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Inactivation of *Escherichia coli* Pyruvate Formate-Lyase by Hypophosphite: Evidence for a Rate-Limiting Phosphorus–Hydrogen Bond Cleavage†

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ABSTRACT: Recently, Knappe and co-workers [Knappe, J., Neugebauer, F. A., Blaschkowski, H. P., & Ganzler, M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 1332] have shown that the catalytically active form of pyruvate formate-lyase from *Escherichia coli* is associated with a protein-bound organic free radical which is quenched upon enzyme inactivation by oxygen or hypophosphite. Our interest in the chemical mechanism of this unusual enzymatic reaction has led us to investigate several key aspects of the inactivation of the lyase by hypophosphite and its relationship to the normal enzymatic reaction. We report here that the inactivation of both the free and acetylated forms of the lyase is subject to a primary kinetic isotope effect using [$^2H_2$]hypophosphite. This suggests that phosphorus–hydrogen bond cleavage is at least partially rate limiting during inactivation. In addition, the inactivated enzyme can be fully reactivated. We have also determined a V_{max}/K_m isotope effect of 3.6 ± 0.7 for pyruvate formation from [^{2}H]formate and acetyl coenzyme A. Thus, carbon–hydrogen bond cleavage is partially rate limiting in the normal reverse reaction. On the basis of our findings, the previous work of Knappe and co-workers, the likelihood that hypophosphite is a formate analogue, the known susceptibility of both hypophosphite and formate to homolysis, and a chemical precedent for homolytic cleavage of pyruvate, we offer a preliminary mechanistic proposal for the lyase reaction.

Pyruvate formate-lyase (EC 2.3.1.54; formate acetyltransferase; PFL)[1] catalyzes the key reaction in anaerobic glucose metabolism—the conversion of pyruvate and coenzyme A (CoA) to acetyl-CoA and formate (Scheme I). The enzyme is a homodimeric protein of M_r 170000 which occurs in both an inactive and active form (Knappe et al., 1969, 1974). The activation process is catalyzed under conditions of anaerobiosis by an Fe(II)-dependent activating enzyme of M_r 30000 (Knappe et al., 1969). In vitro activation also requires py-

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[1] Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EPR, electron paramagnetic resonance; PFL, pyruvate formate-lyase; SAM, *S*-adenosylmethionine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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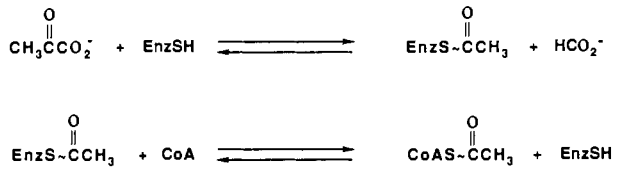
Scheme I



ruvate or oxamate as an allosteric effector, *S*-adenosylmethionine (SAM), and flavodoxin (Blaschkowski et al., 1982) or photoreduced 5-deazariboflavin (Conradt et al., 1984). While the mechanism of activation of pyruvate formate-lyase is not well understood, it appears to result in the stoichiometric cleavage of SAM to 5′-deoxyadenosine and methionine and may involve a transient Fe–adenosyl complex (Knappe & Schmitt, 1976; Knappe et al., 1984).

The enzymatic reaction has been shown to proceed through the intermediacy of an acetyl thio ester–enzyme complex (Knappe et al., 1974). Pertinent data in support of this intermediate include the observation of ping-pong kinetics, isolation of radiolabeled acetyl-enzyme via incubation with [2-^{14}C]pyruvate, and trapping of the complex with hydroxylamine to form acetohydroxamic acid. This is consistent with the long-known, CoA-independent pyruvate–formate carboxylate exchange reaction (Utter et al., 1945). No other cofactors appear to be involved; hence, the overall reaction catalyzed by PFL may be described by two half-reactions (Scheme I).

