Catalytic Power of Pyruvate Decarboxylase. Rate-Limiting Events and Microscopic Rate Constants from Primary Carbon and Secondary Hydrogen Isotope Effects[1]

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Abstract: Isotope effects ([rate constant for light isotopic substrate]/[rate constant for heavy isotopic substrate]) for the action of the thiamin diphosphate dependent pyruvate decarboxylase of *Saccharomyces carlsbergensis* (EC 4.1.1.1) on pyruvate, pyruvate-*1*-^{13}C, pyruvate-*2*-^{13}C, and pyruvate-*3*-d_3 have been determined for each of the steady-state kinetic parameters k/A (second-order in pyruvate), k/B (first-order in pyruvate), and k (zero-order in pyruvate). The 1-^{13}C effects are 1.008 ± 0.010 (k/A), 1.013 ± 0.024 (k/B), and 1.024 ± 0.006 (k). The 2-^{13}C effects are 1.013 ± 0.009 (k/A), 0.951 ± 0.020 (k/B), and 1.039 ± 0.004 (k). The 3-d_3 effects are 0.883 ± 0.013 (k/A), 0.881 ± 0.026 (k/B), and 1.057 ± 0.005 (k). Effects with 2-oxobutanoate and 2-oxobutanoate-*3*-d_2 are 0.951 ± 0.012 (k/A), 0.821 ± 0.096 (k/B), and 1.057 ± 0.005 (k). Pyruvate decarboxylase was already known to be hysteretically activated by the substrate, with pyruvate binding to the regulatory site with dissociation constant 8 mM and producing unimolecular activation (0.46 s^{-1}) and deactivation (0.033 s^{-1}). The isotope effects lead to rate constants for substrate binding to the catalytic site of 8.2×10^4 M^{-1} s^{-1}, for substrate departure from the catalytic site of 120 s^{-1}, for decarboxylation of 640 s^{-1}, and for product release of 640 s^{-1}. Pyruvate decarboxylase increases the rate of decarboxylation of pyruvate by thiamin alone by a factor of 3×10^{12} at pH 6.2, 30 °C. Under these conditions, conversion of activated enzyme and pyruvate to the enzymic species preceding decarboxylation is 4×10^{12} times faster than the specific-base-catalyzed addition of thiamin to pyruvate. The enzymic species preceding decarboxylation reverts to activated enzyme and free pyruvate 6×10^9 times faster than the specific-base-catalyzed reversion of the adduct of thiamin and pyruvate to thiamin and free pyruvate. Enzymic decarboxylation is 10^7 times faster than decarboxylation of the adduct of thiamin and pyruvate.

Introduction

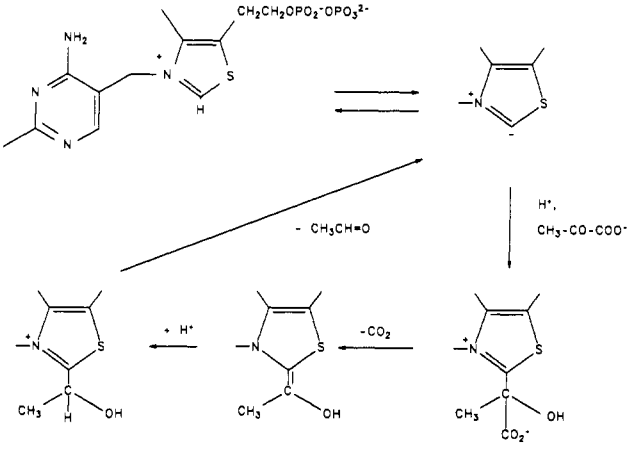
Thiamin diphosphate[2] (TDP; Scheme I) functions in biological systems as a cofactor, a small molecule that participates with an enzyme in performing catalysis. Cofactors are sometimes capable of carrying out the catalytic cycle in the absence of their enzymes, as is true of TDP, but the enzyme commonly produces a substantial further acceleration of the cofactor-induced reaction. Our purpose here is to explore how this happens in the case of the TDP-dependent yeast enzyme pyruvate decarboxylase[3] (PDC; EC 4.1.1.1).

Properties of Thiamin Diphosphate. The capacity of TDP to catalyze the decarboxylation of pyruvate anion (Scheme I) depends mainly on two properties of the thiazolium nucleus of TDP: (a) its capacity to ionize to form a nucleophilic anion and thus generate an adduct with pyruvate anion and (b) its capacity to stabilize the negative charge released upon fission of the substrate C–C bond in decarboxylation. The species formed in decarboxylation (historically known as "active aldehyde") undergoes protonation to give hydroxyethyl-TDP. Carbonyl elimination then regenerates the nucleophilic anion and produces the product acetaldehyde.

The ionization of thiamin compounds has been investigated most recently by Washabaugh and Jencks[4] and the addition and decarboxylation reactions by Kluger, Chin, and Smyth.[5] It will be assumed in the employment of these data for the nonenzymic reaction that any differences between thiamin and thiamin diphosphate can be neglected.

Washabaugh and Jencks[4] found that thiamin behaves as a "normal acid", in the sense that the C–H bond is broken with proton donation to weaker bases in a reaction with rate-limiting diffusion of the protonated base away from the conjugate base of thiamin. This conclusion was drawn from the observation of a unit Broensted coefficient in the general-base-catalyzed deprotonation reaction for relatively weak bases, curvature in the Broensted relationship consistent with greater participation in determining the rate of the actual proton-transfer step as the deprotonating base became stronger, and from kinetic isotope-effect studies consistent with an intrinsic barrier for proton transfer

Scheme I



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(1) This work is taken in part from the doctoral dissertation of F. J. Alvarez (University of Kansas, Lawrence, KS, 1985) and was reported in part in preliminary form: *Thiamin Pyrophosphate Biochemistry*; Schellenberger, A., Schowen, R. L., Eds.; CRC Press: Boca Raton, FL, 1988; Vol. I, pp 101–112. This work was supported by the National Institutes of Health under Grant No. GM-20198.

(2) (a) *Thiamin: Twenty Years of Progress*: Sable, H. Z., Gubler, C. J., Eds.; New York Academy of Sciences: New York, 1982. (b) Kluger, R. *Chem. Rev.* **1987**, *87*, 863–876. (c) *Thiamin Pyrophosphate Biochemistry*; Schellenberger, A., Schowen, R. L., Eds.; CRC Press: Boca Raton, FL, 1988. (d) *Biochemistry and Physiology of Thiamin-Dependent Enzymes*; Bisswanger, H., Ullrich, J., Eds.; VCH Publishers: Weinheim, Germany, 1991.

(3) (a) Zehender, H.; Trescher, D.; Ullrich, J. *Eur. J. Biochem.* **1987**, *167*, 149–154. (b) Uhlemann, H.; Schellenberger, A. *FEBS Lett.* **1976**, *63*, 37–39. (c) Ullrich, J. *Methods Enzymol.* **1970**, *18A*, 109–115. (d) Schellenberger, A. *Angew. Chem., Int. Ed. Engl.* **1967**, *6*, 1024–1035. (e) Holzer, H.; Schulz, G.; Villar-Palasi, C.; Jüntgen-Sell, J. *Biochem. Z.* **1956**, *327*, 331–344. (f) The value of k = 320 s^{-1} calculated from a specific activity of 80 units mg^{-1} for PDC takes the molecular weight per active site to be 242 kDa. There are four TDP binding sites in this mass,[3b] and if all are occupied and catalytically active during the catalytic cycle, the correct value will be 80 s^{-1}. Such a change will alter no qualitative conclusions drawn in this paper.

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similar in magnitude to the barrier for diffusion of the deprotonated thiazolium ion away from its reaction partner. The rate constants for deprotonation, when combined with a diffusional rate constant for reprotonation, lead to a pK_a for thiamin of approximately 18.

Kluger, Chin, and Smyth[5] measured rate constants of 4×10^{-5} s^{-1} (pH 7) and 1.1×10^{-4} s^{-1} (pH 4) for decarboxylation of the thiamin–pyruvate adduct, 1.3 M^{-2} s^{-1} for the specific-base-catalyzed addition reaction, and 1.3 M^{-1} s^{-1} for the specific-base-catalyzed reversion of the adduct to free thiamin and pyruvate. At pH 6.2, where the yeast PDC is optimally active, thiamin is therefore capable of producing a nonenzymic decarboxylation rate of 2×10^{-11} events of decarboxylation per second per molecule of thiamin (standard state of 1 mM for pyruvate).

