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The Substrate Proton of the Pyruvate Kinase Reaction[†]

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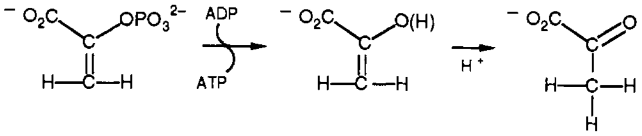
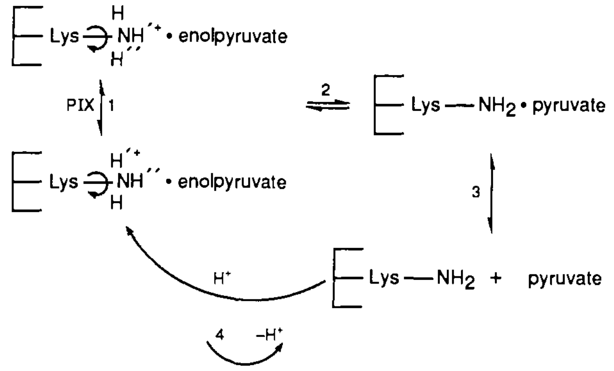
ABSTRACT: The pyruvate kinase reaction occurs in separate phosphate- and proton-transfer stages:



K^+, Mg^{2+}, and Mg·ADP are known to be required for the phosphoryl transfer step, and K^+ and Mg^{2+} with allosteric stimulation by MgATP are important for proton transfer. This paper uses the isotope trapping method with ^3H-labeled water to identify the proton donor and determine when in the sequence of the catalytic cycle it is generated. When the enzyme was allowed to exchange briefly with ^3H_2O (pulse phase) and then diluted into a mixture containing PEP, ADP, and the cofactor K^+, Mg^{2+}, or Co^{2+} in D_2O (chase phase), an amount of [^3H]pyruvate was formed in great excess of the amount expected from steady-state catalysis in the diluted ^3H-labeled water. With K^+, Mg^{2+}, and ADP at pH 6–9.5 in the pulse phase, a limit of 1.25 enzyme equiv of ^3H were trapped. The concentration of PEP required for half-maximum trapping was 14-fold greater than its steady-state K_m. Therefore, the rate constant for dissociation of the donor proton is estimated to be 14 times the steady-state rate of [^3H]pyruvate formation, $\sim$109 s^{-1}, or 1500 s^{-1}. At pD 6.4, Mg^{2+} and ADP were required in the chase, indicating that the ADP in the pulse was not bound tightly enough to be used in the chase. At pD 9.4, ADP was not required in the chase, only Mg^{2+} or Co^{2+}, making it possible to limit the chase to one turnover from hybrid labeled complexes such as E·K·Mg·CoADP or E·K·Co·MgADP and PEP. The ^3H trapped from these complexes are 1 and 1.8, respectively. With Co^{2+} and ADP in both the pulse and chase, 3 enzyme equiv of ^3H were trapped. The value of 1.8 equiv trapped in one turnover is consistent with the preequilibration of a donor containing three ^3H with the substrate, PEP, containing two ^1H to produce pyruvate with three equivalent positions of hydrogen, i.e., $^3/_5$ of 3 = 1.8. Muirhead et al. (1987) have concluded that Lys-269 is likely to be the proton donor on the basis of crystallographic studies of pyruvate kinase of cat muscle. In terms of lysine the amount of label trapped in pyruvate would depend on positional isotope exchange (PIX) to mix the three NH_3^+ protons in the ternary complexes and the extent of exchange that occurs between Lys-$^{\epsilon}NH_2$ and medium before the next recycle of reaction, steps 1 and 4, respectively, of eq 1. With Mg^{2+} step 1 would have to be slow and step 4 rapid compared with the rate



(1)

of product formation to explain the apparent monoprotonic nature of the donor. The opposite would be true with Co^{2+}, allowing 1.8 equiv to be trapped in one turnover and three in multiple turnovers. For the pulse and chase pHs to be as high as 9.5 without a decrease in the amount of ^3H that could be trapped a lysine of very high pK_a is required. To achieve proton-exchange rates $>10^3$ s^{-1} from a group of such high pK_a requires catalysis. Buffer catalysis could be ruled out. Therefore, internal exchange probably occurs with a residue of low pK_a with good access to the medium. The residue may be remote, requiring a proton relay, or it may be a residue in the active-site cavity such as Glu-271, which in the absence of PEP might have direct or indirect access to Lys-269. Upon addition of PEP this circuit would be broken as the Glu-271 serves some important function in the catalyses or is otherwise diverted.

The pyruvate kinase reaction is best described by the succession of two reactions:

$$H^+ + ADP + PEP \rightleftharpoons ATP + enolpyruvate$$
$$enolpyruvate \rightleftharpoons pyruvate$$

[†] This work is presented in memory of Timothy R. Talbot, M.D. (1916–1988), who provided the leadership for development of this Institute and this Center (Weinhouse, 1989). He was a partisan for basic research and a firm advocate of excellence. This work was supported by USPHS Grants GM-20940, CA-06927, and RR-05539 and also by an appropriation from the Commonwealth of Pennsylvania.

