The Control of Pyruvate Kinase of *Escherichia coli*. Binding of Substrate and Allosteric Effectors to the Enzyme Activated by Fructose 1,6-Bisphosphate†

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ABSTRACT: The binding of various regulatory ligands and substrates to the fructose bisphosphate activated pyruvate kinase from *Escherichia coli* has been studied at equilibrium. The allosteric activator, fructose bisphosphate, and the substrate phosphoenolpyruvate bind in a cooperative manner to the enzyme. There is one site for each of these ligands per monomer. In the presence of fructose bisphosphate the binding of phosphoenolpyruvate follows an absorption isotherm, i.e., all homotropic interactions of the substrate are lost. In reciprocal experiments, however, *both* phosphoenolpyruvate and KCl are required in order to facilitate binding of the activator. The allosteric inhibitors of pyruvate kinase, ATP, succinyl-CoA, and GTP compete on the enzyme surface with the binding of the activator, fructose bisphosphate. Inhibitor pairs such as ATP and succinyl-CoA together bring about not cooperative but only additive inhibition of the binding of the activator. The nucleotide substrate GDP and the allosteric inhibitor GTP have in contrast to the activator two seemingly noninteracting sites on each monomer. In the saturating presence of fructose bisphosphate, however, binding of GDP and possibly also of GTP occurs at only one site on each monomer. Magnesium ions inhibit binding of GDP and GTP. KCl which is an activator of the enzyme along with its analogues, such as ammonia, thallium, rubidium, etc., enhances the binding of phosphoenolpyruvate but not of the nucleotides or fructose bisphosphate. The data are analyzed on the basis of a two-site model, where the substrate and fructose bisphosphate bind to one conformation and the inhibitors to the other.

Two allosteric pyruvate kinases are known to exist in *Escherichia coli* (Sanwal, 1970; Waygood and Sanwal, 1974, 1975), one activated by fructose bisphosphate[1] (Waygood and Sanwal, 1974) and another by ribose 5-phosphate and several purine and pyrimidine nucleoside monophosphates (Waygood and Sanwal, 1974). The kinase activated by FDP has been purified to homogeneity, and some of its physicochemical characteristics have been described (Waygood and Sanwal, 1975). It is a tetrameric protein with a molecular weight of 240000 g/mol, and is apparently composed of identical monomers weighing 60000 g/mol. The enzyme activity has been shown to be susceptible to modulation by several effectors, both positive and negative (Waygood and Sanwal, 1971, 1972). In addition to FDP which acts as a positive effector, the enzyme is inhibited by ATP, GTP, and succinyl-CoA (Waygood and Sanwal, 1972). There are remarkable sets of interactions in the regulation of enzyme activity between the negative effectors on one hand and between the various positive and negative effectors on the other (Waygood and Sanwal, 1971, 1972). For instance, the percentage inhibition of the activity of the enzyme in the presence both of succinyl-CoA and ATP is much higher than the sum of percentages of inhibition in the presence of each inhibitor alone. This cooperative modulation has been shown to occur with other enzyme systems, both with regard to positive (Smando et al., 1974) as well as negative effectors (Caskey et al., 1964). In addition to these interactions, some monovalent cations profoundly affect the activity of FDP-activated kinase. In order to elucidate the molecular mechanism by which effectors, ions, and effector pairs influence the activity of the enzyme we decided to investigate the characteristics of binding of the various substrates and regulatory ligands at equilibrium to the FDP-activated pyruvate kinase. These studies, it was expected, would also contribute to a clarification of the mode of action of allosteric enzymes in general.

Materials and Methods

Enzyme Preparation and Assay. Pyruvate kinase was prepared essentially as described earlier (Waygood and Sanwal, 1974) with only minor modifications. The enzyme was judged to be in a high state of purity when only one band was obtained by electrophoresis on dodecyl sulfate polyacrylamide gels. All preparations utilized in various experiments had a specific activity of 48 μmol of P-enolpyruvate utilized per mg of protein per min. The enzyme was assayed by measuring the change in absorbance at 340 nm using a Gilford 2400 recording spectrophotometer. The assay mixture contained in 1 ml, 1.33 μmol of ADP, 1 μmol of PEP, 10 μmol of $MgCl_2$, 0.15 μmol of NADH, 50 μg of lactate dehydrogenase, and 0.03 *M* Hepes buffer. The concentration of the protein was determined by absorbance readings at 280 nm ($E_{1\,cm}$(1%) 1.8).

