Articles

Pyruvate Kinase: Is the Mechanism of Phospho Transfer Associative or Dissociative?†

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ABSTRACT: To test for the possibility that pyruvate kinase proceeds via a dissociative path, we have investigated whether the complex enzyme·ADP·metaphosphate is transiently formed from the complex enzyme·ATP. It is shown that when highly purified pyruvate kinase is used, the rate of positional oxygen isotope exchange in ATP (β,γ bridge and β nonbridge) is about 10^4 times slower in the absence of the cosubstrate pyruvate than it is in the presence of pyruvate. Further, the rate of racemization of the γ-phospho group of [$\gamma(S)$-^{16}O,^{17}O,^{18}O]-ATP is undetectable, being at least 30 times slower even than the rate of positional isotope exchange. These tests thus provide no evidence that pyruvate kinase follows a dissociative mechanism. Indeed, it is argued that the available data are more consistent with an associative path. Evidence is presented that the single, associative, transition state is symmetrical, in which bond making and bond breaking processes are rather precisely balanced.

Pyruvate kinase (EC 2.7.1.40) catalyzes the transfer of a phospho group from phosphoenolpyruvate to ADP, yielding pyruvate and ATP. The overall equilibrium constant is $\sim 3 \times 10^{-4}$ in favor of pyruvate and ATP (Nageswara Rao et al., 1979). From steady-state kinetic work (Dann & Britton, 1978, and references cited therein) and isotope flux studies (Britton & Dann, 1978), it appears that the reaction is sequential, the phospho group being transferred between the bound substrates in ternary complexes with the enzyme. There is no evidence for a phosphoenzyme intermediate, and—in common with most phosphokinases (Knowles, 1980)—the phospho group is transferred with inversion of the configuration at phosphorus (Orr et al., 1978; Blättler & Knowles, 1979). We may therefore presume that the phospho group migrates with "in-line" geometry *directly* from one enzyme-bound substrate to the other. The nature of the phospho group transfer may be probed more deeply, however, and we can ask whether the transfer step is associative or dissociative. In the associative mechanism, bond making precedes bond breaking, and the attack of the acceptor group on phosphorus occurs in a transition state (or, possibly, an intermediate) in which the central phosphorus is pentacovalent and has trigonal bipyramidal geometry. In the dissociative reaction, bond breaking leads, and monomeric metaphosphate is formed as a discrete intermediate before it collapses on to the acceptor nucleophile. These mechanistic extremes are most thoroughly documented in carbon chemistry (as S_N2 and S_N1 reactions), and good evidence exists for the analogous processes in phosphoric ester chemistry.

If the reaction catalyzed by pyruvate kinase was dissociative, we should expect that each of the phospho group donors (phosphoenolpyruvate in one direction and ATP in the other) would, on binding to the enzyme, dissociate partly or wholly to produce enolpyruvate and metaphosphate or ADP and metaphosphate. In the absence of the cosubstrate acceptor, these enzyme-bound species would simply collapse back to the intact donor molecules. Since for a truly dissociative mechanism the acceptor only participates in the second step (the collapse of the metaphosphate formed in the first step), the dissociation of the donor might be catalyzed by the enzyme in the absence of acceptor. Thus, if the dissociative mechanism were to hold for pyruvate kinase, bound ATP might reversibly dissociate on the enzyme to ADP and metaphosphate. Such a dissociation can in principle be probed in two ways, shown in Figure 1. First, if ADP forms on the enzyme, rotation of the β-phospho group about the P_β–$O_{\alpha\beta}$ bond will randomize the three peripheral β oxygens. As was first elegantly demonstrated by Midelfort & Rose (1976) with glutamine synthetase, such randomization can be monitored by positional isotope exchange in reisolated ATP, as shown in Figure 2. This experiment has been performed on pyruvate kinase by Lowe & Sproat (1978), who suggested that pyruvate kinase does catalyze the positional isotope-exchange reaction in the absence of cosubstrate (pyruvate). We show here, however, that the exchange is much slower than has been reported, and most if not all of the observed exchange is due to impurities in the pyruvate kinase used. Second, if ATP dissociates on binding to pyruvate kinase, it is possible that the metaphosphate formed could rotate about a P–O bond (see Figure 1) which, if chiral [γ-^{16}O,^{17}O,^{18}O]ATP was used as the substrate, would lead to the enzyme-catalyzed racemization at the γ-phosphorus center in reisolated ATP (see Figure 3). We report the results of this test for the dissociative pathway and

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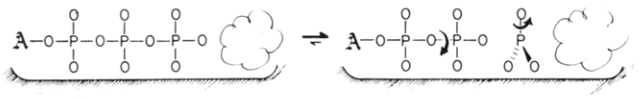

FIGURE 1: Steps involved in a putative dissociative mechanism, in which the enzyme (shaded) catalyzes the dissociation of ATP in the absence of the cosubstrate (cloud). The two testable bond rotations are indicated.