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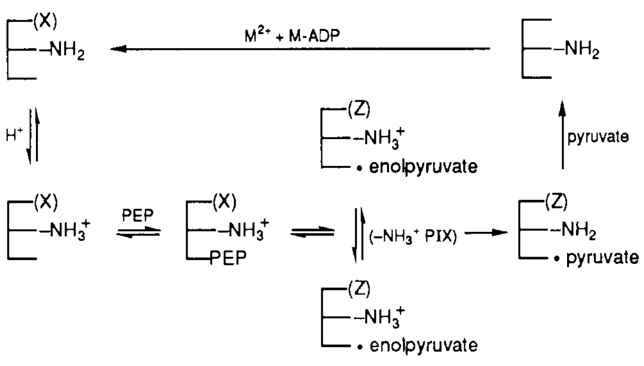
Evidence for this is as follows: ^3H exchange between pyruvate and water occurs in the absence of nucleotides (Rose, 1960). The enzyme catalyzes the stereospecific ketonization of added enolpyruvate (Kuo & Rose, 1978; Kuo et al., 1979) and will phosphorylate a variety of acceptors [see Kayne (1971) for a review]. Most recently we have been able to demonstrate significant amounts of a species with the properties of enolpyruvate in a quenched incubation of enzyme with pyruvate and an ATP analogue and to establish the equilibrium constants for both half-reactions on the enzyme (Seeholzer et al., 1989). Two cofactors are required for the overall pyruvate kinase reaction, K^+ and Mg^{2+} (Gupta et al., 1976), in addition to the nucleotide-bound Mg^{2+}. Both cations are required for the kinase activity and for the ketonization of enolpyruvate generated in solution by the action of phosphatase on PEP[1] (Kuo et al., 1979). Lodato and Reed (1987) used $Mn^{17}O$ spin coupling to demonstrate inner-sphere complexes between enzyme-bound Mn^{2+} and oxalate, a pyruvate analogue, and a non-bridge oxygen–P_γ of ATP. Another P_γ oxygen is part of the chelation structure of ATP with the second metal. Therefore, the enzyme-bound M^{2+} serves to position the substrates and influences the electronic distribution at the C2–O bond of pyruvate and the acidity of the C3–H. The nucleophilic C2–O of enolpyruvate carries out the in-line displacement of the $\beta\gamma$ bridge oxygen of ATP to form PEP and ADP (Hassett et al., 1982). Crystallographic studies by Muirhead et al., (1986, 1987) have identified the M^{2+} binding sites as oxygen atoms of the protein and substrates. These are contained in an $(\alpha/\beta)_8$ barrel domain of the enzyme. A charged Lys-269 is believed to be well-placed for transfer of H^+ to the 2-*si* face of enolpyruvate (Rose, 1970). An anion binding pocket to which FDP or ADP itself can bind in another domain is believed to have allosteric control properties that can be demonstrated under special conditions with the muscle enzyme (Phillips & Ainsworth, 1977) and more generally with other isoenzyme forms.

Concerning the rate profile, a variety of methods have been applied to defining the rate of phosphoryl transfer relative to product formation, including equilibrium isotope exchange and isotope trapping (Dann & Britton, 1978), and ^{31}P NMR line shape (Nageswara & Cohn, 1981; Stackhouse et al., 1985). Conditions for these experiments have varied too much to make a general statement possible. Robinson and Rose (1972) observed using [^3H]PEP that significant amounts of label appeared in water, as much as 30–60%, depending on the pH and M^{2+} used, although lactate dehydrogenase was present to prevent exchange from free pyruvate. For this to occur the labeled pyruvate must undergo positional exchange of its CH_3 hydrogens while on the enzyme. ^3H must then be abstracted from the bound [^3H]pyruvate in spite of a large intrinsic isotope effect and replaced by a proton derived from the medium. The results of the present investigation would amend the general scheme given in the 1972 paper to show that the proton comes from Lys-NH_3^+ instead of the medium, and therefore a positional exchange of ^1H for ^3H on the NH_3^+ group, a form of "interal exchange", precedes the exchange with medium, which only occurs after products are released (Scheme I). An unexpected result of the investigation is the finding that both internal and external exchange are strongly influenced by whether Mg^{2+} or Co^{2+} is used as cofactor. The conclusion that product release is rate limiting will be reexamined in what follows in view of a 4–5-fold inhibition of V_{max} by D_2O.

Scheme I



(X) = (K^+, M^{2+}, M-ADP)
(Z) = (K^+, M^{2+}, M-ATP)

MATERIALS AND METHODS

Pulse/Chase Procedure. The method consists of two phases: the *pulse* phase in which the enzyme with necessary cofactors is incubated in ^3H-labeled water for a short unregulated period, allowing an *equilibrium* phase to be achieved between the medium and the catalytically significant protons. The pulse solution is added to a large volume of buffer (the chase) containing all of the reaction components required to compete by product formation with the exchange loss of ^3H. The reaction is stopped at a time greater than $1/k_{cat}'$, i.e., after many reaction cycles, and much less than the dilution factor/k_{cat}', where k_{cat}' is turnover as measured by ^3H incorporation. Controls are done in which the incorporation of ^3H from the medium is determined by adding substrate(s) *after* the pulse solution has been diluted. The fraction of the bound label that will be trapped in product has been found to be hyperbolically related to the concentration of the substrate used in the chase that completes the reaction mixture (Rose et al., 1974) and to be related to the off-rate of the labeled species by the simple equation $k_{off}/K_{1/2} = k_{cat}/K_m$, where $K_{1/2}$ is the concentration of the trapping substrate required for half-maximal trapping. In other words, if $K_{1/2}$ is 10 times greater than the K_m of the varied substrate, the k_{off} rate of the test substrate will be 10 times greater than k_{cat} in the steady state. This interpretation, of course, assumes that the behavior of the enzyme in the first turnover is typical of its behavior in the steady state. In addition to yielding k for $E{\cdot}{}^3H^+ \rightarrow E + {}^3H^+$, where the ^3H is associated with the donor site, this kind of experiment may be expected to give the pK_a of the donor in the absence of substrate, and if less than 1 enzyme equiv is trapped at pulse pH < pK_a, the loss of ^3H from a liganded form of the enzyme during the chase would be indicated.

