# The Kinetics of Effector Binding to Phosphofructokinase

## THE ALLOSTERIC CONFORMATIONAL TRANSITION INDUCED BY 1,$N^6$-ETHENOADENOSINE TRIPHOSPHATE

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1. The fluorescent ATP analogue 1,$N^6$-etheno-ATP is a good substrate and an efficient allosteric inhibitor of rabbit skeletal-muscle phosphofructokinase. 2. Fluorescence energy transfer occurs between bound 1,$N^6$-etheno-ATP and phosphofructokinase. 1,$N^6$-Etheno-ATP fluorescence is enhanced, intrinsic protein fluorescence is quenched, and the excitation spectrum of 1,$N^6$-etheno-ATP fluorescence is characteristic of protein absorption. 3. The binding reaction of 1,$N^6$-etheno-ATP observed by stopped-flow fluorimetry is biphasic. The fast phase results from binding to the catalytic site alone. The slow phase results from the allosteric transition of the R conformation into the T conformation induced by the binding of 1,$N^6$-etheno-ATP to the regulatory site. 4. The fluorescence signal that allows the transition of the R conformation into the T conformation to be observed does not arise from 1,$N^6$-etheno-ATP bound to the regulatory site. It arises instead from 1,$N^6$-etheno-ATP bound to the catalytic site as a consequence of changes at the catalytic site caused by the transition of the R conformation into the T conformation. 5. In the presence of excess of $Mg^{2+}$, the affinity of 1,$N^6$-etheno-ATP for the regulatory site is very much greater in the T state than in the R state.

Phosphofructokinase (ATP–fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11) plays a major role in the regulation of glycolysis. The activity of the enzyme under physiological conditions is influenced by a wide range of effectors that are stereospecifically distinct, implying that there are several different types of binding site on the enzyme. Accordingly, there have been numerous investigations of the binding of effectors to phosphofructokinase and of their influence on the activity of the enzyme in steady-state assays (see Bloxham & Lardy, 1973). The former have been performed at concentrations of about 1 mg/ml, close to the concentration of the enzyme *in vivo*, whereas the latter have usually been performed at concentrations of less than 1 $\mu$g/ml. A complication in the interpretation of the experiments is therefore the complex association–dissociation behaviour of phosphofructokinase that occurs at the higher concentrations (Aaronson & Frieden, 1972; Leonard & Walker, 1972; Hussey *et al.*, 1977). The regulation of phosphofructokinase by ATP is of particular interest. However, a further complication present at the high enzyme concentrations in binding studies is the minor adenosine triphosphatase activity of the enzyme

Abbreviations used: 1,$N^6$-etheno-ATP, 1,$N^6$-ethenoadenosine triphosphate; [$\beta\gamma$-imido]ATP, adenosine [$\beta\gamma$-imido]triphosphate.

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(Uyeda, 1970; Columbo *et al.*, 1975). It is therefore necessary either to design experiments so that the effect of the adenosine triphosphatase activity is negligible (Columbo *et al.*, 1975) or to use an analogue such as [$\beta\gamma$-imido]ATP, which is not hydrolysed (Wolfman *et al.*, 1978). Despite these difficulties, there is general agreement that there are two classes of site for ATP on the enzyme, one catalytic and one regulatory (Kemp & Krebs, 1967; Wolfman *et al.*, 1978), and that the influence of other inhibitors, such as citrate, is synergistic with ATP (Columbo *et al.*, 1975).

An alternative way of studying the binding of ATP and effectors to phosphofructokinase is to observe the kinetics of the binding reactions directly. Since these processes are very fast, it is necessary to use rapid-reaction equipment to study them. In addition, it is necessary to have a suitable spectroscopic signal that changes on binding. Trentham and co-workers have developed such an approach to the mechanism of kinases based on the use of chromophoric and fluorescent analogues of ATP (Trentham *et al.*, 1972). The fluorescent analogue 1,$N^6$-etheno-ATP has several properties that suggest its potential for use in rapid-reaction studies with phosphofructokinase. These include: (i) a high quantum yield and broad emission band centred on 415 nm (Secrist *et al.*, 1972); (ii) overlap of its absorption spectrum with the

emission spectrum of tyrosine and tryptophan, which may result in energy transfer from protein to nucleotide (Onishi *et al.*, 1973); (iii) its ability to substitute for ATP as a substrate of phosphofructokinase (Secrist *et al.*, 1972). In the present paper we show that $1,N^6$-etheno-ATP is indeed a useful analogue for rapid-reaction studies that give new information about the binding reaction and the resulting allosteric transition of the R conformation into the T conformation.

## Experimental

### Materials

All biochemicals were obtained from either Sigma Chemical Co. (Poole, Dorset, U.K.) or Boehringer (London W.5, U.K.) and used without further purification. All chemicals were AnalaR grade from BDH Chemicals (Poole, Dorset, U.K.).

### Phosphofructokinase

The enzyme from rabbit skeletal muscle was prepared in this laboratory as previously described (Hussey *et al.*, 1977). Preparations with a specific activity in the range 180–200 units ($\mu$mol/min)/mg of enzyme at 26°C were used. The phosphoserine content varied from preparation to preparation as reported over the range 0.02–0.5 mol/mol of monomer. In the early stages of the work only the 30S fraction was used, but in later stages of the work pooled enzyme consisting of a mixture of 30S, 18S and 13S fractions was used (see the Discussion section). All concentrations are expressed in terms of mol of monomer, based on a mol.wt. of 80000 (Walker *et al.*, 1976) and an absorption coefficient at 280 nm of $1.02 \,\text{ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ (Parmeggiani *et al.*, 1966). All experiments described below were performed in 50 mM-potassium phosphate buffer, pH 6.80, containing 0.2 mM-dithiothreitol. $Mg^{2+}$ was maintained at a constant excess of 5 mM over $1,N^6$-etheno-ATP and ATP in all experiments. Assays of the regulatory properties of phosphofructokinase were carried out under the conditions described in the legend to Fig. 1.

### $1,N^6$-Etheno-ATP

$1,N^6$-Etheno-ATP was synthesized as described by Secrist *et al.* (1972) and characterized by its u.v., fluorescence and n.m.r. spectra. These were in excellent agreement with those published. The $1,N^6$-etheno-ATP was stored as a freeze-dried powder and its purity was checked from time to time by t.l.c. on Eastman Chromogram silica gel acetate sheets with isobutyric acid/aq. $NH_3$ (sp.gr. 0.880)/water (66:1:33, by vol.) as solvent. No conversion of $1,N^6$-etheno-ATP into $1,N^6$-etheno-ADP was detected over the period of use, even with gross overloading of the sheets. A

molar absorption coefficient of $5.6 \times 10^3 \,\text{M}^{-1} \cdot \text{cm}^{-1}$ at 275 nm was assumed (Secrist *et al.*, 1972).

### Fluorescence measurements

Static measurements were carried out in a Perkin–Elmer Hitachi MPF-3 fluorimeter with a 1 cm cell. Corrections for the inner filter effect, the self-absorption of exciting light, were made according to the equation:

$$F_{\text{corr.}} = F_{\text{obs.}} \{\text{antilog}\, (dA)\} \qquad (1)$$

where $F_{\text{corr.}}$ and $F_{\text{obs.}}$ are the corrected and observed fluorescence intensities respectively, $A$ is the absorbance for a 1 cm pathlength and $d$ the distance traversed by the exciting light through the solution to the centre of the field of observation of the emission monochromator. For the 1 cm-pathlength cell and the fluorimeter employed, $d$ was determined as 0.545 cm by calibration with glycyltryptophan according to the method of Holland *et al.* (1977). Corrections for the variation of light-intensity with wavelength were made by using a fluorescent screen, Rhodamine B, at a concentration of 3 mg/ml in ethane-1,2-diol, and observing front-face fluorescence at 618 nm.

