The Linkage of Catalysis and Regulation in Enzyme Action. Fluoropyruvate as a Probe of Regulation in Pyruvate Decarboxylases

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Abstract: Pyruvate decarboxylase, a thiamin-diphosphate (ThDP) dependent enzyme from the yeast *Saccharomyces cerevisiae* (SCPDC), catalyzes the conversion of fluoropyruvate to carbon dioxide, fluoride ion and acetate ion, as earlier reported for the wheat-germ enzyme (Gish, G.; Smyth, T.; Kluger, R. *J. Am. Chem. Soc.* **1988**, *110*, 6230−6234). While the fluoropyruvate reaction with the wheat-germ enzyme does not display the sigmoidal kinetics associated with hysteretic regulation by substrate that pyruvate as substrate produces with the wheat-germ enzyme, this feature and the induction period characteristic of hysteretic regulation are preserved when fluoropyruvate acts as substrate and regulator for SCPDC. The $C_1-^{13}C$ kinetic isotope effect for fluoropyruvate with SCPDC is the same as that for pyruvate (1.008−1.009), indicating the decarboxylation step to be about 15% rate limiting in the substrate-binding/decarboxylation part of the reaction. Solvent isotope effects and proton inventories are consistent with the view that fluoropyruvate regulation in SCPDC is not associated with an intracatalytic opening/closing of the active site coupled to sulfhydryl addition of cysteine-221 to the regulator keto group, as is thought to occur in pyruvate regulation of SCPDC. Product-release events in the first few catalytic cycles of the fluoropyruvate reaction, involving enzyme-catalyzed hydrolysis of acetyl-ThDP and release of acetate ion, may bring the wheat-germ enzyme into an ordinarily kinetically inaccessible enzyme-conformational regime analogous to the active form of the bacterial enzyme from *Zymomonas mobilis*, where the active site is permanently open without occupation of the regulatory site. Binding of fluoropyruvate at the regulatory site of SCPDC, in contrast, may remain necessary in the steady state for active-enzyme function but the active site may be permanently opened, thus not requiring the intracatalytic addition−elimination reactions at the regulatory site.

Introduction

Pyruvate decarboxylases catalyze the decarboxylation of pyruvic acid to carbon dioxide and acetaldehyde with use of thiamin diphosphate (ThDP) as a coenzyme. They are the most extensively studied among a number of ThDP-dependent enzymes, and form an ideal system for study of the origins of enzyme catalytic power since the non-enzymic reaction is very well characterized.[1,2] In addition, they show a species variability in their regulatory properties and thus can be used to investigate how regulation and catalysis are linked in enzyme action.

The enzymes from yeast (*Saccharomyces cerevisiae*, SCPDC)[3a−c] and wheat germ[3d] are subject to *hysteretic regulation by the substrate*, while the enzyme from the bacterium *Zymomonas mobilis* (ZMPDC) is unregulated.[3e] Site-directed mutagenesis studies show that cysteine-221 in SCPDC, which is absent in ZMPDC, is essential for enzyme regulation in the catalytic reaction of the normal substrate pyruvate.[4] Alvarez and co-workers[2c] and Hong and co-workers[2e] (see also Schowen[1d]) suggested that a covalent addition and elimination reaction of the sulfhydryl group of cysteine-221 to the regulatory substrate molecule acts as a switch to open and close the active site of the enzyme during the binding of substrate and the release of product within each catalytic cycle. Solvent isotope effects and

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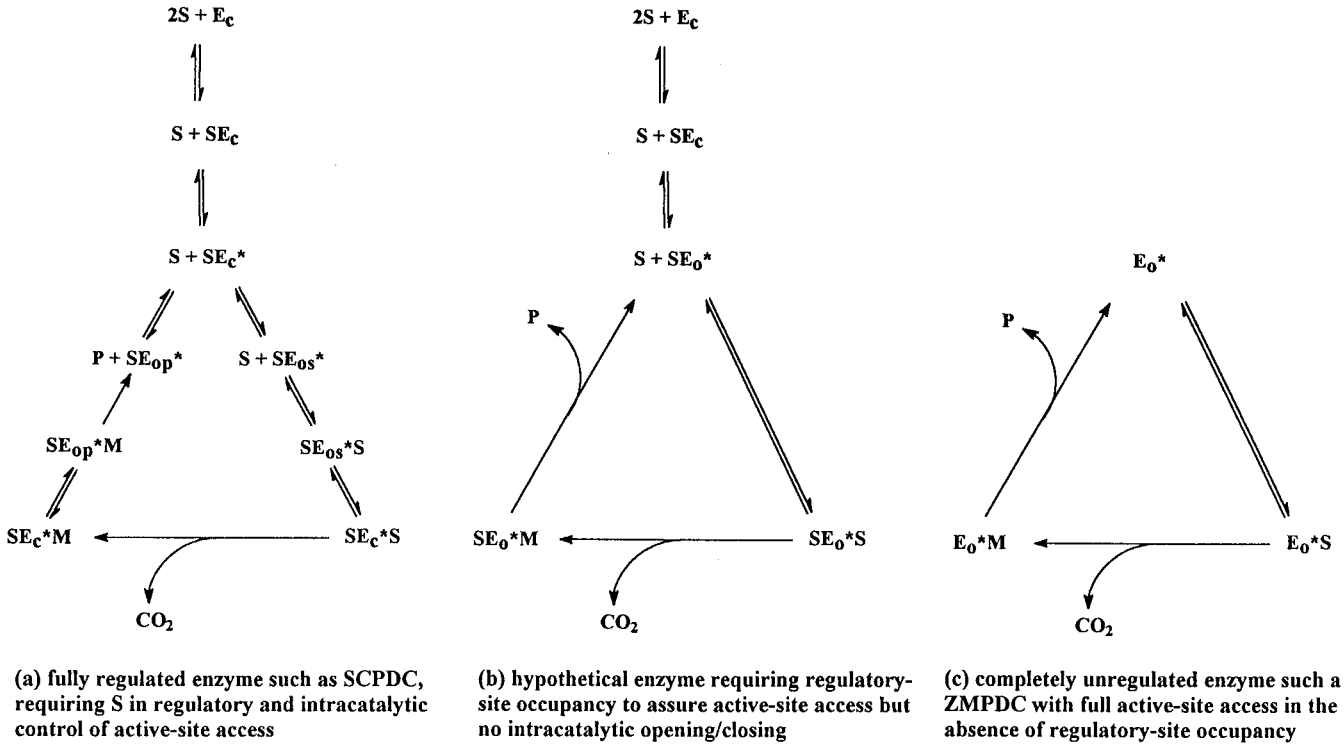