The conditions of the pulse phase are varied as indicated but typically contain in 5 μL pyruvate kinase (2 nmol determined by A_{280nm}), KCl (50 mM), $MgCl_2$ or $CoCl_2$ (5 mM), ADP (20 mM), potassium cacodylate (75 mM), pH 6.0, and ^3H-labeled water (typically $\sim 10^9$ cpm). The mixture was kept on ice for at least 5 min before use. The chase solution typically contained in 2.0 mL of D_2O KCl (100 mM), $MgCl_2$ or $CoCl_2$ (10 mM), ADP (2 mM), PEP (10 mM), and buffer (50 mM, either potassium cacodylate, pD 6.4, or CHES, pD 9.4). D_2O in the chase had some critical consequences not related to the original reason for using it, namely, should we wish to use enolpyruvate as a trap, its stability would be greatly enhanced in D_2O (Kuo et al., 1979). The chase solution, in

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[1] Abbreviations: PEP, phosphoenolpyruvate; M^{2+}, a metal cofactor required in addition to a second one used for nucleotide binding to the enzyme; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; PIX, positional isotope exchange.

a 20-mL counting vial, was stirred magnetically at 4000–5000 rpm (determined with a stroboscope) at ambient temperature (19–21 °C) by using a magnet, vial, and mixer that had been found to give smooth stirring. The 5 μL from the pulse phase was mixed into the chase solution and quenched in 1 s by 160 μL of TCA (1 M) containing pyruvate (10 μmol) as standard, to correct for incomplete recovery. When necessary, PEP was added as carrier for the same reason. The quenched mixture was placed on ice for at least 5 min and neutralized with 75 μL of triethanolamine base (2 N). An accurate sample was taken to determine the total counts. This was used to determine the specific activities of the water in the pulse and chase and to precisely evaluate the amount of enzyme used in each experiment. In control experiments, PEP was omitted from the chase until after mixing with the pulse solution. PEP was then added in small volume followed by the same schedule of stirring and addition of acid. Controls were done with each experimental set. The counts in the control were due to a contaminant in the ^{3}H-labeled water stock (~10^{-5}% of the total) and the turnovers of enzyme during 1 s. A good fume hood and an oxygen mask were used throughout this procedure as well as in the initial steps in the ion-exchange isolation of products.

Table I: Condition for Functional ^{3}H-Labeled Enzyme Formation[a]

	additions	E_T[b] (nmol)	[^{3}H]pyruvate		^{3}H trapped/E_T
			cpm[c]	nmol[d]	
1.	K	1.37	511	0.56	0.41
2.	K, Mg	1.62	443	0.49	0.30
3.	K, Mg, PEP	1.65	437	0.48	0.29
4.	K, Mg, ADP	1.47	1471	1.61	1.1
5.	K, Mg, AMP	1.45	349	0.38	0.26
6.	control for 4[e]	(2.20)	(238)		(0.00)

[a] Each pulse solution contained in 5 μL about 1.5 nmol of enzyme active sites, KCl (75 mM), potassium cacodylate (pH 6.0, 100 mM) and ^{3}H-labeled water (911 cpm/nequiv of H). Further additions included $MgCl_2$ (5 mM), PEP (1 mM), ADP (20 mM), or AMP (20 mM). The chases were alike: pD 6.4, K^+ (100 mM), Mg (20 mM), ADP (2 mM), PEP (10 mM), and potassium cacodylate (50 mM) in 2 mL. Acid was added to stop the reaction at ~1 s. [b] E_T calculated from the total counts introduced into each chase and the counts/nmol of enzyme in the pulse. [c] Counts recovered by ion exchange after subtraction of the counts found in control 6 normalized to the same amount of enzyme. [d] Corrected counts/sa water (911 cpm/nmol). [e] PEP added after pulse diluted 400-fold; ^{3}H-labeled water = 2.3 cpm/nequiv of H.

Isolations. The neutralized reaction sample was added to a column of Dowex 1-X8 acetate form (0.8 × 2 cm), and the columns were washed with cold water until the effluent reached a background level of radioactivity. The pyruvate and PEP were eluted between 5 and 20 mL of cold 50 mM HCl, neutralized with 300 μL of 2 N triethanolamine, and taken to dryness under reduced pressure. ^{3}H activity and pyruvate were determined in the residue. The recovery of pyruvate (determined with lactate dehydrogenase) was routinely quantitative and that of PEP at least 70%. To determine the distribution of trapped counts between pyruvate and PEP, H_2O_2 was added to a sample causing immediate oxidation of the pyruvate without loss of the PEP as determined by assay. When recycled on a Dowex 1-X8 Cl^- column, the label coeluted with a sample of [^{14}C]acetate in 10 mM HCl and the PEP with few counts in 50 mM HCl.

Calculations. The nanomoles of ^{3}H incorporated into pyruvate per nanomole of enzyme was determined from counts isolated minus the control/specific activity of ^{3}H in the pulse/enzyme in pulse. Internal standards were used routinely to correct for variations in counting efficiency. To be aware of any changes in the enzyme during storage its activity was frequently determined and only used when shown to be around 350 units/mg by coupled assay with lactate dehydrogenase (LDH) at 25 °C, pH 7.0, according to Tietz and Ochoa (1958). Reaction conditions included 50 mM imidazole, 100 mM KCl, 4 mM $MgCl_2$, 2 mM ADP, 10 mM PEP, 0.3 mM NADH, and 30 units of LDH. However, the ultimate estimate of the amount of enzyme was based on A_{280} by using $\epsilon_{1\%} = 5.4$ (Bücher & Pfleiderer, 1955) and a molecular weight of 57 000 per subunit (Steinmetz & Deal, 1966) rather than activity. pD was determined from the measured pH + 0.4. When assayed under standard chase conditions, pD 6.4, the K_m of PEP was 45 μM and k_{cat} was 109 s^{-1}.