Recently, Knappe et al. (1984) have made the significant observation that the active form of PFL is associated with an EPR signal (doublet; g = 2.003), consistent with an enzyme-bound organic free radical. The signal was present in both the free and acetylated forms of the active PFL. Disappearance of the signal was observed upon inactivation of PFL by oxygen or by the known inhibitor of the "phosphoroclastic reaction" hypophosphite (Novelli, 1955). Additional studies on the effects of hypophosphite (Knappe et al., 1984) demonstrated that inactivation followed first-order kinetics and that the rate of inactivation was accelerated for acetyl-enzyme as compared with free enzyme. Inactivation with [^{3}H]hypophosphite led to the release of tritium to H_2O in an amount which appeared to be stoichiometric to the amount of active sites present. When [^{32}P]hypophosphite was used, radiolabel was covalently bound to the protein.

Our interest in this unusual enzymatic reaction has prompted us to investigate several critical aspects of the inactivation of PFL by hypophosphite and their relationship to the normal enzymatic reaction. We here report our initial findings.

Experimental Procedures

Chemicals. Unless otherwise noted, all materials were of reagent-grade quality or better and used without further purification. Sodium hypophosphite hydrate was purchased from Aldrich and dried to constant weight in vacuo at 30 °C. [$^2H_3$]Hypophosphorous acid (Aldrich Gold Label, 50 wt % solution in ^2H_2O) was converted to its sodium salt by dilution of the acid solution with ^2H_2O (1:1) and titration to pH 7.5 with NaO^2H. The anhydrous salt was obtained as a white powder following removal of ^2H_2O in vacuo at 30 °C. Isotopic purity of the [$^2H_2$]hypophosphite was greater than 95% on the basis of ^{31}P and ^{1}H NMR analysis, and the chemical purity was greater than 95% by $KMnO_4$ titration (Skoog & West, 1969). [$^2H_2$]Formic acid was obtained from Chemical Dynamics Corp. and converted to its anhydrous potassium salt by titration of a 5-mL aqueous solution of the acid to pH 7.5 with 50% KOH solution and drying in vacuo. The isotopic purity of the potassium [^{2}H]formate was greater than 95% by ^{1}H NMR comparison to a standard sample. 5-Deazariboflavin was a gift from D. T. Ashton, MSD Research Laboratories, Rahway, NJ.

Cell Growth and Protein Purification. Escherichia coli K12 (American Type Culture Collection 10798) were grown under strict anaerobic conditions, although cell harvesting, extraction (French pressure cell; 16 000 psi), and subsequent purification were carried out aerobically (Conradt et al., 1984). All purification steps were performed at 4 °C. The pyruvate formate-lyase used in this work was purified by the procedure of Conradt et al. (1984) through the Sepharose CL-4B chromatography step and estimated to be at least 75% pure on the basis of SDS–PAGE. The activating enzyme was separated from PFL by gel exclusion chromatography on Ultragel AcA44 and was used without further purification.

Activation of Pyruvate Formate-Lyase. Activation of PFL was performed in anaerobic septum-sealed vials and closely followed the procedures set forth by Knappe and co-workers (Knappe et al., 1974). Vials were rendered anaerobic by at least three cycles of evacuation (aspirator pump) and refilling with carrier-grade argon purified by passage through an Oxisorb filter cartridge (MG Scientific Gases). All transfers to and from anaerobic vials were made with gas tight syringes. The complete activation reaction mixture (1.0 mL) contained 100 mM Tris buffer (pH 7.6), 100 mM KCl, 3–6 units of inactive PFL, 0.6 mg of activating enzyme, 0.44 mM ferrous ammonium sulfate [Fe(II)], 5 mM dithiothreitol (DTT), 0.2 mM SAM, 15 μM 5-deazariboflavin, and 10 mM oxamate. Activation reactions were incubated at 30 °C for 1 h with illumination by a 300-W lamp. The activated PFL may be stored at 4 °C for 6 h with a loss of ca. 10% initial activity, while storage at −70 °C results in 50% activity loss after 4 days.