Radioactive Ligands. [^{14}C]FDP (specific activity 496 Ci/mol), [^{3}H]GDP (specific activity 1.43 Ci/mmol), and [^{14}C]PEP (specific activity 6 Ci/mol) were obtained from New England Nuclear. [^{14}C]GTP (specific activity 486 Ci/mol) and [^{14}C]pyruvate (specific activity 7 Ci/mol) were obtained from Amersham-Searle. Before use all radioactive

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[1] Abbreviations used are: FDP, fructose 1,6-bisphosphate; PEP, phosphoenolpyruvate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

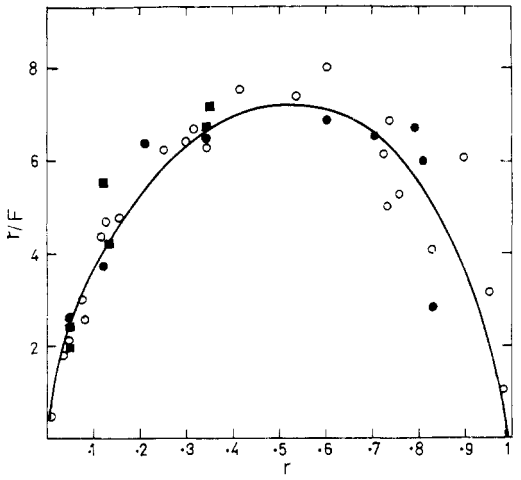

FIGURE 1: Binding of FDP to pyruvate kinase. Equilibrium dialysis method was used. r refers to the number of moles of FDP bound per mole (molecular weight 60000) of subunit, and F refers to the concentration of free FDP (mM). (○) 0.13 mM pyruvate kinase subunit; (●) 0.09 mM subunit and 1 mM PEP; (■) 0.09 mM subunit plus 1 mM PEP and 5 mM $MgCl_2$. The solid line is drawn from fits to the concerted model of Monod et al. (1965) with K_R (dissociation constant of FDP from the high affinity state of the protein) = 0.026 mM ± 0.002 mM, K_T (dissociation constant of FDP from the low affinity state of the protein = 0.5 ± 0.1 mM, and the dimensionless allosteric constant of 68 ± 25. The Hill number is 2.4.

ligands were tested chromatographically for the presence of any contaminants. None were found.

Chemicals and Materials. Pyruvate and lactate dehydrogenase (Type III, beef heart) were obtained from Sigma. PEP (cyclohexylammonium salt) and Hepes were obtained from Calbiochem. ATP, GTP, GDP, and FDP were obtained from Boehringer-Mannheim. Succinyl-CoA was obtained from P.L. Biochemicals. The dialysis tubing was obtained from Fisher Scientific. The Paulus ultrafiltration cell and PM-10 filters were obtained from Metaloglass Inc. All other chemicals were reagent grade.

Binding Studies. Binding of various ligands to the enzyme was measured using two different procedures, equilibrium dialysis and ultrafiltration. Equilibrium dialysis was carried out according to the procedure described by Myer and Schellman (1962) using dialysis cells capable of holding 0.25 ml of solution in each chamber. One chamber contained 0.1 ml of the pyruvate kinase solution in 0.1 M Hepes (pH 7.0) and the other 0.1 ml of various concentrations of radioactive ligands. Other cofactors or ligands whose effect upon the binding was to be tested were added to both chambers bringing the volume to 0.11 ml in each chamber. All solutions were made up in sterile 0.1 M Hepes (pH 7.0). The Visking tubing used to separate the two chambers was boiled before using successively in 0.1 M sodium bicarbonate, double distilled water, and 2 mM EDTA. The tubing was stored in cold (4°C) double distilled water. During an equilibrium dialysis run the cells were rotated in a vertical plane in order to mix the fluid in each chamber and thereby achieve rapid equilibrium. Dialysis was carried out at 4°C for about 18 hr. All ligands were found to have equilibrated across the membrane by this time and the enzyme had retained full enzyme activity. At the end of the run two 0.02-ml samples were routinely withdrawn from each compartment. The radioactivity was measured in glass vials in 5 ml of Aquasol in a liquid scintillation spectrometer. No significant quenching was observed in the presence of protein and various ligands.

