Effect of Substrates and Effectors on the Reversible Inactivation of Pig Spleen Phosphofructokinase by Adenosine Triphosphate

By MAURICE J. WEIDEMANN,* MARYSIA E. KOLBUCH-BRADDON and PETER E. HICKMAN†

*Department of Biochemistry, Faculty of Science, Australian National University,
Canberra, A.C.T. 2600, Australia*

(Received 17 December 1976)

1. To investigate the mechanism of the reversible inactivation of pig spleen phosphofructokinase by ATP, the effect of order of addition of reactants (substrates, effectors and enzyme solution) was studied by preincubating the enzyme before assay with various combinations of its substrates and effectors. 2. Preincubation of the enzyme with MgATP or ATP at pH 7.0 before addition of fructose 6-phosphate caused a rapid and much greater inhibition of activity than that observed when the reaction (carried out at identical substrate concentrations) was initiated with enzyme. 3. The rapid inhibition caused by preincubation with ATP, together with the sigmoidal response to fructose 6-phosphate and activation by AMP, were all blocked by prior photo-oxidation of the enzyme with Methylene Blue, which selectively destroys the inhibitory binding site for ATP [Ahlfors & Mansour (1969) *J. Biol. Chem.* **244**, 1247–1251]. 4. Fructose 6-phosphate, but not Mg^{2+} , protected phosphofructokinase from inhibition during preincubation with ATP in a manner that was sigmoidally dependent on the fructose 6-phosphate concentration. 5. Mg^{2+} , by protecting the enzyme from the inhibitory effect of preincubation at low pH (7.0) and by preventing its activation during preincubation with fructose 6-phosphate, demonstrated both a weak activating effect in the absence of the other substrates and a stronger inhibitory effect in the presence of fructose 6-phosphate. 6. Positive effectors (K^+ , NH_4^+ , AMP and aspartate) protected the enzyme from inhibition during preincubation with MgATP in proportion to their potency as activators, but citrate potentiated the ATP inhibition. P_i significantly slowed the inactivation process without itself acting as a positive effector. 7. The non-linear dependence of the initial rate of the unmodified enzyme on protein concentration (associated with increased positive homotropic co-operativity to fructose 6-phosphate) was intensified by preincubation with ATP and abolished by photo-oxidation. 8. The results are interpreted in terms of an association–dissociation model which postulates that protonation, at low pH, of a photo-oxidation-sensitive inhibitory site for ATP allows more rapid dissociation of an active tetramer to an inactive dimeric species.

In a previous paper we have reported (Hickman & Weidemann, 1975) that pig spleen phosphofructokinase (ATP–D-fructose 6-phosphate 1-phosphotransferase; EC 2.7.1.11) possesses regulatory properties consistent with its fulfilling a rate-controlling role in lymphoid tissue similar to that found in other mammalian tissues (Bloxham & Lardy, 1973). Most prominent amongst its potentially significant effector molecules are AMP, P_i , K^+ , NH_4^+ and

aspartate, as activators, and ATP and citrate as inhibitors.

A factor which has complicated the examination of the regulatory properties of phosphofructokinase is the effect evoked by changing the order of addition of substrates, effectors and enzyme solution to the cuvette, which can cause profound differences in the final activity observed. For example, Ramaiah & Tejwani (1970) found that a hyperbolic curve relating activity to fructose 6-phosphate concentration was obtained when the reaction was initiated by addition of enzyme, but a sigmoidal curve was obtained if the reaction was initiated by addition of fructose 6-phosphate to an incubation medium already containing diluted enzyme in the presence of MgATP. Colombo *et al.* (1975) have reported a long lag in the

* To whom reprint requests should be addressed.
Present address: Max Planck Institut für Immunbiologie,
78 Freiburg-Zähringen, Stübeweg 51, Federal Republic
of Germany.

† Present address: Department of Pharmacology,
Queen Elizabeth II Medical Centre, Nedlands, Western
Australia 6009, Australia.

achievement of a steady rate when the rabbit muscle enzyme was preincubated with MgATP in the presence of citrate before the reaction was initiated with fructose 6-phosphate. Furthermore work with the rabbit muscle enzyme has indicated that the establishment of very low steady-state concentrations of the product (and activator) fructose 1,6-bisphosphate during the coupled assay with aldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase at pH 6.5 may give rise to rates that are non-linear with respect to time and enzyme concentration (Emerk & Frieden, 1975).

During experiments designed to investigate the effect of changing the order of addition of the reactants on the kinetic behaviour of pig spleen phosphofructokinase, which is not activated by fructose 1,6-bisphosphate (Hickman & Weidemann, 1975), we found that the enzyme was rapidly inactivated when preincubated for less than 1 min with ATP. In the present paper we examine the mechanism by which ATP inactivates pig spleen phosphofructokinase; we also demonstrate that other substrates and effectors can influence the rate at which this inactivation occurs, and discuss the results in relation to published work.

Experimental

Materials

All nucleotides, fine chemicals and coupling enzymes were obtained from the sources described previously (Hickman & Weidemann, 1975). Methylene Blue was purchased from British Drug Houses, Poole, Dorset, U.K., and was used without further purification. Pig spleen phosphofructokinase, purified as far as the 'wash' stage of Massey & Deal (1973), was prepared, dialysed and stored in 80 mM-potassium phosphate buffer (pH 8.0) as described previously (Hickman & Weidemann, 1975). Because of the 'aging' effect of storage (described below), which gradually decreases the potency of the reversible ATP inhibition of the partially purified preparation, only freshly prepared batches of enzyme, stored for no more than 7 days at 4°C, were used in the present work.

