The kinetics of effector binding to phosphofructokinase

THE BINDING OF Mg^{2+} -1, N^6 -ETHENOADENOSINE TRIPHOSPHATE TO THE CATALYTIC SITE

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1. The binding of the fluorescent ATP analogue, Mg^{2+} -1, N^6 -etheno-ATP, to the catalytic site of rabbit skeletal muscle phosphofructokinase has been studied by stopped-flow fluorimetry [Roberts & Kellett (1979) *Biochem. J.* **183**, 349–360]. 2. Binding of Mg^{2+} -1, N^6 -etheno-ATP to the catalytic site is consistent with a two-step mechanism of the type:

in which the diffusion-controlled binding of ligand, L, is accompanied by prior inter-conversion of enzyme from one form, E, to another, E^* . 3. The allosteric activators, phosphate and cyclic AMP, which promote an R-type conformation, appear to stabilize slightly different conformations, R and R' respectively. 4. The binding of Mg^{2+} -1, N^6 -etheno-ATP to the catalytic site is strongly affected by its binding to the inhibitory site. The rate constant for the displacement of Mg^{2+} -1, N^6 -etheno-ATP from the catalytic site, k_{32} , is $470 \pm 35 \text{ s}^{-1}$ for the R' conformation, whereas it is $6.0 \pm 0.09 \text{ s}^{-1}$ for the T conformation induced by binding of Mg^{2+} -1, N^6 -etheno-ATP to the inhibitory site.

The importance of ATP in the regulation of phosphofructokinase (ATP-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11), the principal rate-limiting enzyme of glycolysis, is well established (for review, see Bloxham & Lardy, 1973). Mg^{2+} -ATP is able to serve both as a substrate and as an allosteric inhibitor of the enzyme. Thus phosphofructokinase from rabbit skeletal muscle contains two binding sites for Mg^{2+} -ATP per monomer of mol.wt. 80000, one the catalytic site and one an inhibitory site. In addition, there is a third nucleotide binding site, an activating site that binds cyclic AMP, 5'-AMP or ADP (Kemp & Krebs, 1967; Kemp, 1969; Lorenson & Mansour, 1969; Setlow & Mansour, 1972; Wolfman *et al.*, 1978). The inhibition of phosphofructokinase by Mg^{2+} -ATP is attenuated by citrate which also binds to one site per monomer and interacts synergistically with Mg^{2+} -ATP (Columbo *et al.*, 1975).

To examine the reactions at these binding sites, we have made use of the favourable properties of the fluorescent Mg^{2+} -ATP analogue, Mg^{2+} -1, N^6 -

etheno-ATP. This is also an efficient substrate and an effective allosteric inhibitor of phosphofructokinase (Secrist *et al.*, 1972; Roberts & Kellett, 1979) and provides a suitable spectral signal which allows its reaction with phosphofructokinase to be observed in a stopped-flow fluorimeter. When a reaction mixture of Mg^{2+} -1, N^6 -etheno-ATP and phosphofructokinase is excited at 285 nm, the time-course of Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement caused by energy transfer from enzyme to nucleotide is biphasic. The fast phase represents binding to the catalytic site alone. The slow phase results from the allosteric transition of the R conformation into the T conformation induced by binding of Mg^{2+} -1, N^6 -etheno-ATP to the inhibitory site (Roberts & Kellett, 1979). The ability of the stopped-flow fluorimeter to distinguish between the consequences of binding to the catalytic and inhibitory sites permits the study not only of binding to each site independently, but also of their interaction. Such facility is not possible with conventional equilibrium-dialysis experiments.

In this paper, we show that the mechanism of Mg^{2+} -1, N^6 -etheno-ATP binding to the catalytic site alone is consistent with a two-step 'prior-isomeriz-

Abbreviation used: 1, N^6 -etheno-ATP, 1, N^6 -etheno-adenosine triphosphate.

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ation pathway'. A complete kinetic description of this pathway is presented for the R-type conformation induced by cyclic AMP and it is shown how the pathway is affected by the allosteric transition of the R conformation into the T conformation.