In the ultrafiltration procedure (Paulus, 1969), 0.1 ml of a mixture of the enzyme, required ligands, and buffer was equilibrated for 10–15 min at room temperature and then filtered at 40 psi through prewashed (12 hr in double distilled water) PM 10 membranes held in an ultrafiltration cell. When all the solution had clearly passed through the filter, its underside was washed twice with 3 ml of ethylene glycol. The pressure was then released and the filters were counted in Aquasol in a liquid scintillation spectrometer. Blank filters were also counted in which the enzyme solution had been replaced by buffer. For most of the radioactive ligands, the blanks were very constant, and averaged at 2.8 ± 0.03% of the input counts. Phosphoenolpyruvate, for unknown reasons, however, gave blanks of 4.5 ± 0.5% of input counts. The blanks were subtracted from the experimental values before analyzing the data.

Data Analysis. The binding data were analyzed according to Scatchard et al. (1957) using the equation

$$r/F = rk + nk$$

where F is the free concentration of ligand, r is the number of moles of F bound per mole of enzyme subunit with n being the limiting value of r, and k is the association constant. All straight line plots were treated by the method of least squares to obtain best fit to the data. Wherever feasible, nonlinear Scatchard plots were analyzed by curve fitting procedures suggested by Cornish-Bowden and Koshland (1970).

Results

Kinetic Studies

Activation by Monovalent Cations. It had been reported earlier (Waygood and Sanwal, 1974; Maeba and Sanwal, 1968) that the enzyme does not require monovalent cations for activity—an observation at variance with results obtained with pyruvate kinases from other sources (Suelter et al., 1966; Hess and Haeckel, 1970; Tuominen and Bernlohr, 1971). This erroneous conclusion with regard to the *E. coli* pyruvate kinase activated by FDP has been traced to the use of undialyzed lactate dehydrogenase as a coupling enzyme in pyruvate kinase assay. When extensively dialyzed lactate dehydrogenase is used, a requirement for NH_4^+ or another monovalent cation becomes obvious. Apart from NH_4^+, K^+, Tl^+, Rb^+, Cs^+, Li^+, and Na^+ activate the enzyme. The K_m's using the standard assay for K^+, NH_4^+, and Tl^+ are approximately 8, 1.8, and 0.07 mM, respectively. Tl^+ at concentrations higher than 0.8 mM inhibits the activity of the enzyme. The highest V_{max} is exhibited by K^+ cation. Relative to K^+ the velocities for Li^+, Na^+, Cs^+, NH_4^+, and Rb^+ when assayed at saturating concentrations (100 mM) are 0.012, 0.039, 0.326, 0.464, and 0.903, respectively.

Binding of Ligands

Binding of Fructose Bisphosphate. The positive effector of pyruvate kinase, FDP, binds to the enzyme in a positive, cooperative manner (Figure 1). The binding curve yields a linear Hill plot with a slope ($\bar{n}$) of 2.4 (and a half-saturating value for FDP of 0.07 mM). The curve can be fitted reasonably well to the concerted allosteric model of Monod et al. (1965). The data presented in Figure 1 have been obtained using the equilibrium dialysis method; however, identical results are obtained using the Paulus ultrafiltration procedure. There seems to be only one binding site for FDP per