Rapid-reaction measurements were made in a stopped-flow fluorimeter designed according to the recommendations of Gutfreund (described in Bagshaw *et al.*, 1972). The observation chamber was constructed from a 2 mm × 2 mm quartz channel. The distance from the incident surface to the centre of the chamber was thus only 1.0 mm, so that corrections for inner filter effects were usually negligible. The exciting monochromator was a high-intensity Bausch and Lomb u.v. monochromator. To ensure that maximum light-intensity was available, a 3 mm exit slit was used, which resulted in a half-bandwidth of 10 nm. $1,N^6$-Etheno-ATP fluorescence was observed through a combination of two filters, a Kodak Wratten no. 35 and a Schott KVG 380 cut-off filter, creating a window with 1% transmission limits at 374 nm and 466 nm, which was clear of protein fluorescence. Tryptophan emission was observed by using a Schott UV-Line interference filter, centred on 329 nm with a half-bandwidth of 8 nm. No $1,N^6$-etheno-ATP fluorescence was detectable with this filter. The dead time of the stopped-flow fluorimeter was 1.5 ms. Ratio recording was employed for the observation of very small signals to eliminate the effect of noise from the 250 W xenon arc lamp. All experiments were performed at 19 ± 1°C.

### Difference absorption measurements

These were recorded by using twice-5 mm-pathlength tandem-mixing quartz cuvettes (made by Hellma) in a Beckman model 25 spectrophotometer thermostatically maintained at 20°C. In each case the first compartment contained exactly 1.0 ml of the
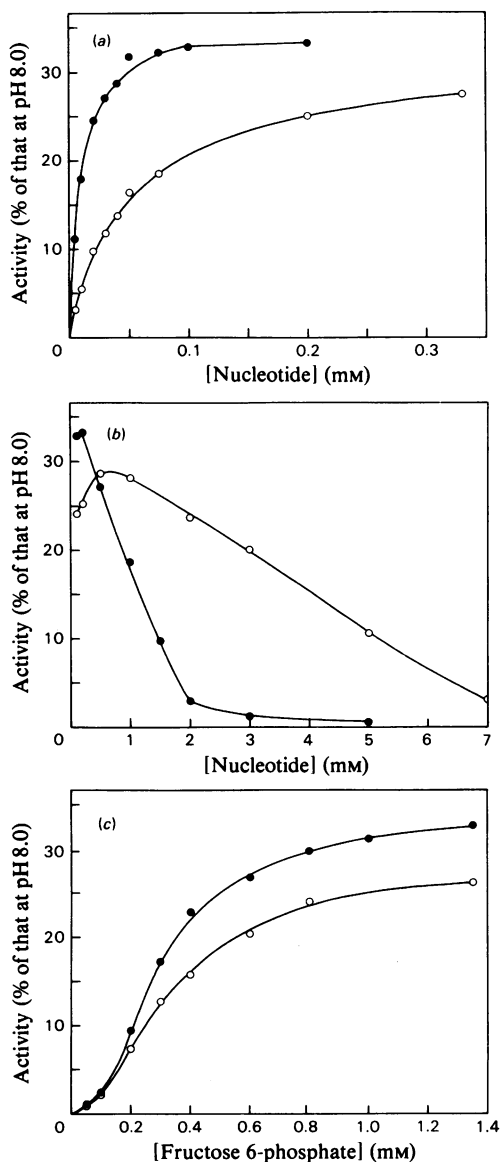
Fig. 1. *Comparison of* 1,$N^6$-*etheno-ATP with ATP as substrate and allosteric inhibitor of phosphofructokinase*
Assay conditions were as follows: 1 ml of assay solution contained 50 mM-phosphate (25 mM-KH$_2$PO$_4$/25 mM-K$_2$HPO$_4$) buffer, pH 6.80, 2.5 units of aldolase, 4.0 units of the mixed enzymes glycerol 1-phosphate dehydrogenase and triose phosphate isomerase (Sigma preparation G1881), 0.16 mM-NADH, 10 mM-(NH$_4$)$_2$SO$_4$ and 1 mM-dithiothreitol. The auxiliary enzymes were dialysed overnight against two changes of the phosphate buffer before use to remove any (NH$_4$)$_2$SO$_4$ carried over from their storage suspension. Mg$^{2+}$ was added as MgCl$_2$ and maintained at a 5 mM constant excess over nucleotide. Enzyme activities are quoted as percentages of the activity at pH 8.0. (*a*) Substrate

stock phosphofructokinase solution (2.0 mg/ml) and the second compartment exactly 1.0 ml of the effector solution (see the legend to Fig. 4). The spectra were corrected for differences in absorption of the two cuvettes. The reactions were started by thorough mixing of the sample cuvette. After mixing, 5 min was allowed to elapse before the spectrum was recorded, to ensure that the reaction was at equilibrium. During the recordings, no time-dependent changes in the spectra occurred.

*Computational analysis*

Enlarged photographic images of the oscilloscope traces were digitized by using a Hewlett Packard HP9864A Digitiser in conjunction with a Hewlett Packard HP9830A mini-computer. Data corrected for the machine dead time were transferred to Disk storage on a DEC system 10 computer via a Hewlett Packard HP2100A computer. The Discrete version IB (April 1976) (Provencher, 1976) was used for fitting up to nine exponentials to the data. It was not necessary to assume a base-line.

Non-linear least-squares analysis of the fast-phase enhancement data (see the Results section) was done on a DEC system 10 computer with BMDP3R, a statistical computer package attributed to the Health Sciences Computing Facility, University of California, Los Angeles, CA, U.S.A.

**Results**

Fig. 1(*a*) shows the steady-state kinetic properties of phosphofructokinase with 1,$N^6$-etheno-ATP as substrate in 50 mM-phosphate buffer, pH 6.80, at 26°C. At low 1,$N^6$-etheno-ATP concentrations the dependence of activity is hyperbolic with Lineweaver–Burk analysis, giving a $K_m$ of 46 μM, about 4-fold that for ATP. $V_{max.}$ was found to be about 84% of that for ATP. At higher concentrations 1,$N^6$-etheno-ATP acts as an allosteric inhibitor of the enzyme, a concentration of about 4.3 mM producing 50% inhibition in the presence of 0.2 mM-fructose 6-phosphate (Fig. 1*b*). At these higher concentrations of 1,$N^6$-etheno-ATP the dependence of activity on fructose 6-phosphate concentration is sigmoidal (Fig. 1c).

