A Rate-Determining Proton Relay in the Pyruvate Kinase Reaction†

Irwin A. Rose,* Donald J. Kuo, and Jessie V. B. Warms
Institute for Cancer Research, Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, Pennsylvania 19111
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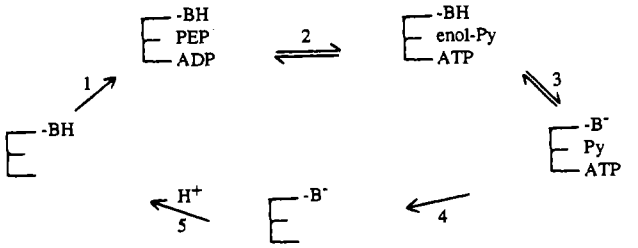
ABSTRACT: This study ascribes the large steady-state D_2O isotope effect on k_{cat} of pyruvate kinase (PEP + ADP → pyruvate + ATP) to the reprotonation of the product form of the enzyme for use in forming pyruvate. Previous tritium trapping experiments [Rose, I. A., & Kuo, D. J. (1989) *Biochemistry 28*, 9579-9585] with muscle pyruvate kinase showed that the proton used for ketonization of enolpyruvate is derived from an enzyme "pool" that contains three kinetically equivalent hydrogens that could be trapped in a nontritiated "chase" medium by high levels of ADP and PEP. The exchange of this pool with the medium was rapid in the free enzyme (~1400 s^{-1}), prior to addition of PEP, and apparently much less in the completed complex. The dissociation rate constant was determined by using the dissociation–competition equation $k_{off}^T = K_{1/2}k_{cat}/K_m$, where k_{cat}/K_m is the steady-state parameter for PEP and $K_{1/2}$ is the concentration of PEP required to trap 50% of the isotope that could be trapped. The present study shows that the competition constant, $K_{1/2}$, is decreased by ~5-fold in D_2O, the same effect seen on k_{cat} under conditions where k_{cat}/K_m, measured in the steady state, is not changed. The common effect of D_2O on k_{cat} in the steady state and k_{off}^T in pulse/chase suggests that the forward reaction rate is determined by hydrogen transfer to the enzyme. Further evidence indicates that the kinetically important proton in question is the proton used for ketonization of enolpyruvate, the substrate proton. This follows from an extract correlation in the conditions that show the D_2O effect on k_{off}^T and that establish the number of T that will be trapped into pyruvate, depending on whether Co or Mg is used as enzyme activator and whether the D_2O is present in the pulse or the chase phase of the trapping experiment. With Co, roughly three T of the pool can be trapped. With Mg, only one T can be trapped from the same pulse solution at the highest concentrations of PEP. Apparently two positions, possibly from a bound water molecule, are rapidly exchanged with water of the chase. The correlations are as follows: when Mg^{2+} is used and only one of the three T can be trapped, the D_2O effect on k_{off}^T is shown when D_2O is present in the chase. Its presence in the pulse medium has no effect. When the pool of protons that will appear in pyruvate is stable, with Co, the D_2O effect on k_{off}^T is seen with D_2O in the pulse and none given with D_2O in the chase. The inference that lysine NH_3^+ is the proton donor group on the basis of the three T trapped is open to question by evidence that one or two of these come from relay positions.

The reaction in which phosphoenolpyruvate (PEP) is converted to pyruvate with generation of ATP occurs in a sequence of two chemical steps:

$$\text{PEP} + \text{ADP} \rightleftharpoons \text{enolpyruvate} \rightleftharpoons \text{pyruvate}$$

The kinase and ketonization component reactions have been verified with appropriate half-reactions, such as the phosphorylation of glycolate and other acceptors [see Kayne (1971) for a review], and the ketonization of added enolpyruvate (Kuo & Rose, 1978; Kuo et al., 1979) and identification of enolpyruvate as a tightly bound component by chemical derivatization (Seeholzer et al., 1991). The overall reaction has an absolute requirement for a monovalent cation such as K^+ and two M^{2+} ions (Gupta et al., 1976), one to bridge the nucleotide to the enzyme and a second, also enzyme-bound, that is used to polarize the carbonyl of pyruvate for enolization and bind to the γ-P of the ATP to guide phosphoryl transfer to the enol or enolate (Lodata & Reed, 1987). The ketonization half-reaction is believed to occur within the domain of the eight alternating helix and sheet sequences, the β-barrel, that constitutes about half the mass of each of the four identical subunits of the muscle enzyme. The immediate source of the H^+ used in the ketonization is believed to be the NH_3^+ of lysine (Lys-269), on the basis of X-ray analysis of crystals infused with PEP (Muirhead et al., 1986, 1987) and pH rate dependence studies (Dougherty & Cleland, 1985), and apparently confirmed by T trapping experiments from this laboratory in which *three* enzyme equivalents of T were found in pyruvate after a solution of enzyme in T water was diluted into a medium containing ADP, PEP, K^+, and Co^{2+} (Rose & Kuo, 1989).