Methods

Enzyme assay. Phosphofructokinase activity was assayed at 23°C by following NADH oxidation at 340 nm in the presence of a 10-fold excess of aldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase activities in the complete reaction mixture, buffered at pH 7.0 with 50 mM-Hepes*/NaOH, described previously (Hickman & Weidemann, 1975). The total capacity of the coupled assay system was tested as a routine by addition of 0.5 mM-

fructose 1,6-bisphosphate. The phosphofructokinase activities reported in this paper were independent of the concentrations of the coupling enzymes, providing the 10-fold excess was maintained. Assays of enzyme activity were conducted in one of two ways: (i) without preincubation, in which the reaction was initiated by adding enzyme solution (approx. 30 µg of protein in 0.05 ml of 80 mM-potassium phosphate buffer, pH 8.0) to cuvettes already containing the complete reaction mixture (Hickman & Weidemann, 1975); or (ii) after various periods of preincubation, in which phosphofructokinase was preincubated at 23°C with combinations of substrate(s) and effectors for the time intervals described in the text before the reaction was initiated by addition of the substrate(s) omitted from the cuvette during the preincubation period. As the preincubation conditions constituted the basic variable in the experimental design, details of the individual experiments are given in the text and in the legends to the appropriate Figures and Tables.

Reactions initiated by addition of enzyme always became linear with respect to time after a very brief initial lag. When phosphofructokinase was preincubated with ATP, however, the reaction rates were not linear and a slow increase in activity was observed throughout the assay period (Fig. 1). The initial velocities were estimated in each case by fitting A_{340} readings obtained during the first 6 min of the assay to a quadratic equation by the method of least squares and using the tangent obtained from the equation at zero time as the initial rate.

Photo-oxidation of phosphofructokinase. Photo-oxidation of pig spleen phosphofructokinase was carried out as described by Ahlfors & Mansour (1969) for the sheep heart enzyme. Enzyme solution (approx. 1.5 mg of protein in 1.0 ml of 80 mM-potassium phosphate buffer, pH 8.0) containing 2.7 µM-Methylene Blue and cooled to 0°C in a small conical glass test tube was placed 19 cm beneath a 250 W reflector bulb and illuminated for periods of up to 36 min while undergoing gentle shaking in a water bath at 0°C. A stream of cool air was blown across the mouth of the tube to prevent excessive heating. The unmodified 'non-photo-oxidized' enzyme, which served as a control in the experiments described, was treated with the same concentration of Methylene Blue and was subjected to the same manipulations as the photo-oxidized sample, but with the lamp switched off. The tube containing the control sample was wrapped in aluminium foil to prevent exposure of the enzyme to background illumination.

Results and Discussion

Effect of photo-oxidation on loss of activity during preincubation with ATP

Fig. 1 contrasts the traces of A_{340} changes versus time obtained when the coupled phosphofructokinase

* Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Fig. 1. Spectrophotometric traces of pig spleen phosphofructokinase activity before and after 40s preincubation with MgATP

Unmodified (a) or 36min-photo-oxidized (b) pig spleen phosphofructokinase activity was measured as a fall in A_{340} under the following conditions: (i) when the assay was initiated by adding enzyme (30 μ g) to cuvettes already containing fructose 6-phosphate (0.2mM), ATP (0.6mM), $MgCl_2$ (3.6 mM) and the coupled assay system buffered at pH7.0 described in the text; and (ii) after preincubation with ATP (0.6mM) and $MgCl_2$ (3.6mM) for 40s in the complete assay system minus fructose 6-phosphate, after which the reaction was initiated by adding fructose 6-phosphate (0.2mM).

assay was initiated either directly with enzyme [Fig. 1a, trace (i)] or with fructose 6-phosphate after preincubation of the enzyme for 40s with 0.6mM-ATP [Fig. 1a, trace (ii)]. Preincubation with ATP led to a severe inhibition of the initial rate that only slowly reverted back to its uninhibited value. The slow restoration of activity in the presence of fructose 6-phosphate demonstrates that the ATP inhibition was at least partially reversible. When the enzyme was photo-oxidized for 36min with Methylene Blue, the inhibitory effect of preincubation with ATP was largely eliminated and there was only a brief lag period after addition of fructose 6-phosphate to the preincubated sample before the two rates become approximately equal [Fig. 1b, traces (i) and (ii)].

Fig. 2 shows the time course of inactivation of both the control and photo-oxidized enzymes during preincubation with 0.6mM-ATP in the absence of other effectors. The control enzyme lost more than 50% of its activity within a few seconds of adding ATP, as judged by changes in the initial rate, whereas the photo-oxidized enzyme was more slowly and less extensively inhibited during the preincubation period with ATP. Plots of $\ln(\text{enzyme activity})$ versus time (not shown) indicate that the inhibition of the non-photo-oxidized enzyme by ATP occurs in two stages that can be described in terms of two first-order (or

Fig. 2. Effect of time of preincubation with ATP on the activity of pig spleen phosphofructokinase

Unmodified (●) or 36min-photo-oxidized (○) phosphofructokinase was preincubated with MgATP (0.6mM) for the time intervals indicated and under the conditions described for Fig. 1 traces (ii), before the reaction was initiated with fructose 6-phosphate (0.2mM). The initial rates (v_0) are expressed, for each enzyme preparation, as percentages of the rates measured in the absence of MgATP preincubation and are given as means $\pm$ S.E.M. for experiments done on three separate batches of freshly prepared enzyme.

pseudo-first-order) rate constants ($k_{+1} = 0.21 \pm 0.004 \text{ s}^{-1}$ and $k_{+2} = 0.03 \pm 0.007 \text{ s}^{-1}$). Photo-oxidation selectively abolished the rapid phase of the inactivation (characterized by k_{+1}) but had little or no effect on the slower phase ($k'_{+2} = 0.03 \pm 0.003 \text{ s}^{-1}$).