The static fluorescence-emission difference spec-

properties: dependence of enzyme activity at low nucleotide concentration with ATP (●) and 1,$N^6$-etheno-ATP (○), in the presence of 0.2 mM-fructose 6-phosphate. (*b*) Inhibitor properties: dependence of enzyme activity at high nucleotide concentration with ATP (●) and 1,$N^6$-etheno-ATP (○), in the presence of 0.2 mM-fructose 6-phosphate. (*c*) Fructose 6-phosphate properties: dependence of activity on fructose 6-phosphate concentration in the presence of 1.5 mM-ATP (●) and 6.0 mM-1,$N^6$-etheno-ATP (○).

trum between unbound $1,N^6$-etheno-ATP $(12.5\,\mu M)$ plus unbound phosphofructokinase $(12.5\,\mu M)$ and a mixture of the two is shown in Fig. $2(a)$. Also given for reference are the emission spectra of unbound phosphofructokinase and unbound $1,N^6$-etheno-ATP (Fig. $2b$). The samples were excited at 280 nm and their spectra corrected as described in the Experimental section for the inner-filter effect before calculation of the difference spectrum. The fluorescence of $1,N^6$-etheno-ATP is enhanced on mixing with phosphofructokinase and has a difference emission maximum centred on 400 nm. In contrast, the tryptophan fluorescence emission from phosphofructokinase is quenched with a minimum in the difference spectrum centred on 330 nm, where $1,N^6$-etheno-ATP does not fluoresce. The coupling of
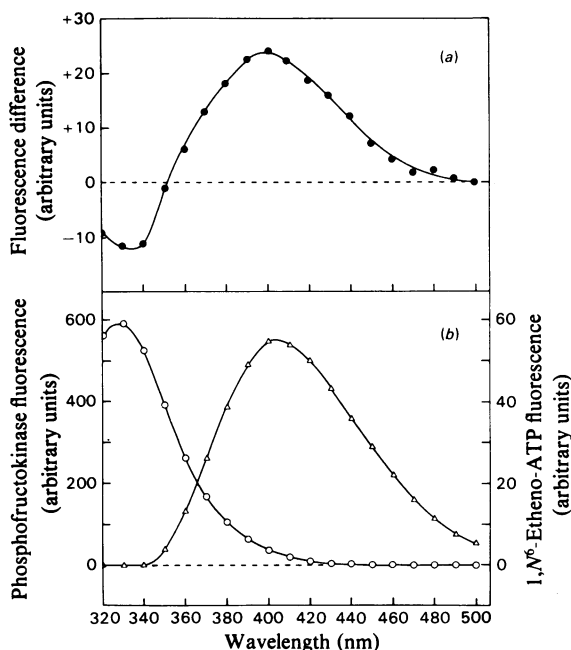


**Fig. 2. Static fluorescence-emission difference spectrum for the reaction of phosphofructokinase with $1,N^6$-etheno-ATP** ($a$) Static fluorescence-emission difference spectrum obtained by subtraction of the fluorescence of unbound phosphofructokinase $(12.5\,\mu M)$ and the fluorescence of unbound $1,N^6$-etheno-ATP $(12.5\,\mu M)$ from the fluorescence of a mixture of the two. The excitation wavelength was 280 nm and each sample fluorescence was corrected for the inner filter effect (see the Experimental section). Conditions were as follows: 50 mM-phosphate buffer, pH 6.80, 0.2 mM-dithiothreitol and 5 mM-MgCl$_2$. ($b$) Fluorescence-emission difference spectra of $12.5\,\mu M$-phosphofructokinase (○) and of $12.5\,\mu M$-$1,N^6$-etheno-ATP (△). Excitation, corrections and conditions were as described for ($a$).

$1,N^6$-etheno-ATP fluorescence enhancement with protein fluorescence quenching suggests the possibility that energy transfer is taking place. However, whereas the relative enhancement of $1,N^6$-etheno-ATP fluorescence at 400 nm is 27 %, the relative quenching of phosphofructokinase fluorescence at 330 nm is only 2 % under these conditions. In view of the relatively large corrections that have to be made for inner-filter effects with each sample, it is therefore necessary to establish whether the enhancement of $1,N^6$-etheno-ATP fluorescence depends on protein absorption. Fig. 3 shows the static excitation difference spectrum corresponding exactly to the emission difference spectrum in Fig. $2(a)$. The fluorescence of $1,N^6$-etheno-ATP was monitored at 405 nm and the spectrum of each sample was corrected for the inner-filter effect and the variation of lamp intensity with wavelength. The difference spectrum is characterized by a maximum at 280 nm and a slight shoulder at 290 nm (Fig. $3a$). Comparison with the absorption spectra of unbound phosphofructokinase and unbound $1,N^6$-etheno-ATP (Fig. $3b$) shows that over the range 260–300 nm the difference spectrum coincides with the protein absorption spectrum. In particular the characteristic peaks in the $1,N^6$-etheno-ATP absorption spectrum at 265 and 275 nm are absent. This result, together with the observation that protein fluorescence is quenched and $1,N^6$-etheno-ATP fluorescence is enhanced on mixing, confirms that enhancement is probably due almost exclusively to energy transfer from protein to nucleotide. Above 300 nm, where protein does not absorb but $1,N^6$-etheno-ATP does, there is a small plateau in the difference spectrum extending up to 350 nm, which is the limit of $1,N^6$-etheno-ATP absorption. This is clearly a minor component of the excitation spectrum. No fluorescence enhancement is seen in the absence of $Mg^{2+}$.

Difference absorption measurements under the same conditions between a mixture of $1,N^6$-etheno-ATP plus phosphofructokinase and the unbound forms show that absorption changes are readily detectable in the range 250–280 nm coinciding with the characteristic peaks of $1,N^6$-etheno-ATP absorption (Fig. 4). However, there is no net change in absorption over the excitation range 280–290 nm used in our fluorescence experiments. There is a small, barely detectable, increase in $1,N^6$-etheno-ATP absorption above 300 nm. When $30\,\mu M$-ATP is substituted for $50\,\mu M$-$1,N^6$-etheno-ATP, changes in absorption are readily observed, not only in the range 250–280 nm, but also in the range 280–290 nm, where no net change was seen with $1,N^6$-etheno-ATP (Fig. 4). In this respect, ATP and $1,N^6$-etheno-ATP are different.

The kinetics of binding of $1,N^6$-etheno-ATP to phosphofructokinase may be followed readily in the stopped-flow fluorimeter. Fig. $5(a)$ shows the
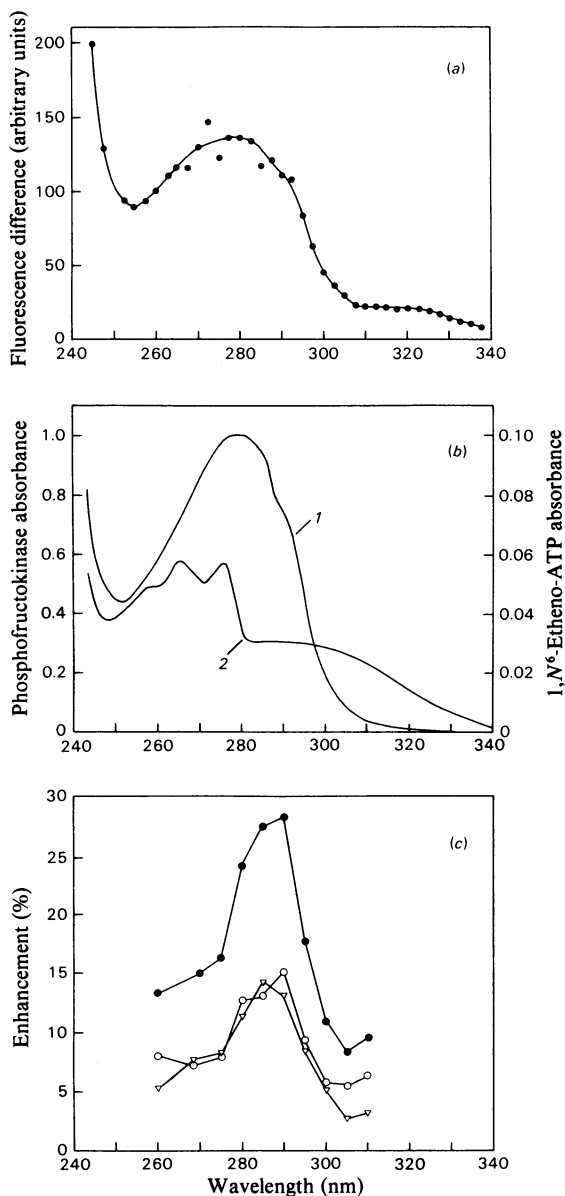
reaction of 100 $\mu$M-1,$N^6$-etheno-ATP with 25 $\mu$M-phosphofructokinase (syringe concentrations). When the reaction mixture is excited at 285 nm and 1,$N^6$-etheno-ATP fluorescence alone is observed with a combination of filters (see the Experimental section), the progress curve is biphasic. Thorough statistical analysis of the data by curve-fitting to a series of exponentials by using the Discrete computer package showed that each phase was fitted well by a single exponential; there was no evidence for any other component in the reaction. The apparent first-order rate constants of the fast and slow phases, $k_f$ and $k_s$, were determined to be 136.0±9.53 s$^{-1}$ and 2.4± 0.08 s$^{-1}$ respectively. Further observations for several seconds afterwards did not reveal any slow fluorescence changes.

