Electron Paramagnetic Resonance Evidence for a Cysteine-Based Radical in Pyruvate Formate-Lyase Inactivated with Mercaptopyruvate†

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ABSTRACT: Pyruvate formate-lyase (PFL) is a glycyl radical-containing enzyme that catalyzes the reversible, nonoxidative conversion of pyruvate and CoA into acetyl-CoA and formate. The radical is located on the α-carbon of glycine 734 and is required for catalysis. Two cysteine residues, C418 and C419, are also essential for catalysis. Mercaptopyruvate, a biologically relevant pyruvate analog, is shown here to be a mechanism-based inactivator of PFL. Upon addition of mercaptopyruvate to active PFL, an EPR spectrum is generated which exhibits components from two sulfur-based radicals. For one of these radicals, a disulfide radical, the hyperfine coupling to a single β-methylene hydrogen is resolved in features at $g = 2.057$ and 2.023. The effects of deuterium labeling of the enzyme on the EPR spectrum for this species are consistent with the new radical being on a cysteine residue, probably cysteine 418 or 419. This spectrum is not formed upon addition of the inactivator to mutant enzymes, C418S and C419S, indicating that both active site cysteines are required for formation of the new radicals. The identity of the second species is also ascribed to be a sulfur-based radical on the basis of the EPR feature found at $g = 2.01$. Our results constitute the first direct evidence of sulfur-based radical formation in an enzyme. A mechanism for formation of the cysteine-based disulfide radical is proposed which requires the participation of the two active site cysteines as well as the glycyl radical.

Escherichia coli, grown under anaerobic conditions, utilizes the enzyme pyruvate formate-lyase (PFL),[1] which provides the cell with acetyl-CoA through a nonoxidative cleavage of pyruvate with concomitant production of formate (Knappe et al., 1974). This enzyme, a homodimer (2 × 85 kDa), is the anaerobic counterpart of pyruvate dehydrogenase and catalyzes a key step in bacterial anaerobic metabolism.

PFL is functional only under anaerobic conditions and exists in active and inactive forms (Sawers & Bock, 1988, 1989). The interconversion between the two forms is achieved by the action of two other enzymes, activase and deactivase. Activase (AE) is a 30 kDa enzyme which requires Fe^{2+}, *S*-adenosylmethionine, and an electron donor (flavodoxin or photoreduced 5-deazariboflavin) as cosubstrates and pyruvate or oxamate as an allosteric effector (Conradt et al., 1988; Wong et al., 1994). Deactivase is a homopolymeric protein (~40 × 96 kDa) which requires Fe^{2+}, NAD, and CoA (Kessler et al., 1991).

The active form of PFL differs from the inactive form in that it contains a stable free radical on the α-carbon of glycine 734 which is required for its biological activity (Unkrig et al., 1989; Knappe et al., 1984; Wagner et al., 1992). An unusual property of this radical is that its α-hydrogen undergoes exchange with solvent (Unkrig et al., 1989; Wagner et al., 1992; Parast et al., 1995). The overall catalytic mechanism exhibits ping-pong kinetics with the intermediacy of an acetyl-enzyme via formation of a thioester with an active site cysteine. Two cysteines, C418 and C419, are implicated to be the site(s) of acetylation (Plaga et al., 1988; Parast et al., 1995). Mutagenesis studies have demonstrated that the two cysteines are not required for generation or subsequent stabilization of the glycyl radical[2] (Knappe et al., 1993; Parast et al., 1995) although both are required for overall catalysis since the mutants C418S and C419S are catalytically inactive.

A mechanism involving formation of substrate radical intermediates following transient formation of a cysteine-based thiyl radical as the first step has been proposed (Brush et al., 1988; Parast et al., 1995). Recent studies have shown that cysteine 419 catalyzes solvent exchange of the α-hydrogen of the glycyl radical (Parast et al., 1995). This residue also mediates the transfer of the glycyl radical to acetylphos-

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[1] Abbreviations: PFL, pyruvate formate-lyase; EPR, electron paramagnetic resonance; CoA, coenzyme A; acetyl-CoA, acetyl coenzyme A; AE, activase; DTT, dithiothreitol; SAM, *S*-adenosyl-L-methionine; Tris, tris(hydroxymethyl)aminomethane; pPFL, protio-PFL; *d*-Cys-PFL, deuteriocysteine-labeled PFL; *d*-PFL, uniformly deuterium labeled PFL; IPTG, isospropyl β-D-thiogalactoside.