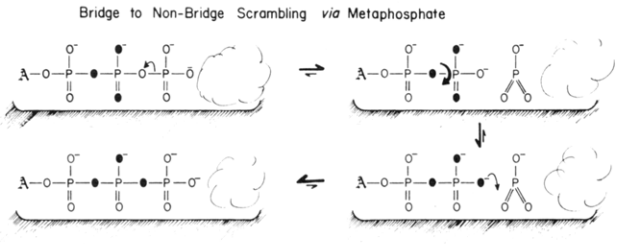

FIGURE 2: Steps involved in the positional isotope-exchange test of Midelfort & Rose (1976).

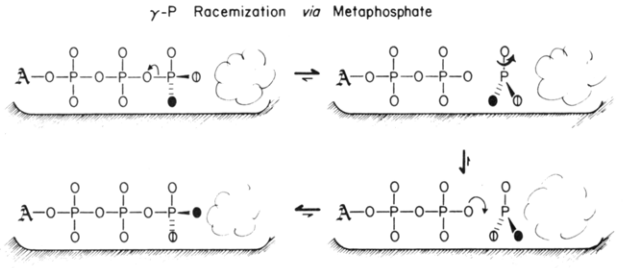

FIGURE 3: Steps involved in the racemization test.

set an upper limit on the rate of racemization catalyzed by pyruvate kinase.

Experimental Procedures

Materials. $KH_2P^{18}O_4$ was synthesized from $H_2{}^{18}O$ (96% enriched, from Monsanto) and PCl_5 by the method of Risley & Van Etten (1978). Mass spectroscopic analysis of the trimethylsilyl derivative showed that the product was 84.3% $^{18}O_4$, 15.0% $^{18}O_3$, and 0.7% $^{18}O_2$. The potassium dihydrogen salt (6.7 mmol) was converted into the mono(tri-*n*-butylammonium) salt by passage down a column (100 mL) of Dowex 50 (pyridinium form). The column was washed with 2 column volumes of distilled, deionized water, and the eluate was concentrated under reduced pressure to 50 mL. Tri-*n*-butylamine (6.7 mmol) was added, and water and pyridine were removed under reduced pressure. Repeated evaporation under N_2 of added dry pyridine yielded the dry tri-*n*-butylammonium salt of [$^{18}O_4$]phosphoric acid. The conversion of sodium salts of nucleotides into their tri-*n*-octylammonium form was achieved analogously. Tri-*n*-octylamine in dioxane was added to the aqueous pyridinium salts, and the water was removed by repeated evaporations of added dry pyridine. Traces of pyridine were removed by the repeated evaporation of added redistilled dry toluene.

Adenosine 5′-[α,β-^{18}O,β-$^{18}O_3$]diphosphate was prepared following the procedure of Michelson (1964). To a solution of adenosine 5′-phosphate [mono(tri-*n*-octylammonium) salt] (0.70 g, 1 mmol) in dry dioxane–dimethylformamide (3:2 v/v; 15 mL) was added diphenyl phosphorochloridate (0.27 g, 1 mmol) and tri-*n*-butylamine (0.34 g, 1.84 mmol), and the mixture was stirred at room temperature under dry N_2 for 2.5 h. The solvent was then removed under reduced pressure to yield a yellow oil. To this material was added dry tri-*n*-butylammonium [$^{18}O_4$]phosphate (0.48 g, 165 mmol) in dry pyridine (10 mL), and the mixture was stirred at room temperature under dry N_2 for 3 h. The solvent was removed under reduced pressure, and the residue was dissolved in 50 mM triethylammonium bicarbonate buffer, pH 7.8 (100 mL), and loaded at 4 °C onto a column (40 × 2.5 cm) of DEAE-Sephadex A-25 (HCO_3^- form) equilibrated in the same buffer. The column was eluted at 4 °C with a linear gradient (1 L plus 1 L, 250–750 mM) of triethylammonium bicarbonate buffer. Fractions containing ADP were pooled, and the solvent and buffer were removed by repeated evaporation of added methanol under reduced pressure. The yield of the triethylammonium salt of ADP was 50% (by enzyme assay).