Pyruvate Decarboxylase (PDC).[2,3] The acceleration produced by the PDC of the yeast *Saccharomyces carlsbergensis*, a tetrameric enzyme of M_r 242000, can be estimated from the kinetic parameters given in the Results section for pH 6.2 and 30 °C. The rate of enzyme-induced decarboxylation under these conditions and at a standard-state pyruvate concentration of 1 mM is 63 events of decarboxylation per second per molecule of enzyme. PDC therefore acceleratees the nonenzymic decarboxylation reaction promoted by free thiamin alone by a factor (at 1 mM pyruvate, 30 °C, and pH 6.2) of $63/(2 \times 10^{-11})$ or 3×10^{12}.

A further interesting feature of PDC is that it is subject to *hysteretic regulation*[6,7] by the substrate. Upon initial combination with pyruvate, PDC is inactive. Pyruvate reversibly combines with a regulatory site, and the enzyme then undergoes a slow unimolecular transition to an active form, which rapidly cycles catalytically.

Microscopic Rate Constants and Isotope Effects. One starting point in an examination of the chemical and biological origins of the catalytic power of PDC is to resolve the overall catalytic cycle into its component parts and to obtain the so-called "microscopic" rate constants for as many as possible of the individual events in the enzymic cycle. These rate constants can then be compared with those for the nonenzymic reaction to deduce in what way the enzymic acceleration is being produced. In the past, kinetic isotope effects have been applied effectively to this kind of problem.[8]

Kinetic isotope effects are useful either in determining transition-state properties, when the isotope effect on the formation of a particular transition state can be isolated, or, if isotope effects are determined on more complex combinations of conversions, in assessing the *kinetic significance* of reactant states, transition states, or of chemical conversions connecting particular reactant states to particular transition states in a chemical reaction. Ideas associated with kinetic significance have been rather thoroughly and clearly rehearsed in the past,[9] but indications of continuing confusion suggest that a brief summary of the situation is necessary here.

Kinetic Significance. The term "kinetic significance" denotes the quantitative degree to which a reactant state or transition state participates in limiting the rate or the quantitative degree to which a particular chemical conversion represents a rate-limiting process. It is a vital point that kinetic significance must be separately evaluated for each different set of experimental conditions. In the simplest case of reagent concentrations, kinetic significances should thus be evaluated for each of the kinetic parameters of a rate law. One cannot, therefore, speak of the "rate-limiting step for an enzymic reaction", but only of "the rate-limiting step (or better, process or conversion) for k_{cat}", "the rate-limiting conversion for k_{cat}/K_m", etc.

Kinetic significance can be defined quantitatively in a particularly simple way for steady-state rate constants, k, for which relationships of this form hold:

$$k^{-1} = k_1^{-1} + k_2^{-1} + \ldots$$

Then the kinetic significance of k_1 is $w_1 = k/k_1$, that of k_2 is $w_2 = k/k_2$, etc. Note that all of the w_i sum to unity, and w_i thus measures the fractional degree to which the conversion (from an initial state or states to a transition state or states) represented by the constant k_i limits the rate for the parameter k. If, as is sometimes the case, the initial state is the same for all of the k_i (this is true for enzyme kinetic parameters of the type k_{cat}/K_m), then w_i measures the kinetic significance of the transition state or states of the k_i conversion.

The particular utility of isotope effects in measuring kinetic significances is that an observed isotope effect on a given kinetic parameter, say the isotope effect k/k^* for the kinetic parameter k, is related to the *intrinsic* isotope effects k_i/k_i^* for the individual conversions that may be kinetically significant for the parameter k by a simple linear combination, as was shown by Stein[10] (here we consider a series of sequential reactions, but Stein treated a more general case):

$$k/k^* = w_1(k_1/k_1^*) + w_2(k_2/k_2^*) + \ldots$$

where the weighting factors $w_i = k/k_i$ *are* the kinetic significances of the individual conversions. Thus, if the intrinsic isotope effects can be measured or estimated, information about kinetic significances can be obtained from the relationship above.

Isotope Effects for PDC Action. The isotope effects employed in this study of PDC action are as follows: the 1-^{13}C effect, for labeling of the carboxyl group carbon of pyruvate; the 2-^{13}C effect, for labeling of the keto group carbon of pyruvate; and the 3-d_3 effect, for labeling of the methyl group hydrogens of pyruvate. These isotope effects give complementary forms of information about kinetically significant processes and transition states in the steady state for PDC action. There are three kinetic parameters, here denoted k, k/B, and k/A, in the steady-state rate law for PDC action, and isotope effects can in principle be determined for each of these parameters.

The 1-^{13}C effect measures the kinetic significance for any kinetic parameter of conversions to the decarboxylation transition state; only conversions to this state are expected to generate a substantial 1-^{13}C isotope effect. While it is not inconceivable that small secondary effects at this center might arise from sources such as desolvation of the carboxylate in the enzyme active site or rehybridization at C_2, these effects should be much smaller than the effect for fission of the C_1–C_2 bond. The 2-^{13}C isotope effect should signal the kinetic significance of conversions to transition states in which binding alterations are occurring at the C_2 of pyruvate. These transition states are transition states for addition

(4) (a) Washabaugh, M. W.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 674–683. (b) Washabaugh, M. W.; Jencks, W. P. *J. Am. Chem. Soc.* **1989**, *111*, 683–692. (c) Washabaugh, M. W.; Jencks, W. P. *Biochemistry* **1988**, *27*, 5044–5053.

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(6) The subject of hysteretic regulation has been reviewed: Frieden, C. *Annu. Rev. Biochem.* **1979**, *48*, 471–489.

(7) The hysteretic regulation of PDC was discovered: (a) Boiteux, A.; Hess, B. *FEBS Lett.* **1970**, *9*, 293–296. (b) Hübner, G.; Fischer, G.; Schellenberger, A. *Z. Chem.* **1970**, *10*, 436–437. (c) Ullrich, J.; Donner, I. *Hoppe-Seyler's Z. Physiol. Chem.* **1970**, *351*, 1026–1029. It has been studied mechanistically most recently by (d) König, S.; Hübner, G.; Schellenberger, A. *Biomed. Biochim. Acta* **1990**, *49*, 465–471. (e) Hübner, G.; König, S.; Schellenberger, A.; Koch, M. H. J. *FEBS Lett.* **1990**, *266*, 17–20. (f) Schellenberger, A.; Hübner, G.; König, S.; Flatau, S.; Neef, H. *Nova Acta Leopold.* **1988**, *61*, 225–242, and studied earlier by (g) Hübner, G.; Weidhase, R.; Schellenberger, A. *Eur. J. Biochem.* **1978**, *92*, 175–181.

(8) (a) *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991. (b) O'Leary, M. H. *Annu. Rev. Biochem.* **1989**, *58*, 377–401. (c) Cleland, W. W. *Bioorg. Chem.* **1987**, *15*, 283–302. (d) Cleland, W. W. *CRC Crit. Rev. Biochem.* **1982**, *13*, 385–428. (e) Klinman, J. P. *Adv. Enzymol.* **1978**, *46*, 415–494. (f) *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum Press: New York, 1978. (g) *Isotope Effects on Enzyme-Catalyzed Reactions*; Cleland, W. W.; O'Leary, M. H., Northrop, D. B., Eds.; University Park Press: Baltimore, MD, 1977.

(9) The basic and complete treatment is that of (a) Noyes, R. M. *Prog. React. Kinet.* **1964**, *2*, 339–362. For enzymic reactions see: (b) Ray, W. J., Jr. *Biochemistry* **1983**, *22*, 4625–4637. See also: (c) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; McGraw-Hill: New York, 1969; pp 474–475. (d) Cleland, W. W. *Acc. Chem. Res.* **1975**, *8*, 145–151. (e) Albery, W. J.; Knowles, J. *Biochemistry* **1976**, *15*, 5631–5640 and the preceding papers.

(10) Stein, R. L. *J. Org. Chem.* **1981**, *46*, 3328–3330.