Enzymes and Chemicals. Rabbit muscle pyruvate kinase was obtained from Boehringer Mannheim Biochemicals. Removal of $(NH_4)_2SO_4$ and buffer adjustment were done by dialysis after which the enzyme at about 1 mM concentration was stored at −75 °C. ^{3}H-Labeled water (5 Ci/mL) was from Amersham. D_2O (99.8%), PEP (both the trisodium and tricyclohexylamine salts), and lactate dehydrogenase were from Sigma.

RESULTS

Pulse Conditions. To determine if pyruvate kinase binds the proton that is used in the ketonization step prior to the phosphoryl transfer, the enzyme was placed in ^{3}H-labeled water briefly in combinations of its cofactors and ADP or PEP but not both. This pulse solution was diluted ~400-fold into a well-stirred solution containing cofactors, ADP, and PEP in D_2O at room temperature. In this way, the enzyme-bound enolpyruvate complex that is generated may have its donor proton enriched with ^{3}H, compared to the medium in which reaction occurs. Acid was added at 1 s, which allowed 40–100 turnovers of the enzyme. Radioactivity in the pyruvate plus PEP fraction that was recovered always exceeded a suitable control, showing that the donor group of the enzyme was labeled in the pulse mixture prior to the kinase step (Scheme I). Greater than 98% of the ^{3}H that was trapped was found in pyruvate as shown by separation of the labeled acetate formed after H_2O_2 treatment from the unreacted PEP.

The amount of ^{3}H in product will depend on many factors: the extent to which the hydrogen donor was labeled in the pulse, i.e., the pH of the pulse relative to the pK_a of the donor, and in the chase the rate of ^{3}H dissociation in competition with its conversion to full reaction complex and the extent to which the ^{3}H is lost from intermediates. The amount of ^{3}H trapped in each turnover will depend on the number of donor equivalents that mix with the two protons of PEP from which pool three protons of the pyruvate will be formed. When a donor is multiprotonic, the amount of ^{3}H in the product that accumulates will depend on the exchange properties of the ^{3}H remaining on the product form of the enzyme. With the chase conditions kept constant, the effect of using different combinations of cofactors and substrate in the pulse was determined (Table I). ADP, probably with its Mg^{2+}, was unique in being able to increase the ^{3}H occupancy to slightly more than 1 enzyme equiv. AMP, FDP, citrate, AsO_4, and FPO_3 were as ineffective as PEP in this respect.

From the dependence of the amount of ^{3}H trapped on ADP concentration in the pulse, one may determine the dissociation constant of MgADP in its complex containing $E{\cdot}{}^3H{\cdot}K^+{\cdot}Mg^{2+}$. The data, omitted for brevity, indicate a linear plot of (^{3}H equiv trapped)$^{-1}$ vs (ADP, mM)$^{-1}$ over the range 0.3–10 mM ADP. The value K_d of ADP = 0.8 mM obtained in this way is greater than has been reported by use of physical methods

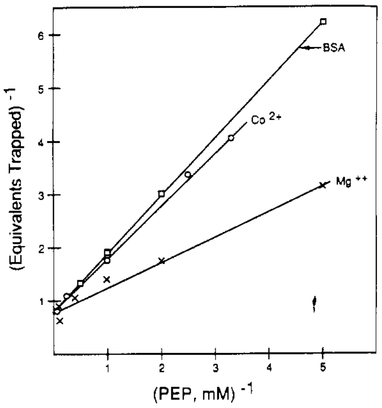


FIGURE 1: Extent and ease of trapping varying Mg or Co in the pulse and K, Mg, and cacodylate (or BSA) at pD 6.4 in the chase: Mg^{2+} (5 mM) in pulse (X)–$K_{1/2}$(PEP) = 0.63 mM, T_{max} = 1.25 equiv. With Co (O) $K_{1/2}$ = 1.18 mM and T_{max} = 1.19. With BSA (20 mg/mL) replacing cacodylate (□) in the chase $K_{1/2}$ = 1.5 mM, T_{max} = 1.35. By use of the steady-state constants K_m(PEP) = 45 μM and k_{cat} = 109 s^{-1} under normal chase conditions and the formula $k_{off} = k_{cat}K_{1/2}/K_m$, k_{off} rates were 1500, 2800, and 3600 s^{-1}.

and from inhibition studies (Mildvan & Cohn, 1966). Although ADP was also present in the chase medium, its role in the pulse was at the substrate site since, as will be shown, its presence on the enzyme assures that product will be formed in a chase lacking ADP.

When the pH of the pulse containing ADP was raised, it was expected that a point might be reached at which less ^3H would be trapped due to lower occupancy of the donor group above its p*K*. By use of the same chase condition (pD 6.4 with $MgCl_2$), no change was found in the pH range 6.0–9.5 (values all between 1.19 and 1.38 enzyme equiv trapped). The high apparent pK_a of the donor group in the pulse solution might be a lysine, tyrosine, a water chelated to Mg^{2+}, or a group having a proton stabilized by additional interactions. A critique of this method for determining the donor pK_a will be given under Discussion.

