The kinetics of effector binding to phosphofructokinase

THE INFLUENCE OF EFFECTORS ON THE ALLOSTERIC CONFORMATIONAL TRANSITION

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1. The extent of the allosteric transition from the R into the T conformation of rabbit skeletal muscle phosphofructokinase induced by Mg^{2+} -1, N^6 -etheno-ATP was determined by stopped-flow fluorimetry from the amplitude of the slow phase of the Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement [Roberts & Kellett (1979) *Biochem. J.* **183**, 349–360]. 2. The amplitude of the slow phase was decreased by low concentrations of the activators cyclic AMP and fructose 1,6-bisphosphate, but increased in a complex manner by the inhibitor citrate. 3. Mg^{2+} -1, N^6 -etheno-ATP and Mg^{2+} -ATP are unable to induce the T conformation to a detectable extent in the presence of saturating cyclic AMP, but can do so readily in the presence of saturating fructose 1,6-bisphosphate. 4. The conformational transitions induced in enzyme alone by different ligands were observed by changes in intrinsic protein fluorescence. In general, an R-type conformation has diminished protein fluorescence compared with a T-type conformation. 5. Mg^{2+} -ATP exerts a complex effect on protein fluorescence; both the enhancement at low concentrations and the quenching at high concentrations of Mg^{2+} -ATP result from the binding of Mg^{2+} -ATP to the inhibitory site and the ensuing allosteric transition. Enhancement reflects the extent of the allosteric transition and involves both tyrosine and tryptophan, probably in the region of the active site; quenching reflects occupation of the inhibitory site and involves tyrosine at the inhibitory site. 6. The mechanism of the allosteric transition from the R into the T conformation induced by Mg^{2+} -1, N^6 -etheno-ATP at low concentrations occurs predominantly by a 'prior-isomerization' pathway; at higher concentrations a limited contribution from a 'substrate-guided' pathway occurs. 7. The allosteric behaviour of phosphofructokinase with respect to Mg^{2+} -ATP and Mg^{2+} -1, N^6 -etheno-ATP binding may be accounted for in terms of the simple, concerted model.

In the previous paper (Roberts & Kellett, 1980), a detailed kinetic description was given of the mechanism of binding of Mg^{2+} -1, N^6 -etheno-ATP to the catalytic site of phosphofructokinase. In this paper we now look at the mechanism of the allosteric transition from the R conformation into the T conformation induced by the binding of Mg^{2+} -ATP or Mg^{2+} -1, N^6 -etheno-ATP to the inhibitory site. Information on the binding of these nucleotides from static-titration and transient-kinetic experiments is interpreted in terms of a simple,

concerted model for the allosteric transition. In addition experiments with other effectors show how the allosteric inhibition by Mg^{2+} -ATP is opposed by the activators cyclic AMP and fructose 1,6-bisphosphate and how it is potentiated by the inhibitor citrate.

Experimental

All materials and methods were as previously described (Roberts & Kellett, 1979, 1980). 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

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

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Fig. 1. Influence of effectors on the reaction of phosphofructokinase with Mg^{2+} -1, N^6 -etheno-ATP

Ordinate, enhancement of Mg^{2+} -1, N^6 -etheno-ATP fluorescence (mV); abscissa, time (ms). Conditions: 50 mM-phosphate (25 mM- KH_2PO_4 /25 mM- K_2HPO_4) buffer, pH 6.80, containing 0.2 mM-dithiothreitol at $19 \pm 1^\circ C$. In each case syringe 1 contained 25 μM -phosphofructokinase and syringe 2

way, no corrections for dilution of enzyme were required. All experiments were performed at $19 \pm 1^\circ C$ 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 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

Phosphofructokinase in 50 mM-phosphate buffer, pH 6.80, at $19^\circ C$ exists effectively entirely in the R conformation (Roberts & Kellett, 1979, 1980). Fig. 1 shows how the biphasic reaction of 25 μM -enzyme under these conditions with 80 μM - Mg^{2+} -1, N^6 -etheno-ATP is influenced by the presence in the Mg^{2+} -1, N^6 -etheno-ATP syringe of either 2 mM-citrate, 5 μM -cyclic AMP or 16 μM -fructose 1,6-bisphosphate. Note that in all reactions the enhancement of Mg^{2+} -1, N^6 -etheno-ATP fluorescence (λ excitation 285 nm) was recorded with the same oscilloscope settings, 10 mV and 50 ms per division respectively, with the exception of that in the presence of citrate which was recorded at 20 mV and 50 ms per division respectively. The influence of these effectors was studied over a wide range of Mg^{2+} -1, N^6 -etheno-ATP concentration. Their presence in the syringe containing Mg^{2+} -1, N^6 -etheno-ATP did not significantly affect the dependence of the amplitude of the fast phase on Mg^{2+} -1, N^6 -etheno-ATP concentration. Since only the catalytic site is involved in the fast phase, the amplitude dependence was analysed by a non-linear least-squares procedure for a single-site model as described previously (Roberts & Kellett, 1979). The dissociation constants determined from the fast phase in these experiments were computed to be $24.1 \pm 2.7 \mu M$ (no effectors), $21.4 \pm 3.9 \mu M$ (0.2 mM-citrate, after mixing), $15.0 \pm 2.4 \mu M$ (1 mM-citrate), $13.3 \pm 3.1 \mu M$ (8 μM -fructose 1,6-bisphosphate).

