reactions will show such effects. Furthermore, the data confirm the utility of chaotropic salts in diagnosing the presence of the hydrophobic effect in such reactions. As in the present case, it is clearly desirable to have such data for a variety of salts to exclude other explanations.

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Mechanism-Based Inactivation of Pyruvate Formate-Lyase by Acetylphosphinate: Evidence for Carbon-Phosphorus Bond Cleavage

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Pyruvate formate-lyase (EC 2.3.1.54; PFL) is an oxygen-sensitive enzyme (MW 170 kDa; dimer) that replaces pyruvate dehydrogenase during anaerobic glucose metabolism in *Escherichia coli* and a number of other prokaryotes.[1] The enzyme converts pyruvate and coenzyme A (CoA) to formate and acetyl coenzyme A via a ping-pong kinetic mechanism with covalent acetyl-PFL as an isolable intermediate.[2] PFL has no known organic or metal cofactor but does contain a protein-based organic free radical which is essential for the catalysis of this remarkable transformation.[3,4] A mechanism styled after a Minisci-type homolysis of pyruvate esters[5] and initiated by the enzyme radical (X•) has been proposed (Scheme I; path A).[6,7] We report here that acetylphosphinate (**1**), a pyruvate analogue, is a mechanism-based inactivator of PFL and present evidence for a novel carbon-phosphorus bond cleavage during the inactivation process.

Hypophosphite ($H_2PO_2^-$) has been the most thoroughly studied inactivator of PFL.[3,6-8] It inactivates both free PFL and acetyl-PFL,[9] quenches the enzyme radical with kinetics similar to the kinetics of inactivation, and covalently labels the enzyme. Both the reaction of acetyl-PFL with formate and the inactivation by hypophosphite are subject to primary kinetic isotope effects consistent with homolytic cleavage of the carbon-hydrogen and phosphorus-hydrogen bonds generating formate radical anion and hypophosphoryl radical anion, respectively.[6] Recent work has suggested that inactivation of acetyl-PFL by hypophosphite may involve carbon-phosphorus bond formation via the hypophosphoryl radical anion.[7,8]

On the basis of the above findings and the structural similarities of **1** to pyruvate, the possible inactivation of PFL by **1**[10] was examined by standard methods (Figure 1).[6] Incubation of PFL with **1** (45 μM) alone afforded inactivation kinetics which were biphasic in nature, comprising an initial rapid phase followed by a slow phase. In contrast to the result with hypophosphite,[9] preincubation of PFL with saturating levels of pyruvate (10 mM) afforded essentially complete protection from inactivation by **1**. Addition of formate (5 mM) or CoA (55 μM) resulted in a dramatic enhancement of inactivation by **1** (25 μM) with clean first-order kinetics ($t_{1/2} \sim 2.2$ min). Inactivated PFL did not regain activity after a 10 000-fold dilution in anaerobic buffer containing 10 mM pyruvate. A K_I of 303 ± 65 μM and k_{inact} of 5.8 ± 0.8 min^{-1} were also determined[15] (Figure 1, inset). All but

