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Hydrogen Exchange of the Glycyl Radical of Pyruvate Formate-Lyase Is Catalyzed by Cysteine 419†

Camran V. Parast,‡,§ Kenny K. Wong,‡,§ Sandra A. Lewisch,‡,|| and John W. Kozarich*,‡,§

Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, and Department of Enzymology, Merck Research Laboratories, Rahway, New Jersey 07065-0900

Jack Peisach and Richard S. Magliozzo

Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, New York 10461

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ABSTRACT: Pyruvate formate-lyase (PFL) catalyzes the reversible conversion of CoA and pyruvate into acetyl-CoA and formate. Active enzyme contains a glycyl radical whose α-hydrogen undergoes rapid exchange with solvent ($t_{1/2} \sim 5$ min at 0 °C). We have investigated this exchange using site-directed mutagenesis and mechanism-based inactivation. Mutation of the active-site cysteine 419 into a serine, which renders the enzyme catalytically inactive, abolishes α-hydrogen exchange in the radical. This suggests that the exchange process is not an intrinsic property of the glycyl radical but is a consequence of its interaction with cysteine 419. This residue is also demonstrated to be involved in the transfer of the radical to acetylphosphinate, a mechanism-based inactivator of the enzyme. In contrast, mutation of the other essential cysteine 418 to a serine has no effect on the hydrogen exchange or the transfer of the radical to acetylphosphinate. A mechanism for the hydrogen exchange catalyzed by cysteine 419 consistent with a redox role for this residue in the normal catalytic reaction is proposed.

Pyruvate formate-lyase (PFL;[1] EC 2.3.1.54) from *Escherichia coli* catalyzes the reversible conversion of pyruvate and CoA into acetyl-CoA and formate ($k_{cat} \sim 800$ s^{-1}) (Knappe et al., 1974). This enzyme is the anaerobic counterpart of pyruvate dehydrogenase and, thus, contributes a key step in bacterial anaerobic metabolism. The reaction exhibits ping-pong kinetics with the intermediary of an acetyl-enzyme via formation of a thioester with an active-site cysteine (Scheme 1). Two cysteines, C418 and C419, have been demonstrated to be essential for catalysis and are implicated to be the site(s) of acetylation (Plaga et al., 1988).

PFL, a homodimer (2 × 85 kDa) when isolated, is catalytically inactive (Sawers & Bock, 1988, 1989). Conversion to the active form is achieved by the action of activating enzyme (AE) under anaerobic conditions which requires *S*-adenosyl-L-methionine (SAM), flavodoxin, or photoreduced 5-deazariboflavin and Fe(II) with pyruvate or oxamate

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* To whom correspondence should be addressed.

‡ University of Maryland.

§ Current address: Merck Research Laboratories.

|| Current address: National Institutes of Health, Bethesda, MD 20892.

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[1] Abbreviations: PFL, pyruvate formate-lyase; EPR, electron paramagnetic resonance; CoA, coenzyme A; acetyl-CoA, acetyl-coenzyme A; AE, activating enzyme; DTT, dithiothreitol; SAM, *S*-adenosyl-L-methionine; Tris, tris(hydroxymethyl)aminomethane; IPTG, isopropyl β-D-thiogalactopyranoside.

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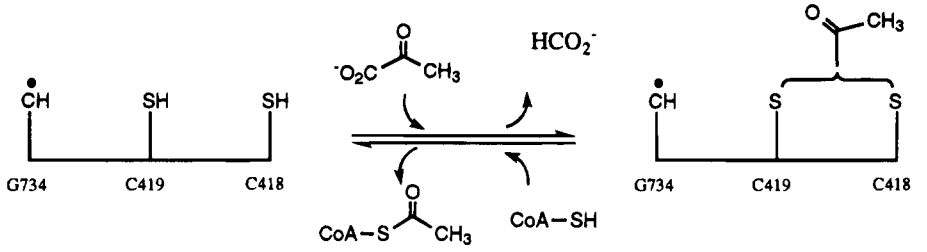
Scheme 1: Reaction Catalyzed by Pyruvate Formate-Lyase[a]



[a] The glycyl radical 734 and both cysteines, 418 and 419, are essential for C–C bond cleavage.