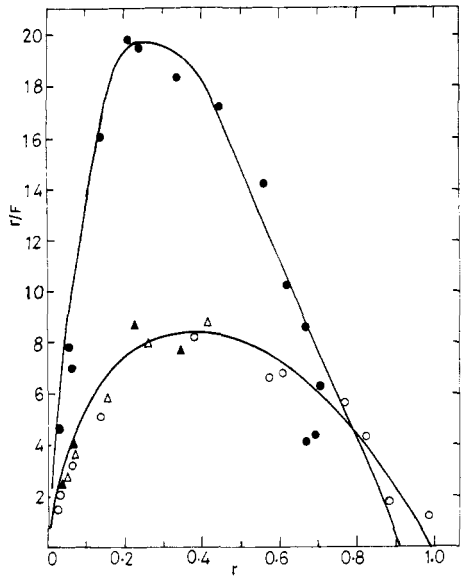

FIGURE 2: Binding of FDP to pyruvate kinase using the ultrafiltration procedure described in the text. 22 μM pyruvate kinase subunit was incubated and filtered with various concentrations of radioactive FDP in the presence or absence of various effectors. (○) No effectors: (△) 20 mM KCl; (▲) 2 mM PEP; (●) 2 mM PEP plus 20 mM KCl. r is the number of moles of FDP bound per mole of subunit and F is the free concentration of FDP (mM). The lines are drawn by eye.

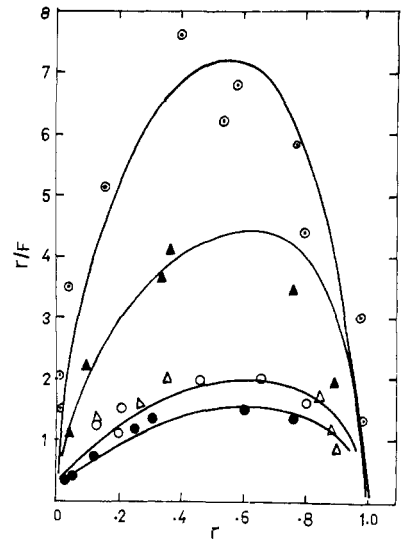

FIGURE 3: Binding of FDP in the presence of various allosteric inhibitors. Using the ultrafiltration procedure, 22 μM pyruvate kinase subunit was incubated and filtered with radioactive FDP as described in Materials and Methods. The incubation mixtures contained in addition to buffer and protein: (⊙) no additions; (▲) 2 mM succinyl-CoA; (△) 2 mM GTP; (○) 2 mM ATP; (●) 2 mM ATP and 2 mM succinyl-CoA. The lines are drawn by fit to the concerted model of Monod et al. (1965) assuming K_R = 0.026 mM; K_T = 0.5 mM; L = 68; L′-succinyl-CoA = 473 ± 117; L′-GTP or ATP, 8440 ± 2070; L′-succinyl-CoA + ATP, 20100 ± 7350.

monomer.

The unusual feature of the activator binding is that it is not effected significantly in the presence of the substrate, PEP, or Mg^{2+}, used singly or together in dialysis or ultrafiltration experiments (Figure 1). In almost all allosteric systems, at least those which have been classified under the K system by Monod et al., the substrate by itself modifies the activator binding from a sigmoid to a hyperbolic mode. In a further search of conditions which would influence FDP binding, we found that PEP and KCl together, but not by themselves separately, enhance the effector binding (Figure 2). It may be noted that at the highest concentration of KCl tried (20 mM) the Scatchard plot of FDP is still nonlinear, but the half-saturation value (judged by Hill plots of the data) drops from 0.07 mM (Figure 1) in the absence of KCl to 0.03 mM in the presence of KCl and PEP. It is possible that higher concentrations of KCl are required in order to yield a noncooperative plot for FDP but we did not investigate binding at high levels because, as will be seen later, PEP binding itself is inhibited at concentrations of KCl higher than 20 mM. It may also be mentioned here that inclusion of 5 mM $MgCl_2$ along with PEP and KCl was without any further effect on the binding of FDP.

