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An Investigation of the Interactions of the Allosteric Modifiers of Pyruvate Kinase with the Enzyme from *Carcinus maenas* Hepatopancreas

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1. Pyruvate kinase purified from the hepatopancreas of *Carcinus maenas* exhibited sigmoidal saturation kinetics with respect to the substrate phosphoenolpyruvate in the absence of the allosteric activator fructose 1,6-bisphosphate, but normal hyperbolic saturation was seen in the presence of this activator. The activation appears to be the result of a decrease in the $s_{0.5}$ (phosphoenolpyruvate) and not to a change in $V_{max.}$. 2. In the presence of ADP and ATP at a constant nucleotide-pool size the results indicate that phosphoenolpyruvate co-operativity is lost on increasing the [ATP]/[ADP] ratio. 3. Paralleling this change is the observation that the fructose 1,6-bisphosphate activation became less as the [ATP]/[ADP] ratio was increased. This was due to the enzyme exhibiting a near-maximal activity in the absence of activator. 4. L-Alanine inhibited the enzyme, but homotropic co-operative interactions were only seen with a cruder (100000*g* supernatant) enzyme preparation. The inhibition by alanine could be overcome by increasing the concentration of either phosphoenolpyruvate or fructose 1,6-bisphosphate, although increasing the L-alanine concentration did not appear to be able to reverse the activation by fructose 1,6-bisphosphate. 5. In the presence of a low concentration of phosphoenolpyruvate, increasing the concentration of the product, ATP, caused an initial increase in enzyme activity, followed by an inhibitory phase. In the presence of either fructose 1,6-bisphosphate or L-alanine only inhibition was seen. 6. The inhibition by ATP could not be completely reversed by fructose 1,6-bisphosphate.

Fructose 1,6-bisphosphate stimulates pyruvate kinase (EC 2.7.1.40) from yeast (Hess *et al.*, 1966) and rat liver (Taylor & Bailey, 1967) with kinetics characteristic of allosteric activation. The allosteric mammalian liver enzyme has been shown to be distinct from the non-allosteric enzyme isolated from skeletal muscle, on the basis of their chromatographic, electrophoretic, immunological and kinetic properties (Tanaka *et al.*, 1967). In general the liver-type enzyme predominates in gluconeogenic tissues (Tanaka *et al.*, 1967), where the regulatory enzyme appears to be involved in the metabolic control of the switch between glycolysis and gluconeogenesis (Llorente *et al.*, 1970; Soling *et al.*, 1973). Studies on the kinetic properties of the enzymes from mammalian liver (Rozengurt *et al.*, 1969) and from yeast (Haeckel *et al.*, 1968; Wieker & Hess, 1971) have shown that most of the observed effects can be explained by the concerted model of allosteric control (Monod *et al.*, 1965), where there is a transition between two states of the enzyme with different affinities for the substrates. There are some discrepancies to this model. Thus the allosteric control by phosphoenolpyruvate, fructose 1,6-bisphosphate, ATP and alanine is not abolished by saturating with K^+ (Rozengurt *et al.*, 1969; Jiminez de Asua *et al.*, 1970), findings that can be accommodated by the sequential conformational-change model of Koshland *et al.* (1966).

The hepatopancreas of the common shore crab *Carcinus maenas* has been demonstrated to be a gluconeogenic tissue (Toghrol, 1969), and, in common with mammalian gluconeogenic tissues, contains a high activity of pyruvate kinase, but a relatively low activity of pyruvate carboxylase (Giles *et al.*, 1975). Pyruvate kinase from this tissue has been extensively purified (Giles *et al.*, 1976*a*).

There is some evidence that in hepatopancreas, this enzyme is a site for metabolic control. The mass-action ratio of 0.53 calculated from the tissue concentrations of substrates and products (Giles *et al.*, 1976*b*) is considerably lower than the equilibrium constant of this reaction, 2000–20000 (McQuate & Utter, 1959; Krimsky, 1959), which indicates a non-equilibrium reaction in the cell. In addition, preliminary studies have shown that fructose 1,6-bisphosphate will activate the enzyme, that L-alanine and ATP will inhibit it, and that in the presence of approximately physiological concentrations of these substrates and effectors the activity of the enzyme is decreased to 5–10% of the total potential activity (Giles *et al.*, 1975).

The present paper reports studies made in the presence of the allosteric effectors, individually and

in combination, on the kinetic properties of the *C. maenas* type-L pyruvate kinase.

Experimental

Materials

Trizma base, Dowex 50 (X8) resin, fructose 1,6-bisphosphate, EGTA, NADH and disodium glycerol 1-phosphate were obtained from Sigma (London) Chemical Co. (London S.W.6, U.K.). ATP, ADP, phosphoenolpyruvate (tricyclohexylamine salt) and pig heart lactate dehydrogenase were supplied by Boehringer Corp. (London W.5, U.K.). All other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K.

Animals

The common shore crab *Carcinus maenas* was collected locally from Southampton Water and kept at 10–12°C in an aquarium containing circulating sea water collected from the same source.