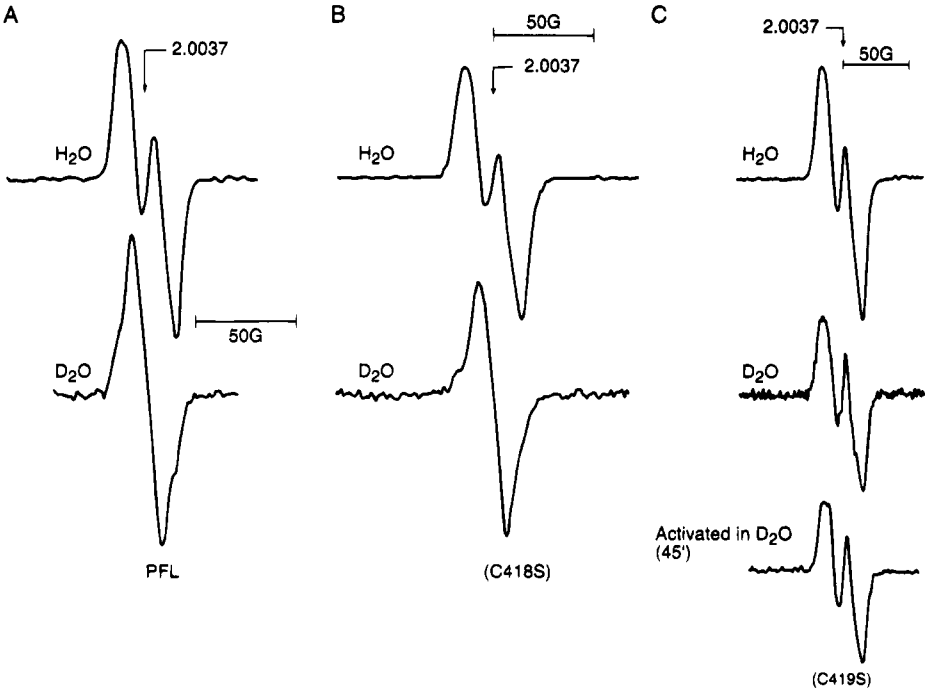


FIGURE 1: First derivative EPR spectra of activated PFL and the C418S and C419S mutants in H_2O and D_2O: (A) PFL in H_2O and in D_2O; (B) C418S mutants in H_2O and in D_2O; (C) C419S mutants in H_2O and in D_2O. The enzyme solutions were activated at 30 °C and D_2O was added at 25 °C. All spectra were recorded at 77 K. The EPR signals of both mutants in H_2O are essentially identical to that of PFL in H_2O. The spectra in D_2O were obtained after addition of D_2O (75% final) into the active enzyme (in H_2O) followed by 2-min incubation at room temperature.

as a positive allosteric effector (Conradt et al., 1988). Recently, another enzyme, deactivase, has been isolated which converts the active form of PFL into the inactive form (Kessler et al., 1991).

The active form of PFL contains a stable free radical which is required for activity (Unkrig et al., 1989; Knappe et al., 1984). Spin quantitation suggests one radical per dimer. This unpaired electron is located on the α-carbon of glycine 734. This residue is the site of fragmentation of the activated protein upon exposure to air (Wagner et al., 1992). The glycyl radical gives rise to an EPR signal ($g = 2.0037$) which consists of a doublet with the α-hydrogen of glycine giving rise to the principal splitting of 15 G (Figure 1A). In contrast, the EPR signal of PFL in D_2O is a singlet, indicating that the α-hydrogen is exchangeable with solvent. The exchange is relatively rapid at room temperature, being complete within 2 min. At 0 °C, the $t_{1/2}$ for exchange is approximately 5 min (Unkrig et al., 1989; Wagner et al., 1992).

Studies by Gautney and Miyagawa (1975) on irradiated glycine crystals have suggested that the glycyl radical undergoes hydrogen exchange by two distinct mechanisms, intramolecular and intermolecular. Intramolecular hydrogen exchange involves the terminal amine group and the α-carbon radical (eq 1) and is too fast to be followed by EPR

$$^{+}D_3N-\dot{\underset{H}{C}}-CO_2^- \rightleftharpoons {}^{+}D_2HN-\dot{\underset{D}{C}}-CO_2^- \quad (1)$$

spectroscopy, while the intermolecular exchange is quite slow, taking place on the order of days.

On the basis of this study, Wagner et al. (1992) suggested an uncharacteristically low pK_a for the α-hydrogen to explain the relatively rapid exchange in active PFL. However, the observation that the α-hydrogen of the putative glycyl radical of the anaerobic ribonucleotide reductase of *E. coli* does not exchange with solvent argues against such a mechanism (Mulliez et al., 1993).