Scheme I



The reaction, as measured by pyruvate production, has long been known to show a large D_2O inhibition (Kayne & Seulter, 1968; Rose, 1977; Rose & Kuo, 1989). The broad pH range, over which the effect is seen (Rose & Kuo, 1989), would seem to rule out some general medium effect acting to alter the distribution of conformational states. Its magnitude, >5-fold, would imply a primary kinetic effect. There are two places in Scheme I from which a primary effect might originate, steps 3 and 5.

Many earlier experiments have suggested that step 3 is rapid and reversible, under most reaction conditions, leading to an

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* Address correspondence to this author.

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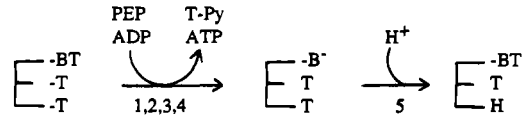
Scheme II



interest in step 5. T trapping experiments (Rose & Kuo, 1989) have provided some information about the proton used in the ketonization that will be useful in defining the origin of the D_2O effect and in its characterization. The hydrogen used for ketonization may bind to the enzyme before the phosphoryl-transfer step and be retained through step 2 in the presence of a high concentration of substrates. When MgADP was used, the number of T that could be trapped from enzyme preincubated with T water depended on the nature of the second metal cofactor, one T with Mg^{2+} and three T with Co^{2+}. The latter value, while supportive of lysine NH_3^+ as the proton donor, has an alternative interpretation (Scheme II) in which one T comes from a monoprotonic donor and two others are derived from a proton relay that is rapidly lost by exchange when Mg^{2+} is used.

In this model an isotope effect in step 5 could come from the transfer of two relay hydrogens, the medium-derived hydrogen, or all three. The model with Lys NH_3^+ as donor would derive its D_2O effect from a single proton transfer from solvent to $E-NH_2$ in step 5. The following evidence favors the relay model: the rate of detritiation of the enzyme shows a large D_2O effect requiring that bond rearrangement of medium-derived hydrogens be important in determining k_{-5}^T. These additional hydrogens are not indirect determinants, but are contributors of protons to the donor since the rules that govern the isotope effect in k_{-5}^T also determine the *number* of T that can be trapped. As direct contributors they may more reasonably show a large primary isotope effect on k_{-5}^T if they are members of a relay than if they are present as ND_2T^+ of the donor.

Materials and Methods

The recent paper by Rose and Kuo (1989) describes in detail the procedures used in the T trapping experiments. Briefly, a mixture of about 1 nmol of rabbit muscle pyruvate kinase (buffered in either H_2O or the same enzyme dialyzed in D_2O) and T water (~3000 cpm/nmol of hydrogen) was made. Other components of the pulse were buffer [usually 50 mM *N,N*-(bis-2-hydroxyethyl)-2-aminoethanesulfonate (BES)], pH 7.0, KCl (100 mM), ADP (5 mM) or dGDP (5 mM), and $MgCl_2$ or $CoCl_2$ at 5 mM. The chase solution in 2 mL contained KCl (100 mM) and varied in the concentration of PEP, nature of buffer in H_2O or D_2O, use of either Co^{2+} or Mg^{2+}, and presence or absence of ADP or dGDP as noted in the text. A 3-μL sample of pulse solution, prepared a few minutes earlier, was added below the surface of the chase solution as it was magnetically stirred at 23 °C. A TCA solution containing 10 μmol of pyruvate and 300 μmol of acid was added after an interval (usually 2 s) sufficient for many more turnovers of the enzyme than required to complete the partition of the T pyruvate and the medium. The acidic solution, a sample of which was taken for total counts, was added to Dowex-1 acetate column (0.7 × 2 cm) washed with water until counts came to background. The pyruvate was eluted with 20 mL of 50 mM HCl adjusted to pH ~5 with triethanolamine base and taken to dryness at 30 °C. Recovery was >90% without loss of T. Counts found in pyruvate were converted to enzyme equivalents trapped by using the T specific activity of the pulse solution and the amount of enzyme after counts incorporated from the chase solution per se were corrected for, using a control in which PEP was added after dilution of the pulse. The amount of pyruvate kinase present was based on A_{280} as before (Rose & Kuo, 1989).