Chase Conditions. The concentration of PEP used in the chase at pD 6.4 was varied, keeping the K^+, Mg^{2+}, and ADP constant (Figure 1). K^+, Mg^{2+} or Co^{2+}, and ADP were present in the pulse. Linear double-reciprocal plots were obtained as expected for simple competition between exchange and trapping (Rose et al., 1974). The limit value of ^3H that could be trapped was ~1.25 enzyme equiv. When Mg^{2+} was used in the pulse, half of maximum trapping required 630 μM PEP or 14 times the K_m of 45 μM found under the same reaction conditions when LDH was used in the assay where k_{cat} was found to be 109 s^{-1}. Therefore, ^3H exchanges from the E·K·Mg·ADP complex at 14k_{cat} or ~1500 s^{-1}. The significant difference in $K_{1/2}$ values when Co^{2+} or Mg^{2+} was used in the preequilibrium shows that Co^{2+} and CoADP were not fully exchanged for Mg or MgADP in the chase. Buffer catalysis is not important since the ^3H exchange rate was about the same if the cacodylate (50 mM) in the chase was decreased to 5 mM or, if following Pocker and Janjik (1988), bovine serum albumin (20 mg/mL, containing 5 mM histidine residues, pD 6.4) was used as the main buffer source (Figure 1).

To determine if components of the ternary substrates complex dissociate in the chase phase at significant rates in competition with product formation, specific components of the chase solution were either omitted (K^+, M^{2+}, or ADP) or changed (Mg^{2+} to Co^{2+}), keeping the PEP concentration high at 10 mM. The dilution of the pulse solution would have decreased the free components 400-fold to K^+ (0.5 mM), Mg^{2+} (25 μM), and ADP (50 μM), concentrations unable to maintain a full complex in competition with detritiation. As seen in Table II, at pD 6.4, 65–75% of the complex containing K, Mg, and ADP was not trapped by very high PEP at pD 6.4, if Mg^{2+} or ADP was omitted from the chase, possibly showing loss of MgADP from the central equilibrium complex before product release. This would be consistent with equilibrium exchange studies of Dann and Britton (1978), showing that ADP/ATP exchange is greater than PEP/ATP exchange in the same experiment and is not suppressed by PEP. The complex was more stable in this respect in the chase at pD 9.4. The bound MgADP component of the pulse complex was retained long enough for the kinase and ketonization steps to occur as shown by ^3H trapped in the virtual absence of ADP in the chase as indicated by low extents of labeling in parallel controls. K^+ was not lost from the complex before PEP binds and the catalytic cycle is completed (experiment 2 of Table II). The differences seen between the Mg^{2+} and Co^{2+} chases suggest that E–Mg^{2+} of the pulse dissociates significantly before being fixed into the ternary substrates complex at the high pH using 10 mM PEP. The presence of Co^{2+} in the chase more than compensates for the dilution of the Mg^{2+}.

Table II: Components Required in the Chase[a]

	^3H trapped/ E_T		^3H trapped/ E_T
1. pD 6.4		2. pD 9.4	
K, Mg, ADP	1.11	K, Mg, ADP	1.04
ADP	0.34	TMA, Mg, ADP	1.18
K, Mg	0.29	K, Mg	0.89
K, ADP	0.26	K	0.61
		3. pD 9.4	
		K, Co, ADP	1.62
		K, Co	1.83

[a] The normal pulse, pH 6.0 with K (100 mM), Mg (5 mM), and ADP (20 mM), was diluted 400-fold. The chases contained either potassium cacodylate (50 mM), pD 6.4, or CHES (50 mM), pD 9.4, with or without KCl (100 mM), $MgCl_2$ or $CoCl_2$ (10 mM), ADP (2 mM), and PEP (10 mM). Although not added, the concentrations of missing components were K^+ (0.25 mM), Mg^{2+} (12 μM), and ADP (50 μM) derived from the pulse. In experiments in which ADP is not present in the chase it is assumed that the trapping process could not have gone beyond one catalytic cycle.

Nature of the Proton Donor. How completely does the ^3H that is trapped represent the whole content of the donor in the limit of high PEP? The difference between 1.83 in experiment 3 of Table II with CO^{2+} and pD 9.4 and the limit of ~1.25 in Figure 1 with Mg^{2+} and pD 6.4 is out of the range of error and suggested that some of the ^3H of the donor was lost on the way to product when Mg^{2+} and pD 6.4 were used. A test of this is to evaluate the discrimination that would be found against ^3H from the medium in the formation of product in a steady-state experiment. If trapping of the donor is incomplete, a large intrinsic isotope effect in the ketonization step will result in pyruvate with a lower specific activity than that of the water. Figure 2 shows the maximum velocities from pH 6 to 9 in H_2O and D_2O and the extent of discrimination against ^3H from the medium in both cases. As reported previously (Simon et al., 1968; Robinson & Rose, 1972), a 6–8-fold discrimination against ^3H is seen in H_2O. This can only occur if ^3H that is not used in the ketonization step is able to exchange into the medium or exchange with ^1H of the donor. A corresponding discrimination in D_2O of ~2.3 would be predicted (Swain et al., 1958). However, an average value of ~1.2 was found (Figure 2). Therefore, in trapping 1.25 enzyme equiv of ^3H as in Figure 1 the enzyme is not losing isotope by discrimination, and at the highest substrate con-

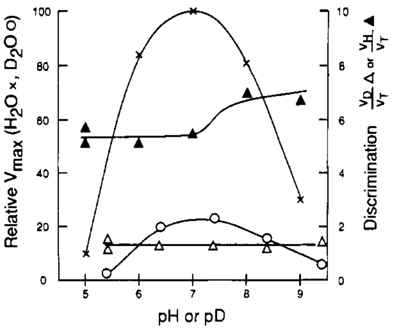


FIGURE 2: Effect of D_2O on V_{max} and ^3H into pyruvate as a function of pH. V_{max} was determined in 1 mL at 21 °C in 50 mM buffers (pH): acetate (5.0); cacodylate (6.0); 4-morpholinepropanesulfonic acid (MOPS) (7.0); N-(2-hydroxyethyl)piperazine-N'-3-propanesulfonic acid (HEPPS) (8.0); CHES (9.0) with KCl (100 mM), $MgCl_2$ (10 mM), PEP (2 mM), ADP (2 mM), NADH (0.3 mM), LDH (70 units), and pyruvate kinase (40 milliunits). Absorbance at 340 nm was followed. Incorporation of ^3H into pyruvate used the same conditions except PEP (10 mM), ADP (20 mM), NADH (13.5 mM), LDH (18 units), pyruvate kinase (50 milliunits), and 0.5 mCi of ^3H in 0.1 mL of H_2O or D_2O. Samples were placed in acid, lactate isolated on Dowex 1-X8 acetate, counted, and assayed with acetylpyridine-NAD at pH 9.5 with LDH.