[2] S. A. Lewisch, K. K. Wong, and J. W. Kozarich, manuscript in preparation.

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phinate, a pyruvate analog (Parast et al., 1995). Although the formation of thiyl radicals has been postulated for several enzymes (Stubbe, 1988, 1994), no direct evidence for their existence has been reported. In this work, we report direct EPR detection of an enzymic cysteine-based radical upon mechanism-based inactivation of PFL by mercaptopyruvate.

EXPERIMENTAL PROCEDURES

Materials. 5-Deazariboflavin was the gift of D. T. Ashton of Merck Research Laboratories, Rahway, NJ. Deuterium-labeled cystine (3,3,3′,3′-d_4, 98%), succinic acid (d_4, 98%), and D_2O (99.8%) were purchased from Cambridge Isotope Laboratories. Deuterium-labeled mercaptopyruvate was prepared by dissolving sodium mercaptopyruvate in acetate buffer in D_2O (pH 4.5, meter reading not corrected for deuterium isotope effect) and incubating for 3−4 h at room temperature (Cooper et al., 1982). Cysteine auxotroph *E. coli* strain JM15 (sex, F^-; chromosomal markers, cysE50, tfr-8) was obtained from Dr. Barbara J. Bachmann, *E. coli* Genetic Stock Center, Department of Biology, Yale University. All other chemicals including sodium mercaptopyruvate were obtained from Aldrich or Sigma and were used without further purification.

Enzyme Preparations. Recombinant AE was purified from *E. coli* strain N4830 (Wong et al., 1993). AE in the soluble fraction was purified by gel filtration chromatography and used without further purification. Recombinant PFL was purified from *E. coli* JM109 (Parast et al., 1995). C419S and C418S mutant enzymes were purified from *E. coli* strain N4830.[2] The purification of PFL was based on a modification of the procedure of Conradt et al. (1988). Protein concentrations were determined by the method of Bradford (1976) using a kit purchased from Bio-Rad.

Perdeuteriated PFL (d-PFL). *d*-PFL was obtained from cells grown on minimal media prepared in D_2O with succinate-d_4 (5 g/L) as the sole carbon source . Cells were acclimated to D_2O by successive growth in 10%, 50%, and 99.8% D_2O with succinate-d_4. At each step, cells were grown to OD ~0.9, pelleted, and resuspended in fresh medium (OD ~0.1) containing successively higher D_2O content.

Deuteriocysteine-Labeled PFL (d-Cys-PFL). *d*-Cys-PFL was purified from *E. coli* strain JM15, a cysteine auxotroph, bearing pKKBWM5.5C, a construct of pKK223-3 (Pharmacia) and *pfl* under the control of the *tac* promoter. The cells were grown in rich media (OD ~0.9) and were pelleted and resuspended in fresh minimal media (final OD ~0.1) containing cystine-d_4 (85 μM) as the cysteine source. The cells were then grown to an OD ~1.0 prior to harvesting. No IPTG induction was required for overproduction of PFL.

Activation of PFL for Kinetic Studies (Low Enzyme Concentration). Typically, 0.5-mL solutions containing 0.13 mg/mL PFL were activated in anaerobic septum-sealed vials according to a previously described procedure (Brush et al., 1988).

Activation of PFL for EPR Experiments (High Enzyme Concentration). Recombinant PFL and the C418S and C419S mutant enzymes were activated by the procedure described by Parast et al. (1995). The extent of activation was estimated from the intensity of the EPR signal due to the glycyl radical.

EPR Spectroscopy. EPR spectroscopy measurements were performed on a Varian E112 spectrometer equipped with a Systron-Donner frequency counter and an NMR gaussmeter. First derivative spectra were obtained at 77 K on 200-μL samples in 4-mm quartz EPR tubes equipped with septum-sealed caps. The microwave frequency was 9.23 GHz.