Assay of Pyruvate Formate-Lyase. The activity of PFL was measured spectrophotometrically (Knappe et al., 1974) with a Gilford Response II spectrophotometer, equipped with a thermostated six-position cell compartment. All assays were carried out in septum-sealed anaerobic cuvettes containing 1.0 mL of the appropriate assay mixture in 100 mM Tris buffer (pH 8.1) temperature equilibrated to 25 °C. The complete assay mixtures contained the following: (A) for the forward reaction, 2 units of citrate synthase, 30 units of malic dehydrogenase, 10 mM DTT, 0.1 mM Fe(II), 3.0 mM NAD, 55 μM CoA, 10 mM pyruvate, 0.1 mg of BSA, and 10 mM DL-malate; (B) for the reverse reaction, 15 units of lactate dehydrogenase, 5 units of phosphotransacetylase, 55 μM CoA, 10 mM acetyl phosphate, 0.3 mM NADH, 0.1 mg of BSA, 10 mM DTT, 0.1 mM Fe(II), and formate at the given concentrations. Reaction was initiated by injection of a 20-μL aliquot of active PFL (0.06–0.12 unit), and the reduction of NAD (forward reaction) or oxidation of NADH (reverse reaction) was monitored at 340 nm. Initial rates were calculated from the slope of the absorbance vs time curve. Kinetic constants were determined from reciprocal plots of specific activity vs substrate concentration. Oxygen leakage into the assay mixture was indicated by a red coloration of the solution due to the Fe(II)–DTT oxygen scavenging system (Knappe et al., 1974). Protein concentrations were determined by the Bio-Rad protein dye binding assay (Bradford, 1976). One unit of activity is defined as the amount of enzyme required to consume 1 μmol of substrate per minute at pH 8.1 and 25 °C.

Chemical Inactivation of Pyruvate Formate-Lyase. Time-dependent inactivation studies were carried out in

anaerobic vials containing up to 3 units of PFL. Hypophosphite, at concentrations given in the text, was injected into the active PFL solution and the mixture incubated at 25 °C. Twenty-microliter aliquots of the inactivation reaction mixture were removed at various time intervals and assayed for residual PFL activity as described above. In all cases the concentration of hypophosphite was diluted at least 50-fold when residual activity was measured. Inactivation reactions were followed through at least two half-lives. Stock solutions of hypophosphite were prepared in Tris buffer (pH 8.1) and rendered anaerobic by passage of argon through the solution for at least 10 min before use. Half-lives were determined from plots of log residual enzyme activity vs time. Control studies indicated that hypophosphite (5 mM) had no effect on the activities of citrate synthase or malic dehydrogenase in the activity assay.

Reactivation Studies on Chemically Inactivated Pyruvate Formate-Lyase. Anaerobic samples of active PFL (5–12 units, 1 mg of total protein) were inactivated by treatment with 10 mM hypophosphite (60 min at room temperature) or with oxygen (stirring in air for 60 min). Activity assay (forward reaction) of the treated samples revealed no detectable pyruvate consumption. The inactive PFL samples were injected onto a septum-sealed anaerobic column of Sephadex G-25 (1.4 × 19.5 cm) previously equilibrated with an anaerobic buffer containing 100 mM Tris (pH 7.6), 10 mM DTT, and 0.1 mM Fe(II). Elution was conducted at 4 °C at a flow rate of 0.5 mL/min. Protein fractions were collected into anaerobic vials and assayed for PFL activity. Prior to reactivation studies, the eluted protein was pooled and concentrated if the protein concentration was less than 0.2 mg/mL. Anaerobic concentration was accomplished on Amicon Centricon 10 microconcentrators, modified by sealing of the sample reservoir with a subaseal septum (Aldrich). The sample reservoir was made anaerobic by being purged with argon for 10 min and the membrane then washed with 500 μL of the G-25 anaerobic column buffer. Filtration through the membrane was accomplished by connecting a vacuum source to the septum on the concentrator base via a syringe needle. The G-25 protein effluent was injected into the sample reservoir and concentrated to ca. 1 mg/mL under a pressure head of argon from an argon-filled balloon. Suitable aliquots (0.05 mg) of the G-25-treated protein solutions were then activated as described above and assayed to determine activity recovery. A control sample of active PFL was treated similarly and its activity following G-25 treatment determined. The specific activity of PFL in these studies was calculated by use of the total protein concentration of each sample containing both PFL and activating enzyme.