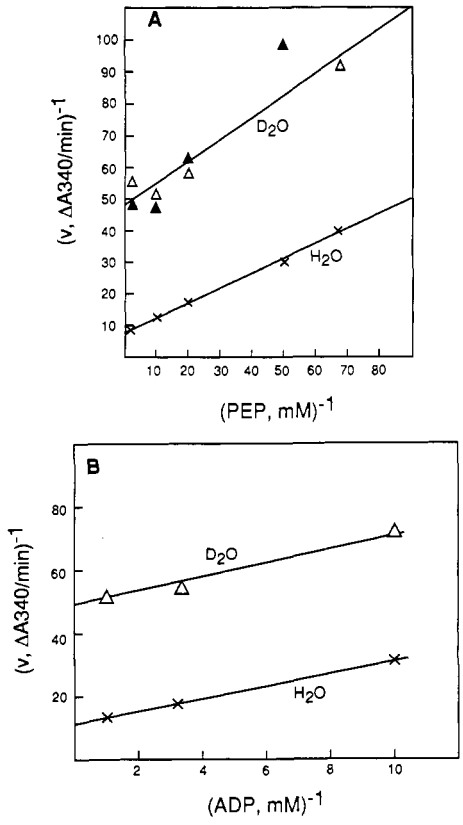


FIGURE 1: D_2O effect on initial velocity with Mg^{2+}, pH 7.0 (A), and Mn^{2+}, pH 8.0 (B). (A) Incubations in H_2O (×) or D_2O (Δ) contained PIPES (100 mM, pH 7.0), $MgCl_2$ (50 mM), KCl (100 mM), ADP (2 mM), NADH (0.3 mM), lactate dehydrogenase (30 units), varied PEP, and 1.8 pmol of pyruvate kinase. Initial rates were determined at 20 °C by change at 340 nm: $^Dk_{cat} = 196\ s^{-1}/30s^{-1} = 6.5$; $^D(k_{cat}/K_m) = 2.9 \times 10^6\ M^{-1}s^{-1}/2.1 \times 10^6\ M^{-1}s^{-1} = 1.3$. (B) Conditions modified from (A): buffer triethanolamine (100 mM, pH 8.0), Mn^{2+} (1 mM), PEP (constant at 0.5 mM), 0.8 pmol of pyruvate kinase, and ADP varied. $^Dk_{cat} = 4.4$; $^D(k_{cat}/K_m) = 1$.

Double-reciprocal plots of equivalents trapped versus PEP were always linear, giving the value $K_{1/2}$, the concentration of PEP in the chase required to trap half the upper limit of counts. Experiments done in D_2O were adjusted to a pH meter reading 0.4 unit greater than those in H_2O.

Initial rate measurements of pyruvate formation were made in H_2O or D_2O (1 mL) at 23 °C with NADH and lactate dehydrogenase; other conditions were as noted.

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. ^3H-Labeled water (5 Ci/mL) was from Amersham. D_2O (99.8%), PEP, ADP, dGDP, and lactate dehydrogenase were from Sigma.

