Kinetic Isotope Effects on Substrate Association: Reactions of Phosphoenolpyruvate with Phosphoenolpyruvate Carboxylase and Pyruvate Kinase†

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ABSTRACT: Kinetic isotope effects on association have been measured using the remote label methodology developed by O'Leary and Marlier (1979). The isotope effect on V/K_A for the first substrate in an obligatorily ordered mechanism is an isotope effect on its second-order rate constant for association with the enzyme. With phosphoenolpyruvate carboxylase the $^{18}(V/K_{PEP})$ when the bridging O is labeled decreases from 1.0056 ± 0.0007 to 0.9943 ± 0.0002 as the concentration of bicarbonate, the second substrate, increases from 2 to 200 mM. With pyruvate kinase the $^{18}(V/K_{PEP})$ decreases from 1.0024 ± 0.0014 to 0.9928 ± 0.0027 as the concentration of ADP increases from 1.5 to 30 mM. These inverse kinetic isotope effects are best understood as arising from an isotope effect on the rate constant for forming the Michaelis complex of enzyme and substrate. The inverse value suggests that the bridging oxygen is in a vibrationally stiffer environment in the transition state for the association reaction.

The binding of a substrate to the active site of an enzyme can potentially activate the substrate for the reaction catalyzed by the enzyme. The extent of the activation that can be observed in the Michaelis complex is a matter of some discussion. Initial observation of vibrational changes were made on the activated carbonyls in triose-phosphate isomerase and citrate synthase (Belasco & Knowles, 1980; Kurz et al., 1985). The catalytic importance of the vibrational changes in carbonyl frequencies was unequivocally demonstrated by the correlation of carbonyl frequency with the rate of deacylation of the chymotrypsin acyl-enzyme intermediate (Tonge & Carey, 1992). Callendar, Burgner and co-workers (Deng et al., 1989, 1992) have further shown that there can be significant changes in the Raman spectra, both of the pyridine nucleotide and of pyruvate when bound at the active site of lactate dehydrogenase. One corollary of the change in the vibrational spectra of a substrate when it binds at the active site is that there will be an equilibrium isotope effect on the association reaction. These equilibrium isotope effects on association have been directly observed (LaReau et al., 1989).

For the association of a substrate with an active site, the simplest kinetic scheme is given in eq 1. If there is an

$$E \underset{k_{off}}{\overset{k_{on}A}{\rightleftharpoons}} E{\cdot}A \qquad K_A = k_{on}/k_{off} \tag{1}$$

isotope effect on the equilibrium association constant, K_A, there must be an isotope effect on either one or both of the defining kinetic constants as well. In this scheme k_{on} is a second-order rate constant and is determined by the diffusion-limited number of collisions between substrate and active site and the fraction of collisions that generate a stable complex. In many cases this second-order rate constant approaches the diffusion limit of about 10^9 M^{-1} s^{-1}. This implies that the fraction of productive collisions is near unity. If diffusion is the sole factor determining k_{on}, then there should be a negligible isotope effect on the second-order rate constant, and any isotope effect on the equilibrium constant would have to be reflected directly in k_{off}. However, if the substrate is desolvated or if changes in the conformation of the substrate (with associated changes in vibration spectra) must occur to reach the transition state for association, kinetic isotope effects on k_{on} would be anticipated. We undertook a study of isotope effects on association rate constants to discriminate between these two possibilities and to consider the differences between equilibrium and kinetic isotope effects on association.

Measuring a kinetic isotope effect on association requires a system either where the association can be directly monitored or where the product of association can be quantitatively trapped. In order to use the precision available from isotope ratio mass spectrometry, we have chosen to kinetically trap the initial complex and convert it to product. This is possible in the obligatorily ordered two substrate kinetic mechanism with PEP being the initial substrate shown in eq 2. In this ordered mechanism the apparent V/K_A is

$$E \underset{k_{off\text{-}PEP}}{\overset{k_{on}PEP}{\rightleftharpoons}} E{\cdot}PEP \underset{k_{off\text{-}B}}{\overset{k_{on}B}{\rightleftharpoons}} E{\cdot}PEP{\cdot}B \xrightarrow{k_5} E + \text{products} \tag{2}$$

dependent on the concentration of the second substrate required to form the catalytic ternary complex. In the limit of saturating B, the association of PEP becomes completely rate determining; i.e., V/K_{PEP} for an obligatorily ordered reaction is the true second-order rate constant for the addition of PEP to the enzyme to form the kinetically competent

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binary complex. Under these circumstances the kinetic isotope effect on the association rate constant, k_{on}, should be determinable by the standard competitive methods developed to determine *V/K* heavy atom isotope effects on enzyme-catalyzed reactions by isotope ratio mass spectrometry (O'Leary, 1980).