Results and Discussion

Inactivation of PFL by Hypophosphite. The inhibition of pyruvate formate-lyase by hypophosphite was first observed by Novelli (1955) in his work on the CoA-independent carboxylate exchange reaction between pyruvate and formate. In a more detailed study, Knappe and co-workers (1984) found that inactivation of PFL by hypophosphite follows first-order kinetics and is accelerated when the enzyme is in its acetylated state. As shown in Figure 1, we have corroborated these results. At 25 °C PFL has a half-life of ~7.9 min in the presence of 0.5 mM hypophosphite. Furthermore, when PFL was incubated with pyruvate in the absence of CoA, the resulting acetyl-enzyme was inactivated 3.6-fold faster ($t_{1/2}$ ~ 2.2 min) than the free enzyme. Using slightly different conditions, Knappe et al. (1984) observed a similar acceleration with pyruvate. The enhancement of inactivation is best explained by a facilitated binding of hypophosphite to the formate binding site in the acetyl-enzyme. Our observation that formate (20 mM) affords partial protection to PFL from inactivation only when the enzyme is acetylated is consistent with this idea (data not shown). The high concentration of formate required for protection is similar to the K_m of formate (24.5 mM) in the reverse reaction (Knappe et al., 1974).

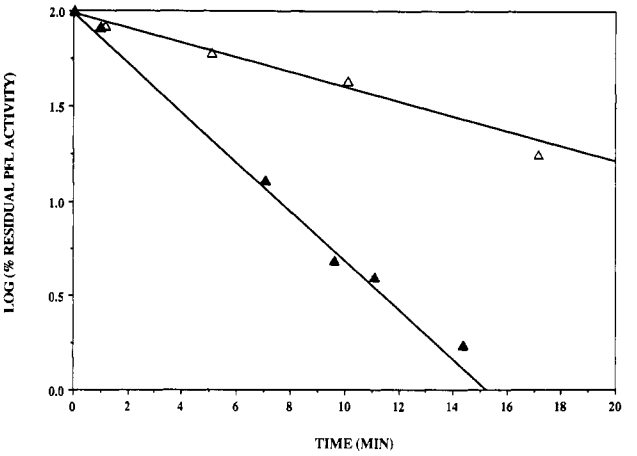


FIGURE 1: Time-dependent inactivation of PFL by hypophosphite and the acceleration of inactivation by pyruvate. Reaction mixtures contained 0.5 mM hypophosphite and no pyruvate (△) or 5 mM pyruvate (▲). Samples were preincubated with active PFL for 10 min at 25 °C prior to the addition of hypophosphite.

Table I: Recovery of Activity of Chemically Inactivated PFL by Anaerobic Gel Filtration and Subsequent Reactivation with the PFL Activating System[a]

	% activity recovery[b] following anaerobic gel filtration[c]		% activity recovery[b] following reactivation of gel-filtered inactive protein[d]	
sample	total units	sp act.	total units	sp act.
active PFL control	74	96		
oxygen- (air) inactivated PFL	0	0	77	95
hypophosphite-inactivated PFL	0	0	76	90

[a] The data reported here represent the average of at least two separate experiments. For complete details see Experimental Procedures. [b] Activity recovery is relative to the PFL activity observed (forward reaction) immediately prior to chemical inactivation reactions. Recovery of total protein varied from 72 to 86%. [c] No PFL activity (forward reaction) was detected for inactivated samples prior to G-25 chromatography. [d] A sample of active PFL boiled for 5 min could not be reactivated.

Reactivation of PFL. The nature of inactivation was investigated by anaerobic Sephadex G-25 gel filtration chromatography of hypophosphite-treated PFL. These results are shown in Table I and are compared with those of a sample of oxygen-inactivated enzyme and a control containing active PFL. While the recovery of activity from the control sample was nearly quantitative, the protein fractions from the enzyme samples inactivated by hypophosphite or oxygen remained inactive. While this suggests irreversible enzyme inactivation, we subsequently discovered that reactivation of hypophosphite-inactivated, gel-filtered protein samples afforded nearly quantitative restoration of PFL activity (Table I). As expected, oxygen-inactivated enzyme samples could also be restored to full activity.