Results

Origin of the Kinetic D_2O Effect. Mg^{2+}-activated pyruvate kinase is ~5 times more active in H_2O than in D_2O (Rose & Kuo, 1989). In the neutral pH range with Mg this is observed as a purely V_{max} effect (Figure 1). V_{max}/K_m is not significantly affected by use of either PEP or ADP as the varied substrate with the other held constant at high concentration. From this it may be concluded that the D_2O effect must occur in a step subsequent to the first irreversible step or subsequent to release

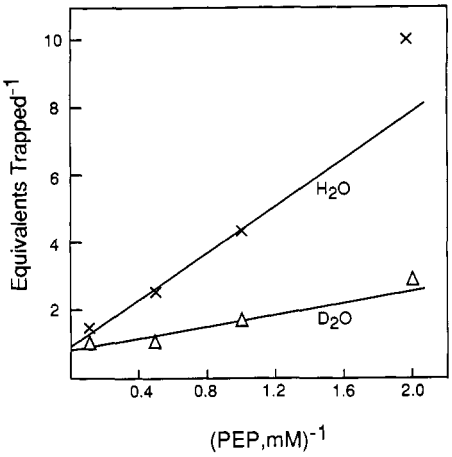


FIGURE 2: With Mg^{2+}, D_2O lowers k_{-5}^T when present in the chase. Pulses of 1.4 nmol of E in PIPES (100 mM, pH 7), KCl (100 mM), $MgCl_2$ (10 mM), and ADP (20 mM) were chased at 20 °C in an H_2O (×) or D_2O (△) medium with the same components, pH = pD = 7.0, except ADP (2 mM) and PEP varied as shown.

of the first product. Previous studies (Robinson & Rose, 1972) tend to characterize step 3 as rapid.

D_2O Effect on k_{-5} As Shown by Trapping Experiments. If the D_2O effect is caused by a decrease in rate at step 5, $E^{B-} \rightarrow E^{BH}$, a comparable effect can be expected for the same step in the reverse direction. This would lead to a D_2O effect on the loss/capture ratio of T as a function of PEP in pulse/chase experiments using the equation $k_{off} = K_{1/2}(k_{cat}/K_m)$ (Rose et al., 1974). A decrease in k_{-5}^T due to D_2O would result in a parallel decrease in $K_{1/2}$, the concentration of PEP required for half-maximal trapping, if there is no D_2O effect on k_{cat}/K_m for PEP, as is shown with Mg^{2+} at pH 7 (Figure 1A). A typical experiment testing the effect of D_2O on k_{-5}^T is reported in Figure 2. With D_2O in the chase, $K_{1/2}$ went from 3.7 to 0.87 mM, consistent with a 4-fold decrease in k_{-5}^T. Therefore, the dissociation of donor-related T of the pulse is linked to a chase-derived H transfer acting at the level either of solvent or of enzyme at positions derived from solvent. If the latter, they must be acquired from solvent by exchange of protons of the enzyme as it enters the chase. Furthermore, one may ask if these positions are themselves proton sources for the donor.

With Co^{2+}, three enzyme equivalents of T are trapped. Therefore, two positions having exchanged when Mg^{2+} is used make it possible for D_2O of the chase to affect k_{-5}^T from positions on the enzyme that supply the proton for the ketonization, in accordance with Scheme II. If these are the source of the D_2O effect, one expects the nature of the chase medium to have little effect on k_{-5}^T when Co^{2+} is used. This is seen (Figure 3A, compare × and ● symbols). If the two additional T trapped with Co^{2+} are part of a relay responsible for the D_2O effect, the nature of the medium used in the pulse should have a marked effect on k_{-5}^T as found in Figure 3A (× and △). On the other hand, with Mg^{2+}, use of D_2O versus H_2O in the pulse had no effect (data not shown). By use of D_2O in the chase and either H_2O or D_2O in the pulse, $K_{1/2}$ values of 1.4 and 1.25 mM were found.

It seemed likely that the solvent effect on k_{-5}^T due to D_2O in the pulse using Co^{2+} might be an underestimate since a significant effect of D_2O on k_{cat}/K_m was observed in initial rate experiments from pH 7 to pH 9. Correcting for these differences did not seem reliable since the Co^{2+} enzyme does show a pH-invariant region of maximum velocity (Kwan et al., 1975), which complicates such comparisons (Bender & Hamilton, 1962). A solution to this problem came with the