Adenosine 5′-[α,β-^{18}O,β-$^{18}O_2$]triphosphate was prepared following the method of Ott et al. (1967). The tris(triethylammonium) salt of adenosine 5′-[α,β-^{18}O,β-$^{18}O_3$]diphosphate was converted into the mono(tri-*n*-butylammonium) salt by the method described above for orthophosphate. To the mono(tri-*n*-butylammonium) salt (500 μmol) in anhydrous hexamethylphosphoramide (10 mL) under N_2 was added, by cannula, carbonyl diimidazole (3 mmol) in anhydrous hexamethylphosphoramide (10 mL). The solution was stirred at room temperature under dry N_2 for 12 h. Anhydrous methanol (200 μL, 4.62 mmol) was then added, and the mixture was stirred for a further 3 h. To this solution was then added, by cannula, the mono(tri-*n*-butylammonium) salt of [$^{16}O_4$]-phosphoric acid in anhydrous hexamethylphosphoramide (10 mL), and the pale yellow solution was stirred at room temperature for 12 h. Water (40 mL) and triethylamine (200 μL) were then added, and after 30 min the solution was adjusted to pH 8.0. The mixture was loaded on to a column (40 × 2.5 cm) of DEAE-Sephadex A-25 (HCO_3^- form), which was eluted with a linear gradient (1 L plus 1 L, 450–950 mM) of aqueous triethylammonium bicarbonate. Fractions containing ATP were pooled, and the solvent and buffer were removed by repeated evaporation of added methanol under reduced pressure. The yield of the triethylammonium salt of [α,β-^{18}O,β-$^{18}O_2$]ATP was 30% (by enzyme assay).

Adenosine [γ(*S*)-^{16}O,^{17}O,^{18}O]triphosphate was prepared by the method described earlier (Blättler & Knowles, 1979).

P^1,P^5-Bis(5′-adenosyl)pentaphosphate (trilithium salt) was obtained from Boehringer Mannheim (Indianapolis, IN).

Pyruvate kinase (ex rabbit muscle) for the positional isotope-exchange experiments was obtained from Sigma Chemical Co. (St. Louis, MO) as a lyophilized, salt-free powder (lot no. 71F-9570 and 90F-9665). The specific catalytic activity of the two batches was 205 and 214 units/mg, respectively, on the basis of $A^{0.1\%}_{280nm} = 0.54$ (Bücher & Pfleiderer, 1955). The enzyme (15 mg) was subjected to ion-exchange chromatography on a column (20 cm × 1 cm) of carboxymethyl-Sephadex, equilibrated with 50 mM potassium phosphate buffer, pH 5.5. The column was then eluted with a pH gradient (100 mL plus 100 mL, 50 mM potassium phosphate buffer, pH 5.5–7.5). The specific catalytic activity of the enzyme after this treatment was 221 units/mg. The enzyme was then subjected to preparative isoelectric focusing on a bed of Sephadex–IEF (Pharmacia, Piscataway, NJ) prepared from a slurry of Sephadex (4 g) in a solution of ampholyte (5 mL of a 40% w/v mixture, pH range 4–9) in distilled, deionized water (95 mL). The bed dimensions were 11 cm × 24 cm × 0.5 cm, and the protein solution (approximately 25 mg in 1.8 mL of 1% glycine–HCl buffer, pH 6.0) was applied to a trough

in the center of the bed. The bed was focused for 12–14 h at 8 W and 4 °C. After focusing was complete, the bed was divided into 30 strips, and the protein was eluted from each gel fraction with 100 mM triethanolamine–HCl buffer, pH 7.6 (8–10 mL). Fractions containing pyruvate kinase of the highest specific catalytic activity were dialyzed against the same buffer and then concentrated by using Amicon filter cones (Lexington, MA). Only the fraction having the highest specific activity (of 347 units/mg) was used in the positional isotope-exchange experiments.

Pyruvate kinase (ex rabbit muscle) for the ATP racemization experiments was a kind gift from David Ash and Mildred Cohn. The enzyme was subjected to ion-exchange chromatography on carboxymethyl-Sephadex as described by Bondar & Pon (1969) before use and had a specific activity of 185 units/mg.

Methods. Mass spectroscopic measurements were made on an AEI MS-9 instrument. Ultraviolet measurements were done with a Perkin-Elmer 554 recording spectrophotometer. ^{31}P NMR spectra were recorded on a Bruker WM-300 WB instrument at 121.5 MHz with a deuterium field lock. A spectral width of 1500 Hz was used with a pulse width of 25 μs and an acquisition time of 5.45 s. A total of 4000–9000 scans was taken.

