Biochimica et Biophysica Acta, 525 (1978) 230—251

BBA 68464

ELEMENTARY STEPS OF THE ($Na^+ + K^+$)-ATPase MECHANISM, STUDIED WITH FORMYCIN NUCLEOTIDES

S.J.D. KARLISH [a], D.W. YATES [b] and I.M. GLYNN [c]

[a] *Department of Biochemistry, Weizmann Institute, Rehovoth (Israel),* [b] *Department of Biochemistry, University of Bristol, Bristol BS8 1TD and* [c] *Physiological Laboratory, University of Cambridge, Cambridge CB2 3EG (U.K.)*

(Received September 22nd, 1977)
(Revised manuscript received February 16th, 1978)

Summary

1. Formycin triphosphate (FTP), a fluorescent analogue of ATP, is a substrate for ($Na^+ + K^+$)-ATPase (ATP phosphohydrolase, EC 3.6.1.3), with properties similar to those of ATP.

2. FTP and formycin diphosphate (FDP) bind to the enzyme with high affinity and, on binding, the nucleotide fluorescence is enhanced 3—4-fold. It is therefore possible, with a stopped-flow fluorimeter, to measure the rates of binding and release of FTP and FDP under conditions in which turnover does not occur.

3. When the enzyme · FTP complex is exposed to conditions permitting turnover (Mg^{2+}, $Na^+ \pm K^+$), changes in fluorescence occur which can be explained by supposing that they reflect the interconversion of states with or without bound nucleotides. A rapid fall in fluorescence, that we attribute to the rapid release of FDP from newly phosphorylated enzyme, is followed by a steady state in which low fluorescence suggests that little nucleotide is bound. Eventually, exhaustion of FTP allows rebinding of FDP to the enzyme, which is signalled by a rise in fluorescence.

4. The estimated rate of FDP release from newly formed phosphoenzyme is unaffected by the presence of K^+ (0—2 mM) or the concentration of FTP (1—20 μM).

5. Experiments with [γ-^{32}P]FTP show that about 1 mol of ^{32}P is incorporated per mol of enzyme. The rate of phosphorylation of the enzyme by [γ-^{32}P]FTP has been measured with a rapid-mixing-and-quenching apparatus.

6. Kinetic data from the fluorescence and phosphorylation experiments

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Abbreviations: FTP, formycin triphosphate; FDP, formycin diphosphate; FMP, formycin monophosphate; CDTA, (*trans*-1,2-cyclohexylenedinitrilo)tetraacetic acid.

show that the behaviour of the enzyme, at least at the low nucleotide concentrations employed, is consistent with the Albers-Post model, and is difficult to reconcile with models in which K^+ acts at or before the step in which FDP is released during turnover.

Introduction

In the last few years, ($Na^+ + K^+$)-ATPase (($Na^+ + K^+$)-dependent ATP phosphohydrolase, EC 3.6.1.3) has been purified from a number of sources, and much information about its structural, biochemical and transporting properties is now available (for reviews see refs. 1—4). Until very recently, most information about intermediate biochemical steps in the pump cycle has been obtained by following the formation and disappearance of phosphoryl enzyme derivatives. Since the interpretation of these experiments is still controversial [1,2,4], it is useful to have an independent method for looking at intermediate steps in the cycle.

We recently reported experiments with a purified ($Na^+ + K^+$)-ATPase prepared from pig kidney, showing that formycin nucleotides, which are fluorescent analogues of adenine nucleotides [5—9], bind to the enzyme with high affinity, and that the binding is associated with an increase in the nucleotide fluorescence [10]. In the presence of suitable ligands, binding of FTP is followed by hydrolysis, and a characteristic pattern of changes in fluorescence is observed. These features make it possible to follow steps in the cycle previously inaccessible, and to resolve some of the uncertainties left by the phosphorylation experiments.

In this paper we describe experiments with formycin nucleotides which fall into five groups:

(i) Experiments showing that the behaviour of the enzyme with FTP as substrate parallels the behaviour with ATP as substrate.

(ii) Experiments in which changes in the steady-state level of fluorescence have been used to measure the binding of formycin nucleotides to the enzyme in conditions not allowing turnover.

(iii) Experiments with a stopped-flow fluorimeter, in which changes in fluorescence associated with binding or displacement of formycin nucleotides, under conditions not allowing turnover, have enabled us to estimate the rate constants for the binding and release of FTP and of FDP.

(iv) Experiments in which the stopped-flow fluorimeter has been used to record transient changes in fluorescence accompanying hydrolysis of FTP.

(v) Experiments with a rapid-mixing-and-quenching apparatus, in which [γ-^{32}P]FTP has been used to measure the rates of phosphorylation and dephosphorylation of the enzyme.

The experiments designed to measure the transient changes in fluorescence accompanying FTP hydrolysis are especially interesting, since, if we assume that the changes in fluorescence reflect binding and release of nucleotide rather than changes in the fluorescence of bound nucleotide, they provide a method for monitoring steps in the ($Na^+ + K^+$)-ATPase cycle that have previously not been observable.

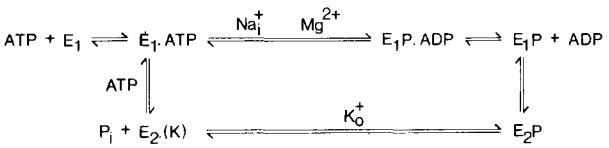

Fig. 1. An updated version of the Albers-Post scheme for the mechanism of $(Na^+ + K^+)$-ATPase (see refs. 1, 4, 11—13). E_1 is a form of the enzyme with a high affinity for ATP which predominates in Na^+-containing K^+-free media. The presence of Mg^{2+} brings about phosphorylation to the primary form of the phosphoenzyme $E_1P \cdot ADP$. Dissociation of ADP yields E_1P, a form of phosphoenzyme which can transfer the phosphoryl group back to ADP. A conformational change is thought to produce E_2P, a form of the phosphoenzyme which is hydrolysed slowly spontaneously but rapidly in the presence of K^+. $E_2 \cdot (K)$ is a dephosphoenzyme (containing occluded K^+) formed by the K^+-catalyzed hydrolysis of E_2P. A second conformational change converts $E_2 \cdot (K)$ back to the E_1 form of the enzyme. This change is supposed to be slow at low ATP concentrations. but rapid if ATP is bound at a low-affinity binding site. Note that the steps immediately responsible for moving Na^+ or K^+ across the membrane are not defined.

In reading through the accounts of the experiments, it is helpful to bear in mind the salient features of the Albers-Post scheme for the $(Na^+ + K^+)$-ATPase mechanism [1,4,11—13] (see Fig. 1), which we believe to be consistent with the results. It should be noted, however, that though the scheme specifies the chemical steps which require Na^+ or K^+, it does not specify which steps move the ions across the membrane. That question is considered in the following paper [14].

A preliminary account of some of the experiments reported here has already been published [10].

Materials and Methods

Preparation of enzyme. $(Na^+ + K^+)$-ATPase was purified from the outer medulla of pig kidney by the simpler of Jørgensen's two procedures [15]. The particles were washed twice in a solution containing 25 mM histidine (pH 7.5 at 20°C) and 1 mM EDTA, to remove traces of ATP, and were then stored in this solution at 0°C. The specific activities of different preparations were in the range 14—23 μmol ATP hydrolysed $\cdot$ (mg protein)$^{-1} \cdot$ min^{-1}. Ouabain-insensitive ATPase activity was usually not detectable (<0.5% of total).

The molar concentration of enzyme could be estimated to an accuracy of about 20% from the measured specific activity, assuming that pure $(Na^+ + K^+)$-ATPase preparations have an activity of 40 μmol $\cdot$ (mg protein)$^{-1} \cdot$ min^{-1}, and that the molecular weight is 250 000 [15,16].

Preparation of formycin nucleotides. Formycin was obtained from Meiji Seika Kaisha Ltd., Kawasaki, Japan. FMP was prepared chemically using a procedure described by Eccleston and Trentham [17] for the preparation of thio-IMP, which is itself based on a procedure of Yoshikawa et al. [18]. FTP and FDP were either prepared chemically [17], or were made from FMP by an adaptation of a published enzymic method [8]. FTP was prepared by incubating, for 1 h at 25°C, 2 ml of a solution containing: 17 mM FMP, 20 μM FTP (to prime the system), 100 mM Tris/Tris $\cdot$ HCl (pH 8.0 at 25°C), 20 mM $MgCl_2$, 50 mM creatine phosphate, 100 units of creatine kinase, and 40 units of adenylate kinase. Quantitative conversion of FMP to FTP was checked by thin-layer chromatography on polyethyleneimine-impregnated (PEI) cellulose sheets

[17]. FTP was then separated from the other components by elution from Dowex-1-Cl. FDP was prepared from FTP by incubating it with glucose and hexokinase [8], or was isolated as a by-product in the preparation of FTP.

[γ-^{32}P]FTP of high specific activity (50 Ci/mol) was prepared either by photophosphorylation of FDP using lettuce chloroplasts [19] or by the method of Glynn and Chappell [20].

