Dioxygen Inactivation of Pyruvate Formate-Lyase: EPR Evidence for the Formation of Protein-Based Sulfinyl and Peroxyl Radicals

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Received August 22, 1997; Revised Manuscript Received November 4, 1997[⊗]

ABSTRACT: We here report EPR studies that provide evidence for radical intermediates generated from the glycyl radical of activated pyruvate formate-lyase (PFL) during the process of oxygen-dependent enzyme inactivation, radical quenching, and protein fragmentation. Upon exposure of active PFL to air, a long-lived radical intermediate was generated, which exhibits an EPR spectrum assigned to a sulfinyl radical (RSO•). The EPR spectrum of a sulfinyl radical was also generated from the activated C418A mutant of PFL, indicating that Cys 418 is not the site of sulfinyl radical formation. Exposure of the activated C419A mutant or C418AC419A double mutant to air on the other hand, resulted in a new EPR spectrum that we assign to the α-carbon peroxyl radical (ROO•) of the active-site glycine, G734. These findings suggest that C419 is the site of sulfinyl radical formation and that replacement of this cysteine with alanine results in the accumulation of the carbon peroxyl radical. The results also support the proposal that the peroxyl radical and the sulfinyl radical are intermediates in the oxygen-dependent inactivation and cleavage of the protein. Moreover, these observations are consistent with the hypothesis that C419 and G734 are in close proximity in the activated enzyme and may participate in a glycyl/thiyl radical equilibrium. A mechanism that accounts for the formation of the radical intermediates is proposed.

Pyruvate formate-lyase from *Escherichia coli* (EC 2.3.1.54; PFL) is a glycyl-radical-containing enzyme (*1*, *2*) that catalyzes the anaerobic conversion of pyruvate and coenzyme A (CoA)[1] into acetyl-CoA and formate (*3*). This reaction is reversible and follows a ping-pong kinetic mechanism with acetyl-PFL as an isolable intermediate (*3*). Under strictly anaerobic conditions, active PFL contains a relatively stable radical, localized at the α-carbon of glycine 734 (G734), which is essential for catalytic activity (*4*). Two cysteines, C418 and C419, are also essential for catalysis (*5*). Site-directed mutagenesis studies have shown that the two cysteines are not involved in generation or subsequent stabilization of the glycyl radical (*5*, *6*). C419 is required for solvent hydrogen exchange at the α-carbon of the radical at G734 (*6*), suggesting a close proximity between these residues. C418 has been established as a site required for acetylCoA/CoA thioester exchange (*5*).

Purified PFL, a homodimer (2 × 85 kDa), is catalytically inactive when isolated under aerobic conditions (*7*, *8*). Anaerobic conversion to active enzyme is achieved by the action of PFL-activating enzyme (AE), which requires *S*-adenosyl-L-methionine (AdoMet). Flavodoxin or photoreduced 5-deazariboflavin, Fe(II), and pyruvate or oxamate as an allosteric effector are also required (*9*). Generation of the glycyl radical is coupled to a stoichiometric cleavage of AdoMet to yield 5′-deoxyadenosine and methionine.

The inactivation of PFL upon exposure to dioxygen (O_2) has been used to define the site of the radical in active enzyme. The inactivation process results in cleavage of the protein yielding 82 and 3 kDa fragments (*4*). Amino acid sequencing of the fragments established G734 as the site of the enzymic radical. In order to elucidate the mechanism of the inactivation process, we have attempted to identify by EPR the formation of protein-based radicals that are generated after oxygen exposure, leading to enzyme inactivation and fragmentation. We report here the EPR detection of a sulfinyl radical (RSO•), formed during O_2-inactivation, that is derived from C419, and a peroxyl radical (ROO•) that we suggest is located at G734. The findings provide compelling evidence for a close spatial relationship between G734 and C419, and for at least two radical intermediates formed during inactivation by dioxygen.

MATERIALS AND METHODS

All chemicals 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.

Site-Specific Mutagenesis. The plasmid containing the PFL gene, pKKBWM5.5C (6), was modified changing the TGC codon for cysteine 418, 419 to a GCT for alanine.