Assay for PFL Activity. PFL activity was measured by a coupled enzyme assay described previously (Knappe et al., 1974; Brush et al., 1988). Aliquots (10 μL) were taken from the activation mixture with a gas-tight syringe at indicated time intervals and assayed for PFL activity. The production of acetyl-CoA was monitored by the appearance of NADH ($\epsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$).

Inactivation of PFL by Mercaptopyruvate. Time-dependent inactivation studies were carried out in anaerobic vials (Pierce, 1.5 mL) containing 0.5 mL of enzyme solution (0.13 mg/mL active PFL). Mercaptopyruvate stock solutions were freshly prepared by dissolving its sodium salt in water and adjusting the pH to ~3 by dropwise addition of 1 N HCl and rendered anaerobic with argon. Mercaptopyruvate, at indicated concentrations, was then injected into active PFL solution and incubated at 25 °C. At indicated time intervals, 10-μL aliquots were removed and assayed for residual PFL activity as described above. The concentration of the inactivator was diluted 100-fold or more when residual activity was being measured. Inactivation reactions were typically followed to at least 2 half-lives. Control studies indicated that mercaptopyruvate had no effect on the activities of the coupling enzymes, citrate synthase and malic dehydrogenase, used in the activity assay. The rates of inactivation of PFL with mercaptopyruvate were either fitted to a single exponential decay (eq 1) or a double exponential decay (eq 2) using Sigma Plot (Jandel Scientific). In eqs 1

$$f(x) = ae^{-bx} \quad (1)$$

$$f(x) = ae^{-bx} + ce^{-dx} \quad (2)$$

and 2, a and c are the amplitude of the first and second exponential; b and d are the rate constants for the single and double exponential. Details for EPR sample preparation are given in the figure legends.

RESULTS

Inactivation of PFL by Mercaptopyruvate. Incubation of active PFL with mercaptopyruvate resulted in a time- and concentration-dependent inactivation of the enzyme. The kinetics of inactivation (Figure 1) was clearly biphasic with an initial fast rate of inactivation followed by a slower rate (●). Conversion of PFL to acetyl-PFL by the addition of pyruvate (Knappe et al., 1974) protected the enzyme against inactivation (▲), indicating that mercaptopyruvate and pyruvate compete for the same site on the enzyme. The addition of formate, however, simplified the kinetics of inactivation by mercaptopyruvate to a pseudo-first-order process (■), the rate of which corresponded well to the initial rate of inactivation observed in the presence of the inhibitor alone. An analysis of [^{14}C]formate exchange into mercaptopyruvate during inactivation revealed that the inhibitor was also a substrate requiring 5−10 turnovers per inactivation (data not shown).

A New EPR Spectrum Is Generated in Mercaptopyruvate-Inactivated PFL. When active PFL was mixed with mer-