Concentrations of formycin nucleotides were estimated from absorption measurements at 295 nm ($\epsilon_{295} = 1 \cdot 10^4\ M^{-1} \cdot cm^{-1}$).

FTPase activity. FTP hydrolysis was followed by measuring the release of ortho[^{32}P]phosphate from [γ-^{32}P]FTP. Reaction mixtures, of volume 0.5 ml, containing enzyme, [γ-^{32}P]FTP, salts and buffer, as indicated in Table I or in the text, were incubated in duplicate at room temperature (20 ± 2° C). Conditions were arranged so that not more than 10% of the FTP was hydrolysed. Incubations were terminated by the addition of 0.1 ml of 50% (w/v) trichloroacetic acid followed by 1 ml of water, 1 ml of ammonium molybdate (5% (w/v) in 2 M H_2SO_4) and 2 ml of a 4 : 1 (v/v) mixture of isobutanol and benzene. The tubes were shaken and centrifuged, and 1-ml aliquots were removed for scintillation counting. Total counts in FTP were usually estimated by allowing all of the FTP to be hydrolysed and then extracting the ^{32}P as phosphomolybdate. Substrate blanks were run to allow for non-enzymic hydrolysis and for ortho-[^{32}P]phosphate contamination of the [γ-^{32}P]FTP.

Fluorescence measurements. Changes in steady-state levels of fluorescence were measured with a Farrand fluorimeter, equipped with an overhead stirrer and injection port which allowed additions to be made during continuous stirring and recording. Formycin nucleotide fluorescence was excited at 310 nm, and the emission at 380 nm was measured. A Wratten 18A stray-light filter was placed after the excitation monochromator, and a Schott WG-365 UV cut-off filter was placed before the emission monochromator. Changes in fluorescence resulting from binding or release of nucleotide had to be measured against a background made up of intrinsic protein fluorescence, the fluorescence of unbound nucleotide, and scattered light. The excitation and emission wavelengths chosen gave the best compromise between sensitivity and background rejection. At optimal protein and FTP of FDP concentrations, the change in signal caused by nucleotide binding was as much as 10% of the total emission. At formycin nucleotide concentrations greater than about 30 μM, however, the problem of measuring small relative changes in fluorescence against the high background became insuperable.

Stopped-flow fluorescence measurements. Experiments were done at room temperature (20 ± 2° C) with the instrument described by Bagshaw et al. [21], but modified for split-beam operation. Fluorescence was excited at 310 nm using a 10-nm bandpass monochromator, and the emitted light was detected after passage through Schott U.G. 11 and K.V.370 filters which excluded most of the light below 360 nm. Signals were recorded either on a Tektronix type 564 storage oscilloscope or on a Datalab DL 905 transient recorder.

Phosphorylation experiments. Steady-state levels of phosphorylation were measured by mixing enzyme (50—100 μg protein) with [γ-^{32}P]FTP (4 or 40 μM) in about 1 ml of a solution containing 80 mM NaCl, 20 mM Tris/Tris · HCl (pH 7.2 at 20° C) and 10 mM CDTA. Enough $MgCl_2$ was added to give a final

free Mg^{2+} concentration of 2 mM, and the reaction mixture was quenched 5 s later with 5 ml of ice-cold 20% $HClO_4$ containing 2 mM ATP and 2 mM K_2HPO_4. After standing for at least 10 min at 0°C, the denatured protein was collected on 25-mm Whatman GF/C glass filter discs on a vacuum manifold and counted as described by Yates and Duance [22]. To correct for traces of [γ-^{32}P]FTP adhering to the denatured protein or the filter, [γ-^{32}P]FTP was added to quenching solution containing protein, and, after the suspension had been taken through the standard filtering procedure, the residual counts were subtracted from the counts of the other samples. The correction was usually 2—3%.

Rates of phosphorylation were measured with the quenched-flow machine described by Gutfreund [23]. The length of the delay tube, and the speed of the syringe plungers, were varied to produce samples with ages from 5 to 200 ms after mixing. These were quenched into tared vials, the volume of enzyme/FTP mixture delivered (0.7—1.3 ml) being calculated from the final weight of each vial.

Results

FTP hydrolysis

Table I shows that FTP was hydrolysed at rates which were greatly affected by its own concentration and by the concentrations of Na^+ and K^+. At high concentrations of FTP (300 μM), Na^+ and K^+ acted synergistically to activate hydrolysis; at low concentrations of FTP, K^+ inhibited hydrolysis.

The rate of FTP hydrolysis in the absence of K^+ was measured at different FTP concentrations (0.025—2.5 μM) with the Na^+ concentration fixed at 125 mM, and at different Na^+ concentrations (5—125 mM) with the FTP concentration fixed at 10 μM. (Tris was used to keep the ionic strength constant.) With 125 mM Na^+, the dependence of hydrolysis rate on FTP concentration could be described by a Michaelis curve with a K_m of 0.5 μM (not shown). With 10 μM FTP, the relation between hydrolysis rate and Na^+ concentration was

TABLE I

HYDROLYSIS OF FTP BY (Na^+ + K^+)-ATPase

The reaction mixtures, 500 μl in volume, contained 1.15 μg enzyme protein/ml; [γ-^{32}P]FTP, NaCl and KCl as shown; Tris/Tris · HCl (pH 7.5 at 20°C) 25 mM in Expt. 311275 and sufficient to make the total cation concentration 100 mM in Expt. 191275; 4 mM $MgCl_2$; 1 mM EDTA. Incubation was at 21°C, and the duration was varied between 5 and 60 min so that not more than 10% of the FTP was hydrolysed.

Experiment	Concentrations (mM) of alkali-metal ions		Concentration of FTP (μM)	Rate of FTP hydrolysis (nmol · mg^{-1} · min^{-1})
	Na^+	K^+		
311275	165	0	300	147
	165	5	300	644
191275	80	0	10	220
	80	1	10	77.6
	10	0	10	49.1

complex with a plateau at intermediate Na^+ concentrations (5—10 mM Na^+) and a progressive rise, without saturation, at higher concentrations (not shown).

When K^+ was present, the rate of FTP hydrolysis increased with FTP concentration over a much wider range, and was not maximal at 300 μM FTP. We did not determine the full activation curve, however, because the experiment required more FTP than was available.

Prevention of FTP hydrolysis. Since most of the fluorescence experiments described in this paper involved the binding of FTP to the enzyme before the addition of some other agent, such as Mg^{2+} or ATP, it was necessary to verify that FTP premixed with enzyme in media nominally free of Mg^{2+} was not in fact hydrolysed.

This was tested in the experiment of Fig. 2, in which [γ-^{32}P]FTP was mixed with a high concentration of enzyme (about 1.3 μM in sites), in the presence of different concentrations of ions and EDTA or (*trans*-1,2-cyclohexylenedinitrilo) tetraacetic acid (CDTA). Surprisingly, in Na^+-containing media without added Mg^{2+}, relatively rapid hydrolysis occurred in the presence of 1 mM EDTA, 1 mM CDTA or even 10 mM EDTA. Very high levels of CDTA (>10

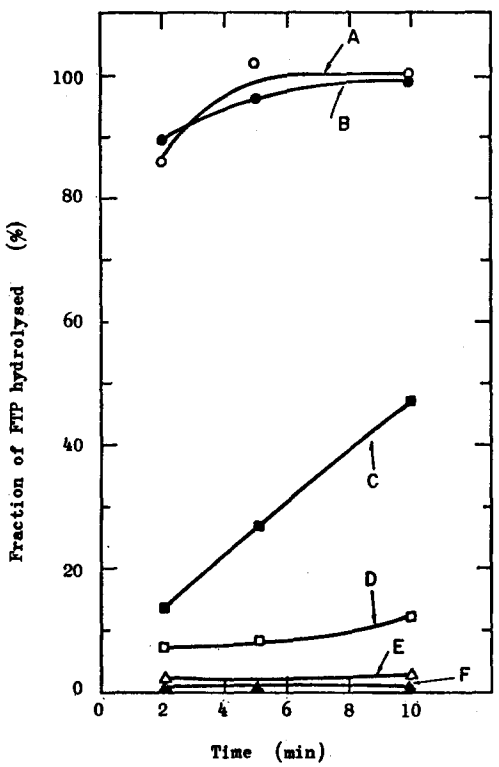

Fig. 2. Na^+-dependent hydrolysis of FTP by (Na^+ + K^+)-ATPase in media nominally free of Mg^{2+} and containing divalent metal ion chelators. Each tube contained, in a volume of 100 μl: 90 μg enzyme protein (specific activity 14 $\mu mol \cdot mg^{-1} \cdot min^{-1}$), 4 μM [$\gamma$-^{32}P]FTP, and at least 22 mM Tris (pH 7.5 at 20°C). NaCl was 80 mM in tubes A—D, 20 mM in E, and absent in F. EDTA (Tris salt) was 1 mM in A, and 10 mM in C and F. CDTA (Tris salt) was 1 mM in B, 10 mM in D, and 25 mM in E. Incubation was at 20°C. At the times indicated in the figure, 30-μl aliquots were withdrawn and squirted into 1.2-ml portions of 5% (w/v) trichloroacetic acid at 0°C.

mM), or the absence of Na^+, were necessary to reduce the FTP hydrolysis to a low level. These findings seem to confirm the existence of tightly bound Mg^{2+} reported by Skou [24]. For most fluorescence experiments involving prebound FTP, 10 mM CDTA was therefore included in the medium, and the enzyme concentrations used were not more than a third of that used in the experiments of Fig. 2.