The extent to which the non-photo-oxidized enzyme was inactivated by ATP was found to be variable. The

purification method used (Massey & Deal, 1973) enabled kinetic experiments to be performed on the dialysed enzyme only 24h after homogenization and extraction. Batches of enzyme tested immediately after preparation were almost completely inactivated by low concentrations of ATP (e.g. 0.6mM) within 20–30s, whereas batches stored at 4°C for more than 12 days were inhibited by less than 50% by the same ATP concentration. In other kinetic studies, where more extensively purified phosphofructokinases have been preincubated with ATP as a routine, striking losses of activity have not been reported (e.g. Kemp, 1971). With comparatively long purification procedures the 'aging' process may obliterate the inhibitory response to ATP before commencement of kinetic work, or, alternatively, the observations reported in the present paper may be unique for the spleen enzyme. Whatever the mechanism of the 'aging' process is, the loss of sensitivity to ATP on prolonged storage was not accompanied by a corresponding loss of specific activity, nor did storage greatly alter the characteristics of the sigmoidal response to fructose 6-phosphate when assays were initiated with non-photo-oxidized enzyme.

Fig. 3 shows the effect of time of photo-oxidation on the activity of phosphofructokinase, assayed both before and after preincubation with ATP. Photo-oxidation progressively destroyed the sensitivity of the enzyme to the rapid phase of the inactivation by ATP until, at approx. 18 min, it was no longer observed. Some loss of activity (approx. 30%) accompanied the loss of sensitivity to ATP during photo-oxidation, which is perhaps attributable to enzyme denaturation brought about by shaking the solution with Methylene Blue. Ahlfors & Mansour (1969) and Lee *et al.* (1973) have also reported a lower specific activity for the photo-oxidized enzyme.

In a conventional assay, initiated with enzyme, non-photo-oxidized phosphofructokinase gave the expected strongly sigmoidal response to variation in fructose 6-phosphate concentration that became more pronounced as the MgATP concentration was increased (Fig. 4). It should be noted that, in these experiments, the free Mg^{2+} , fixed at 3 mM in excess of the added ATP concentration (see Hickman & Weidemann, 1975), would have undergone only a small diminution due to complex-formation with fructose 6-phosphate (Achilles *et al.*, 1973), amounting to 5.3% at 1.0mM- (Fig. 4) and to 27% at 5.0mM-fructose 6-phosphate (Fig. 5). At both concentrations of MgATP, 'desensitization' of the photo-oxidized enzyme to inhibition by ATP was demonstrated by its reversion to a conventional hyperbolic response to variation in fructose 6-phosphate concentration (Hill coefficient $h = 1.04$). The loss of co-operative behaviour with respect to fructose 6-phosphate, and the decrease in ATP binding from 3.6 to 2.0mol per 100000g of enzyme (Lorenson & Mansour, 1969)

Fig. 3. Effect of time of photo-oxidation on the activity of phosphofructokinase measured before and after 40s preincubation with MgATP

The enzyme (1.5mg/ml) was photo-oxidized at 0°C in the presence of Methylene Blue (2.7 μ M) under the conditions described in the text. At the time intervals indicated, samples of photo-oxidized enzyme were withdrawn and tested for activity after initiation of the reaction with enzyme (○) or after 40s preincubation with MgATP and initiation of the reaction with fructose 6-phosphate (●) as described for Fig. 1.

that has been observed in the absence of a concomitant change in the catalytic capacity of the photo-oxidized enzyme (Ahlfors & Mansour, 1969), has led to the suggestion (Mansour, 1972) that there are two catalytic sites and two allosteric inhibitory sites for ATP per 100000g of enzyme, of which the two inhibitory sites are selectively lost after photo-oxidation. This suggestion is consistent with the behaviour of the spleen enzyme in that its activation by AMP, which is known to be dependent on competition with ATP at its inhibitory binding site (Kemp & Krebs, 1967), was totally abolished, along with ATP inhibition and the sigmoidal response to fructose 6-phosphate, by 18min of photo-oxidation (Fig. 5).

Fig. 4. Effect of photo-oxidation on the sigmoidal response of phosphofructokinase to fructose 6-phosphate

Unmodified (●) or 18 min-photo-oxidized (○) phosphofructokinase activity was measured as described in the text as a function of fructose 6-phosphate concentration at two fixed concentrations of MgATP: (a) 0.6 mM-ATP plus 3.6 mM-MgCl₂; (b) 1.5 mM-ATP plus 4.5 mM-MgCl₂. In each case the reaction was initiated by addition of enzyme. The Hill coefficients (*h*) for each of the conditions studied are given on the appropriate curves.

Fig. 5. Effect of photo-oxidation on the activation of phosphofructokinase by AMP

(a) Unmodified (●) or 18 min-photo-oxidized (○) phosphofructokinase activity was measured as a function of fructose 6-phosphate concentration under conditions where the two other substrates were fixed at 1.5 mM-ATP and 4.5 mM-MgCl₂ and the reaction was initiated by addition of enzyme. (b) Assay conditions identical with those for Fig. 5(a) were used except that the cuvettes contained, in addition, 0.5 mM-AMP in the presence of unmodified (▲) or 18 min-photo-oxidized (△) phosphofructokinase. The Hill coefficients (*h*) for each of the conditions studied are given on the appropriate curves.