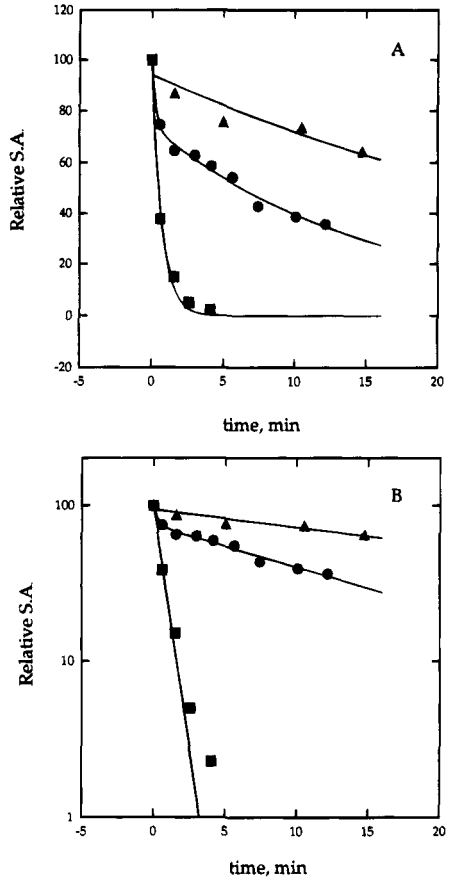


FIGURE 1: (A) Kinetics of inactivation of PFL (0.13 mg/mL) by mercaptopyruvate (8.60 μM) alone (●), in the presence of 10 mM pyruvate (▲), and in the presence of 25 mM formate (■). The K_m for pyruvate and formate are 2 mM and 25 mM, respectively (Knappe et al., 1974). The inactivation data in the presence of pyruvate (▲) and formate (■) were fitted to a single exponential decay with calculated rate constants of 0.027 ± 0.006 min^{-1} and 1.43 ± 0.12 min^{-1}, respectively. The inactivation data with mercaptopyruvate alone (●) were fitted to a double exponential decay with a calculated rate constant of 3.6 ± 1.8 min^{-1} for the first, fast phase and 0.062 ± 0.006 min^{-1} for the second, slow phase. (B) Semilogarithmic plot of the data in A.

captopyruvate, a complicated EPR spectrum was obtained within 20 s (Figure 2B) which was a composite of the signal due to the original glycyl radical (Figure 2A) and that of new radical species. This spectrum did not change significantly after incubation of up to 1 min at 25 °C, and the fractional loss of the glycyl radical was consistent with the rapid partial inactivation of PFL observed with inhibitor alone (Figure 1). The effect of formate on the generation of the new spectrum was investigated to correlate the kinetics of inactivation (Figure 1) with the EPR data. With formate present, the EPR signal due to the original glycyl radical was lost within 10 s of incubation, and the spectrum exhibited only those features due to the new species (Figure 2C). The faster rate of loss of the glycyl radical compared to the rate of inactivation discussed above reflects the higher concentration of mercaptopyruvate (5 mM vs 8.6 μM) used for the EPR studies due to the higher protein concentrations required (70 μM). The biphasic kinetics in the absence of formate are retained since the slow deacylation is essentially independent of mercaptopyruvate concentration. Pretreatment of active PFL with excess pyruvate prevented the appearance of the new EPR species with mercaptopyruvate (data not shown). Estimates of spin concentration in samples of active

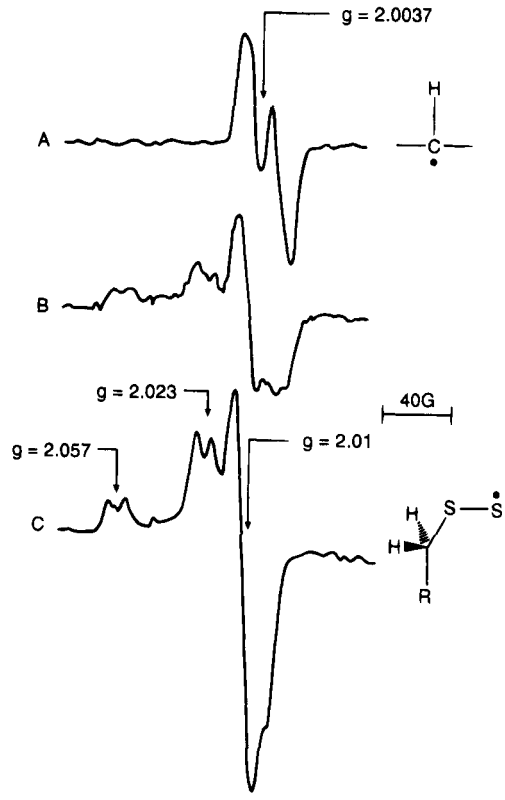


FIGURE 2: EPR spectra (first derivative) of (A) PFL alone (10 mg/mL), (B) PFL and mercaptopyruvate (5 mM), and (C) the reaction mixture in (B) after addition of formate (25 mM, final). All spectra were recorded at 77 K. Mercaptopyruvate was added to the active enzyme solution at room temperature, and the sample was refrozen in liquid N_2 after 20 s. The sample was thawed at room temperature prior to addition of formate. The sample was quickly refrozen in liquid N_2 10 s after addition of formate. Spectrum A was recorded at 0.05 mW while both spectra B and C were recorded at 2 mW. The central feature of the glycyl radical is missing in (B) because the spectrum was recorded using 2 mW power, a condition that broadens and distorts the components due to the glycyl radical signal (Unkrig et al., 1989). The proposed structure of the disulfide radical responsible for the features at g = 2.057 and 2.023 is shown.