Equilibrium binding

Fluorescence titrations of binding were performed by premixing enzyme with FTP or FDP at different concentrations, and then recording the fall in fluorescence when the bound formycin nucleotide was displaced with an excess of ATP. Control experiments showed that, at the wavelengths used, changes in protein fluorescence did not contribute to the observed changes in fluorescence.

If there is only one kind of binding site, and no interaction between sites, the relation between the fractional saturation of sites (R) and the total concentration of FTP (free and bound) is given by:

$$\frac{1}{1-R} = \frac{1}{K_D} \cdot \frac{[FTP_{tot}]}{R} - \frac{nE_{tot}}{K_D}$$

where K_D is the dissociation constant of the FTP-site complex, E_{tot} is the total concentration of enzyme, and n is the number of binding sites per enzyme molecule (see page 71 of ref. 25). R, at each FTP concentration, is given by the ratio of the fall in fluorescence using that concentration to the estimated fall in fluorescence using saturating FTP concentrations, and a plot of $1/(1-R)$ against $[FTP]/R$ allows us to estimate K_D and n. Data from an experiment to measure FTP binding are shown in Fig. 3. The slope gives a value of 0.91 μM for K_D; values of 1.05 and 1.1 μM were obtained in two other experiments. From the intercept in Fig. 3 and the estimated enzyme concentration (E_{tot}) of 0.36 μM, n was calculated to be 1.36. Given the uncertainty in the estimate of enzyme concentration and in the magnitude of the intercept, this value is not significantly different from 1.

With FDP it is possible to measure binding in the presence as well as in the absence of Mg^{2+}, since hydrolysis does not occur and the enzyme is not contaminated with adenylate kinase. K_D for the enzyme · FDP complex was estimated to be 3.5 μM in the absence of Mg^{2+} and 4.8 μM when Mg^{2+} was present.

Competition between FTP and ATP or its non-hydrolysable analogue adenyl-5[1]-yl imido diphosphate (AMP-P(NH)P) has been investigated in experiments, like that of Fig. 4, in which different concentrations of the competing ligand were used to displace FTP bound to the enzyme. If there is simple competition between FTP and the ligand, we may write

$$\frac{E_{tot}}{[E \cdot FTP]} = 1 + \frac{K_D}{[FTP]}\left(1 + \frac{I}{K_i}\right)$$

where I is the concentration of ligand, and K_i is the dissociation constant of the ligand.

Since $[E \cdot FTP]$ is proportional to $(\Delta_\infty - \Delta_I)$, where Δ_I is the fall in fluores-

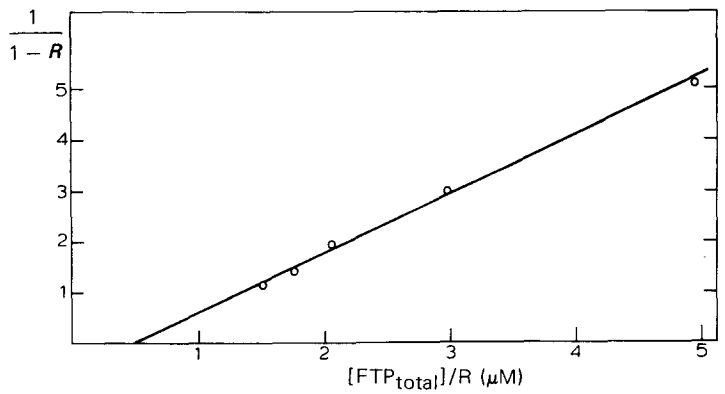

Fig. 3. The relation between FTP concentration and the binding of FTP to $(Na^+ + K^+)$-ATPase. The fluorescence cell contained, in a volume of 2.1 ml: 374 μg enzyme (specific activity 20.1 μmol · mg^{-1} · min^{-1}), 80 mM NaCl, 70 mM Tris/Tris · HCl (pH 7.8 at 20°C), 5 mM EDTA, and FTP at concentrations in the range 0.25—4 μM. Bound FTP was displaced from the enzyme by the addition of 2.5 μl of 50 mM ATP solution, and the drop in fluorescence (ΔF) was recorded; a second addition of ATP produced no further change in fluorescence. (When the FTP concentration was 4 μM, the fraction of the total signal attributable to the enhancement of FTP fluorescence on binding was about 10%; at this concentration of FTP, light scattering and protein fluorescence accounted for about 40% of the total signal.) The temperature was 20°C. R, the fraction of the high-affinity nucleotide binding sites occupied by FTP, was assumed to be equal to $\Delta F/\Delta F_\infty$, where ΔF was the observed fluorescence change at each concentration of FTP, and ΔF_∞ was calculated by extrapolation to infinite FTP concentration of a graph of $1/\Delta F$ against $1/[FTP_{free}]$. Because of the high concentration of the enzyme, $[FTP_{free}]$ was significantly less than $[FTP_{total}]$ but, for values of $[FTP_{total}]$ of 1 μM and above, a satisfactory estimate could be obtained by using a rough value for K_D (1 μM) suggested by the raw data. (An alternative basis for estimating ΔF_∞, which gave a very similar answer, is the observation that, in experiments in which a higher range of FTP concentrations was investigated, ΔF at 4 μM FTP was about 0.8 × ΔF at FTP concentrations at which the high-affinity nucleotide binding sites must have been nearly saturated.) The slope of the graph of $1/(1 - R)$ against $[FTP_{total}]/R$ gives a value of 0.91 μM for K_D. See text for further details and a discussion of the significance of the intercept.

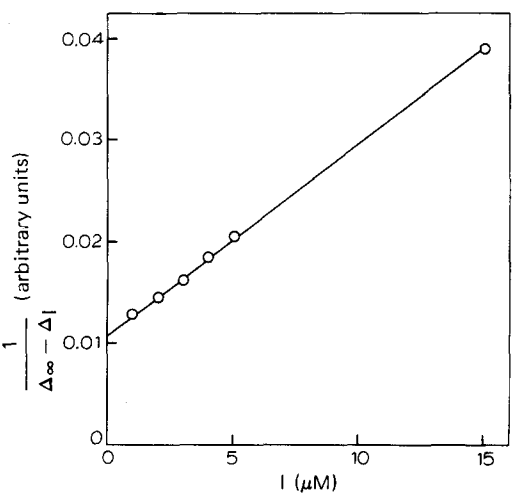

Fig. 4. Competition between FTP and AMP-P(NH)P for binding to $(Na^+ + K^+)$-ATPase. The fluorescence cell contained, in a volume of 2 ml: 300 μg enzyme protein (specific activity 15 μmol · mg^{-1} · min^{-1}), 4 μM FTP, 50 mM NaCl, 5 mM EDTA (Tris salt). The temperature was 20°C and the pH was 7.8. 2 μl of AMP-P(NH)P solution (1—15 mM) were added, and the drop in fluorescence Δ_I at each concentration (I) of AMP-P(NH)P was noted. Δ_∞, the drop in fluorescence as $I \to \infty$, was estimated from a "direct linear" plot [41]. (Changes in the concentration of free FTP were calculated to be always <5%.) The linearity of the graph of $1/(\Delta_\infty - \Delta_I)$ against I shows that AMP-P(NH)P competes with FTP for the enzyme binding sites (see text), and, from the intercept and slope, K_i has been calculated to be 1.1 μM.

cence caused by the ligand at concentration I, and Δ_∞ is the fall in fluorescence as $I \to \infty$, a graph of $1/(\Delta_\infty - \Delta_I)$ against I gives a straight line, from the slope and intercept of which K_i may be calculated. The straight line of Fig. 4 suggests that displacement of FTP by AMP-P(NH)P is competitive, and it leads to a value of 1.1 μM for K_i. A similar experiment with ATP gave $K_i = 0.15$ μM, which is close to the published values of 0.12 μM [26] and 0.22 μM [27] for the dissociation constant of the enzyme · ATP complex determined from binding studies.

Binding and release rates of formycin nucleotides

The enhancement of formycin fluorescence that occurs when formycin nucleotides bind to the enzyme, allows us to measure binding and release rates. For the reaction

$$E + FTP \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot FTP$$

the observed rate constant for net binding (k_{obs}), when enzyme and FTP are mixed in the stopped-flow fluorimeter, is given by the expression

$$k_{obs} = k_{+1}[FTP] + k_{-1}$$

(see page 121 of ref. 25).

Theoretically, measurements of net binding rates over a range of FTP concentrations would yield estimates of both k_{+1} and k_{-1}. In practice satisfactory results can be obtained only near an optimal FTP concentration which turns out to be approx. 4 μM. At lower concentrations the fraction of enzyme bound becomes too small; at higher concentrations the background fluorescence becomes too high.