Figure 1. Models for the action of (a) the hysteretically regulated yeast enzyme SCPDC, (b) a hypothetical enzyme that exhibits some regulatory properties, and (c) the unregulated bacterial enzyme ZMPDC. In (a) SCPDC is depicted as exhibiting intracatalytic control of active-site access.[1d,2a,c−e] The vertical manifold at the top depicts the binding of a "regulatory substrate" molecule in the regulatory site (left of the letter E), followed by a slow, reversible transition that converts the inactive species SE into the active species SE^*_c, where the subscript c indicates the active site to be closed. Addition of cysteine-221-SH to the keto group of the "regulatory substrate" molecule opens the active site in such a manner as to permit substrate access (SE^*_{os}) and substrate then enters; elimination of cysteine-221-SH from the hemithioacetal is then coupled to closure of the active site. Decarboxylation now occurs within the closed active site. After decarboxylation, sulfhydryl addition is again coupled to active-site opening to a form appropriate for product release, SE^*_{op}, which then re-closes before onset of the next cycle of catalytic action. In (b) a hypothetical enzyme is considered in which occupation of the regulatory site by a substrate molecule leads to a permanently open active site. In (c) is shown the action of the unregulated ZMPDC, which effects substrate binding, decarboxylation, and product release in a sequence that is qualitatively and quantitatively similar to the catalytic sequence of SCPDC, except that the active site is permanently open to substrate entry and product departure without the requirement for occupancy of the regulatory site by a substrate molecule.[2d]

proton inventories[2a,d] for SCPDC (the regulated enzyme) and ZMPDC (the unregulated enzyme) are consistent with this hypothesis: an inverse isotope-effect contribution of about 3, arising from addition of a reactant-state sulfhydryl group or its equivalent[5] to the keto group of a regulatory-site pyruvate molecule, is the signature of the regulatory addition event. It is observed in substrate binding and product release for SCPDC but not for ZMPDC.

The catalytic power of the unregulated ZMPDC is similar to and probably larger than that of the regulated SCPDC.[2d] Primary ^{13}C isotope effects indicate that the kinetic significances (relative importance in determining the rate) of substrate binding, decarboxylation, and product release are similar in ZMPDC and SCPDC.[2d] The regulatory features of SCPDC seem to have been superimposed on a catalytic mechanism common to both SCPDC and ZMPDC, probably at some loss in catalytic power for SCPDC. Thus the control of active-site access in this case cannot be a feature that contributes to catalytic power. It is instead a wholly negative feature, available to deprive the enzyme of its power to catalyze substrate reactions when the enzyme is "inactive" but enabling only the normal level of catalytic power, freely available in unregulated pyruvate decarboxylases, when the enzyme becomes "active". The biological role of this kind of regulation may derive from the need for metabolic switching between aerobic and fermentative pathways in the yeast but not in bacteria that are obliged to use the fermentative pathway.

Figure 1 summarizes the situation for the regulated yeast enzyme SCPDC (Figure 1a, with enzyme activity requiring occupancy of the regulatory site and intracatalytic addition−elimination at cysteine-221 coupled to active-site opening and closing), for the bacterial enzyme ZMPDC (Figure 1c, with enzyme activity requiring neither occupancy nor reaction at a regulatory site), and a hypothetical enzyme (Figure 1b) in which it is imagined that active-site occupancy is required for activity but no intracatalytic reaction is necessary. The hypothesis of Figure 1b will be useful at a later point.

Fluoropyruvate, studied by Leung and Frey[6] as a substrate of the pyruvate-decarboxylase component of the pyruvate-dehydrogenase enzyme complex, is an analog of pyruvate that can act potentially as both substrate and regulator of pyruvate decarboxylases. It was applied as a probe of catalysis and regulation in the wheat-germ pyruvate decarboxylase system

(5) Jordan and his co-workers (Jordan, F.; Baburina, I.; Gao, Y.; Guo, A.; Kahyaoglu, A.; Nemeria, N.; Volkov, A.; Yi, J. Z.; Zhang, D.; Machado, R.; Guest, J. R.; Furey, W.; Hohmann, S. In *The Biochemistry and Physiology of Thiamin Diphosphate Enzymes*; Bisswanger, H., Schellenberger, A., Eds.; Intemann: Prien, Germany, 1996; pp 53−69) observed no change in absorption in the SH region (2545−2570 cm^{-1}) of the FT-IR spectrum of free SCPDC at pH 6 when cysteine-221 was mutated to serine. This was interpreted to indicate that cysteine-221 was ionized at pH 6 and thus not contributing to the absorption in this region. If cysteine-221 is ionized in SCPDC at pH 6, the reactant-state fractionating site generating the inverse isotope effects around 3 could not be the sulfhydryl group itself, but could possibly be a low-barrier hydrogen bond involving the thiolate anion.

(6) Leung, L. S.; Frey, P. A. *Biochem. Biophys. Res. Commun.* **1978**, *81*, 274−279.

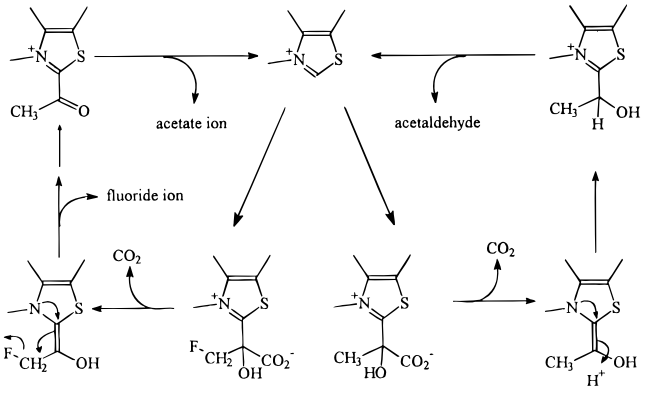

Figure 2. Chemistry of the catalytic cycle for ThDP-dependent decarboxylases with the substrates pyruvate (right-hand side) and fluoropyruvate (left-hand side). With pyruvate, the sequence is addition, decarboxylation to the enamine intermediate, protonation of the intermediate, and elimination to generate acetaldehyde. With fluoropyruvate, the sequence is addition, decarboxylation to the enamine intermediate, elimination of fluoride ion and protonation to generate acetyl-ThDP, and hydrolysis of acetyl-ThDP to generate acetate.

by Gish, Smyth, and Kluger.[7] As a substrate (Figure 2), it shares with pyruvate the part of the normal reaction pathway leading through the substrate-binding and decarboxylation steps (early stage of the reaction), and then differs in the sequence of steps following decarboxylation and leading through product release (later stage of the reaction). With pyruvate acetaldehyde is formed as product, but with fluoropyruvate fluoride ion is released and acetate is formed with no generation of aldehyde.[7] Comparison of catalysis and regulation with fluoropyruvate and pyruvate should thus provide information about the linkage of catalysis and regulation, allowing a distinction between the earlier, shared stage of the reaction and the later, divergent stage of the reaction.