PFL treated with mercaptopyruvate were consistent with an efficient 1-to-1 conversion of the glycyl radical (one radical per enzyme dimer; Unkrig et al., 1989) to the new radicals.

The new EPR spectrum contains a signal having g values and hyperfine splittings closely related to those reported for disulfide radicals formed in irradiated cysteamine hydrochloride (Nelson et al., 1977) and crystalline cysteine or cystine (Hadley & Gordy, 1974a,b, 1975). The presence of this signal containing two clearly resolved doublets (splittings ~10 G) at g = 2.057 and 2.023 and a third feature near g ~2.00 allowed its assignment to a disulfide radical. A component due to a second species is also observed at g = 2.01. Upon further incubation, the intensity of all features in the new spectrum decreased without the appearance of any new features until no EPR signal could be detected after ~2 min.

A Cysteine-Based Radical Is Formed. The EPR spectrum generated in the presence of mercaptopyruvate was identical in H_2O and D_2O (Figure 3A), indicating that the hyperfine splittings in the components at g = 2.057 and 2.023 are not due to solvent-exchangeable hydrogens. In addition, deuterium substitution for the methylene hydrogens of mercaptopyruvate did not change or abolish the splitting pattern (data not shown). However, perdeuteriation of PFL by cell

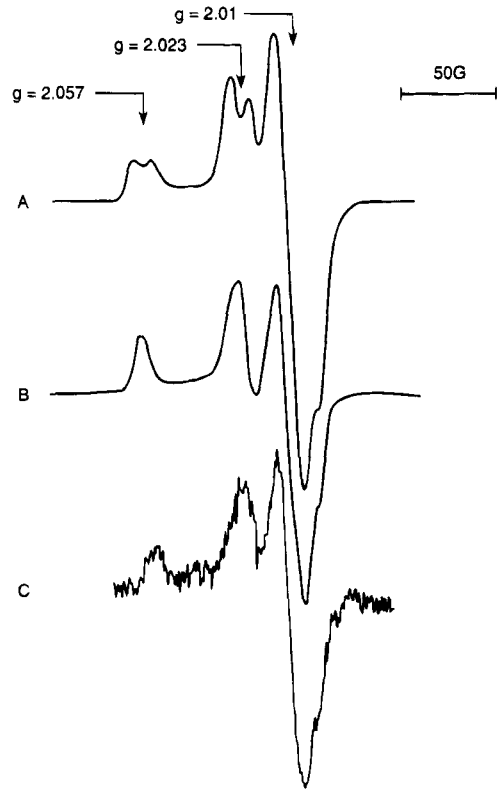


FIGURE 3: EPR spectra (first derivative) of the radical intermediate observed upon addition of mercaptopyruvate (5 mM) and formate (25 mM) to (A) protio-PFL (10 mg/mL), (B) perdeuteriated PFL (7 mg/mL), and (C) *d*-cysteine-labeled PFL (2 mg/mL). All spectra were recorded at 77 K and at 2 mW.

growth and protein expression in deuteriated medium resulted in conversion of the doublets into singlets, indicating that the radical was enzyme-based (Figure 3B). The identity of the radical was established by specific deuterium labeling of the β-methylene hydrogens of cysteines in PFL by protein expression in *E. coli* JM15, a cysteine-requiring auxotroph. The resulting EPR spectrum of the inactivated enzyme was identical to that of perdeuteriated PFL (Figure 3C), demonstrating that the origin of the radical is a cysteine residue on the enzyme. Previous assignments of the β-hydrogen hyperfine splittings in EPR spectra of disulfide radicals formed in irradiated thiols and disulfides are consistent with our assignment (Nelson et al., 1977; Hadley & Gordy, 1974a,b, 1975).