Pyruvate kinase (PK)[1] and phosphoenolpyruvate carboxylase from *Zea mays* (PEP-C) are ideal model systems for these studies because prior kinetic studies (Dann & Britten, 1978; Dougherty & Cleland, 1985, for PK) (Janc et al., 1992) have provided strong evidence that both enzymes have effectively obligatorily ordered kinetic mechanisms with PEP adding first as shown in eqs 3 and 4. Both of these reactions

$$E \underset{k_2}{\overset{k_1PEP}{\rightleftharpoons}} E{\cdot}PEP \underset{k_4}{\overset{k_3HCO_3^-}{\rightleftharpoons}} E{\cdot}PEP{\cdot}HCO_3^- \xrightarrow{k_5} E + \text{oxalacetate} + \text{phosphate} \quad (3)$$

$$E \underset{k_2}{\overset{k_1PEP}{\rightleftharpoons}} E{\cdot}PEP \underset{k_4}{\overset{k_3ADP}{\rightleftharpoons}} E{\cdot}PEP{\cdot}ADP \xrightarrow{k_5} E + \text{pyruvate} + \text{ATP} \quad (4)$$

have PEP as the initial substrate, which allows the various isotope effects to be measured by the remote label method (O'Leary & Marlier, 1979) using the carboxylate of PEP as the carrier of the remote ^{13}C label.

MATERIALS AND METHODS

Materials. Pyruvate kinase (EC 2.7.1.40) from rabbit muscle, L-lactic dehydrogenase (EC 1.1.1.27) from rabbit muscle, pyruvate decarboxylase (EC 4.1.1.1) from yeast, hexokinase (EC 2.7.1.1) from yeast, oxalacetate decarboxylase (ODC, EC 4.1.1.3) from *Pseudomonas* species, ADP as potassium salt, thiamin pyrophosphate chloride (TPP), NADH as disodium salt, PEP as monocyclohexylammonium salt, glucose, $NH_2OH{\cdot}HCl$, L-lactate as lithium salt, and 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (bistris-propane, BTP) were purchased from Sigma and were used without further purification. Ascarite was purchased from Aldrich. Citric acid monohydrate, $MgCl_2$, and KCl were from Fisher Scientific. Carbonic anhydrase (bovine erythrocytes), glucose-6-phosphate dehydrogenase (G6PDH, *Leuconostoc mesenteroides*), and oxalacetate decarboxylase (ODC) were from Sigma. PEP-C was purified from *Z. mays* to homogeneity as described by O'Leary et al. (1981) and Diaz (1986) and had a specific activity of 20 units/mg.

Labeled PEP. [1-^{13}C,3-^{13}C]PEP, [1-^{13}C,2-^{18}O]PEP and [1-^{12}C]PEP (^{13}C-depleted) as tris(cyclohexylammonium) salts were synthesized as previously described (Caldwell, 1986). The [1-^{13}C]PEP species were mixed with a 90-fold excess of [1-^{12}C]PEP to approximate natural abundance of ^{13}C in the PEP carboxylate.

PEP-C Reactions. Labeled PEP (160 μmol) was mixed with varying amounts of $KHCO_3$ and incubated with 2.5 units of PEP-C and 25 units of ODC in 0.1 M BTP·HCl, pH 7.5 or 9.5, and 5 mM $MgCl_2$ in a total volume of 12 mL. The product oxalacetate was converted to pyruvate and bicarbonate by the action of ODC so that the concentration of the second substrate would not change during the reaction. The reaction was monitored at fixed times by enzymatically assaying for pyruvate, OAA, and PEP. After 15 min, 1.2 mL of the solution was removed and allowed to react overnight to reach 100% conversion. The other 10.8 mL was titrated to pH 2 to allow the substrate HCO_3^- to be converted to CO_2 and removed from solution. After 30 min, the pH was readjusted to 6.4 with BTP and thiamin pyrophosphate was added. The solution was transferred to a reaction flask sealed with a septum and connected to both a vacuum line and a side arm containing 2 units of PDC via stopcocks. CO_2-free nitrogen (generated by passing cylinder nitrogen through a column of ascarite) was bubbled through the solution for 1 h to remove any traces of CO_2, and then the PDC from the side arm was mixed with the pyruvate solution. Quantitative conversion to CO_2 and acetaldehyde was assured by removing 20-μL aliquots and assaying for remaining pyruvate. At completion the PDC reaction was acidified, and the nitrogen atmosphere was removed in three freeze−thaw cycles. CO_2 was distilled cryogenically using dry ice-2-propanol mixtures to trap the CO_2. Repeated cryodistillations yielded purified CO_2 which was quantified manometrically, and the isotopic composition was measured as described below.