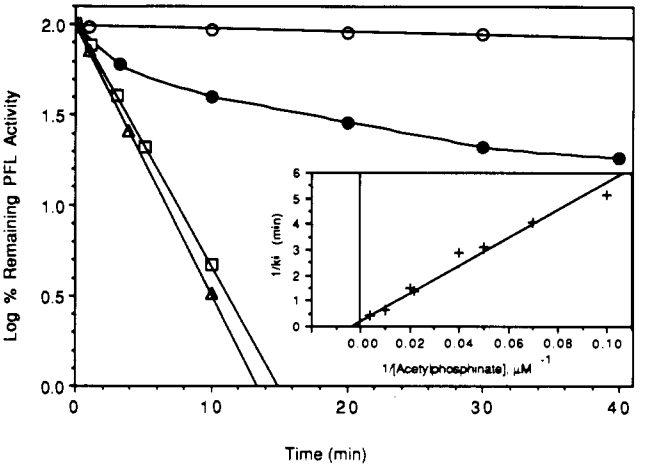
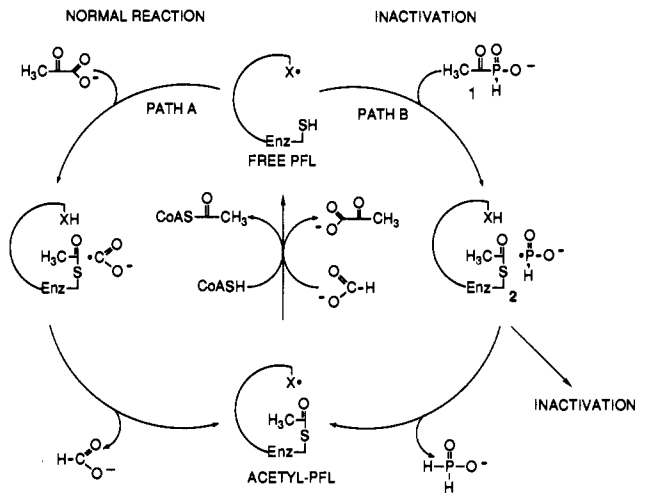


Figure 1. Time course for inactivation of PFL by acetylphosphinate (**1**). Active PFL (4 units) in anaerobic, 100 mM Tris buffer pH 8.1 (500 μL) at 25 °C was incubated with **1** under the following conditions: (●) 45 μM **1** alone; (○) preincubation with 10 mM pyruvate before addition of 45 μM **1**; (□) 55 μM CoA or (△) 0.5 mM formate and 25 μM **1**. Aliquots (20 μL) were removed at indicated times and quantitated for residual PFL activity.[6] Inset: Determination of K_I and k_{inact} for **1**. Rates of inactivation were determined in the presence of 55 μM CoA and 10-250 μM **1**. The line plot was determined by nonlinear regression.[15]

Scheme I



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(9) Inactivation of acetyl-PFL by hypophosphite is 4-6-fold faster than that of free PFL, suggesting that hypophosphite is a formate analogue.[3,6] As expected, formate protects against inactivation.

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(12) Acetylphosphonate and methyl acetylphosphonate, inhibitors of thiamine-dependent, pyruvate metabolizing enzymes,[13] are at 10 mM neither inhibitors nor substrates for PFL.

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one of the criteria for a mechanism-based inhibition of PFL by **1** have been fulfilled.[11]

A proposal explaining these phenomena is outlined in Scheme I (path B). We suggest that **1** is recognized by PFL as a catalytically viable pyruvate anlogue.[12] Homolysis of the C-P bond from the putative tetrahedral intermediate affords acetyl-PFL containing a quenched enzyme radical and the hypophosphoryl radical anion. This complex (**2**), essential equivalent to one generated by hypophosphite with active acetyl-PFL, can partition between inactivation involving the hypophosphoryl radical anion and regeneration of the enzyme-bound radical with concomitant formation of hypophosphite. Dissociation of hypyophosphite leaves the enzyme in its active acetylated form which is protected against further inactivation by **1** until deacylation occurs. Dithiothreitol (DTT; 4 mM), present in the reaction mixture, causes a slow deacylation, accounting for the biphasic kinetics with **1** alone. Formate or CoA causes a rapid deacylation affording active PFL which is available for additional cycles of inactivation/protection by **1** until complete inactivation is achieved.

A key prediction of this hypothesis is the formation of pyruvate from **1** and formate during the cycling to inactivation. Preliminary studies with [^{14}C]formate and unlabeled **1** have shown that inactivation is accompanied by the formation of 5 enzyme equivalents of [^{14}C]pyruvate, suggesting ~5 turnovers per inactivation and verifying C-P bond cleavage. The inhibition of PFL by **1** differs significantly from its reversible, slow, tight-binding inhibition of pyruvate dehydrogenase.[14] In this case, a dead-end complex between **1** and thiamine pyrophosphate is formed which cannot heterolytically dephosphinylate.