Methods

Assay of pyruvate kinase. The assay system used was as previously described (Giles *et al.*, 1976*a*), except that 50mM-dipotassium glycerol 1-phosphate was included. This salt served both as the source of K^+ (100mM) and, in conjunction with sufficient $MgSO_4$, buffered the Mg^{2+}_{free} concentration at 8mM (Boyer, 1969). The amount of $MgSO_4$ required was calculated by using the known dissociation constants of the chelating species (Boyer, 1969). Reaction, which was linear with time, was initiated by addition of phosphoenolpyruvate and was followed at 340nm and 25°C in a Perkin–Elmer 356 spectrophotometer operating in either the split-beam or dual-wavelength (wavelength couple 340:420nm) mode. The data presented in the Figures are the mean values of at least duplicate determinations of each point, the individual values of which differ by less than 5%. In the experiments using the 100000*g* supernatant of hepatopancreas homogenate a small blank rate was observed in the absence of phosphoenolpyruvate. This was subtracted from the rate measured in the full assay system. One unit of enzyme is defined as that amount that catalyses the formation of 1 μmol of pyruvate/min at 25°C.

The present studies were simplified by conducting all the experiments at constant concentrations of K^+_{total} and Mg^{2+}_{free}. Pilot experiments showed that the K^+_{total} concentration used (100mM) was sufficiently high that no increase in enzyme activity occurred on addition of more K^+. [The Mg^{2+}_{free} concentration used (8mM) was similar to that used in previous kinetic studies of pyruvate kinase, and in the light of recent evidence approximates to the intracellular Mg^{2+}_{free} concentration in squid axon (Brinley & Scarpa, 1975).] Use of a constant Mg^{2+}_{free} concentration ensures that the adenine nucleotides will not produce their effects by altering the Mg^{2+}_{free} concentration and that the magnesium chelate forms of the nucleotides will always be in a constant ratio to the total nucleotide concentration added.

Substrate assays. ADP (Adam, 1963), phosphoenolpyruvate (Czok & Eckert, 1963), ATP (Lamprecht & Trautschold, 1963) and fructose 1,6-bisphosphate (Bücher & Hohorst, 1963) solutions were prepared daily and assayed by standard enzymic techniques. Analysis for cross-contamination of the substrates indicated that the ATP contained appreciable amounts of ADP. Allowance for the presence of this ADP was made when both nucleotides were added together.

Enzyme. Peak-1 pyruvate kinase (specific activity 220units/mg of protein) purified by method A of Giles *et al.* (1976*a*) was used as the source of purified enzyme. The other enzyme source used was a 100000*g* supernatant of a hepatopancreas homogenate. The hepatopancreas from one animal was removed, homogenized in 10ml of ice-cold 67mM-Tris/HCl (pH7.4)/0.67mM-EDTA/1mM-2-mercaptoethanol/33% (v/v) glycerol, and the homogenate clarified by centrifugation for 1h at $100000g_{av.}$. The homogenate was prepared fresh each day. No significant decay of the enzyme activity was observed when assayed in standard conditions over the period taken to perform an experiment.

Data analysis. Results of the kinetic experiments were fitted to the Hill (1910) equation by least-squares regression analysis by using a programme based on the method of Atkins (1973) run on a Hewlett–Packard 9810 desk-top computer. Hill plots of $\log[v/(V-v)]$ against $\log[S]$ were constructed by using the least-squares estimates of V, $s_{0.5}$ and h.

Preparation of dipotassium glycerol 1-phosphate. Disodium glycerol 1-phosphate (5g) was dissolved in 10ml of water and deionized by chromatography on a column (1.5cm×16cm) of Dowex 50 (X8; H^+ form). The free acid was adjusted to pH8 with 10M-KOH and its concentration determined by hydrolysing a known quantity in $H_2SO_4/HClO_4$ (3:2, v/v) and measuring the P_i released by the method of Berenblum & Chain (1938). The prepared salt contained less than 1% Na^+ with respect to K^+ when determined by flame photometry. Before use the solution was adjusted to pH7.4 with HCl.

Results

Activation by phosphoenolpyruvate and fructose 1,6-bisphosphate

At low phosphoenolpyruvate concentrations fructose 1,6-bisphosphate activated the purified *C. maenas* pyruvate kinase. Variation of the phosphoenolpyruvate concentration in the presence and absence of