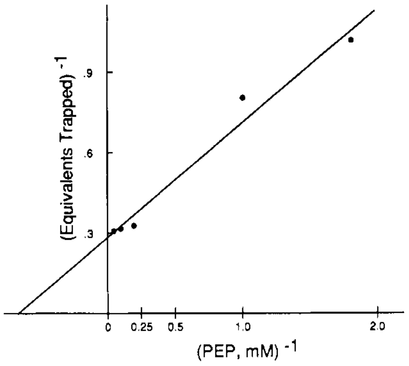


FIGURE 3: With CO^{2+} in the pulse and chase 3 equiv are trapped: The pulse contained KCl (100 mM), $CoCl_2$ (5 mM), ADP (20 mM), and potassium cacodylate (50 mM, pH 6). The chase (K·CHES, 50 mM, pD 9.4), KCl (100 mM), $CoCl_2$ (10 mM), ADP (2 mM), and PEP varied as noted. In the limit 3.3–3.5 equiv of ^3H were trapped with $K_{1/2}$(PEP) = 1.54 mM.

centration the enzyme is behaving like a monoprotonic donor. This would seem to rule out lysine-NH_3^+ as the donor unless the NH_3^+ group is torsionally restrained, allowing only one of its hydrogens to be used in the keto–enol equilibration (Scheme I) and therefore only one ^3H/enzyme to form the pyruvate product.

When Co^{2+} was used in the pulse instead of Mg^{2+}, about 3 enzyme equiv of ^3H could be trapped in a chase containing K^+, Co^{2+}, ADP, and PEP, at pD 9.4 (Figure 3). Therefore, a lysine seems quite likely as the donor after all. This is consistent with the finding 1.8 ^3H trapped when Co^{2+} is used at pD 9.4 during a single turnover (experiment 3 of Table II). This is the value expected if a torsionally free $[^3H]NH_3^+$ donor and PEP were to equilibrate, the pyruvate would carry off three-fifths of three ^3H of the donor or 1.8 enzyme equiv in one cycle. From this and other evidence for equilibration it is not possible to attribute the large D_2O effect seen for V_{max} to the ketonization step.

DISCUSSION

When the limit of ~1.2 enzyme equiv of ^3H trapped was found by using Mg^{2+} (Figure 1), it was reasonable to propose a monoprotonic donor or that trapping could only occur in one cycle of reaction, i.e., other hydrogens of the donor would have equilibrated in the chase in a manner not competitive with PEP. Apparently, the monoprotonic donor did not exchange with the other protons during the course of reaction since in a steady-state reaction in $[^3H]D_2O$ the pyruvate had about 1 ^3H/mol, neither less, as expected from the intrinsic isotope effect if product release is faster than enolization, nor up to 3 times more, as would be expected if pyruvate had fully exchanged with the medium prior to free product formation. This is consistent with a monoprotonic donor that is in equilibrium with bound pyruvate, an equilibrium that is isolated from the medium. The 4–5-fold slower catalytic rate in D_2O (Figure 2) cannot simply be due to a kinetic isotope effect that is expressed in the ketonization step.

When it was found that 3 equiv of ^3H could be trapped in the limit by using Co^{2+} rather than Mg^{2+}, it was clear that the donor group contained, or had access to, at least 3 enzyme equiv of ^3H from the pulse. Two hydrogen equivalents must be lost by exchange with the medium in every cycle under the conditions of Figure 1. The difference between the Mg^{2+} and Co^{2+} experiments depended on the makeup of the chase and could not be attributed to an effect of M^{2+} on the pool size in the pulse as shown in Table II, in which the same pulse solution was used in all experiments. The results with Co^{2+} again indicate that ketonization is in equilibrium and occurs without access to the medium; neither isotope discrimination nor enrichment was seen in a steady-state experiment using ^3H in D_2O: k^D/k^3H = 1.2 (pD 9.4/Co^{2+} with PEP at 10 mM). Of further interest is the realization that with Co^{2+} the donor pool must have stability through several turnovers. By use of a reaction time of 1 s, at least 40 turnovers of the enzyme occur under the Co^{2+}/D_2O conditions, allowing plenty of opportunity to transfer 60% of the ^3H present on the enzyme to the pyruvate formed in each cycle. If there are more than 3 equiv in the pool of protons that include the donor and any residues able to provide protons to the donor, these additional hydrogens must not be labeled, either because they do not become labeled in the pulse or because, once labeled, they exchange rapidly into the chase medium by processes with which high PEP cannot compete. Medium-exchanged residues that become members of the "donor pool" would not be detected because of their low specific activity. The trapping experiment alone would then not be definitive in identifying the total number of hydrogens available in excess of three for transfer to enolpyruvate.

That three is the real limit for the number of hydrogens that either constitute or are in rapid exchange with the donor when Co^{2+} is used is indicated by the observation that the limit at high PEP of ^3H that is trapped in one enzyme cycle (ADP omitted from the chase but present in the pulse) is 1.8 equiv. This is the value to be expected from equilibration of three ^3H of the enzyme and two ^1H of PEP with three of the five taken randomly to label pyruvate. One unlabeled hydrogen added to this pool would lower this value to 1.5 equiv, and two would lower it to 1.3 equiv.

