REVIEW OF THE COMPARATIVE BIOCHEMISTRY OF PYRUVATE KINASE

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INTRODUCTION

Pyruvate kinase (EC 2.7.1.40 ATP: pyruvate phosphotransferase) from mammalian sources functions predominantly in an aerobic environment. Basically two forms of the enzyme are found, one in gluconeogenic tissues which has allosteric properties; the other in non-gluconeogenic tissues which exhibits classical Michaelis–Menten kinetics.

By contrast, pyruvate kinase from many invertebrate sources functions in an oxygen-depleted or oxygen-free environment, while some invertebrates undergo alternate periods of aerobic and anaerobic metabolism, e.g. the intertidal bivalve molluscs. Adaptations to environments such as these have meant that under anaerobic conditions dramatic changes in the overall metabolism of the organism have taken place. Often little of the glycolytic end product, lactate, accumulates in the organism. This is associated with an alternative route for phosphoenolpyruvate metabolism such that additional reactions occur that produce ATP by substrate level phosphorylation of ADP. Obviously under conditions such as these the activity of pyruvate kinase must be diminished below that which is observed under aerobic conditions. Concomitantly alternative compound(s) must be produced that will act as an electron sink for the oxidation of NADH since the formation of pyruvate will no longer be stoichiometric with that of the reduced pyridine nucleotide.

PHYSICAL PROPERTIES

Isoenzymes

In mammals at least three distinct isoenzymes are found. The isoenzyme, type M, is found in brain, heart and skeletal muscle. It is electrophoretically and kinetically distinct from, but immunologically similar to, the type A isoenzyme which occurs in most other tissues except erythrocytes. Liver and kidney contain a third isoenzyme, type L, which is electrophoretically and immunologically distinct from type A but kinetically some properties are common to both isoenzymes (Tanaka et al., 1965; Imamura & Tanaka, 1972; Imamura et al., 1972). The erythrocyte pyruvate kinase probably represents a fourth isoenzyme (Carbonell et al., 1973).

Type L pyruvate kinase exhibits positive homotropic co-operativity with respect to phosphoenolpyruvate but not with ADP and it is activated by relatively low concentrations of fructose-1,6-biphosphate. Pig kidney type A isoenzyme is also activated by this ligand but in contrast to the type L pyruvate kinase it does not exhibit positive co-operativity with respect to phosphoenolpyruvate (Berglund & Humble, 1979). Rabbit muscle, type M isoenzyme is activated by high concentrations of fructose-1,6-biphosphate provided that the concentration of free Mg 2+ is less than 1 mM (Phillips & Ainsworth, 1977).

Pyruvate kinase from the adductor muscle of Mytilus edulis (de Zwann, 1972; de Zwann & Holwerda, 1972; de Zwann et al., 1975) and from mantle tissue (Livingstone & Bayne, 1974) both showed a sigmoidal saturation curve with respect to phosphoenolpyruvate and were both activated by fructose-1,6-biphosphate. Similar findings for the enzyme isolated from the hepatopancreas of Carcinus maenas have been reported (Giles et al., 1976a). Pyruvate kinase from a number of other invertebrates have been shown to be activated by fructose-1,6-biphosphate, these include locust (Bailey & Walker, 1969), cricket (Hoffman, 1975) and a number of platyhelminthes (Kohler, 1974; Carter & Fairbairn, 1975), while the enzyme from the trematode, Schistosoma mansoni, and the arctic blowfly, Protophornia terranovae were unaffected by this ligand (Brazier & Jaffe, 1973; Wood et al., 1977).

Pyruvate kinase from the leg muscle of the Alaskan king crab, Paralithodes camtschatica, gave one band on electrophoresis but existed as kinetically distinct forms at different temperatures. These variants were thought to be interconvertible with a change in temperature (Somero, 1969). The cold variant of the enzyme had hyperbolic kinetics with a minimum $K_m$ for phosphoenolpyruvate at 5°C while the "warm" variant had a minimum $K_m$ at 12°C and displayed a sigmoidal saturation curve for phosphoenolpyruvate. Thus these variants of the enzyme are not true isoenzymes in the conventional sense. Pyruvate kinase isolated from the hepatopancreas of C. maenas could also be separated into two distinct forms after chromatography on DEAE-cellulose (Giles et al., 1976a) but the interconversion of these two forms was dependent on the presence of fructose-1,6-bisphosphate suggesting that they were different conformers of the same protein.