Experimental

All materials and methods were as previously described (Roberts & Kellett, 1979). Static titrations of rabbit skeletal muscle phosphofructokinase were carried out with a stock solution of Mg^{2+} -1, N^6 -etheno-ATP containing enzyme at the same concentration as in the titration cell. In this way, no corrections for dilution of enzyme were required. All experiments were performed at $19 \pm 1^\circ\text{C}$ in 50 mM-potassium phosphate buffer, pH 6.80, containing 0.2 mM-dithiothreitol. $[\text{Mg}^{2+}]$ was maintained at a constant excess of 5 mM over $[\text{Mg}^{2+}$ -1, N^6 -etheno-ATP] in all experiments. Phosphofructokinase concentrations are expressed in terms of mol of monomer, based on a mol.wt. of 80 000 (Walker *et al.*, 1976). Concentrations quoted in stopped-flow experiments are syringe concentrations unless otherwise specified.

Results

When 25 μM -phosphofructokinase was mixed with 100 μM - Mg^{2+} -1, N^6 -etheno-ATP in 50 mM-phosphate buffer at pH 6.80 and the reaction was observed at an excitation wavelength of 285 nm, the time-course for the enhancement of Mg^{2+} -1, N^6 -etheno-ATP fluorescence was biphasic. Each phase is fitted well by a single exponential and the dependence of reaction amplitude on Mg^{2+} -1, N^6 -etheno-ATP concentration was hyperbolic for the fast phase, whereas it was sigmoidal for the slow phase [Fig. 5(a) in Roberts & Kellett, 1979]. Fig. 1(a) of the present paper shows the dependence of the apparent first-order rate constant of the fast phase for binding to the catalytic site, k_f , on Mg^{2+} -1, N^6 -etheno-ATP concentration. The standard error in k_f values tended to be a little larger at the extremes of the concentration range. This was because the data at low Mg^{2+} -1, N^6 -etheno-ATP concentrations were determined at the limit of the stopped-flow technique, whereas at high concentrations the free concentration of Mg^{2+} -1, N^6 -etheno-ATP was large compared to that bound and the signal-to-noise ratio consequently poor.

In the presence of cyclic AMP, Mg^{2+} -1, N^6 -etheno-ATP binds only to the catalytic site and the allosteric transition of the R conformation into the T conformation induced by Mg^{2+} -1, N^6 -etheno-ATP is blocked, so that the enzyme remains entirely in the R-type conformation stabilized by cyclic AMP (Wolfman *et al.*, 1978; Roberts & Kellett, 1979).

Fig. 1. Dependence of k_f on Mg^{2+} -1, N^6 -etheno-ATP concentration and the effect of preincubation of phosphofructokinase with cyclic AMP

Ordinate, k_f (s⁻¹); abscissa, total concentration (μM) of Mg^{2+} -1, N^6 -etheno-ATP after mixing. (a) Enzyme alone: syringe 1, 25 μM -phosphofructokinase; syringe 2, Mg^{2+} -1, N^6 -etheno-ATP + 10 mM- MgCl_2 excess. Dashed line is the empirical line fitted by eye. (b) Enzyme preincubated with cyclic AMP: syringe 1, 25 μM -phosphofructokinase + 50 μM -cyclic AMP; syringe 2, Mg^{2+} -1, N^6 -etheno-ATP + 10 mM- MgCl_2 excess. The solid line is the theoretically predicted line fitted as described in the text to the 'prior-isomerization pathway'. Values of the rate constants were taken as $k_{12} = 450 \text{ s}^{-1}$, $k_{23} = 2 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$, $k_{21} = 4.7 \times 10^3 \text{ s}^{-1}$, $k_{32} = 470 \text{ s}^{-1}$. Conditions: 50 mM-phosphate (25 mM- KH_2PO_4 /25 mM- K_2HPO_4) buffer, pH 6.80, containing 0.2 mM-dithiothreitol at $19 \pm 1^\circ\text{C}$. Observations were made as described previously (Roberts & Kellett, 1979).

Thus when 25 μM -phosphofructokinase was first dialysed against phosphate buffer containing 50 μM -cyclic AMP and then mixed with 100 μM - Mg^{2+} -1, N^6 -etheno-ATP, only the fast phase was observed; the slow phase was completely absent even if

observations were continued for several seconds or more [Fig. 5(d) in Roberts & Kellett, 1979]. At high concentrations of Mg²⁺-1,N⁶-etheno-ATP, the half-time of the reaction in the presence of cyclic AMP, $t_{\frac{1}{2}}$ was much larger than the dead-time of the apparatus, so that almost all of the reaction could be observed. Detailed analysis of the reaction record showed that it was a monophasic, first-order process, the data being fitted very well by a single exponential. At low concentrations of Mg²⁺-1,N⁶-etheno-ATP, the $t_{\frac{1}{2}}$ approached the dead-time (approx. 1 ms) of the stopped-flow fluorimeter. Because only about 50% of the reaction record could be observed, it was not possible to extrapolate the data to zero-time to produce reaction amplitudes that were sufficiently precise to allow determination of the equilibrium constant for Mg²⁺-1,N⁶-etheno-ATP binding (Roberts & Kellett, 1979). However, the $t_{\frac{1}{2}}$, and therefore also k_f , for this apparent first-order reaction could still be determined accurately. Fig. 1(b) shows the dependence of k_f on Mg²⁺-1,N⁶-etheno-ATP concentration when the enzyme has been preincubated with cyclic AMP.