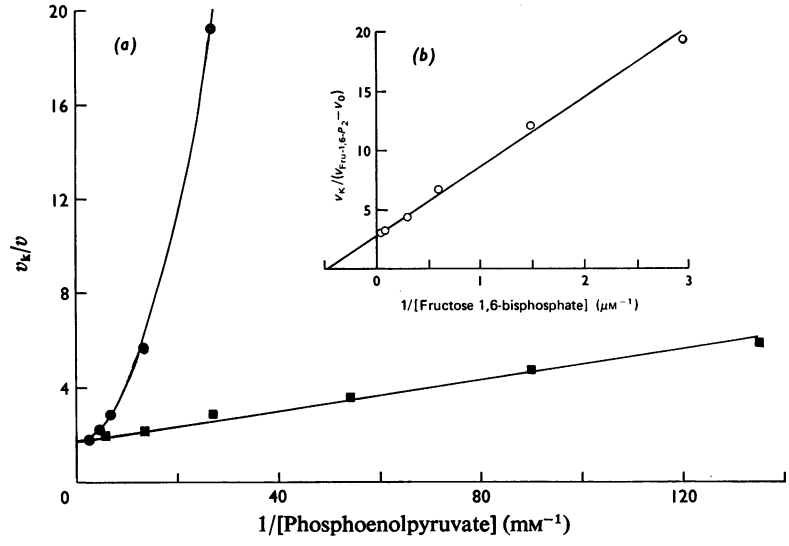

Fig. 1. *Allosteric activation of purified C. maenas pyruvate kinase by fructose* 1,6-*bisphosphate*

(*a*) Effect of fructose 1,6-bisphosphate on the homotropic co-operative interactions of phosphoenolpyruvate with the enzyme. The ADP concentration was 0.125 mM. All the points are the average of duplicate determinations. Two points, one at 740 μM-phosphoenolpyruvate in the absence of fructose 1,6-bisphosphate and one at 3.7 μM-phosphoenolpyruvate in the presence of the activator, have been omitted from the graph for clarity but were included in the data analysis. The least-squares values for the Hill coefficient and $s_{0.5}$ (phosphoenolpyruvate) were 2.06 and 115 μM in the absence (●) and 0.85 and 22 μM in the presence (■) of 0.5 mM-fructose 1,6-bisphosphate. (*b*) Double-reciprocal plot of activation against fructose 1,6-bisphosphate concentration showing hyperbolic saturation of the activator. The concentrations of ADP and phosphoenolpyruvate were 0.22 mM and 78 μM respectively. The apparent activation constant for fructose 1,6-bisphosphate was 2.1 μM. The initial rate of enzyme activity, v, was expressed as a ratio to that measured in standard assay conditions, v_k, i.e. when the concentrations of ADP, phosphoenolpyruvate and fructose 1,6-bisphosphate were 0.56, 0.80 and 0.50 mM respectively. $v_{Fru\text{-}1,6\text{-}P_2}$ and v_0 are the enzyme activities measured in the presence and absence of fructose 1,6-bisphosphate.

the activator (Fig. 1*a*) showed that the positive homotropic binding of phosphoenolpyruvate was abolished on adding the activator. The fructose 1,6-bisphosphate appears to activate the enzyme by decreasing the $s_{0.5}$ (phosphoenolpyruvate), although there is no apparent effect on $V_{max.}$. This result, which is similar to that found for the rat liver (Rozengurt *et al.*, 1969) and yeast (Haeckel *et al.*, 1968) enzymes, is consistent with a conformational change of the 'K' type as defined by Monod *et al.* (1965). The binding of fructose 1,6-bisphosphate has no effect on the binding of ADP, hyperbolic saturation kinetics occurring in the presence and absence of the activator. This is unlike the yeast enzyme, where ADP also exhibits positive homotropic kinetics (Haeckel *et al.*, 1968). Variation of the concentration of fructose 1,6-bisphosphate in the presence of a low concentration of phosphoenolpyruvate (Fig. 1*b*) shows a hyperbolic saturation of the enzyme with the activator. In the conditions quoted the apparent activation constant for fructose 1,6-bisphosphate was 2.1 μM.

In the experiments described above, ATP, a known inhibitor of the enzyme, was absent. Experiments were therefore designed to investigate the nature of the activations by fructose 1,6-bisphosphate and phosphoenolpyruvate in the presence of ATP.

Measurement of the pyruvate kinase activity at a constant adenine nucleotide concentration of 1 mM (approximately physiological for hepatopancreas), but with a variation in the [ATP]/[ADP] ratio, showed that the positive homotropic co-operativity with respect to phosphoenolpyruvate was lost as the concentration of ATP increased (Fig. 2*a*). Repeating the experiment in the presence of 500 μM-fructose 1,6-bisphosphate showed that the positive homotropic interactions of phosphoenolpyruvate with the enzyme were absent at all times, but that the $s_{0.5}$ (phosphoenolpyruvate) did increase with the [ATP]/[ADP] ratio (Fig. 2*b*). A plot of activation by fructose 1,6-bisphosphate, at various phosphoenolpyruvate concentrations, as a function of the [ATP]/[ADP] ratio shows that the effect of the allosteric activator