In order to understand the mechanism of this reaction, we have investigated hydrogen exchange in the glycyl radical of PFL using site-directed mutagenesis and mechanism-based inactivation studies. In this report, we show that hydrogen exchange of the glycyl radical of PFL with solvent is not spontaneous but is catalyzed by the active-site cysteine 419. A direct role in hydrogen exchange as well as a redox role in the normal catalytic reaction is proposed for this residue.

EXPERIMENTAL PROCEDURES

Materials. All chemicals including deuterium oxide (99.9% D) were obtained from Aldrich or Sigma and were used without further purification. 5-Deazariboflavin was the gift of D. T. Ashton of Merck Research Laboratories, Rahway, NJ. Acetylphosphinate was prepared by the method of Baille and co-workers (1980, 1988). In aqueous solution it is in equilibrium with its hydrate in a 2.4:1 ratio, favoring the free carbonyl. $C_2H_4O_3PNa$: ^{1}H-NMR (400 MHz, D_2O) hydrate δ 1.32 (d, 3H, J = 10.3 Hz), 6.58 (d, 1H, J = 509 Hz); ketone δ 2.3 (d, 3H, J = 4.2 Hz), 6.67 (d, 1H, J = 547 Hz); ^{31}P-NMR (D_2O) hydrate δ 25.51 (dq, J = 10.3 Hz, J = 510.3 Hz); ketone δ 12.70 (dq, J = 4.2 Hz, J = 546.7 Hz); ^{13}C-NMR (D_2O, H-decoupled) hydrate δ 94.3 (d, J = 140 Hz), 22.4 (d, J = 13.6 Hz); ketone δ 224.5 (d, J = 110 Hz), 27.9 (d, J = 46.5 Hz). Incubation with 10 mM DTT overnight caused no change in the ^{1}H-NMR spectrum.

Enzyme Preparations. Recombinant AE was purified from *E. coli* strain N4830 bearing pMGA1-2, a construct of pMG27NS (Gross et al., 1985) which contains *act*, the gene for activating enzyme under the control of the P_L promoter (Wong et al., 1993). AE in the soluble fraction was purified by gel filtration chromatography and used directly without further purification. Recombinant PFL was purified from *E. coli* JM109 (Pharmacia) bearing pKKBWM5.5C, a construct of pKK223-3 (Pharmacia) and *pfl* under the control of the *tac* promoter. Induction with IPTG was not required, and PFL was estimated to constitute about 50% of the soluble cellular protein under conditions of aerobic growth. C419S and C418S mutant enzymes were purified from *E. coli* strain N4830 bearing pMG27NS containing *pfl*, the gene for pyruvate formate-lyase containing the indicated mutation under the control of the P_L promoter.[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.

Activation of PFL. Recombinant PFL and the C418S and C419S mutant enzymes were activated by a modification of a previously described procedure (Brush et al., 1988). Typically, the activation mixture contained 100 mM Tris/100 mM KCl, pH 7.6, 1.5 mM SAM, 50 μM 5-deazariboflavin, 10 mM DTT, 20 mM oxamate, 0.4 mM Fe(II), 50 μg/mL AE, and 10 mg/mL PFL or the mutant enzyme in a final volume of 1.0 mL. After all the components were mixed, the pH was adjusted to 7.6, and 200-μL aliquots were taken out and placed in EPR tubes equipped with septum-sealed caps (Pierce). Activation mixtures were purged with oxygen-free argon and were incubated at 30 °C in EPR tubes held in a water bath until anaerobic as indicated by a color change from red to clear or greenish (due to ferric to ferrous DTT conversion). Activation was initiated by photoreduction of 5-deazariboflavin using a combination of a 300-W halogen lamp and a 100-W xenon lamp situated 5 cm from the sample. The activation time was typically 1 h, after which the samples were quickly frozen in liquid nitrogen and placed in a liquid N_2 cold finger dewar for EPR measurements at 77 K.

EPR Spectroscopy. EPR spectra were obtained on a Varian E112 spectrometer equipped with a Systron-Donner frequency counter and an NMR gaussmeter. First derivative spectra were recorded at a microwave frequency of 9.229 GHz. The microwave power was 50 μW for spectra of the glycyl radical and 2 mW for the radical formed from activated enzymes plus acetylphosphinate.