The use of filters is necessary to obtain a strong enough fluorescence signal from 1,$N^6$-etheno-ATP. Since the filters create a window with 1 % transmission limits at 374 and 466 nm, it is not possible to obtain an emission spectrum of 1,$N^6$-etheno-ATP. However, an excitation difference spectrum may be obtained by observing the 1,$N^6$-etheno-ATP signal
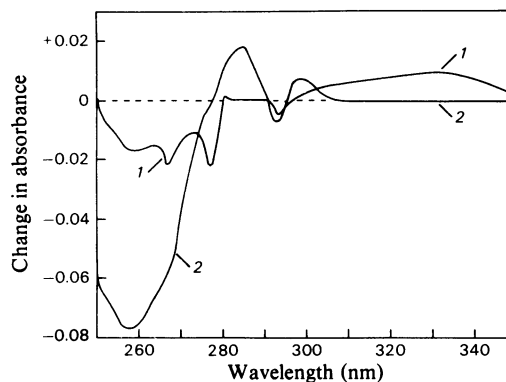


Fig. 4. *Absorption difference spectrum for the reaction of phosphofructokinase with 1,$N^6$-etheno-ATP and ATP* Difference spectra for the reaction of 12.5 $\mu$M-phosphofructokinase with 50 $\mu$M-1,$N^6$-etheno-ATP (curve *1*) and 30 $\mu$M-ATP (curve *2*) are shown. Other conditions were as described for Fig. 2.

Fig. 3. *Fluorescence-excitation difference spectrum for the reaction of phosphofructokinase and 1,$N^6$-etheno-ATP* (*a*) Static fluorescence-excitation difference spectrum obtained by subtraction of the fluorescence of unbound phosphofructokinase (12.5 $\mu$M) and the fluorescence of unbound 1,$N^6$-etheno-ATP (12.5 $\mu$M) from the fluorescence of a mixture of the two. The emission wavelength was 405 nm and each sample fluorescence was corrected for the variation of lamp intensity with wavelength and the inner filter effect (see the Experimental section). (*b*) Absorption spectra of phosphofructokinase (12.5 $\mu$M) (curve *1*) and 1,$N^6$-etheno-ATP (12.5 $\mu$M) (curve *2*). (*c*) Stopped-flow fluorescence-excitation difference spectrum for the reaction of phosphofructokinase with 1,$N^6$-etheno-

ATP. Syringe 1, 25 $\mu$M-phosphofructokinase; syringe 2, 100 $\mu$M-1,$N^6$-etheno-ATP and 10 mM-MgCl$_2$. 1,$N^6$-Etheno-ATP fluorescence was observed through filters (see the Experimental section): ●, total enhancement of 1,$N^6$-etheno-ATP fluorescence; ○, fast-phase enhancement; ▽, slow-phase enhancement. Error bars have been omitted for clarity. The standard error based on a sample size of six was approx. 5%. Other conditions were as described for Fig. 2.
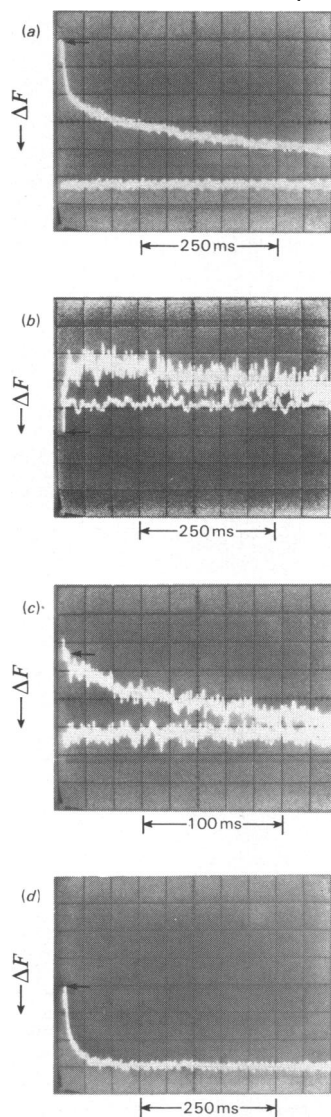
Fig. 5. *Reaction of phosphofructokinase and* 1,N$^6$*-etheno-ATP observed in the stopped-flow fluorimeter*
(a) Enhancement of 1,N$^6$-etheno-ATP fluorescence. Syringe 1, 25 μM-phosphofructokinase; syringe 2, 100 μM-1,N$^6$-etheno-ATP and 10 mM-MgCl$_2$. (b) Quenching and enhancement of phosphofructokinase fluorescence by 1,N$^6$-etheno-ATP. Concentrations were as described for (a). (c) Enhancement of phosphofructokinase fluorescence by ATP. Concentrations were as described for (a) except that syringe 2 contained 60 μM-ATP and 10 mM-MgCl$_2$. (d) Effect of cyclic AMP preincubation on enhancement of 1,N$^6$-etheno-ATP fluorescence. Concentrations were as described for (a), except that syringe 1 contained 25 μM-phosphofructokinase dialysed against 50 μM-cyclic

and varying the excitation wavelength in the usual way. If the results are expressed as percentage enhancement, corrections for the variation of both lamp intensity and self-absorption with wavelength are automatically eliminated. In addition, since there is no net change in absorption during the reaction, there is no need to correct for changes in the inner-filter effect. The amplitude of the reaction gives the amplitude of the difference spectrum between un-changed components and those that have reacted directly. The excitation difference spectrum may therefore be readily obtained from the stopped-flow fluorimeter without the requirement for any of the corrections that have to be made for static spectra. The excitation difference spectrum obtained from the stopped-flow fluorimeter with 100 μM-1,N$^6$-etheno-ATP and 25 μM-phosphofructokinase, shown in Fig. 3(c), is directly comparable with the static spectrum shown in Fig. 3(a). There are slight differences between the two. In particular, the maximum of the static spectrum is at 280 nm compared with 285 nm for the stopped-flow spectrum. This difference probably reflects the difference in the bandwidths of the exciting monochromators in the Perkin–Elmer Hitachi MPF-3 and stopped-flow fluorimeters, 2 nm and 10 nm respectively. It is clear that the stopped-flow excitation spectrum is similarly dependent almost exclusively on phosphofructokinase absorp-tion, although, once more, there is a minor component above 300 nm resulting from 1,N$^6$-etheno-ATP. There is thus good agreement between the static and stopped-flow excitation difference spectra, demon-strating energy transfer. Interestingly, the difference spectra for the fast and slow phases behave ident-ically, suggesting that they originate for the same reasons (Fig. 3c).