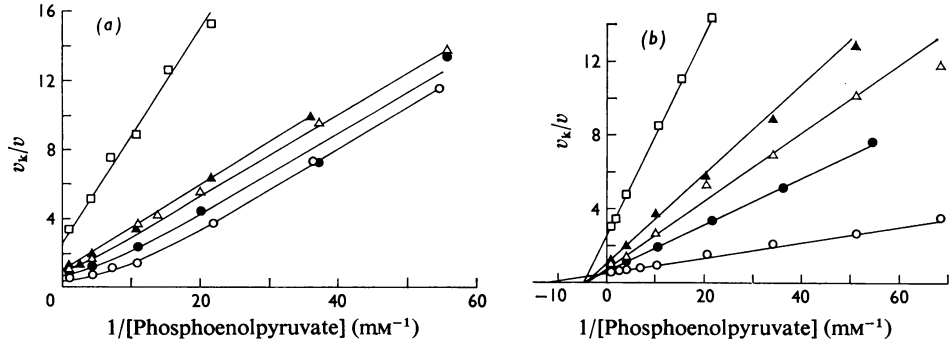

Fig. 2. *Double-reciprocal plot showing the relationship between the activity of the purified enzyme and phosphoenolpyruvate concentration at different [ATP]/[ADP] ratios, but with a constant 1 mM-total adenine-nucleotide pool, in the presence and absence of 0.5 mM-fructose 1,6-bisphosphate*

The [ATP]/[ADP] ratios used were: ○, 0:1; ●, 1:1; △, 7:3; ▲, 4:1; □, 19:1. (*a*) The absence of fructose 1,6-bisphosphate. The Hill coefficients and $s_{0.5}$ (phosphoenolpyruvate) values calculated were respectively: ○, 1.63 and 137 μM; ●, 1.39 and 139 μM; △, 1.21 and 209 μM; ▲, 1.33 and 149 μM; □, 1.03 and 214 μM. (*b*) The presence of 0.5 mM-fructose 1,6-bisphosphate. The Hill coefficients calculated were all unity, and the $s_{0.5}$ (phosphoenolpyruvate) values were: ○, 77 μM; ●, 206 μM; △, 285 μM; ▲, 350 μM; □, 230 μM.

became less as the [ATP]/[ADP] ratio increased (Fig. 3).

Inhibition by L-*alanine*

The actions of the negative effector L-alanine were reversed by increasing the concentration of either fructose 1,6-bisphosphate or phosphoenolpyruvate (Fig. 4). The positive effector fructose 1,6-bisphosphate caused a shift of the $I_{0.5}$ (alanine) (inhibitor concentration giving half-maximal inhibition) towards higher concentrations. The Hill plots of these data, in the modification of Jensen & Nester (1966), show no homotropic interactions of alanine. This is not in agreement with the predictions of the concerted allosteric model, where the homotropic cooperativity of a negative modifier should be strengthened in the presence of a positive modifier.

Llorente *et al.* (1970), using a crude enzyme extract from rat liver, found significant differences in the effects of alanine from those found by Schoner *et al.* (1970), who used a partially purified rat liver enzyme preparation. Subsequent investigations led Seubert & Schoner (1971) to suggest that a partial desensitization of the enzyme occurred on purification. It was reasonable therefore to investigate whether a similar phenomenon occurred with the *C. maenas* enzyme. Repeating the alanine-inhibition experiments, but with a fresh 100000*g* hepatopancreas supernatant as the enzyme source, showed that an increase in the concentration of the positive effector fructose 1,6-bisphosphate caused a strengthening of the homotropic interactions of alanine (Fig. 5). The Hill coefficient increased from 0.85 in the absence, to 2.5 in the presence, of 3.3 μM-fructose 1,6-bisphosphate.

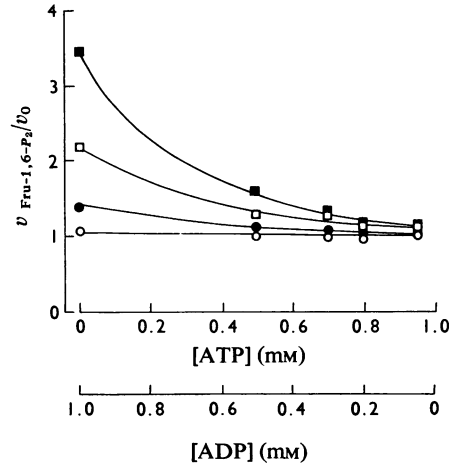

Fig. 3. *Magnitude of the activation by fructose 1,6-bisphosphate of the purified C. maenas pyruvate kinase at different phosphoenolpyruvate concentrations as a function of the [ATP]/[ADP] ratio with a constant 1 mM-total adenine-nucleotide pool*

The enzyme activity was measured in the presence ($v_{Fru-1,6-P_2}$) and absence of 0.5 mM-fructose 1,6-bisphosphate (v_0) at four concentrations of phosphoenolpyruvate; ○, 200 μM; ●, 100 μM; □, 50 μM; ■, 25 μM.