Molecular weight

The molecular weight of rabbit muscle pyruvate kinase, type M, is generally accepted as 237,000 with a subunit molecular weight of 57,000 (Cottam et al., 1969) suggesting that the enzyme exists as a tetramer. Rat liver enzyme, type I, has been given as 208,000 (Tanaka et al., 1967b). The type A enzyme, isolated from pig kidney, gave a figure of 210,000 when estimated by the use of sedimentation equilibrium centri-
Mechanism of reaction

Pyruvate kinase from every animal source so far studied has shown a requirement for both a monovalent and a divalent metal ion. Physiologically, these are considered to be $K^+$ and $Mg^{2+}$ respectively, though their exact function in the overall catalytic mechanism is still uncertain despite considerable effort by many investigators.

A major area of confusion which delays the elucidation of the mechanism of pyruvate kinase is the nature of the true substrates of the reaction. Since $ADP^3-$ readily chelates $Mg^{2+}$ in solution (to a lesser extent this is true also of phosphoenolpyruvate$^3-$) it is uncertain whether MgADP or $ADP^3-$ and $Mg^{2+}$ and/or both species bind to the protein. Though with the yeast, pig liver and rabbit muscle enzyme evidence has been presented that the true substrates of the enzyme are $ADP^3-$, PEP$^3-$ and $Mg^{2+}$ (Macfarlane & Ainsworth, 1972, 1974; Ainsworth & Macfarlane, 1973).

Phosphoryl transfer from phosphoenolpyruvate to ADP catalysed by the type M rabbit muscle pyruvate kinase has been shown to occur by a direct transfer mechanism (sequential) and not via a stable phosphorylated enzyme intermediate (non-sequential or ping-pong) (Reynard et al., 1961).

The type L enzyme from liver shows a sigmoidal relationship between initial velocity and the concentration of phosphoenolpyruvate unless it is activated by the addition of relatively low concentrations of fructose-1,6-bisphosphate. This brings about a lowering of the $S_{0.5}$ value for phosphoenolpyruvate. Evidence obtained by steady state kinetics for the pig liver enzyme indicated that the mechanisms may be of the ping-pong type (Macfarlane & Ainsworth, 1974). Isotopic exchange studies on pyruvate kinase isolated from rabbit liver (Dunn & Britton, 1977) failed to demonstrate an exchange between $[2-^3H]ADP$ and ATP or phosphoenol-$[1-^{13}C]pyruvate$ and pyruvate suggesting that the reaction does not proceed via a phosphorylated enzyme intermediate.

The mechanism of reaction of the type M rabbit muscle pyruvate kinase has been designated as a rapid equilibrium random type mechanism (Reynard et al., 1961; Ainsworth & Macfarlane, 1973) on the basis of steady-state initial velocity measurements. This type of mechanism is characterized by the random addition of substrates and release of products from their respective enzyme complexes with which they are in thermodynamic equilibrium, a consequence of the interconversion of the central complexes being the rate limiting step of the reaction mechanism. Type M pyruvate kinase isolated from the pincer and leg muscle of $C. maenas$ gave product inhibition patterns in which both ATP and pyruvate were competitive against the variable substrate, phosphoenolpyruvate (Newton et al., 1976a). These observations suggest the random release of products from the central complex and is consistent with the rapid equilibrium random type mechanism. Type L pyruvate kinase isolated from $C. maenas$ hepatopancreas gave initial rate data and product inhibition patterns which suggested that the mechanism of reaction had many similarities to that proposed for the $C. maenas$ muscle enzyme (Giles et al., 1976c).

However, the validity of assigning this mechanism to pyruvate kinase is contentious. Data showing that the release of pyruvate from the enzyme could be rate limiting in the forward reaction was obtained from studies of the rate of detrinitiation of pyruvate catalysed by rabbit muscle pyruvate kinase (Robinson & Rose, 1972). The rate of exchange of tritium from tritiated water into phosphoenolpyruvate also was limited, possibly by the release of ADP or phosphoenolpyruvate from the enzyme.