In contrast with the fast phase, the slow phase, which represents the allosteric transition from the R conformation into the T conformation, shows great

80 μM -1, N^6 -etheno-ATP + 10 mM- $MgCl_2$. Syringe 2 also contained the following additions: (a) no addition; (b) 2 mM-citrate; (c) 5.0 μM -cyclic AMP; (d) 16 μM -fructose 1,6-bisphosphate. Excitation wavelength was 285 nm. Observations were made through a combination of two filters, Kodak Wratten No. 35 and a Schott KVG 380 cut-off filter.

sensitivity of its amplitude to the presence of effectors in the syringe containing Mg^{2+} -1, N^6 -etheno-ATP (Fig. 2). The amplitude is decreased by the activators cyclic AMP and fructose 1,6-bisphosphate, even at sub-stoichiometric concentrations, 2.5 and $8\mu\text{M}$ respectively. However, the most pronounced effect is seen with the inhibitor citrate, when there is a dramatic increase in the amplitude of the slow phase, particularly at low Mg^{2+} -1, N^6 -etheno-ATP concentrations. Thus, at $10\mu\text{M}$ - Mg^{2+} -1, N^6 -etheno-ATP (after mixing), the amplitude is increased about 8.8-fold in the presence of 1 mM-citrate (Figs. 1a, 1b and 2). At higher concentrations of Mg^{2+} -1, N^6 -etheno-ATP, however, the amplitude of the slow phase actually decreases and appears to approach a value similar to that observed at saturating Mg^{2+} -1, N^6 -etheno-ATP concentrations in the absence of citrate.

In the above experiment, enzyme was titrated with Mg^{2+} -1, N^6 -etheno-ATP in the presence of a fixed concentration of citrate. The alternative experiment, in which $12.5\mu\text{M}$ -enzyme is titrated with citrate at a fixed concentration of Mg^{2+} -1, N^6 -etheno-ATP ($10\mu\text{M}$ after mixing) is shown in Fig. 3. It is again apparent that the amplitude of the fast phase is unaffected. In contrast, the amplitude of the slow phase increases markedly with increasing citrate concentration and is described by a rectangular hyperbola characterized by ΔF_{max} of

Fig. 3. Influence of citrate on the biphasic enhancement of Mg^{2+} -1, N^6 -etheno-ATP fluorescence

Ordinate, phase amplitude (mV); abscissa, total concentration (mM) of citrate after mixing. Syringe 1, $25\mu\text{M}$ -phosphofructokinase; syringe 2, $20\mu\text{M}$ -1, N^6 -etheno-ATP + 10 mM- MgCl_2 + citrate. O, Fast-phase amplitude, discontinuous line fitted by eye; ●, slow-phase amplitude, theoretical curve fitted to a rectangular hyperbola characterized by ΔF_{max} of 382 ± 11.8 mV and a citrate concentration at half-saturation of 0.7 ± 0.06 mM. Other conditions were as described for Fig. 1.

Fig. 2. Influence of effectors on the amplitude of the slow phase of Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement. Ordinate, slow-phase amplitude (mV); abscissa, total concentration (μM) of Mg^{2+} -1, N^6 -etheno-ATP after mixing. The conditions and experiments correspond exactly to those of Fig. 1. ●, No additions; ■, 1 mM-citrate; ○, $5.0\mu\text{M}$ -cyclic AMP; ▽, $8\mu\text{M}$ -fructose 1,6-bisphosphate. The effector concentrations are those after mixing.

382.0 ± 11.8 mV and a half-saturation concentration of 0.7 ± 0.06 mM-citrate.

The dependence of the apparent first-order rate constant of the fast phase, k_f , on Mg^{2+} -1, N^6 -etheno-ATP concentration has the same general form whether or not the syringe containing Mg^{2+} -1, N^6 -etheno-ATP also contains effectors (results not shown). Although there are differences in detail, there is no obvious pattern: in each case, k_f decreases monotonically with increasing Mg^{2+} -1, N^6 -etheno-ATP concentration to an asymptotic limit, as reported in the preceding paper (Roberts & Kellett, 1980). This dependence is distinct from that

of the apparent first-order rate constant of the slow-phase, k_s ; in the absence of effectors, the latter shows a minimum (Fig. 4a). The effect of 1 mM-citrate is not to alter the form of the dependence, but rather to move the whole curve to lower Mg^{2+} -1, N^6 -etheno-ATP concentrations. Cyclic AMP (2.5 μM) has little effect on the position of the curve, although k_s is somewhat decreased at low concentrations of Mg^{2+} -1, N^6 -etheno-ATP, so that the minimum is less pronounced. In the presence of 8 μM -fructose 1,6-bisphosphate, the values of k_s are up to about 4-fold greater, and no minimum is now observed (Fig. 4b). If the curve is simply expanded