Paralleling this change was a small decrease in the $I_{0.5}$ (alanine) value from 1.34 mM in the absence, to 0.85 mM in the presence, of 3.3 μM-fructose 1,6-bisphosphate. Extrapolation to a saturating alanine con-

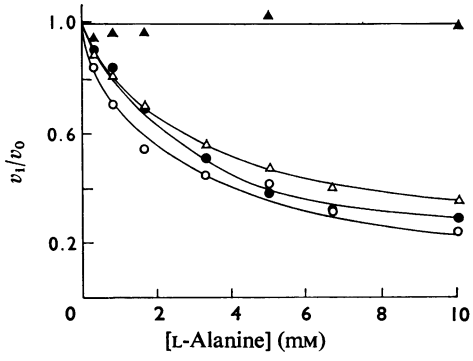

Fig. 4. *Effect of fructose* 1,6-*bisphosphate and phosphoenolpyruvate concentration on the inhibition of the purified C. maenas pyruvate kinase by* L-*alanine*
Plot of the enzyme activity in the presence of inhibitor (v_i) relative to that in its absence (v_0), as a function of inhibitor concentration. The concentrations of ADP and phosphoenolpyruvate used were 114 and 36 μM respectively. The lines correspond to fructose 1,6-bisphosphate concentrations of: ○, 0 μM; ●, 1 μM; △, 2 μM. The fourth line (▲) was obtained in the absence of fructose 1,6-bisphosphate but at a high (980 μM) concentration of phosphoenolpyruvate.

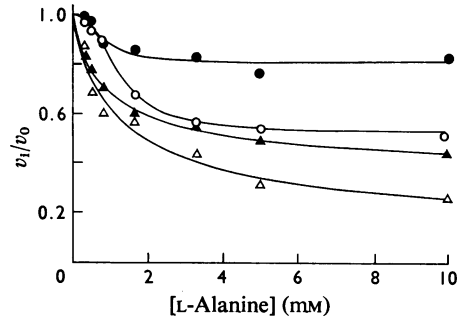

Fig. 5. *Effect of fructose* 1,6-*bisphosphate concentration on the inhibition by* L-*alanine of the pyruvate kinase activity in a* 100000*g supernatant of C. maenas hepatopancreas*
Plot of the enzyme activity in the presence of the inhibitor (v_i) relative to that in its absence (v_0) as a function of inhibitor concentration. The concentrations of ADP and phosphoenolpyruvate used were 116 and 37.4 μM respectively. The lines correspond to fructose 1,6-bisphosphate concentrations of: △, 0 μM; ▲, 1 μM; ○, 2 μM; ●, 3.3 μM.

centration indicated that there was always a finite rate. Addition of a low concentration of fructose 1,6-bisphosphate to the crude enzyme affected the catalytic activity that remained at high alanine concentrations. Saturation with alanine did not completely reverse the activation caused by fructose 1,6-bisphosphate. Conversely, saturation with fructose 1,6-bisphosphate did reverse the inhibition by alanine.The different responses of the crude extract and purified enzyme are most unlikely to be due to the presence of extraneous ions. The assay conditions were identical, except for the two enzyme sources, with the only possible extraneous ions being those in the cell extract. As the overall dilution of this in the assay medium was greater than 1:3000 this would effectively eliminate any effects due to extraneous ions.

Repeating the experiments in the presence of a 1 mM or 2 mM total nucleotide pool, with an [ATP]/[ADP] ratio of 7:3, showed that alanine was inhibitory. A greater proportion of the activity remained, however, on saturation with alanine compared with the activity obtained under similar conditions in the absence of ATP. The results also showed that there was little difference between the effects at the two nucleotide-pool sizes used.

Inhibition by ATP

The effect of ATP, in the presence of a constant 300 μM-ADP, was investigated by using a 100000*g* supernatant as the enzyme source. At a low concentration of phosphoenolpyruvate, and in the absence of other effectors, a biphasic action was

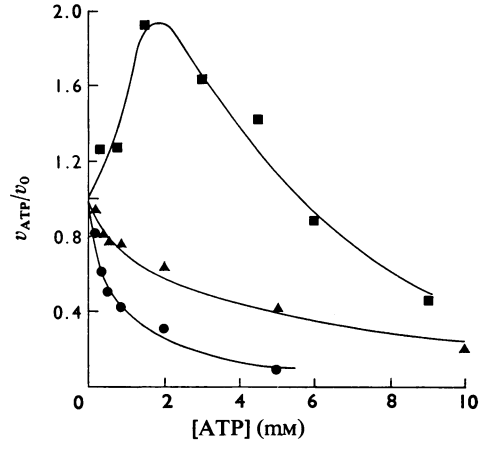

Fig. 6. *Effect of ATP on the pyruvate kinase activity in a* 100000*g supernatant of C. maenas hepatopancreas*
The enzyme activity in the presence of ATP (v_{ATP}) relative to that in its absence (v_0) plotted as a function of ATP concentration. The concentrations of ADP and phosphoenolpyruvate used were 119 and 37.4 μM respectively. The other additions were: ■, none; ▲, 1.67 mM-L-alanine; ●, 3.3 μM-fructose 1,6-bisphosphate.

obtained on increasing the concentration of ATP (Fig. 6). Initially, the enzyme activity increased as the ATP concentration rose, until a maximal doubling in enzyme activity was measured at 2mM-ATP. This phase of activation was followed by inhibition. In the presence of either fructose 1,6-bisphosphate or L-alanine no initial stimulation was seen, only the inhibitory phase, and the Hill coefficients for the ATP inhibition were 1. A decrease in the $I_{0.5}$ (ATP) occurred in the presence of either alanine ($I_{0.5}$ = 2.8mM) or fructose 1,6-bisphosphate ($I_{0.5}$ = 0.62mM) compared with that seen for the inhibitory phase obtained in the absence of effectors. The activation of the *C. maenas* enzyme is similar to that observed for the yeast enzyme, where a stimulation in activity was observed in the presence of low concentrations (2.5mM) of ATP (Haeckel *et al.*, 1968).