Giles et al. (1976c) studied the kinetics of rabbit muscle pyruvate kinase but in the reverse direction. The possibility that the reaction was not completely rapid equilibrium was investigated by measuring the initial rates of isotopic exchange for five of the six possible exchange reactions at chemical equilibrium. In a truly rapid-equilibrium random reaction all the exchange rates should be identical. The rate of isotopic exchange between a substrate-product pair in the forward and reverse directions gave identical values, indicating that the system was at chemical equilibrium. However, when the initial rates for the different exchanges were determined under two sets of equilibrium conditions for any substrate-product pair it was observed that in both conditions the phosphoenolpyruvate-pyruvate exchange was the slowest. This result suggested that the steps involving binding and/or release of pyruvate contribute towards the rate determining step(s) of the reaction, which directly supports the conclusions reached by Robinson & Rose (1972).

Another feature of the pyruvate kinase reaction is the apparent ease with which the enzyme forms dead-end complexes. The existence of the dead-end complex, enzyme-ADP-pyruvate, has been suggested many times for enzymes isolated from both vertebrate and invertebrate sources. There are relatively few reports on the existence of substrate inhibition. Jan-
with rabbit muscle pyruvate kinase at high concentrations of ADP. The existence of two types of binding sites for ADP had previously been suggested for this enzyme as a consequence of kinetic protection studies (Mildvan & Cohn, 1966). More recently the X-ray crystallographic studies of cat muscle enzyme have directly indicated the presence of two ADP binding sites (Stammers & Muirhead, 1975).

Giles et al. (1976c) in their study of the reverse reaction catalysed by the rabbit muscle enzyme obtained direct evidence for substrate inhibition of the enzyme by ATP.

Substrate inhibition for both the type M and L enzyme from C. maenas, with respect to both ADP and phosphoenolpyruvate has been reported (Newton et al., 1976b; Giles et al., 1976c). In addition for the type L C. maenas enzyme the multiple inhibition of the reaction by ATP (Giles & Post, 1980) would be consistent with substrate inhibition by ATP if the reaction were studied in the reverse direction. In fact with this enzyme the only reactant not exhibiting substrate inhibition was pyruvate. Thus it is possible to speculate that for substrate inhibition to occur, the presence of a phosphate group is required. Evidence, though not conclusive, has been obtained supporting the existence of the mixed substrate product dead-end complex of the type enzyme–ATP–ADP with both type L and type M enzymes isolated from C. maenas (Giles et al., 1976c; Newton et al., 1976a).

The physiological control of pyruvate kinase

The identification of control sites in glycolysis has depended on a variety of experimental approaches. These include comparison of the mass-action ratio of a reaction (calculated from the intracellular steady state metabolite concentrations) with its thermodynamic equilibrium constant, observing changes in the concentrations of intermediates on initiating a metabolic change, measuring maximal enzyme activities in a cell extract, and studying the physical and kinetic properties of the component enzymes (for a more detailed discussion see Newsholme & Start, 1973).

As is to be expected most of these studies have been applied to mammalian species and the results suggest that phosphofructokinase and pyruvate kinase are prime regulatory sites (Scrutton & Uter, 1968). Phosphofructokinase has been implicated as being the rate-limiting enzyme of glycolysis, a role favoured by both its potential regulatory characteristics and the fact that it is the one enzyme that is unique to glycolysis. The physiological significance of regulation of pyruvate kinase (one of the last enzymes of glycolysis) has been much greater than one.

Comparison of the mass-action ratio with the equilibrium constant for the pyruvate kinase reaction in those species with a ratio greater than one, however, indicated that within the cell the reaction is non-equilibrium (Beis & Newsholme, 1975). Therefore, in order for the enzyme to catalyse a non-equilibrium reaction in vitro the activity of the enzyme must be considerably less than the observed maximal activity obtained in optimal conditions in vivo. This can result from decreases in concentrations of the substrates phosphoenolpyruvate and ADP, increases in the concentration of one (but not both) of its products, or by allosteric modifiers. As the reaction is taken to be a non-equilibrium reaction in vitro it cannot be the result of changes in the concentrations of both its products.

In the few species (e.g. Ascaris lumbricoides, Ostrea edulis and M. edulis) that have a ratio of pyruvate kinase to phosphofructokinase activities that is less than one the low ratio is the consequence of a lower activity of pyruvate kinase. Indeed, it may be the low enzyme activity which is creating the non-equilibrium state in these species. Some form of control on pyruvate kinase in these species is still required however to allow the transition from aerobic metabolism utilising glycolysis and the tri-carboxylic acid cycle to an anaerobic metabolism utilising the "succinate" pathway (Awapara & Simpson, 1967; Saz, 1971).