An alternative way to determine the equilibrium constant for Mg²⁺-1,N⁶-etheno-ATP binding was by static titration. Fig. 2 shows a representative titration of 12.5 μ M-phosphofructokinase in the presence of 50 μ M-cyclic AMP. Since only one catalytic site per monomer is available for Mg²⁺-1,N⁶-etheno-ATP, it may be shown that:

$$F = \frac{(F_b - F_f)}{2} \cdot \{[E]_t + [L]_t + K_d - \sqrt{([E]_t + [L]_t + K_d)^2 - 4[L]_t[E]_t}\} + F_f[L]_t \quad (1)$$

where F is the observed fluorescence and F_b and F_f the intrinsic fluorescence of the bound and free forms of ligand (Mg²⁺-1,N⁶-etheno-ATP) respectively; $[E]_t$ and $[L]_t$ are the total concentrations of enzyme and ligand respectively and K_d is the dissociation constant of ligand from enzyme. The solid line in Fig. 2 is the theoretical line fitted to eqn. (1) by using a non-linear least-squares procedure (Roberts & Kellett, 1979). The values of F_b and F_f in arbitrary fluorescence units were determined to be 4.72 ± 0.059 and 1.44 ± 0.003 units/ μ M respectively and K_d to be 27 ± 1.0 μ M.

The rate constant for the displacement of Mg²⁺-1,N⁶-etheno-ATP from the R-type conformation induced by cyclic AMP was determined to be 470 ± 35 s⁻¹ by displacing 100 μ M-Mg²⁺-1,N⁶-etheno-ATP with 2400 μ M-Mg²⁺-ATP in the presence of a free concentration of cyclic AMP of 50 μ M (Fig. 3a). As shown previously, this ratio of Mg²⁺-ATP to Mg²⁺-1,N⁶-etheno-ATP is more than sufficient to cause total and irreversible displacement (Roberts & Kellett, 1979). When the same experiment was performed in phosphate buffer in the

Fig. 2. Static Mg²⁺-1,N⁶-etheno-ATP fluorescence titration of phosphofructokinase preincubated with cyclic AMP

Ordinate, Mg²⁺-1,N⁶-etheno-ATP fluorescence enhancement in arbitrary units, corrected as previously described (Roberts & Kellett, 1979); abscissa, total concentration (μ M) of Mg²⁺-1,N⁶-etheno-ATP in the cuvette. The cuvette contained 12.5 μ M-phosphofructokinase. [Mg²⁺] was maintained at a constant excess of 5 mM. Excitation wavelength, 285 nm; emission wavelength, 405 nm. The solid line is the theoretically predicted line fitted as described in the text to eqn. (1) with a dissociation constant of 27 ± 1.0 μ M. Other conditions were as described for Fig. 1.

absence of cyclic AMP, so that enzyme was locked in T state throughout, the rate constant for displacement was 6.0 ± 0.09 s⁻¹ (Fig. 3b).

Fig. 4 shows a single-turnover experiment in the presence of phosphate alone. In this experiment 50 μ M-phosphofructokinase was mixed with buffer containing 60 μ M-1,N⁶-etheno-ATP, 60 μ M-fructose 6-phosphate and 10 mM-Mg²⁺. The rate constants for the fast and slow phases were 150 ± 17 s⁻¹ and 5.9 ± 0.2 s⁻¹ respectively.