The inactivation of PFL by hypophosphite is known to involve the loss of the EPR doublet observed for active enzyme (Knappe et al., 1984). In addition, Knappe and co-workers

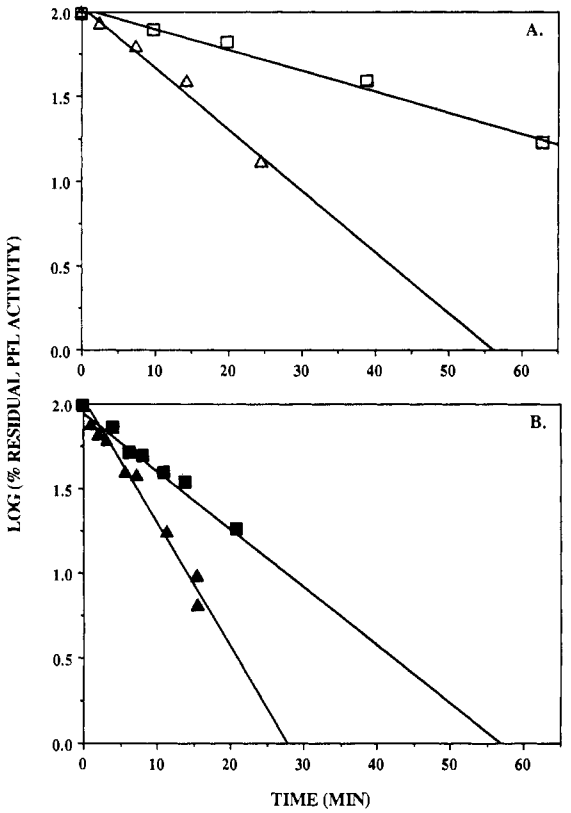
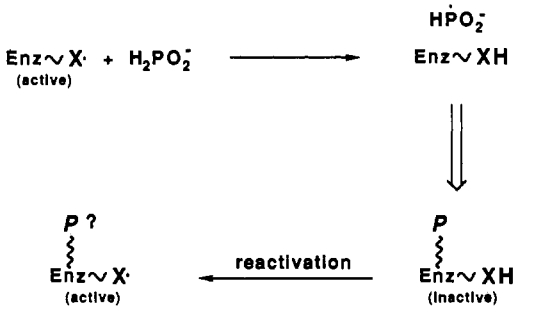


FIGURE 2: Isotope effects on the rate of inactivation of PFL by (A) [$^1H_2$]- (△) and [$^2H_2$]- (□) hypophosphite and (B) [$^1H_2$]- (▲) and [$^2H_2$]- (■) hypophosphite in the presence of 2 mM pyruvate. The reaction mixtures contained 0.25 mM hypophosphite. In (B), active PFL was preincubated with pyruvate for 10 min at 25 °C prior to the addition of hypophosphite.

discovered that inactivation by [^{32}P]hypophosphite led to a covalent radiolabeling of the protein. The label was reasonably stable to neutral or acid conditions but very labile in alkali (80% hydrolysis in 1 M NaOH in 15 min at 20 °C). Hence, the reactivation observed by us could involve prior cleavage of the protein–phosphinate bond. The relatively neutral conditions to which the inactivated protein is exposed would tend to disfavor quantitative loss of the phosphinate moiety by chemical means; however, a cleavage by the activating system is a possibility. Of course, the modified protein could be capable of being fully reactivated in which case the covalent labeling of the protein is peripheral to the primary event, quenching of the enzyme-bound radical. Detailed investigations of these possibilities are in progress with [^{32}P]hypophosphite.

Isotope Effects. Knappe and co-workers (Knappe et al., 1984) also reported that inactivation of PFL with [^{3}H]hypophosphite led to a release of tritium to solvent determined by ion-exchange chromatography. The release appeared to be stoichiometric to the amount of enzyme active sites, and no tritium was bound to the inactive protein. In light of the potential for proton exchange from hypophosphite (Jenkins & Yost, 1959) or an enzyme-bound derivative during the workup, we felt that it was crucial to determine the temporal relationship between inactivation and the observed washout of tritium. To this end, we have discovered that inactivation of PFL with [$^2H_2$]hypophosphite introduces a primary kinetic isotope effect on the process. As shown in Figure 2A, an isotope effect of 2.6 was observed for free enzyme with 0.25 mM hypophosphite. This result strongly suggests that a hydrogen abstraction from hypophosphite is at least partially rate limiting during inactivation. A smaller, but clearly primary,

Scheme II[a]



[a] P = phosphorus of unknown oxidation state; P? = actual presence to be determined.

isotope effect of 1.6 was also observed for acetyl-enzyme (Figure 2B). We have also established by ^{31}P NMR that the chemical exchange of the hypophosphite deuterons was not significant under the usual assay conditions (data not shown).