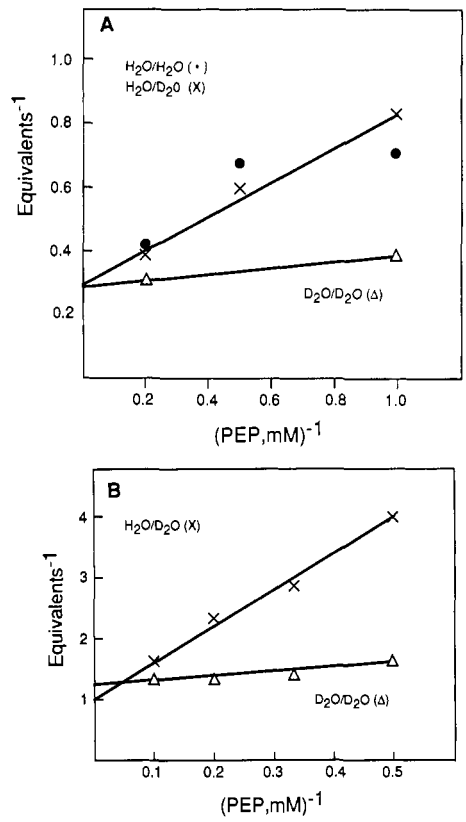


FIGURE 3: With Co^{2+}, D_2O lowers k_{-5}^T from the pulse having no effect from the chase. (A) Pulses of 1.4 nmol of E, BES [50 mM, pH 7.0 (● and ×) or pD 7.0 (△)], KCl (100 mM), $CoCl_2$ (10 mM), and ADP (20 mM) were chased at 20 °C in H_2O (●) or D_2O (× and △) medium with the same components, pH = pD = 7.0, except ADP (2 mM) and PEP varied. (B) Pulses with 1 nmol of E in BES [50 mM, pH 7.0 (×) or pD 7.0 (△)], KCl (100 mM), $CoCl_2$ (5 mM), and dGDP (10 mM) were chased into D_2O buffer (TEA, 50 mM, pD 8.0), KCl (100 mM), $CoCl_2$ (5 mM), dGDP (1 mM), and PEP varied. TCA was added at 5 s, resulting in ~10 turnovers on the basis of T incorporated in the controls.

use of dGDP instead of ADP in these experiments. In previous studies with the alternate acceptor and Mg^{2+} a 20 times slower V_{max} was observed with complete loss of the D_2O effect (Rose, 1977). By use of Co^{2+}, the rate with dGDP was ~10% the rate with ADP. Presumably step 2, the phosphoryl-transfer step, has become rate-limiting for the whole reaction cycle. Although step 5 would no longer be rate-limiting for the overall cycle, the dissociation–competition equation would be used to determine k_{-5}^T. Figure 3B shows that when dGDP was used, the $K_{1/2}$ for PEP was 10 times greater with enzyme pulsed in H_2O, 5 versus 0.5 mM. The importance of the medium of the pulse was again shown with enzyme chased in H_2O by using either H_2O or D_2O in the pulse: $K_{1/2}$ values were 2.5 and 0.5 mM, respectively (data not shown).

Do the Hydrogens of the Relay Mix with the Donor Hydrogen at Step 3? The above data suggest that the E^{BH} of scheme I, step 1, is better represented in Scheme II. Although the loss of two of the three enzyme equivalents of T when Mg^{2+} was used indicates that the donor position does not equilibrate with the others as they are being lost in the chase, there is reason to believe that the three positions do equilibrate during the enol → keto step 3. This is shown by T trapping experiments limited to a single turnover by the absence of ADP in the chase. As shown previously allowing only one turnover, the enzyme traps more than one T by using Co^{2+} and less than one T by using Mg^{2+} (Rose & Kuo, 1989). In a single-reaction turnover, a monoprotic donor would be able to supply exactly 1 equiv of T to form pyruvate unless the donor hydrogen

Table I: Comparing T Trapped in One and Many Turnovers[a,d]

M^{2+}	equiv of T trapped		
	one turnover[b]	many turnovers[c]	one/many
Mg^{2+}	0.77 ± 0.07	1.15 ± 0.03	0.65 ± 0.3
Co^{2+}	2.5 ± 0.05	3.3 ± 0.1	0.76 ± 0.04
Co/Mg	3.5	2.9	

[a] Enzyme (0.95 nm) was pulsed with T at pD 7.5 in BES (50 mM), KCl (100 mM), ADP (5 mM), and Co^{2+} or Mg^{2+} (5 mM). Chases with Mg (5 mM) contained imidazole (50 mM, pD 7), KCl (100 mM), PEP (10 mM), and ADP absent (one turnover) or present at 2 mM. Chases with Co (5 mM) were done in the same way at pD 9.0 (CHES, 50 mM). [b] No ADP in chase; final ADP will be 7 μM. [c] With ADP present in the chase, the controls (PEP added after dilution of the pulse) indicated that 15 and 100 turnovers of the enzymes occurred in the Mg and Co experiments, respectively. In the absence of ADP, turnovers, indicated by counts into pyruvate in controls, were nil. [d] Average of duplicate experiments.