The first trace (A) of Fig. 5 is representative of seven consecutive experiments which allowed calculation of an average net binding rate constant (k_{obs}) of 84.4 ± 6.3 s^{-1} at 4 μM FTP. We were not able to follow the kinetics of FDP binding because the higher concentrations of FDP necessary to achieve adequate binding led to loss of the signal in the background noise.

Release of FTP or FDP has been monitored as a fall in fluorescence following displacement of the bound nucleotide by an excess of ATP (Fig. 5B and 5C). The following rates have been obtained: for FTP, $k_{-1} = 32.6 \pm 1.6$ s^{-1} (five measurements); for FDP, $k_{-1} = 112 \pm 2$ s^{-1} (three measurements). In experiments of this kind, it is necessary that the binding rate of the displacing agent is fast compared with the dissociation rate being measured, and that the affinity is high so that displaced ligand has no opportunity to rebind (see page 206 of ref. 25). Since the equilibrium dissociation constant of the enzyme · ATP complex is only about 0.15 μM (see above), a final ATP concentration of 20 μM should have been sufficient; and, in fact, similar rates of dissociation were observed when the ATP concentration was increased 50-fold.

The second-order rate constant (k_{+1}) describing the real (as distinct from the net) rate of binding of FTP to the enzyme can be calculated from the rate constant for FTP release ($k_{-1} = 32.6$ s^{-1}), and either the net binding rate constant at 4 μM FTP ($k_{obs} = 84.4$ s^{-1}) or the equilibrium dissociation constant ($K_D = 1.02$ μM). The first method gives a value of $1.2 \cdot 10^7$ $M^{-1} \cdot s^{-1}$; the second a

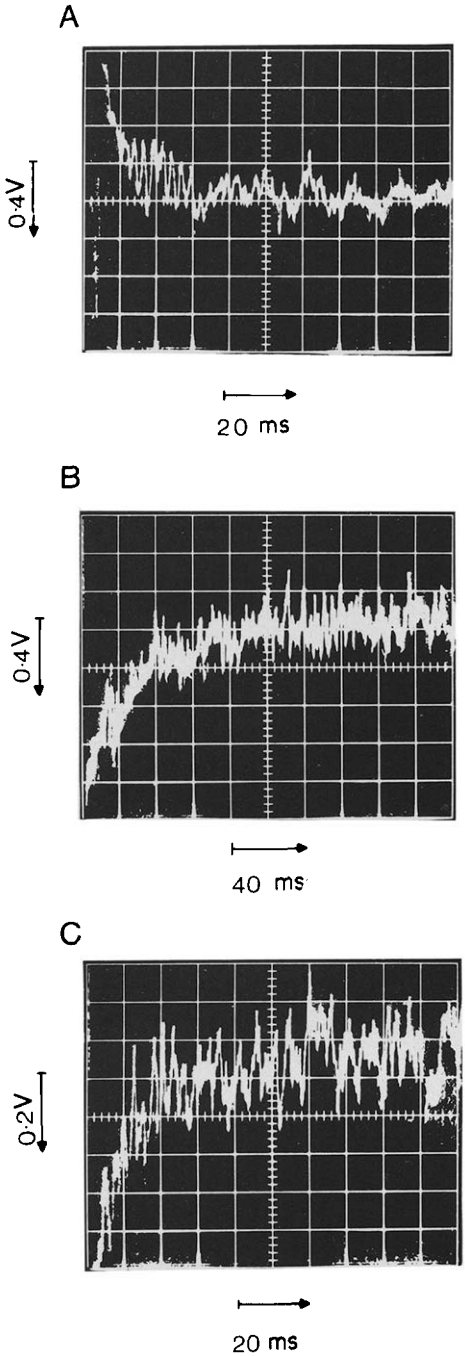

Fig. 5. Rate of binding and release of formycin nucleotides by $(Na^+ + K^+)$-ATPase, in Mg^{2+}-free media, monitored with the stopped-flow fluorimeter. An upward deflection indicates a fall in fluorescence. (A) FTP binding. Syringe I contained, in a volume of 2.5 ml: 2.1 mg enzyme protein (specific activity 23 μmol · mg^{-1} · min^{-1}), 40 mM NaCl, 60 mM Tris/Tris · HCl (pH 7.7 at 20°C); 10 mM CDTA (Na^+ salt). Syringe II contained 2.5 ml of the same basic medium but with 8.2 μM FTP and no enzyme. After mixing, the FTP concentration was 4.1 μM. The temperature was 18°C. The fluorimeter time constant was 1 ms. (B) FTP displacement by ATP. Syringe I contained, in a volume of 2.5 ml: 860 μg enzyme protein, 4 μM FTP, 40 mM NaCl, 60 mM Tris/Tris · HCl (pH 7.7 at 20°C); 10 mM CDTA (Na^+ salt). Syringe II contained 2.5 ml of the same basic medium but with no enzyme and with 40 μM ATP instead of 4 μM FTP. The temperature was 21°C. The fluorimeter time constant was 2 ms. (C) FDP displacement by ATP. Syringe I contained the same mixture as in B but with 10 μM FDP instead of 4 μM FTP. Syringe II contained the same mixture as in B. The temperature was 21°C. The fluorimeter time constant was 1 ms.

value of $3.16 \cdot 10^7\ M^{-1} \cdot s^{-1}$. For FDP, only the second method is available, and it gives a value of $3.20 \cdot 10^7\ M^{-1} \cdot s^{-1}$, which is not significantly different from the binding rate constant for FTP calculated in the same way.

Rates of binding and release of FTP in the presence of magnesium may be inferred from the phosphorylation experiments and will be discussed later.

Fluorescent changes accompanying the hydrolysis of FTP

Fig. 6 shows typical transient changes in fluorescence that accompany turn-

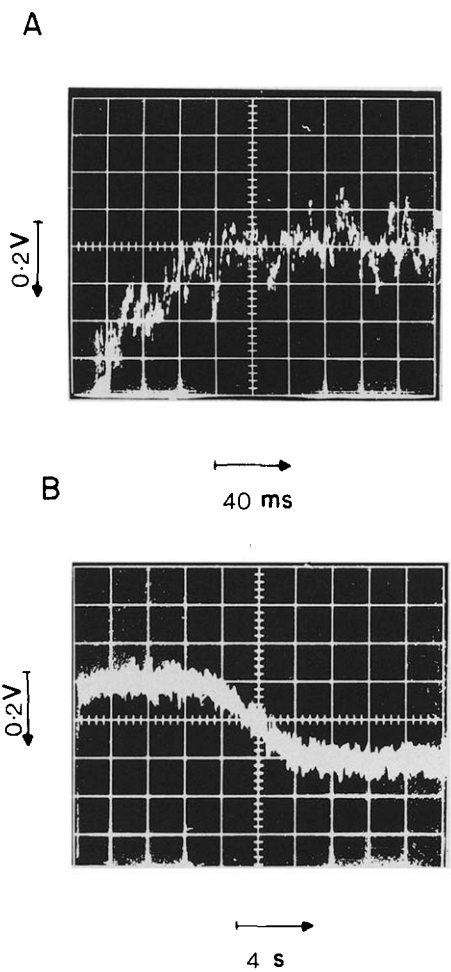

Fig. 6. Fluorescence changes accompanying hydrolysis of FTP prebound to $(Na^+ + K^+)$-ATPase. An upward deflection indicates a fall in fluorescence. Scan A has a duration of 200 ms, and shows the rapid fall in fluorescence that we attribute to the release of FDP from newly formed phosphoenzyme. Scan B has a duration of 20 s, and shows the low level of fluorescence that accompanies steady-state hydrolysis, and the increase in fluorescence that occurs as FTP becomes exhausted and the FDP formed during the reaction recombines with the enzyme. Syringe I contained, in a volume of 2.5 ml: 850 μg enzyme protein (specific activity 23 $\mu mol \cdot mg^{-1} \cdot min^{-1}$), 4.2 μM FTP, 40 mM NaCl, 60 mM Tris/Tris · HCl (pH 7.7 at 20°C); 10 mM CDTA (Na^+ salt). Syringe II contained 2.5 ml of the same basic medium and, in addition, 4.2 μM FTP and 22 mM $MgCl_2$. After mixing, the equilibrium concentration of free Mg^{2+} was 1 mM, though because net binding of Mg^{2+} by CDTA is slow [42] the concentration of Mg^{2+} during the scans may have been higher. The temperature was 17°C. The fluorimeter time constant was 2 ms for the fast scan (A), and 20 ms for the slow scan (B).

over, when enzyme that has bound FTP in a medium containing Na^+ and CDTA is suddenly mixed with excess Mg^{2+}. Control experiments showed (i) that changes in protein fluorescence, or in the fluorescence characteristics of free nucleotide, did not contribute to the observed changes in fluorescence, and (ii) that the magnitude of the drop in fluorescence associated with hydrolysis was essentially the same as the magnitude of the drop in fluorescence caused by displacing the bound FTP with ATP. If we assume that the changes accompanying hydrolysis reflect binding or release of nucleotide, and not changes in the fluorescence of bound nucleotide, the three phases may be interpreted simply as (a) dissociation of FDP from newly formed phosphoenzyme ($E_1P \cdot FDP \rightarrow E_1P + FDP$), (b) a steady state in which the enzyme molecules spend most of the time in a form or forms with low affinities for FTP and FDP, and (c) rebinding of FDP to free enzyme as the concentration of FTP approaches zero.