On the basis of the EPR studies of Knappe et al. (1984) and the isotope effects reported here, a reasonable hypothesis for the inactivation of PFL is shown in Scheme II. Enzyme-catalyzed homolytic phosphorus–hydrogen bond cleavage would quench the enzyme-bound radical and generate a hypophosphite radical anion which could react covalently with the enzyme and ultimately be quenched by solvent hydrogens, perhaps via DTT. The chemical generation of the radical anion has been well studied. Hydroxyl radicals produced by Fenton chemistry (Walling, 1975) readily abstract a hydrogen from hypophosphite in aqueous solution to yield the radical anion. The EPR spectrum of the radical species has been characterized (g = 2.0028; Gilbert et al., 1972). Irradiation of hypophosphite crystals has resulted in the detection of stabilized radicals with a half-life of 24 h (Morton, 1962). The excellent reducing properties of hypophosphite are well-known in organic chemistry (Kornblum et al., 1950), consistent with the relatively low energy of the P–H bond (79 kcal/mol; Dean, 1987).

This hypothesis does not easily explain the observations of Knappe et al. (1984) using [^{3}H]hypophosphite. The stoichiometric release of tritium upon inactivation implies no isotope effect; however, the use of tritium as a tracer complicates the analysis. An intramolecular discrimination by the enzyme between the tritium and the hydrogen on a labeled hypophosphite molecule would necessarily dampen the intermolecular selection effect. Since our deuterium isotope effect was determined under nonsaturating conditions with nearly completely dideuteriated hypophosphite, any intramolecular discrimination is suppressed. However, the stoichiometric release of tritium would also necessarily require that both hydrogens of the hypophosphite be labilized. Three possibilities which could account for the tritium release are (1) an enhanced susceptibility to heterolytic exchange with solvent from an enzyme-bound species, (2) a mechanistic scenario for inactivation which requires the sequential homolytic abstraction of both hypophosphite hydrogens, and (3) a less likely requirement for multiple turnovers of hypophosphite per inactivation which, when the tritium selection effect is factored in, gives the appearance of stoichiometric tritium release. The ultimate distinction among these possibilities could be made by a reexamination of the inactivation by [^{3}H]hypophosphite and by a determination of the oxidation state of the phosphorus chemically released from inactivated enzyme with [^{32}P]-hypophosphite. These studies are in progress.

The obvious extrapolation of the homolysis of hypophosphite to formate activation prompted us to measure the isotope effect