Hence if adequate control were not exerted, it was argued that a substrate cycle could develop preventing efficient gluconeogenesis. As has been mentioned earlier a distinct form of pyruvate kinase (the type L isoenzyme) capable of allosteric interactions is found in mammalian gluconeogenic tissues, whilst the non-allosteric type M isoenzyme is found in non-gluconeogenic tissues.

Control of muscle pyruvate kinase

Cross-over type experiments (Chance et al., 1958) have indicated that the pyruvate kinase reaction is a potential control site in glycolysis. This has been found to be true for many species including the following invertebrates; the Alaskan King Crab (Hochachka et al., 1971), the blowfly Phormia regina (Hansford, 1978), Urechis unicintus (Yamasu et al., 1975) and Crassostrea gigas (Yasumatsu et al., 1975). Muscle from a large number of different species (both vertebrate and invertebrate) has been shown to contain large amounts of pyruvate kinase when measured by the maximal enzymic activity observable in tissue extracts (Zammit et al., 1978). The information for these species allowed calculation of the ratio of the maximal activity of pyruvate kinase to that of phosphofructokinase (the proposed rate-limiting enzyme of glycolysis). In the vast majority of cases this ratio was much greater than one.

The factors that contribute to the low activity of pyruvate kinase in mammalian skeletal muscle have not been fully established. It has been shown that phenylalanine inhibits the enzyme from rabbit muscle (Carminatti et al., 1971) but only at concentrations far in excess of those expected within the cell. A more likely mediator of activity is phosphocreatine. This has been shown to inhibit the rabbit muscle enzyme at physiological concentrations (Kemp, 1973). Reports of inhibition by NADH (Weber et al., 1965; 1967a).
and acetyl-CoA (Weber et al., 1967b) have been shown to be artefactual (Ibsen & Trippet, 1973; Seubert et al., 1968).

The present explanation of the low activity of pyruvate kinase expressed within the mammalian muscle cell is the inhibition by phosphocreatine coupled with low intracellular concentrations of substrates. In these conditions considerable product inhibition by ATP is likely to occur. The normal physiological concentrations of ADP and phosphoenolpyruvate are of the same magnitude as their apparent $K_m$ values whilst the ATP concentration is high compared to its apparent $K_m$ value (0.14--0.9 mM) (Dyson et al., 1975; Giles et al., 1976c).

The pyruvate kinase reaction requires Mg for activity. Without concerning ourselves here as to why Mg is required it is a possibility that changes in the intracellular free Mg$^{2+}$ concentration could cause changes in the activity of the enzyme. The regulatory significance of changes in free Mg$^{2+}$ within the cell have been discussed by Ainsworth & Phillips (1976). These authors conclude that the regulatory significance of such changes will depend on the nature of the enzyme mechanism involved. With regard to rabbit muscle pyruvate kinase changes in free Mg$^{2+}$ can only influence the metabolic activity to a limited extent. The changes are likely to result solely from a redistribution of the various substrate-species present.

The activation of rabbit muscle pyruvate kinase by fructose-1,6-bisphosphate at low (0.1 mM) concentrations of Mg$^{2+}$ (Phillips & Ainsworth, 1977) is unlikely to have any physiological significance, as full activation only occurs at unphysiologically high concentrations of fructose-1,6-bisphosphate (>1 mM). This observation, however, is interesting in that it provides a link to several other vertebrate and invertebrate species whose muscle pyruvate kinase is activated by fructose-1,6-bisphosphate at physiological levels (Zammit et al., 1978).

This last study showed fructose-1,6-bisphosphate activation of pyruvate kinase from the muscle of fish, amphibia (except for skeletal muscle of the frog and toad) and reptiles in conditions approaching the physiological state. No activation was seen with either avian or mammalian muscle enzymes. All the marine invertebrates investigated in this study showed stimulation, whereas no activation was observed for the enzyme from insect flight muscle. Activation of muscle pyruvate kinase of other marine invertebrates have been reported (M. edulis, Orconectes limosus, C. maenas) (M. edulis, Orconectes limosus, C. maenas) (Mustafa & Hochachka, 1972; de Zwaan & Holwerda, 1972). Thus the sensitivity of the enzyme to fructose 1,6-diphosphate appears to be present in most invertebrate muscles. The exceptions include the enzymes isolated from insect flight muscle (Zammit et al., 1976; Hoffman, 1977; Bailey & Walker, 1969), the squid (Storey & Hochachka, 1975), the octopus (Guderley et al., 1976a), the pelagic shrimp Oplophorus gracilis (Guderley et al., 1976b), the crayfish Orconectes limosus (Lesick, 1976) and the crab C. maenas (Newton, 1977).