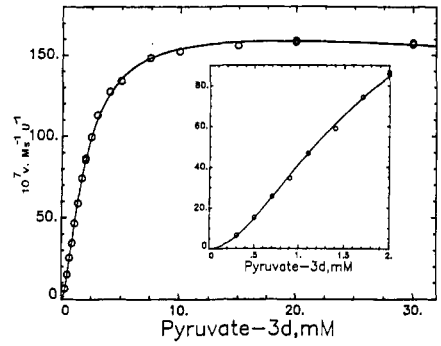

Figure 1. Typical initial-rate steady-state kinetic results for the action of pyruvate decarboxylase. Initial velocities are plotted against the concentration of the substrate pyruvate-*3*-d_3 for pH 6.2, 30 °C, the conditions of Table III. At high concentrations of substrate, the slight decrease in rate from weak substrate inhibition is observable. The inset shows the data for low concentrations of substrate, displaying the sigmoid character associated with substrate activation of the enzyme.

or elimination reactions at the keto group of pyruvate, including those for addition of the TDP-derived anion to the pyruvate keto group, for the decarboxylation event, and for other processes such as the elimination of the TDP anion from its adduct with the acetaldehyde product in the course of product release from the enzyme. The β-deuterium secondary isotope effect at the methyl group of pyruvate can differentiate conversions in which the C_2 of pyruvate or acetaldehyde is taken either from an sp^2 reactant-state configuration to a transition-state configuration that is partially or wholly sp^3 (addition) or from conversions in which the reactant-state configuration is sp^3 and the transition-state configuration is wholly or partially sp^2 (elimination). Previous work[11] has shown that addition reactions give inverse β-deuterium isotope effects ($k_H/k_D < 1$) while elimination reactions give normal β-deuterium isotope effects ($k_H/k_D > 1$).

In addition to these qualitative distinctions, good quantitative estimates of the expected intrinsic effects can also be made. The 1-^{13}C isotope effect for the nonenzymic decarboxylation has been measured by Jordan, Kuo, and Monse[12] as 1.051 ± 0.001. For a variety of other reactions,[13] decarboxylation isotope effects fall in the range 1.03–1.06, so that this appears to be a safe range in which to estimate the intrinsic effect for the PDC-catalyzed decarboxylation of pyruvate with a probable value of 1.05. The studies of Marlier and O'Leary[14] suggest that rate-limiting addition–elimination at carbonyl centers can be accompanied by normal isotope effects of up to at least 1.04. It is not known how these effects will depend on the exact nature of the transition state, so that the safe assumption here is that normal effects in the approximate range of 1.01–1.04 might well be expected. Finally, trideuteration of the position β to a carbonyl or carbonyl adduct group is expected[11] to produce inverse isotope effects between 1.00 and 0.83 for addition to carbonyl and normal effects of 1.00 to 1/0.83 for elimination to generate carbonyl.

Table I. Velocity Ratios v_{12}/v_{13} for Labeled Pyruvates with Pyruvate Decarboxylase as a Function of Pyruvate Concentration (30.00 ± 0.05 °C)

[pyruvate], mM	v_{12}/v_{13} - $(CH_3CO^*CO_2^-)$ ± SD $(N_{12},N_{13})^b$	v_{12}/v_{13} - $(CH_3{}^*COCO_2^-)$ ± SD $(N_{12},N_{13})^b$
0.50	1.010 ± 0.017 (5,5)	1.000 ± 0.025 (8,8)
1.00	1.012 ± 0.007 (7,7)	1.015 ± 0.009 (5,5)
1.00		1.008 ± 0.012 (7,7)
2.00	1.020 ± 0.007 (6,6)	1.009 ± 0.009 (8,7)
3.00		1.007 ± 0.013 (8,8)
4.00	1.013 ± 0.012 (8,6)	
5.00	1.024 ± 0.012 (6,6)	1.020 ± 0.006 (6,6)
5.00		1.017 ± 0.012 (8,4)
10.00		1.034 ± 0.010 (6,8)
20.00	1.024 ± 0.008 (5,5)	1.037 ± 0.013 (7,5)
20.00		1.031 ± 0.010 (4,4)
20.00[c]	1.028 ± 0.010 (7,6)	1.041 ± 0.013 (7,6)

[a] Citric acid/sodium citrate buffer, total concentration 0.100 M, pH 6.20 ± 0.03; pyruvate decarboxylase 0.05 unit mL^{-1}; thiamin diphosphate 5.0 mM; $MgSO_4$ 5.0 mM; alcohol dehydrogenase 16 unit mL^{-1}; NADH 0.2856 mM. [b] SD = standard deviation, N = number of runs. [c] Temperature 15.00 ± 0.03 °C.

Results

Our study has yielded the effects of three kinds of isotopic substitution for pyruvate and the related substrate 2-oxobutanoate (indicated by structures **1**–**4** on each of the kinetic constants in

$CH_3CO^{13}CO_2^-$ **1** $CH_3{}^{13}COCO_2^-$ **2** $CD_3COCO_2^-$ **3**

$CH_3CD_2COCO_2^-$ **4**

the rate law for action of brewer's yeast (*Saccharomyces carlbergensis*) pyruvate decarboxylase. The rates were measured by coupling the decarboxylation system to the action of alcohol dehydrogenase[3e] (eqs 1a,b) so that the conversion could be monitored by the spectral change at 340 nm when NADH is converted to NAD^+.

$$CH_3COCO_2^- + H^+ \xrightarrow{PDC} CH_3CHO + CO_2 \quad (1a)$$

$$CH_3CHO + H^+ + NADH \xrightarrow{ADH} CH_3CH_2OH + NAD^+ \quad (1b)$$

Kinetics. With a relatively minor modification, the well-known steady-state initial rate law[7] was confirmed. Typical data, in this case for the d_3-substrate **3**, are shown in Figure 1. The pyruvate-induced activation of PDC, indicated by the sigmoid curve at low pyruvate concentration, is known.[7] The slight fall-off at high pyruvate concentration corresponds to substrate inhibition. When a constant for substrate inhibition is incorporated into the rate law (see below), the residual sum of squares drops by a factor of 2; an F test[15] indicates a confidence level of 98% for inclusion of this constant. The value of the constant lies in the range 0.2–0.3 M and is not well-determined by the data. In all fits, this constant (K_i) was therefore fixed at the mean value of 264 mM. A schematic mechanism in accord with these and a variety of other observations to be described below is given in Figure 2.

The phenomenological rate law is given in eq 2, where v is velocity, e_0 the total enzyme concentration and S the substrate concentration. Equation 2 includes a provision for the weak

(11) (a) Cleland, W. W. *Isot. Org. Chem.* **1987**, *7*, 61–133. (b) Kovach, I. M.; Hogg, J. L.; Raben, T.; Halbert, K.; Rodgers, J.; Schowen, R. L. *J. Am. Chem. Soc.* **1980**, *102*, 1991–1999. (c) More recent measurements by F. J. Alvarez and I. M. Kovach suggest that 0.83 (three deuteriums) may be a better equilibrium isotope effect for ketone addition than the previously recommended[11b] value of 0.87.

(12) Jordan, F.; Kuo, D. J.; Monse, E. U. *J. Am. Chem. Soc.* **1978**, *100*, 2872–2878. The same authors (*J. Org. Chem.* **1978**, *43*, 2828–2830) found the effect to be about 1.058 in 50% aqueous ethanol.

(13) G. E. Dunn (*Isot. Org. Chem.* **1977**, *3*, 1–40) has reviewed ^{13}C isotope effects on decarboxylation reactions, most of which were carried out at high temperatures and under conditions where the decarboxylation event was not cleanly rate-limiting. For those cases where C–C bond breaking appears likely to be rate-limiting, extrapolation of the effects to about 300 K on the assumption that the isotope effects are enthalpic in origin yields values of about 1.03–1.05. J. F. Marlier and M. H. O'Leary (*J. Am. Chem. Soc.* **1986**, *108*, 4896–4899) measured effects from 1.057 to 1.064 for decarboxylation of pyridyl-4-acetic acid as the medium was changed from pure water to 75% dioxane/25% water. C. B. Grissom and W. W. Cleland (*J. Am. Chem. Soc.* **1986**, *108*, 5582–5583) found values of 1.04–1.05 for the decarboxylation of oxalacetate in the presence and absence of metals. Because decarboxylation is rarely completely rate-limiting in enzymic reactions, the examples of enzymic decarboxylations discussed by M. H. O'Leary (*Acc. Chem. Res.* **1988**, *21*, 450–455) give isotope effects that are mostly smaller, but occasionally are in the range 1.04–1.05.

(14) J. F. Marlier and M. H. O'Leary measured carbonyl-^{13}C isotope effects of 1.0262 ± 0.0025 for the acidic hydrolysis of methyl benzoate (*J. Org. Chem.* **1981**, *46*, 2175–2177), 1.0426 ± 0.0026 for the basic hydrolysis, and 1.0410 ± 0.0022 for the hydrazinolysis (pH 7.9) of methyl benzoate (*J. Am. Chem. Soc.* **1979**, *101*, 3300–3306).