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[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1997.
[1] Abbreviations: Acetyl-CoA, acetyl coenzyme A; AdoMet, *S*-adenosyl-L-methionine; AE, PFL activase; C418A, C419A, and C418A/C419A, amino acid replacement mutants of PFL; CoA, coenzyme A; DTT, dithiothreitol; EPR, electron paramagnetic resonance; IPTG, isopropyl β-D-thiogalactoside; PFL, pyruvate formate-lyase; Tris, tris(hydroxymethyl)aminomethane.

S0006-2960(97)02086-2 CCC: $15.00
Published on Web 01/13/1998

Plasmid DNA was prepared using QIAGEN Midiprep Kit (Chatsworth, CA). C418A, C419A, and C418AC419A mutants were constructed using a Quickchange site-directed Mutagenesis Kit (Stratagene Cloning Systems, La Jolla, CA) and the primer sets 5′-C GCT ATT GCT GCT TGC GTA AGC CCG-3′, 5′-CGG GCT TAC GCA AGC AGC AAT AGC G-3′, 5′-C GCT ATT GCT TGC GCT GTA AGC CCG-3′, 5′-CGG GCT TAC AGC GCA AGC AAT AGC G-3′, and 5′-C GCT ATT GCT GCT GCT GTA AGC CCG-3′, 5′-CGG GCT TAC AGC AGC AGC AAT AGC G-3′, respectively. The PCR reaction mixtures contained 50 ηg of plasmid DNA template, 125 ηg of each primer, and nucleoside triphosphates, reaction buffer, and *Pfu* DNA polymerase supplied by the manufacturer of the kit. After overlaying each sample with mineral oil, they were placed in a thermal cycler and denatured for 30 s at 95 °C and then subjected to 16 cycles of 95 °C for 30 s, 55 °C for 1 min, and 68 °C for 14 min. The reaction mixtures were then cooled and digested with 1 μL of the restriction enzyme *Dpn* I to digest the parental DNA template. A 4 μL aliquot of the *Dpn* I-treated DNA was used to transform into *E. coli* XL-I Blue competent Cells (Stratagene). Transformed cells were plated on LB agar containing 50 μg/mL ampicillin, and after overnight growth, colonies were picked and tested for PFL production. Plasmid mini preps were prepared using mini prep kit and analyzed by agarose gel electrophoresis. DNA sequencing of the 2 kb insert to confirm the incorporation of the mutations at 418 and 419 and lack of PCR errors was performed with an ABI prism Model 377 automated DNA sequencer using dye-labeled dideoxy nucleotides.

Enzyme Preparations. Recombinant AE was purified from *E. coli* strain N4830 bearing pMGA1-2, a construct of pMG27NS (*10*) that contains *act*, the gene for activating enzyme under the control of the P_L promotor (*11*). AE in the soluble fraction was partially purified by gel filtration chromatography and used directly without further purification.

Recombinant PFL was purified from *E. coli* JM 109 (*6*) (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. The purification of PFL was based on a modification of the procedure of Conradt et al. (*9*). C418A, C419A, and C418A/C419A mutant enzymes were purified similar to WT PFL. Protein concentrations were determined by the method of Bradford (*12*) using a kit purchased from Bio-rad.

Activation of PFL. Recombinant PFL, C418A, C419A, and C418AC419A mutant enzymes were activated by a modification of a published procedure (*13*). Typically, the activation mixture contained 100 mM Tris/100 mM KCl, pH 7.6, 1.5 mM AdoMet, 50 μM 5-deazariboflavin, 10 mM DTT, 20 mM oxamate, 0.4 mM Fe(II), 50 mg/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 transferred to septum-sealed EPR tubes (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 colorless or pale green. Activation was initiated by photoreduction of 5-deazariboflavin using illumination from both 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. Several control samples were activated under similar experimental conditions. These control samples usually contained all the activation components except one that was omitted such as AdoMet, AE, or deazariboflavin. A final control sample contained all components but was not illuminated.