In a single turnover isotope trapping experiment with Mg^{2+} close to 1 equiv of ^3H was trapped, requiring again that there not be unlabeled protons in equilibrium with a single ^3H of the enzyme. Assuming that Lys-NH_3 is the donor, this requires that positional exchange of its hydrogens (step 1 of eq 1) be slow relative to the rate of product release (step 3), leaving 2 equivalents of ^3H in the E–NH_2. These are apparently lost by exchange from the product form of the enzyme when Mg^{2+} is used but retained with Co^{2+}. These differences between Mg^{2+} and Co^{2+} explain the observation in H_2O

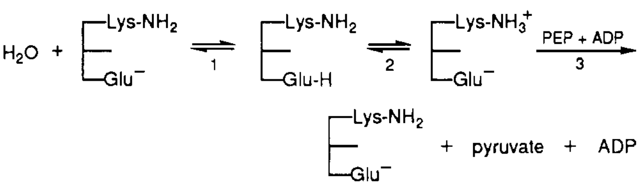
(Robinson & Rose, 1972, Table IV) that the exchange of ^{3}H of [^{3}H]PEP into water was greater with Co^{2+} than Mg^{2+}, whereas the exchange from water in PEP is less with Co^{2+}. Both exchange processes require PIX in bound pyruvate, but only the exchange into water requires PIX in the Lys-$NH_2{}^3H$. When the published results are normalized to the appearance of label in pyruvate in both experiments, the effect that one could attribute to an increase in PIX of Lys-NH_3^+ would be 10–30-fold greater with Co^{2+}.

What are the properties of the M^{2+} cofactor that contribute to freezing out the Lys-NH_3^+ group? In a comparison of four metal cofactors the ratios of ^{3}H-labeled water to ^{3}H-labeled pyruvate formed from [^{3}H]PEP with different M^{2+} followed in the sequence Co > Ni > Mn > Mg (Robinson & Rose, 1972, Figure 3) in linear inverse to the solution pK_a of water coordinated to each metal (Chaberek et al., 1952). At pH 7.5 the fraction, F, of [^{3}H]PEP that was used in the exchange path was $\log F = -0.3\mathrm{p}K_a$. In addition, from data of that paper, the fraction of [^{3}H]PEP that exchanged increased with pH, $\log F = 0.3\mathrm{pH}$. The exchange rate of [^{3}H]pyruvate was dependent on M^{2+} and pH in the same way as [^{3}H]PEP. Mn^{2+} and Mg^{2+} were equally effective as cofactors, but the pH for half-maximum rate was 1.5 units higher for Mg^{2+} (Robinson & Rose, 1974). From these results it is proposed that an ionizable group with pK_a determined by the M^{2+} cofactor interacts with the donor Lys-NH_3^+ such that its torsional freedom is restricted when the ligand to the metal is in the protonated form.

The influence of M^{2+} on the stability of ^{3}H in the E–NH_2 formed is critical for trapping after the first cycle. With Mg^{2+}, trapping the 2 equiv of ^{3}H by additional reaction cycles must be very inefficient, perhaps $(1.25 - 1)/2 = 12\%$. Stabilization by Co^{2+} of ^{3}H in the presumed Lys-NH_2 base is therefore orders of magnitude greater than by Mg^{2+}. An inner-sphere complex of Co^{2+} with the NH_2 group after product release, much weaker with Mg^{2+}, could protect the amine from proton exchange. The Co^{2+} would be displaced by PEP and ADP in the next catalytic cycle, allowing the amine to become protonated and, in the limit of high substrate concentration, all of the remaining ^{3}H would be trapped into pyruvate. Spectroscopic evidence for an inner-sphere amine ligand with Mn^{2+} (Reed & Cohn, 1973) and Co^{2+} (Kwan et al., 1975) of muscle pyruvate kinase under specific conditions may be related to stabilization of the donor in its product form.

Since the large inhibition of D_2O on V_{max} and discrimination (Figure 2) cannot be explained as a primary isotope effect on the ketonization step, another source must be found. The mechanism of entry of hydrogen to replace the one used in each cycle may provide the explanation. The observation of complete trapping, absence of exchange, in the pulse/chase limit implies that the regeneration of the donor must occur before the ternary substrates complex is formed. Either PEP or ADP can add first as shown by significant isotope trapping of PEP (Dann & Britton, 1978) and single-turnover ^{3}H trapping in a chase with PEP but without ADP (Table II). Exchange of ^{3}H can occur from either binary substrate complex: from E·PEP as shown by the incomplete trapping of ^{3}H from that complex (Table I) and from E·ADP in competition with PEP-dependent formation of ternary complex (Figure 2). The amount of PEP required for trapping is determined by the rate that ^{3}H per se dissociates from E·ADP rather than that of other components of that complex, K^+ and Mg^{2+}, which either do not dissociate or are present at high concentration in the chase (Table II). From the $K_{1/2}$ concentration of PEP, the dissociation rate constant of ^{3}H from E–Lys-NH_3^+·ADP is approximately 1500 s^{-1} (Figure 1). The value for ^{1}H exchange should be ~6 times greater (Figure 2), ~10^4 s^{-1}. Assuming a diffusion limit for reprotonation of 10^{10} M^{-1} s^{-1}, the highest pK_a the donor could have is ~6. Insensitivity of ^{3}H trapped to pulse pH up to pH 9.5, consistent with lysine as the donor, requires that the rate of uncatalyzed exchange be no more than 100 s^{-1} (Grunwald et al., 1957; Eigen et al., 1969). It may be that the distribution of ^{3}H on the enzyme in the pulse is sensitive to the pH of the chase. Evidence for this has been obtained in studies in progress with fumarase. This would be expected if the exchange of ^{3}H-labeled water from the active site were slower than the adjustment of this compartment to the pH of the chase medium with which it must be in continuity. In this case the data cited for the high pK_a of the donor on the basis of pulse pH alone would be misleading. Supporting this conclusion, however, is the experiment of Figure 3, where 3 equiv were trapped from a chase of pD 9.4.