Pyruvate Kinase Reactions. The pyruvate kinase reactions were run in the presence of glucose and hexokinase so that the ATP formed during the reaction would be reconverted to ADP, thus maintaining a constant ADP concentration during the reaction. This ensured that the conversion of PEP to pyruvate would be quantitative and that the back-reaction (phosphorylation of pyruvate by ATP) would be minimized. The reactions contained 15 mM PEP and glucose, varying concentrations of ADP in 20 mL of a reaction buffer containing 0.1 M BTP·HCl, titrated to either pH 6.8 or pH 8.4 with KOH, 0.1 M KCl, 10 mM $MgCl_2$, and 40 μM TPP. The reaction solutions were placed in the previously described vacuum line reaction flask and sparged for 30 min (pH 6.8) or 4 h (pH 8.4) with CO_2-free nitrogen. Pyruvate kinase (1 unit), PDC (10 units), and hexokinase (10 units) were added anaerobically via hypodermic syringe. The sample was quenched after the time estimated for 10% conversion by acidifying to pH 1−2 with phosphoric acid and the CO_2 isolated by cryodistillation as described above. The fraction of reaction was determined by manometrically quantifying the product CO_2. Because of the limited supply of some of the double-labeled PEP samples, the initial concentration of PEP was sometimes reduced to 2.5 mM and the extent of reaction allowed to increase to about 30% to maintain the amount of product CO_2. For all of the complete conversion samples, the reaction was left overnight and the PEP concentration was reduced to 1.67 mM.

Acetaldehyde is volatile and its molecular mass (44 amu) matches that of CO_2. Consequently, its presence would interfere with the precise isotope ratio determinations. An alternative process was devised to chemically remove acetaldehyde. Several samples were quenched by thermal denaturation by microwaving for 5 s at high power. These samples were acidified to pH 4.5, hydroxylamine (0.1 mL of 6 M) was added anaerobically, and the temperature was raised to 40 °C for 2 h. This converted all of the product acetaldehyde into a nonvolatile oxime. The CO_2 was then isolated by cryodistillation. The efficiency of the two methods of eliminating of acetaldehyde, i.e., "freeze-and-thaw" method and the chemical conversion to the oxime,

[1] Abbreviations: BTP, 1,3-bis[[tris(hydroxymethyl)methyl]amino]-propane; OAA, oxalacetate; ODC, oxalacetate decarboxylase; PEP-C, phosphoenolpyruvate carboxylase; PK, pyruvate kinase; TPP, thiamin pyrophosphate chloride.

was controlled by monitoring the abundance of peaks at *m/e* 29 and 43, known to be due to acetaldehyde. No difference in the results was observed for the two procedures.

Isotopic Ratio Analysis. The isotopic composition of the CO_2 samples was measured using a Finnigan Delta S isotope ratio mass spectrometer, equipped with a dual inlet system that allows alternation between a standard of known isotopic composition and the sample to be measured. Thus, the output from this instrument is an isotopic ratio $R = {}^{13}C/{}^{12}C$ of the sample compared to a laboratory standard and is expressed as δ. The δ value is related to the isotopic ratios as

$$\delta = (R/R_{st} - 1) \times 1000 \quad (5)$$

where R is the ${}^{13}C/{}^{12}C$ ratio for the measured sample and R_{st} is the ${}^{13}C/{}^{12}C$ ratio for the standard. Reported δ values were recalculated against an interlaboratory standard which is CO_2 obtained from the Pee Dee belemnite with a known ${}^{13}C/{}^{12}C$ of 0.01124 (Craig, 1957).

The ${}^{13}C/{}^{12}C$ isotopic ratios are obtained from the ratio of the intensity of peaks at *m/e* 45 and 44. These ratios must be corrected for the presence of a small contribution due to ${}^{12}C{}^{17}O{}^{16}O$ in the *m/e* 45 peak. The correction is made by subtracting from the 45/44 ratio a value that corresponds to the isotopic abundance of ${}^{17}O$ in the sample based on the measurement of ${}^{18}O$ abundance in the peak at *m/e* 46 as described by Craig (1957). In order to monitor the presence of acetaldehyde in the samples, the intensities of interfering peaks were measured at *m/e* 29 and 43, which correspond to loss of a methyl group or hydrogen, respectively, from acetaldehyde.