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Evidence of Vibrational Mode Coupling in 2-Fluoroethanol via High-Resolution Infrared Spectroscopy in a Molecular Beam

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The rotationally resolved spectrum of 2-fluoroethanol (2FE) has been measured to determine the extent of mode-selective vibrational coupling. The photoisomerization of 2FE in low-temperature matrices has been studied extensively.[1-10] By excitation of the OH or CH stretch in matrix-isolated 2FE it is possible to induce isomerization from the more stable Gg′ to the Tt conformer. Controversial evidence from some experiments suggests that mode-selective vibrational coupling occurs between hydrogen stretching modes and modes that participate in the Gg′-to-Tt isomerization of 2FE.[1-4] The relative quantum efficiencies for isomerization in an Ar matrix show that excitation of the OH stretch is 7 times more efficient in inducing isomerization than excitation of a CH stretch.[4] Thus far, studies have failed to determine conclusively whether the discrepancy in isomerization efficiencies is caused by mode-selective coupling of the hydrogen stretches to the isomerization coordinate[1-4] or by matrix effects involving the surrounding media.[8-10] By examining 2FE in a molecular beam we remove the ambiguity of the environmental effects, and the role of mode-selective coupling can be definitively and quantitatively evaluated.

High-resolution infrared spectroscopy in molecular beams measures the degree of vibrational coupling by identifying the molecular eigenstates directly. The molecular eigenstates in larger molecules are often superposition states of the zeroth-order vibrational states. The number and intensities of the transitions present in a rotationally resolved spectrum provide a direct measurement of the extent of mixing between different vibrational modes. de Souza, Kaun, and Perry first used high-resolution infrared spectroscopy to determine the molecular eigenstates in 1-butyne,[11,12] and this study was later extended by McIlroy and Nesbitt.[13,14] The spectra of McIlroy and Nesbitt clearly resolve transitions to many molecular eigenstates within the acetylenic stretch of 1-butyne. From analysis of the spectra it was determined that extensive coupling occurs between vibrational modes. Since 2FE is of a similar size and has a similar density of states, mode coupling should be spectrally apparent in 2FE as well. The extent of the coupling would be expected to correlate with the rate of the isomerization, if mode-selective coupling does in fact determine the rate of isomerization.

The rotationally resolved spectrum of the asymmetric CH stretch of the fluorinated carbon[3] was measured covering the spectral region from 2978 to 2989 cm^{-1} with a spectral resolution of 12 MHz. The experimental apparatus will be described in detail elsewhere[15] but, briefly, consisted of a color-center laser pumped by a dye laser, which in turn was pumped by an argon ion laser. The laser was crossed with a skimmed molecular beam. The method of optothermal detection was used after the design of Miller and co-workers.[16] Helium carrier gas at a pressure of 6 psig was passed over the liquid sample of 2FE and expanded through a nozzle of 50-μm diameter.

To analyze the data, our experimental spectrum was compared to a calculated spectrum, using a rigid rotor model and exact diagonalization of the rotational Hamiltonian.[17] The details of the data analysis, as well as a complete discussion of the results of this analysis, will be presented elsewhere.[15] When the fit is optimized, there are more peaks in the experimental data than are calculated by using the rigid rotor model. The additional peaks are characteristic of vibrational mode coupling. Many, although not all, of the individual peaks in the calculated spectrum correspond to a small cluster of peaks in the experimental data. A cluster of peaks result when the intensity of a single peak from one vibrational mode is distributed over a number of vibrational modes. Thus, the presence of clusters of peaks is indicative of the presence of vibrational mode coupling between the asymmetric CH stretch and other vibrational modes in 2FE.

The extent of mode coupling was determined from the intensities and the spacing of peaks within a cluster of peaks. The number of peaks in the cluster depends on the density of states, as well as the degree of mixing between the optically active light state and the manifold of dark states. From the location and intensities of the individual transitions in a cluster of peaks, we can evaluate the magnitude of the matrix element which couples the light state to specific individual dark states. Note that the average separation

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