Fig. 4. Influence of effectors on k_s for Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement. Ordinate, k_s (s⁻¹); abscissa, total concentration (μM) of Mg^{2+} -1, N^6 -etheno-ATP after mixing. The conditions and experiments correspond exactly to those of Fig. 2. (a) ●, No additions; ■, 1 mM-citrate; ○, 5.0 μM-cyclic AMP. (b) □, 8 μM-Fructose 1,6-bisphosphate. Error bars have been omitted from (a) for clarity. Each point is the mean for six determinations and the mean S.E.M. is ± 0.09 s⁻¹.

Table 1. Influence of effectors on the reaction of Mg^{2+} -1, N^6 -etheno-ATP with 25 μ M-phosphofructokinase when enzyme is predialysed against saturating concentrations of the effectors

Addition to dialysis medium	[Mg^{2+} -1, N^6 -etheno-ATP] (μ M) ...	Proportion of phase of reaction (%)			
		30		100	
		Fast	Slow	Fast	Slow
No addition		55	45	50	50
Cyclic AMP (50 μ M)		100	0	100	0
Fructose 1,6-bisphosphate (50 μ M)		100	0	80	20

along the concentration axis, it may be that at higher Mg^{2+} -1, N^6 -etheno-ATP concentrations k_s would increase again. Unfortunately this concentration range was not accessible.

In the previous experiments, effectors were included in the syringe containing Mg^{2+} -1, N^6 -etheno-ATP. The complementary experiments are those in which enzyme is preincubated with effectors before reaction with Mg^{2+} -1, N^6 -etheno-ATP. Unfortunately, it is not possible to obtain a complete picture of how effectors in these experiments influence the initial state of the enzyme and the extent of the allosteric transition as determined from the relative percentages of the slow and fast phases. This is because k_f increases markedly with decreasing Mg^{2+} -1, N^6 -etheno-ATP concentration, becoming so great in the presence of the activators cyclic AMP and fructose 1,6-bisphosphate that a large proportion of the fast phase is lost in the dead-time of the apparatus (Roberts & Kellett, 1980). As a consequence, preincubation experiments with these effectors are reported here only for a limited set of conditions, namely high Mg^{2+} -1, N^6 -etheno-ATP and saturating effector concentrations, from which useful information can be readily obtained. Table 1 compares the effect of first dialysing the enzyme against either 50 μ M-cyclic AMP or 50 μ M-fructose 1,6-bisphosphate before reaction with 30 μ M- or 100 μ M- Mg^{2+} -1, N^6 -etheno-ATP. It is apparent that both cyclic AMP and fructose 1,6-bisphosphate stabilize an R-type conformation, since in both cases the slow phase is either abolished or vastly decreased. The most striking effect of preincubation is seen in the case of citrate, however, especially at low concentrations of Mg^{2+} -1, N^6 -etheno-ATP. Thus, when 25 μ M-enzyme is mixed with 30 μ M- Mg^{2+} -1, N^6 -etheno-ATP + 2 mM-citrate, the proportions of the fast and slow phases are 24 ± 0.7 and $76 \pm 0.7\%$ respectively, with corresponding rate constants of 124 ± 13.3 and 2.6 ± 0.03 s⁻¹ (Fig. 5a). When in comparison the experiment is repeated with enzyme predialysed against 2 mM-citrate, the reaction is no longer biphasic. Instead, three phases are observed with rate constants 121 ± 5.1 s⁻¹ (k_1), 23.0 ± 1.81 s⁻¹ (k_2) and 1.3 ± 0.4 s⁻¹ (k_3). Their corresponding

Fig. 5. Effect of preincubation with citrate on the reaction of phosphofructokinase with Mg^{2+} -1, N^6 -etheno-ATP

Ordinate, Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement (mV); abscissa, time (ms). (a) Syringe 1, 25 μ M-phosphofructokinase; syringe 2, 30 μ M-1, N^6 -etheno-ATP + 12 mM-MgCl₂ + 2 mM-citrate. (b) Syringe 1, 25 μ M-phosphofructokinase previously dialysed against 1 mM-citrate; syringe 2, 30 μ M-1, N^6 -etheno-ATP + 12 mM-MgCl₂ + 1 mM-citrate. The arrows indicate the point at which flow is stopped. Other conditions were as described for Fig. 1.

amplitudes as a percentage of the total change were 47, 30 and 23 respectively.