Calculations. Experimental values of the kinetic isotope effects were calculated using a modified equation of Bigeleisen (Bigeleisen & Wolfsberg, 1958) to accommodate the definition of δ:

$${}^{13}(V/K) = \frac{\ln(1-f)}{\ln[1 - f(1000 + \delta_f)/(1000 + \delta_0)]} \quad (6)$$

where δ_f and δ_0 are the relative isotopic compositions of the product at fraction of reaction f and that of the reactant at the beginning of the reaction, respectively. The latter value was determined from the isotopic composition of the CO_2 from the quantitative conversion samples based on the assumption that the initial isotopic composition of a substrate is the same as that of the corresponding product after 100% conversion. From the experimentally determined values of the remote label kinetic isotope effects, ${}^{3-13}(V/K_{PEP})$ and ${}^{2-18}(V/K_{PEP})$[2] were calculated according to eq 7, which assumes that the rule of the geometric mean holds (Bigeleisen, 1955):

$${}^{I}(V/K_{PEP}) = {}^{I,1-13}(V/K_{PEP})/{}^{1-13}(V/K_{PEP}) \quad (7)$$

${}^{I}(V/K_{PEP})$ is the calculated isotope effect at the isotopically substituted position, ${}^{I,1-13}(V/K_{PEP})$ is the experimental isotope effect determined for the doubly labeled material, and ${}^{1-13}(V/K_{PEP})$ is the experimentally determined isotope effect at the remote label position.

[2] The nomenclature of Northrop (1982) as expanded by Cleland (1982) is used to denote isotope effects. A leading superscript refers to an isotope effect on the following kinetic or thermodynamic parameter. Double isotope effects are indicated by two leading superscripts. If the site of isotopic substitution is ambiguous, the superscript contains a number indicating the site of isotopic substitution.

Table 1: Kinetic Isotope Effects on V/K_{PEP} for Phosphoenolpyruvate Carboxylase Determined from the Isotope Ratio of CO_2 Obtained from C-1 of PEP[a]

pH	$[HCO_3^-]$ (mM)	${}^{1-13}(V/K_{PEP})$	${}^{2-18,1-13}(V/K_{PEP})$	${}^{2-18}(V/K_{PEP})$
7.5	2	0.9950 ± 0.0002	1.0006 ± 0.0005	1.0056 ± 0.0007
7.5	8	0.9975 ± 0.0007	1.0018 ± 0.0002	1.0043 ± 0.0009
7.5	200	0.9951 ± 0.0001	0.9894 ± 0.0001	0.9943 ± 0.0002
9.5	8	0.9970 ± 0.0004	0.9966 ± 0.0003	0.9996 ± 0.0007

[a] ${}^{1-13}(V/K_{PEP})$ and ${}^{2-18,1-13}(V/K_{PEP})$ are experimental values determined from [1-^{13}C]PEP and [1-^{13}C,2-^{18}O]PEP, respectively, and ${}^{2-18}(V/K_{PEP})$ is calculated as their ratio as described in eq 7.

Table 2: Experimental Data Used for Determination of the Kinetic Isotope Effect (KIE) with [1,3-${}^{13}C_2$]PEP at pH 6.8 with 1.5 mM ADP[a]

no.	δ_0	f	δ_f	${}^{13}k$
1	−72.342	0.294	−75.112	1.0035
2	−72.031	0.391	−76.967	1.0064
3	−72.759	0.258	−75.869	1.0044
4		0.266	−76.005	1.0046
av:	−72.377		av:	1.0047 ± 0.0012

[a] The KIEs were calculated using the averaged δ_0 value.

RESULTS

The apparent ${}^{2-18}(V/K_{PEP})$ and ${}^{1-13}(V/K_{PEP})$ isotope effects on the PEP-C reaction as a function of bicarbonate at pH 7.5 are shown in Table 1. ${}^{1-13}(V/K_{PEP})$ is near unity and independent of the bicarbonate concentration. This makes extracting ${}^{2-18}(V/K_{PEP})$ from the observed isotope effect with [1-^{13}C,2-^{18}O]PEP less ambiguous. It is clear that the increasing bicarbonate concentration reduces ${}^{2-18}(V/K_{PEP})$ from a normal effect at low bicarbonate concentrations to a significantly inverse effect at high bicarbonate concentrations. We argue in the discussion that this observed inverse effect is unequivocal evidence of a kinetic isotope effect on the association rate constant.

Data from a typical remote label isotope effect determination of ${}^{3-13,1-13}(V/K_{PEP})$ on the pyruvate kinase reaction using [1,3-^{13}C-2]PEP are shown in Table 2. The data demonstrate the reproducibility of the experimental protocol and the precision of the isotope ratio mass spectrometer. The isotope effects on the pyruvate kinase reaction are presented in Table 3. The 3-^{13}C kinetic isotope effects measured at two different pH values, 8.4 and 6.4, differ significantly. Both the 3-^{13}C and 2-^{18}O isotope effects are dependent on the concentration of ADP. While the ^{13}C isotope effect becomes unity at high ADP concentration, the ^{18}O isotope effect becomes more inverse with increasing ADP concentration. Again, in the discussion it is argued that this is unequivocal evidence for a kinetic isotope effect on the second-order association rate constant, k_1, of eq 4.