Table II summarises the results of an experiment in which we looked at the effects of changing the concentrations of K^+, Na^+ and FTP on the rates of the fluorescence changes accompanying hydrolysis. The first point to note is that the rate of the initial drop in fluorescence, which we attribute to the release of FDP from $E_1P \cdot FDP$, was not significantly affected by the presence of K^+, and was affected only slightly by a reduction in the Na^+ concentration from 80 to 10 mM. In contrast, the presence of 1 mM K^+, or the reduction in Na^+ concentration, led to a marked prolongation of the period of low fluorescence, reflecting presumably the reduction in the rate of turnover (see lines 3—5 in Table I). In the experiment of Table II, unlike those reported earlier [10], K^+ was present in both solutions before mixing, and therefore had access to the enzyme even before the first turnover. (A separate experiment showed that 1 mM K^+ does not displace bound FTP in the presence of 80 mM Na^+.) The second point to note is that the rate of the initial fall in fluorescence was not significantly affected by an increase in the FTP concentration. The duration of the period of low fluorescence was roughly proportional to the initial concentration of FTP, and this is understandable since, in the absence of K^+, even 4 μM FTP gives a nearly maximal turnover rate. Because it was not possible to measure the changes in fluorescence satisfactorily at FTP concentrations much

TABLE II

RATES OF THE FLUORESCENCE CHANGES ASSOCIATED WITH FTP HYDROLYSIS BY ($Na^+ + K^+$)-ATPase AT DIFFERENT Na^+, K^+ AND FTP CONCENTRATIONS

The procedure was similar to that described in the legend to Fig. 6. KCl, when present, was included in both syringes. The 10 mM Na^+ medium contained the Tris salt of CDTA instead of the sodium salt of CDTA, and 100 mM Tris/Tris · HCl instead of 60 mM Tris/Tris · HCl.

Concentrations (mM) of alkali-metal ions		Concentration of FTP (μM)	k_{obs} for FDP release (±S.E.) (s^{-1})	Approx. duration of steady state of low fluorescence (s)
Na^+	K^+			
80	0	4	32.7 ± 3.5 (n = 3)	8
80	0	12	33.6 ± 2.5 (n = 5)	22
80	0	20	42.9 ± 5.4 (n = 4)	35
80	1	4	31.1 ± 4.2 (n = 4)	36
10	0	4	23.0 ± 2.3 (n = 3)	22

above 20 μM, we also did a separate experiment in which sufficient ATP was included in the syringe containing the Mg^{2+} to give final ATP concentrations of 20 μM or 1 mM. In these conditions (see Fig. 7B), the prebound FTP phosphorylates the enzyme during its first turnover, after which occupation of the enzyme by ATP prevents further access by FTP. The rate of the initial drop in fluorescence was not significantly affected by the presence of ATP (40.6 ± 3.3 s^{-1} in the controls; 40.9 ± 2.6 s^{-1} with 20 μM ATP; 48.2 ± 5.5 s^{-1} with 1 mM ATP). In the presence of the excess of ATP, the final rise in fluorescence associated with the rebinding of FDP was, of course, not observed.

Experiments to investigate the rate of formation of phosphoenzyme

Phosphorylation of $(Na^+ + K^+)$-ATPase by [γ-^{32}P]FTP closely resembled

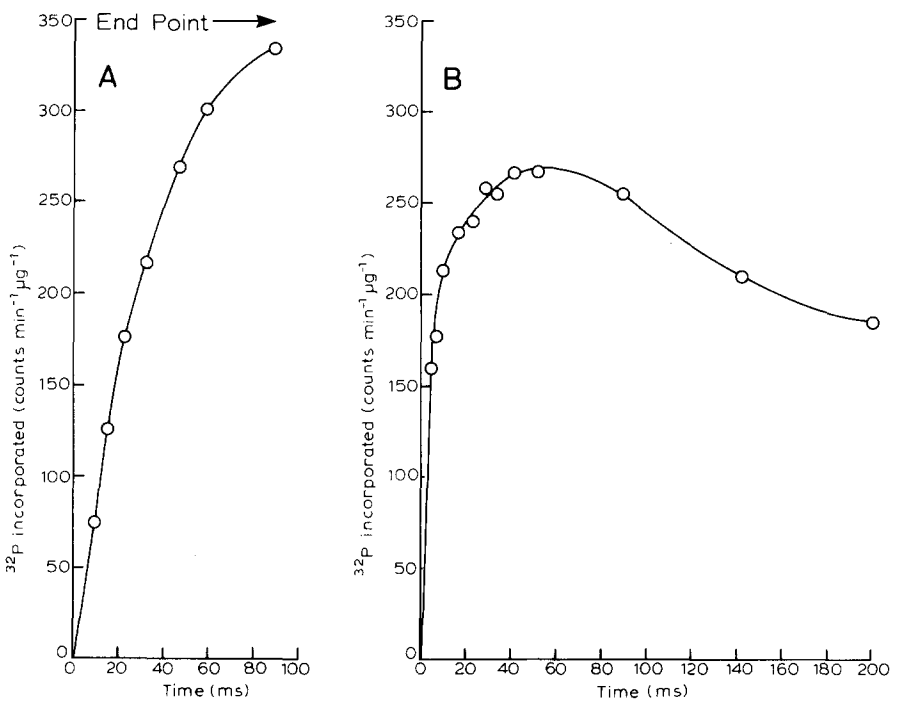

Fig. 7. (A) Time course of phosphorylation of $(Na^+ + K^+)$-ATPase by [γ-^{32}P]FTP which was not prebound. Syringe I of the quenched-flow apparatus contained, in a volume of 2.5 ml, 250 μg enzyme protein (specific activity 23 μmol · mg^{-1} · min^{-1}), 80 mM NaCl, 20 mM Tris/Tris · HCl (pH 7.7 at 20°C), 2 mM $MgCl_2$, 1 mM EDTA. Syringe II contained 2.5 ml of the same medium without enzyme but with 8 μM [γ-^{32}P]FTP. The temperature was 20°C. The end point shown in the figure was determined by mixing enzyme and substrate in the flow apparatus, and quenching the mixture 1—2 s later. (B) Phosphorylation of $(Na^+ + K^+)$-ATPase by prebound [γ-^{32}P]FTP in a single turnover. Syringe I contained, in a volume of 2.5 ml: 212 μg enzyme protein (specific activity 23 μmol · mg^{-1} · min^{-1}), 4 μM [γ-^{32}P]FTP, 40 mM NaCl, 60 mM Tris/Tris · HCl (pH 7.7 at 20°C); 10 mM CDTA (Na^+ salt). Syringe II contained 2.5 ml of the same medium without enzyme or FTP but with the addition of 22 mM $MgCl_2$ and 40 μM ATP. The temperature was 20°C. Incorporation of radioactivity when the enzyme was fully phosphorylated (determined in a separate experiment by mixing enzyme, FTP and Mg^{2+} in the flow apparatus and quenching 1—2 s later) was 354 cpm/μg enzyme protein. The maximal incorporation of radioactivity during the single turnover was 77% of this value. Since, with 4 μM FTP, only about 80% of the enzyme would have been binding FTP, virtually all of the prebound FTP must have succeeded in phosphorylating the enzyme.

phosphorylation by [γ-^{32}P]ATP. Incorporation of ^{32}P into $HClO_4$-precipitable protein required the presence of Na^+ and Mg^{2+}, and was reduced by at least 90% in media containing K^+ (not shown). In experiments in which the enzyme was allowed to hydrolyse FTP (4 or 40 μM) at 0 or 20°C in the absence of K^+, the average number of phosphorus atoms incorporated per enzyme molecule in the steady state was 0.92. Given the uncertainty in the estimate of enzyme concentration (see Materials and Methods), this is not significantly different from 1.

Fig. 7A shows the time course of phosphorylation of the enzyme when it was mixed with [γ-^{32}P]FTP in the presence of Mg^{2+} and Na^+. The end-point marked on the graph represents the incorporation observed 1—2 s after mixing. A pseudo first-order rate constant may be derived from the slope of a semi-logarithmic plot of ^{32}P incorporation against time. Data from two experiments, covering a range of FTP cencentrations, are collected together in Table III. At low concentrations, the observed rate of phosphorylation increased as the concentration was raised, because binding of FTP was rate limiting, but at higher concentrations the observed phosphorylation rate approached a maximum limited, presumably, by the rate of net phosphoryl transfer. The relation between observed rate of phosphorylation and FTP concentration, at low FTP concentrations, gave an estimated second-order rate constant for FTP binding which was $5 \cdot 10^6\ M^{-1} \cdot s^{-1}$. Extrapolation of the observed rate of phosphorylation to infite FTP concentration gave estimates of the rate constant for net phosphoryl transfer. For the two experiments in Table III these estimates were 96 and 76 s^{-1}.

