosphorylation of E_2P [26]. The interactions of Na^+ ions with the phosphoenzyme are rather complex and are probably connected with regulatory effects of Na^+ ions at the external aspect of the Na pump (discussed in detail in refs. 1, 16, 27).

Behaviour at high ATP concentrations

The remarkable feature of the conformational transition $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ is of course its very slow rate in the absence of ATP and the large acceleration caused by ATP binding to a low affinity site. This confirms our findings with the formycin nucleotides [3,6] but the present results are a significant advance because the measured rates of the transition and the range of ATP concentrations at which observations could be made have been greatly extended.

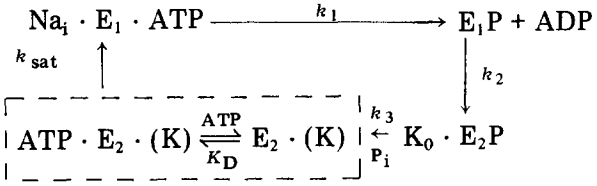
Provided that ATP equilibrates with $E_2 \cdot (K)$ much more rapidly than the rate of the conformational transition, one may write

$$k_{obs} = k_{sat} \cdot \frac{[ATP]}{[ATP] + K_D} + k_0 \qquad (1)$$

K_{obs} is the observed rate constant for the conformational change at a particular concentration of ATP. k_{sat} is the rate constant at saturating concentration of ATP. k_0 is the rate constant in the absence of ATP. $k_{sat} + k_0 = k_{max}$. K_D is the dissociation constant for binding of ATP to the low affinity site on $E_2 \cdot (K)$. At ATP concentrations much lower than the K_D a straight line relationship should hold, with a slope equal to k_{sat}/K_D (see fig. 5). The calculation of k_{sat} is ham-

pered by the lack of direct estimates of K_D. An accessible experimental constant such as the K_m for ATP is sustaining $(Na^+ + K^+)$-ATPase activity, is unsatisfactory as a substitute for K_D, because its value will depend on the rate constants of all the steps in the cycle. One can estimate the K_D and so k_{sat} by an indirect procedure using the experimental constant K_m and the rate constant of the individual steps measured by rapid kinetic techniques. (see below.)

With optimal concentrations of Na^+ (120 mM), K^+ (10 mM) Mg (3 mM) and variable ATP in the concentration range of the low affinity sites (100—2000 μM), the steps of the turnover cycle are effectively irreversible and can be depicted simply as follows:



The irreversibility of the phosphorylation and dephosphorylation steps is due to the effective absence of ADP and P_i. Reversal of the step $E_1P \rightarrow E_2P$ is known to require the absence of K^+ ions and binding of Na^+ to phosphoenzyme at low affinity binding sites [28]. Finally the conformational change ATP · E_2 · (K) → E_1 · ATP should also be quite irreversible in the above conditions for $(Na^+ + K^+)$-ATPase activity (refs. 6 and 7). The scheme assumes again rapid equilibration of ATP with $E_2 \cdot (K)$ and also that the rate of the transition $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ in the absence of ATP is negligible by comparison with the rate in the fully ATP bound state (see Fig. 5). A steady state analysis shows that the Km for ATP is equal to

$$\frac{K_D}{1 + k_{sat}(1/k_1 + 1/k_2 + 1/k_3)} \qquad (2)$$

The phosphorylation and dephosphorylation steps of $(Na^+ + K^+)$-ATPase have been studied by a number of workers, with the use of rapid flow apparatus (see refs. 29—35). The most comprehensive studies, in conditions similar to those of our experiments, are those of Mardh et al. [29—31] using a brain enzyme. Rate constants for k_1, k_2 and k_3 at 20°C derived from these studies are respectively approx. 11 000 min^{-1}, approx. 4500 min^{-1} and >14 000 min^{-1}. The Km for activation of the ATPase at 20°C is approx. 200 μM ATP for both brain [29,36] and the kidney enzyme (SK unpublished). Thus assuming similarity in the values of the individual rate-constants for the two tissues, one can calculate K_D and k_{sat} using the relationship in Eqn. 2 and the slope of the line k_{obs} vs. [ATP] in Fig. 5 (i.e. $k_{sat}/K_D = 0.12$). This leads to a K_D of 450 μM and k_{sat} is about 54 s^{-1}. The value of k_{sat} is slightly overestimated because the value of k_3 is a minimal one, but this does not affect the conclusion below. Note also that the values of K_m, k_1, k_2 and k_3 apply to conditions with Mg^{2+} present and the rates of $E_2 \cdot (K) \rightarrow E_1 \cdot Na$ are measured in the absence of Mg^{2+}, but this should not affect the calculation of k_{sat} because as we have shown with the formycin nucleotides [6], the rate of the conformational change is not affected by addition of Mg^{2+}.

The turnover number of the kidney enzyme in optimal conditions of ATP hydrolysis at 20°C was estimated to be 30 s^{-1} (±1 S.E.M.) from measurements of ATP hydrolysis and maximal levels of the phosphoenzyme (SK unpublished). The value is roughly 1/5 of that estimated similarly at 37°C [37]. This leads one to the striking conclusion that a large fraction i.e. 30/54 or 0.55 of the enzyme is present as the K-form in the steady-state of ATP hydrolysis. Similarly one can calculate roughly the fractions of E_1, E_1P and E_2P from the experiments of Mardh et al. [29—31] with the brain enzyme, using the measured rate constants and turnover number approx. 20 s^{-1}. These proportions are $E_1 \approx 0.11$, $E_1P \approx 0.27$ and $E_2P < 0.86$. Assuming that $E_2 \cdot (K)$ is the only other non-phosphorylated intermediate it should constitute at least 53% of the brain enzyme molecules. The excellent agreement with the prediction above may be fortuitous. Nevertheless, it is encouraging that results from both phosphorylation experiments, in which $E_2 \cdot (K)$ is not directly identified, and the direct fluorescence approach, are compatible with the hypothesis that a large fraction of the enzyme will be $E_2 \cdot (K)$ in optimal conditions of ATP hydrolysis.

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