(15) Draper, N. R.; Smith, H. *Applied Regression Analysis*, 2nd ed.; Wiley: New York, 1981; pp 101–102.

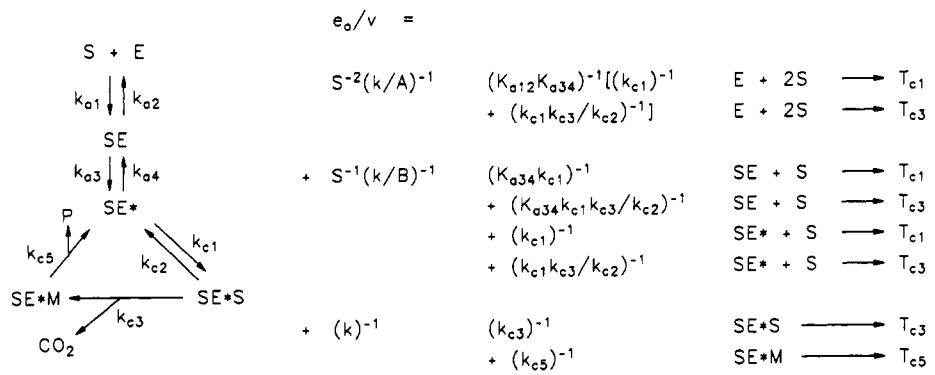

Figure 2. At left is a mechanistic scheme consistent with observations on PDC action, incorporating rate constants for the activation phase (subscript a) and the catalytic cycle (subscript c). To the right of the scheme, the phenomenological rate law is written vertically; substrate inhibition is omitted from both the mechanistic scheme and the rate law. To the right of each of the terms in the rate law (shown in reciprocal form) is the definition of the corresponding kinetic parameter (also shown in reciprocal form) in terms of the microscopic rate constants; $K_{a12} = (k_{a1}/k_{a2})$, $K_{a34} = (k_{a3}/k_{a4})$. Finally, to the right of each term that contributes to one of the kinetic parameters is indicated the net transformation that it describes. The species E (free PDC), SE (PDC with S in the regulatory site), SE* (activated PDC), SE*S (activated PDC with S in the catalytic site), and SE*M (species following decarboxylation) are reactant states in the scheme at left; the Ts are transition states for the steps indicated by the subscript (T_{c1} is the transition state for the k_{c1} step, etc.).

Table II.[a] Carbon Isotope Effects for Individual Kinetic Constants in PDC Action (pH 6.20, 30.00 ± 0.05 °C)

position of label	$J_{k/A}$	$J_{k/B}$	J_k
$CH_3CO^*CO_2^-$	1.008 (SD 0.010)	1.013 (SD 0.024)	1.024 (SD 0.006)
$CH_3{}^*COCO_2^-$	1.013 (SD 0.009)	0.951 (SD 0.020)	1.039 (SD 0.004)

[a] Calculated by a least-squares fit of the data of Table I to eq 3 with use of weighting factors from eqs 5a–c, $A = 2.73$ mM^2, $B = 1.37$ mM, $K_i = 264$ mM.

substrate inhibition described above. This feature, which is of minimal mechanistic interest, is not included in Figure 2.

$$v/e_0 = (k)S^2/\{A + BS + S^2[1 + (S/K_i)]\} \quad (2)$$

Figure 2 also shows the relationship between the microscopic rate constants and the three rate constants of the phenomenological rate law, k/A, k/B, and k. On the assumption[3c] that the activity of pure yeast PDC is 80 units/mg, the constants at 30 °C and pH 6.2 are those shown[3f] at the bottom of Figure 2 with $k = 320$ s^{-1}. We obtain $A = 2.73 \pm 0.06$ mM^2 and $B = 1.37 \pm 0.07$ mM; thus, $k/A = (1.17 \pm 0.03) \times 10^8$ M^{-2} s^{-1} and $k/B = (2.34 \pm 0.12) \times 10^5$ M^{-1} s^{-1}.

Carbon Isotope Effects. In order to obtain isotope effects on the individual kinetic constants, direct determinations of the rates vs S for $CH_3CO^{13}CO_2^-$ (90.0% ^{13}C) and $CH_3{}^{13}COCO_2^-$ (91.4% ^{13}C) had to be made and compared with measurements for unlabeled $CH_3COCO_2^-$. Mass spectrometric or radiometric determinations of isotope effects in competition experiments, since they involve competitive interaction of both isotopic substrates with enzyme, cannot yield the isotope effects on all individual kinetic parameters. Instead, competition experiments yield only isotope effects on parameters of the type k_{cat}/K_m, which reflect both binding and subsequent events. In spite of the lower precision of the direct-measurement technique used here, this technique is the only way isotope effects on k can be obtained. Table I presents the results of pairwise determinations of rates, in which alternating runs employed the two isotopic modifications of the substrate.

The most effective way to calculate the isotope effects on k/A, k/B, and k from the dependence on S of the observed isotope effects is to make use of Stein's method.[10] Equations 3–5 (derived in Appendix I, supplementary material) express the fact that the values of measured velocity ratios (v/v^*), where v is for the unlabeled and v^* for the labeled substrate (e.g., v_{12}/v_{13} in Table I), are weighted averages of the isotope effects $J_{k/A}$ (on k/A), $J_{k/B}$ (on k/B), and J_k (on k). The weighting factors of eq 3 are defined in eqs 5a–c as functions of kinetic parameters A and B and substrate concentration S. The weighting factors w depend on values of A and B *for the* ^{12}C *(or protium) substrate only*, and thus depend identically on substrate concentration S for all isotope effects (carbon and hydrogen) for a given substrate.

Table III.[a] Velocity Ratios for Labeled Pyruvates and 2-Oxobutanoates with Pyruvate Decarboxylase as a Function of Substrate Concentration (30.00 ± 0.05 °C)

substrates	v_{nH}/v_{nD} ([substrate], mM)[b]
$CH_3COCO_2^-$/ $CD_3COCO_2^-$ ($n = 3$)	0.886 (0.30), 0.883 (0.50), 0.903 (0.70), 0.909 (1.10), 0.975 (1.40), 0.948 (1.70), 0.963 (2.00), 0.987 (2.50), 0.975 (3.00), 1.011 (4.00), 1.046 (5.00), 1.044 (7.50), 1.065 (10.0), 1.078 (15.0), 1.060 (20.0), 1.072 (30.0)
$CH_3CH_2COCO_2^-$/ $CH_3CD_2COCO_2^-$ ($n = 2$)	0.962 (0.300), 0.979 (0.60), 1.004 (1.00), 1.029 (1.50), 1.041 (2.00), 1.046 (5.00), 1.054 (15.0)

[a] [TPP] = [$MgSO_4$] = 5.0 mM; citrate buffer 0.10 M, pH 6.20 ± 0.03; [PDC] = 0.04 unit mL^{-1} (pyruvate), 0.7 unit mL^{-1} (2-oxobutanoate). [b] Average of one to three runs. Errors in isotope effects around 1–2%, always <5%.

$$(v/v^*) = w_{k/A}J_{k/A} + w_{k/B}J_{k/B} + w_kJ_k \quad (3)$$

$$J_{k/A} = (k/A)/(k/A)^* \quad (4a)$$

$$J_{k/B} = (k/B)/(k/B)^* \quad (4b)$$

$$J_k = k/k^* \quad (4c)$$

$$w_{k/A} = A/\{A + BS + S^2(1 + [S/K_i])\} \quad (5a)$$

$$w_{k/B} = BS/\{A + BS + S^2(1 + [S/K_i])\} \quad (5b)$$

$$w_k = S^2(1 + [S/264])/\{A + BS + S^2(1 + [S/K_i])\} \quad (5c)$$

The values $A = 2.73$ mM^2 and $B = 1.37$ mM were employed to calculate $w_{k/A}$, $w_{k/B}$, and w_k at each value of S from eqs 5a–c. It was assumed that $K_i = 264$ mM for all isotopic species. The weighting factors were then inserted into eq 3 with $v/v^* = v_{12}/v_{13}$, and values of $J_{k/A}$, $J_{k/B}$, and J_k were obtained by a least-squares fit of the data in Table I to eq 3. The results are given in Table II. The corrections for incomplete labeling of the substrates are within the standard deviations of the isotope effects.