EPR Spectroscopy. EPR spectra were recorded at 77 K on a Varian E-112 spectrometer.[2] Microwave frequency was measured using a Systron-Donner frequency counter. Precision bore quartz EPR tubes were used to ensure a uniform filling factor for spin quantitation. Experimental conditions were as follows: microwave frequency 9.230 GHz; modulation amplitude, $\leq$3.2 G; microwave power, 1.0 mW except where noted otherwise. The noise in spectra of wild-type PFL + O_2 and C419A + O_2, which were of low intensity, was smoothed using an *n*-point smoothing algorithm. Spin intensity was evaluated by double integration of the first-derivative EPR signals using the trapezoidal rule after application of a linear base-line correction. The EPR spectra of activated mutant enzymes were the same as that of the glycyl radical of wild-type PFL and are not shown. Simulation of EPR spectra was achieved using the program "qpow" (*14*−*16*).

Dioxygen Inactivation Reactions. The protocol for inactivation experiments involved recording the EPR spectrum of the glycyl radical in activated PFL or PFL mutant enzyme samples followed by thawing and gently mixing the sample in the EPR tube opened to air. In general, the samples were refrozen in liquid nitrogen after 20 s, and an EPR spectrum was rerecorded. This procedure usually sufficed to elicit components of only the new signals studied here without interference from residual glycyl radical but was occasionally repeated if the glycyl radical signal remained evident. EPR signal intensities for the new radical species are reported as percentages of the starting glycyl radical signal intensity. The oxygen inactivation of control samples (mentioned above) was carried out under the same experimental conditions used for PFL and mutant samples.

RESULTS

The EPR spectrum of activated PFL is a doublet at g = 2.0037 and arises from a glycyl radical on residue G734 (*1*) (Figure 1A). The principal splitting (15 G) is due to the α-H of G734. Spin quantitation of active PFL is consistent with one radical per dimer, suggesting half-site reactivity (*4*). Though the radical in the active enzyme is stable under anaerobic conditions, the enzyme is rapidly inactivated and cleaved when it is exposed to oxygen. The identity of the fragments indicates that the polypeptide is cleaved at G734 (*4*). These results suggest that oxygen reacts with the glycyl radical in active PFL and that a new radical or radicals might be formed during inactivation.

[2] The software used for data acquisition and manipulation is available upon request to the NIH Biotechnology Research Resource in Pulsed EPR at the Albert Einstein College of Medicine, Bronx, NY.

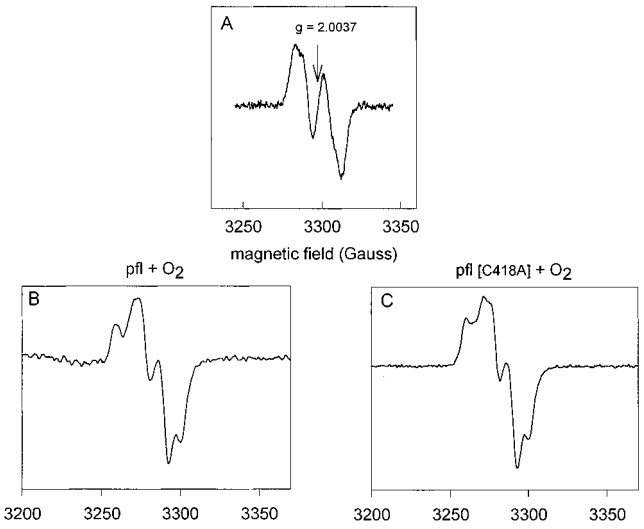


FIGURE 1: (A) EPR spectrum of the glycyl radical (first derivative) of PFL and (B) EPR spectrum of the radical formed from activated WT-PFL after 20 s exposure to air. The intensity of this spectrum was 30% that of the starting glycyl radical. (C) EPR spectrum of the radical formed from activated C418A PFL after 20 s exposure to air. The intensity of this signal was 55% that of the starting radical.