Irradiated thiols and disulfides give additional EPR signals due to monosulfide (thiyl) and disulfide radicals having components at $g = 2.01$ (Nelson et al., 1977). In our experiments, the site and the origin of the second radical giving this feature remain to be elucidated since the components at $g \leq 2.01$ were apparently unchanged in both deuterium-labeled and protio-PFL. Furthermore, no components expected at lower field (g ~2.2) for a thiyl radical could be consistently found.

To address whether either of the active site cysteines are involved in the reaction producing the new radicals, mercaptopyruvate was added to the activated mutant enzymes, C418S and C419S,[2] under the same conditions as for native PFL. The EPR signal of the glycyl radical in both mutants remained unchanged even after 30 min of incubation with mercaptopyruvate at room temperature (data not shown), indicating that neither of the mutant enzymes was capable of generating the new radicals. Therefore, both active site cysteines are required for the production of the enzyme-bound disulfide as well as the second radical species.

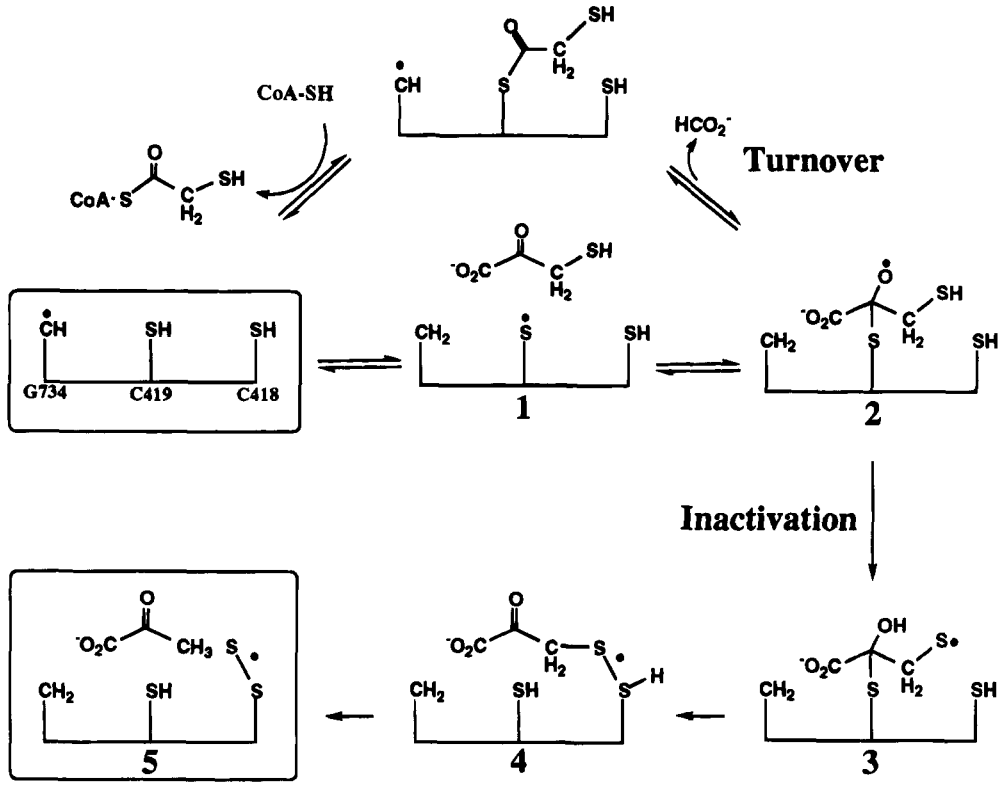
DISCUSSION

Mercaptopyruvate is a naturally occurring α-keto acid which is believed to form, in vivo, from transamination as well as oxidative deamination of L-cysteine. Mercaptopyruvate is the substrate for the enzyme mercaptopyruvate sulfurtransferase (Cooper, 1983). This enzyme produces pyruvate and an enzyme−persulfide intermediate which could transfer the sulfur to a variety of groups (Jarabak & Westley, 1980; Westley, 1977). Mercaptopyruvate is also a substrate for glutamine transaminase (Cooper & Meister, 1974), asparagine transaminase (Cooper, 1977), and lactate dehydrogenase (Meister, 1965). Mercaptolactate, the product of reduction of mercaptopyruvate by lactate dehydrogenase, is a normal urinary constituent found in mixed disulfide linkage with cysteine (Cooper et al., 1982; Ubuka et al., 1968). While the interaction of mercaptopyruvate with PFL has not been reported previously, the biochemistry of this metabolite may have relevance to the mechanism of inactivation of PFL.