Zammit et al. (1978) suggested that the stimulation of pyruvate kinase enzyme activity by fructose-1,6-bisphosphate occurs in those invertebrate muscles in which the oxygen supply is poor. In such muscles the change in the rate of glycolysis between rest and activity needs to be large enough to provide sufficient energy for the requirements of the organism. In tissues with a poor oxygen supply this extra energy will have to come from an energetically less efficient anaerobic pathway. Therefore, in these species the activation of pyruvate kinase by fructose-1,6-bisphosphate may provide a feed-forward mechanism to ensure that the activity of pyruvate kinase is increased in relation to the activity of phosphofructokinase. Furthermore, in those invertebrates muscles that utilize the "succinate" pathway for anaerobic metabolism, such a feed-forward activation may create an initial burst of pyruvate kinase activity at the onset of anaerobiosis. This initial activation could, in its turn, permit the accumulation of alanine which is thought to be essential for the dual regulation of phosphoenolpyruvate carboxykinase and pyruvate kinase in these muscles (Zammit & Newsholme, 1976; Mustafa, 1972, 1973; de Zwaan, 1972; de Zwaan et al., 1976; Hochachka, 1976).

Amongst the invertebrate species there have been several reports of inhibition of pyruvate kinase by the invertebrate equivalent of phosphocreatine, phosphoarginine (Guderley et al., 1976b; Wieser, 1977; Wieser & Lackner, 1977). In the snail Helix pomatia it was found that the activity of pyruvate kinase in a crude extract was less in the presence of phosphoarginine. The inhibition was not competitive in nature and it was suggested that the inhibition was modulated by a protein in the extract that transfers either the phosphagen or a phosphate group onto the enzyme (Wieser & Lackner, 1977). Subsequent studies on the crude enzyme from M. edulis (de Zwaan & Ebberink, 1978) lead these authors to suggest that the apparent inhibition by phosphoarginine is probably due to a side reaction involving arginine kinase. The results of experiments using extensively purified phosphoarginine and the type M pyruvate kinase from the walking leg and pincer muscle of C. maenas has confirmed this view (Poat et al., 1980). These workers showed that it was only in the presence of trace amounts of arginine kinase that any inhibition was seen. Indeed sufficient arginine kinase was found to survive the normal purification procedures used to isolate phosphoarginine that addition of this phosphoarginine resulted in an apparent inhibition. A heat inactivation step was required in the protocol before phosphagen, free of arginine kinase, could be prepared. The apparent inhibition was ascribed to a side reaction involving arginine kinase and phosphoarginine resulting in ADP depletion.

The factors that affect the activity of the enzyme from M. edulis have been investigated by a number of workers using crude tissue extracts (de Zwaan, 1972; de Zwaan & de Bont, 1975; de Zwaan et al., 1975; Holwerda et al., 1973; Livingstone & Bayne, 1974). This enzyme shows sigmoidal kinetics with respect to phosphoenolpyruvate and hyperbolic saturation with respect to ADP. The enzyme is activated by fructose-1,6-bisphosphate and inhibited by L-alanine and ATP; the inhibitions being countered by fructose-1,6-bisphosphate. These effects are all pH-dependent, such that at lower pH values fructose-1,6-bisphosphate stimulation is increased. In many respects this muscle enzyme has characteristics similar to those found for the mammalian type L enzyme.
The effect of pH on fructose 1,6-bisphosphate stimulation, however, is the opposite to that found for either the mammalian type L enzyme (Rozengurt et al., 1969) or yeast enzyme (Wiekher & Hess, 1971). As described above these particular properties may be of use to this species in the transition from aerobic metabolism of glucose to the anaerobic "succinate" pathway.

Control of pyruvate kinase from gluconeogenic tissues

The most intensively studied pyruvate kinase of this kind is the type L isoenzyme from mammalian liver. Rapid changes in the activity of this enzyme can be readily explained as this isoenzyme is activated by both the glycolytic intermediate fructose-1,6-bisphosphate and by its substrate phosphoenolpyruvate (Taylor & Bailey, 1967; Tanaka et al., 1967a; Rozengurt et al., 1969). In addition, ATP and alanine inhibit the enzyme at physiological concentrations (Suebert et al., 1968; Lorette et al., 1970). Over a longer time scale control may be exerted by altering the amount of active protein present in the cell. It has recently been shown that a reversible phosphorylation–dephosphorylation mediated by a cyclic-AMP-dependent protein kinase can modulate the activity of this isoenzyme (for a recent review see Engstrom (1978)). No such inhibition of a type M isoenzyme has yet been demonstrated.