EPR spectroscopy was used to test this hypothesis in wild-type PFL and in three mutant enzymes, C418A, C419A, and C418A/C419A. Figure 1B shows the EPR spectrum of a new radical formed from activated wild-type PFL after 20 s exposure to air at room temperature. The same radical signal was generated in a sample of active PFL diluted 15% with oxygenated buffer and refrozen after 30 s (not shown). The intensity of the new signal varied with time of incubation in the presence of air, and incubation at room temperature for longer than 30 s led to decay of the signal without appearance of other EPR signals. The intensity of the spectrum shown (Figure 1B) was 30% of that of the starting glycyl radical, demonstrating a good yield of the new radical species. The use of $^{17}O_2$ for inactivation led to broadening of the EPR spectrum consistent with the presence of ^{17}O in the radical (not shown). No paramagnetic species were observed in control samples that were photoreduced but did not contain AdoMet, AE, or deazariboflavin or in samples that contained all components but were not photoreduced. Furthermore, when control samples were thawed and exposed to air for 20 s similar to activated PFL samples, no EPR signals were detected. These observations are consistent with the formation of a new enzyme-based radical species from active enzyme and oxygen and with its subsequent decay into nonparamagnetic products.

Our ongoing studies of PFL have illustrated the importance of C419 in mediating catalysis by what we have proposed to be a glycyl/thiyl radical exchange (*6*, *17*). Therefore, the oxygen inactivation experiment was repeated using the activated mutant enzyme, C418A. Figure 1C shows that the spectrum produced after exposure of C418A to air is similar to that produced from the wild-type enzyme. The intensity of this signal was 55% of that of the starting radical. Control samples again excluded the possibility of adventitious radical formation.

Computer simulations of the spectrum of the new radical (Figure 1B,C) allowed evaluation of **g**-tensor and hyperfine coupling values in order to make a structural assignment

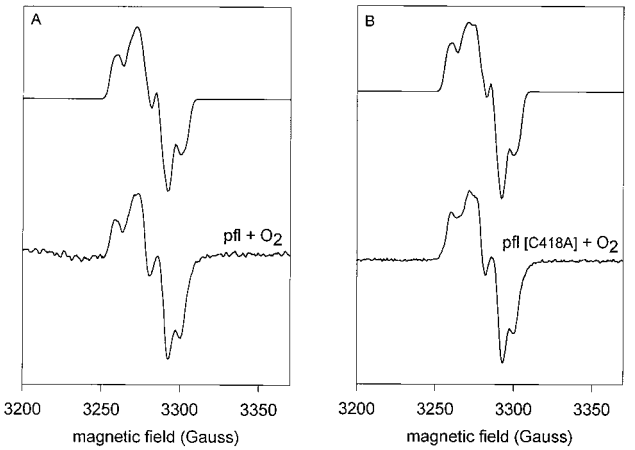


FIGURE 2: Overlay of computer-simulated EPR spectra and experimental data for the radical formed in wild-type PFL (A) and C418A PFL (B) upon exposure to air. The *g*-values and hyperfine couplings (see text) are consistent with assignment of the spectra to a sulfinyl radical.

(Figure 2A,B). The simulation parameters for the wild-type PFL radical were $g_{x,y,z} = 2.0204, 2.0084, 2.0005$ with hyperfine couplings for two, S = 1/2 nuclei; $a_{iso}(1) = 29.0$ MHz, $a_{iso}(2) = 11.3$ MHz. For the EPR signal of the C418A mutant, $g_{x,y,z} = 2.0204, 2.0082, 2.0005$, $a_{iso}(1) = 26.5$ MHz, $a_{iso}(2) = 11.0$ MHz. These parameters are similar to those reported for frozen solution spectra of the sulfinyl radical formed from cysteine ($g_{x,y,z} = 2.0203, 2.0080, 2.0027$; $a_{iso}(H) = 39.2$ MHz) and other sulfhydryl containing molecules (*18*). The differences in *g* values and the hyperfine couplings for the protein spectra compared to those for cysteine-sulfinyl radical suggest that the geometry and/or chemical environment of the PFL-based sulfinyl radicals differ from that of the corresponding small molecule radical in frozen aqueous solution. The possibility that the observed sulfinyl radical observed in the PFL experiments is an artifact due to the reducing buffer system that contained DTT was ruled out by the absence of paramagnetic species after photoreduction of the samples lacking one of the activation components. Furthermore, no paramagnetic species were observed when these control samples were subjected to oxygen exposure. Finally, the signal intensity of the sulfinyl radical always constituted a substantial fraction of the signal intensity of the starting glycyl radical and was never greater than the glycyl radical intensity. This would generally not be the case if the radical arose from the reaction of dioxygen with the small molecule components in the reaction mixture (e.g., Fe(II), DTT, etc.) present at relatively high concentrations.