The inactivation of PFL by mercaptopyruvate is a mechanism-based process (Figure 1). The kinetics of inactivation observed in the presence and absence of formate is reminiscent of our previous analysis of the inactivation of PFL by acetylphosphinate, another pyruvate analog (Ulissi-Demario et al., 1991). We, therefore, propose a similar mechanism for inactivation by mercaptopyruvate (Scheme 1). Mercaptopyruvate is recognized by PFL as a substrate, and the enzyme acts on the inhibitor partitioning between inactivation and formation of mercaptoacetyl-PFL as a product of a normal reaction. In the absence of excess formate or CoA to efficiently deacylate the mercaptoacetyl-PFL, this form of the enzyme is protected against further inactivation. The biphasic kinetics in Figure 1 suggests that normal processing of mercaptopyruvate is favored and that adventitious deacylation by nucleophiles such as DTT (present in the reaction mixture) is quite slow. In the case of acetylphosphinate, the processing to acetyl-PFL is favored by 5 to 1 over inactivation (Ulissi-Demario et al., 1991), and the partition ratio for mercaptopyruvate appears to be similar (5−10 to 1). The addition of excess formate to deacylate the protected enzyme allows for additional cycles with the inhibitor until complete inactivation is achieved.

EPR studies of mercaptopyruvate inactivation of PFL provide direct evidence for a radical mechanism and permit a correlation with the inactivation kinetics discussed above. The composite EPR spectrum of Figure 2B is consistent with the protection of the major fraction of glycyl radical from inactivation by the inhibitor unless excess formate is added (Figure 2C). Previous work has established that the EPR spectra of the glycyl radicals of PFL and acetyl-PFL are identical (Parast et al., 1995); thus, the residual glycyl radical in Figure 2B can be attributed to mercaptoacetyl-PFL. Upon addition of formate, rapid deacylation permits multiple cycling of the enzyme with the inhibitor, thus maximizing the formation of the new radical species by complete inactivation. These observations establish the mechanistic relationship between enzyme inactivation and the generation of new radical species.

Scheme 1. Proposed Mechanism for PFL Inactivation by Mercaptopyruvate[a]



[a] The enzyme partitions between turnover and inactivation through species **2**. Inactivation is proposed to be a result of rearrangement of the radical via an intramolecular hydrogen transfer to give species **3** followed by addition to cysteine 418 giving disulfide radical **4**. This species is proposed to undergo S−C bond cleavage to yield disulfide radical **5** which is observed by EPR.

The spectrum of the inhibitor-treated PFL is similar to that presented by Nelson et al. (1977) for irradiated cysteamine hydrochloride, which contains signals due to both mono-(thiyl) and disulfide radicals. The EPR signal for one of the new radical species reported here matches that of the disulfide radical. Our data, however, did not allow an unambiguous identification of the g_z component expected near $g = 2.2$ assigned for the thiyl radical (Nelson et al., 1977).

Isotopic substitution allowed localization of the disulfide radical intermediate (Figure 3). These studies demonstrate conclusively that the radical resides on a cysteine residue of the enzyme. The resolved splitting for only one β-hydrogen nucleus in the cysteine residue is consistent with the observations of Hadley and Gordy (1974a,b, 1975) and Nelson et al. (1977) for orientationally constrained disulfide radicals. Experiments using PFL containing chirally mono-deuteriated cysteine at the C-3 position could establish the identity of the prochiral hydrogen responsible for the observed coupling. Furthermore, since mutation of either of the active site cysteines into a serine eliminates the formation of both new radicals with mercaptopyruvate, we suggest that the disulfide radical is located on one or both of the active site cysteines. The location of the second radical will require further experiments.