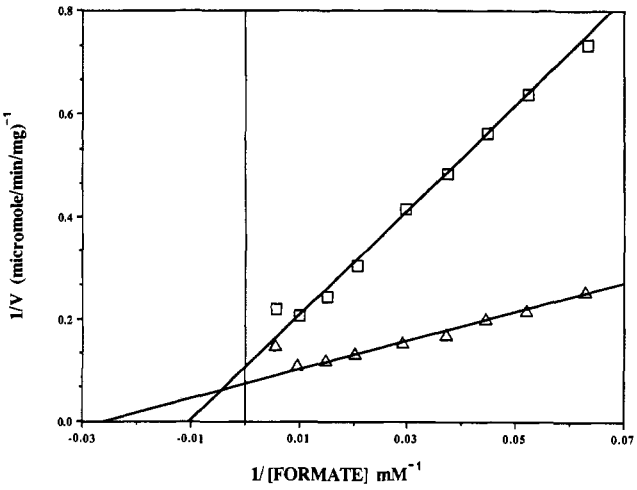


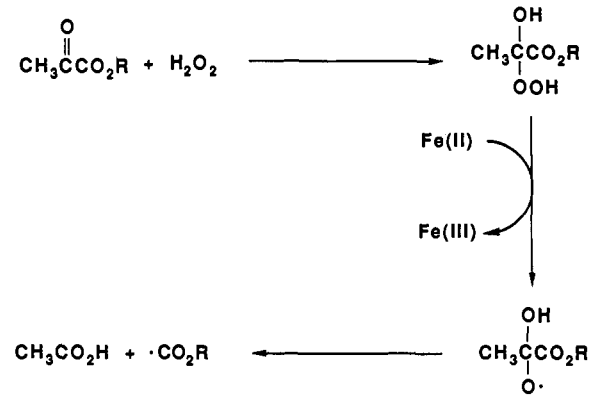
FIGURE 3: Lineweaver–Burk plot of the initial velocity of the conversion of [^{1}H]- (▲) and [^{2}H]- (□) formate to pyruvate (reverse reaction) catalyzed by PFL. The concentration of formate was varied between 16 and 200 mM at a fixed concentration of CoA (55 μM). Lines were generated by a least-squares fit of data excluding the 100 and 200 mM points due to substrate inhibition.

on pyruvate formation using [^{2}H]formate (Figure 3). In three independent experiments, an isotope effect of 3.6 ± 0.7 was found to be expressed on V_{max}/K_m. An isotope effect on V_{max} was more difficult to accurately determine for two reasons. First, at high formate concentrations (200 mM), substrate inhibition was observed. In light of the ping-pong mechanism proposed for the enzyme, this is not surprising. Second, V_{max} may be ultimately limited by the rate of acetyl-CoA-dependent thio ester exchange to generate acetyl-enzyme, the enzyme form required for reaction with formate. This would dampen the magnitude of the V_{max} isotope effect. The value of the V_{max} effect for the experiment shown in Figure 3 was ~1.4 and was generally less than 2.0 in other experiments. Since the V_{max}/K_m isotope effect is less sensitive to these factors, the value of 3.6 was quite reproducible. Thus, carbon–hydrogen bond cleavage is a rate-limiting step in the normal reverse reaction and suggests a low commitment to catalysis in this direction. The homolytic abstraction of a hydrogen atom from formate has chemical precedent and is a facile process as suggested by the C–H bond energy of 90 kcal/mol (Dean, 1987). The formate radical anion has been readily generated via Fenton chemistry and its EPR spectrum (g = 2.000) characterized in aqueous solution (Norman & West, 1969; Beckwith & Norman, 1969; Anderson et al., 1971).

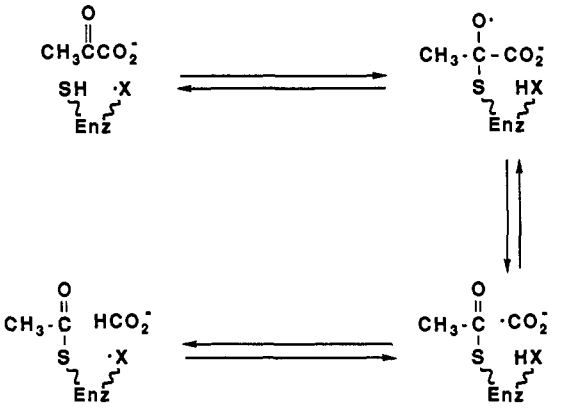
Conclusions. On the basis of our findings and the previous work of Knappe et al. (1984), we believe that the evidence for a homolytic mechanism for PFL is persuasive. A chemical precedent is the H_2O_2/Fe(II)-dependent homolytic cleavage of ethyl pyruvate to ethyl formate radical and acetate, the Minisci reaction (Bernardi et al., 1973; Heinisch & Lotsch, 1985a,b), which has been used in the selective carboxylation of heteroaromatic bases (Scheme III). The initial steps in this process involve the formation of a hydroperoxy hemiketal with ethyl pyruvate and H_2O_2 and its reduction with Fe(II) to the alkoxy radical. Subsequent homolytic β-scission yields acetate and the ethyl formate radical synthon.

Therefore, we would like to offer the following preliminary proposal for the mechanism of PFL (Scheme IV). The enzyme-based radical (structure unknown) could initiate the reaction by generation of a thiyl radical at the active site cysteine. Addition of a thiyl radical to the carbonyl of pyruvate would lead to a tetrahedral alkoxy radical intermediate reminiscent of the putative intermediate in the Minisci reaction.