ADP was assayed by the addition of a sample (50 μL) to a solution of 50 mM triethanolamine–HCl buffer, pH 7.6 (950 μL), containing KCl (100 mM), $MgCl_2$ (5 mM), phosphoenolpyruvate (1.8 mM), NADH (0.12 mM), pyruvate kinase (12 units), and lactate dehydrogenase (44 units). The absorbance change at 340 nm was measured.

ATP was assayed by the addition of a sample (50 μL) to a solution of 50 mM triethanolamine–HCl buffer, pH 7.4 (950 μL), containing KCl (100 mM), $MgCl_2$ (5 mM), glucose (75 mM), $NADP^+$ (2 mM), hexokinase (45 units), and glucose-6-phosphate dehydrogenase (33 units). The absorbance change at 340 nm was measured.

Pyruvate kinase was assayed at 37 °C, by the addition of a sample (10 μL) to a solution of 100 mM triethanolamine–HCl buffer, pH 7.60 (990 μL), containing KCl (100 mM), $MgCl_2$ (10 mM), phosphoenolpyruvate (0.75 mM), ADP (4.7 mM), NADH (0.12 mM), and lactate dehydrogenase (44 units). One unit of enzyme activity is defined as that amount which catalyzes the consumption of 1 μmol of phosphoenolpyruvate/min, under these conditions.

The rate of positional isotope exchange was determined from the rate of equilibration of the two labeled peripheral β oxygens of adenosine 5′-[α,β-^{18}O,β-$^{18}O_2$]triphosphate with the unlabeled β–γ-bridge oxygen. The appearance of ^{18}O label in the β–γ bridge was monitored by ^{31}P NMR of samples (2 mL) withdrawn from incubation mixtures (6 mL) at 37 °C in 100 mM triethanolamine–HCl buffer, pH 7.6, containing KCl (100 mM), $MgCl_2$ (15 mM), bovine serum albumin (0.14–0.6 mg/mL), labeled ATP (5.6 mM), P^1,P^5-bis(5′-adenosyl)-pentaphosphate (0.58 mM), and pyruvate kinase (8–1215 units). Each sample was quenched by the addition of methanol (5 volumes) and then evaporated to dryness under reduced pressure. After redissolution in buffer, the sample was applied to a column (1.8 cm × 15 cm) of DEAE-Sephadex (A-25) equilibrated with 200 mM triethanolamine–bicarbonate buffer, pH 8.0, and eluted with a linear gradient (150 mL plus 150 mL, 200–800 mM) of the same buffer. The fractions containing ATP were pooled and freeze-dried. The sample was then dissolved in 50 mM triethanolamine–HCl buffer, pH 7.6 (2 mL), containing EDTA (20 mM), and D_2O (1:1 v/v). The H_2O used for all NMR experiments was distilled–deionized and was treated with Chelex resin before use. The final concentration of ATP in the samples for NMR was thus about 6 mM. The undecoupled ^{31}P NMR spectrum of the region of the γ-phosphorus resonance was taken at ambient temperature (see Figure 4). The maximum amount of ADP formed during NMR examination was 4.6% of the ATP present. The rate constants for the positional isotope-exchange process were calculated as follows. The fraction, F, of the ATP that has undergone positional isotope exchange is defined as $[(^{18}O/^{16}O)_t - (^{18}O/^{16}O)_{t=0}]/[(^{18}O/^{16}O)_{t=\infty} - (^{18}O/^{16}O)_{t=0}]$, where the ratio $(^{18}O/^{16}O)$ is determined from the ratio of the peak height of the upfield-shifted (^{18}O-perturbed) resonance for the γ-phospho group to the sum of the peak heights of the shifted and the unshifted resonances for the γ-phospho group. The rate constant for the exchange process was then calculated from $k = -\ln [(1 - F)[ATP]/([E]t)]$, where [E] is the concentration of enzyme subunits. The small upfield peaks in the NMR spectra at $t = 0$ derive from the presence of low levels of ^{18}O labeling of the peripheral oxygens of the γ-phospho group of the synthetic ATP sample. This was demonstrated by transfer of the γ-phospho group to glycerol, mediated by glycerol kinase, followed by mass spectroscopic analysis of the resulting *sn*-glycerol 3-phosphate.