Hydrogen Isotope Effects. Table III shows v_{3H}/v_{3D} for $CH_3COCO_2^-$ vs $CD_3COCO_2^-$ and v_{2H}/v_{2D} for $CH_3CH_2COCO_2^-$ vs $CH_3CD_2COCO_2^-$ obtained, as with the carbon-labeled substrates, as a function of the substrate concentration S. The isotope effects on k, k/A, and k/B were again determined by Stein's method.[10] For pyruvate isotope effects, the weighting factors of eqs 5a–c depend only on the values of A and B for the unlabeled substrate

Table IV.[a] Hydrogen Isotope Effects for Individual Kinetic Constants in PDC Action (pH 6.20, 30.00 ± 0.05 °C)

substrate	$J_{k/A}$	$J_{k/B}$	J_k
$C^*H_3COCO_2^-$	0.883 (SD 0.013)	0.881 (SD 0.026)	1.085 (SD 0.006)
$CH_3C^*H_2$-$COCO_2^-$	0.951 (SD 0.012)	0.821 (SD 0.096)	1.057 (SD 0.005)

[a] From a least-squares fit of eq 3 to the data of Table III, with the weighting factors w calculated from eqs 5a–c with A = 2.73 mM², B = 1.37 mM, and K_i = 264 mM for pyruvate and A = 0.45 mM², B = 0.145 mM, and K_i = 1364 mM for 2-oxobutanoate.

and on the substrate concentration; they are, therefore, identical with those used for the carbon isotope effects. These factors were inserted into eq 3, with $v/v^* = v_{3H}/v_{3D}$, and the isotope effects determined as above. For the 2-oxobutanoate isotope effects, weighting factors were calculated from A = 0.45 mM², B = 0.145 mM, and K_i = 1364 mM. The measurements from Table III were again fit to eq 3 by a least-squares calculation to yield isotope effects on k, k/A, and k/B. The calculated secondary hydrogen isotope effects for both pyruvate and 2-oxobutanoate are presented in Table IV.

Discussion

Interpretation of the 1-^{13}C Isotope Effect on k: The Kinetic Significance of Decarboxylation and Product Release when PDC Is Saturated by Pyruvate and the Rate Constants for Decarboxylation and Product Release. When PDC is saturated with pyruvate, the reaction is kinetically first-order in total enzyme with rate constant k. As can be seen from Figure 2, k is given by $k_{c3}k_{c5}/(k_{c3} + k_{c5})$, where k_{c3} is the rate constant for decarboxylation and k_{c5} is the rate constant for product release.[16] As explained in the introduction, we take the probable intrinsic isotope effect for decarboxylation to be 1.05. We will later consider the effect on our conclusions of assuming other values for this intrinsic isotope effect. Since the labeled carbon has already departed as carbon dioxide before the onset of product release, we set the intrinsic isotope effect for product release to 1.00.

Applying Stein's method (Appendix I of the supplementary material), we have

$$J_k = [k/k_{c3}]J_{dec} + [k/k_{c5}]J_{rel} \quad (6)$$

Taking J_{dec} = 1.05 and J_{rel} = 1.00, noting that $[k/k_{c5}] = (1 - [k/k_{c3}])$, and taking from Table II that J_k = 1.024 ± 0.006, we obtain k/k_{c3} = 0.48 ± 0.12 and k/k_{c5} = 0.52 ± 0.12. Therefore, decarboxylation and product release are essentially equal in kinetic significance. Stated otherwise, they each determine the rate for k to the extent of 50%. Since k = 320 s^{-1}, $k_{c3} = k_{c5}$ = ca. 640 s^{-1}.

If the actual intrinsic effect for decarboxylation were as small as 1.03, the values of the rate constants would be k_{c3} = 400 s^{-1} and k_{c5} = 2000 s^{-1}. If the intrinsic effect were as large as 1.06, then k_{c3} = 800 s^{-1} and k_{c5} = 533 s^{-1}. We therefore conclude that k_{c3} is between 400 and 800 s^{-1}, with a probable value of 640 s^{-1}, and that k_{c5} is between 533 and 2000 s^{-1}, with a probable value of 640 s^{-1}. Conceivable secondary carbon isotope effects at this center could also introduce error in this estimate, but it is quite unlikely that such an error will be significant in comparison to the limits just considered.

(16) The rate constants k_{c3} and k_{c5} of course may, and almost surely do, describe processes more complex than the simple unimolecular decarboxylation event and the simple unimolecular migration of acetaldehyde out of the active site, respectively. In effect, k_{c3} describes the conversion, by way of a possibly multistep process, of the pool of species following the arrival of substrate in the active site (referred to in Figure 2 as SE*S) to the pool of species following the departure of carbon dioxide but preceding the departure of acetaldehyde (SE*M in Figure 2). Conversion, also possibly by a multistep process, of the SE*M pool to the free product and SE* is described by k_{c5}. The assignment of an intrinsic 1-^{13}C isotope effect of 1.00 to the product-release step depends only on the fact that decarboxylation is complete; the complexity of the product-release process thus in no way affects the validity of this assignment. The transition-state structural features deduced in the following sections are, furthermore, strongly supportive of the view that the decarboxylation event is the most kinetically significant event in the k_{c3} manifold. The argument given in the text, while simplified, is therefore likely to be correct.

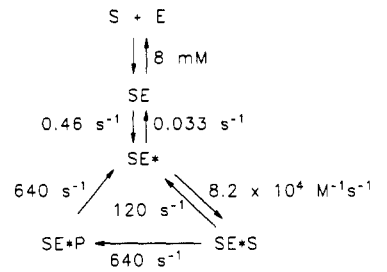

Figure 3. Scheme for the action of PDC (see Figure 2) with values for each of the microscopic rate constants. See the text and ref 16 for cautionary remarks on the meaning of the rate constants and symbols for intermediates.

Interpretation of the 1-^{13}C Isotope Effect on k/A: The Kinetic Significance of Decarboxylation and Substrate Binding when PDC Is Nearly Free of Substrate. At very low pyruvate concentrations, the enzyme exists free of substrate and the steady-state rate constant becomes k/A. The value of the 1-^{13}C isotope effect on k/A was measured as 1.008 ± 0.010 (Table II), with the experimental error thus being uncomfortably large. This isotope effect is, however, similar to the competitive isotope effects determined by three groups in the past.[12,17] In Appendix II of the supplementary material, it is shown that the competitive isotope effect is equal to the isotope effect on k/A multiplied by the equilibrium isotope effect on generation of SE* from S and E (Figure 2). This process, which does not involve bonding changes at C_1 of pyruvate, should exhibit no 1-^{13}C isotope effect. Thus the competitive isotope effect should be equal to the isotope effect on k/A. Under the conditions closest to our conditions of pH 6.2 and 30 °C, the competitive values are 1.0083 ± 0.0003 (ref 17a), 1.0065 ± 0.0016 (ref 12), and 1.0063 ± 0.0005 (ref 17b). These measurements were not conducted under conditions completely identical with our experiments, but the isotope effects are nevertheless close to our mean value. We shall therefore adopt the mean value of 1.008 obtained in our work for the isotope effect $J_{k/A}$ and assign an error limit of 0.002, which is somewhat more generous than the largest value for the competitive measurements.

The definition of k/A in Figure 2 shows that

$$k/A = (k_{a1}/k_{a2})(k_{a3}/k_{a4})[k_{c1}k_{c3}/(k_{c2} + k_{c3})] \quad (7)$$

Transient kinetic studies[7g,18,19] give (k_{a1}/k_{a2}) = 125 ± 13 M^{-1} and (k_{a3}/k_{a4}) = 14 ± 2. From k/A = (1.2 ± 0.1) × 10^8 M^{-2} s^{-1}, we obtain $[k_{c1}k_{c3}/(k_{c2} + k_{c3})]$ = (6.9 ± 1.4) × 10^4 M^{-1} s^{-1}.

Taking the 1-^{13}C isotope effect on (k_{a1}/k_{a2}) and on (k_{a3}/k_{a4}) to be unity, the isotope effects on k_{c1} and k_{c2} also to be unity, and the isotope effect on $(k_{c1}k_{c3}/k_{c2})$ to be 1.05, we obtain as above, by use of Stein's method:

$$J_{k/A} = [k_{c2}/(k_{c2} + k_{c3})](1.05) + [k_{c3}/(k_{c2} + k_{c3})] \quad (8)$$

From $J_{k/A}$ = 1.008, we obtain a kinetic significance for decarboxylation in the process described by k/A of 0.16 ± 0.04 = $[k_{c2}/(k_{c2} + k_{c3})]$. Decarboxylation is therefore 16 ± 4% rate-limiting at low substrate concentrations. This conclusion is subject again to relatively small errors that might arise from secondary carbon isotope effects and to the complications that arise from uncertainties about whether cofactor addition at C_2 occurs as part of the k_{c1} step or the k_{c3} step.[16]

We already know that k_{c3} = 640 s^{-1}, so we also calculate k_{c2} = ca. 120 s^{-1}. In addition, from $[k_{c1}k_{c3}/(k_{c2} + k_{c3})]$ = (6.9 ± 1.4) × 10^4 M^{-1} s^{-1}, we calculate k_{c1} = 8.2 × 10^4 M^{-1} s^{-1}.