DISCUSSION

Are the Observed Isotopic Fractionations Kinetic Isotope Effects on Association? For the ordered mechanism of eq 2, the expected limit of the apparent ${}^{18}(V/K_{PEP})$ as the second substrate is increased is ${}^{18}k_{on}$. This is because the high concentration of the second substrate traps all of the bimolecular complexes formed, independent of isotopic

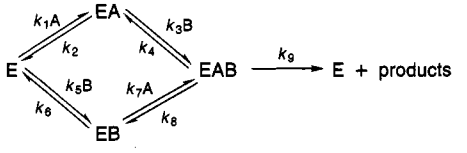
Table 3: Isotope Effects on the Pyruvate Kinase Reaction as a Function of ADP Concentration[a]

	[ADP] (mM)			
	pH = 6.8			pH = 8.4
labeled PEP	1.5	15	30	1.5
1-^{13}C	1.0003 ± 0.0010(13)			0.9966 ± 0.0006(5)
1,3-$^{13}C_2$	1.0047 ± 0.0012	1.0009 ± 0.0011		1.0303 ± 0.0014(3)
$^{3-13}(V/K_{PEP})$	1.0044 ± 0.0016	1.0006 ± 0.0015		1.0338 ± 0.0015
1-^{13}C,2-^{18}O	1.0027 ± 0.0010(3)	0.9933 ± 0.0015(3)	0.9931 ± 0.0025(3)	
$^{2-18}(V/K_{PEP})$	1.0024 ± 0.0014	0.9930 ± 0.0018	0.9928 ± 0.0027	

composition, and "commits" the binary complex to product formation (algebraically $k_3B \gg k_{off\text{-}PEP}$). The change from normal to inverse values for $^{18}(V/K_{PEP})$, observed as the concentration of bicarbonate or ADP increases, was unexpected. The common assumption has been that only steps with changes in the chemical bonding will give rise to isotope effects, and consequently the kinetic isotope effect on the association of a substrate with the active site of an enzyme would be unity (Cleland, 1982). Although the simplest explanation of the observed effects is that they are true isotope effects on the second-order rate constant, $k_{on\text{-}PEP}$, several alternative explanations need to be considered.

The most common source of inverse kinetic isotope effects is when there is a preequilibrium where the isotopic substitution introduces an inverse equilibrium isotope effect. Thus an inverse apparent $^{D}(V/K_{NAD})$ is expected in dehydrogenase reactions when substrate release is rate determining because there is an inverse equilibrium isotope effect on the conversion of [4-^{2}H]NAD$^+$ to [4-^{2}H]NADH (Cook et al., 1980). At pH 6.8, product release is thought to be rate determining for pyruvate kinase (Dougherty & Cleland, 1985). A trivial explanation of our results would be that the mechanism is actually a rapid equilibrium random mechanism and the observed inverse $^{18}(V/K_{PEP})$ reflects the $^{18}K_{eq}$ for the phosphoryl transfer from PEP to ADP. For the $^{18}K_{eq}$ to be reflected in the $^{18}(V/K_{PEP})$, the rapid equilibrium assumption is required. This explanation, however, cannot be accepted for our results because the apparent $^{18}(V/K_{PEP})$ is independent of the concentration of the second substrate in a rapid equilibrium mechanism (Cook, 1991), contrary to the results presented in Tables 1 and 3.

The assumption of obligatorily ordered kinetic schemes for both enzyme reactions is certainly an oversimplification. The full random mechanism shown in eq 8 needs to be



(8)

considered. Cleland (1982) has considered this mechanism with the assumption that there is only a kinetic isotope effect on k_9. The standard expression for the observed isotope effect, assuming that only k_9 is isotope sensitive, is given by eqs 9 and 10. The key feature of eq 10 is that as the

$$\text{app } {}^{D}(V/K_A) = \frac{{}^{D}k_9 + c_{f\text{-}ex}}{1 + c_{f\text{-}ex}} \quad (9)$$

$$c_{f\text{-}ex} = k_9/[k_8 + k_4k_2/(k_2 + k_3B)] \quad (10)$$

concentration of B increases, the external commitment factor, $c_{f\text{-}ex}$, increases. This feature is shared with the obligatorily ordered mechanism, but in the random mechanism $c_{f\text{-}ex}$ only reaches a limit of k_9/k_4 instead of becoming infinite. Thus the observed isotope effect will still express some part of the kinetic isotope effect on k_9. In our two reactions k_9 must be normal because initially increasing $c_{f\text{-}ex}$ results in smaller normal isotope effects. Thus, if there is any randomness to the pyruvate kinase or PEP-C kinetic mechanisms, the observed $^{18}(V/K_{PEP})$ will be closer to unity than the true $^{18}k_{on\text{-}PEP}$; i.e., allowing for a small amount of random character to the kinetic mechanism results in the conclusion that $^{18}k_{on\text{-}PEP} < 0.993$ for pyruvate kinase and $^{18}k_{on\text{-}PEP} < 0.994$ for PEP-C.