became mixed with other hydrogens of the enzyme in the course of reaction. Assuming equilibration between the donor and these other hydrogens to be complete, the results should indicate the size of the pool, or, knowing the size of the pool, the single-turnover result should indicate the extent of equilibration in step 3. Such information is not available from normal trapping experiments, where many enzyme turnovers are allowed, and, in the limit of high trapping agent, all relevant T will be found in the product. When pyruvate is formed in a single turnover, it will carry off three-fifths of the T that participate in step 3 if that step is at isotope equilibrium with two hydrogens that remain on the enzyme; i.e., the ratio trapped in one/many cycles of trapping should be 0.6. Table I shows this ratio for Mg^{2+} and Co^{2+} to be 0.67 and 0.76, respectively. Incomplete isotopic equilibration in step 3 would give lower values as would complete equilibrium with more than two hydrogens proximal to the monoprotonic donor. The increase in counts trapped with multiple turnovers with Mg^{2+} shows that the loss of two of three that occurs in the first step is atypical and does not occur in subsequent turnovers.

Configurations of the Proton Relay. In Scheme II the distribution of the protons that can be used in pyruvate formation is that of one T associated with the donor and two others as components of the relay. At the interface of the relay and the medium a residue of low pK_a may act to shuttle protons between the two. To determine the number of bonds to H or D that are directly involved in the process of T dissociation, the effect of using D_2O at 0, 50, and 100% mole fraction was determined (Kresge, 1964; Gold, 1969; Albery, 1973). The steady-state maximum forward velocity measured with 50% D_2O was usually exactly midway between the 0 and 100% D_2O values, giving no indication of multiple proton transfers in the same process.

Discussion

At an earlier time, when the D_2O effect on pyruvate kinase was attributed to making the C–H bond in the forward direction, the loss of the D_2O effect when ADP was replaced by dGDP and V_{max} was decreased 20-fold was taken as evidence against a mechanism in which phosphoryl and proton transfer occur synchronously (Rose, 1977). In fact, we now believe that the D_2O effect observed on the forward maximum velocity of pyruvate kinase should indeed be assigned to the hydrogen derived from solvent that is used for ketonization of enolpyruvate. However, the source of the isotope effect is not at the ketonization step, step 3, which is believed to be at equilibrium on the enzyme in the steady state. Rather, the isotope effect comes from the reprotonation of the product form of the enzyme, step 5. The quasi-equilibrium status of step 3 was first inferred from the observation of exchange into water of T from PEP (Robinson & Rose, 1972), suggesting that release of products is slow relative to the exchange process. Consistent with this is the observation that the methyl group of pyruvate formed in one turnover acquires 65–76% of the T that can be trapped from approximately three positions on the enzyme (Table I). Without equilibration this value would be ~20% of all that could be trapped. The failure to observe a D_2O effect on k_{cat}/K_m of PEP (Figure 1) indicates clearly that any isotope effect on proton transfer in step 3 is masked by rapid reversal of that reaction. Finally, in unpublished studies, [^{32}P]PEP/ATP exchange at equilibrium over a range of conditions was only ~1.5 times slower in D_2O, possibly reflecting an equilibrium effect, ruling out steps 1–4 as sources of the kinetic D_2O effect on k_{cat}.

That step 5 may be *the* rate-limiting step in the steady state is supported by the large D_2O effect on k_{-5}^T measured by the isotope trapping method and seen as a decrease in the concentration of PEP required to trap in D_2O. Were the effect of D_2O on k_{cat} derived from some influence on steps 1–4, more PEP would be required to achieve 50% trapping in D_2O, not less. Since k_{cat}/K_m values of PEP and ADP were unaffected by D_2O (Figure 1), it is likely that reprotonation of the enzyme in the steady state occurs prior to addition of either substrate as shown in Scheme I. The possibility that such alternatives occur significantly without a D_2O effect is unlikely considering the size of the solvent effect seen at substrate saturation.