The allosteric inhibitors of pyruvate kinase (Waygood and Sanwal, 1974), GTP, and ATP both inhibit FDP binding (Figure 3). The inhibition appears to be competitive. In a previous publication (Waygood and Sanwal, 1972) we had shown that succinyl-CoA is also an inhibitor of the activity of the enzyme and it brings about cooperative inhibition when simultaneously present with ATP. As seen from Figure 3 succinyl-CoA does inhibit the binding of FDP, albeit feebly, but together with ATP the inhibition is at best additive rather than cooperative. The reasons for this discrepancy between kinetic and binding data are not known.

Binding of Phosphoenolpyruvate. Using equilibrium dialysis, it was possible to detect some PEP binding in the presence of 5 mM FDP and 20 mM KCl. However, the binding was not consistent. One of the possible explanations for the poor binding of PEP in dialysis experiments was that an enzyme–phosphate complex is produced with PEP as the phosphoryl donor. The complex if fairly stable would not be available for binding with PEP and would result in "poor" and possibly inconsistent binding results. It is pertinent to mention that exhaustive studies with the rabbit muscle pyruvate kinase have failed to indicate the presence of an enzyme–phosphate complex in the reaction pathway of the enzyme (Boyer, 1962). In the present work no detectable hydrolysis of PEP was detected by enzymatic assay in about 18 hr normally required for the completion of dialysis under our experimental conditions.

Using the Paulus ultrafiltration cell a weak binding of PEP in the absence of FDP is discernible. The binding appears to be cooperative (Figure 4), but due to the wide scatter of the data this point cannot be emphasized. KCl at a concentration of 20 mM had no detectable affect on the binding of PEP, but the large errors involved would obscure a small change, if such a change were to occur in its presence. Cooperative binding of PEP is not observed when FDP is present but PEP binds much more tightly to the enzyme (Figure 4). KCl (20 mM) does not significantly affect PEP binding at the FDP concentration used. The dissociation constant for PEP in the presence of saturating FDP can be calculated from Figure 4 to be 0.17 ± 0.08 mM. This value is to be contrasted with a K_m of 0.03 mM for PEP obtained in kinetic experiments in the presence of FDP (Waygood and Sanwal, 1974). There seems to be one PEP binding site per monomer weighing 60000 g/mo

Binding of GDP and GTP. The binding of nucleotides was studied both by equilibrium dialysis as well as ultrafiltration. In contrast to FDP and PEP, the binding data obtained with GDP in dialysis experiments yield a linear Scatchard plot (Figure 5) with a site dissociation constant of 0.52 mM. Unexpectedly, however, the limiting value of r comes out to be 1.7 which suggests that there are two GDP binding sites per monomer. Judging by the linearity of the

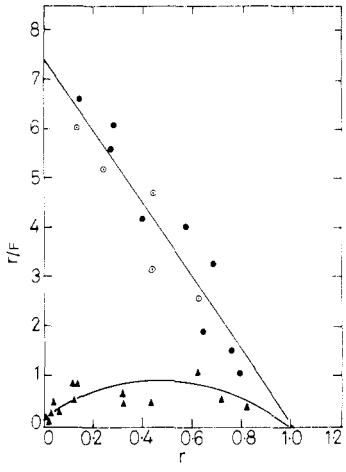

FIGURE 4: Binding of phosphoenolpyruvate. Using the ultrafiltration cell, 26 μM pyruvate kinase subunit was incubated and filtered with phosphoenolpyruvate-C^{14} (▲). In the presence of 1 mM FDP, 13 μM pyruvate kinase subunit was used (●). r is the number of moles bound per mole of subunit and F is the free phosphoenolpyruvate concentration (mM). The straight line was drawn by the method of least squares. In addition, results obtained in the presence of 1 mM FDP and 20 mM KCl are shown (⊙). The r error is ±20% as a result of the blank filter variability. The dissociation constant given by the straight line is about 0.17 mM.