Gish, Smyth, and Kluger[7] found that, with the wheat-germ enzyme, regulation by substrate, observed with pyruvate, was abolished in the reaction of fluoropyruvate as substrate: the fluoropyruvate reaction exhibited simple hyperbolic kinetics rather than the sigmoidal kinetics seen with pyruvate. The enzyme produced 1 equiv of fluoride ion, measured by a fluoride-specific electrode, and no fluoroacetaldehyde as product (tested for by alcohol dehydrogenase of which it is a known substrate), showing that all of the enamine intermediate (Figure 2) underwent fluoride-ion elimination and generation of acetate ion, with none undergoing the normal protonation reaction leading to fluoroacetaldehyde. Since the fluoropyruvate reaction diverged from the normal pyruvate route only in the product-release stage of the reaction, this suggested[7] that the regulatory event giving rise to sigmoidal kinetics occurs during product release.

This finding, if it also applies to the yeast enzyme SCPDC, is consistent with the model of Figure 1 in that part of the regulation is connected with reversible opening of the active site during product release. However, some regulatory effect from a similar event in reactant binding is still expected on the basis of the SCPDC model. We have therefore measured the kinetic parameters, carbon isotope effect, and solvent isotope effects for the fluoropyruvate−SCPDC system.

Results

Transient Kinetics for Fluoropyruvate with SCPDC. Under the conditions used here, fluoropyruvate is more than

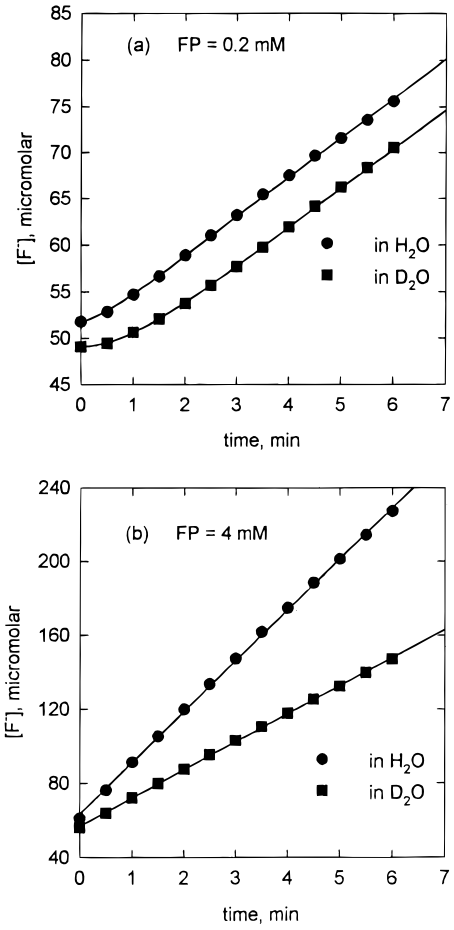

Figure 3. Time courses of the fluoride-ion concentration with SCPDC at pH 6.0, 30 °C, with two different initial concentrations of fluoropyruvate (FP) as regulator and substrate. (a) [FP] = 0.2 mM: a distinct induction period, extending over 1−3 min, is observable in protium oxide as solvent and much more clearly observable in deuterium oxide as solvent. Although the induction period is longer in deuterium oxide, the steady-state rates are equal in the two isotopic solvents at this low fluoropyruvate concentration. (b) [FP] = 4 mM: induction periods can no longer be detected in either isotopic solvent. The steady-state rate is substantially reduced in deuterium oxide at this high fluoropyruvate concentration.

95% in the hydrate form (see Experimental Section). Nevertheless, the generation of fluoride ion remains strictly first order in enzyme under all conditions used, so the dehydration reaction never becomes rate limiting. No aldehyde formation is detected when it is attempted to couple the SCPDC-catalyzed decarboxylation of fluoropyruvate to either yeast alcohol dehydrogenase or horse liver alcohol dehydrogenase. Furthermore, a full equivalent of fluoride ion is recovered from the SCPDC-catalyzed decarboxylation of fluoropyruvate.

An activation phase in the time course of decarboxylation of fluoropyruvate is observed at low concentrations of fluoropyruvate in both H_2O and D_2O (Figure 3; data in Table S1 of the Supporting Information). The activation phase becomes diminished at high concentrations of fluoropyruvate. The kinetics of the time-dependent activation of SCPDC by fluoropyruvate has been characterized by us and will be discussed elsewhere, but it is apparent from Figure 3 that slow activation of SCPDC, of the type observed with pyruvate as substrate-regulator, occurs also with fluoropyruvate.

Steady-State Kinetics for Fluoropyruvate with SCPDC. The steady-state specific velocity, $v/[\text{enzyme}]_o$, as a function of fluoropyruvate concentration, is plotted in Figure 4 (data in Table S2 of the Supporting Information). The inset confirms

(7) Gish, G.; Smyth, T.; Kluger, R. *J. Am. Chem. Soc.* **1988**, *110*, 6230−6234.

Table 1. Kinetic Parameters and Isotope Effects for Fluoropyruvate and Pyruvate as a Substrate/Regulator of Yeast Pyruvate Decarboxylase (SCPDC) at pH 6.0 (pD 6.5) and 30 °C

				solvent isotope effect (H_2O/D_2O)	
parameter	fluoropyruvate, H_2O	pyruvate, H_2O	fluoropyruvate, D_2O	fluoropyruvate	pyruvate
$10^{-6}k/A$, $M^{-2}\ s^{-1}$	285 ± 9	156 ± 9	297 ± 19	0.97 ± 0.07	0.53 ± 0.05
$10^{-3}k/B$, $M^{-1}\ s^{-1}$	106 ± 4	212 ± 18	135 ± 14	0.79 ± 0.09	0.57 ± 0.11
k, s^{-1}	44.2 ± 0.2	320 ± 4	23.5 ± 0.2	1.88 ± 0.02	1.59 ± 0.03
10^3K_i, M	(156)	(264)	(156)		
10^9A, M^2	155 ± 5	2050 ± 120	79 ± 5		
10^6B, M	417 ± 16	1510 ± 130	174 ± 18		

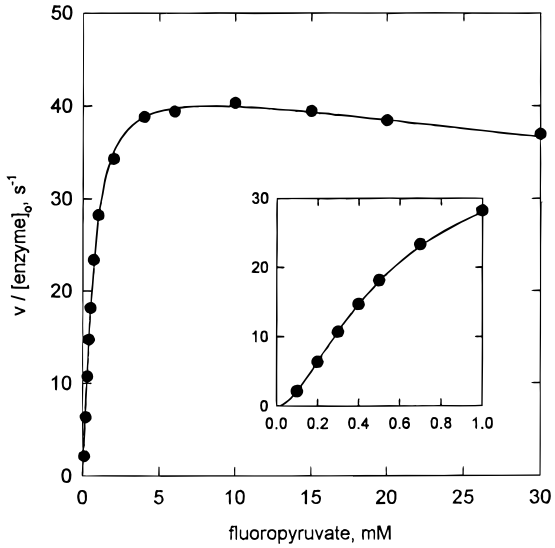

Figure 4. Steady-state specific rate of fluoride-ion production in the SCPDC-catalyzed decarboxylation of fluoropyruvate at pH 6.0, 30 °C, as a function of fluoropyruvate concentration. The inset shows the sigmoidal characteristic of substrate activation, visible at low fluoropyruvate concentrations. Substrate inhibition is visible at high fluoropyruvate concentrations. The line corresponds to eq 1 with the parameters shown in Table 1.

the sigmoidal characteristic of substrate activation. The Hill coefficient is 1.5−1.6. Substrate inhibition is observed at high concentrations of fluoropyruvate. Such kinetic behavior[2a] is described by eq 1:

$$v/[\mathrm{SCPDC}]_o = kS^2/[A + BS + S^2(1 + S/K_i)] \qquad (1)$$

Here S represents the added concentration of fluoropyruvate and no account is taken of hydration. The values of A, B, k, and K_i are obtained by fitting the initial rate vs fluoropyruvate concentration S to eq 1. K_i is not well defined in either H_2O (183 ± 17 mM) or D_2O (129 ± 16 mM), so we have followed our previous practice[2] of employing the average value of 156 mM to fit the data for both isotopic solvents, leading to changes in the calculated kinetic parameters that are within a single standard deviation.