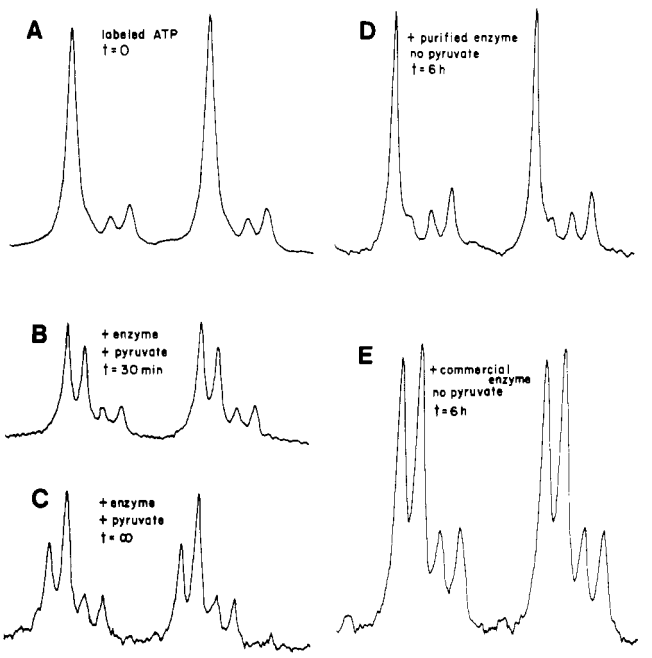

FIGURE 4: ^{31}P NMR spectra of the signals from the γ phosphorus of [β,γ-^{18}O,β-$^{18}O_2$]ATP after incubation with pyruvate kinase under different conditions: (A) starting material; (B) after incubation with commerical pyruvate kinase (8 units) in the presence of pyruvate (50 mM) for 30 min; (C) as (B), after incubation for 26 h; (D) after incubation with highly purified pyruvate kinase (1215 units) in the *absence* of pyruvate for 6 h; (E) after incubation with commercial pyruvate kinase (1215 units) in the *absence* of pyruvate for 6 h.

The racemization of the γ-phosphoryl group of [$\gamma(S)$-^{16}O,^{17}O,^{18}O]ATP catalyzed by pyruvate kinase was determined as follows. [$\gamma(S)$-^{16}O,^{17}O,^{18}O]ATP (sodium salt, 276 μmol) was incubated at 37 °C with pyruvate kinase (see Materials) (3100 units) in 50 mM K-Hepes buffer, pH 8.0, (27 mL) containing EDTA (1 mM), $MgCl_2$ (15 mM), and potassium chloride (100 mM), for 120 min. Under these conditions, it had been shown (Mildred Cohn and David Ash, personal communication) that 185 units of this enzyme sample catalyzed the complete positional scrambling (of β,γ-bridge and β-nonbridge oxygens) of ^{18}O-labeled ATP (0.4 μmol) in 1 min. In our experiment, therefore, the incubation time (120 min) was 3 times longer than is necessary for complete positional

Table I: Rate Constants for Positional Isotope Exchange in ^{18}O-Labeled ATP, Catalyzed by Pyruvate Kinase

	rate constant[a] (min^{-1})		
	+pyruvate	−pyruvate	ratio[b]
commercial enzyme[c]	563	1.98	280
highly purified enzyme[d]	524	0.064	8200

[a] For reaction conditions, see Methods. [b] +pyruvate/−pyruvate. [c] After dialysis and concentration. [d] After ion-exchange chromatography and preparative isoelectric focusing.

isotope scrambling. After the incubation, the ATP was reisolated by ion-exchange chromatography on DEAE-cellulose in 92% yield. The enantiomeric excess at the γ phosphorus of the reisolated adenosine [$\gamma(S)$-^{16}O,^{17}O,^{18}O]triphosphate was determined by transfer to (*S*)-propane-1,2-diol catalyzed by *Escherichia coli* alkaline phosphatase (Jones et al., 1978) and subsequent determination of the enantiomeric excess at phosphorus in the derived 1-[^{16}O,^{17}O,^{18}O]phospho-(*S*)-propane-1,2-diol by the metastable ion mass spectrometric method described earlier (Abbott et al., 1978, 1979).

Results

The rate constants for the positional isotope-exchange reaction in the presence and in the absence of added pyruvate are reported in Table I. In this table are reported the rate constants obtained by using commercial enzyme that had only been dialyzed and concentrated before use and using enzyme that had been highly purified by ion-exchange chromatography on CM-Sephadex followed by preparative isoelectric focusing. It is evident that, with highly purified pyruvate kinase, positional isotope exchange in ATP is nearly 10^4 times slower in the absence of the acceptor cosubstrate than in its presence.