Solvent Hydrogen Exchange. Typically, a 200 μL of sample containing about 10 mg/mL PFL or the mutant enzymes was activated and frozen at 77 K, and the EPR spectrum was recorded. The enzyme solution was thawed at room temperature under a stream of argon. Argon-saturated D_2O (600 μL) was injected into the sample, and the solution was rapidly mixed and frozen in liquid nitrogen at the indicated time. All additions were performed using gas-tight syringes.

Acetylphosphinate Reactions. A PFL sample, activated in an EPR tube as described above, was frozen in liquid nitrogen, and the EPR signal of the resting radical was measured. The sample was then thawed, acetylphosphinate (7 mM, final concentration) and formate (25 mM, final) were added at room temperature, and the solution was refrozen in liquid nitrogen within 30 s for reexamination by EPR. The solutions of acetylphosphinate and formate were made anaerobic by purging with argon for at least 10 min in septum-sealed vials. All transfers were made using gas-tight syringes.

RESULTS

Cysteine 419 Is Required for Hydrogen Exchange. The EPR spectrum of activated PFL is essentially a doublet with additional hyperfine splittings poorly resolved in the spectrum recorded in frozen solution (Figure 1A) (Knappe et al., 1984). The principal splitting has been assigned to the α-hydrogen of the glycyl radical (Wagner et al., 1992). This hydrogen readily undergoes exchange with solvent deuterium, a process which leads to conversion of the doublet into a singlet in the EPR spectrum (Unkrig et al., 1988).

The possible roles of the two active-site cysteines, C418 and C419, in this exchange were investigated by site-directed mutagenesis. C418S and C419S mutants (10 mg/mL) were activated in H_2O. After the EPR spectra of the mutant enzymes were recorded, D_2O was added into the solutions to achieve a final D_2O content of 75% (Figure 1B,C). The EPR signal of C418S changed from a doublet into a singlet, indicating exchange of the hydrogen for a deuterium (Figure 1B). The radical of the C419S mutant, on the other hand, remained a doublet after addition of D_2O (Figure 1C), demonstrating that no exchange had occurred even after 2-h incubation at room temperature. The inherent instability of the enzyme radical precluded longer incubation. The EPR signal for the C419S mutant, however, remained a doublet even when the activation was carried out in D_2O, indicating that hydrogen exchange was either totally abolished in this mutant or its rate of exchange was significantly reduced. Therefore, cysteine 419 is required for the hydrogen exchange to occur.

Acetyl-PFL Is Capable of Hydrogen Exchange. In order to assess the effect of acetylation of PFL on hydrogen exchange in the glycyl radical, activated PFL (10 mg/mL, 60 μM) was incubated with 20 mM pyruvate in the absence of CoA and formate. Here, the ratio of acetyl-PFL/PFL is $\sim 1 \times 10^4$ as calculated according to the equilibrium constant of 50 for the first half-reaction (Knappe et al., 1974) and