(17) (a) O'Leary, M. H. *Biochem. Biophys. Res. Commun.* **1976**, *73*, 614–618. (b) DeNiro, M. J.; Epstein, S. *Science* **1977**, *197*, 261–263.

(18) Hübner, G. Dissertation B, University of Halle, Halle, Germany, 1978.

(19) Ermer, J. Dissertation A, University of Halle, Halle, Germany, 1988. This dissertation also contains evidence and discussion relating to the role of the imino N of the cofactor as general base in decomposition of the product–TDP adduct and of the role of sulfhydryl groups in substrate binding.

This completes the determination of the microscopic rate constants for the catalytic cycle. When our values are combined with results given above for the activation phase taken from the transient kinetic studies of Hübner[18] and Ermer,[19] we have the account shown in Figure 3.

Interpretation of the 2-^{13}C and Secondary Deuterium Isotope Effects on k: Transition-State Features in Product Release. Some characteristics of the transition state for product release from PDC can now be inferred from other isotope effects on the rate constant k.

The 2-^{13}C isotope effect on k (Table II) is 1.039 ± 0.004. This is a weighted average of the effects on decarboxylation (J_{dec}, weight 0.5) and product release (J_{rel}, weight 0.5):

$$1.039 = (0.5)J_{dec} + (0.5)J_{rel} \quad (9)$$

The magnitude of J_{dec} is not known, but almost surely a normal isotope effect will be produced at C_2 when the C_1–C_2 bond breaks; the effect might well be approximately equal to the 1-^{13}C isotope effect of 1.05, but in any case seems unlikely to be larger than ca. 1.06. Equation 9 suggests then that J_{rel} is in the range of about 1.02 to 1.07; if the isotope effects at the two ends of the breaking C_1–C_2 bond should in fact be equal (J_{dec} = 1.05), then J_{rel} is about 1.03. A structure for the product-release transition state that involves bonding changes in progress at the carbonyl group of C_2 is therefore probable. The isotope effect of 1.03 is quite close, for example, to the values of 1.026 to 1.043 obtained by Marlier and O'Leary[14] for reactions at the carbonyl group of methyl benzoate. Here again, caution must be exercised with respect to the steps in which the individual molecular events occur.[16]

The simplest candidates for a transition state of the type described above are those for the elimination of the TDP anion from the acetaldehyde adduct formed by protonation of the newly decarboxylated "active aldehyde", i.e., 1-hydroxyethyl-TDP, or for the attack of a nucleophile at the carbonyl group of an already formed acetaldehyde molecule. Such a nucleophile might be water if the species actually released from PDC is the hydrate of acetaldehyde and not acetaldehyde itself. Alternatively, acetaldehyde might be undergoing conversion to or formation from a sulfhydryl adduct if an adduct of acetaldehyde to an enzyme sulfhydryl group is somehow involved as an intermediate structure in product release.[20] The 2-^{13}C isotope effect does not discriminate between addition and elimination but merely signals bonding changes at the carbonyl carbon.

The secondary deuterium isotope effect enables discrimination between addition and elimination processes to be made, since addition processes generate inverse isotope effects and elimination processes generate normal isotope effects. The secondary deuterium isotope effect on k (Table IV) is 1.085 ± 0.006. This, like the other isotope effects, is a weighted average:

$$1.085 = (0.5)J_{dec} + (0.5)J_{rel} \quad (10)$$

Kluger and Brandl[21] have measured an isotope effect of 1.11 ± 0.01 for the nonenzymic decarboxylation of the thiamin adduct of pyruvate, which can be taken as a reasonable approximation to the intrinsic effect for enzymic decarboxylation. Setting J_{dec} = 1.11, we obtain J_{rel} = 1.05. This calculation assumes that the adduct at C_2 has already been formed in the effective reactant state for decarboxylation. If it has not, then the effect of 1.11 would need to be multiplied by an effect of 0.83 for addition, yielding an intrinsic effect of J_{dec} = 0.92. This would produce J_{rel} = 1.25. Thus there is little doubt that J_{rel} is normal.

This normal isotope effect strongly supports the idea that the most kinetically significant event in product release is a carbonyl elimination reaction. This may be the elimination to form acetaldehyde from the 1-hydroxyethyl-TDP intermediate. Alternatively, if a sulfhydryl adduct of acetaldehyde intervenes in product release,[20] then its decomposition to free acetaldehyde is likely to be rate-limiting.

The measured value of the secondary deuterium isotope effect on k of 1.085 ± 0.006, determined with pyruvate-*3*-d_3, corresponds to an isotope effect of $(1.085)^{1/3}$ = 1.028 per deuterium. The secondary deuterium isotope effect was also measured with 2-oxobutanoate-*3*-d_2 as substrate, thus with only two deuteriums in the 3-position. If the intrinsic isotope effects and the kinetic significances for decarboxylation and product release were the same for 2-oxobutanoate as for pyruvate, then the predicted isotope effect for 2-oxobutanoate-*3*-d_2 would be $(1.028)^2$ = 1.056. This agrees well with the measured value (Table IV) of 1.057 ± 0.005. This tends to confirm the conclusions reached for pyruvate and to suggest that the transition-state structures and balance among rate-determining steps for k are not affected by the change from pyruvate to 2-oxobutanoate.

Interpretation of the 2-^{13}C and Secondary Deuterium Isotope Effects on k/A: Transition-State Features in Reactant Binding. These two isotope effects can be employed in a manner similar to that just illustrated in order to characterize the transition states for reactant binding. The rate constant k/A, as we have seen (eq 7 and Figure 2), describes the conversion of the initial state E + 2S to the transition states for reactant binding (transition state of the k_{c1} step, kinetic significance 0.84 ± 0.04) and for decarboxylation (transition state of the k_{c3} step, kinetic significance 0.16 ± 0.04).

Prior to reactant binding, contributions are included for equilibrium formation of the activated enzyme SE* from E + S (equilibrium constant $k_{a1}k_{a3}/k_{a2}k_{a4}$). This will generate both 2-^{13}C and secondary deuterium equilibrium isotope effects if the formation of SE* from E + S involves bonding changes at the pyruvate carbonyl. In fact, the regulatory activation of PDC is thought to follow upon the addition of the regulatory pyruvate molecule to an enzymic sulfhydryl group.[7b,g,18-20] Such a process should produce an equilibrium secondary deuterium isotope effect[11] of about 0.83; the 2-^{13}C equilibrium isotope is of uncertain magnitude but is probably inverse and quite small.[22]

The observed isotope effects on k/A should then be given by

$$J_{k/A} = E_{reg}[(0.84)J_{bin} + (0.16)J_{dec}] \quad (11)$$

where E_{reg} is the equilibrium isotope effect for the generation of SE* from E + S, J_{bin} is the isotope effect for the conversion of S + SE* to the transition state for the substrate-binding (k_{c1}) step, and J_{dec}, as before, is the isotope effect for the process leading from S + SE* to the decarboxylation transition state.

For the secondary deuterium isotope effect, we take E_{reg} = 0.83 and $J_{k/A}$ = 0.883 ± 0.013 (Table IV). To obtain J_{dec} we note that TDP must add to the keto group of the substrate pyruvate molecule (isotope effect 0.83) and then decarboxylation must occur (isotope effect 1.11) for an isotope effect of 0.92. This yields a value of J_{bin} = 1.04. If the uncertainties are considered, a reasonable statement is that the isotope effect on binding is close to unity, either slightly inverse or slightly normal. A small isotope effect, either slightly inverse or slightly normal, would suggest that the transition state for the k_{c1} step in fact involves little or no progression of nucleophilic attack at the substrate keto group. The most logical transition-state structure may be that for a conformational reorganization preceding nucleophilic attack of the TDP anion at the substrate carbonyl group. Alternatively, a very "early" transition state for nucleophilic attack, in which the keto group had been deprived of interaction with other electron donors but binding by the nucleophile had not yet begun, could be reconciled with either a slightly normal or slightly inverse isotope effect. An "early" structure for such a transition state would be consistent with the high basicity[4] of the TDP anion, if

(20) Substantial evidence, summarized by G. Hübner, S. König, and A. Schellenberger (*Biomed. Biochem. Acta* **1988**, *47*, 9–18), implicates the sulfhydryl groups of PDC in the reaction mechanism. One possible role is for a sulfhydryl group near the opening of the active site to function as a relay station, assisting the transport of acetaldehyde from the TDP site to the external solution or even (in vivo) to the next enzyme in the metabolic pathway. We therefore consider it desirable to at least entertain the hypothesis that a sulfhydryl adduct of acetaldehyde may be an intermediate in product release.