Discussion

To interpret the present experiments, it is necessary to define the initial conformational state(s) in which phosphofructokinase exists before reaction

Fig. 3. Displacement of Mg^{2+} -1, N^6 -etheno-ATP by Mg^{2+} -ATP from the catalytic site of phosphofructokinase in the R' and T conformations

Ordinate, quenching of Mg^{2+} -1, N^6 -etheno-ATP fluorescence (mV); abscissa, time (ms). (a) R' conformation: syringe 1, 25 μM -phosphofructokinase previously dialysed against 50 μM -cyclic AMP, 100 μM - Mg^{2+} -1, N^6 -etheno-ATP + 5 mM- MgCl_2 ; syringe 2, 2.4 mM-ATP + 7.5 mM- MgCl_2 . (b) T conformation: as described for (a) except that syringe 1 did not contain cyclic AMP. Other conditions were as described for Fig. 1. The arrows indicate the point at which flow was stopped.

with Mg^{2+} -1, N^6 -etheno-ATP. It would be intuitively expected that, in the presence of powerful activators such as cyclic AMP or phosphate, phosphofructokinase would exist effectively only in an R-type conformation. Nevertheless, the values of the allosteric constants derived by fitting steady-state kinetic data to a concerted model predict that, under both these conditions, enzyme in the absence of substrates exists in a mixture of R and T forms (Goldhammer & Hammes, 1978). It seems likely,

Fig. 4. Single turnover of phosphofructokinase. Ordinate, Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement (mV); abscissa, time (ms). Syringe 1, 50 μM -phosphofructokinase; syringe 2, 60 μM - Mg^{2+} -1, N^6 -etheno-ATP, 60 μM -fructose 6-phosphate + 10 mM- MgCl_2 . The arrows indicate the point at which flow was stopped. Other conditions were as described for Fig. 1.

however, that the reason for this conclusion is that the steady-state data were obtained by using an ATP-regenerating system containing large concentrations of phosphoenolpyruvate, which is an inhibitor of phosphofructokinase and is competitive with citrate. Since the latter is able to promote a T-type conformation of the enzyme even in the absence of substrates (Roberts & Kellett, 1980), it is to be expected that phosphoenolpyruvate will do the same. Thus steady-state kinetic data obtained in the presence of the ATP-regenerating system would not reflect the real situation for cyclic AMP and phosphate alone.

In view of the steady-state kinetic data, however, it is necessary to re-emphasize our earlier conclusion that, under the conditions of the experiments reported here, phosphofructokinase exists effectively only in an R-type conformation, both in the presence of cyclic AMP and in the presence of phosphate alone. This is most clearly seen in the case of cyclic AMP, saturating concentrations of which completely inhibit the allosteric transition. In full agreement with the direct binding data of Wolfman *et al.* (1978), only the catalytic site is available to Mg^{2+} -1, N^6 -etheno-ATP for enzyme in the presence of saturating cyclic AMP; no detectable binding occurs to the inhibitory site (Roberts & Kellett, 1979). In the presence of phosphate alone, the dissociation constant of Mg^{2+} -1, N^6 -etheno-ATP from the catalytic site, $24.1 \pm 2.7 \mu\text{M}$, is identical within experimental error to that of $27 \pm 1.0 \mu\text{M}$ for

enzyme in the presence of cyclic AMP. Thus enzyme in the presence of phosphate alone also exists in an R-type conformation. Further evidence on this point is presented below that is consistent with the idea that the conformations of the enzyme that exist in the presence of phosphate alone and in the presence of cyclic AMP are in fact slightly different R-type conformations.

The dependence of the apparent first-order rate constant of the fast phase, k_f , on Mg²⁺-1,N⁶-etheno-ATP concentration thus gives information about the mechanism of Mg²⁺-1,N⁶-etheno-ATP binding to the catalytic site in an R-type conformation. For enzyme in the presence of either cyclic AMP alone or phosphate alone, k_f decreases monotonically to an asymptotic limit (Fig. 1). Such behaviour is consistent with the 'prior-isomerization pathway' for the binding of ligand, L, and enzyme, E, (Koshland & Neet, 1968):

When enzyme and substrate are mixed and the total concentration of ligand, [L]_t, is much greater than that of enzyme, [E]_t, this mechanism predicts that:

$$k_f = k_{12} + \frac{k_{21}}{1 + \frac{[L]_t}{K^*}} \quad (2)$$

where

$$K^* = \frac{[E^*][L]}{[E^*L]} \quad (3)$$

Thus k_f should decrease monotonically from $(k_{12} + k_{21})$ to an asymptotic limit of k_{12} with increasing ligand concentration.