More formally, if we now assume nonunity isotope effects on the association and dissociation of PEP as well as on k_9, the equation for app$^{I}(V/K_{PEP})$ for the random mechanism is given by eq 11. For simplicity we assume that the isotope

$$ {}^{I}(V/K_A) = \{{}^{I}k_9{}^{I}k_7(k_3k_6k_1/k_7 + k_2k_5 + k_3k_5B)[k_2'(k_4 + k_8' + k_9') + k_3B(k_8' + k_9')]\}/\{(k_3k_6k_1'/k_7' + k_2'k_5 + k_3k_5B)[k_2(k_4 + k_8 + k_9) + k_3B(k_8 + k_9)]\} \quad (11)$$

$$ {}^{I}(V/K_A) = \frac{{}^{I}K_A{}^{I}k_9 + {}^{I}k_7k_9/k_8}{1 + k_9/k_8} \quad (12)$$

effects on the association and dissociation of PEP were independent of whether it added first or second; i.e., $^{18}k_1 = {}^{18}k_7$. This equation has the appropriate limits in that the external forward commitment factor reduces to $k_9/(k_4 + k_8)$ at low B to k_9/k_8 at high concentrations of B and becomes infinite when the mechanism is obligatorily ordered, i.e., if k_8 is zero. One important feature of eq 12 is that an isotope effect on association will always be expressed on V/K_A. Under circumstances where the forward commitment is low, the observed effect will be an equilibrium effect, but when the forward commitment becomes large, i.e., when k_3B/k_2 is large for an obligatorily ordered reaction or when k_9/k_8 is large for the random mechanism of eq 10, the true kinetic isotope effect on the association rate constant will be observed.

What Processes Are Included in the Bimolecular Association Rate Constant? The common representation of sequential enzymatic reactions as two sequential bimolecular steps, as shown in eqs 2 and 10, oversimplifies our growing understanding of the alterations in enzyme structure induced by substrate binding. This is most clearly observed in the differences between the crystal structures of enzymes with and without ligands bound at the active site. Citrate synthase has a obligatory ordered mechanism with oxalacetate binding before acetyl-CoA. The obligate order of addition is enforced by the dramatic conformational change in the

enzyme structure induced by the binding of OAA. These changes in the enzyme conformation may also be kinetically observed in transient kinetic studies of ligand binding (Hammes, 1982). Thus the single bimolecular steps indicated in eq 2 must more realistically be viewed as having a bimolecular association to form a collision complex followed by a unimolecular isomerization to form the Michaelis complex, as shown for PEP in eq 13. The rate constant for

$$E + PEP \underset{k_{off}}{\overset{k_{col}}{\rightleftharpoons}} E\text{- - -}PEP \underset{k_{r\text{-}iso}}{\overset{k_{f\text{-}iso}}{\rightleftharpoons}} E{\cdot}PEP \xrightarrow{k_{on}B} E{\cdot}PEP{\cdot}B \quad (13)$$

the second-order formation of the collision complex is k_{col}, while $k_{f\text{-}iso}$ and $k_{r\text{-}iso}$ are the unimolecular rate constants for the isomerization of the collision complex to form the Michaelis complex, E·PEP. The experimentally observed association rate constant for PEP is given by eq 14. If raising

$$\text{app } k_{on\text{-}PEP} = k_{col}k_{f\text{-}iso}/(k_{off} + k_{f\text{-}iso}) \quad (14)$$

the concentration of the second substrate does not trap the initial complex, but only the isomerized Michaelis complex (as shown in eq 13), the ^{18}O isotope effects on association we observed may reflect the isotope effect on $k_{f\text{-}iso}$, the forward rate constant for the unimolecular isomerization. This will be true if $k_{off} \gg k_{f\text{-}iso}$. This seems probable for most enzymes. Since the initial collision complex lacks many of the specific interactions leading to high affinity, the association constant will be very modest, in the range of 10−100 M^{-1}. Assuming a diffusion-limited k_{col} of 10^9 M^{-1} s^{-1}, k_{off} will be 10^7−10^8 s^{-1}. Under these conditions the kinetics of the reaction will still appear to be first order in PEP because the usual concentrations employed are well below the 10−100 mM dissociation constant for the collision complex. While the dissociation rate constant of 10^7−10^8 s^{-1} is slower than rotation about most single bonds, indicating that the substrate will be able to explore a large number of conformations during the lifetime of the collision complex, it is faster than the rates of many loop motions which more typically are on the order of the turnover number for the enzyme (Hammes, 1982). Under these conditions, the observed $^{18}(V/K_{PEP})$ will be given by eq 15 and the equilibrium isotope effect on association given by eq 16. These two equations