The kinetic parameters are given in Table 1 along with those previously measured for pyruvate.

Carbon Isotope Effect by Competitive Measurement for Fluoropyruvate with SCPDC. Natural-abundance isotope-ratio mass spectrometry of the product carbon dioxide provides the $C_1-{}^{13}C$ isotope effect of 1.0058 ± 0.005. Since fluoropyruvate exists in aqueous solution mainly (>95%) as the hydrate, the observed isotope effect must be corrected for the equilibrium hydration isotope effect[8] (1.0028 ± 0.0009) if, as expected, it is the keto form of fluoropyruvate that reacts with ThDP in the active site of the enzyme. The corrected isotope effect is 1.0086 ± 0.0006.

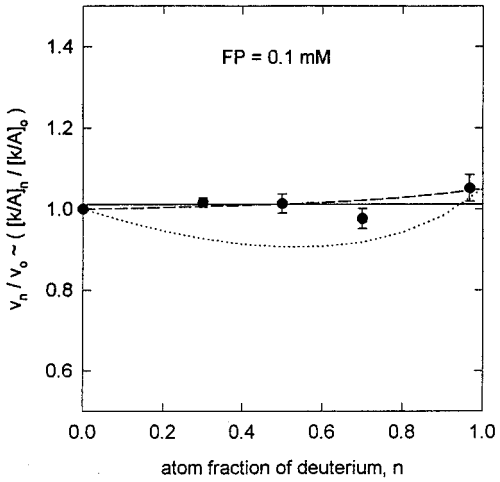

Figure 5. Proton inventory for the catalytic conversion of fluoropyruvate to fluoride ion and acetate ion by SCPDC at a constant fluoropyuvate concentration of 0.1 mM, so that the rate ratios in the isotopic solvents approximate those for the rate constant k/A. The ratio v_n/v_o does not vary with atom fraction of deuterium and the data are best fit by a the solid line drawn through the average value of the rate ratios. In principle, the isotope effect of unity could result from cancellation of inverse and normal isotope effects. The dotted line, passing well below the data, shows the predicted proton inventory for a model similar to that for k/A in the pyruvate−SCPDC system, with a sulfhydyl-addition event (inverse isotope effect of 3) occurring in the substrate-binding step (85% rate limiting) and being canceled by a normal isotope effect of 2.83, arising from many sites in the protein or solvent [thus $v_o/v_n = 0.15 + 0.85(2.83)^n(1 - n + n/3)$]. The dashed line passing among the data is a modification of this model with inverse isotope of 3 being canceled by a normal isotope effect of 2.83, arising from a single transition-state site [thus $v_o/v_n = 0.15 + 0.85(1 - n + n/3)/(1 - n + n/2.83)$]. The solid line passing among the data corresponds to the average value of the data (1.01) and represents a model in which no isotope effects arise in the binding of substrate or in decarboxylation.

Solvent Isotope Effects and Proton Inventories for Fluoropyruvate with SCPDC. The steady-state kinetics for decarboxylation of fluoropyruvate with catalysis by SCPDC was also conducted in D_2O, yielding the kinetic parameters and isotope effects shown in Table 1. The reaction rate as a function of atom fraction of deuterium (proton inventory[9]) was determined in mixtures of H_2O/D_2O buffers. In one series of experiments, a very small value of [fluoropyruvate] = 0.1 mM (1/4.2-fold B, 1/3.9-fold $\sqrt{A}$) was used so that the proton inventory approximates that for the rate constant k/A (Figure 5, data in Table S3 of the Supporting Information). In a second

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(9) Schowen, K. B.; Schowen, R. L. *Methods Enzymol*. **1982**, *87c*, 551−606. Quinn, D. M.; Sutton, L. D. In *Enzyme Mechanism from Isotope Effects*; Cook, P. F., Ed.; CRC Press: Boca Raton, FL, 1991.

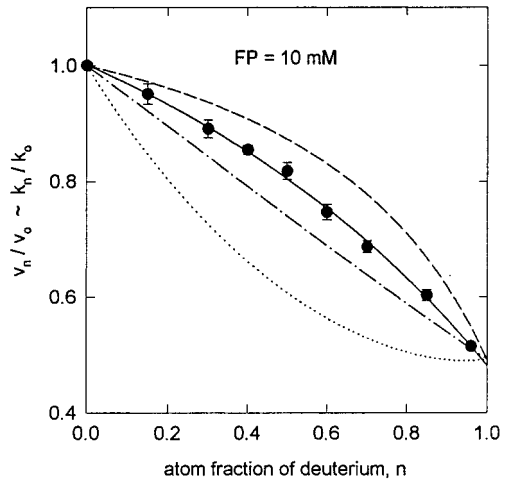

Figure 6. Proton inventory for the catalytic conversion of fluoropyruvate to fluoride ion and acetate ion by SCPDC at a constant fluoropyuvate concentration of 10 mM, so that the rate ratios in the isotopic solvents approximate those for the rate constant *k*. Three of the lines, none of which fits the data, represent models in which *k* is taken to describe product release, with an inverse isotope effect of 3 on product release canceled by a normal isotope effect of 6.1 [thus $v_n/v_o = \Gamma/(1 - n + n/3)$]: dotted line, normal isotope effect of 6.1 from many protein or solvent sites as in the pyruvate−SCPDC system [thus $\Gamma = (1/6.1)^n$]; dashed-dotted line, normal isotope effect of 6.1 from two transition-state sites [thus $\Gamma = (1 - n + n/\sqrt{6.1})^2$]; dashed line, normal isotope effect of 6.1 from one transition-state site [thus $\Gamma = (1 - n + n/6.1)$]. The line passing through the points represents a model in which two steps are described by *k*, with a normal isotope effect arising from a single transition-state site for one of the steps and no isotope effect on the other step [thus $v_o/v_n = 0.56 + 0.44/(1 - n + n/3.4)$]. The values of 0.56 and 0.44 for the kinetic significances and the value of 3.4 for the isotope effect are best-fit values but the numerical values are not highly significant (see text).

series of experiments, a saturating value of [fluoropyruvate] = 10 mM (24-fold *B*, 25-fold $\sqrt{A}$) was used, thus approximating the proton inventory for the rate constant *k* (Figure 6, data in Table S3 of the Supporting Information).