It was possible to obtain a complete kinetic description for the binding of Mg²⁺-1,N⁶-etheno-ATP to the catalytic site of enzyme locked in the R-type conformation induced by cyclic AMP. For these conditions, values of the rate constants were assigned as follows: k_{12} was taken as 450 s⁻¹ from the asymptotic value of k_f at high Mg²⁺-1,N⁶-etheno-ATP concentration (Fig. 1b); k_{32} was taken as 470 s⁻¹, the rate constant for the displacement of Mg²⁺-1,N⁶-etheno-ATP by Mg²⁺-ATP. The rate constant, k_{23} , for the diffusion-controlled binding of L to E* was assumed to be $2 \times 10^8 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Eigen & Hammes, 1968). Finally, k_{21} was estimated from a knowledge of the dissociation constant, K_d , given by the expression:

$$K_d = \left(1 + \frac{k_{21}}{k_{12}} \right) \frac{k_{32}}{k_{23}} \quad (4)$$

The value of K_d , determined by static titration to be $27 \pm 1.0 \mu\text{M}$, corresponds to a value for k_{21} of $4.7 \times 10^3 \text{ s}^{-1}$. The solid line in Fig. 1(b) was computed by using these assigned values of the rate constants.

The values of k_{12} and k_{21} imply that initially about 90% of the enzyme is in the E form and about 10% is in the E* form which is removed within the dead-time of the stopped-flow fluorimeter by the diffusion-controlled binding of nucleotide. The corresponding burst of E*L production is therefore not seen; only the first-order production of E*L caused by depletion of E is observed. In contrast with the stopped-flow fluorimeter results, the amplitudes obtained with the static fluorimeter reflect not only binding to E but also to E*. The dissociation constants obtained by these techniques are therefore in principle distinct, and the numerical differences will, of course, depend upon the initial percentage of E* present. In practice, an initial percentage of E* greater than 10 would be relatively easy to detect. The fact that the dissociation constant determined for enzyme in the presence of cyclic AMP by static titration also describes the stopped-flow data well confirms that the initial percentage of E* is very low.

The nature of the enzyme interconversion, $E \rightleftharpoons E^*$, in the 'prior-isomerization pathway' has not been specified. An obvious interpretation is that the interconversion is simply a local conformational change at the catalytic site. However, an alternative interpretation arises from the studies of Frieden and co-workers on the pH-dependent inactivation of the enzyme that occurs below pH 6.8 (Frieden *et al.*, 1976). These investigators propose that the enzyme exists in a proton-linked isomerization equilibrium between an active, unprotonated tetramer and an inactive protonated tetramer that subsequently dissociates slowly to dimer. Such an isomerization would be expected to be slower than the accompanying protonation-deprotonation steps, so that the latter would not be rate-limiting. The relative proportion of the protonated form increases with decreasing pH and decreasing temperature in such a way that, under the conditions of the present experiments, 50 mM-phosphate buffer, pH 6.80 at 19°C, not more than a few percent of the enzyme would exist in the protonated form. Moreover, Bock & Frieden (1976) have shown that it is the protonated form that preferentially binds ATP. Thus the 'prior-isomerization pathway' would appear to be consistent with the pH-dependence data if E and E* represent the unprotonated and protonated forms respectively. We have not investigated the pH dependence of k_f , because the tetramer at low pH dissociates to dimer, which reacts differently from tetramer (Roberts & Kellett, 1980) and which can under some circumstances isomerize to yet another form (Frieden *et al.*, 1976). In addition it is not

possible to measure proton uptake under the present experimental conditions because of the phosphate present.

R-type conformations

It has been argued above that both phosphate and cyclic AMP effectively stabilize phosphofructokinase entirely in an R-type conformation. The question arises as to whether the conformations of the enzyme under each of these conditions are the same or whether they represent slightly different conformations, say R and R'. The K_d for Mg^{2+} -1, N^6 -etheno-ATP binding to the catalytic site determined by stopped-flow fluorimetry in the presence of phosphate alone is $24.0 \pm 2.7 \mu M$ (Roberts & Kellett, 1979). This value is not significantly different from the value of $27.0 \pm 1.0 \mu M$, determined by static titration and stopped-flow fluorimetry respectively, for enzyme in the presence of phosphate + cyclic AMP. As noted before, then, identity of dissociation constants suggests that the same conformation is present under each condition. However, the K_d comprises four rate constants (eqn. 4) and the rate data of Fig. 1 show that values of individual rate constants change with conditions. Thus the value of k_{12} determined by the asymptotic value of k_f at high Mg^{2+} -1, N^6 -etheno-ATP concentrations is about $120 s^{-1}$ for phosphate alone compared with about $450 s^{-1}$ for phosphate plus cyclic AMP. These values for k_{12} are remarkably constant from preparation to preparation so that the difference does not reflect any variation between preparations. Other differences between enzyme in the presence of phosphate buffer with and without cyclic AMP are seen. For example, when enzyme in 50 mM-phosphate buffer, pH 6.80, is mixed with saturating cyclic AMP, a small but significant quenching of protein fluorescence (about 7%) occurs in a first-order process characterized by a rate constant of $18 \pm 1.3 s^{-1}$ (Pettigrew & Frieden, 1978; Roberts & Kellett, 1980). In addition, Goldhammer & Hammes (1978) have reported that the turnover number of phosphofructokinase in the presence of phosphate is about 2-fold less than that in the presence of cyclic AMP. None of the differences quoted are particularly large. However, they are consistent and suggest that phosphate and cyclic AMP stabilize slightly different R-type conformations, that will be referred to as R and R' respectively.