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

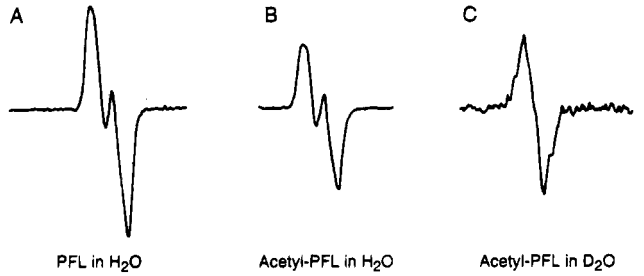


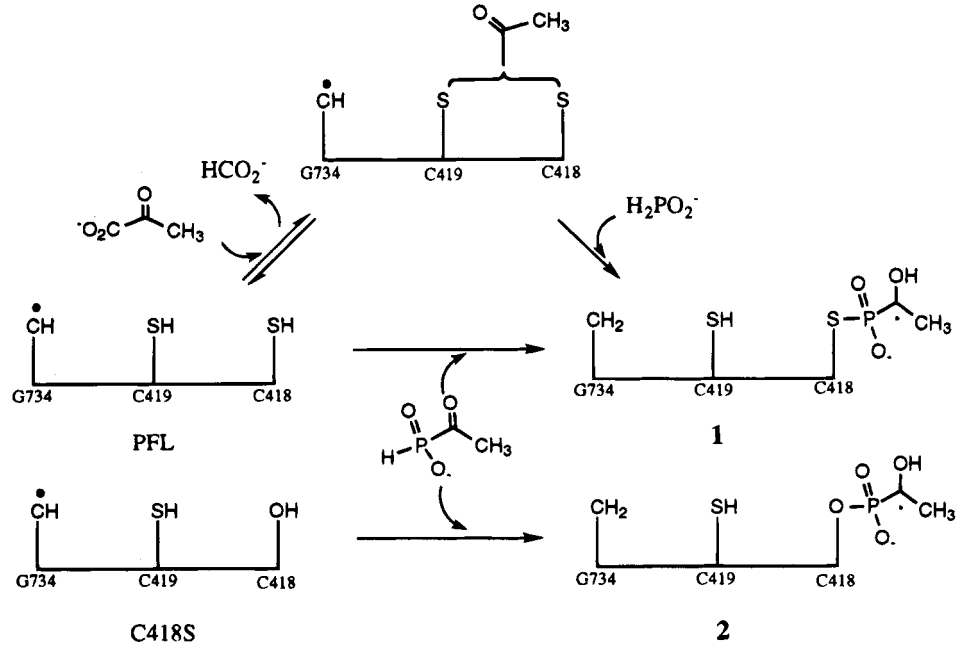
FIGURE 2: EPR spectra of PFL in H_2O (A), acetyl-PFL in H_2O (B), and acetyl-PFL in D_2O (C). All spectra were recorded at 77 K. PFL was acetylated by addition of 20 mM pyruvate. The spectrum in D_2O was obtained as described in Figure 1.

the concentrations of added pyruvate and stoichiometrically generated formate (~60 μM). Therefore, the enzyme was essentially in the acetylated form. The EPR signal of the acetylated enzyme was identical to that of the free enzyme, consistent with an earlier report (Unkrig et al., 1989) (Figure 2A,B). After addition of D_2O, the EPR signal of acetyl-PFL changed into a singlet, indicating that hydrogen exchange was unaffected by enzyme acetylation (Figure 2C). No significant difference in the rate of hydrogen exchange was detectable between free and acetylated enzyme under the conditions employed here, both being complete within 2 min at room temperature. This result suggests that a significant population of cysteine 419 in the acetyl-PFL is free to catalyze the hydrogen exchange of the glycyl radical.

Cysteine 419 Mediates the Transfer of the Radical to Acetylphosphinate. Hypophosphite is known to be a mechanism-based inactivator of acetyl-PFL (Knappe et al., 1984; Brush et al., 1988). In the presence of pyruvate or acetyl-CoA, hypophosphite reacts with the radical-containing PFL to give a new long-lived radical (Unkrig et al., 1989). This radical has been assigned to species **1** (Scheme 2) localized on cysteine 418 on the basis of the effects of perdeuteriation of the acetyl group on the EPR signal of the long-lived radical and by isolation of a modified peptide bearing a (hydroxyethyl)phosphonate moiety on this residue. Alternatively, acetylphosphinate has been shown to be a mechanism-based inactivator of the free PFL (Ulissi-Demario et al., 1991). Turnover of acetylphosphinate was also observed via P–C bond cleavage (~5 turnovers per inactivation). In this case, a long-lived radical intermediate is also generated which has an EPR absorption identical to that generated by the reaction of hypophosphite with acetyl-PFL (Figure 3A). This observation supports the proposal that inactivation of acetyl-PFL by hypophosphite and inactivation of free PFL by acetylphosphinate converge mechanistically to afford a common radical intermediate (Ulissi-Demario et al., 1991; Knappe et al., 1993).

To address whether both or only one of the active-site cysteines is required for transfer of the radical to the inactivator, acetylphosphinate was added to the radical-containing C418S and C419S mutant enzymes under anaerobic conditions. No radical intermediate was observed with the C419S mutant. In fact, the EPR absorption of the glycyl radical of this mutant remained unchanged even after prolonged incubation (up to 15 min) with the inactivator (data not shown). The C418S mutant enzyme, on the other hand, reacted rapidly (<30 s) with acetylphosphinate to give a long-lived intermediate. The new radical intermediate exhibited a slightly different splitting pattern than that for PFL (Figure 3B), indicating cysteine 418 is not required for generation of the radical intermediate by acetylphosphinate. Though the structure of this intermediate formed with the inactivator and C418S mutant has yet to be determined, preliminary EPR spectral simulations suggest that the splitting pattern could arise from a structure similar to that of species **1** (Scheme 2). A proposed structure, shown as species **2** (Scheme 2), contains an oxygen–phosphorus bond in place of the sulfur–

Scheme 2: Proposed Reaction of Hypophosphite and Acetylphosphinate with PFL and the C418S Mutant[a]



[a] In the presence of pyruvate, hypophosphite reacts with PFL to give a long-lived radical intermediate, species **1** (Figure 3), which is covalently attached to cysteine 418. Alternatively, acetylphosphinate reacts with the free enzyme to give the same radical intermediate. The C418S mutant also reacts with acetylphosphinate to give a similar radical intermediate (Figure 3). Species **2** is suggested as the possible structure for this new radical intermediate.