Activation by phosphoenolpyruvate in the presence of ATP and L-*alanine*

The interactions of phosphoenolpyruvate with the enzyme in a crude homogenate in the presence of the other allosteric modifiers of the enzyme are shown in Fig. 7. The results show that as the concentration of phosphoenolpyruvate is increased, the enzyme activity tends to the same value, irrespective of the presence of alanine or fructose 1,6-bisphosphate.

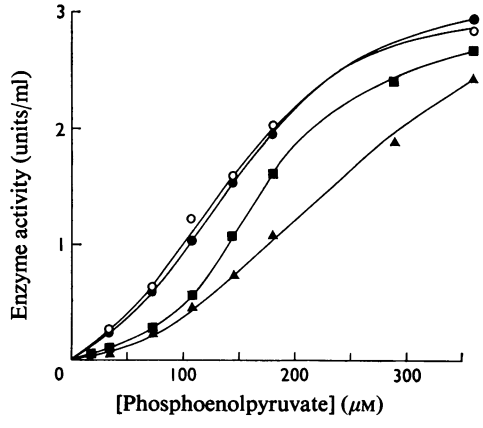

Fig. 7. *Actions of* L-*alanine and fructose* 1,6-*bisphosphate on the pyruvate kinase activity in a* 100000*g supernatant of C. maenas hepatopancreas when phosphoenolpyruvate was the variable substrate*

The enzyme was assayed as given in the text in the presence of 300μM-ADP and 700μM-ATP. The concentrations of L-alanine and fructose 1,6-bisphosphate used were: ○, 0mM and 2μM; ●, 1mM and 2μM; ■, 10mM and 2μM; ▲, 10mM and 1μM respectively. The Hill coefficients and $s_{0.5}$ (phosphoenolpyruvate) values calculated were: ○, 1.71 and 148μM; ●, 1.86 and 174μM; ■, 2.83 and 177μM; ▲, 1.83 and 384μM respectively.

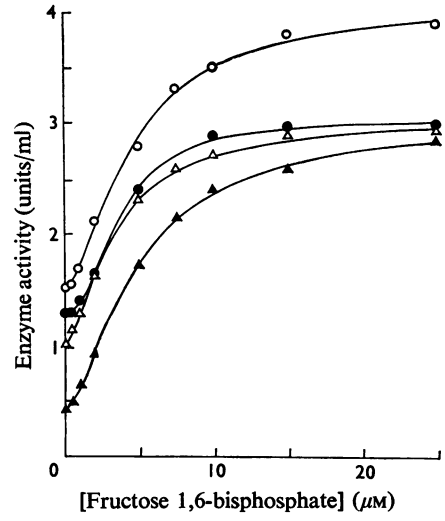

Fig. 8. *Actions of* L-*alanine and ATP on the activation of the pyruvate kinase activity in a* 100000*g supernatant of C. maenas hepatopancreas by the activator fructose* 1,6-*bisphosphate*

The enzyme was assayed as given in the text in the presence of 300μM-ADP and 144μM-phosphoenolpyruvate. The concentrations of L-alanine and ATP used were: ○, 0 and 0mM; ●, 0 and 700μM; △, 1mM and 700μM; ▲, 10mM and 700μM respectively. The Hill coefficients and the concentration of the activator that gave half-maximal activation were calculated as: ○, 1.62 and 4.55μM; ●, 2.17 and 3.71μM; △, 1.49 and 3.38μM; ▲, 1.56 and 4.73μM respectively.

Increasing the concentration of L-alanine, at a fixed fructose 1,6-bisphosphate concentration, resulted in an increase in the positive homotropic co-operative actions of phosphoenolpyruvate. A decrease in the fructose 1,6-bisphosphate concentration at a constant alanine concentration also led to an increase in the positive homotropic co-operative actions of phosphoenolpyruvate as measured by an increase in the Hill coefficient. An increase in the inhibitor concentration, or a decrease in the activator concentration, caused the $s_{0.5}$ (phosphoenolpyruvate) to shift towards a higher value.

Activation by fructose 1,6-*bisphosphate in the presence of ATP and* L-*alanine*

The effect of fructose 1,6-bisphosphate on the 100000*g*-supernatant enzyme at a constant concentration of phosphoenolpyruvate (144μM) and in the presence of ATP and L-alanine are shown in Fig. 8. In the absence of ATP and alanine, fructose 1,6-bisphosphate activated the enzyme with kinetics that exhibit positive homotropic co-operativity. The inhibition observed on addition of ATP to give an [ATP]/[ADP]

ratio of 7:3 could not be completely reversed by increasing the fructose 1,6-bisphosphate concentrations. At the same time, however, the positive co-operative interactions of fructose 1,6-bisphosphate with the enzyme increased as shown by the increasing Hill coefficient. Addition of alanine in the presence of ATP produced an additional inhibition that depended on the amount of the amino acid added. Increasing the concentration of the fructose 1,6-bisphosphate showed that saturation with fructose 1,6-bisphosphate was able to reverse the alanine inhibition, although the enzyme activity measured was less than that in the absence of ATP. In the presence of alanine, the enzyme appears to lose some of its co-operative interactions with fructose 1,6-bisphosphate, as seen by the decrease in the Hill coefficients.