Discussion

Catalysis and Regulation in the Fluoropyruvate−SCPDC System. As can be seen in Figures 3 and 4, SCPDC exhibits with fluoropyruvate the same indicators of hysteretic substrate regulation as it does with pyruvate: the slow onset of catalytic activity (in the case of fluoropyruvate, over a period of 10−60 s; Figure 3) and sigmoidal steady-state kinetics (Figure 4, inset). Fluoropyruvate thus acts as both substrate and regulator of SCPDC in a reaction that leads to carbon dioxide, fluoride ion, and acetate ion as products.

Table 1 compares the rate constants for SCPDC catalysis of the pyruvate and fluoropyruvate reactions (see Figures 1 and 2 for structures and mechanisms). Three rate constants are obtained. A third-order rate constant *k*/*A* describes conversion of the reactant-state assembly {2S + E} to the effective transition state for substrate binding and decarboxylation. A second-order rate constant *k*/*B* describes conversion of the assembly {S + SE, SE*} to the effective transition state for substrate binding and decarboxylation (SE and SE* are at equilibrium in the steady-state and the contribution of each to the reactant state is proportional to its equilibrium population). A first-order rate constant *k* describes both conversion of SE*S to the transition state for decarboxylation and conversion of SE*M to the transition state for product release. The rate constants *k*/*A* and *k*/*B* thus in principle describe events early in the reaction (up to and through the decarboxylation step) and the rate constant *k* describes events late in the reaction (from SE*S through decarboxylation and product release). In the pyruvate−SCPDC system,[2a] the effective transition state for *k*/*A* and *k*/*B* is that for substrate binding (85%), while the value of *k* is determined equally by decarboxylation (50%) and product release (50%).

Table 1 shows that fluoropyruvate and pyruvate, which share the early part of the reaction pathway, exhibit rate constants *k*/*A* and *k*/*B* which are very little different for the two substrate regulators: *k*/*A* is about half as large and *k*/*B* about twice as large for fluoropyruvate as for pyruvate. However, fluoropyruvate is present in solution at >95% as the hydrate. If the keto form of fluoropyruvate is the "true" substrate, the effective values of *k*/*A* and *k*/*B* will be larger than those in Table 1. Whether this is the case is unknown, but it is clear that fluoropyruvate is at least as competent as pyruvate as both substrate and regulator of SCPDC.

The rate constant *k*, describing the part of the reaction in which the mechanistic pathways for pyruvate and fluoropyruvate diverge, shows a larger effect of structure: the value for fluoropyruvate is reduced by around 8-fold from that for pyruvate. Fluoropyruvate is likely to undergo SCPDC-catalyzed decarboxylation more rapidly than pyruvate[10] so this rate constant is now likely to describe chiefly the product-release event. After decarboxylation of the fluoropyruvate adduct of ThDP, the resulting enamine (Figure 2) is expected to eliminate fluoride ion. The resulting enolic species should then undergo protonation and isomerization to acetyl-ThDP. Acetyl-ThDP will react with water to release acetate ion and complete the catalytic cycle. Some of these events may be compressed into single transition states for concerted reactions, but all of these sequential processes must occur with first-order rate constants larger than 44 s^{-1}, the value of *k*. The first-order rate constant for nonenzymic hydrolysis[11] of acetyl-ThDP is 1.2×10^{-2} s^{-1} at pH 7.0, 24 °C. The value should become somewhat smaller at the lower pH of 6 and should become somewhat higher at the higher temperature of 30 °C used in the present enzymic study, so a value around 0.01 s^{-1} is probably correct for the enzymic conditions. It thus appears SCPDC must be catalyzing the hydrolysis of acetyl-ThDP by a factor in excess of 4000-fold.

Carbon Isotope Effect and Kinetic Significance of Decarboxylation vs Substrate Binding. Competitive measurements of the $C_1-{}^{13}C$ isotope effect, which yield[2a] the effect on the rate constant *k*/*A*, lead to the kinetic significance of the decarboxylation step for the early stages of the reaction, i.e., substrate binding and decarboxylation. These are the steps common to the reaction pathways of fluoropyruvate and pyruvate. The $C_1-{}^{13}C$ isotope effect for pyruvate with SCPDC has been measured by several groups by competitive techniques.[8] The effect under most nearly comparable conditions[2a] is around 1.008, and if the intrinsic isotope effect for decarboxylation is estimated[2a] at around 1.05, then, for the early part of the reaction, the decarboxylation transition state is the rate-

(10) Arylgloxylates, including those with *para* substituents, are substrate regulators of SCPDC (Lehmann, H.; Fischer, G.; Hübner, G.; Hohnert, K. D.; Schellenberger, A. *Eur. J. Biochem.* **1973**, *32*, 83−87). Generally, values of the rate constant *k* increase with electron withdrawal, the data having been fitted by Lehmann and co-workers against σ^+ to obtain a value of $\rho^+ = 0.85$. The data also agree with a biphasic fit against σ with a change in rate-limiting step from a step with $\rho = 2.8$ to a step with $\rho = 0$, the former step being 26% rate limiting for the unsubstituted phenylglyoxalate. It is not certain what the rate-limiting step for *k* is with these substrates but the biphasic fit to the substituent effect is consistent with decarboxylation being largely rate limiting ($\rho = 2.8$) for electron-donating substitutents and product release ($\rho = 0$) becoming rate limiting for electron-withdrawing substituents.

(11) Lienhard, G. E. *J. Am. Chem. Soc.* **1966**, *88*, 5642−5649.

limiting transition state only to the extent of about 15% (~0.008/0.05), while the substrate-binding transition state (no carbon isotope effect) is rate limiting to around 85%.

A very similar isotope effect of 1.0086 is here obtained with fluoropyruvate, and thus the decarboxylation step is again about 15−20% rate limiting for the fluoropyruvate reaction with SCPDC at low substrate concentrations, where the early part of the reaction dominates the rate. The relative free energies of the transition states for decarboxylation and for the events connected with entry of the substrate into the active site (and perhaps formation of the substrate−ThDP adduct) are therefore quantitatively similar with pyruvate and fluoropyruvate. As mentioned above, the decarboxylation transition state in the fluoropyruvate system is expected to be stabilized compared to that for the pyruvate system. Apparently the transition state(s) involved in substrate binding are similarly stabilized.