Protection of the enzyme by substrates and positive effectors, and potentiation of the ATP inhibition by citrate

It has been noted previously, by many workers, that fructose 6-phosphate blocks the inhibition of phosphofructokinase by MgATP (Kühn *et al.*, 1974; Tejwani *et al.*, 1974; Lowry & Passonneau, 1966). In Fig. 6 we demonstrate that the inhibition of phospho-

Fig. 6. Effect of fructose 6-phosphate concentration during preincubation with ATP on the subsequent activity of phosphofructokinase

Phosphofructokinase was preincubated for 40s with either (●) 0.5mM- or (○) 2.0mM-ATP plus the indicated concentration of fructose 6-phosphate. At the end of the preincubation period the reaction was initiated with either 3.5mM- or 5mM-MgCl₂ plus sufficient fructose 6-phosphate to adjust its final assay concentration to 3.0mM (or, in one case, to 5.0 mM). The results are expressed as percentages of the initial velocity observed at each ATP concentration when the enzyme was fully 'protected' by 3.0mM-fructose 6-phosphate. Hill coefficients (*h*) which relate to the ability of fructose 6-phosphate to 'protect' the enzyme from inactivation are given on the appropriate curves.

fructokinase during 40s of preincubation with ATP and fructose 6-phosphate before initiation of the reaction with Mg²⁺ was less effective if higher concentrations of fructose 6-phosphate were present during the preincubation period. In the absence of fructose 6-phosphate and Mg²⁺, free ATP inactivated the enzyme in a very similar manner to that shown previously for MgATP. The design of this experiment should be noted: the enzyme was preincubated with either 0.5mM- or 2.0mM-ATP in the presence of defined concentrations of fructose 6-phosphate and then, after 40s, the reaction was initiated with MgCl₂ plus sufficient additional fructose 6-phosphate to bring its final concentration to 3.0mM (or, in one case, to 5.0mM). Thus, although all of the assays were conducted at the same final concentration of MgATP and fructose 6-phosphate, we were able to demonstrate that fructose 6-phosphate present during the preincubation period had a marked protective effect on the subsequent activity of the enzyme that was both positively co-operative and distinct from its function as a substrate. Because in conventional assays, initiated with the pig spleen enzyme, the extent of the ATP inhibition is decreased as the fructose 6-phosphate concentration is increased (Hickman & Weidemann, 1975), it is possible to attribute the decreased inhibition by ATP during the preincubation period (Fig. 6) to decreased binding of ATP to its allosteric inhibitory site in the presence of fructose 6-phosphate. Thus all of the results involving fructose 6-phosphate and ATP, alone and together, that pertain to alterations in the rapid phase of ATP inhibition (Fig. 6 and Table 1) may simply reflect alterations in the affinity of the allosteric inhibitory site for ATP.

In Table 1 the effect of preincubating phosphofructokinase with all of its substrates, both singly and together, is examined. The control value without preincubation (set at 100% relative activity) was obtained by adding enzyme (stored at pH 8.0) to the cuvette last in the sequence of additions to initiate the reaction. Unexpectedly, 40s preincubation with the reaction buffer alone, at pH 7.0, caused a 44% fall in the initial rate when the reaction was initiated by addition of all three substrates; this effect can be attributed to exposure of the enzyme to low pH before the addition of substrates, as the effect was absent at pH 8.0 but became more pronounced as the pH was lowered to 6.5 (Fig. 7).

As expected, preincubation of the enzyme with ATP alone caused a marked additional inhibition of enzyme activity, preincubation with fructose 6-phosphate caused a marked activation, and preincubation with both substrates together restored the activity to near the control value (Table 1). Although the addition of Mg²⁺ during the preincubation period had no significant effect on the inhibition produced by preincubation with free ATP, it did significantly

Table 1. *Effect of 40 s preincubation with various substrate combinations on the activity of pig spleen phosphofructokinase*
 Pig spleen phosphofructokinase was preincubated at pH 7.0 for 40 s with the substrates indicated; after the preincubation period the reaction was initiated by adding the remaining substrate(s) to the cuvette. Where preincubation is given as 'None', the reaction was initiated by adding enzyme to cuvettes already containing all of the substrates at the fructose 6-phosphate concentrations indicated. Results are expressed as percentages of the initial rate determined at 0.2 mM-fructose 6-phosphate when the reaction was initiated with enzyme, and are given as means $\pm$ S.E.M. with the numbers of enzyme batches tested given in parentheses.

	Relative activity (%)
1. Fructose 6-phosphate concentration 0.2 mM	
Preincubated with:	
None	100.0 $\pm$ 8.8 (4)
Buffer alone (pH 7.0)	56.4 $\pm$ 6.4 (8)
ATP (0.6 mM)	2.0 $\pm$ 0.8 (3)
Mg ²⁺ (3.6 mM)	97.2 $\pm$ 10.8 (3)
Fructose 6-phosphate (0.2 mM)	193.6 $\pm$ 9.2 (3)
ATP (0.6 mM) + Mg ²⁺ (3.6 mM)	3.2 $\pm$ 2.0 (3)
ATP (0.6 mM) + fructose 6-phosphate (0.2 mM)	91.2 $\pm$ 14.8 (4)
Mg ²⁺ (3.6 mM) + fructose 6-phosphate (0.2 mM)	119.6 $\pm$ 13.6 (3)
2. Fructose 6-phosphate concentration 1.0 mM	
Preincubated with:	
None	216.4 $\pm$ 8.8 (3)
Buffer alone (pH 7.0)	117.6 $\pm$ 25.6 (3)
ATP (0.6 mM) + fructose 6-phosphate (1.0 mM)	175.6 $\pm$ 5.2 (3)
Mg ²⁺ (3.6 mM) + fructose 6-phosphate (1.0 mM)	184.4 $\pm$ 7.6 (3)