Discussion

The first steady-state kinetic studies reported in the literature for the allosteric pyruvate kinases (Haeckel *et al.*, 1968; Rozengurt *et al.*, 1969) were explained by using the concerted model of allosteric control as suggested by Monod *et al.* (1965). The results of a more rigorous analysis of the interactions of fructose 1,6-bisphosphate, phosphoenolpyruvate and ATP with the yeast enzyme (Hess *et al.*, 1973) could not be adequately explained by this model, and the formation of a hybrid polymeric state of the enzyme, participating in parallel to the R–T equilibrium of the Monod *et al.* (1965) model, was proposed. The inclusion of this hybrid invalidated the symmetry assumption of the Monod *et al.* (1965) model and the mechanism proposed became a simple example of the sequential-conformational-change model of Koshland *et al.* (1966).

The theories of allosteric interactions (Monod *et al.*, 1965; Koshland *et al.*, 1966) as developed explain the binding properties of a protein. To date no direct physical binding studies have been conducted on the *C. maenas* pyruvate kinase from hepatopancreas. One reason for this, in addition to the technical difficulties that exist in obtaining sufficient protein, is that the results of such experiments would not be unambiguous, since it has been demonstrated that the kinetic properties of the enzyme in a crude tissue extract and after purification are different. Since the homogeneous protein is required for binding studies it is possible that the results would be different from those for the native enzyme. We were therefore compelled to use a kinetic approach to study the allosteric interactions of this enzyme. Such studies assume that the initial rate of the enzyme reaction is directly proportional to the fractional saturation of the enzyme. If this assumption is not valid the data become very difficult, if not impossible, to analyse. Nevertheless much of the information gathered on allosteric proteins in the literature has been implied from kinetic studies.

The change in the kinetic properties of the *C. maenas* enzyme on purification is intriguing and gives some credence to the suggestion that the sigmoidal kinetics observed are the result of subunit interactions. Irrespective of its purity the enzyme always showed positive homotropic kinetics with respect to phosphoenolpyruvate, and fructose 1,6-bisphosphate always activated the enzyme. The purified enzyme, however, gave hyperbolic saturation kinetics for the fructose 1,6-bisphosphate binding, whereas the crude enzyme gave a sigmoidal response to this ligand. Similar observations were made for the binding of the inhibitor L-alanine. That the homotropic co-operative interactions of fructose 1,6-bisphosphate and alanine alone were lost suggests that it is the transmission of the site–site interactions that are altered in the purified enzyme. These results suggest that phosphoenolpyruvate exerts its co-operative effect at a different site from that for fructose 1,6-bisphosphate.

A simple model to explain the co-operative kinetics of phosphoenolpyruvate binding involves the substrate binding at the active site and triggering a conformational change from this site. The apparently anomalous effects of ATP can be explained, without the assumption of a second allosteric binding site, if this ligand can induce a conformational change comparable with that produced by phosphoenolpyruvate by its binding to the active site (it is a product of the reaction). From this hypothesis it can be predicted that at a low concentration of phosphoenolpyruvate, and in the absence of other activators, increasing the concentration of ATP will cause an increase in enzyme activity as a result of the favourable subunit interactions. Increasing the concentration of ATP past an optimal value will cause a fall in the enzyme activity, as it acts as a product inhibitor. In the presence of the allosteric activator the enzyme will already be in the fully activated state and ATP will only show inhibition. Further, because of the nature of this inhibition, saturation with fructose 1,6-bisphosphate will not reverse it. In the presence of a high concentration of ATP the enzyme will be in the fully activated state and will not show an increase in enzyme activity on adding the allosteric activator fructose 1,6-bisphosphate. All these predictions were verified experimentally with the *C. maenas* enzyme. It appears unnecessary therefore to invoke a second inhibitory binding site for ATP to explain its actions. If this hypothesis is correct, it implies that the γ-phosphate group of ATP is involved in triggering the conformational change of the enzyme, as ADP exhibits no co-operative effects.

It is possible to determine the allosteric constants of an enzyme that can be described by the Monod *et al.* (1965) model by using the procedure developed by

Blangy *et al.* (1968) if the affinity of the two conformers of the enzyme for the substrate can be measured. The procedure has been applied to yeast pyruvate kinase (Hess *et al.*, 1973), by using fructose 1,6-bisphosphate and ATP to drive the enzyme into the two conformers, from which the non-exclusive binding constant was measured. It has been reported, however, that low concentrations of ATP activate the yeast enzyme (Haeckel *et al.*, 1968). If this activation occurs in a similar way to that suggested for the *C. maenas* enzyme, it is possible that the reported values of the allosteric constants may need revision, as the ATP could be causing the transition to the high-affinity, rather than the low-affinity, state.