Scheme II



Buffer ion catalysis of ^{3}H exchange from the donor NH_3^+ seems to be ruled out since no decrease in exchange was observed if the cacodylate used was decreased 10-fold. Furthermore, a macromolecular buffer, BSA, used instead of cacodylate, did not decrease the exchange rate (Figure 1). It is therefore likely that a protein functional group of lower pK_a catalyzes the dissociation of the donor and its regeneration in the catalytic cycle. From its location in the crystal structure (Muirhead et al., 1986, 1987) Glu-271, a conserved residue in the active site, could be the group in question. A proton relay including Lys-269, bound waters, Glu-271, and external water would be interrupted when the bound water was displaced by PEP.

Kinetic consequences of adding extra hydrogen-transfer steps prior to ternary complex formation can be discussed with reference to Scheme II. Isotope effects in both steps 1 and 2 could contribute to the 4–5-fold decrease seen when V_{max} is measured in D_2O. As mentioned, the ketonization step should not be the source of this effect. The phosphoryl-transfer step cannot be the site of a D_2O effect since, when a 20 times poorer acceptor, such as 2-deoxy-GDP, is used, the D_2O effect disappears instead of increasing (Rose, 1977). Discrimination against ^{3}H, 6-fold in H_2O, disappeared in D_2O (Figure 2). If the heavy atom isotope effect in step 2 was larger than in step 1, as might be expeected if more than one hydrogen is transferred in step 2, the discrimination, due to step 1, would decrease in D_2O much more than expected from the Swain relationship. The value for k^D/k^T predicted from $k^H/k^T = 6$ would be 2.2; found was 1–1.2. Discrimination against ^{3}H in normal water was not decreased if the phosphoryl-transfer step was made rate limiting by using dGDP (Rose, 1977). This might be explained by the relay hypothesis if high substrate makes step 2 irreversible so that the discrimination due to step 1 could be expressed.

The difference in behavior of pyruvate kinase with Co^{2+} or Mg^{2+} with respect to the size of the donor pool available for hydrogen exchange with PEP should be useful in characterizing the enzyme in vivo. When [6-$^2H_2$]glucose was fermented by yeast, 50% of the ethanol, the half coming from C1 and C2, had no deuterium, the rest contained CDH_2 and CD_2H in the ratio 0.21:1 (Saur et al., 1968). Sequence homology makes it probable that lysine in a similar environment is the hydrogen donor in yeast pyruvate kinase. As with the muscle enzyme [^{3}H]PEP is extensively detritiated during formation of pyruvate by yeast pyruvate kinase to an extent determined by pH and whether Mg^{2+} or Mn^{2+} is used (Ford & Robinson, 1976). Assuming the ketonization step to be at equilibrium as with the muscle enzyme, the extent of exchange should reflect the extent of positional exchange possible in a single turnover. Full positional exchange prior to product release, as observed for the muscle enzyme activated by Co^{2+}, would result in a ratio of 2:1 of mono- to dideuterated ethanol. The low ratio found requires a very low extent of positional exchange characteristic of a Mg^{2+}-activated enzyme.

CONCLUSIONS

(1) The enzyme, not the medium, is the immediate source of protons for the ketonization step.

(2) The donor group is fully formulated prior to the kinase step, especially if ADP is present.

(3) The donor group is a Lys-NH_3^+, probably the Lys-269 suggested by Muirhead (1987).

(4) Chimeric complexes can be generated for one turnover, which shows that it is the non-nucleotide M^{2+} that determines the PIX properties of NH_3^+ and protection of the NH_2 from exchange.

(5) With Mg^{2+} the donor is torsionally constrained to act as a monoprotonic donor. With Co^{2+} the donor acts as though it has three equivalent protons that can be used for the ketonization step.

(6) The E-Lys-NH_2 protons, present after liberation of products, are quite stable to exchange with the medium when Co^{2+} is present, suggesting formation of an inner complex. With Mg^{2+} exchange of these protons is more rapid than recycling.

(7) From the amount of PEP required to trap the donor proton, its exchange rate is calculated to be 10–100 times too fast for a lysine residue. Glu-271 seems appropriately placed to conduct protons between the medium and the donor.

(8) Methodological aspects of the isotope trapping method not previously realized may be mentioned here: Trapping of labeled substrate can be used to determine the binding constant of other components that govern the affinity of the labeled species for enzyme in the pulse. If the cofactor can be omitted from the chase without loss of hydrogens trapped, the cofactor will have been shown not to dissociate in each cycle of activity. By redefinition of $K_{1/2}$, dissociation rate constants of unlabeled components of the pulse can be determined if they are released in the chase. By deleting a substrate or cofactor from the chase that is suppled in the pulse, one may be able to limit the extent of catalysis of enzyme to one turnover. This may give information about the size of the donor pool without requiring that the label survive through additional reaction cycles. Finally, the methodology is simple, inexpensive, and reproducible. The reproducibility results in large part from faster mixing of the bulk of the ^3H_2O during the chase than of the active site associated ^3H_2O. Because of this difference and the overriding rate of pH equilibration in solution, the pH of the chase solution may be dominant in determining the ^{3}H occupancy of the active site rather than the pH of the pulse phase.

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