The observation that this phosphorylation is stimulated by glucagon (van Berkel et al., 1977; Feliu et al., 1977; Ishibashi & Cottam, 1978) lead to the suggestion that control of this enzyme is related to the entry of precursors into gluconeogenesis and not to the rate of glycolysis. There is evidence for the in vivo existence of a substrate cycle involving the reactions catalysed by pyruvate kinase, pyruvate carboxylase and phosphoenolpyruvate carboxykinase (Rognstad & Katz, 1977). The measurements involved in establishing such a cycle are technically complex and there has been much discussion of the validity of the results obtained (Katz & Rognstad, 1976). If it does transpire that there is a functional cycle operating in vivo then inhibition of pyruvate kinase would decrease the rate of the cycling. This would allow an increased production of phosphoenolpyruvate, and enhanced gluconeogenesis would follow.

A more recent suggestion is that the control of pyruvate kinase in mammalian liver by phosphorylation–dephosphorylation is closely related to the regulation of fatty acid synthesis from glycogen, rather than to the control of gluconeogenesis (Hopkirk & Bloxham, 1979). The liver is a major site of fatty acid synthesis (Braynerger et al., 1973) and the rates of fatty acid synthesis can exceed those of gluconeogenesis (Clark et al., 1974). Experiments have indicated that although glucose is a poor precursor for fatty acids (Salmon et al., 1974; Hems et al., 1975) that glycogen depletion is correlated with fatty acid synthesis (Woods & Krebs, 1971; Hems et al., 1975). In spite of the fundamental problems that exist in explaining these observations the implication is that glycogen (but not glucose) is a good precursor for fatty acid synthesis. In this situation pyruvate kinase could have a major influence on the rate of passage of glycosyl units (from glycogen) into fatty acids.

A development of the control of the enzyme by phosphorylation–dephosphorylation has recently been proposed (Hall et al., 1979). This proposal stems from the observation that the specific activity of the enzyme isolated from the livers of rats fasted for 72–84 hr is approximately one third of that of the enzyme isolated from animals fed a high carbohydrate diet. These results are extensions of earlier studies which showed that the total pyruvate kinase content of rat liver varies with diet (Krebs & Eggleston, 1965; Tanaka et al., 1965; Bailey et al., 1968). The current observations, therefore, suggest that this variation in activity is the result of a change in the specific activity of the protein, not necessarily the amount. Subsequent experiments (Hall et al., 1979) indicated that the enzyme is susceptible to a proteolytic cleavage, or limited digestion, causing inactivation of the enzyme. It would appear that only the phosphorylated form of the enzyme is susceptible to this irreversible proteolytic modification. Thus the phosphorylation system may be involved in several ways in altering the amount of active enzyme within the cell.

The long-term control of activity of the enzyme in the invertebrates has not really been investigated yet. It is known, for example, that there are changes in total tissue content of pyruvate kinase, and of glycogen, in response to seasonal or other environmental factors (de Zwaan, 1970; Livingstone & Bayne, 1974; Wieser & Wright, 1979; Lesicki, 1977). The existence of a cyclic-AMP-dependent phosphorylation system in any but mammalian species has not been reported for this enzyme. It would be mentioned, however, that in at least one species of crab (Eriocheir sinensis) variations in cyclic-AMP levels have been measured in response to changes in the external salinity (Schoffeniels, 1976). It is a real possibility, therefore, that a similar mechanism operates in these species. This will obviously be an area of active research in the future.