Fig. 7B presents the results of a "radioactive chase" experiment using [γ-^{32}P]FTP, based on the kind of experiment first performed with [γ-^{32}P]-ATP by Post et al. [12]. Enzyme was mixed with [γ-^{32}P]FTP at a concentration of 4 μM, so that about 80% of the enzyme was bound to FTP. Na^+ was present, and hydrolysis of the FTP was prevented by 10 mM CDTA. Portions of the suspension were rapidly mixed in the flow machine with an excess of both Mg^{2+} and ATP and, after various time intervals, were quenched with $HClO_4$. As soon as the enzyme was mixed with ATP, any free enzyme (whether

TABLE III

RATE OF APPEARANCE OF PHOSPHOENZYME AT DIFFERENT FTP CONCENTRATIONS

The procedure in these experiments was similar to that described in the legend to Fig. 7A except that (i) the FTP concentrations in syringe II were double the concentrations shown below, and (ii) in Experiment 030976 the specific activity of the enzyme was only 14 μmol · (mg protein)$^{-1}$ · min^{-1}.

Experiment	Concentration of FTP (μM)	Observed rate constant (s^{-1})	Estimated rate constant as FTP → ∞ (s^{-1})
290976	4	33.3	96
	25	72.5	
	75	87.6	
030976	1	4.8	76
	4	21.5	
	9.5	36.3	
	16	45.3	

present initially or formed by dissociation of the enzyme · FTP complex) would have been occupied by ATP and so no longer available for phosphorylation by [γ-^{32}P]FTP. As can be seen from Fig. 7B, the level of incorporated ^{32}P rose quickly and reached a maximum which was about 80% of the level obtained in a separate experiment in the absence of ATP (not shown). The observed net phosphorylation rate constant, derived from the rising part of the curve in Fig. 7B, is 87 s^{-1}. Since the fraction of enzyme which became phosphorylated is approximately the same as the fraction which bound FTP, virtually all of the enzyme that bound FTP must have been phosphorylated. It follows that the rate constant for release of FTP from the enzyme must be much smaller than the observed rate constant for the phosphoryl transfer. If we assume that our estimate of the maximal level of phosphorylation is not more than 10% out, the rate constant for FTP release in the presence of Mg^{2+} must be less than 10 s^{-1}. As the phosphoenzyme was hydrolysed, the level of incorporated ^{32}P fell, and the large excess of ATP saturated the enzyme so that essentially only the first turnover involved the incorporation of ^{32}P. The fall in incorporated ^{32}P between 140 and 200 ms shows that the turnover rate must have been about 1—2 s^{-1}.

Discussion

FTP as a substrate for (Na^+ + K^+)-ATPase

The first group of experiments described in Results show that, in a variety of conditions, the behaviour of (Na^+ + K^+)-ATPase with FTP as substrate closely parallels the known behaviour with ATP as substrate [1]. More particularly (i) at high FTP concentrations, Na^+ and K^+ act synergistically to promote hydrolysis; at low FTP concentrations, K^+ slows hydrolysis. (ii) When Na^+ is present without K^+, FTP is hydrolysed slowly, and with a K_m that is very low and comparable with the dissociation constant of the enzyme · FTP complex (determined in separate binding experiments under conditions not permitting hydrolysis). (iii) When hydrolysis occurs in the absence of K^+, the complex Na^+ activation curve resembles that reported with ATP as substrate [13], with features that are thought to reflect three separate effects of Na^+ [28]. It therefore seems justifiable to use the behaviour observed with FTP as a clue to the mechanism of the (Na^+ + K^+)-ATPase when it is hydrolysing ATP.

Nucleotide binding

The features of formycin nucleotide binding revealed by the fluorescence titrations are in line with previous experience of nucleotide binding to the (Na^+ + K^+)-ATPase [26—28]. The high binding affinity of formycin nucleotides presumably reflects the fact that the six-membered ring in the purine base, which is known to be a major determinant of the strength of nucleotide binding to (Na^+ + K^+)-ATPase, is identical in formycin and adenosine (see ref. 9). The order of affinities FTP > FDP, and ATP > FTP > AMP-P(NH)P is not unexpected, in view of the differences between the nucleotides. As far as the rather poor estimate allows us to judge, only one molecule of FTP is bound to the enzyme with a high affinity, and this, too, is what would be expected from studies of ATP binding [26,27,30].

For the interpretation of transient changes in fluorescence, it is helpful to know not only equilibrium binding constants but also rate constants for the binding and release of nucleotides. The available estiamtes are collected together in Table IV. For FTP in the absence of Mg^{2+}, the quotient k_{-1}/k_{+1} (where k_{-1} and k_{+1} are the measured rate constants) is equal to 2.66 μM. Although this is greater than the measured value of K_D (1.03 μM), the uncertainty of the estimate of k_{+1} makes the difference insignificant; indeed, a more reliable value of k_{+1} ($3.16 \cdot 10^7$ $M^{-1} \cdot s^{-1}$) may be obtained from the measured values of K_D and k_{-1}. The 3—4-fold difference between k_{-1} for FTP and k_{-1} for FDP parallels the difference in the K_D values, and implies that, at equal concentrations, the two nucleotides bind at similar rates.

The estimates of rate constants for the binding and release of FTP, derived from measurements of the rates of change of phosphoenzyme concentration, are significantly lower than those derived from the fluorescence experiments (see Table IV). This is almost certainly because the phosphorylation experiments were done in the presence of Mg^{2+} and the fluorescence experiments in their absence. Very recently Mårdh and Post [31] have shown that the rate of dissociation of ATP from a (Na^+ + K^+)-ATPase preparation is reduced nearly 4-fold by the presence of Mg^{2+}. No direct estimate of K_D for FTP in the presence of Mg^{2+} is available, but if we take estimates of k_{+1} and k_{-1} obtained from the phosphorylation experiments (see Table IV), we get an upper limit for K_D of 2 μM. This is not unreasonable, since (i) the observed K_m for Na^+-FTPase activity was 0.5 μM (p. 234), and (ii) K_D for FTP in the absence of Mg^{2+} is about 1 μM, and the effect of Mg^{2+} on K_D for FDP does not lead us to expect a large change when Mg^{2+} is present.

Turnover experiments

When enzyme that has bound FTP in a medium containing Na^+ and CDTA is suddenly mixed with excess Mg^{2+}, the expected sequence of events may be

TABLE IV

EQUILIBRIUM AND KINETIC CONSTANTS DESCRIBING THE BINDING OF FORMYCIN NUCLEOTIDES TO (Na^+ + K^+)-ATPase

Constant	Units	Mg^{2+}	FTP	FDP	Type of evidence
K_D	μM	Absent	1.03	3.5	Equilibrium fluorescence titration. Fig. 3
K_D	μM	Present	—	4.8	Equilibrium fluorescence titration
K_m (Na^+-FTPase)	μM	Present	0.5	—	Steady-state FTP hydrolysis
k_{+1}	$M^{-1} \cdot s^{-1}$	Absent	$1.2 \cdot 10^7$	—	Stopped-flow fluorescence. See p. 238
k_{+1}	$M^{-1} \cdot s^{-1}$	Absent	$3.16 \cdot 10^7$	$3.20 \cdot 10^7$	Calculated from K_D and k_{-1}
k_{+1}	$M^{-1} \cdot s^{-1}$	Present	$5 \cdot 10^6$	—	Phosphorylation. See p. 243
k_{-1}	s^{-1}	Absent	32.6	112	Stopped-flow fluorescence. See p. 238
k_{-1}	s^{-1}	Present	<10	—	Single turnover phosphorylation. See p. 244 and Fig. 7B.

written

$$E \cdot FTP \underset{b}{\overset{a}{\rightleftharpoons}} E_1P \cdot FDP \xrightarrow{c} EP + FDP,$$

where EP represents both E_1P and E_2P, and a, b and c are the rate constants for the various reactions.

Equations describing the appearance of phosphoenzyme, and the release of FDP from $E_1P \cdot FDP$, are derived in Appendix I. It should be noted that each of these processes is, strictly, described by the sum of two exponentials. Because the experimental curves (see for example Figs. 6 and 7) are not accurate enough to be resolved into two exponentials, however, the values of observed rate constants given in the text are obtained by treating the curves as single exponentials. This procedure does not allow us to determine the individual rate constants a, b and c, but by comparing observed rate constants in different conditions we can still reach important conclusions.

Fluorescence measurements. If we assume that changes in fluorescence simply reflect changes in nucleotide binding, the low level of fluorescence during steady-state hydrolysis (Fig. 6) implies that release of FDP from $E_1P \cdot FDP$ precedes the rate-limiting step of the turnover cycle. The same conclusion follows from the invariance of the rate of the initial fall in fluorescence, when the addition of K^+ or a change in the concentration of Na^+ caused large changes in the rate of turnover (Table II). If our assumption is correct, K^+ and changes in the concentration of Na^+ must, therefore, affect the rate of turnover by acting at a step or steps after that at which FDP is released.