Solvent Isotope Effect and Proton Inventory for *k*/*A* (Substrate Binding) in the Fluoropyruvate−SCPDC System. Table 1 shows the solvent isotope effect on *k*/*A* for the fluoropyruvate−SCPDC system to be unity. This is in contrast to the effect for the pyruvate−SCPDC system,[2c,d] where *k*/*A* is 2.3-fold larger in D_2O than in H_2O. The proton inventory for the pyruvate−SCPDC system can best be described[2d] by taking the inverse effect of 2.3 to arise completely from the substrate-binding step, which is 85% rate limiting. This assigns an isotope effect (rate constant in H_2O/rate constant in D_2O) of (1/3) to formation of the substrate-binding transition state (85% rate limiting) and an effect of 1.0 to formation of the decarboxylation transition state (15% rate limiting), so that the observed effect of 1/2.3 is then the weighted average effect ({0.85 × [1/3]} + {0.15 × [1.0]}). The proton inventory then further shows the effect of 1/3 in substrate binding to arise from the conversion of a loosely bound reactant-state site (fractionation factor 0.33, appropriate to sulfhydryl or sulfhydryl equivalent[5] at cysteine-221) to a transition-state site with isotope fraction similar to bulk water (fractionation factor 1.0, appropriate to the OH group of an adduct of cysteine-221 at the keto group of the regulatory pyruvate molecule). This isotope effect and proton inventory for the pyruvate−SCPDC system are an indicator of the regulatory sulfhydryl addition thought to be coupled to substrate entry into the active site.[2a,c−e]

The absence of a solvent isotope effect for *k*/*A* in the fluoropyruvate−SCPDC system appears to suggest that no such addition-coupled opening of the active site is needed, although substrate binding remains about 85% rate limiting. It is, however, possible that an inverse isotope effect of 3 from the regulatory addition reaction is being cancelled by a normal isotope effect near three from another source.

A proton inventory is shown in Figure 5 in which such cancellation is considered. The simplest model consistent with the data is that no isotope effect on either substrate binding or decarboxylation is occurring, since no variation of the rate with atom fraction of deuterium is occurring (solid line, Figure 5). However, the model that generates the dashed line is also acceptable: here the cancelling normal isotope effect comes from a single site in the substrate-binding transition state. A model in which the inverse isotope effect is cancelled by an effect from many sites in the protein or solvent, which might be considered the most reasonable cancellation model since it resembles the model for product release in the pyruvate−SCPDC system,[2a,d] generates the dotted line, which does not fit the data.

Thus regulatory-site addition in exactly the manner that it occurs in the pyruvate−SCPDC system, with no cancelling isotope effects, can be excluded for the fluoropyruvate−SCPDC system, as can a combination of regulatory-site addition and protein or solvent reorganization, such as was observed for product release in the pyruvate−SCPDC system. Only if the regulatory-site addition is accompanied by an event that generates a one-proton isotope effect of just the needed magnitude to cancel an isotope effect from sulfhydryl addition is the regulatory-site addition permitted by the data. While this is possible, the simplest interpretation of the data is that no isotope effect is being generated and that no addition reaction is coupled to substrate entry into the active site in the fluoropyruvate−SCPDC system.

Solvent Isotope Effect and Proton Inventory for *k* (Product Release) in the Fluoropyruvate−SCPDC System. In the pyruvate−SCPDC system, the directly measured $C_1-{}^{13}C$ isotope effect on the rate constant[2a] *k* of 1.025 shows that decarboxylation and product release each determine the rate equally. The solvent isotope effect is a normal effect of approximately 1.5 and can be interpreted[2c,d] by assuming the entire effect to arise from the product release step: this assigns an effect of 2.0 to the product-release step and an effect of 1.0 to the decarboxylation step, with the rate constant then exibiting the mean effect of 1.5. The product-release isotope effect of 2.0 appears from the proton inventory to result from an inverse isotope effect of 3, arising from a regulatory sulfhydryl-addition event, that is canceled by a normal isotope effect around 6, arising from many sites in the protein or solvent, such as might occur in connection with a protein conformational change. This model is required to account for an extraordinary curvature in the proton inventory ("hypercurvature").[2c,d]

For the fluoropyruvate−SCPDC system, Table 1 gives the solvent isotope effect on *k* as 1.9, essentially equal to the isotope effect of 2.0 estimated for the product-release step in the pyruvate−SCPDC system. Since *k* is 8-fold smaller with fluoropyruvate than with pyruvate, and the decarboxylation rate constant is expected to be larger with fluoropyruvate than with pyruvate,[10] it is reasonable to assume that *k* for fluoropyruvate is essentially the rate constant for product release. The product-release step in the fluoropyruvate reaction could have the same mechanism as with pyruvate (active-site opening coupled to regulatory-site addition, accompanied by a protein conformation change), if the divergent events following decarboxylation of the fluoropyruvate−ThDP adduct (fluoride elimination, formation of acetyl-ThDP, hydrolysis of acetyl-ThDP) were all relatively rapid compared to opening of the active site and release of the products. This would agree with the similar values of the solvent isotope effects. However, the proton inventory shows this not to be the case.

The proton inventory for *k* shown in Figure 6 exhibits a mild curvature *opposite in sense* to the hypercurvature that signals regulatory-site addition of sulfhydryl or its equivalent in the pyruvate system.[2c,d] The dotted line is that which fits the data for the pyruvate system and is strongly inconsistent with the data for the fluoropyruvate system. Furthermore, the data are inconsistent with all simple modifications of the pyruvate product-release model. The dotted line describes cancellation of the inverse isotope effect of 3 for regulatory addition by a multi-site effect of 6.1; the dashed-dotted line assumes cancellation by a two-site isotope effect of 6.1 and the dashed line cancellation assumes cancellation by a one-site isotope effect of 6.1. Lines for cancellation by isotope effects from numbers of sites greater than two will lie between the dashed-dotted line and the dotted line. The data are thus inconsistent with all models similar to the regulatory-addition model for the pyruvate system that involve a cancelling isotope effect from any integral number of sites up to an "infinite" number (the multi-site model).

This result suggests the following: (a) active-site opening, coupled to regulatory-site addition of sulfhydryl or its equivalent, is not required for product release in the fluoropyruvate system; and (b) the normal solvent isotope effect of 1.9 must arise from completely different sources than the effect of 2.0 for product release in the pyruvate system.

One simple concept is that the normal isotope effect of 1.9 for fluoropyruvate may be connected with a proton-transfer event in the formation of acetyl-ThDP. If this is so, the sense of the curvature in Figure 6 (a "dome-shaped" proton inventory) requires[9] that another step with a smaller isotope effect (or none at all) be partially rate limiting. The expression for such a proton inventory,[9] if the isotope effect arises from a single site in the transition state, is:

$$v_o/v_n = w/(1 - n + n/J) + (1 - w) \qquad (2)$$

where w is the kinetic significance of the isotopically sensitive step with one-site isotope effect J and $(1 - w)$ is the kinetic significance of the isotopically insensitive step with isotope effect 1.0. This expression was fitted to the data in Figure 6, yielding $w = 0.44 \pm 0.06$, $J = 3.4 \pm 0.2$. These values inserted into eq 2 generate the solid line in Figure 6. The parameters w and J are statistically correlated and the fitted values are not highly significant, but it is fair to say that the data are consistent with a two-step process with each step proceeding at about the same rate, one step with no isotope effect or only a small one, one with an isotope effect of 3−4.