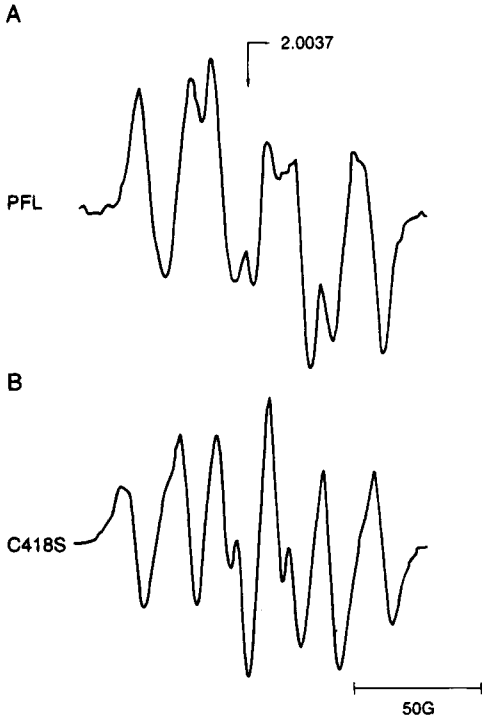


FIGURE 3: EPR spectra of the stable intermediate formed upon addition of acetylphosphinate to (A) PFL and (B) the C418S mutant. All spectra were recorded at 77 K. Acetylphosphinate (7 mM final) was added to the activated enzyme solutions at room temperature, and the sample was refrozen in liquid N_2 after 30 s. Both spectra were recorded at 2 mW. Preliminary estimates based on spectral simulations of the hyperfine coupling parameter for the radical in (A) are 83.5 MHz for ^{31}P and 104 and 53 MHz for two strongly coupled hydrogens (^1H). The corresponding values for the radical in (B) are 93.5 MHz for ^{31}P and 115 and 58 MHz for two strongly coupled hydrogens (^1H). Any additional hydrogen couplings are <15 MHz.

phosphorus bond (species **1**). This new feature could be responsible for the larger hyperfine coupling to the phosphorus and the methyl protons (in C418S) as manifested in the general increase in spectral width for species **2**.[3]

DISCUSSION

Previous studies have shown that both mutant enzymes, C419S and C418S, can be converted to the radical form as efficiently as PFL, indicating that the two cysteines are not involved in generation or subsequent stabilization of the glycyl radical[2] (Knappe et al., 1993). Furthermore, the EPR signals of the glycyl radical generated in both mutants are identical to that of PFL. However, both mutant enzymes are catalytically inactive, suggesting that both cysteines are required for the overall catalysis[2] (Knappe et al., 1993). In other words, formation of the glycyl radical is necessary but not sufficient for activity.