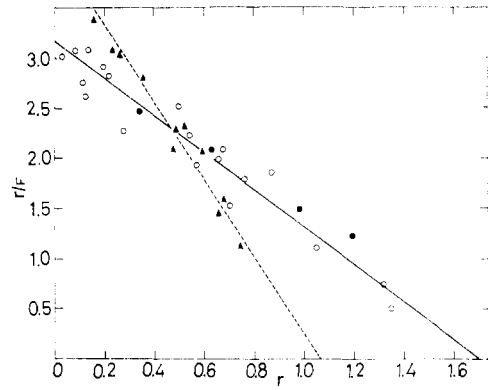

FIGURE 5: Binding of GDP to pyruvate kinase in the absence and presence of the activator. Dialysis method was used. The concentration of the subunit in experiments without FDP was 0.12 mM, and in its presence 0.038 mM. Whenever used, the concentration of FDP was 5 mM. The binding of [^{3}H]GDP was measured in the absence (○) and presence of 41 mM KCl (●). The solid line obtained in the absence of activator was drawn assuming r = 1.7 and K_D = 0.52 mM. The broken line obtained in the presence of FDP (▲) was drawn assuming r = 1.06 and K_D = 0.275 mM.

Scatchard plot, it would seem that the two binding sites are identical and noninteracting. The binding of GDP is affected in the presence of FDP, the allosteric activator, in two ways (Figure 5). First, the K_D changes to about 0.27 mM and, second, only one site seems to remain available for the binding of GDP. Essentially, quantitatively similar binding data are obtained using the ultrafiltration procedure (not shown). Thus, in the absence of FDP the limiting value of r is 1.8 and K_D is 0.5 mM, and in the presence of the activator the value of r is 1 and K_D is about 0.33 mM.

The binding characteristics of GTP on the enzyme surface are almost similar to GDP. With both the dialysis as well as the ultrafiltration procedures two noninteracting binding sites are discernible in the absence of FDP each having a dissociation constant of about 0.34 mM (Figure 6). In the presence of FDP the number of GTP binding sites are certainly reduced but despite our repeated efforts the limiting value of r did not reach below 1.2 (Figure 6). It is quite possible that GTP binds nonspecifically to some other site in addition to binding to an allosteric and the GDP-specific site.

The binding data presented above were obtained in the absence of Mg^{2+}. Since pyruvate kinase requires Mg^{2+} for catalytic activity and could conceivably modify the binding of the nucleotides we tested its effect on the binding of GDP and GTP. Magnesium drastically reduced the binding of both of the nucleotides to the enzyme (Figure 7).

Pyruvate Binding. No consistent binding of any significance could be detected by use of either equilibrium dialysis or the Paulus ultrafiltration cell. A variety of conditions were used such as addition to the dialysis or filtration buffer of various concentrations of KCl, FDP, and Mg^{2+} either separately or in various combinations, but the maximum amount of binding achieved was hardly larger than the calculated errors. The concentrations of pyruvate kinase used for equilibrium dialysis and the ultrafiltration procedure were 0.22 and 0.12 mM subunit, respectively. It would thus appear that the dissociation constant of pyruvate is greater than 5 mM.

Effect of KCl on the Binding of Other Ligands. It has been noted earlier that KCl affects the binding of FDP only in the presence of PEP. Up to a certain concentration of KCl (20 mM) in the presence of a fixed amount of PEP the binding of FDP becomes successively tighter (see Figure 2) but becomes looser if the concentration of KCl is increased further. This fact coupled with the observation that both PEP and KCl are required for the enhanced binding of FDP to pyruvate kinase suggested to us that KCl may indeed be affecting primarily the binding of PEP to the enzyme. This does seem to be the case as is readily apparent from Figure 8. The binding of PEP measured in the presence of FDP is significantly enhanced up to a concentration of 20–40 mM but decreases gradually thereafter at higher concentrations (Figure 8).

In contrast to the binding of PEP, KCl at low concentrations (up to 50 mM) does not increase binding of GDP or GTP to the enzyme. At very high concentrations of KCl (>100 mM) the binding of both of the nucleotides is significantly decreased.

Discussion

The studies presented here were largely undertaken to ascertain the physical basis of the regulatory effects exerted on the activity of the pyruvate kinase from *E. coli* by various ions and effectors, both positive and negative. Some kinetic results obtained with the fructose bisphosphate activated pyruvate kinase (Waygood and Sanwal, 1974) were sufficiently unusual to warrant equilibrium binding studies presented here.