The invariance of the rate of the initial fall in fluorescence when the FTP concentration was increased, or when ATP was added with the Mg^{2+} (Table II), is interesting, since it is difficult to reconcile with the suggestion (see refs. 1 and 32) that release of FDP from newly phosphorylated enzyme is accelerated by the binding of ATP at a separate low-affinity site. (An effect of FTP concentration on the rate of the initial fall in fluorescence, reported earlier [10], can be disregarded, since in the early experiments FTP was not prebound to the enzyme, and at the lower FTP concentrations building of FTP would have been partially rate limiting.)

The duration of the low-fluorescence steady state is a function both of the turnover time and of the number of turnovers necessary to exhaust the FTP (see the rough proportionality between duration and FTP concentration in Table II). In the absence of K^+, the rate-limiting step is the hydrolysis of E_2P, and the increase in turnover rate caused by increasing the Na^+ concentration from 10 to 80 mM (see Table II) can probably be explained by the effect of Na^+ acting at low-affinity external sites on the rate of hydrolysis of E_2P (see ref. 28). In the presence of K^+, at the low nucleotide concentrations used in these experiments, the rate-limiting step is thought to be the conversion of $E_2 \cdot (K)$ to $E_1 \cdot Na$ (see ref. 13 and the following paper [14]).

The final rise in fluorescence associated with rebinding of FDP occurs over a number of cycles as the FTP become exhausted and FDP accumulates. The observed rate of net rebinding of FDP cannot, therefore, be identified with any particular step. If our interpretation is correct, however, the rate of the final rise in fluorescence should be roughly correlated with the steady-state hydrolysis rate, for the rate of net rebinding of FDP will be limited largely by the rate

of turnover. This rough correlation was found to hold.

Measurements of ^{32}P incorporation. These measurements complement the fluorescence studies in two ways. First, the finding that approx. 1 mol of phosphate is incorporated per mol of enzyme shows that FTP phosphorylates the enzyme to the same extent as ATP [33], and suggests that E_2P was the major steady-state intermediate in our experiments, when K^+ was absent. Secondly, the experiments show that phosphoenzyme (presumably $E_1P \cdot FDP$) is formed from FTP at a rate which is compatible (a) with the hypothesis that the enzyme is phosphorylated before FDP is released, and (b) with the hypothesis that the phosphoenzyme is an intermediate in the hydrolysis of FTP. The actual rate constants describing the appearance of phosphoenzyme (96 and 76 s^{-1} in the experiments of Table III, and 87 s^{-1} in the "ATP chase" experiment of Fig. 7) are somewhat lower than the corresponding values reported for ATP, namely 180 s^{-1} at 21°C with bovine brain microsomes [34] and 140 s^{-1} at 21°C with *Electrophorus* electric organ microsomes [35]. It is not clear whether this is because FTP is slightly less effective at transferring its terminal phosphate to the enzyme, or whether the discrepancy simply reflects the use of a different enzyme preparation.

Discussion of alternative mechanisms

Skou [2], and more recently Whittam and Chipperfield [36], have proposed that the phosphoenzyme formed when preparations of $(Na^+ + K^+)$-ATPase are incubated with ATP, Mg^{2+} and Na^+ in the absence of K^+ is not an intermediate under physiological conditions, when K^+ is present and ATP hydrolysis is accompanied by Na^+-K^+ exchange. Skou suggests that, under physiological conditions, the pump binds Na^+ internally and K^+ externally, and that in this state it is not phosphorylated by ATP but forms a complex "in which the γ-phosphate of ATP is bound to the system in a bond which is intermediary between a covalent and an electrostatic bond". The complex is supposed to exist only transiently, while the bond between the γ- and the β-phosphate is being broken and the concerted translocation of the cations is occurring. Since this scheme predicts that the major steady-state intermediate would include bound nucleotide and that K^+ would affect the rate of release of FDP from the enzyme, it is unattractive, at least at the low nucleotide concentrations that we have used. It is conceivable that different chemical steps occur at high nucleotide concentrations, and the fact that K^+ accelerates ATP hydrolysis when the nucleotide concentrations are high, and retards it when they are low, makes this suggestion plausible. The slowing of hydrolysis by K^+ at low nucleotide concentrations can, however, be explained more economically in terms of the slow conversion between K- and Na-forms of the enzyme (see ref. 13 and the following paper [14]). The finding that, under appropriate conditions, the hydrolysis of ATP at very low concentrations may be accompanied by an outward movement of Na^+ with a normal stoichiometry [28] also makes one reluctant to accept the suggestion that hydrolysis of ATP at very low concentrations is by a substantially different mechanism.

Tonomura and his colleagues [37,38] have rejected schemes involving the formation first of an ADP-sensitive and then of a K^+-sensitive phosphoenzyme, and propose instead a scheme in which the main steady-state intermediate is a

high-energy phosphoenzyme with bound ADP, which is in equilibrium with an unphosphorylated form of the enzyme with tightly bound ATP. Alkali-metal ions are supposed to affect both this equilibrium and the rate of breakdown of the phosphoenzyme. This scheme, too, is awkward to reconcile with the relatively low steady-state fluorescence, and the lack of effect of K^+ on the apparent rate of FDP release, observed in our experiments.

Appendix 1

Derivation of equations describing the net incorporation of ^{32}P into the enzyme, and the fall in fluorescence associated with the release of FDP from the phosphoenzyme

When enzyme that has bound FTP in a medium containing Na^+ and CDTA is suddenly mixed with excess Mg^{2+}, the expected sequence of events may be written

$$E \cdot FTP \underset{b}{\overset{a}{\rightleftharpoons}} E_1P \cdot FDP \xrightarrow{c} EP + FDP ,$$

where EP represents both E_1P and E_2P, and a, b, and c are the rate constants for the various reactions. The conversion of $E_1P \cdot FDP$ to EP plus FDP is effectively irreversible, because the concentration of FDP in the mixture is initially zero. The dissociation of $E \cdot FTP$ to E plus FTP may also be ignored, because the "chase" experiment of Fig. 7B showed that virtually all of the enzyme that had bound FTP became phosphorylated.

If x, y and z denote respectively the concentrations of $E \cdot FTP$, $E_1P \cdot FDP$ and EP, at time t, and x_0 is the initial concentration of $E \cdot FTP$, we may write

$$\frac{dx}{dt} = by - ax \tag{1}$$

$$\frac{dy}{dt} = ax - (b + c)y \tag{2}$$

$$z = x_0 - x - y \tag{3}$$

From Eqns. 1 and 2, by eliminating y and dy/dx, integrating, and introducing the boundary conditions: $x = x_0$ when $t = 0$, and $x = y = 0$ when $t = \infty$, we have

$$x = \frac{x_0}{\alpha - \beta} \{(a - \beta)\, e^{-\alpha t} - (a - \alpha)\, e^{-\beta t}\} \tag{4}$$

where α and β are the two roots of the equation: $x^2 - (a + b + c)x + ac = 0$. Now, the total concentration of phosphoenzyme at any time is equal to $x_0 - x$. The equation describing the net incorporation of ^{32}P is therefore

$$[E_1P \cdot FDP]_t + [EP]_t = [E \cdot FTP]_0 \left[1 - \frac{1}{\alpha - \beta} \{(a - \beta)\, e^{-\alpha t} - (a - \alpha)\, e^{-\beta t}\}\right] \tag{5}$$

If fluorescence enhancement merely reflects nucleotide binding, and is not affected by the phosphorylation state of the nucleotide or of the enzyme, the drop in fluorescence will be proportional to z.

From Eqns. 1, 3 and 4,

$$z = x_0\left\{1 + \frac{1}{\alpha - \beta}(\beta\, e^{-\alpha t} - \alpha\, e^{-\beta t})\right\} \tag{6}$$

The fall in fluorescence is therefore described by the equation

$$[E \cdot FTP]_t + [E_1P \cdot FDP]_t = [E \cdot FTP]_0\left\{\frac{1}{\beta - \alpha}(\beta\, e^{-\alpha t} - \alpha\, e^{-\beta t})\right\} \tag{7}$$

It will be noticed that Eqn. 5 and Eqn. 7 both contain two exponential terms, and that, in both equations, each exponential constant is a function of all three rate constants.
If $b << a$, or $b << c$, eqn. 5 simplifies to

$$[E_1P \cdot FDP]_t + [EP]_t = [E \cdot FTP]_0(1 - e^{-at}) \tag{8}$$

which is obviously correct, as either assumption implies that phosphorylation is effectively irreversible. Similarly, Eqn. 7 simplifies to

$$[E \cdot FTP]_t + [E_1P \cdot FDP]_t = [E \cdot FTP]_0\left\{\frac{1}{c - a}(c\, e^{-at} - a\, e^{-ct})\right\} \tag{9}$$

Appendix 2, however, shows that b is unlikely to be much smaller than a, and may well be rather greater. That being so, the fact that the observed rate constant for the appearance of the phosphoenzyme (approx. 87 s^{-1}, see Fig. 7 and Table III) is significantly greater than the observed rate constant for the decrease in fluorescence (approx. 43 s^{-1}, in an experiment done on the same day and in the same conditions as those in Fig. 7) shows that c cannot be very much greater than a and b. We are therefore not justified in using the simplified equations.