protect the enzyme from the inhibitory effect of the buffer at pH 7.0. However, when the enzyme was preincubated with fructose 6-phosphate, the final activity was significantly lower if Mg²⁺ was present during the preincubation period. The effect of preincubation with free Mg²⁺ is evidently complex, leading to either weak activation associated with the protection of the enzyme from the inhibitory effect of low pH (but not that due to low pH and ATP together) in the absence of the other substrates, or to a somewhat stronger inhibition in the presence of fructose 6-phosphate (see also Kühn *et al.*, 1974). This latter effect, which may be due to direct or indirect interference with the binding of fructose 6-phosphate to the catalytic site, may explain why free Mg²⁺ at concentrations in excess of 5 mM has been found consistently to be inhibitory (Lowry & Passonneau, 1966); this process evidently has a low affinity for free Mg²⁺, as an apparent K_i of 20 mM was determined when the ATP concentration was fixed at 1.0 mM and that of fructose 6-phosphate at 0.2 mM (Hickman, 1974). Under similar assay conditions, pig spleen phosphofructokinase has an apparent K_i of 1.5 mM for MgATP (Hickman & Weidemann, 1975). If it is assumed that incubation with free Mg²⁺ causes occupation of a separate bivalent-cation-binding site (Mathias, 1972, quoted by Colombo *et al.*, 1975), the resulting stabilization may 'fix' the enzyme into a conformation that is resistant to the action of effectors that might otherwise convert it into a more active (e.g. fructose 6-phosphate) or less active (e.g. H⁺) form. The additional inhibition of the enzyme by

ATP, however, is not significantly relieved by Mg²⁺, even when its concentration is fixed at 3.0 mM in excess of that of the added ATP.

Fig. 8 shows the ability of five different positive effector molecules and one negative effector (citrate) either to protect the enzyme or to potentiate its inactivation during 40 s preincubation with 0.6 mM-ATP. Fig. 8(a) presents the results as absolute activities, where the effects of the modulators in the absence of preincubation (i.e. at zero time) can be compared with their effects after 40 s of preincubation; in Fig. 8(b) the data have been replotted to emphasize the relative effectiveness of the individual modulators in maintaining the initial rates established before preincubation with ATP. Except for citrate, which was tested at its apparent K_i concentration (1.2 mM), the effectors were examined at saturating concentrations at least 4 times higher than the apparent K_a values determined under conditions identical with those used in the experiment. This would tend to eliminate the possibility that the relative potency of the activators in protecting the enzyme from ATP inhibition (Fig. 8b) merely reflects an arbitrary choice of concentration.

The following conclusions can be drawn from Fig. 8: (i) the efficacy of positive effectors in slowing the inactivation process was approximately proportional to their potency as activators in the absence of preincubation with ATP (i.e. $K^+ > NH_4^+ = AMP > aspartate$); (ii) P_i, which had no significant activating effect when the reaction was initiated with enzyme, was almost as powerful as K⁺ in its capacity to

protect the enzyme from ATP inactivation [SO_4^{2-} appears to play an analogous 'protective' role (Hickman, 1974)]; (iii) the negative effector, citrate, strongly potentiated the inhibition by ATP.

Fig. 7. Effect of pH on the activity of phosphofructokinase preincubated with buffer alone before initiation of the reaction with MgATP plus fructose 6-phosphate.

Phosphofructokinase was preincubated with 50mM-Hepes/NaOH buffer at pH 8.0 (Δ), pH 7.5 ($\circ$), pH 7.0 ($\bullet$) and pH 6.5 ($\blacktriangle$) for the time intervals indicated, and the reaction was initiated by a mixture of the three substrates (0.2mM-fructose 6-phosphate, 0.6 mM-ATP and 3.6mM-MgCl₂). The initial velocities for the time course of the inactivation are expressed as percentages of the rates determined, at each pH, in the absence of preincubation. The inset shows the pH-dependence of the rate in the absence of preincubation ($\blacksquare$) and after 40s of preincubation with the appropriate buffer ($\square$), taking the initial velocity at pH 8.0, when the reaction was initiated with enzyme, as 100%.

It is noteworthy that, even in the presence of 'protective' ligands, the initial rates determined on addition of fructose 6-phosphate continued to decline as the period of preincubation with ATP increased from 10 to 40s; this is in harmony with the view that the rapid phase of the inactivation, characterized by rate constant k_{+1} , is at least partly blocked by the appropriate positive effectors and is potentiated by citrate ($k_{+1} = 0.38\text{s}^{-1}$), whereas the slower phase, characterized by k_{+2} , occurs independently of the presence of 'protective' ligands during the short preincubation times used.

Irrespective of the order of addition of reactants, activators of phosphofructokinase shift the rate curve for fructose 6-phosphate to the hyperbolic form, but inhibitors shift it to the sigmoidal form (Bloxham & Lardy, 1973). It is well documented that adenine nucleotide activators (such as ADP and AMP) act by competing directly with ATP for binding to the allosteric inhibitory site, but inhibitors such as citrate, which do not share a binding site with ATP, act indirectly by potentiating the binding of ATP to the same allosteric site (Kemp & Krebs, 1967; Colombo *et al.*, 1975). Not all of the positive effectors act by direct competition with ATP at the allosteric site, however. NH_4^+ , which is a powerful protector of the enzyme during preincubation with ATP (Fig. 8), also increases the activity markedly in the presence of ITP (Hickman & Weidemann, 1973), which binds poorly to the inhibitory site for ATP (Kemp, 1969). P_i partially relieves the ATP inhibition without exerting an independent activating effect in the absence of preincubation, which is compatible with the suggestion by Hill & Hammes (1975) that potassium phosphate competes directly with fructose 6-phosphate for substrate-binding sites on the rabbit muscle enzyme and may thus have an indirect 'protective' effect associated with making the allosteric inhibitory sites less accessible to ATP during the preincubation period (Lorensen & Mansour, 1969), similar to the effect exerted by fructose 6-phosphate. By analogy, fructose 1,6-bisphosphate, which binds to the catalytic site (Mansour, 1972), has no direct stimulatory effect on the spleen enzyme (Hickman & Weidemann, 1975), but appears to play only a stabilizing and 'protective' role similar to that of P_i and SO_4^{2-} (Wakid & Mansour, 1965; Hickman, 1974). For this reason, the objections raised by Emerk & Frieden (1975) to the use of a fructose 1,6-bisphosphate-removing coupled assay with the rabbit muscle enzyme do not apply to the present study.