(21) Kluger, R.; Brandl, M. *J. Am. Chem. Soc.* **1986**, *108*, 7828–7832.

(22) For instance, J. F. Marlier and M. H. O'Leary (*J. Am. Chem. Soc.* **1984**, *106*, 5054–5057) found an equilibrium effect K_{12}/K_{13} of 0.991–0.993 for the conversion of aqueous carbon dioxide to aqueous bicarbonate ion.

this anion is indeed an intermediate in the enzymic reaction. In principle, a general-base-catalyzed addition[4] of TDP to pyruvate (also with an "early" transition state) could be occurring. The studies reported here cannot distinguish these possibilities.

Here again, a comparison with 2-oxobutanoate is possible. The predicted value of the secondary isotope effect for 2-oxobutanoate, assuming the transition-state structures and kinetic significances for reactant binding and decarboxylation in k/A are unaffected by the change from pyruvate to 2-oxobutanoate, is $(0.883)^{2/3} = 0.92$. The measured isotope effect (Table IV) is 0.951 ± 0.012. These are qualitatively similar but quantitatively different, suggesting some change in intrinsic isotope effects for regulatory interaction, substrate binding or decarboxylation, or some change in the kinetic significance of substrate binding and decarboxylation in k/A between pyruvate and 2-oxobutanoate as substrates for PDC.

In principle, a further test of the structure is provided by the 2-^{13}C isotope effect of 1.013 ± 0.009 (Table II). This will also be given by eq 11, but the equilibrium and intrinsic effects are uncertain. If we take $J_{dec} = 1.05$ (equal isotope effects at the two ends of the rupturing bond), we obtain $E_{reg}J_{bin} = 1.006$. If E_{reg} is, as anticipated, slightly inverse, then J_{bin} would be slightly normal. Since the magnitudes of the two effects remain unknown, the result cannot distinguish a conformational reorganization transition state from an "early" carbonyl-addition transition state.

Isotope Effects on k/B. This kinetic parameter attains kinetic significance only at intermediate concentrations of pyruvate, and therefore isotope effects are not well-determined for it. The constant itself is complex, describing four different processes first-order in pyruvate (Figure 2).

Because of the unreliability of the apparent isotope effects on k/B, we do not wish to make use of them in drawing mechanistic conclusions. A brief comment is useful, however, to assure that they do not strongly conflict with the other findings. As Figure 2 shows, k/A and k/B differ only in the contributions of equilibria in the activation phase of the reaction. Activation events involve no changes in bonding at the carboxyl carbon and should, therefore, produce no 1-^{13}C isotope effects. As a result, we expect the 1-^{13}C isotope effects on k/A and k/B to be equal and the effect on k/B to add no mechanistic information to the effect for k/A. In fact, within experimental uncertainties, the two effects are equal (Table II).

The activation equilibria, if interaction at the pyruvate keto group occurs,[22] should generate both 2-^{13}C isotope effects and secondary deuterium isotope effects, so that these effects should be different for k/A and k/B. This may be the case (Table II), but the effects on k/B are poorly determined.

Furthermore, if the two effects on k/B are constrained to unity, little or no change in the fitted isotope effects on k and k/A results. For example, constraining the 2-^{13}C isotope effect for k/B to unity generates values of 1.013 ± 0.005 (in place of 1.008 ± 0.010) for the k/A isotope effect and 1.026 ± 0.003 (in place of 1.024 ± 0.006) for the k effect. Constraining the k/B secondary isotope effect for 2-oxobutanoate-3-d_2 to unity generates isotope effects of 0.932 ± 0.007 (in place of 0.951 ± 0.012) for k/A and 1.051 ± 0.004 (in place of 1.057 ± 0.005) for k. The fact that little or no change in the other calculated effects is found when the k/B effects are constrained to unity shows that (a) the other results are insensitive to assumptions about k/B and (b) the values obtained for the k/B effects are not reliable enough for careful interpretation.

The measured value of k/B provides a rough check on the magnitudes we have estimated for the rate constants. From the definition of k/B in Figure 2 and our estimates of the catalytic-cycle rate constants combined with the value of k_{a3}/k_{a4} taken from transient kinetic studies,[7g,18,19] we calculate $k/B = 0.6 \times 10^5$ M^{-1} s^{-1} while the measured value is 2.3×10^5 M^{-1} s^{-1}, so that our calculated value is 4-fold smaller than the measured value. One source of error is the cumulative uncertainties in the estimated microscopic constants for the catalytic cycle. Another is the fact that the transient kinetic studies were conducted at somewhat different pH and temperature from the steady-state investigation, introducing cumulative errors into the interpolated and extrapolated values of the activation rate constants.

Table V. Distribution of the Catalytic Power of Pyruvate Decarboxylase Among the Mechanistic Steps of the Reaction[a]

conversion	enzymic rate constant	nonenzymic rate constant	acceleration factor
formation of the TDP-pyruvate adduct	82 s^{-1}	2×10^{-11} s^{-1}	$10^{12.6}$
return of the adduct to reactants	120 s^{-1}	2×10^{-8} s^{-1}	$10^{9.8}$
decarboxylation of the adduct	640 s^{-1}	5×10^{-5} s^{-1}	$10^{7.1}$

[a] Rate constants for the enzymic reaction are taken from Figure 3; the second-order rate constant k_{c1} was multiplied by the standard-state concentration of 1 mM for pyruvate. Nonenzymic rate constants were calculated from the values measured by Kluger, Chin, and Smyth[5] for the specific-base-catalyzed addition of thiamin to pyruvate (1.3 M^{-2} s^{-1}, multiplied by standard-state concentrations of pyruvate (1 mM) and hydroxide ion ($10^{-7.8}$, pH 6.2)), specific-base-catalyzed reversion of the adduct to reactants (1.3 M^{-1} s^{-1}, multiplied by the standard-state concentration of hydroxide ion ($10^{-7.8}$)), and decarboxylation of the adduct (value of 5×10^{-5} s^{-1} interpolated for pH 6.2).

Conclusions

We are now in a position to examine the manner in which PDC accelerates the thiamin-promoted decarboxylation of pyruvate by a factor of $10^{12.5}$. The events of carbonyl addition, reversion of the adduct to reactants, and decarboxylation will be considered individually. The product-release step will not be considered because of the lack of appropriate data for a nonenzymic reaction. The distribution of the catalytic acceleration among the processes considered is summarized in Table V.

Conversion of the activated enzyme SE* and pyruvate to the transition state for the k_{c1} step is the enzymic transformation most analogous to the nonenzymic specific-base-catalyzed addition of thiamin to the keto group of pyruvate, although we do not know whether carbonyl addition is actually complete in the enzymic state SE*S. Under the standard-state conditions of 1 mM pyruvate and pH 6.2, this process is accelerated by a factor of $10^{12.6}$, about the same as the overall enzymic acceleration factor of $10^{12.5}$. It is apparently in increasing the rate of formation of the adduct of TDP with pyruvate that a very large part of the catalytic power of PDC is exerted.

The reversion of SE*S to SE* + S is analogous to the specific-base-catalyzed reversion of the thiamin–pyruvate adduct to reactants. The enzyme also accelerates this process but by the smaller factor of $10^{9.8}$. This has the effect of making the steady-state ratio of SE*S/SE* higher than the steady-state ratio of [adduct]/[thiamin] by a factor of $10^{12.6}/10^{9.8} = 10^{2.8}$. This may occur in whole or in part by an enzyme-induced increase in the acidity of TDP, a matter considered in some detail by Washabaugh and Jencks.[4]

Finally, the enzymic decarboxylation (conversion of SE*S to SE*M) is faster than the nonenzymic decarboxylation by a factor of $10^{7.1}$. This factor is a negligible part of the total enzymic acceleration of $10^{12.5}$. Nevertheless, if PDC had not produced a considerable part of this acceleration, the decarboxylation process would have become rate-limiting for the enzymic reaction, leading to an overall smaller acceleration than is observed. In the nonenzymic reaction, the kinetic significance of the decarboxylation transition state under the standard-state conditions of Table V is 10^{-4}, while for the enzymic reaction, the kinetic significance of decarboxylation is about 10^{-1}. It is therefore possible to think of a factor of about 10^6 of the total acceleration of about 10^7 as "preventing" the decarboxylation from becoming the rate-limiting event for the enzymic reaction. While the suggestion of acceleration of decarboxylation by a hydrophobic environment[23] is both reasonable and consistent with our findings, a test of the concept is not provided.