The same reaction between 100 μM-1,N$^6$-etheno-ATP and 25 μM-phosphofructokinase may also be followed by observing only the change in intrinsic protein fluorescence with a Schott UV-Line inter-ference filter centred on 329 nm (see the Experimental section). The reaction progress curve is again bi-phasic (Fig. 5b). As expected from the static-spectrum data (Fig. 2a), the overall quenching is very low, approx. 2%. However, this small overall amplitude is the result of two compensating changes in protein fluorescence. The fast phase results from quenching (about 6%) whereas the slow phase results from enhancement (about 4%). The rate constants, $k_f = 148.0 \pm 8.79\,\mathrm{s}^{-1}$ and $k_s = 2.6 \pm 0.2\,\mathrm{s}^{-1}$, are not signi-ficantly different from those determined when 1,N$^6$-etheno-ATP fluorescence was observed. Thus the

AMP. Other conditions were as described for Fig. 2. The arrows indicate the point at which flow is stopped.
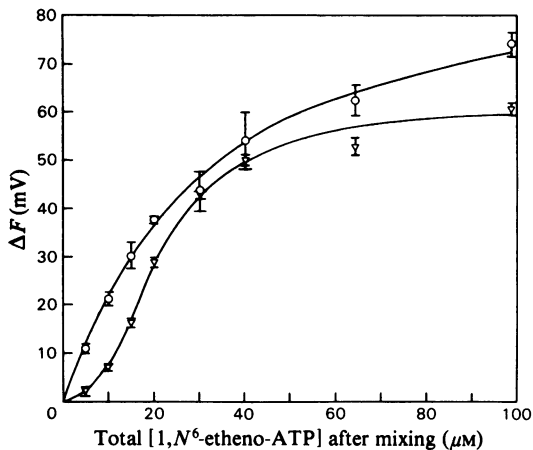
Fig. 6. *Dependence of the amplitudes of the fast and the slow phases of* 1,$N^6$-*etheno-ATP fluorescence enhancement on total* 1,$N^6$-*etheno-ATP concentration*

The reaction was observed in the stopped-flow fluorimeter. Syringe 1, 25 $\mu$M-phosphofructokinase; syringe 2, 1,$N^6$-etheno-ATP plus 10 mM excess MgCl$_2$. Because of the basis on which the calculations with eqn. (2) are performed the concentrations of 1,$N^6$-etheno-ATP on the abscissa are the total 1,$N^6$-etheno-ATP concentrations after mixing, ○, Fast phase; ▽, slow phase. Other conditions were as described for Fig. 2. The error bars represent standard errors based on a sample size of six.
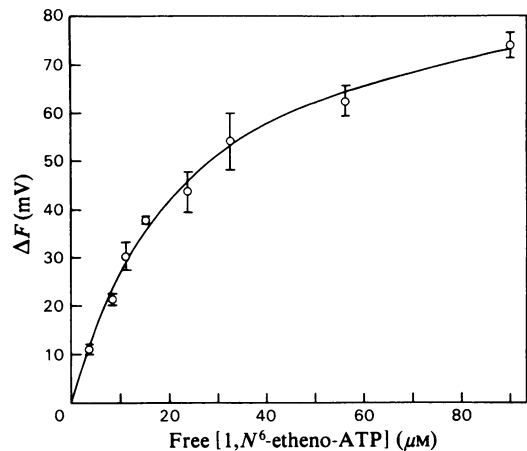


Fig. 7. *Dependence of the amplitude of the fast phase of* 1,$N^6$-*etheno-ATP fluorescence enhancement on free* 1,$N^6$-*etheno-ATP concentration*

The fast-phase data presented in Fig. 6 were analysed by non-linear least squares according to eqn. (2) on the assumption that a single class of independent equivalent sites, one per monomer, is involved in the fast-phase reaction (see the text). The continuous line is the theoretically fitted line for a dissociation constant of 24.1 ± 2.7 $\mu$M, and the 1,$N^6$-etheno-ATP concentrations in the Figure are the free concentrations calculated with this constant.

fast and the slow phases of the protein fluorescence change represent the same reactions as the corresponding phases of the 1,$N^6$-etheno-ATP fluorescence enhancement. Substitution of ATP for 1,$N^6$-etheno-ATP abolishes the fast phase of protein fluorescence quenching, so that, when 25 $\mu$M-phosphofructokinase is mixed with 60 $\mu$M-ATP, only the slow phase of protein fluorescence enhancement is seen (Fig. 5c).

Preincubation of phosphofructokinase with cyclic AMP, a powerful activator of phosphofructokinase, abolishes the slow phase of 1,$N^6$-etheno-ATP fluorescence enhancement. When 25 $\mu$M-phosphofructokinase, previously dialysed against 50 $\mu$M-cyclic AMP, is mixed with 100 $\mu$M-1,$N^6$-etheno-ATP, only the fast phase is seen (Fig. 5d). Its amplitude remains unchanged. In addition, when in the same experiment only protein fluorescence is observed, the slow phase is absent, leaving only the fast-phase quenching (results not shown). Moreover, when ATP is substituted for 1,$N^6$-etheno-ATP, no change in protein fluorescence at all is seen (results not shown).

At pH 6.80 and 50 $\mu$M-1,$N^6$-etheno-ATP, the rate constants $k_f$ and $k_s$ are independent of phosphofructokinase concentration over the range 2.5–25 $\mu$M (concentrations after mixing). At pH 8.0, the slow

phase is completely absent over the same concentration range (results not shown).

The dependences of the amplitudes of the fast and the slow phases of 1,$N^6$-etheno-ATP fluorescence enhancement on 1,$N^6$-etheno-ATP concentration within the range 5–100 $\mu$M are shown in Fig. 6 for experiments performed at pH 6.80 and 12.5 $\mu$M-phosphofructokinase. The amplitudes were determined at constant photomultiplier gain, and there was no need to correct for changes in the inner-filter effect from sample to sample at these low concentrations since the pathlength of the exciting beam to the centre of the observation chamber was only 1 mm. As expected, the amplitudes of both phases increase with increasing 1,$N^6$-etheno ATP concentration. However, the dependences of the two phases are quite different. Whereas the fast phase appears hyperbolic, the slow phase is markedly sigmoidal. Furthermore, the appearance of the slow phase lags behind that the fast phase. Thus, at concentrations (after mixing) of 1,$N^6$-etheno-ATP below 10 $\mu$M, the proportion of the total amplitude seen as slow phase is very small. At ATP concentrations above 10 $\mu$M, the proportion of the slow phase increases rapidly, to become comparable with the fast phase at about 30–40 $\mu$M. It is apparent therefore that an increase in 1,$N^6$-etheno-ATP concentration results in a complex

increase in the amplitude of the fluorescence change associated with the slow-phase reaction.