Scheme III



Scheme IV



This species could also be generated by a thiolate addition to pyruvate followed by one-electron oxidation by the radical species. Although thiolate addition would be preferred on thermodynamic grounds, we favor a radical process since the production of thiyl radicals is a facile reaction and the resulting transiently quenched enzyme radical is immediately positioned to deliver a hydrogen atom to the formate radical anion. Thiyl radicals have been proposed as intermediates in the mechanism of styrene–glutathione conjugate formation catalyzed by prostaglandin H synthase and horseradish peroxidase (Stock et al., 1986) and of pyruvate:ferredoxin oxidoreductase (Docampo et al., 1987) and inferred for the B_{12}-dependent ribonucleotide reductase[2] (Babior & Krouwer, 1979). Collapse of the alkoxy radical would yield the acyl-enzyme and the formate radical anion which is reduced to formate by hydrogen atom abstraction from the quenched enzyme radical, thereby regenerating this species. CoA-dependent thio ester exchange would complete the reaction cycle. Our continuing efforts are directed toward testing this hypothesis.

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Rat Liver γ-Butyrobetaine Hydroxylase Catalyzed Reaction: Influence of Potassium, Substrates, and Substrate Analogues on Hydroxylation and Decarboxylation†

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ABSTRACT: Interaction of rat liver γ-butyrobetaine hydroxylase (EC 1.14.11.1) with various ligands was studied by following the decarboxylation of α-ketoglutarate, formation of L-carnitine, or both. Potassium ion stimulates rat liver γ-butyrobetaine hydroxylase catalyzed L-carnitine synthesis and α-ketoglutarate decarboxylation by 630% and 240%, respectively, and optimizes the coupling efficiency of these two activities. Affinities for α-ketoglutarate and γ-butyrobetaine are increased in the presence of potassium. γ-Butyrobetaine hydroxylase catalyzed decarboxylation of α-ketoglutarate was dependent on the presence of γ-butyrobetaine, L-carnitine, or D-carnitine in the reaction and exhibited $K_{m(app)}$ values of 29, 52, and 470 μM, respectively. γ-Butyrobetaine saturation of the enzyme indicated a substrate inhibition pattern in both the assays. Omission of potassium decreased the apparent maximum velocity of decarboxylation supported by all three compounds by a similar percent. β-Bromo-α-ketoglutarate supported γ-butyrobetaine hydroxylation, although less effectively than α-ketoglutarate. The rat liver enzyme was rapidly inactivated by 1 mM β-bromo-α-ketoglutarate at pH 7.0. This inactivation reaction did not show a rate saturation with increasing concentrations of β-bromo-α-ketoglutarate. None of the substrates or cofactors, including α-ketoglutarate, protected the enzyme against this inactivation. Unlike β-bromo-α-ketoglutarate, β-mercapto-α-ketoglutarate did not replace α-ketoglutarate as a cosubstrate. Both β-mercapto-α-ketoglutarate and β-glutathione-α-ketoglutarate were noncompetitive inhibitors with respect to α-ketoglutarate.

The enzyme γ-butyrobetaine hydroxylase [4-trimethylammoniobutyrate,α-oxoglutarate:oxygen oxidoreductase (3-hydroxylating), EC 1.14.11.1] catalyzes the crucial, final step in the biosynthesis of L-carnitine (eq 1). It belongs to a unique

$$\gamma\text{-butyrobetaine} + \alpha\text{-ketoglutarate} + O_2 \xrightarrow{Fe^{2+}} \text{L-carnitine} + CO_2 + \text{succinate} \quad (1)$$

class of non-heme ferrous iron dioxygenases in which the hydroxylation of substrate is linked to the oxidative decarboxylation of α-ketoglutarate (Abbott & Udenfriend, 1974; Hayaishi et al., 1976). A partial reaction, namely, α-ketoglutarate decarboxylation uncoupled from hydroxylation, occurs with all α-ketoglutarate-coupled dioxygenases, especially in the presence of substrate analogues (Counts et al., 1978; Rao & Adams, 1978; Hsu et al., 1981; Holme & Lindstedt, 1982). Such a decarboxylation in the absence of γ-butyrobetaine hydroxylation was demonstrated for human liver and

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