The kinetic control of the enzyme from the hypodermis of Cancer magister (Guderley, 1976; Guderley & Hochachka, 1977) and the hepatopancreas of C. maenas (Giles et al., 1975, 1975a, b, 1977) have been investigated in some detail. In the latter species it is known that there are at least two distinct isoenzymes, depending on the tissue used to isolate the enzyme. The pincer and walking-leg muscles contain a type M isoenzyme, whilst the hepatopancreas contains a type L isoenzyme. This is a tissue distribution reminiscent of that found in mammals. The pyruvate kinase from both of these species is activated by fructose 1,6-bisphosphate at low concentrations of phosphoenolpyruvate, and inhibition is shown on addition of ATP or alanine. Using physiological concentrations of the substrates and effectors the effective activity of the hepatopancreatic enzyme was decreased to less than 5% of the potential activity present (Giles et al., 1976b). From these and other studies (Giles et al., 1977) the authors concluded that pyruvate kinase in hepatopancreas can be regulated by changes in concentration of phosphoenolpyruvate and fructose-1,6-bisphosphate. The latter, however, would only modulate the activity in the presence of a continuous inhibition exerted by ATP and alanine.

In the continued presence of ATP (i.e. the physiological situation) the allosteric inhibitor alanine had to be present before fructose 1,6-bisphosphate activation was seen. This contrasts to the potent acti-
vation seen in a simpler assay system containing only ADP and phosphoenolpyruvate. It was proposed that this difference arises because ATP, like phosphoenolpyruvate and fructose-1,6-bisphosphate, causes conversion of the enzyme to its fully active conformer (Giles et al., 1977). It was found experimentally that at low phosphoenolpyruvate concentrations and in the absence of fructose-1,6-bisphosphate addition of ATP caused an initial increase in enzyme activity. Only at higher concentrations was inhibition observed. A similar response has been reported for the yeast enzyme (Haeckel et al., 1968).

A closely related observation was that in the presence of physiological concentrations of ATP and ADP but absence of fructose-1,6-bisphosphate no homotropic co-operativity was seen on varying phosphoenolpyruvate. This contrasts to the marked co-operativity seen in the absence of ATP. All these observations are consistent with the thesis that ATP causes transition to the fully active conformer of the enzyme. As ADP shows no co-operative interactions the implication is that it is the γ-phosphate of ATP that is involved in the conformational change. It is this phosphate group that is transferred from phosphoenolpyruvate, and as the enzyme has a sequential mechanism will occupy the same locus on the enzyme.

It is clear, therefore, that it will only be in the presence of an allosteric inhibitor, alanine, that activation could be seen in normal physiological conditions. Interestingly the I0.5 values for alanine obtained for the C. maenus enzyme are considerably larger (≥ 1 mM) than those measured for the mammalian enzyme (≥ 100 μM, Llorente et al., 1970). This is consistent with the higher concentrations of alanine found in this euryhaline species.

A potential complication is the known variation of alanine concentration in C. maenus in response to external salinity (Duchateau et al., 1959). There is, however, little difference in the mass-action ratios for the pyruvate kinase reaction between animals acclimated to either 50 or 100% seawater (Poat & Giles, unpublished observations). As this implies little variation of the in vivo pyruvate kinase activity, compensatory changes must occur in the remaining ligands. It is unlikely on this evidence for the changes that are thought to occur in C. maenus metabolism on variation of salinity to be the result of alteration of the activity of pyruvate kinase intracellularly.

CONCLUSIONS

Comparative studies, either between tissues on one species or between species, have contributed a great deal to our knowledge of the way that pyruvate kinase functions, and of the role that it plays within the cell. There are still many questions to be answered, but the direction that future studies will take can be seen. Two such possibilities follow. The first area will be the further investigation of the cAMP-dependent phosphorylation–dephosphorylation process. These studies may well indicate further mechanistic studies on how it operates, investigations into those species that possess such a control system. One possible development resulting from this type of study would be an appreciation of when, in evolutionary terms, this type of control originated. The second area for further study is to find a satisfactory explanation to account for the low activity of the enzyme that is expressed in muscle cells.

The conclusion of the comparative studies that have been conducted to date show that there is a less clear demarcation between the M type and L type isoenzymes in invertebrates. Thus many M type isoenzymes from these species show activation of fructose-1,6-bisphosphate. It is significant, therefore, that recent X-ray crystallographic evidence (Stuart et al., 1979) has demonstrated a binding site for ADP in a binding pocket between two domains that is distant from the active site. As the type L isoenzyme is subject to allosteric regulation by nucleotides an obvious inference is that this is a vestigial binding site for allosteric effectors. The position of the ADP site is compatible with it being able to influence the transmission of co-operative conformational changes from one active site to another. This interpretation is reinforced by the demonstration of fructose-1,6-bisphosphate activation of the rabbit muscle enzyme at very low Mg2+ concentrations (Phillips & Ainsworth, 1977).

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