It is shown below in the Discussion section that the fast phase represents binding of $1,N^6$-etheno-ATP to the enzyme, whereas the slow phase represents the allosteric transition of the R conformation into the T conformation. The fast phase can be analysed on the assumption that only a single class of independent sites, one per monomer, is involved in the binding reaction (see the Discussion section). For such a class of sites it may be shown that:

$$\Delta F = \frac{\Delta F_{\text{max.}}}{2[E]_t} \cdot \Big\{ [E]_t + [L]_t + K_d$$
$$- \sqrt{([E]_t + [L]_t + K_d)^2 - 4[L]_t[E]_t} \Big\} \quad (2)$$

where $\Delta F$ is the change in fluorescence observed at given total concentrations of enzyme and ligand, $[E]_t$ and $[L]_t$ respectively, $K_d$ is the dissociation constant of ligand from the enzyme and $\Delta F_{\text{max.}}$ is the maximum change in fluorescence observed at saturating concentrations of ligand. Eqn. (2) assumes that the fluorescence change is proportional to binding. In the analysis of the fast-phase amplitudes there is no need to consider complications from the slow phase, since the fast-phase binding reaction is complete before the slow-phase conformational transition has effectively begun. For the same reason, the total concentration of $1,N^6$-etheno-ATP in the observation chamber after mixing can be used directly in eqn. (2). The data were analysed by non-linear least-squares procedure by using the BMDP computer package (see the Experimental section) to search for the two unknown parameters, $\Delta F_{\text{max.}}$ and $K_d$. The results are displayed in Fig. 7, in which we are now able to present the data in more usual form by giving the amplitude as a function of the free (not total) concentration of $1,N^6$-etheno-ATP. The dependence is clearly hyperbolic, and it is apparent that the model of a single class of independent sites fits the data well with a dissociation constant of $24.1 \pm 2.7 \mu M$ and a $\Delta F_{\text{max.}}$ value of $92.5 \pm 1.53$ mV. $k_f$ varies from about $220 s^{-1}$ to $120 s^{-1}$ and $k_s$ from about $4 s^{-1}$ to $2 s^{-1}$ over the $1,N^6$-etheno-ATP concentration range $5-100 \mu M$. The analysis of these dependences is complex and is not presented here. However, $k_f \gg k_s$ over the entire range, justifying the assumption that the binding reaction equilibrium is achieved rapidly compared with the conformational transition.

$1,N^6$-Etheno-ATP is readily displaced from phosphofructokinase by ATP. When $25 \mu M$-enzyme plus $100 \mu M$-$1,N^6$-etheno-ATP are mixed with $500 \mu M$-ATP the reaction is monophasic, with a first-order rate constant of $5.9 \pm 0.08 s^{-1}$. An identical result was also obtained with ATP at a concentration of $1250 \mu M$ (Fig. 8). These concentrations of nucleotides are sufficiently high that even with the 2 mm-path-length observation cell employed it was necessary to
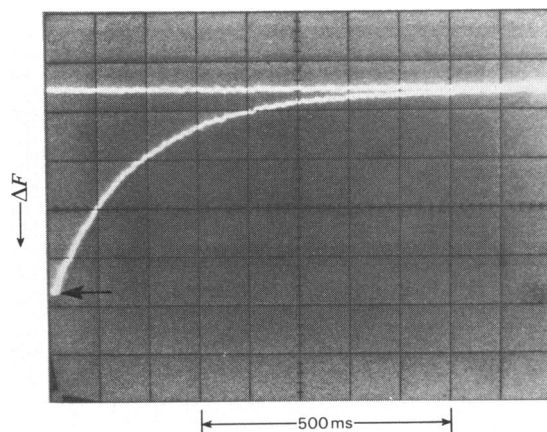


Fig. 8. *Displacement of $1,N^6$-etheno-ATP by ATP from phosphofructokinase 'locked' in the T conformation*
The quenching of $1,N^6$-etheno-ATP fluorescence caused by displacement of bound $1,N^6$-etheno-ATP from phosphofructokinase by ATP. Syringe 1, $25 \mu M$-phosphofructokinase, $100 \mu M$-$1,N^6$-etheno-ATP and 5 mM-MgCl$_2$; syringe 2, $2500 \mu M$-ATP and 7.5 mM-MgCl$_2$. Other conditions were as described for Fig. 2. The arrow indicates the point at which flow is stopped.

make a correction of the amplitude of reaction for the inner filter effect. When this was done, it was found that the amplitudes at $500 \mu M$- and $1250 \mu M$-ATP were the same, showing that all the $1,N^6$-etheno-ATP had been displaced. This was to be expected, since the ATP concentration was an order of magnitude greater than that of $1,N^6$-etheno-ATP and ATP binds more tightly. Thorough analysis of the data showed that they are fitted very well by a single exponential and that there is no evidence for any other component.

## Discussion

### Phosphofructokinase preparation

The phosphofructokinase used in this study was prepared by the method of Hussey et al. (1977). We have shown previously that this preparation, in common with all preparations from rabbit skeletal muscle, is heterogeneous. The enzyme consists of a mixture of 30S, 18S and 13S species (Aaronson & Frieden, 1972; Hussey et al., 1977). In addition, the enzyme is partially phosphorylated (Hussey et al., 1977), a finding that has been confirmed by other workers (Riquelme et al., 1978; Uyeda et al., 1978). The degree of phosphorylation varies from an average of about 0.2 mol of phosphate/mol of monomer for 30S species to an average of about 0.4 mol/mol for a mixture of 18S and 13S species. There is no evidence as yet, however, that differences

in phosphorylation determine differences in molecular weight, or vice versa; the difference might arise from distinct fibre types, for example (Peter *et al.*, 1972). However, no functional differences between the enzyme species have been established (Hussey *et al.*, 1977; Riquelme *et al.*, 1978). Because of the demand for large amounts of enzyme for stopped-flow experiments, therefore, pooled enzyme consisting of a mixture of 30S, 18S and 13S species was used for much of the present work. However, a number of the experiments have been repeated with only a purified 30S fraction of phosphofructokinase. No differences in reaction behaviour were observed between pooled and 30S enzyme.

### 1,$N^6$-Etheno-ATP as an analogue of ATP

Fig. 1(*a*) confirms the report by Secrist *et al.* (1972) that 1,$N^6$-etheno-ATP is a potentially useful substrate analogue of ATP for studies on phosphofructokinase. In 50mM-phosphate buffer, pH 6.80, the $K_m$ of the enzyme for 1,$N^6$-etheno-ATP is 46 $\mu$M, compared with 11 $\mu$M for ATP. Furthermore the $V_{max.}$ for 1,$N^6$-etheno-ATP is 84% of that determined for ATP.

As well as being a good substrate, 1,$N^6$-etheno-ATP also displays with phosphofructokinase the essential regulatory properties seen for ATP. At high concentrations 1,$N^6$-etheno-ATP funtions as an allosteric inhibitor and, in the notation of Monod *et al.* (1965), brings about the transition from an active R conformation into an inactive T conformation. At a fructose 6-phosphate concentration of 0.2mM, 50% inhibition is attained by about 4.3mM-1,$N^6$-etheno-ATP (Fig. 1*b*). In addition, the dependence of activity on fructose 6-phosphate concentration is sigmoidal (Fig. 1*c*).

Phosphate at 50mM was used in the present experiments to stabilize the enzyme. A slightly acid pH, 6.80, was used to ensure that 1,$N^6$-etheno-ATP is an effective inhibitor, although it is somewhat less effective than ATP under the same conditions, since only 1.1mM-ATP is required to produce 50% inhibition.

$Mg^{2+}$ is present at constant excess of 5mM in all experiments reported in the present paper. The species involved is therefore Mg–1,$N^6$-etheno-ATP, although it is referred to as 1,$N^6$-etheno-ATP throughout.