The mechanism of formation of these sulfur-based radicals is speculative at this point; however, it is reasonable to propose a mechanism for inactivation based on the normal reaction. Transient formation of a thiyl radical has been proposed as the first step in the reaction catalyzed by PFL (Brush et al., 1988). In fact, Parast et al. (1995) have obtained strong evidence for involvement of cysteine 419 in a homolytic (radical) process. The second step has been proposed to be the formation of a tetrahedral oxy-radical intermediate from pyruvate and the enzymic thiyl radical which then leads to C−C bond homolysis. We suggest a similar tetrahedral oxy-radical intermediate can form from the enzymic thiyl radical and mercaptopyruvate (species **2**, Scheme 1). This intermediate can partition between turnover and inactivation since formation of an inhibitor-based thiyl radical (species **3**) by an intramolecular hydrogen transfer could occur in addition to C−C bond cleavage. This step is feasible on the basis of thermodynamic arguments as the chemical generation of thiyl radicals from the oxy radical (i.e., hydroxyl radicals) is energetically favorable, having an activation energy close to 0 (Asmus, 1990). The inhibitor-based thiyl radical could then form a more stable disulfide radical with the adjacent cysteine 418 (species **4**). Hadley and Gordy (1974a,b, 1975) as well as Nelson et al. (1977) have demonstrated that thiyl radicals are reactive and give rise to disulfide radicals at room temperature. Species **4** could collapse with expulsion of the sulfur of the inhibitor to generate pyruvate and an enzyme-bound disulfide radical (species **5**). The S−C bond in mercaptopyruvate is known to be labile as demonstrated by its spontaneous cleavage in aqueous solutions (Cooper et al., 1982). Desulfurylation of mercaptopyruvate by mercaptopyruvate sulfurtransferase has also been postulated to be catalyzed by an active site cysteine (Westley, 1977). Finally, the lack of hyperfine splittings due to the β-hydrogens of mercaptopyruvate also suggests S−C bond cleavage. Thus species **5** is an attractive candidate for the disulfide radical detected in our observed EPR spectrum.

The key prediction of this hypothesis is the availability of a hydrogen atom donor (−SH) to provide an alternate

route for the single electron after formation of the tetrahedral oxy-radical intermediate (species **2**). There are several lines of evidence in support of this prediction. First, hydroxypyruvate, the oxygen analog of mercaptopyruvate, is also a mechanism-based inactivator of PFL, though no radical intermediate is observed in this case (data not shown). Second, neither *S*-methylmercaptopyruvate nor *O*-methylhydroxypyruvate is an inactivator of PFL (data not shown) in spite of the observation that both α-ketobutyrate and α-ketopentanoate are substrates (Chase & Rabinowitz, 1968). In fact, preliminary data indicate that *O*-methylhydroxypyruvate and *S*-methylmercaptopyruvate are substrates (data not shown). These findings are consistent with the prediction that the inactivation mechanism involves rerouting of the single electron via an appropriate hydrogen atom donor.

According to this mechanism, the observed cysteine-based disulfide radical is formed between cysteine 418 and the sulfur atom from mercaptopyruvate (species **5**). This prediction could be tested by the use of [^{33}S]mercaptopyruvate. The unassigned sulfur-based radical species could be either the thiyl radical of the enzyme or the inhibitor (species **1** or **3**, respectively) or the cation radical of species **5**, each of which would show features at $g \sim 2.01$ in their EPR spectra (Nelson et al., 1977). Another prediction is that both active-site cysteines are required for inactivation, which is supported by resistance of both mutants, C418S and C419S, to trapping of the glycyl radical by the inhibitor.

In summary, we have shown that, upon inactivation of PFL by mercaptopyruvate, a cysteine-based disulfide as well as a second sulfur-based radical intermediate is generated. This is the first report of direct detection of an enzymic sulfur-based radical by mechanism-based inactivation. Although the mechanism of inactivation may only be in part related to the normal catalytic process, the observations reported here provide further support for a homolytic mechanism for PFL and argue persuasively for the role of the thiyl radical in catalysis.

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