(23) (a) Crosby, J.; Stone, R.; Lienhard, G. E. *J. Am. Chem. Soc.* **1970**, *92*, 2891–2900. (b) Crosby, J.; Lienhard, G. E. *J. Am. Chem. Soc.* **1970**, *92*, 5707–5716.

Comparison of the free energy diagrams for the enzymic and nonenzymic reactions and the roles of reactant-state and transition-state stabilization in the generation of the catalytic power of PDC will be considered in the context of models for the molecular evolution of enzymes.[24]

Experimental Section

Materials. Thiamin diphosphate hydrochloride, NADH, sodium pyruvate (anhydrous, Type II), and sodium 2-oxobutanoate (monohydrate) were purchased from Sigma Chemical Co., anhydrous citric acid from Fisher Scientific Co., sodium citrate dihydrate from J. T. Baker Chemical Co., and magnesium sulfate (anhydrous, analytical reagent) from Mallinkrodt Chemical Co. Water was deionized and double-distilled.

Isotopic Substrates. Sodium pyruvate-*1*-^{13}C (anhydrous, 90.0 atom % ^{13}C) and sodium pyruvate-*2*-^{13}C (anhydrous, 91.4 atom % ^{13}C) were obtained from MSD Isotopes.

Sodium pyruvate-*3*-d_3 was prepared by exchange of pyruvic acid (Aldrich, 95%, 5–6 g) with 80 g of deuterium oxide (Aldrich Gold Label, 99.8 atom % deuterium) in a sealed bottle for 9 h at 120 °C. The product was obtained by removal of water at reduced pressure, distillation of pyruvic-*3*-d_3 acid, dissolution in ethanol, and precipitation of sodium pyruvate-*3*-d_3 with sodium acetate. GC–MS analysis of the methyl ester showed no detectable d_0 compound (*m/e* 102), 0.42% d_1 (*m/e* 103), 4.83% d_2 (*m/e* 104), and 89.37% d_3 (*m/e* 105). Sodium pyruvate was purified in an identical manner and found to give kinetic behavior identical with the purchased material.

Sodium 2-oxobutanoate-*3*-d_2 was prepared by exchange with deuterium oxide in a procedure similar to that for sodium pyruvate-*3*-d_3 and was purified similarly except that the final precipitation was accomplished with sodium formate. The product was more than 95 atom % deuterated at the 2-position (NMR).

Enzymes. Pyruvate decarboxylase (EC 4.1.1.1) from *Saccharromyces carlsbergensis* was purchased from Sigma Chemical Co. with a specific activity of 11–16 units mg^{-1}, suspended in a solution of 5% glycerol, 3.2 M ammonium sulfate, 5.3 mM potassium phosphate, 1 mM magnesium acetate, and 0.5 M ethylenediamine tetraacetic acid. Alternatively, the enzyme was isolated from dried brewer's bottom yeast (Sigma) according to the procedure of Sieber et al. (sonication, protamine precipitation, acetone fractionation, ammonium sulfate precipitation, CM-Sephadex chromatography) to achieve a specific activity of 64 units mg^{-1}. The two preparations gave identical results. The enzyme was stored at 4 °C and showed no change in activity over 9 months. Yeast alcohol dehydrogenase (Sigma) had a specific activity of 320–400 units mg^{-1}.

Kinetic Measurements. The production of acetaldehyde in the PDC reaction was coupled to its reduction by NADH with catalysis by yeast alcohol dehydrogenase (ADH). The reaction was initiated by addition of 0.600 mL of a solution of sodium pyruvate in citrate buffer to a 1-cm cuvet containing 0.400 mL of a solution of citrate buffer, PDC, TDP, NADH, ADH, and magnesium sulfate, both solutions at pH 6.20 and 30.00 ± 0.01 °C. The final concentrations were as follows: citrate buffer, 0.10 M (total); PDC, 0.16 unit mL^{-1} (ca. 8 nM); TDP, 5.0 mM; Mg^{2+}, 5.0 mM; ADH, 8 units mL^{-1}; NADH, 0.256 mM; and sodium pyruvate, 0.300–30.0 mM. The cuvet was maintained in the thermostated cell compartment of a Cary 118 spectrophotometer, of which the photometric output was directed to a Heathkit H-11A computer. Absorbance data at 340 nm were collected at intervals of 0.5 s for a total of 300–600 s with an average of 800 points being stored. A background rate was determined by omission of PDC; its value was generally around 0.050 ± 0.003 mAU s^{-1}.

Measurements of isotope effects were made by measuring rates in identical solutions, with the two isotopic modifications of the substrate added in identical concentrations in paired experiments. The ratio of velocities v_1/v_2 provided the observed isotope effect for the chosen substrate concentration. Isotope effects on kinetic parameters were calculated as described in the next section.

Data Reduction. Rates were calculated by fitting the absorbance vs time data to the least-squares straight line. Points more than three standard deviations from the calculated value were eliminated and the rate recalculated; commonly no change occurred in the calculated rate. Subtraction of the background rate and division by the enzyme concentration in units mL^{-1} provided the final reaction velocity v. We tabulated v in dimensions of M s^{-1} (units mL^{-1})$^{-1}$ or the equivalent mol s^{-1} unit^{-1}.

For cases in which the values of kinetic parameters were to be determined from the dependence of v on S, the data were fitted to eq 2 by the nonlinear least-squares program BMDP-AR, with each value of v being weighted by its inverse variance (relative standard deviations in v being about 5% for points at [pyruvate] < 1 mM and about 2% for higher concentrations).

To calculate isotope effects on kinetic parameters from observed isotope effects at a series of pyruvate concentrations (obtained in paired experiments), eqs 4–6 of the Results section were employed. Weighting factors were calculated from the values of A, B, and K_i, as measured for the unlabeled pyruvate, and the isotope effects on the parameters k, k/A, and k/B were then calculated as least-squares best-fit values by the BMDP-AR program.

Deuterium Exchange in Pyruvate. In order to be sure that deuterium exchange did not render the secondary isotope effect experiments erroneous, the initial rates of enolization of pyruvic acid and pyruvic-*3*-d_3 acid were determined at 25 °C, pH 6.2, since this reaction is likely to be the route of isotope exchange. The reaction was followed by the decrease in absorbance at 353 nm in solutions 2 mM in iodine and 1.0 M in potassium iodide. The decrease was linear during the 1–2% of reaction, and the apparent first-order rate constant (ratio of zero-order rate constant to pyruvate concentration) was constant for pyruvate concentrations from 3 to 10 mM. The reactions were first-order in the trianion of citrate and exhibited an isotope effect of 5.4 ± 0.3. The half-lifetime for loss of protium was 8 days and for loss of deuterium 44 days. This reaction did not, therefore, interfere in the secondary isotope effect measurements.

Concentrations of 2-Oxobutanoate and 2-Oxobutanoate-*3*-d_2. The protiated substrate was obtained as a monohydrate; the deuterated compound was presumed to be unhydrated, having been precipitated from absolute ethanol. To avoid errors in the isotope effects arising from incorrect concentrations, the concentrations of both isotopic modifications in their stock solutions were determined from the absorbance change at 340 nm upon addition of an aliquot of stock solution to a solution containing 75 units mL^{-1} of lactate dehydrogenase (rabbit muscle; EC 1.1.1.27; Sigma), 0.256 mM NADH, and 0.10 M phosphate buffer at pH 8.0. the change in extinction coefficient was taken as 6220 M^{-1} cm^{-1}.

Registry No. TDP, 154-87-0; PDC, 9001-04-1; D, 7782-39-0; ^{13}C, 14762-74-4; CH_3COCO_2, 127-17-3; $CH_3CH_2COCO_2$, 600-18-0.

Supplementary Material Available: A derivation of weighting-factor equations for the isotope effects on kinetic parameters and of the relation of competitive isotope effects for PDC to microscopic rate constants and kinetic parameters (2 pages). Ordering information is given on any current masthead page.

(24) Alvarez, F. J.; Ermer, J.; Heckenthaler, T.; Huhta, D.; Hübner, G.; Schellenberger, A.; Schowen, R. L. Manuscript in preparation.