### Fluorescence energy transfer from phosphofructokinase to 1,$N^6$-etheno-ATP

When phosphofructokinase and 1,$N^6$-etheno-ATP are mixed, 1,$N^6$-etheno-ATP fluorescence is enhanced. The difference emission spectrum has a maximum at 400 nm (Fig. 2*a*),and the maximum extent of enhancement is about 27% at an excitation wavelength of 280 nm (Figs. 3*a* and 3*c*). Static-spectrum measurements in the Perkin–Elmer Hitachi MPF-3 fluorimeter at 280 nm show that the intrinsic protein

fluorescence of phosphofructokinase is quenched by about 2% at 330 nm. In addition, the excitation spectrum of 1,$N^6$-etheno-ATP fluorescence enhancement measured either in the static fluorimeter (Fig. 3*a*) or in the stopped-flow fluorimeter (Fig. 3*c*) is determined by phosphofructokinase absorption. These observations are consistent with the occurrence of energy transfer from protein to nucleotide. Liou & Anderson (1978) have previously reported that there is little, if any, quenching of phosphofructokinase fluorescence by 1,$N^6$-etheno-ATP. However, as shown in Fig. 5(*b*), the overall protein fluorescence quenching is very small only because it comprises two compensating changes.

### Assignment of the fast-phase and slow-phase reactions

When 1,$N^6$-etheno-ATP is mixed with phosphofructokinase, the enhancement of 1,$N^6$-etheno-ATP fluorescence occurs in a biphasic reaction (Fig. 5*a*). Over the entire range of 1,$N^6$-etheno-ATP concentration from 5 to 100 $\mu$M used in the present study, each phase was fitted well by a single exponential and there was no evidence for any other component in the reaction record. The fast-phase enhancement of 1,$N^6$-etheno-ATP fluorescence (Fig. 5*a*) is accompanied by a quenching of intrinsic protein fluorescence, which has an identical time course within experimental error (Fig. 5*b*) and which is absent when ATP is substituted for 1,$N^6$-etheno-ATP (Fig. 5*c*). Similarly, the slow phase of 1,$N^6$-etheno-ATP fluorescence enhancement is matched by a slow phase of protein fluorescence enhancement that has an identical time course within experimental error (Fig. 5*b*) but that, in contrast with the fast phase, also occurs when ATP is substituted for 1,$N^6$-etheno-ATP (Fig. 5*c*).

It is well known that the binding of ATP results in an allosteric transition from an active R conformation into an inactive T conformation (see Bloxham & Lardy, 1973). A simple explanation for the data is therefore that the fast phase represents binding of 1,$N^6$-etheno-ATP to the enzyme, giving rise to protein fluorescence quenching and 1,$N^6$-etheno-ATP fluorescence enhancement by energy transfer, whereas the slow phase represents the resulting allosteric conformational changes induced in phosphofructokinase, giving rise to enhancement of protein fluorescence. This would in turn cause increased enhancement of 1,$N^6$-etheno-ATP fluorescence by energy transfer.

An alternative explanation for the slow phase might be that it represents enzyme dissociation, for, in 50mM-phosphate buffer, pH 6.80, phosphofructokinase exists as a mixture of tetramer and higher polymers that dissociate to tetramer on mixing with ATP (Liddle *et al.*, 1976, 1977). However, the slow phase does not represent this dissociation reaction, since $k_s$ for the protein fluorescence change in the

presence of ATP, $10.7 s^{-1}$, is significantly faster than the rate constant for the ATP-induced dissociation of superaggregate to tetramer, $4.0 s^{-1}$ (Liddle et al., 1976, 1977). This conclusion is strengthed by the observation that $k_s$ measured at $50 \mu M$-$1,N^6$-etheno-ATP is independent of phosphofructokinase concentration over a 10-fold range from $2.5 \mu M$, where phosphofructokinase exists predominantly as tetramer, to $25 \mu M$, where phosphofructokinase exists predominantly as a mixture of 30S and 18S species. Furthermore, the slow phase is completely absent at pH 8.0 throughout the same concentration range, even though once again the predominant form of the enzyme changes from tetramer to 30S and 18S species (Leonard & Walker, 1972; Aaronson & Frieden, 1972; Pavelich & Hammes, 1973; Hussey et al., 1977).

Support for the idea that the slow phase represents the allosteric conformational change from the R into the T conformation comes from the observation that the proportion of the slow phase increases with increasing $1,N^6$-etheno-ATP concentration in a complex manner (Fig. 6). Furthermore, the preincubation of phosphofructokinase with cyclic AMP results in the abolition of the slow phase (Fig. 5d). Thus a powerful activator of the enzyme is able to block the inhibitory conformational change. The finding that the fast binding reaction also occurs in the presence of cyclic AMP confirms the supposition that the enzyme is initially in the R conformation when cyclic AMP is absent, and is in agreement with the conclusion by Goldhammer & Hammes (1978) that phosphate promotes the R conformation. The slow phase is also absent at pH 8.0, where phosphofructokinase does not display regulatory properties since it cannot undergo the allosteric transition (see Bloxham & Lardy, 1973). In contrast, the fast phase is still present at pH 8.0.

Fig. 5(c) shows that intrinsic protein fluorescence is enhanced by about 6.6 % on mixing with $30 \mu M$-ATP. This finding agrees well with the static-spectrum observations made by Liou & Anderson (1978), who found about 5 % enhancement at this concentration, especially in view of the fact that the static-spectrum data have to be corrected for the inner filter effect. As explained in the Results section, the stopped-flow data need no such correction. In agreement with the observation that the R conformation has diminished intrinsic fluorescence compared with the T conformation, Hill & Hammes (1975) have reported that the activators fructose 6-phosphate and fructose 1,6-bisphosphate cause quenching of tryptophan emission.

We have so far ascribed the fast phase of the binding of $1,N^6$-etheno-ATP and the slow phase to the allosteric transition of the R conformation into the T conformation induced by $1,N^6$-etheno-ATP. We must now consider which sites are involved in

the reactions and from where the fluorescence signals originate. It is well known from a variety of studies on binding, steady-state kinetics and changes in the chemical reactivity of thiol groups that there are two classes of ATP-binding site on phosphofructokinase (Kemp & Krebs, 1967; Kemp, 1969; Lorenson & Mansour, 1969; Setlow & Mansour, 1972). Wolfman et al. (1978) have measured directly the binding of the non-hydrolysable analogue [$\beta\gamma$-imido]ATP to the enzyme. They reported that only two molecules of [$\beta\gamma$-imido]ATP are bound per 80000-dalton monomer. These differ greatly in their affinities, the 'tight' catalytic site having a dissociation constant of about $1 \mu M$, in contrast with a value of about $100 \mu M$ for the 'loose' regulatory site. In addition, Liou & Anderson (1978) reported a value of $2.3 \pm 0.3$ binding sites per 80000-dalton monomer for $1,N^6$-etheno-ATP. It is therefore tempting to assign the fast and the slow phases to the catalytic and regulatory sites respectively. However, since the fast phase represents binding of $1,N^6$-etheno-ATP to the enzyme, it is possible that it originates from both sites, which might well have distinct signals. In fact, the fast-phase assignment appears to be straightforward. Non-linear least-squares analysis of the dependence of fast-phase amplitude on $1,N^6$-etheno-ATP concentration shows that the data are fitted very well on the assumption that only a single class of independent equivalent sites, one per monomer, is involved that have a dissociation constant for $1,N^6$-etheno-ATP of $24.1 \pm 2.7 \mu M$ (Fig. 7). The success of the analysis justifies the additional assumptions that for this site fluorescence enhancement is proportional to binding and that the binding equilibrium is established rapidly compared with the transition from the R conformation into the T conformation, so that analytical complications from the slow phase do not have to be considered. Several attempts were made to fit the data with two-site models, including one in which the signal originated from one site only while binding was permitted at both sites. Even so, none of these models was as successful as the single-site model. Clear confirmation that only a single site is involved in the fast-phase reaction is provided by the fact that preincubation with cyclic AMP abolishes the slow phase but does not affect the fast phase. Wolfman et al. (1978) have shown that in the presence of cyclic AMP phosphofructokinase binds only one molecule of [$\beta\gamma$-imido]ATP per monomer, the only conditions under which they were able to observe a single binding isotherm. Since phosphofructokinase is fully active in the presence of cyclic AMP, the fast phase represents binding of $1,N^6$-etheno-ATP to the catalytic site. The slow-phase allosteric conformational change from the R form into the T form must therefore be caused by binding to the regulatory site. However, the signal that allows this transition to be observed does not originate from the regulatory site.