The configuration at the γ phosphorus of [γ-^{16}O,^{17}O,^{18}O]ATP that had been incubated with pyruvate kinase for a time 3 times longer than would have been required to effect the complete positional isotope exchange of the β-nonbridge oxygens and the β,γ-bridge oxygen, was found to be *S* and of 99.2 ± 8% enantiomeric excess. That is, within experimental error, there is no detectable racemization of the γ-phospho group of [$\gamma(S)$-^{16}O,^{17}O,^{18}O]ATP on incubation with pyruvate kinase in the absence of cosubstrate. Quantitatively, if there is in fact 1% of racemization, a racemization event occurs less than once in every 300 times that a positional isotope-exchange event occurs. If (at the extreme of the experimental uncertainty of the stereochemical measurement) there is 8% of racemization, a racemization event occurs less than once in every 30 positional isotope-exchange events.

Discussion

If the mechanistic pathway followed by pyruvate kinase were to be a dissociative one proceeding via a monomeric metaphosphate intermediate, the enzyme would need to catalyze the heterolytic bond cleavage between the donor molecule and the phospho group being transferred. In one direction, one might expect that enzyme-bound phosphoenolpyruvate would dissociate into bound enolpyruvate and bound metaphosphate and, in the other direction, bound ATP would dissociate into bound ADP and bound metaphosphate. In this paper, two tests have been made of the latter possibility. First, if pyruvate kinase were to catalyze the heterolysis of bound ATP, literature precedent (Rose, 1979) would lead one to expect that the three peripheral oxygen atoms of the β-phospho group of the resulting bound ADP would become equivalent, resulting in catalysis of the positional isotope exchange illustrated in Figure 2. From the results shown in Table I it is clear that the rate of positional isotope exchange catalyzed by pyruvate kinase is a very slow process. With commercial enzyme, the exchange reaction is nearly 300 times slower in the absence of pyruvate than in its presence. [The rate constant of ~550 min^{-1} in the presence of pyruvate (Table I) is comparable with the minimum value (Rose, 1979) of 780 min^{-1} calculated from the data of Dann & Britton (1978).] When the pyruvate kinase is further purified by ion-exchange chromatography and then by preparative isoelectric focusing, the positional isotope-exchange rate in the absence of pyruvate falls to about 10^{-4} times the rate observed in the complete reaction system (of enzyme plus ATP plus pyruvate). More than 96% of the exchange reaction observed with the commercial kinase is therefore caused by contaminating enzyme(s). An earlier report (Lowe & Sproat, 1978, 1979) that pyruvate kinase does catalyze the positional isotope-exchange reaction was based upon experiments with unpurified commercial enzyme, which evidently contains a catalytic contaminant or contaminants. It appears, then, that pyruvate kinase has little or no ability to catalyze positional isotope exchange in ATP in the absence of pyruvate. This finding is consistent with the facts that neither creatine kinase (Lowe & Sproat, 1980), purified hexokinase (Rose, 1980), nor phosphoglycerate kinase (M. Cohn, private communication) has any significant ability to catalyze the positional isotope exchange of ATP in the absence of cosubstrate.

The second test of the dissociation of ATP when bound alone to pyruvate kinase is shown in Figure 3 and evaluates the possible rotation of the putative enzyme-bound metaphosphate about one of its P–O bonds. When [$\gamma(S)$-^{16}O,^{17}O,^{18}O]ATP is incubated with pyruvate kinase in the absence of pyruvate for a period long enough to allow (according to the positional isotope-exchange test) metaphosphate to form many times over, no racemization of the γ-phospho group is observed. Racemization at the γ phosphorus, if it occurs at all, proceeds at a rate that is *at least* 30 times slower even than the very slow positional isotope exchange process. *It seems, in summary, that catalysis of neither positional isotope exchange nor racemization at the γ phosphorus of ATP is an intrinsic property of pure pyruvate kinase in the absence of pyruvate.* Neither of these processes can therefore be used to suggest that this enzyme proceeds via a dissociative mechanism for phospho transfer.

Is the Phospho Transfer Associative? It may be argued that a phosphokinase would not activate the phospho group for transfer until and unless the cosubstrate was bound, in order to avoid the possibilty of the fruitless reaction of the activated phospho group with water. These arguments predict that the cosubstrate (in our case, pyruvate) must be bound in order that transfer be initiated, that is, that any partial reaction such as positional isotope exchange should be subject to "substrate synergism" (Bridger et al., 1968). According to this view, *even if* the reaction mechanism were a dissociative one, we should not expect to observe any cleavage of the enzyme-bound donor molecule in the absence of enzyme-bound acceptor, and the results of the tests applied in this paper are unsurprising. Nevertheless, in the absence of any evidence to support a dissociative pathway, what can be said about the nature of the phospho transfer? Phosphotransferases are, even by enzyme standards, extraordinarily effective catalysts, and we may ask how the rate enhancements are achieved. The simplest approach to this question is to ask how the enzyme could stabilize the rate-limiting transition state(s) for the actual phospho transfer step(s). If the transfer follows a dissociative