Quantitatively, the data presented in this communication are not precise enough to distinguish between sequential (Koshland et al., 1966) and concerted models (Monod et al., 1965). Data given in Figure 1, for instance, can be approximated by both of the models. The results, however, can be qualitatively interpreted on the basis of a two-state model, where the enzyme in the absence of any ligands is assumed to be present predominantly in a state which binds the activator, FDP, and the substrate, PEP, with a very low affinity. This would explain the positive cooperative Scatchard plots obtained with FDP or with PEP singly and also the fact that the cooperativity in the binding of PEP is com-

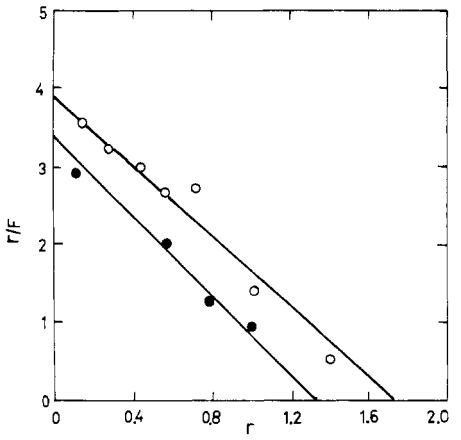

FIGURE 6: The binding of GTP to pyruvate kinase using the ultrafiltration method. The concentration of subunit was 38 μM. The binding was measured in the absence (○) as well as the presence of 5 mM FDP (●). The straight lines are fitted by the method of least squares.

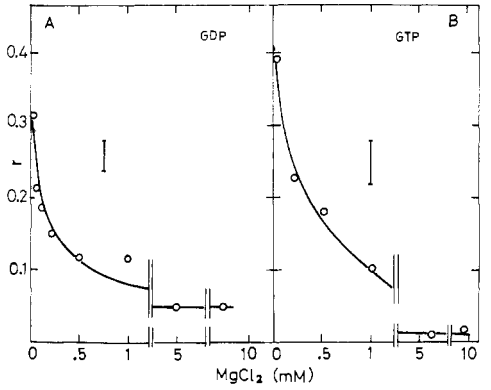

FIGURE 7: Effect of $MgCl_2$ on nucleotide binding. Binding was measured by ultrafiltration. The concentration of pyruvate kinase subunit was 11 μM and that of [^{3}H]GDP (panel A) or [^{3}H]GTP (panel B) was 0.1 mM. Error bars are given.

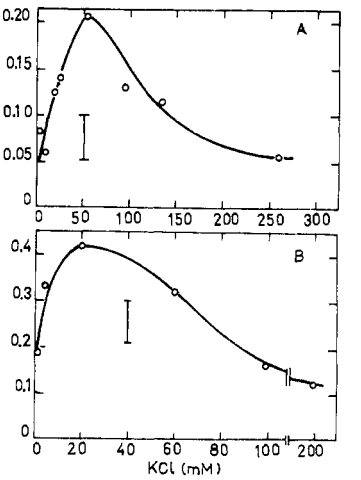

FIGURE 8: Effect of KCl on PEP binding. Data in panel A are obtained using equilibrium dialysis and in panel B by using ultrafiltration procedure. In dialysis experiments 0.15 mM subunit was dialyzed against 0.5 mM PEP at different KCl concentrations. In ultrafiltration experiments 11 μM subunit was incubated and filtered in the presence of 0.1 mM [^{14}C]PEP, 1 mM FDP, and different concentrations of KCl. r is the number of moles bound per mole of subunit. The error bar is given.

pletely abolished in the presence of saturating concentrations of FDP. The reciprocal effect of PEP on the binding of the activator is only observed in the presence of KCl as reported for the yeast enzyme (Kuczenski and Suelter, 1971). If, as is assumed by the two-state models generally (Monod et al., 1965; Koshland et al., 1966), the form of the enzyme which binds the activator and substrate poorly is the same form which binds the allosteric inhibitors with high affinity, then competition is expected to occur between the binding of the inhibitor and the activator. Such is indeed found to be the case. The binding of FDP is competitive with all of the inhibitors tested, viz., GTP, ATP, and succinyl-CoA. Surprisingly, however, in marked contrast to the kinetic results obtained earlier (Waygood and Sanwal, 1974) ATP and succinyl-CoA together do not cause synergistic or cooperative inhibition but rather additive inhibition. The reason for this discrepancy is not known.