*Origin of the 1,$N^6$-etheno-ATP fluorescence enhancement signal of the slow-phase reaction*

Fig. 8 shows the reaction record of the displacement of 1,$N^6$-etheno-ATP from phosphofructokinase by ATP under conditions such that the enzyme is almost fully in the T conformation at the start of the reaction and remains locked there by the displacing ATP. Both the catalytic and regulatory sites are therefore fully occupied initially by 1,$N^6$-etheno-ATP and subsequently displaced completely by ATP. The two sites have very different affinities from each other for ATP and its analogues (Kemp & Krebs, 1967; Kemp, 1969; Lorenson & Mansour, 1969; Setlow & Mansour, 1972). Yet the displacement reaction shows no evidence of heterogeneity; the data are fitted very well by a single exponential defining a homogeneous first-order reaction with a rate constant of $5.9 \pm 0.08$ $s^{-1}$. One possible explanation for this observation is that the rate constant for the displacement of 1,$N^6$-etheno-ATP, $k_{off}$, is the same for both the catalytic and regulatory sites. However, it seems extremely unlikely that the great differences between them would be reflected solely in the 'on' constant, $k_{on}$. Indeed, differences in the reactivity of ligands with proteins are usually expressed in $k_{off}$ rather than $k_{on}$ (Gutfreund, 1972). A far more likely explanation is that the displacement signal originates from one site only. This must be the catalytic site, since a binding signal is seen in the presence of cyclic AMP. The natural conclusion of this argument is that the slow-phase enhancement of 1,$N^6$-etheno-ATP fluorescence, caused as a consequence of binding to the regulatory site and the subsequent conformational change of the R form into the T form, arises from concomitant changes in the fluorescence of 1,$N^6$-etheno-ATP bound at the catalytic site. This interpretation is consistent with the observed identity of the excitation spectra for the fast and the slow phases (Fig. 3c). Both are dependent on tyrosine absorption and both show a small plateau above 300 nm. This similarity would not be expected for two such different sites.

The changes in 1,$N^6$-etheno-ATP fluorescence at the catalytic site as a result of the transition of the R conformation into the T conformation could arise from: (i) increased efficiency of energy transfer caused by alteration in the relative orientation of acceptor and donor or the distance between them; (ii) a change in acceptor/donor absorption; (iii) a change in acceptor/donor quantum yield. An increase in the efficiency of energy transfer would require that as 1,$N^6$-etheno-ATP fluorescence is enhanced so protein fluorescence is quenched. This is not the case for the slow phase; both are enhanced (Fig. 5). Difference absorption measurements between mixed and un-mixed reactants show that, although there are significant differences in the range 250–280 nm, there are no differences in the excitation range 280–290 nm

used in the present experiments (Fig. 4a). It is unlikely therefore that the slow-phase enhancement of 1,$N^6$-etheno-ATP fluorescence is caused by a change in acceptor/donor absorption, although perfectly compensating changes in the two cannot be ruled out. Since the protein fluorescence is enhanced, the most probable explanation is that the quantum yield of the donor is increased. Increased energy transfer follows, although the efficiency of this process may be unchanged.

It should be noted that the intrinsic protein fluorescence changes induced by ATP are actually more complicated than has so far been implied, for Liou & Anderson (1978) have shown that at concentrations above about 60 $\mu$M-ATP binding causes quenching rather than enhancement of fluorescence. This observation is in agreement with our own static measurements and also stopped-flow measurements in this concentration range (results not shown). We have demonstrated above that the transition of the R conformation into the T conformation enhances fluorescence in the general region of the catalytic site. These observations may be reconciled, therefore, if the allosteric transition is at least partially concerted (Goldhammer & Hammes, 1978) and actual occupation of the regulatory site by ATP causes limited quenching. Thus at low concentrations the conformational-state function would precede the binding-state function for the regulatory site, giving rise to increased protein fluorescence; subsequent filling of the remaining vacant regulatory sites would result in quenching. In contrast with ATP, occupation of the regulatory site at high concentrations by 1,$N^6$-etheno-ATP does not lead to quenching (Fig. 6). In this respect ATP and 1,$N^6$-etheno-ATP are different. C.d. measurements by Liou & Anderson (1978) have already emphasized that the two nucleotides have quite subtle differences in their effect on phosphofructokinase conformation.

*General comments*

$k_f$ and $k_s$ are both dependent on 1,$N^6$-etheno-ATP concentration. The values of the apparent second-order rate constant for the fast phase based on $k_f$ at different 1,$N^6$-etheno-ATP concentrations are much less than would be expected for a diffusion-controlled reaction and are consistent with a two-step binding mechanism. The dependence of $k_s$ on 1,$N^6$-etheno-ATP concentration is complex and may not conform to either the simple concerted or sequential models for co-operativity (Monod *et al.*, 1965; Koshland *et al.*, 1966). However, knowledge of the detailed mechanisms of the fast and the slow phases does not materially affect the reaction assignments proposed above.

All solutions contained a constant excess of 5 mM-$Mg^{2+}$ over 1,$N^6$-etheno-ATP. The species involved in these reactions is therefore actually Mg–1,$N^6$-etheno-

ATP. The affinity of Mg-1,$N^6$-etheno-ATP for the regulatory site is far greater in the T conformation than in the R conformation, in parallel with the behaviour of Mg-ATP (Mathias & Kemp, 1972). Binding to this site in the R conformation is so weak that it cannot be detected by the present methods in the concentration range 5–100 $\mu$M-1,$N^6$-etheno-ATP. Higher concentrations were not investigated, because the observed enhancement signal of bound 1,$N^6$-etheno-ATP becomes very small compared with the fluorescence of the large concentration of free 1,$N^6$-etheno-ATP. Wolfman *et al.* (1978) have suggested that cyclic AMP reverses the inhibitory effect of ATP by 'blocking' the regulatory site in R conformation, possibly by direct binding to that site. The present data imply that at the low 1,$N^6$-etheno-ATP concentrations the site is effectively unavailable to 1,$N^6$-etheno-ATP in the R conformation, binding to the T conformation is exclusive and 'blocking' occurs by an allosteric mechanism.

For the moment at least it is possible only to speculate about the changes that occur at the catalytic site as a result of the transition of the R conformation into the T conformation. Columbo *et al.* (1975) have shown that inhibitors other than ATP may increase the affinity of ATP not only for the regulatory site but also for the catalytic site. Since ATP is itself an inhibitor, of course, then it is possible that ATP binding to the regulatory site and the subsequent transition of the R conformation into the T conformation may increase the affinity of ATP for the catalytic site. This suggestion is consistent with the finding by Liou & Anderson (1978) that at low 1,$N^6$-etheno-ATP concentrations ATP promotes binding of 1,$N^6$-etheno-ATP. Such an increase, together with an alteration in the extent of the transition of the R conformation into the T conformation, would explain why the isotherm for the binding of [$\beta\gamma$-imido]ATP to the 'loose' regulatory site is complex (Wolfman *et al.*, 1978). The alteration of the fluorescence of 1,$N^6$-etheno-ATP bound to the catalytic site as a result of 1,$N^6$-etheno-ATP binding to the regulatory site is a striking example of the allosteric phenomenon.

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