Effect of protein concentration on the co-operative behaviour of phosphofructokinase

In addition to the effect of the order of addition of reactants, the concentration of phosphofructokinase in the cuvette is known to influence its co-operative

Fig. 8. Time course of inactivation of phosphofructokinase when preincubated with various effector molecules in the presence of 0.6 mM-MgATP

The enzyme was preincubated at pH 7.0 for the time intervals indicated with 0.6 mM-MgATP alone (○) or with 0.6 mM-MgATP plus individual effector molecules at the following concentrations: 50 mM-K⁺ (■); 5 mM-NH₄⁺ (▲); 0.5 mM-AMP (●); 2.7 mM-aspartate (▽); 7.0 mM-P_i (□); 1.0 mM-citrate (▼). After preincubation, the reaction was initiated with 0.5 mM-fructose 6-phosphate and the initial rate determined as described in the text. (a) The rates are all expressed as percentages of the initial rate determined, in the absence of added effector molecules, when the reaction was initiated with enzyme. (b) The results given in (a) are replotted, for each set of experimental conditions, as percentages of the individual rates determined in the absence of preincubation. Each point represents the mean value of six independent observations made with three separate batches of freshly prepared enzyme.

behaviour (Hulme & Tipton, 1971; Wenzel *et al.*, 1976). For instance, Table 2 shows that, with the MgATP concentration fixed at 1.0 mM, increasing the dilution of the stock solution of phosphofructokinase 5-fold raised the Hill coefficient (*h*) from 1.2 to 1.9 when the activity was titrated with fructose 6-phosphate. Consistent with this observation, the initial rates of the non-photo-oxidized enzyme measured at a fixed fructose 6-phosphate concentration were not directly proportional to enzyme concentration when the reaction was initiated with enzyme (Fig. 9a). Preincubation of the enzyme with ATP for 40 s

greatly exaggerated the non-linearity of the response to protein concentration by causing very strong inhibition (almost 100%) over the low enzyme-concentration range studied in the present paper and very little inhibition when the enzyme solution used was more concentrated. Most strikingly, the inhibitory response to ATP became apparent over a very narrow range of protein concentrations, with an abrupt threshold. In contrast, photo-oxidation modified the enzyme so that the non-linear response of the initial rate to increased protein concentration and the inhibitory response to ATP were both

Table 2. *Effect of dilution on the sigmoidal response of phosphofructokinase to fructose 6-phosphate*

Initial rates were determined, after initiation of the reaction with enzyme, as a function of fructose 6-phosphate concentration at pH 7.0 in the presence of a fixed concentration of MgATP (1.0 mM). Hill coefficients (h) were determined for the undiluted enzyme (10 μ g/ml) and after 2.5- and 5-fold dilution of the stock solution with potassium phosphate buffer (80 mM, pH 8.0) containing EDTA (1.0 mM) so that the enzyme plus diluting buffer was added to the cuvette in a total volume of 0.05 ml.

Dilution factor	Hill coefficient (h)
Undiluted	1.24
Diluted 2.5-fold	1.65
Diluted 5.0-fold	1.87

eliminated over the entire enzyme-concentration range tested (Fig. 9b). Thus the decreased co-operativity to fructose 6-phosphate, the loss of the inhibitory response to ATP and the disproportionately large increase in activity observed when the enzyme solution was concentrated or when the regulatory binding site for ATP was blocked by photo-oxidation all appear to be related phenomena. In particular, it appears that increasing the protein concentration is equivalent, in some way, to blocking selectively the inhibitory site for ATP.

Protonation and dissociation of phosphofructokinase as the basis of the sigmoidal response to fructose 6-phosphate: a possible model

Mansour (1972) has summarized the evidence that inactivation of phosphofructokinase by inhibitors that promote the binding of ATP to its inhibitory site, by low pH, or by dilution, is related to the dissociation of the enzyme from an active tetrameric form (360 000 daltons) to an inactive dimer. If this is also true of the pig spleen enzyme, the basis of the sigmoidal response to fructose 6-phosphate (see Scheme 1) may reside in the pre-existing equilibrium between two conformational isomers (tetramers and dimers) that have intrinsically different catalytic activities (Monod *et al.*, 1965). Scheme 1 proposes that both conformational isomers can be protonated at low pH. The two dimeric forms are inactive and, for the purpose of the argument developed below, the two tetrameric forms are considered to have equivalent catalytic activities, irrespective of protonation. The evidence favouring a polymerizing-depolymerizing mechanism for the spleen enzyme has two main bases: (i) the non-linear increase in activity (Fig. 9) and (ii) the strong decrease in the Hill coefficient (h), as the protein concentration of the non-photo-oxidized enzyme is increased (Table 2).

Fig. 9. *Effect of varying the concentration of unmodified and photo-oxidized phosphofructokinase on the degree of inactivation achieved by preincubation with MgATP for 40 s*

(a) With non-photo-oxidized enzyme, the reaction was initiated with either enzyme (○) or 0.5 mM-fructose 6-phosphate after 40 s preincubation at pH 7.0 with 0.6 mM-ATP (●) at the enzyme concentrations indicated. (b) Conditions were as described for (a) except that 36 min-photo-oxidized enzyme was used. The results are all expressed as percentages of the initial rate (v_0) measured, at the highest concentration of non-photo-oxidized enzyme, when the reaction was initiated with enzyme.