Since preincubation experiments give only limited information about the way in which effectors alone influence the conformational state of the enzyme, further information on this point was obtained by studying the binding of effectors directly by monitoring the concomitant changes in the intrinsic protein fluorescence of phosphofructokinase. The activators cyclic AMP and fructose 1,6-bisphosphate cause quenching of the intrinsic fluorescence in the absence of nucleotide. With saturating cyclic AMP, quenching occurs to the extent of about 7% in a first-order process having a rate constant of $18 \pm 1.3 \text{ s}^{-1}$ (Fig. 6a), whereas with saturating fructose 1,6-bisphosphate about 14% quenching occurs at a rate constant of $147 \pm 12.6 \text{ s}^{-1}$ (Fig. 6b). In contrast, the inhibitor citrate at a concentration of 1 mM causes about 4% enhancement overall in a biphasic process with rate constants of $13.7 \pm 1.68 \text{ s}^{-1}$ and $1.5 \pm 0.20 \text{ s}^{-1}$.

Unlike the other effectors, Mg^{2+} -ATP exerts a complex effect on protein fluorescence, causing enhancement at low concentrations and quenching at high concentrations (Liou & Anderson, 1978). The static titrations shown in Fig. 7, corrected as described previously (Roberts & Kellett, 1979), confirm their observations and show further that the extent of enhancement, and especially of quenching, is strongly dependent upon the excitation wavelength at which measurements are made. Thus at 280, 285 and 298 nm, the extents of enhancement at $10 \mu\text{M}$ - Mg^{2+} -ATP are approx. 3.1, 5.0 and 3.7% respectively, whereas the extents of quenching at $100 \mu\text{M}$ - Mg^{2+} -ATP are approx. 11.7, 4.6 and 1.8% respectively. Also shown in Fig. 7 is the fact that cyclic AMP abolishes both the enhancement and the quenching of the fluorescence changes induced by Mg^{2+} -ATP. As noted previously, in stopped-flow measurements, where it is not necessary to make corrections for inner-filter effects, no changes in protein fluorescence induced by Mg^{2+} -ATP were detectable in the presence of cyclic AMP (Roberts & Kellett, 1979). The very small quenching, up to 0.7%, shown in the static titration with cyclic AMP present probably reflects the considerable difficulty of making precisely the large corrections necessary for static titrations.

Stopped-flow measurements at 285 nm are in satisfactory agreement with the corresponding static titration of the enzyme by Mg^{2+} -ATP alone, when allowance is made for the fact that the slit width of the exciting monochromator is 10 nm for the stopped-flow fluorimeter and 2 nm for the static fluorimeter (Fig. 8a). The equivalent titrations with Mg^{2+} -1, N^6 -etheno-ATP have not been performed, because the changes in protein fluorescence are too small to be readily measurable (Roberts & Kellett,

Fig. 6. Conformational transitions of phosphofructokinase induced by effectors alone

Ordinate, intrinsic protein fluorescence (mV); abscissa, time (ms). In each case, syringe 1 contained $25 \mu\text{M}$ -phosphofructokinase. Syringe 2 contained: (a) $100 \mu\text{M}$ -cyclic AMP; (b) $100 \mu\text{M}$ -fructose 1,6-bisphosphate; (c) 2 mM-citrate. The excitation wavelength was 285 nm and fluorescence was observed by using a Schott UV-line filter centred on 329 nm. The arrows indicate the point at which flow is stopped. Other conditions were as described for Fig. 1.

Fig. 7. Static fluorescence titration of phosphofructokinase by Mg^{2+} -ATP

Ordinate, percentage change in intrinsic protein fluorescence; abscissa, total concentration (μM) of Mg^{2+} -ATP in the cuvette. The cuvette contained $12.5 \mu M$ -phosphofructokinase and $[Mg^{2+}]$ was maintained at a constant excess of 5 mM over ATP. Enzyme in the presence of phosphate alone, excited at (●) 280 nm , (▲) 285 nm and (○) 298 nm . □, Enzyme in the presence of $50 \mu M$ -cyclic AMP excited at 285 nm . Observation wavelength, 329 nm . Other conditions were as described for Fig. 1.

1979). The dependence of k_s upon ATP concentration for protein fluorescence changes is illustrated in Fig. 8(b). Unfortunately these data are subject to relatively large errors because of the very small amplitudes, especially at high concentrations, and the consequently poor signal-to-noise ratio.

Discussion

Influence of effectors on the binding of Mg^{2+} -1, N^6 -etheno-ATP to phosphofructokinase

When both enzyme and nucleotide are excited at 285 nm , the time-course of Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement is biphasic. As shown previously (Roberts & Kellett, 1979, 1980) the slow phase represents the allosteric transition from the R conformation into the T conformation induced by binding of Mg^{2+} -1, N^6 -etheno-ATP to the inhibitory site. The fluorescence signal which allows the allosteric transition to be followed does not arise from Mg^{2+} -1, N^6 -etheno-ATP bound at the inhibitory site, but from that bound at the catalytic site as a consequence of changes resulting from the allosteric transition.