$$^{18}(V/K_{PEP}) = {}^{18}K_{col}\,{}^{18}k_{f\text{-}iso} \quad (15)$$

$$^{18}K_{assoc} = {}^{18}K_{col}({}^{18}k_{f\text{-}iso}/{}^{18}k_{r\text{-}iso}) \quad (16)$$

stress the difference in the kinetic isotope effect on association from the equilibrium isotope effect on association. Equilibrium isotope effects on association can be measured by equilibrium dialysis (LaReau et al., 1989) and are inferred to exist from Raman and infrared spectroscopic studies that detail differences in the vibrational spectra of free and bound substrates (Deng et al., 1989; Tonge & Carey, 1992). Equation 15 also indicates that an observed kinetic isotope effect on association not only depends on the isotope effect for diffusion, k_{col}, but may largely reflect the isotope effect on the unimolecular isomerization leading to the formation of the Michaelis complex. Thus the kinetic isotope effect on association reflects to what extent the interactions leading to the observed equilibrium isotope effects have been developed in the transition state for binding. When understood, these kinetic isotope effects on association may help inform and corroborate molecular mechanics modeling of the dynamics of ligand binding.

One might argue that the viscosity dependence of V/K_{PEP} would allow the determination of the partition ratio for the collision complex, $k_{f\text{-}iso}/k_{off}$, by assuming that increasing viscosity would only decrease k_{col} and k_{off} and would not affect the unimolecular isomerization rate constants. The assumption that the rate constants for protein conformational changes are independent of viscosity has been shown clearly to be unwarranted (Ansari et al., 1994). The size of the viscogenic agent employed may play a deciding role, with small viscogens like glycerol, ethylene glycol, and even D_2O having a significant effect on the rate of protein conformational changes (Kurz et al., 1987, 1992).

A second potential contribution to the bimolecular rate constant is selection of a subpopulation of the free substrate pool. An obvious example of this selection comes if only one protonation state of the substrate forms a productive complex with the enzyme. The kinetic mechanism and the equations for V/K_A and the $^I(V/K_A)$ for the hypothetical case where only the dianion, and not the trianion, of substrate A forms a Michaelis complex are given in eqs 17−20, where

$$HPEP^{2-} \overset{K_a}{\rightleftharpoons} PEP^{3-} + H^+ \quad (17)$$

$$E + HPEP^{2-} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}HPEP^{2-} \underset{k_4}{\overset{k_3B}{\rightleftharpoons}} E{\cdot}HPEP^{2-}{\cdot}B \quad (18)$$

$$V/K_{PEP} = k_1[1/(1 + K_a/H^+)] \quad (19)$$

$$\text{at } H^+ < K_a \quad {}^I(V/K_{PEP}) = {}^Ik_1/{}^IK_a \quad (20)$$

the leading superscript I refers to any isotope effect. If the minor population of the substrate, e.g., the protonated form above the pK_a, is the form which preferentially binds to the enzyme, then the equilibrium isotope effect on the preequilibrium step [in this case $^I(1/K_a)$], is reflected in $^I(V/K_{PEP})$ as shown in eq 20. In general, there can be significant ^{18}O and ^{15}N equilibrium isotope effects on protonation (Jones et al., 1991; Hermes et al., 1985) so this is a potential explanation of our observed inverse $^{18}(V/K_{PEP})$. We discount this artifactual explanation for both enzymes because kinetic evidence suggests that the trianion of PEP is the substrate for both pyruvate kinase (Dougherty & Cleland, 1985) and PEP-C (Janc et al., 1992). Protonation of the PEP phosphate will probably not generate an appreciable isotope effect since there are not significant isotope effects on the nonbridging phosphate oxygens on protonation or on complexation with Mg^{2+} (Jones et al., 1991).

What Can Be Inferred about PEP Association from the Kinetic Isotope Effects on Association? The inverse isotope effect on association indicates that the labeled bridging oxygen is in a "stiffer" environment in the transition state for binding than it is free in solution. Because the magnitude of the effect is small, it could be attributed to a large number of different phenomena. Two general phenomena are attractive explanations: (1) increased H-bonding to the labeled bridging O and (2) restricted torsions about the C−O and O−P bonds. Full protonation of water leads to a stiffer environment of the oxygen (Thornton, 1962), even though the stretching frequencies to the other hydrogen atoms are significantly decreased. In PEP where the O−H bonds have been replaced by O−C and O−P bonds, the protonation or

strengthened hydrogen bonding is expected to lead to a stiffer environment. If this bonding interaction is present at the binding transition state, it would lead to the observed inverse $^{18}(V/K_{PEP})$ values. In PEP-C the bridging oxygen plays a key role in binding as the phosphonate substrate where the bridging O is replaced with a methylene (O'Leary, 1983). Interaction of the ionized phosphate with a positively charged group at the active site could potentially restrict rotation about both the C−O and P−O bonds. Cleland has shown that restriction of rotation about a C−S bond can lead to significant inverse isotope effects and that the restricted rotation does not alter other vibrations in the molecule significantly (Cleland, 1987). Unfortunately, isotope effects are not yet capable of discriminating between different factors that lead to the same result, in this case a stiffening of the environment of the bridging ^{18}O.