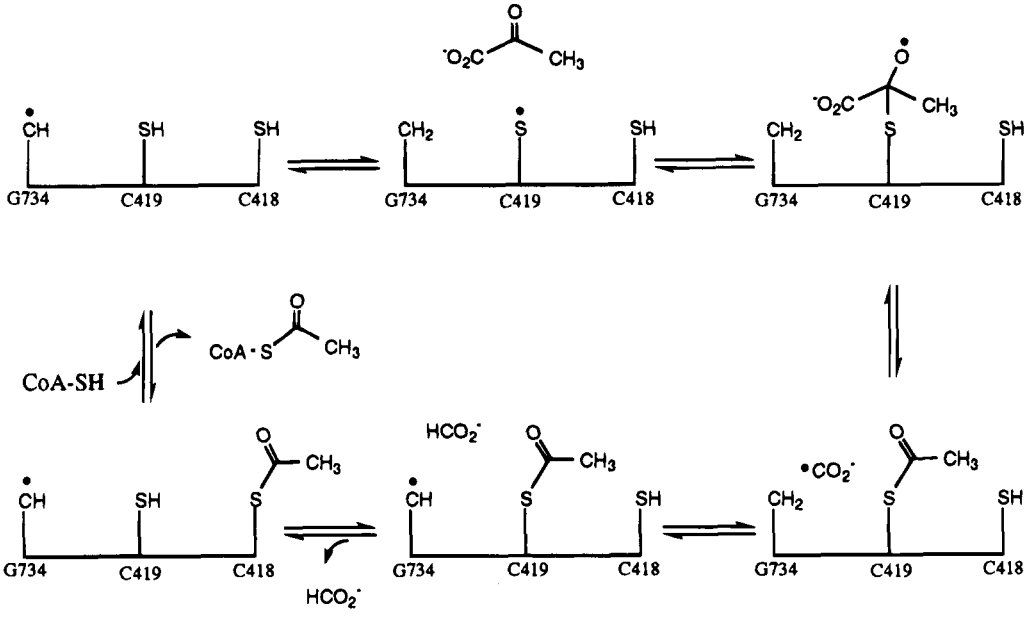
Mutation of the active-site cysteine 419 into a serine abolishes the exchange property of the glycyl radical. No exchange was observed upon prolonged incubation (~2 h) in D_2O, suggesting that serine was ineffective in replacing the role of cysteine in this exchange within the time frame of the experiment. On the other hand, mutation of cysteine 418 into a serine had no detectable effect on the hydrogen exchange, as compared to the native enzyme. Therefore, the glycyl radical of PFL does not undergo spontaneous exchange with solvent. Instead, exchange requires the proximity and chemical reactivity of the active-site residue cysteine 419.

The mutation of cysteine 419 into a serine also renders the radical of the enzyme resistant to trapping by acetylphosphinate, a mechanism-based inactivator of PFL (Scheme 2). However, the C418S mutant enzyme can react with acetylphosphinate (Figure 3), and the observed radical intermediate is similar to the one obtained by the inactivation of PFL with acetylphosphinate. These observations suggest that cysteine 419 mediates the transfer of the enzyme radical to acetylphosphinate. The dual function of cysteine 419 both in hydrogen exchange of the glycyl radical and in the catalytically relevant radical formation with acetylphosphinate suggests that the ability of this residue to participate in a homolytic process is essential to an understanding of its enzymatic function. Indeed, participation of thiol groups in homolytic processes has precedence both in organic chemistry and in protein chemistry. Properties of thiol radicals in relevant biological systems have been reviewed by Asmus (1990). Even though the existence of thiol radicals in enzymes has not been directly demonstrated, their ubiquity in organic reactions has lead to proposed mechanisms involving thiol radicals in enzymes (Stubbe, 1988).

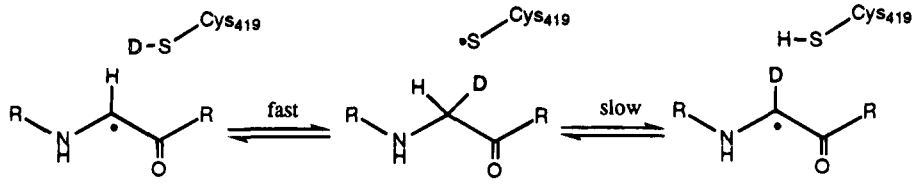
Experimental evidence suggests a different role for cysteine 418. Experiments with the C419S mutant indicate that it is as efficient as PFL in catalyzing thioester exchange with CoA/acetyl-CoA[2] (Knappe et al., 1993). However, this activity is abolished in the C418S mutant, suggesting that cysteine 418 is the primary residue participating in the thioester exchange with CoA. In our experiment, when PFL is converted into acetyl-PFL by addition of pyruvate, the glycyl radical still undergoes hydrogen exchange (Figure 2). The rate of the hydrogen exchange does not appear to be significantly decreased, as compared with the free (unacetylated) PFL. In light of the requirement for a free cysteine 419 for hydrogen exchange, this result suggests that cysteine 418 is a major site of localization of the acetyl group in the acetylation of PFL by pyruvate, consistent with the thioester exchange result with CoA. On the other hand, Plaga et al. (1988) have determined by peptide analysis that cysteine 419 is a major site of acetylation of PFL by pyruvate. It is possible, however, to reconcile these observations by proposing a transesterification of the acetyl group from cysteine 419 to cysteine 418 after pyruvate C−C bond cleavage and prior to thioester exchange with CoA. This transesterifaction would be required to reposition the acetyl group from the site of homolytic pyruvate cleavage (C419) to the site of