Under the conditions of the experiment (50 mM -phosphate buffer, $\text{pH } 6.80$) phosphofructokinase initially exists effectively only in the R conformation (Wolfman *et al.*, 1978; Roberts & Kellett, 1979, 1980) and the extent of the allosteric transition

depends on the concentration of Mg^{2+} -1, N^6 -etheno-ATP and the presence or absence of other effectors. Preincubation of enzyme with saturating concentrations of the activators, cyclic AMP or fructose 1,6-bisphosphate, shows that they both stabilize an R-type conformation since the slow phase is either totally abolished or vastly decreased (Table 1). This interpretation is confirmed by complementary experiments in which enzyme was reacted with Mg^{2+} -1, N^6 -etheno-ATP plus sub-stoichiometric concentrations of the activators. As expected, since Mg^{2+} -1, N^6 -etheno-ATP stabilizes the T conformation, the amplitude of the slow phase was decreased by this inclusion (Fig. 2). Moreover, the amplitude of the slow phase was increased by the inhibitor citrate. The dependence of the amplitude of the slow phase in the presence of activators on Mg^{2+} -1, N^6 -etheno-ATP concentration is sigmoidal, similar to that in the absence of effectors. However, for citrate the dependence is complex and will be discussed in more detail below.

In preincubation experiments, the amplitude or nature of the fast-phase binding reaction to the catalytic site is changed by effectors when compared with that seen with phosphate alone. For example, as noted earlier in the Results section, it is no longer possible to measure the amplitude of the fast phase directly at low concentrations of Mg^{2+} -1, N^6 -etheno-ATP for enzyme in the presence of

Fig. 8. Reaction of phosphofructokinase with Mg^{2+} -ATP. Syringe 1, 25 μM -phosphofructokinase; syringe 2, ATP + Mg^{2+} at a constant 10 mM excess. (a) Dependence of reaction amplitude on ATP concentration: ordinate, percentage enhancement of intrinsic protein fluorescence; abscissa, total ATP concentration (μM) after mixing. (b) Dependence of the rate constant on ATP concentration: conditions were as described for (a). Excitation wavelength 285 nm; observations were made by using a Schott UV-line filter centred on 329 nm. Other conditions were as described for Fig. 1.

activators, because k_f becomes so great that a large proportion of the fast phase is lost in the dead-time of the apparatus (see also Roberts & Kellett, 1980). Similarly, for citrate, preincubation increases the amplitude of the fast phase (Fig. 5) although the interpretation is altogether more complicated (see below). In contrast with the preincubation experi-

ments, when effectors are included in the Mg^{2+} -1, N^6 -etheno-ATP syringe, the amplitude of the fast phase is not affected and the measured dissociation constant for Mg^{2+} -1, N^6 -etheno-ATP from the catalytic site are the same within experimental error as that for enzyme in the presence of phosphate alone. The fact that in these experiments the amplitude of the slow phase is affected by the presence of effectors in the Mg^{2+} -1, N^6 -etheno-ATP syringe, although the fast phase is not, implies that the conformational changes induced by the binding of the different effectors are slow compared with the fast-phase binding reaction to the catalytic site, but fast compared with the slow-phase allosteric transition from the R into the T conformation induced by Mg^{2+} -1, N^6 -etheno-ATP. Direct observation of the fluorescence changes (Fig. 6), which accompany the conformational transitions on binding, shows that this is indeed the case for saturating cyclic AMP ($k = 18.0 \pm 1.3 s^{-1}$) and citrate ($k_1 = 13.7 \pm 1.68 s^{-1}$ and $k_2 = 1.5 \pm 0.20 s^{-1}$). The transition induced by saturating fructose 1,6-bisphosphate occurs somewhat faster, and the rate constant ($k = 147 \pm 12.6 s^{-1}$) is actually comparable with the lower limit of k_f observed at high Mg^{2+} -1, N^6 -etheno-ATP concentrations. The fast phase of Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement in the above experiments therefore represents the same reaction regardless of the effector in the Mg^{2+} -1, N^6 -etheno-ATP syringe; that is, the binding of Mg^{2+} -1, N^6 -etheno-ATP to the catalytic site in the R conformation stabilized by phosphate, since the effectors change the conformation of the enzyme at a rate slower than the binding of Mg^{2+} -1, N^6 -etheno-ATP to the catalytic site. In preincubation experiments with effectors, the enzyme is of course able to change conformation in response to the binding of effector before the reaction with Mg^{2+} -1, N^6 -etheno-ATP, so that the fast phase reaction is modified as described above. As a consequence, the information obtained from preincubation experiments is limited.