Catalytic site in R and T conformations

As noted earlier, the rate constant for the displacement of Mg^{2+} -1, N^6 -etheno-ATP from the catalytic site measures k_{32} in the 'prior-isomerization pathway'. In the presence of cyclic AMP, the enzyme remains in the R' conformation at high Mg^{2+} -1, N^6 -etheno-ATP concentrations and even at the very high displacing concentrations of Mg^{2+} -

ATP (approx. 1.2 mM). Thus k_{32} for the catalytic site in the R' conformation is $470 \pm 35 s^{-1}$ (Fig. 3a). In phosphate alone, the enzyme adopts the T conformation at high Mg^{2+} -1, N^6 -etheno-ATP concentrations. Even though both the catalytic and inhibitory sites are occupied in these circumstances, the rate constant for displacement, $6.0 \pm 0.09 s^{-1}$, still reflects k_{32} for the catalytic site in its T state because the observed displacement signal arises only from the catalytic site (Roberts & Kellett, 1979). This value is in excellent agreement with that of $5.9 \pm 0.08 s^{-1}$ determined previously. For the catalytic site in the R' conformation k_{32} is thus approx. 80-fold greater than that in the T conformation.

Attempts to determine k_{32} for the catalytic site of the enzyme in the R conformation stabilized by phosphate alone were unsuccessful. In these experiments, enzyme was first preincubated with Mg^{2+} -1, N^6 -etheno-ATP at low concentrations (5–10 μM) so that the enzyme was initially predominantly in the R conformation. However, the value of k_{32} turned out to be identical to that for T conformation, implying that under the conditions of the experiment the allosteric transition into the T conformation occurred at a rate faster than the displacement of Mg^{2+} -1, N^6 -etheno-ATP from the catalytic site of the R conformation. Independent measurements show that the apparent first-order rate constant of the allosteric transition is dependent on the concentration of Mg^{2+} -ATP (Roberts & Kellett, 1980). At a concentration of 1 mM- Mg^{2+} -ATP, the rate constant is approx. $140 s^{-1}$. Since k_{32} could not be determined for enzyme in the presence of phosphate alone, a complete kinetic description of the 'prior-isomerization pathway' could not be obtained in the same way that was possible for cyclic AMP. The dashed line in Fig. 1(a) was therefore simply fitted by eye to the data obtained for enzyme in phosphate alone.

Single turnover of phosphofructokinase

Finally it should be emphasized that the above experiments refer to the binding at the catalytic site of Mg^{2+} -1, N^6 -etheno-ATP in the absence of fructose 6-phosphate. Fig. 4 shows a single-turnover experiment in the absence of cyclic AMP. In this experiment enzyme is mixed with fructose 6-phosphate and Mg^{2+} -1, N^6 -etheno-ATP simultaneously. The fast phase for Mg^{2+} -1, N^6 -etheno-ATP binding has a rate constant of approx. $150 s^{-1}$. This is very similar to the value for k_{12} of $120 s^{-1}$ so that, in this particular experiment, fructose 6-phosphate appears to have little, if any, effect on the fast-phase binding reaction of Mg^{2+} -1, N^6 -etheno-ATP to the catalytic site. The slow phase has a rate constant of approx. $5.9 s^{-1}$, although it is not known whether this reflects dissociation of Mg^{2+} -1, N^6 -etheno-ADP or some prior conformational change. We have not studied

the effect on turnover of preincubation of the enzyme with fructose 6-phosphate. The single-turnover experiment has potential for looking at the binding interactions of Mg²⁺-1,N⁶-etheno-ATP and fructose 6-phosphate and also for investigating the catalytic-centre mechanism of phosphofructokinase.

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