What is the likely origin of a one-proton isotope effect of 3−4? Following decarboxylation, three steps occur: (1) elimination of fluoride ion, which is not expected to have a solvent isotope effect as large as 3−4, leading to the enol form of acetyl-ThDP; (2) ketonization of the enol form of acetyl-ThDP, which could readily exhibit a large isotope effect, e.g., from proton transfer to carbon; (3) hydrolysis of acetyl-ThDP, which is likely[11,12] to occur with rapid, reversible hydration of the carbonyl, followed by rate-limiting C−C bond fission, probably with specific-base catalysis, which also should not generate a large normal solvent isotope effect. The isotope-sensitive step is thus likely to be the ketonization step, with the isotope effect arising from proton donation to carbon and/or deprotonation of the hydroxyl group. The non-isotope-sensitive process may be one or both of the other two steps.

Conclusions for the Fluoropyruvate−SCPDC System. Fluoropyruvate appears to be a substrate/regulator for SCPDC but to function as a regulator in a different manner from pyruvate. Like pyruvate, fluoropyruvate binds in the regulatory site and occasions formation of active enzyme in a process slow compared to the catalytic cycle (induction period before the steady state); like pyruvate, fluoropyruvate is required to remain in the regulatory site to permit binding and catalytic destruction of fluoropyruvate in the active site (sigmoidal steady-state kinetics); like pyruvate, fluoropyruvate binds into the active site by way of a transition state about 1 kcal/mol less stable than that for decarboxylation, so that binding is about 85% rate limiting and decarboxylation about 15% rate limiting.

Unlike pyruvate, fluoropyruvate does not appear to control active-site access through intracatalytic addition−elimination reactions with cysteine-221 in the regulatory site. The simplest interpretation of the solvent isotope effects for fluoropyruvate is that no solvent isotope effect at all accompanies substrate binding, while the only solvent isotope effect in product release comes from donation of an exchangeable proton to carbon during the ketonization step leading to acetyl-ThDP. This reaction occurs at a similar rate to fluoride-ion elimination, acetyl-ThDP hydrolysis, or some combination of them. In contrast, with pyruvate, the solvent isotope effect on substrate binding is dominated by an inverse effect of 3 from addition of cysteine-221 or its equivalent[5] to pyruvate carbonyl in the regulatory site. A similar inverse effect, along with a large normal effect from a protein conformational change, arises during product release. These inverse isotope effects of 3 are the signatures of the regulatory sulfhydryl-addition events suggested to be coupled to active-site opening to allow substrate entry or product departure. Their absence in the case of fluoropyruvate thus suggests that with SCPDC, fluoropyruvate does not undergo sulfhydryl addition−elimination reactions at the regulatory site coupled to opening and closing of the active site.

The high catalytic activity of SCPDC in accelerating conversion of fluoropyruvate to carbon dioxide, fluoride ion, and acetate ion, however, indicates the absence of any barrier of notable size to active-site access. This leads to the suggestion that *in the fluoropyruvate−SCPDC system, regulatory-site binding and slow activation are required for enzyme activity but intracatalytic opening and closing of the active site do not occur, so that fluoropyruvate, when it binds in the regulatory site, must render the active site permanently open*. Fluoropyruvate does not "block" the active site in an open configuration by means of permanent sulhydryl addition at cysteine-221: if this were so, the signature of the inverse isotope effect would appear in k/A, which has the free enzyme as initial state. Instead fluoropyruvate—or its hydrate—must achieve the permanent opening of the active site by binding in the regulatory site without sulfhydryl addition at cysteine-221. This corresponds to the situation in Figure 1b. Either direct binding of fluoropyruvate or its hydrate in the active site must "freeze" the active site open, or the passage of the enzyme through a small number of catalytic cycles must lead to such a freezing.

In principle, fluoropyruvate might alkylate the sulfhydryl group of Cys-221, producing an unregulated enzyme analogous to that obtained by alkylation with bromopyruvate.[13] This irreversible modification would, however, produce a hyperbolic dependence of the steady-state velocity on fluoropyruvate concentration, in contrast to the sigmoidal dependence seen at low concentrations of the substrate. Such irreversible modifications are therefore excluded.

Fluoropyruvate with Wheat-Germ PDC and SCPDC. Gish, Smyth, and Kluger[7] have presented conclusive evidence that fluoropyruvate acts as a substrate with wheat-germ PDC but in a reaction that requires no second molecule of fluoropyruvate to bind in the regulatory site. This circumstance is similar to that in the pyruvate−*Zymomonas* PDC system:[2d] here the enzyme is active in catalyzing the decarboxylation of pyruvate but requires no regulatory-site occupancy by pyruvate. Wheat-germ PDC with fluoropyruvate thus seems to act according to the scheme of Figure 1c. Either the direct binding of fluoropyruvate to the wheat-germ enzyme must convert it into a form resembling (or at least behaving similarly to) the *Zymomonas* enzyme, or more likely passage through a relatively small number of catalytic cycles draws the wheat-germ enzyme permanently into a form with properties similar to those of the *Zymomonas* enzyme.

Overall Conclusions. These observations may be taken to suggest a protein−conformational relationship among the

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systems of Figure 1a (intracatalytic control of active-site access through sulhydryl addition−elimination at the regulatory site: exemplified by pyruvate with SCPDC and wheat-germ PDC), Figure 1b (occupancy of the regulatory site blocks the active site open: exemplified by fluoropyruvate with SCPDC), and Figure 1c (no occupancy of a regulatory site required, active site permanently open: exemplified by fluoropyruvate with wheat-germ PDC, pyruvate with *Zymomonas* PDC, and pyruvate with the cysteine-221-serine mutant constructed by Baburina and co-workers[4]). If this is so then interaction of SCPDC with fluoropyruvate would carry it from its normal, intracatalytically regulated conformation into a conformation with the active site frozen open by the presence of fluoropyruvate in the regulatory site. The enzyme could be driven into this form either directly or by passage through a number of abnormal catalytic cycles involving formation and hydrolysis of acetyl-ThDP in the active site. Interaction of fluoropyruvate with wheat-germ PDC would similarly carry that enzyme into a form with permanently open active site not requiring a regulator molecule in the regulatory site, again either directly or by way of a number of abnormal catalytic cycles. If this model were correct, it could emerge that the *Zymomonas* enzyme would exhibit mainly conformational differences from SCPDC. Such differences might involve only the disposition of groups forming the access machinery of the active site; these groups might consitute as small an array as the side chains of one or a few residues, or as large an array as the domains of a subunit or the subunits themselves.[14]

Experimental Section

Materials. Thiamin diphosphate hydrochloride ("cocarboxylase"), sodium fluoropyruvate, potassium fluoride, and NADH were purchased from Sigma, sodium fluoride standard solution (0.1000 M), phosphoric acid, and anhydrous citric acid from Fisher, and sodium citrate dihydrate and magnesium sulfate (anhydrous) from J. T. Baker. Deuterium oxide was obtained from Aldrich (99.9% D).