The D_2O effect on k_{-5}^T is seen in an H_2O medium with Co^{2+} if the D_2O is present in the pulse (Figure 3). Therefore, the D_2O effect on k_{-5}^T and therefore also on k_5 does not depend on the nature of the medium but on the nature of at least one enzyme-bound hydrogen. This enzyme H is itself used in the ketonization step as shown by the correspondence of the D_2O effect on k_{-5}^T with the incorporation into pyruvate of T from the pulse using either Mg^{2+} or Co^{2+} as activators (Figures 2 and 3). When Mg^{2+} is used and only one T can be trapped, only D_2O in the chase affects k_{-5}^T. This is attributed to replacement of two hydrogens of the enzyme H_3 by protons of the chase. If Co^{2+} is used in the chase with the same pulse solution, three T are trapped. Presumably, none of the functional hydrogens exchange in the presence of Co^{2+}. Now the chase has little effect on k_{-5}^T; D_2O in the pulse has a large effect. For this reason, the enzyme H is pictured as one of the protons of a proton relay structure that acts to carry protons from the medium to the active-site base. A second proton either functions in the relay directly or may be bonded to the heteroatom that supports the relay and enters the system by exchange. From the proton inventory analysis, no more than one H is required to move in the rate-limiting step.

The observation of about three T trapped seemed easily explained (Rose & Kuo, 1989) by the three hydrogens of a lysine NH_3^+ without reference to additional hydrogens. As nondonor sources of T are identified, the number of T remaining to characterize the donor grows smaller. Both the finding that the D_2O effect on k_{-5}^T comes from two hydrogens of the pulse using H_2O and Co^{2+} in the chase (Figure 3) and the problem of attributing a 5-fold D effect on k_{-5}^T to the difference between ND_2T^+ and NH_2T^+ undermine the assignment of all three T to this donor. The combination of Lys-269 as donor on the basis of the crystal structure (Muirhead et al., 1986) *and* two hydrogens in the relay should allow five T to be trapped in the limit. Uncertainty as to the fractionation of H and T in the pulse weakens assessment of the total number of T positions based on counts trapped alone. This problem was approached by comparing the counts trapped

in a one and many turnover experiment (Table I): In one turnover, assuming step 3 to be at equilibrium, three hydrogens as pyruvate are selected from a pool containing 2 H from the PEP and n T from the enzyme; i.e., $3/(2 + n)$ = ratio of counts trapped in one/many turnovers. The values from Table I correspond to $n = 2.6$ for Mg^{2+} and 1.9 for Co^{2+}. These fit best the model of a monoprotic donor and at least one hydrogen of the enzyme in exchange equilibrium with it. Again, at least one relay hydrogen is required to explain the D_2O effect on k^T_{-5}.

The assignment of Lys-269 as the donor was made from a crystal infused with PEP since no other prototropic amino acid residue seemed suitably located in the region designated as the active site (Muirhead et al., 1986). It may be worth remembering that the molecule on which the donor will act is not PEP but enolpyruvate and that the process of getting to enolpyruvate from PEP might produce significant changes in the PEP binding site. It can be questioned whether enolpyruvate itself added to a crystal would induce the proper orientation of the donor group since the maximum rate of ketonization of enolpyruvate, per se, was only ~11% of the rate found with PEP plus ADP (Kuo et al., 1979). That a single bound H_2O may serve a role in the proton relay is suggested by the loss of 66% of the functional T when Mg^{2+} is used instead of Co^{2+} (Table I). The failure to show a buffer ion effect on k^T_{-5} (Rose & Kuo, 1989) suggests that the relay may be terminated at the solvent end by a residue of pK_a below the pH used in the pulse, pH 7.0, and is therefore not responsible for any of the T trapped. Nevertheless, transfers through this group could be responsible for some of the solvent isotope effect noted in k_5 and k_{-5}. It is interesting to note that, to the extent that step 5 is rate limiting in the catalytic cycle, the enzyme will be unoccupied by anything derived from the substrates even at saturation. This remains to be tested.

Registry No. D_2, 7782-39-0; Co, 7440-48-4; Mg, 7439-95-4; Mn, 7439-96-5; PEP, 138-08-9; ADP, 58-64-0; pyruvate kinase, 9001-59-6; lysine, 56-87-1.

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