Alanine is an inhibitor of the mammalian type-L pyruvate kinases (Seubert *et al.*, 1968), but little detailed information is available about its interactions with the enzyme and the effects that it manifests on the steady-state kinetics of the enzyme. The actions of alanine on the activity of the *C. maenas* enzyme are more complicated than those of the other ligands investigated. In the absence of fructose 1,6-bisphosphate and ATP an apparent value of 0.85 was obtained for the Hill coefficient when the concentration of L-alanine was varied (Fig. 5). If this value is a reliable estimate it would imply that the Monod *et al.* (1965) model is invalid for this enzyme, as negative homotropic co-operative interactions cannot be accommodated in this model; that is the Hill coefficient must be equal to, or greater than, 1 (Dalziel & Engel, 1968).

It is critical, therefore, to decide whether the Hill coefficient measured is significantly less than 1, as this will have important mechanistic implications. The value of the Hill coefficient was 0.85 when estimated by the method of Atkins (1973), but was near 1 when estimated by the classical graphical method of analysis (Koshland, 1970). This discrepancy arises from the different methods used to determine the enzyme activity at saturating concentrations of L-alanine, $v_{sat.}$, and in the error that exists in the difference between the inhibited (v_i) and uninhibited (v_0) rates. At low inhibitor concentrations this is a small difference between two large numbers, both of which are experimentally determined. Thus a large percentage error may be associated with this difference, the percentage error getting smaller as the difference gets larger. Significant error may be introduced, therefore, at both the low inhibitor concentrations (due to that in v_0-v_i) and at high inhibitor concentrations (due to that in $v_{sat.}$). Consequently the value of the slope of the Hill plot may show significant variation, depending on the method of analysis. The data available at the moment therefore do not indicate that the value of this Hill coefficient is significantly less than 1.

In the eventuality that this value is less than 1 the negative co-operativity can be explained either by the 'pseudoconservative' model of Viratelle & Seydoux (1975) or the sequential conformational-change model of Koshland *et al.* (1966). Negative co-operativity is seen in the latter model when the binding of a ligand hinders the binding of subsequent molecules of the ligand to the enzyme. Two well-documented enzymes that have been suggested to have this type of mechanism are glyceraldehyde 3-phosphate dehydrogenase (Conway & Koshland, 1968) and glutamate dehydrogenase (Engel & Dalziel, 1969).

Another anomalous observation of the alanine-inhibition experiments is that the alanine inhibition can be reversed by saturating with fructose 1,6-bisphosphate, whereas alanine will not reverse the activation by fructose 1,6-bisphosphate. A partial inhibition is occurring. Incomplete antagonism between allosteric activators and allosteric inhibitors has been reported for several enzymes and is well documented for *Escherichia coli* L-threonine deaminase (Changeux, 1962). Partial inhibitions are difficult to explain and no obvious explanation for the effects of the *C. maenas* enzyme can be suggested on the basis of the present results. The partial inhibition of L-threonine deaminase has been suggested to be due to a preferential non-exclusive binding of either or both of the ligands involved to the enzyme (Rubin & Changeux, 1966). The results for the *C. maenas* enzyme may be explained by a non-exclusive binding of the substrate that occurs in the presence of alanine, as extrapolation of the enzyme activity obtained to a saturating concentration of alanine gives a value greater than zero. An alternative explanation for this observation requires the existence of two types of binding site for fructose 1,6-bisphosphate. At one type, L-alanine can compete with the fructose 1,6-bisphosphate binding, whereas at the other only the activator can bind.

The physiological implications of the results presented are difficult to assess. The physical nature of the tissue and the fact that *C. maenas* possesses an open circulation means that perfusion studies of hepatopancreas are not feasible. As a result no reliable estimates of the rates of the glycolytic or gluconeogenic fluxes are available for this tissue. This leaves only a scant idea of the kind of enzyme-activity changes that may occur physiologically. It is evident, however, that at physiological concentrations of all the substrates and effectors the enzyme activity is likely to be considerably less than that obtained in optimal conditions (Giles *et al.*, 1976*b*).

The present studies can offer a possible explanation for the previous observation (Giles *et al.*, 1976*b*) that fructose 1,6-bisphosphate only activates the enzyme at a physiological [ATP]/[ADP] ratio in the presence of alanine. In the absence of alanine, the ATP favours formation of the high-affinity state of the enzyme, the form on which fructose 1,6-bisphosphate

has no action. In the presence of alanine, however, some of the enzyme may be in a low-affinity conformational state, the form which fructose 1,6-bisphosphate can activate. This finding illustrates the caution that must be exercised before the results of experiments *in vitro* can be meaningfully transposed to the situation *in vivo*. The simultaneous presence of modifiers can alter the response of an enzyme to variation of one of the ligands. The results in the present paper, however, do indicate that, in the presence of the modifiers studied, the enzyme activity can vary over a relatively wide range if there are concerted changes in the concentrations of the controlling ligands around their measured total tissue concentrations.

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