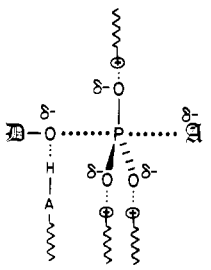

FIGURE 5: Expected transition state for a symmetrical, associative, in-line process.

path, it is difficult to envisage a disposition of enzyme catalytic groups that would stabilize a dissociative transition state *in preference to* the starting material or the products. In a dissociative step, there is a movement of electron density *away from* the peripheral oxygens of the phospho group toward the leaving group. So that the relative stabilization of a transition state with this electron distribution could be achieved, the enzyme would have to *destabilize* the charge on the peripheral oxygens of the starting material. This would run counter to the known binding and recognition features of phospho transfer enzymes and would create problems, for example, in the interpretation of the action of the nonspecific phosphatases. Further, metaphosphate is a species of rather high free energy with respect to its precursors, and unless the metaphosphate intermediate itself can be stabilized, there is little scope for accelerating the rate of its formation, since this rate is principally determined by the accompanying change in the equilibrium free energy (Guthrie, 1977).

In contrast, in the transition state for an *associative* reaction, there can be movement of electron density *onto* the peripheral oxygens (depending, of course, on the looseness or tightness of the transition state), and it is easy to imagine an appropriate constellation of electron sinks (positively charged amino acid side chains, metal cations, and hydrogen-bond donors) that would preferentially stabilize the trigonal bipyramidal transition state of an associative process (Figure 5). It is, indeed, gratifying that the two enzymes that involve nucleophilic displacements at phosphorus for which crystal structure determinations have been carried to very high resolution [*Staphylococcus* nuclease by Cotton et al. (1979); ribonuclease A by G. Petsko and co-workers (private communication)] each show a distribution of charged groups that is consonant with this view of a trigonal bipyramidal, associative transition state.

What Is the Symmetry of the Transition State? Finally, the *symmetry* of the transition state for phospho group transfer can be probed, and we may ask about the relative extents of bond formation and bond cleavage at the critical transition state. Some years ago, we proposed on theoretical grounds that an enzyme catalyst would be most efficient if "the kinetically significant transition state is flanked by kinetically significant intermediates of equal free energy" (Albery & Knowles, 1976). For a phosphokinase, this amounts to the prediction that the two ternary complexes (here, enzyme·phosphoenolpyruvate·ADP and enzyme·pyruvate·ATP) will be of *equal* Gibbs free energy: the "internal" equilibrium constant (K_{int}, for enzyme-bound species) will be close to unity even if the equilibrium constant for the free species (K_{eq}) is far from 1. In the past 2 years or so, the internal equilibrium constants for a number of phospho transfer enzymes have been determined, largely by Cohn and her group, and it is gratifying to see that while the K_{eq} values vary over nearly 9 orders of magnitude, the values of K_{int} are all quite close to 1. These data are collected in Table II. On the basis that a single associative transition state links the two ternary complexes of a phosphotransferase, simple application of the ideas on free energy relationships of Brønsted, Hammett, and Hammond suggests that two species of equal free energy will interconvert by way of a *symmetrical* transition state. It appears, then, that the phosphokinases have evolved to the point where there is almost perfect compensation between bond-making and bond-breaking processes in a symmetrical transition state of in-line geometry at phosphorus.

Table II: Overall Equilibrium Constants (K_{eq}) and Internal Equilibrium Constants (K_{int}) for Phosphotransferases

enzyme	K_{eq}[a]	K_{int}[b]	ref
pyruvate kinase	3×10^{-4}	1	*c*
phosphoglycerate kinase	3×10^{-4}	0.8	*d*
glutamine synthetase	$\sim 3 \times 10^{-4}$	2	*e*
Ile-tRNA synthetase	4×10^{-4}	0.2	*f*
Met-tRNA synthetase	4×10^{-4}	1.7	*g*
arginine kinase	0.1	1.2	*d*
creatine kinase	0.1	1	*d, h*
phosphoglucomutase	17	0.4	*i, j*
hexokinase	2×10^{3}	1	*h, k*
myosin ATPase	2×10^{5}	10	*h, l*

[a] [MgADP][donor]/[MgATP][acceptor]. [b] [enzyme·MgADP·donor]/[enzyme·MgATP·acceptor]. [c] Nageswara Rao et al. (1979). [d] Nageswara Rao et al. (1978). [e] T. D. Meek, K. A. Johnson and J. J. Villafranca (private communication). [f] Holler & Calvin (1972). [g] Fayat et al. (1980). [h] Lawson & Veech (1979). [i] Ray & Long (1976a). [j] Ray & Long (1976b). [k] Wilkinson & Rose (1979). [l] Gutfreund & Trentham (1975).