On the basis of the two-state model, the binding of an allosteric inhibitor in the absence of any other ligands is expected not to be cooperative (i.e., follow an adsorption isotherm) but become cooperative in the presence of an activator or any substrate which gives rise to homotropic interactions. Such, however, is not the case with the binding of GTP, one of the allosteric inhibitors of the enzyme. In the absence of other substrates or effectors, the binding data for GTP not only produce a linear Scatchard plot, but, surprisingly, yield two binding sites per monomer of 60000 molecular weight. This is not only a characteristic of GTP binding but also of the binding of the substrate, GDP, which again seems to have two binding sites. These anomalous data can only be rationalized on the assumptions that both GTP and GDP bind to the allosteric as well as the active nucleoside diphosphate binding site and, further, that the binding affinity of both of these compounds for the two states of the protein as well as the two sites is nearly the same. There is some evidence for these assumptions. Our earlier kinetic experiments (Waygood and Sanwal, 1974) had demonstrated that GDP brings about substrate inhibition. This may indeed be due to the postulated binding of GDP not only to its catalytic but also to the allosteric site. If the assumptions made above to fit the data qualitatively to a two-state model are valid, it is expected that the binding of GDP and GTP to the enzyme would be antagonized by the activator, FDP and, furthermore, only one site per monomer for the inhibitors would remain in its presence. This is the case when the binding of GDP is done in the presence of FDP. Only one site per monomer is discernible in the presence of the activator. For GTP, there is certainly a reduction in the number of sites in the presence of the activator but for unknown reasons the extrapolated number of binding sites in this case does not reach 1. It is pertinent to point out here that the binding data are not sufficiently incisive to decide whether the allosteric inhibitor and the allosteric activator sites are identical or different. What seems certain is the fact that there is only one site on each of the monomers for the substrates and effectors. At least the activator and substrate sites are distinct from each other. The results obtained with the *E. coli* enzyme are somewhat different from the yeast enzyme in this regard (Fell et al., 1972). In the latter case it has been suggested that half-site saturation may occur, i.e., only half as many molecules of the substrate may bind as there are monomers. However, it is becoming increasingly evident that pyruvate kinases from different sources may indeed have different allosteric mechanisms. Binding of FDP to the rabbit liver enzyme, for example, in contrast to the *E. coli* and yeast enzyme (Kuczen-

ski and Suelter, 1971), is not cooperative (Irving and Williams, 1973).

There are several other conclusions that can be drawn from the binding data. The allosteric model notwithstanding it is clear that both substrates of the enzyme bind independently of each other on the enzyme surface. This supports the inference that the kinetic mechanism is quite possibly Rapid Equilibrium Random as has been shown for the muscle pyruvate kinase by Mildvan and Cohn (1966). Magnesium ions do not seem to be involved in the binding of effectors or substrates to the enzyme. This finding is somewhat unexpected because it has been suggested on the basis of proton relaxation enhancement studies of the yeast pyruvate kinase that Mn^{2+} is involved in enzyme–metal–substrate (Cottam et al., 1972) and enzyme–metal–FDP complexes (Mildvan and Cohn, 1966). However unlikely it may be, our experiments have not excluded the possibility that tightly bound Mn^{2+} may, however, already be present in the enzyme preparations.

The mechanism of the activation of the enzyme by K^+ ions is not clarified by our results. Up to a concentration of 20 m*M* it facilitates the binding of PEP without having any effect on the binding of GDP or GTP. It may be suggested that K^+ ions possibly favor the formation of a high affinity (for substrate) state of the enzyme. Kuczenski and Suelter (1971) have suggested that K^+ may be involved in producing an unique active conformer of the enzyme from yeast.

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