Scheme 1. Possible equilibrium mixture of protonated and unprotonated tetramers and dimers of pig spleen phosphofructokinase

PFK represents a phosphofructokinase subunit. It is postulated that equilibrium constant $K_1 = \frac{[\text{H PFK}_2]^2}{[\text{H PFK}_4]} \gg K_2 = \frac{[\text{PFK}_2]^2}{[\text{PFK}_4]}$

Mansour (1972) has shown that photo-oxidation of the sheep heart enzyme and/or high pH both decrease the number of ATP-binding sites (from four to two) and the number of citrate-binding sites (from two to zero) without affecting the number of binding sites (two) for fructose 6-phosphate. Because catalytic activity is retained after photo-oxidation, the three histidine residues destroyed must be at inhibitory binding sites for ATP and not at the catalytic site. Histidine residues at these sites are likely to become protonated, and capable of forming hydrogen bonds with two of the ionized phosphate moieties of the ATP^{4-} molecule, at slightly acid pH. At alkaline pH, or after photo-oxidation, the histidine residues would be unprotonated or unavailable for protonation and ATP would no longer bind to the inhibitory site. Thus two equilibrium constants (K_1 and K_2) are necessary to describe the dissociation of the protonated (H PFK_4) and unprotonated (PFK_4) active tetramers of the unmodified enzyme (where PFK represents a subunit of phosphofructokinase) to their respective inactive dimers. Because a sigmoidal response to fructose 6-phosphate is observed with dilute enzyme at pH 7.0 (Fig. 4), but not at pH 8.0 (Hickman & Weidemann, 1975), it is necessary to predict that K_1 is much larger than K_2 so that a large excess of the inactive isomer (H PFK_2) is generated at low pH in the absence of fructose 6-phosphate. This difference provides the basis for the positively co-operative response observed at pH 7.0. Conversely, at pH 8.0, in the absence of protonation of the inhibitory site for ATP, K_2 must be smaller than K_1 so that the active (PFK_4) predominates over the inactive (PFK_2) species and a simple hyperbolic response to fructose 6-phosphate is obtained. Finally, at high protein concentration, the association of both protonated and unprotonated dimers to active tetramers will be favoured and the corresponding shift in the positions of the two equilibria will remove the remaining basis for positive co-operativity at low pH.

According to Scheme 1, the positions of the two equilibria governed by K_3 and K_4 , which are under the control of the pH, will determine whether ATP binds to its inhibitory site. The photo-oxidized enzyme, which lacks ionizable histidine residues at the ATP inhibitory site (Mansour, 1972) and does not dissociate to dimers under conditions, such as dilution, that normally promote dissociation of the unmodified enzyme (Ahlfors & Mansour, 1969), will be permanently 'locked' into the unprotonated conformations. Its dissociation to inactive dimers at all protein concentrations, controlled by K_2 alone, will be insufficient to give rise to sigmoidal kinetics.

The rapid inhibition of the unmodified enzyme by ATP and low pH can be explained readily by this scheme. If ATP binds preferentially to the protonated (H PFK_4) form of the enzyme, and stabilizes it, the enzyme will dissociate more rapidly to the inactive dimer (H PFK_2) in the presence of ATP according to the postulate that $K_1 \gg K_2$. This is consistent with a number of observations reported in the present paper: (i) lowering the pH alone is sufficient to inactivate the enzyme rapidly in the absence of fructose 6-phosphate (Fig. 7); (ii) the enzyme is inactivated more and more rapidly as the preincubation pH is lowered from 8.0 to 6.0 (Fig. 7); (iii) when the pH is fixed at 7.0, the presence of ATP or MgATP greatly accelerates the rate of inactivation (Table 1); (iv) citrate, which binds to the enzyme only at low pH (Mansour, 1972), causes a further acceleration of the inactivation rate at pH 7.0 (Fig. 8). These results suggest that the rapid phase of the inactivation ($k_{+1} = 0.21 \text{ s}^{-1}$) may be due to the dissociation of the protonated tetramer.

The simplest explanation for the stabilizing effect of the substrate fructose 6-phosphate (Fig. 6; Table 1) is that it binds to the unprotonated (PFK_4) form of the enzyme preferentially. This would upset the equilibrium position of the protonation step, governed by the equilibrium constant K_3 , and lower the concentra-

tion of H PFK₄ sufficiently to prevent significant dissociation. In principle, fructose 1,6-bisphosphate and P_i, both of which 'protect' the enzyme but do not activate it when the reaction is initiated with enzyme (Fig. 8; Hickman & Weidemann, 1975), could mimic the effect of fructose 6-phosphate by binding at the catalytic site. In contrast, AMP probably protects the enzyme by inhibiting ATP binding at the protonated inhibitory site. The remaining activators (K⁺, NH₄⁺ and aspartate) may bind at regulatory sites distinct from the ATP inhibitory site, the occupation of which stabilizes the non-protonated form of the tetramer, or may prevent ATP binding to its protonated inhibitory site in the same manner as AMP.