The preincubation experiments do nevertheless yield further information about the relative influence of the activators on phosphofructokinase. Thus, although cyclic AMP and fructose 1,6-bisphosphate are both powerful activators of the enzyme and stabilize an R-type conformation, they also appear to behave distinctly. Thus when the enzyme is predialysed against 50 μM -fructose 1,6-bisphosphate the slow phase in the reaction of 12.5 μM -phosphofructokinase with 30 μM - Mg^{2+} -1, N^6 -etheno-ATP is completely abolished, whereas it is only decreased with 100 μM - Mg^{2+} -1, N^6 -etheno-ATP. Hence Mg^{2+} -1, N^6 -etheno-ATP at high concentrations is able to overcome the effect of saturating fructose 1,6-bisphosphate. This is not the case for saturating cyclic AMP; the slow phase remains abolished at 100 μM - Mg^{2+} -1, N^6 -etheno-ATP. Indeed, even

1250 μM - Mg^{2+} -ATP is unable to induce the allosteric transition from the R conformation into the T conformation with enzyme dialysed against 50 μM -cyclic AMP (Roberts & Kellett, 1980). This is true also for 5'-AMP (data not shown). These observations with fructose 1,6-bisphosphate and cyclic AMP correlate well with the equilibrium dialysis experiments of Wolfman *et al.* (1978) on the binding of [β -imido]ATP. They found that nucleotide could occupy the 'loose' inhibitory site in the presence of saturating fructose 1,6-bisphosphate, but not in the presence of saturating cyclic AMP.

Binding of Mg^{2+} -ATP

Mg^{2+} -ATP causes both enhancement and quenching of protein fluorescence (Liou & Anderson, 1978). Since enhancement occurs at low Mg^{2+} -ATP concentrations and quenching at high concentrations (Fig. 7), a simple explanation is that enhancement represents binding directly to the high-affinity catalytic site and quenching represents binding directly to the low-affinity inhibitory site (Pettigrew & Frieden, 1979). However, within the Mg^{2+} -ATP concentration range of the present experiments, both enhancement and quenching are abolished either when the enzyme is saturated with cyclic AMP or when the enzyme is at pH 8. (Fig. 7; see also Roberts & Kellett, 1979; Pettigrew & Frieden, 1979). In both of these conditions phosphofructokinase is unable to undergo the allosteric transition from the R conformation into the T conformation as judged by (i) the availability only of the high-affinity catalytic site either at pH 7 in the presence of cyclic AMP (Wolfman *et al.*, 1978; Roberts & Kellett, 1979) or at pH 8 alone (Pettigrew & Frieden, 1979); (ii) the observation only of simple Michaelis-Menten kinetics (Bloxham & Lardy, 1973); (iii) the abolition of the slow phase of Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement which results from the allosteric transition when phosphofructokinase is mixed with Mg^{2+} -1, N^6 -etheno-ATP (Roberts & Kellett, 1979). It follows then that both the enhancement and the quenching of protein fluorescence caused by Mg^{2+} -ATP must arise as a consequence of binding to the inhibitory site and the ensuing allosteric transition. Thus enhancement does not result from binding to the catalytic site directly. Support for this conclusion is provided by the observation (Fig. 8*b*) that the protein fluorescence changes induced by Mg^{2+} -ATP, both enhancement and quenching, occur at a rate that is not only very much slower than a diffusion-controlled encounter reaction, but also that is much slower even than the two-step binding reaction of nucleotide to the catalytic site (Fig. 8*b*; see also Roberts & Kellett, 1979, 1980). The activators cyclic AMP, fructose 6-phosphate and fructose 1,6-bisphosphate stabilize an R-type con-

formation and cause quenching of protein fluorescence (Fig. 2; see also Hill & Hammes, 1975; Pettigrew & Frieden, 1979; Roberts & Kellett, 1979, 1980). The inhibitor citrate, on the other hand, stabilizes a T-type conformation and causes enhancement of protein fluorescence (Fig. 6). In general, then, a T-type conformation has enhanced protein fluorescence compared with an R-type conformation. However, for Mg^{2+} -ATP there is an additional complication, for quenching occurs at high Mg^{2+} -ATP concentrations because of increased occupation of the inhibitory site. Hence there is a distinction between the fluorescence quenching, which reflects the extent of the occupation of the inhibitory site by Mg^{2+} -ATP and is a measure of the 'binding function' to that site, and the fluorescence enhancement, which measures the extent of the transition of the R conformation into the T conformation induced as a consequence of Mg^{2+} -ATP binding to the inhibitory site, and is a measure of the 'conformational-state function' in the terminology of Monod *et al.* (1965).