If the observed $^{18}(V/K_{PEP})$ isotope effects in the presence of saturating amounts of the second substrate are kinetic isotope effects on association, both the $^{1-13}(V/K_{PEP})$ and $^{3-13}(V/K_{PEP})$ isotope effects reported under the same conditions must also be kinetic isotope effects on association. For pyruvate kinase, both the carboxylate and terminal carbon effects are not different from unity, indicating that there is no detectable difference in their environments between solution and the transition state for association. Since the ^{13}C equilibrium isotope effect on protonating a carboxylate is unity (O'Leary & Yapp, 1978), ^{13}C isotope effects cannot be used to determine the protonation state of the carboxylate. The decrease of the observed $^{3-13}(V/K_{PEP})$ to unity as the ADP concentration was increased provides confirmation that the observed effects are not residual isotope effects from the chemical reaction where $^{13}V/K_{PEP}$ is significantly greater than unity. The methylene carbon will not be affected by H-bonding, restricted rotation, or other presumed interactions of PEP with the active site on association so the value of unity is predicted for the kinetic isotope effect on association.

For PEP-C the carboxylate carbon shows a small but significant inverse isotope effect at all concentrations of bicarbonate. As for the bridging O, this effect could arise from several different sources, but the inverse value is suggestive of a more torsionally or vibrationally restricted environment on the enzyme. Since this same inverse carboxylate $^{13}V/K_{PEP}$ is observed when the terminal $^{13}V/K_{PEP}$ is 1.053 (O'Leary & Paneth, 1987), any change in the carboxylate environment between the binding transition state and the transition state for carboxylation must be insensitive to isotopic substitution.

Caveats for Interpretation of Isotope Effects. The existence of kinetic isotope effects on association makes the quantitative and qualitative analyses of isotope effects more limited. As we argued, an inverse isotope effect on *V/K* cannot necessarily be assumed to arise from an equilibrium isotope effect generated by a large reverse commitment factor. Normal isotope effects on association could qualitatively alter the conclusion of operative kinetic mechanism from obligatorily ordered to random. For example, in an ordered mechanism with a normal ^{18}O isotope effect of 1.01 on association of A [which generates an $^{I}(V/K_A)$ of 1.01], an $^{I}(V/K_B)$ of 1.06, and an $^{I}k_{cat}$ of 1.07, the standard conclusion would be that the kinetic mechanism was steady-state random, instead of ordered, with B dissociating 20-fold times more readily from the ternary complex than A (Cook, 1991). It is also possible that kinetic isotope effects on association will introduce modest errors into calculations of intrinsic isotope effects determined by multiple isotope substitution (Cleland, 1991). Using the simplest unimolecular mechanism shown in eq 21, it can be demonstrated that

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}A \xrightarrow{k_3} E + P \qquad (21)$$

a kinetic isotope effect on k_1 introduces errors into the calculation of the intrinsic isotope effect. Assuming $k_2 = k_3$, $^{13}k_3$ and $^{D}k_3$ of 1.06 and 6, respectively, and $^{13}k_1$ of 1.01, the observed $^{13}(V/K_H)$, $^{13}(V/K_D)$, and $^{D}(V/K)$ can be calculated to be 1.037, 1.052, and 3.5, respectively. These values can be used in the equations provided by Cleland (1991) to calculate the intrinsic $^{13}k_3$ and $^{D}k_3$ values of 1.058 and 4.95, respectively, representing the introduction of errors of 3% and 20%.

Conclusions. We have demonstrated that the inverse $^{18}(V/K_{PEP})$ values observed for phosphoenolpyruvate carboxylase and pyruvate kinase can be understood as kinetic isotope effects on association. They reflect the stiffer environment of the bridging phosphate O when it is in the transition state for association than when it is free in the bulk solution. This stiffer environment may develop in the transition state for a unimolecular conformational change that occurs after an initial collision complex is formed. These small, but significant kinetic isotope effects on steps other than the chemical transformation step may complicate the unambiguous interpretation of small nonunity isotope effects on *V/K*. The effect of potential randomness in the kinetic mechanism and preequilibria in solution on the observed kinetic binding isotope effects have been explored. Kinetic binding isotope effects are predicted by the current understanding of protein−ligand interactions, can be measured precisely by isotope ratio mass spectrometry under favorable conditions, and although arising from the same interactions are quantitatively different from equilibrium isotope effects on association.

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