Alternative explanations for the ATP inhibition

Emerk & Frieden (1975) have argued that the rate of polymerization of inactive muscle phosphofructokinase to the active four-subunit enzyme was insufficiently fast under their assay conditions to account for the sigmoidal kinetics observed at pH 6.5. Further, the rather slow depolymerization ($t_{\frac{1}{2}}$ = approx. 1.5h) of muscle phosphofructokinase, which has been observed by Lad *et al.* (1973) and which is implicit in the concept put forward by Frieden (1970) that phosphofructokinase undergoes a slow hysteretic response to rapid changes in ligand concentration, makes it conjectural whether all of the effects described in the present paper, some of which are very rapid indeed, can be attributed solely to the influence of ligands on the relative rates of dissociation of the protonated and unprotonated tetramers (Scheme 1). At high enzyme concentration, for example, phosphofructokinase aggregates readily into active higher-molecular-weight forms (Zimmermann *et al.*, 1973), which appear to be integral multiples of the dimer and which may have both decreased accessibility to ATP at the inhibitory sites (Lorenson & Mansour, 1969) and decreased co-operativity between the fructose 6-phosphate-binding sites situated on different subunits (Hill & Hammes, 1975). Polymerization of this type is an alternative explanation for the progressive loss of ATP inhibition (Fig. 9) and diminished positive co-operativity (Table 2) observed at high protein concentration.

Colombo *et al.* (1975) have shown that citrate has a potentiating effect on the fall in the initial rate observed when the rabbit muscle enzyme is preincubated with ATP before initiation of the reaction with fructose 6-phosphate. Citrate appears to increase the affinity of the inhibitory site for MgATP at protein concentrations that resist significant dissociation of the enzyme to dimers. It is possible that the synergistic inhibition caused by citrate in the presence of MgATP may be associated with conformational changes that can occur in the absence of dissociation of phosphofructokinase tetramers to dimers, although citrate

does intensify the non-linear response of the ox heart enzyme to changes in protein concentration (Hulme & Tipton, 1971) and, at 5 mM, greatly accelerates the dissociation of the muscle enzyme to the dimeric form (Lad *et al.*, 1973). The present results do not exclude the possibility that the protonated form of the tetramer may have an intrinsically lower catalytic capacity than the unprotonated form. If this is the case, the rapid phase of the inactivation (k_{+1} = 0.21 s⁻¹) promoted by low pH and ATP and potentiated by citrate might represent the protonation step itself, whereas the slower phase of the inactivation (k_{+2} = 0.03 s⁻¹) might represent the preferential dissociation of the relatively inactive protonated tetrameric form to a wholly inactive dimer. If there are intrinsic differences in the catalytic activities of the protonated and unprotonated forms of the active tetramer, it is possible that some effectors (such as H⁺) can exert 'fine' and very rapid control over enzyme activity by influencing the equilibrium position of the two conformational isomers of the active tetramer.

Part of this work was conducted during the tenure of a Commonwealth Postgraduate Studentship by P. E. H. and was supported by Australian Research Grants Committee Grants no. D70-17436 and D2 70-17436 to M. J. W. We are indebted to Dr. G. D. Smith of this Department for many helpful discussions.

References

- Achilles, W., Cumme, G. A. & Hoppe, H. (1973) *Acta Biol. Med. Ger.* **31**, 763-770
- Ahlfors, C. E. & Mansour, T. E. (1969) *J. Biol. Chem.* **244**, 1247-1251
- Bloxham, D. P. & Lardy, H. A. (1973) *Enzymes 3rd Ed.* **8**, 239-278
- Colombo, G., Tata, P. W., Girotti, A. W. & Kemp, R. G. (1975) *J. Biol. Chem.* **250**, 9404-9412
- Emerk, K. & Frieden, C. (1975) *Arch. Biochem. Biophys.* **168**, 210-218
- Frieden, C. (1970) *J. Biol. Chem.* **245**, 5788-5799
- Hickman, P. E. (1974) Ph.D. Thesis, Australian National University
- Hickman, P. E. & Weidemann, M. J. (1973) *FEBS Lett.* **38**, 1-3
- Hickman, P. E. & Weidemann, M. J. (1975) *Biochem. J.* **151**, 327-336
- Hill, D. E. & Hammes, G. G. (1975) *Biochemistry* **14**, 203-213
- Hulme, E. C. & Tipton, K. F. (1971) *FEBS Lett.* **12**, 197-200
- Kemp, R. G. (1969) *Biochemistry* **8**, 3162-3168
- Kemp, R. G. (1971) *J. Biol. Chem.* **246**, 245-252
- Kemp, R. G. & Krebs, E. G. (1967) *Biochemistry* **6**, 423-434
- Kühn, B., Jacobasch, G., Gerth, C. & Rapoport, S. M. (1974) *Eur. J. Biochem.* **43**, 443-450
- Lad, P. M., Hill, D. E. & Hammes, G. G. (1973) *Biochemistry* **12**, 4303-4309

- Lee, L. M. Y., Krupka, R. M. & Cook, R. A. (1973) *Biochemistry* **12**, 3503–3508
- Lorenson, M. Y. & Mansour, T. E. (1969) *J. Biol. Chem.* **244**, 6420–6431
- Lowry, O. H. & Passonneau, J. V. (1966) *J. Biol. Chem.* **241**, 2268–2279
- Mansour, T. E. (1972) *Curr. Top. Cell. Regul.* **5**, 1–46
- Massey, T. H. & Deal, W. C. (1973) *J. Biol. Chem.* **248**, 56–62
- Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118
- Ramaiah, A. & Tejwani, G. A. (1970) *Biochem. Biophys. Res. Commun.* **39**, 1149–1156
- Tejwani, G. A., Kaur, J., Ananthanarayanan, M. & Ramaiah, A. (1974) *Biachim. Biophys. Acta* **370**, 120–129
- Wakid, N. & Mansour, T. E. (1965) *Mol. Pharmacol.* **1**, 53–59
- Wenzel, K. W., Kurganov, B. I., Zimmermann, G., Yakovlev, V. A., Schellenberger, W. & Hofmann, E. (1976) *Eur. J. Biochem.* **61**, 181–190
- Zimmermann, G., Wenzel, K. W., Gauer, J. & Hofmann, E. (1973) *Eur. J. Biochem.* **40**, 501–505