To explain the dependence of enhancement and quenching on Mg^{2+} -ATP concentration, it is necessary to propose that the allosteric transition induced by Mg^{2+} -ATP is at least partially concerted, so that at low concentrations of Mg^{2+} -ATP the change in conformational-state function precedes that of the binding function; subsequent occupation of the remaining vacant inhibitory sites in the T state at high Mg^{2+} -ATP concentrations results in quenching as the binding function approaches the conformational-state function (Roberts & Kellett, 1979). Goldhammer & Hammes (1978) have shown that the steady-state kinetic properties of phosphofructokinase at pH 7.0 are consistent with the fully-concerted allosteric model of Monod *et al.* (1965). In addition, the dependence of k_s upon Mg^{2+} -1, N^6 -etheno-ATP concentration provides evidence that at low concentrations of nucleotide the allosteric transition can precede binding in the kinetic pathway (see below).

Although enhancement and quenching both arise as a consequence of the binding of nucleotide to the inhibitory site, the origins of their fluorescence changes are distinct; enhancement and quenching show different dependences on excitation wavelength (Fig. 7). Both tyrosine and tryptophan contribute to enhancement, an observation that is consistent with the fact that these residues contribute also to the excitation spectrum of the slow phase of Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement caused by energy transfer from protein to nucleotide (Roberts & Kellett, 1979). As reported previously, the latter signal arises from Mg^{2+} -1, N^6 -etheno-ATP bound at the catalytic site, but does so as a consequence of changes in the environment of that site caused by the allosteric

transition. The major, and possibly sole, contributor to quenching is tyrosine. Circumstantial evidence for the location of tyrosine at the inhibitory ATP site is provided by the acetylation studies of Chapman *et al.* (1969). Presumably then it is this tyrosine residue(s) whose fluorescence is quenched on occupation of the inhibitory site by Mg^{2+} -ATP.

One puzzling feature of our earlier study on Mg^{2+} -1, N^6 -etheno-ATP binding (Roberts & Kellett, 1979) was that Mg^{2+} -ATP displacement experiments did not reveal any signal change at the inhibitory site, that is, there was no detectable energy transfer to nucleotide at this site. The simple explanation would be that the orientation of donor and acceptor are incompatible. In view of the above results, however, an alternative explanation might be that the binding of Mg^{2+} -1, N^6 -etheno-ATP causes additional quenching of the tyrosine at the inhibitory site over and above that by energy transfer, and that this additional quenching compensates for increased Mg^{2+} -1, N^6 -etheno-ATP fluorescence caused by energy transfer. The protein fluorescence changes induced by Mg^{2+} -1, N^6 -etheno-ATP are very small, so that we have not been able to investigate the matter further. In so far as enhancement of Mg^{2+} -1, N^6 -etheno-ATP fluorescence reflects enhancement of protein fluorescence, no quenching occurs up to $100\text{ }\mu\text{M}$ - Mg^{2+} -1, N^6 -etheno-ATP, in the absence of citrate (Fig. 2).

Binding of citrate

The effect of citrate is to increase the amplitude of the slow phase very markedly, especially at low concentrations of Mg^{2+} -1, N^6 -etheno-ATP (Figs. 2 and 3). It is known that, at low concentrations of ATP, citrate actually increases the affinity of the catalytic site for Mg^{2+} -ATP (Randle *et al.*, 1968; Columbo *et al.*, 1975). The large increase in Mg^{2+} -1, N^6 -etheno-ATP fluorescence at low concentrations therefore possibly reflects some increased binding of Mg^{2+} -1, N^6 -etheno-ATP at the catalytic site. However, this is unlikely to be the principal explanation for the increase in amplitude, because, at about $20\text{ }\mu\text{M}$ - Mg^{2+} -1, N^6 -etheno-ATP, the fluorescence amplitude exceeds that at higher concentrations. Most of the amplitude increase must therefore result from an increase in energy transfer compared with that in the absence of citrate. Citrate alone, of course, promotes a T-type conformation with enhanced protein fluorescence (Fig. 6c). Moreover, citrate inhibits phosphofructokinase synergistically with Mg^{2+} -ATP (Columbo *et al.*, 1975). In terms of the explanation advanced above for Mg^{2+} -ATP, therefore, the increase in amplitude at lower concentrations of Mg^{2+} -1, N^6 -etheno-ATP is caused by an increase in energy transfer resulting from increased protein fluorescence in the region of the catalytic site. The latter is the consequence of a

much greater change in the extent of the allosteric transition from the R conformation into the T conformation at a given concentration of Mg^{2+} -1, N^6 -etheno-ATP because of the synergism between citrate and Mg^{2+} -1, N^6 -etheno-ATP. Interestingly, at higher concentrations, the fluorescence becomes slightly quenched. The parallel with the effects of Mg^{2+} -ATP alone on protein fluorescence is apparent.