[3] The larger hyperfine couplings in Figure 3B compared to Figure 3A would be consistent with replacing a P−S bond (PFL) with a P−O bond (C418S) in the radical shown in Scheme 2. According to theoretical calculations applied to the investigation of second row element compounds, d-function can serve to facilitate π back-donation to P, though sulfur achieves this less efficiently than oxygen (Magnusson, 1990). Furthermore, a mechanism for π bonding based on "negative hyperconjugation" also suggests that sulfur is less donating than oxygen (Reed & Schleyer, 1990). Both mechanisms would lead to predictions consistent with the observation of weaker hyperfine couplings in the radical formed with acetylphosphinate and PFL. In calculations of Mulliken populations in phosphinate compounds using d-functions on both phosphorus and sulfur (oxygen), it was reported that the charge on P goes from +1.428 to +1.286 upon substituting one oxygen with a sulfur (Rasanen et al., 1994). This trend would predict a result opposite to our observation though these calculations also reveal large changes in bond angles for C−P−S−C. It seems likely that torsional angle changes could directly alter the hyperfine couplings in the two carbon-centered π-radicals and could be the most important effect responsible for the EPR changes.

Scheme 3: Mechanism for PFL Reaction As Proposed by Brush et al. (1988) with Modification To Include Participation of Cysteine 418 in Thioester Exchange with CoA



Scheme 4: Proposed Homolytic Mechanism for Hydrogen/Deuterium Exchange of the Glycyl Radical of PFL Catalyzed by Cysteine 419[a]



[a] The slow exchange of deuterium into the glycyl radical relative to a kinetically competent glycyl/thiyl radical interchange is proposed to be due to the high degree of stereoselectivity for the reabstraction of deuterium from the α-carbon of the transiently quenched glycine.

CoA acetylation (C418), which is expected to be a heterolytic process although this point remains to be established.

The proximity of cysteine 419 to the glycyl radical is relevant to its catalytic function. Although C418S is inactive with respect to the overall conversion of pyruvate to acetyl-CoA and to pyruvate-formate exchange, this mutant is capable of mediating the transfer of the radical to acetylphosphinate. This result suggests that cysteine 419 can function in the absence of cysteine 418 in a radical-mediated process. It has been suggested that the role of cysteine 419 may be the formation of a catalytically functional thiyl radical by transient quenching of the glycyl radical (Brush et al., 1988) (Scheme 3). Thus, the transiently formed thiyl radical (generated from the thiol group of cysteine 419) could add to the keto group of pyruvate and serve as the initial site of acetylation after the subsequent expulsion of formate radical anion. The function of cysteine 418, however, appears to be predominantly in mediating thioester exchange to CoA, since C419S is functional in CoA/acetyl-CoA thioester exchange but C418S is not. Cysteine 418 does appear to play an auxiliary role in the C-C bond cleavage of pyruvate since C418S cannot mediate pyruvate−formate exchange. However, the result with acetylphosphinate suggests that cysteine 418 is not essential for initiating radical transfer to this inactivator. Taken together, the findings strongly argue for a role for cysteine 419 as a direct mediator of the glycyl radical action while cysteine 418 plays a major role in the transfer of the acetyl moiety to CoA. The key mechanistic connection between cysteine 419 and cysteine 418 can be accommodated by a required, reversible transesterification of the acetyl group between both residues.

We, therefore, propose a homolytic mechanism for hydrogen exchange of the glycyl radical which is catalyzed by cysteine 419 (Scheme 4). The glycyl radical could abstract a hydrogen (deuterium) from the thiol group of cysteine 419, generating a transient thiyl radical and the quenched glycine. The chemical quenching of α-carbon-based radicals of glycine by thiols has been documented (Pryor et al., 1973). In addition, the chemical generation of glycyl radicals from thiyl radicals has recently been reported (Zhao et al., 1994). The incorporation of deuterium into the glycyl radical of PFL would, thus, require a nonstereospecific abstraction of hydrogen by the thiyl radical of cysteine 419 from the presumably chirally deuteriated glycine 734 (Scheme 4). The slow rate of deuterium incorporation relative to catalysis (k_{cat} ~ 800 s^{-1}) is best considered a reflection of the very high degree of stereoselectivity of hydrogen transfer between the glycyl and thiyl radicals and does not rule out the kinetic competency of this process in the normal catalytic reaction.

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