Appendix 2

The poise of the equilibrium between E · ATP and E_1P · ADP

Eqns. 5 and 7 of Appendix 1 would be much simpler if the rate constant a for the transfer of a phosphoryl group from FTP to the enzyme were much greater than the rate constant b for the reverse reaction. Information about the probable relative magnitudes of a and b can be derived from the results of an experiment by Post et al. [39], in which enzyme pretreated with oligomycin was exposed briefly to different concentrations of ATP and ADP, in the presence of Mg^{2+} and Na^+, and the extent of phosphorylation was measured. About half of the enzyme was found to be phosphorylated when [ATP] was 30 μM and [ADP] was 150 μM. Because the enzyme had been pretreated with oligomycin, nearly all of the phosphoenzyme must have been either E_1P · ADP or E_1P.

Consider the reaction sequence

$$E + ATP \overset{K_1}{\rightleftharpoons} E \cdot ATP \overset{K_2}{\rightleftharpoons} E_1P \cdot ADP \overset{K_3}{\rightleftharpoons} E_1P + ADP$$

And the competing reactions

$$E + ADP \overset{K_i}{\rightleftharpoons} E \cdot ADP$$

$$E_1P + ATP \overset{K_j}{\rightleftharpoons} E_1P \cdot ATP$$

where K_1, K_3, K_i and K_j are the dissociation constants of the various complexes, in the conditions of the experiment, and K_2 is defined as $[E_1P \cdot ADP]/[E \cdot ATP]$.

When half of the enzyme is phosphorylated, we may write

$$[E] + [E \cdot ATP] + [E \cdot ADP] = [E_1P \cdot ADP] + [E_1P] + [E_1P \cdot ATP]$$

From this equation and the definitions of the five constants it is easy to show that

$$K_2 = \frac{1 + (K_1/[ATP^*])(1 + [ADP^*]/K_j)}{1 + (K_3/[ADP^*])(1 + [ATP^*]/K_j)}$$

where $[ATP^*]$ and $[ADP^*]$ represent any pair of concentrations of ATP and ADP at which half of the enzyme is phosphorylated.

To calculate K_2 we need the choose values for the various constants. K_1 is not precisely known in the presence of Mg^{2+}, but 0.2 mM will be of the right order of magnitude, and the calculated value of K_2 is insensitive to the precise value of K_1. For K_i we assume that the relative affinities of the enzyme for ATP and ADP in the presence of Mg^{2+} are similar to the relative affinities in the absence of Mg^{2+} [27,29], and hence we put $K_i = 10 \times K_1$. K_j we put $= \infty$, on the grounds that the rate of Na^+-Na^+ exchange in red cells was found to be insensitive to changes in the ATP concentration in the range 300—1200 μM, implying that ATP does not complete effectively with ADP for the phosphoenzyme [40]. We do not know the value of K_3, so we have calculated K_2 for a series of possible values of K_3. It turns out that if K_3 is assumed, in turn, to be 0, 3, 30, 300 and 3000 μM; K_2 works out as 1.51, 1.48, 1.26, 0.50 and 0.072. The estimates are not very different if K_1 is assumed to be 2 μM, or even 20 μM, rather than 0.2 μM. In other words, the reaction $E \cdot ATP \rightleftharpoons E_1P \cdot ADP$ cannot be poised more than slightly to the right, and is probably poised to the left. If FTP behaves like ATP, if follows that b cannot be much smaller than a.

Incidentally, without knowing or making assumptions about K_3, it is impossible to use the results of Post et al. to calculate the difference between the free-energy of hydrolysis of the phosphoryl group in E_1P and the free-energy of hydrolysis of the terminal phosphoryl group in ATP (Cf. ref. 38). This is because the poise of the overall reaction depends not only on the free energy change associated with the bond-splitting step, but also on the difference between the free energies of binding of the reactant (ATP) and the product (ADP).

Acknowledgements

We are grateful to Professor H. Gutfreund, Dr. D.R. Trentham and Dr. J.F. Eccleston for advice and help. Different aspects of the work were supported by grants from the British M.R.C. and S.R.C, and by a grant from the American M.D.A. to Dr. Trentham. S.J.D. K. held E.M.B.O. short-term Fellowships and was also supported by the Royal Society.

References

1 Glynn, I.M. and Karlish, S.J.D. (1975) Ann. Rev. Physiol. 37, 13—55
2 Skou, J.C. (1975) Q. Rev. Biophys. 7, 401—434
3 Jørgensen, P.L. (1975) Q. Rev. Biophys. 7, 239—274
4 Albers, R.W. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 3, pp. 283—301, Wiley, New York
5 Hori, M., Ito, E., Takita, T., Koyama, G., Takeuchi, T. and Umezawa, H. (1964) J. Antibiot. (Tokyo) A, 17, 96—99
6 Robins, R.K., Townsend, L.B., Cassidy, F., Gerster, J.K., Lewis, A.F. and Miller, R.L. (1966) J. Heterocyclic Chem. 3, 110
7 Koyama, G., Macda, K., Umezawa, H. and Iitaka, Y. (1966) Tetrahedron Lett. 597
8 Ward, D.C., Cerami, A., Reich, E., Acs, G. and Altwerger, L. (1969) J. Biol. Chem. 244, 3243—3250
9 Yount, R.G. (1975) Adv. Enzymol. 43, 1—56
10 Karlish, S.J.D., Yates, D.W. and Glynn, I.M. (1976) Nature 263, 251—253
11 Albers, R.W. (1967) Annu. Rev. Biochem. 36, 727—756
12 Post, R.L., Kume, S., Tobin, T., Orcutt, B. and Sen, A.K. (1969) J. Gen. Physiol. 54, 306s—326s
13 Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530—6540
14 Karlish, S.J.D., Yates, D.W. and Glynn, I.M. (1978) Biochim. Biophys. Acta 525, 252—264
15 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36—52
16 Kepner, G.R. and Macey, R.I. (1968) Biochim. Biophys. Acta 163, 188—203
17 Eccleston, J. and Trentham, D.R. (1977) Biochem. J. 163, 15—29
18 Yoshikawa, M., Kato, T. and Takenshi, T. (1969) Bull. Chem. Soc. Jap. 42, 3505—3508
19 Avron, M. (1961) Anal. Biochem. 2, 345—353
20 Glynn, I.M. and Chappell, J.B. (1964) Biochem. J. 90, 147—149
21 Bagshaw, C.R., Eccleston, J.F., Trentham, D.R., Yates, D.W. and Goody, R.S. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 127—135
22 Yates, D.W. and Duance, V.C. (1976) Biochem. J. 159, 719—728
23 Gutfreund, H. (1969) Methods Enzymol. 16, 229—249
24 Skou, J.C. (1974) Biochim. Biophys. Acta 339, 234—245
25 Gutfreund, H. (1972) Enzymes: Physical Principles, Wiley-Interscience, London
26 Nørby, J.S. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 1004—1016
27 Hegyvary, C. and Post, R.L. (1971) J. Biol. Chem. 246, 5235—5240
28 Glynn, I.M. and Karlish, S.J.D. (1976) J. Physiol. Lond. 256, 465—496
29 Jensen, J. and Nørby, J.C. (1971) Biochim. Biophys. Acta 233, 395—403
30 Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 53—67
31 Mårdh, S. and Post, R.L. (1977) J. Biol. Chem. 252, 633—638
32 Blostein, R. (1975) J. Biol. Chem. 250, 6118—6124
33 Jørgensen, P.L. (1977) Biochim. Biophys. Acta 466, 97—108
34 Mårdh, S. and Zetterqvist, Ö. (1974) Biochim. Biophys. Acta 350, 473—485
35 Froehlich, J.R., Albers, R.W., Koval, G.J., Goebel, R. and Berman, M. (1976) J. Biol. Chem. 251, 2186—2188
36 Whittam, R. and Chipperfield, A.R. (1975) Biochim. Biophys. Acta 415, 149—171
37 Kanazawa, T., Saito, M. and Tonomura, Y. (1970) J. Biochem. Tokyo 67, 693—711
38 Fukushima, Y. and Tonomura, Y. (1975) J. Biochem. Tokyo 78, 749—755
39 Post, R.L., Kume, S. and Rogers, F.N. (1973) in Mechanisms in Bioenergetics (Azzone, G.F., Ernster, L., Papa, S., Quagliariello, E. and Silliprandi, N., eds.), pp. 203—218, Academic Press, New York
40 Glynn, I.M. and Hoffman, J.F. (1971) J. Physiol. Lond. 218, 239—256
41 Eisenthal, R. and Cornish-Bowdon, A. (1974) Biochem. J. 139, 715—720
42 Klodos, I. and Skou, J.C. (1977) Biochim. Biophys. Acta 481, 667—679