The enhancement of protein fluorescence that accompanies the conformational change induced by saturating citrate concentrations occurs in a biphasic process characterized by rate constants of $13.7 \pm 1.68\text{ s}^{-1}$ and $1.5 \pm 0.20\text{ s}^{-1}$. We have not investigated in detail precisely what these two phases represent. For present purposes, it is sufficient to say that both these constants are much faster than that for the citrate-induced dissociation of phosphofructokinase tetramer to dimer, $4.6 \times 10^{-2}\text{ s}^{-1}$, under these conditions (Liddle *et al.*, 1977). Thus conformational change precedes dissociation. Moreover, since k_s for Mg^{2+} -1, N^6 -etheno-ATP fluorescence enhancement in the presence of 1 mM -citrate ranges from about 6 s^{-1} to 3 s^{-1} (Fig. 4a), the dissociation reaction is not involved in the slow phase in any way.

However, dissociation of tetramer to dimer is an important consideration in the interpretation of experiments in which enzyme has been preincubated with citrate. In these circumstances, the initial enzyme form is inactive dimer, which can be regarded as existing in a T-type conformation (Goldhammer & Hammes, 1978). Hence the fast phase at $15\text{ }\mu\text{M}$ - Mg^{2+} -1, N^6 -etheno-ATP in Fig. 5(b) ($k_1 = 121 \pm 5.1\text{ s}^{-1}$) presumably represents binding to the catalytic site of this T-type dimer; it does not reflect binding to a tetramer or higher polymer in an R-type conformation as in all other experiments described in this paper. The progress curve for the T-type dimer is in fact triphasic, and more experimentation is needed to resolve this problem.

Mechanism of the allosteric transition

The analysis of the dependence of k_s on Mg^{2+} -1, N^6 -etheno-ATP concentration is potentially very complex, since it concerns the allosteric transition of a tetrameric protein, both the catalytic and inhibitory Mg^{2+} -ATP sites can be occupied at the same time, and the observed signal originates only from the catalytic site. It is therefore not appropriate to make other than very tentative and rather simple comments about the mechanism involved.

The allosteric transition induced by Mg^{2+} -1, N^6 -etheno-ATP binding to the inhibitory site could occur either by a 'prior-isomerization' or by a 'substrate-guided' pathway (Koshland & Neet, 1968). In fact, it appears that both these pathways are involved. In the present experiments, phos-

phosphofructokinase initially exists effectively only in the R conformation because of the effect of phosphate (Roberts & Kellett, 1979, 1980). However, within the limits of experimental error, analysis of the fast phase amplitude dependence on Mg^{2+} -1, N^6 -etheno-ATP concentration has shown that only a single binding site, the catalytic site, is effectively available to Mg^{2+} -1, N^6 -etheno-ATP at low concentrations in this conformation. The affinity of Mg^{2+} -1, N^6 -etheno-ATP for the inhibitory site is far greater in the T conformation; indeed, binding to this site in the R conformation is sufficiently weak that it could not be detected by analysis of the amplitudes of reaction within the experimental concentration range, up to $100\mu\text{M}$ (Roberts & Kellett, 1979). The transition of the R conformation into the T conformation must therefore occur on the kinetic pathway before the binding of Mg^{2+} -1, N^6 -etheno-ATP to the inhibitory site, at least when occupancy of the latter is low. Indeed, at low concentrations of Mg^{2+} -1, N^6 -etheno-ATP, k_s decreases with increasing concentration in a manner consistent with a 'prior-isomerization' pathway (Fig. 4a; see also Roberts & Kellett, 1980).

At higher concentrations, however, k_s then begins to increase with increasing concentration, suggesting that, in addition to the 'prior-isomerization' pathway, a 'substrate-guided' pathway may now also be beginning to contribute, so that the dependence of k_s upon Mg^{2+} -1, N^6 -etheno-ATP concentration shows a minimum. The value of k_s at $100\mu\text{M}$ - Mg^{2+} -1, N^6 -etheno-ATP is only about 3s^{-1} and the continuing upward trend shows that saturation of k_s has not been achieved; indeed, a recent single measurement has shown that, at a concentration of 1mM - Mg^{2+} -ATP, the apparent first-order rate constant of the allosteric transition is approx. 140s^{-1} . Hence, at the low concentrations used in the present experiments, the 'substrate-guided' pathway is likely to be making only a limited contribution to the binding of the early molecules (first or second) of nucleotide to the inhibitory site. This explains why binding at this site was not detected in the analysis of the fast-phase amplitudes.

The above conclusions are consistent with the equilibrium measurements (Fig. 7), which show that at very low concentrations of Mg^{2+} -ATP the extent of the allosteric transition (protein fluorescence

enhancement) precedes binding to the inhibitory site (quenching). Thus there is an appreciable population of tetrameric molecules of the type T_4L_1 and T_4L_2 with nucleotide, L, bound at the inhibitory site.

Goldhammer & Hammes (1978) have previously shown that the steady-state kinetics of Mg^{2+} -ATP utilization by phosphofructokinase can be explained satisfactorily in terms of the model of Monod *et al.* (1965). The above experiments show further that this simple, concerted, model can account also for the allosteric behaviour of phosphofructokinase with respect to the binding of Mg^{2+} -ATP and Mg^{2+} -1, N^6 -etheno-ATP.

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