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Structure–Function Relationships in Lactose Synthase. Structural Requirements of the Uridine 5′-Diphosphate Galactose Binding Site†

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ABSTRACT: The structural requirements for the donor pyranosyl moiety of UDP-Gal in either galactosyl transfer or "lactose" biosynthesis have been determined. The 4″-deoxy analogue, UDP-4″-deoxyglucose, was synthesized and fully characterized as a donor substrate for galactosyltransferase. The relative rate of deoxyglucosyl transfer to glucose or GlcNAc acceptors was 5.5 ± 0.6% of that of UDP-Gal as the substrate, with K_m values in the same range as that for UDP-Gal or UDP-Glc. Several conclusions may be drawn as to the detailed structural requirements of the UDP-Gal binding site: an *axial* 4″-hydroxyl group on the pyranosyl moiety is necessary for precise substrate alignment as is also an *equatorial* 6″-CH_2OH moiety. Where one or the other moiety was lacking (UDP-dGlc or UDP-Arab), the maximal rate of glycosyl transfer was ca. $^1/_{20}$th that of UDP-Gal.

Galactosyltransferase (uridine diphosphate D-galactose:D-glucose 1-galactosyltransferase, EC 2.4.1.22) catalyzes the lactose synthase reaction

$$\text{UDP-Gal} + \text{Glc} \xrightarrow[\alpha\text{LA}]{\text{Mn(II)}} \text{UDP} + \text{lactose} \qquad (1)$$

where αLA[1] is the modifier protein α-lactalbumin. It also catalyzes a general glycosyl transferase reaction, not restricted to lactating mammary glands

$$\text{UDP-Gal} + \text{GlcNAc} \xrightarrow{\text{Mn(II)}} \text{UDP} + \text{LacNAc} \qquad (2)$$

where the acceptor, GlcNAc, is a (nonreducing end) residue in complex carbohydrate biosynthesis. The metal ion specificity is broad, although the optimal cation is Mn(II) which binds to two metal ion sites per galactosyltransferase molecule (O'Keefe et al., 1980; Andree & Berliner, 1980). Until Andree & Berliner (1978) first showed that UDP-Glc was an acceptable (yet marginally active) substrate in both reactions 1 and 2, UDP-Gal was believed to be the only (donor) substrate with the correct pyranosyl stereochemistry to bind properly for productive glycosyl transfer. It had also been ascertained that the uridylyl moiety was most important in binding UDP-Gal substrate and inhibitor analogues (Andree & Berliner, 1978; Berliner & Wong, 1975).

Since the small, yet observable activity of UDP-Glc vs. UDP-Gal (0.3% vs. 100%) suggested that an equatorial hydroxyl moiety in the 4″ position of the pyranosyl position was (barely) tolerable for productive binding, we have examined the consequences of the absence or presence (axial or equatorial) of hydroxyl substituents at this position.

Materials and Methods

UDP-Gal, UDP-Glc, uridine 5′-phosphomorpholidate, phosphoenolpyruvate, NADH, pyruvate kinase, and α-lactalbumin were from Sigma Chemical Co. Pyridine and *N*,*N*-dimethylformamide (reagent grade) were dried over CaH_2 and distilled immediately before use.

TLC was performed on Baker-Flex (IB-F) silica gel with UV or H_2SO_4 charring for visualization. Descending paper chromatography (Whatman No. 1) utilized periodate–$AgNO_3$ (Trevelyan et al., 1950) or phosphate spray (Hanes & Isherwood, 1949) for sugar and nucleotide visualization, respectively. The solvent systems used were (A) 7% (v/v) ethyl

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[1] Abbreviations: UDP-Gal, uridine 5′-diphosphate galactose; UDP-Glc, uridine 5′-diphosphate glucose; UDP-dGlc, uridine 5′-diphosphate 4″-deoxyglucose; UDP-Arab, uridine 5′-diphosphate L-arabinopyranose; αLA, α-lactalbumin; Glc, glucose; GlcNAc, *N*-acetylglucosamine; LacNAc, *N*-acetyllactosamine; dGlc, 4-deoxyglucose or 4-deoxyxylohexose; $R_{UDP\text{-}Gal}$ or R_{ref}, the R_f value relative to a UDP-Gal or reference standard; TLC, thin-layer chromatography.

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