Enzymes. Pyruvate decarboxylase (EC 4.1.1.1) from *Saccharomyces cerevisaie* (specific activity 9 units/mg; specific activity[15] of the pure enzyme, 80 units/mg) was purchased from Sigma, suspended in a solution of 5% glycerol, 3.2 M ammonium sulfate, 5 mM potassium phosphate, 1 mM magnesium acetate, 0.5 mM EDTA, and 25 μM ThDP, pH 6.5. Alcohol dehydrogenase (EC 1.1.1.1) from baker's yeast with a specific activity of 360 units/mg and lactate dehydrogenase from rabbit muscle Type II were also obtained from Sigma. Alcohol dehydrogenase (EC 1.1.1.1) from horse liver was purchased from Fluka with a specific activity of 1.6 units/mg of protein.

Kinetic Method. The decarboxylation of fluoropyruvate was followed by measurement of the fluoride ion product with use of a fluoride ion electrode (Fisher) vs a calomel reference electrode with a Beckman model 4500 pH meter (read to 0.1 mV) interfaced to an IBM compatible computer (data acquisition at 10−30-s intervals for a period of 5−10 min). Data reduction is described below. The reaction was conducted at 30.0 °C in a glass vessel contained in a jacketed beaker through which thermostated water was circulated (reaction solution volume 25.5 mL). The fluoride electrode was calibrated at 30 °C by a standard NaF solution in 0.1 M citrate buffer containing 5 mM $MgSO_4$ and 5 mM ThDP over the range of fluoride ion concentrations produced in the reaction before each experiment, and 50 μM potassium fluoride was added to the reaction solution to provide a stable background reading. The reaction was started by addition of a SCPDC solution to the reaction vessel. The final concentrations for the reaction were as follows: SCPDC = 11.5 nM, ThDP = 5 mM, $MgSO_4$ = 5 mM, KF = 50 μM, fluoropyruvate = 0.1−30 mM, 0.1 M citrate buffer (all at pH 6.0).

Detection of aldehyde formation from decarboxylation of fluoropyruvate was attempted by coupling to the yeast or horse liver alcohol dehydrogenase reaction, followed at 360 nm with a Shimadzu 160U UV/vis spectrophotometer interfaced to an IBM-compatible computer.

Hydration of Fluoropyruvate. The proton NMR spectrum of fluoropyruvate in D_2O at 30 °C in 0.1 M citrate buffer (pD 6.5) shows doublets at δ = 4.5 ppm (CH_2 of the hydrate form) and 5.5 ppm (CH_2 of the keto form), both peaks being split by a hydrogen−fluorine nuclear spin−spin interaction with a splitting of J = 46 Hz. From the relative height of the two doublets, the percentage of hydration of fluoropyruvate was calculated to be about 90%. Similar results were obtained by Kokesh[16] (85% hydration) and by Hurley and co-workers[17] (95% hydration). The predominance of the hydrate in aqueous solution is confirmed by the natural-abundance ^{13}C NMR: doublet at δ = 86 ppm (CH_2F, J = 159 Hz); doublet at δ = 93 ppm ($C(OH)_2$, J = ca. 15 Hz); singlet at δ = 175 ppm (CO_2^-). No keto-carbonyl signal was detected above the noise level, in agreement with Hurley and co-workers.[17]

If the keto form of fluoropyruvate is the substrate for SCPDC, at sufficiently high enzyme concentrations the dehydration reaction should become rate limiting. This does not occur under the conditions used here. When the SCPDC concentration was increased by 1.20-fold, the observed rate was increased by a factor of 1.12 ± 0.03 ([fluoropyruvate] = 1 mM), 1.23 ± 0.06 ([fluoropyruvate] = 10 mM), and 1.23 ± 0.08 ([fluoropyruvate] = 40 mM), with the rate becoming independent of [fluoropyruvate] at ca. 5 mM. The observed rates are therefore not the non-enzymic dehydration rates.

Competitive Measurements of ^{13}C Isotope Effects. Procedures for the competitive measurements followed those described by O'Leary.[18] A solution containing 30 mM fluoropyruvate, 1 mM $MgCl_2$, and 10 μM ThDP in 0.1 M potassium phosphate buffer, pH 6.5, was divided into two portions. Each portion was placed in a closed vessel, and then purged with N_2 gas for 3 h to remove residual CO_2. After the solutions were incubated in a water bath at 25 °C for 30 min, pyruvate decarboxylase was injected into both solutions to initiate the reactions. The so called "low conversion" solution (20 mL) was allowed to decarboxylate to the extent of approximate 10%, and then the reaction was terminated by addition of concentrated phosphoric acid to lower the pH below 2. The "high conversion" solution (2 mL) was allowed to decarboxylate completely before addition of acid. The carbon dioxide formed from the reactions was isolated on a vacuum line using dry ice−isopropyl alcohol and liquid nitrogen traps. The vacuum line maintained a vacuum of 400−800 μTorr throughout the distillation. The extent of the reaction was checked by assay of the remaining fluoropyruvate with lactate dehydrogenase. Isotopic ratios for isolated CO_2 were measured with a Finnigan Delta S isotope ratio mass spectrometer, with appropriate correction for ^{17}O. Isotope ratios and isotope effects were calculated as described previously.[19]

Kinetic Technique for Solvent Isotope Effects. Citrate buffers (0.1 M) were prepared with use of either pure H_2O or 99.9% D_2O as solvent. Solvent isotope effects were calculated from the kinetic parameters measured in 99.9% D_2O buffers and H_2O buffers. In proton inventory studies, a mixture of appropriate volumes of D_2O buffer and H_2O buffer gave the required atom fraction of deuterium n. All reactions in H_2O/D_2O mixtures used the same substrate solution (prepared in H_2O), and were started by addition of the same enzyme solutions (prepared in H_2O), so that the errors from enzyme and substrate solutions would tend to cancel. The value of n was corrected for protium introduced in buffer preparation and by enzyme and substrate solutions. Calibrations of fluoride electrode were done in H_2O, D_2O, and 50% D_2O (see Data Reduction section below).

Data Reduction. A calibration curve for the fluoride ion electrode was obtained for each set of experiments by fitting the potential in millivolts (mV) as a function of fluoride concentration [F^-] at constant ionic strength to the Nernstian line: $mV = A - B \log [F^-]$. The slope B and intercept A were obtained for n = 0, 0.5, and 1, with B essentially constant at 59 mV and A typically a few percent larger in D_2O. Values of A and B at n between 0 and 0.5, or n between 0.5 and 1, were calculated assuming a linear relation of A or B vs n. The fluoride ion

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concentration was calculated from the resulting calibration curve and plots of [F^-] vs time were constructed. The activation part of the SCPDC reaction was allowed to pass, and initial rates were then calculated from the fit of the concentration of fluoride ion vs time to a straight line using the SIGMA-PLOT (least-squares fitting) program. Kinetic parameters were determined by fitting velocity vs substrate concentration to eq 1 using the SIGMA-PLOT program (nonlinear least-squares fitting), with a weighting factor of $1/v$ applied.

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Supporting Information Available: Tables of data for kinetic studies and proton inventories (3 pages). See any current masthead page for ordering and Internet access instructions.

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