The small differences between the spectrum of the radical formed in wild-type PFL compared to that of the C418A mutant suggests that while both spectra arise from the same species, the environment and/or geometry of the sulfinyl group differs. The differences may reflect those chemical and structural changes accompanying replacement of the sulfhydryl of C418 with a methyl group and further suggests that a likely location of the sulfinyl radical is at C419 in these two proteins. If the spectra of the wild-type and mutant enzymes were identical, it might be taken as evidence that the site of the radical was far removed from the site of amino acid replacement. Another conclusion that may be drawn from the observation that spectra for aerated wild-type PFL and C418A are similar but not identical is that the new radical

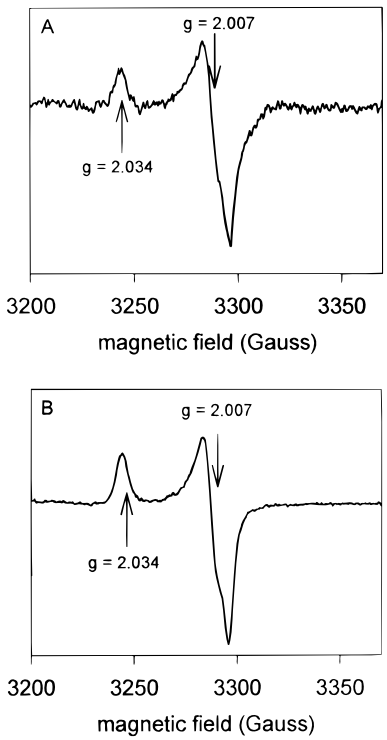


FIGURE 3: (A) EPR spectrum of a radical formed from activated C419A PFL after 20 s exposure to air. The intensity of this signal was 11% that of the starting glycyl radical. (B) EPR spectrum of a radical formed from activated C418AC419A PFL after 20 s exposure to air. The intensity of this signal was 30% that of the starting glycyl radical.

resides on the enzyme and is not due to production of a sulfinyl radical in solution.

A different EPR spectrum was obtained when the activated mutant enzymes, C419A and C418A/C419A, were exposed to air (Figure 3A,B). Here, an axial signal lacking hyperfine splittings was found ($g = 2.034, 2.007$). The spectrum could be observed superimposed on that of the starting glycyl radical for samples exposed to air for less than 20 s (data not shown), suggesting that the new radical signal evolves as the glycyl radical disappears. The g values for this signal are similar to those for peroxyl radicals (*19*) including alkyl and sulfur peroxyl groups, though individual values for g_y and g_z could not be resolved (*18*, *20*−*22*).

In general, it is difficult to distinguish between carbon-based and sulfur-based peroxy radicals by EPR (*18*). The observation of a peroxy radical in the C418A/C419A double mutant clearly excludes these essential cysteines as candidates. The most reasonable possibility then is that the peroxyl radical is associated with G734 and is formed by direct addition of dioxygen to the glycyl radical. The low-field feature of this peroxyl radical signal ($g = 2.034$) was also transiently observed in a spectrum (not shown) of wild-type PFL exposed to air, recorded at high power (2 mW). This observation is consistent with Scheme 1 outlined below for production of peroxyl and sulfinyl radicals from the glycyl radical in wild-type PFL and mutant enzymes.

DISCUSSION

The conversion of pyruvate and CoA to acetyl-CoA and formate catalyzed by PFL constitutes a novel fragmentation of pyruvate reminiscent of the radical-mediated, chemical cleavage of pyruvate esters (*23*). Consistent with a radical mechanism, catalysis by PFL requires the presence of glycyl radical on G734, as well as two cysteine residues, C418 and C419 (*6*, *17*, *24*). On the basis of previous studies, C419 was proposed to mediate radical transfer from G734 during inactivation of the enzyme by mechanism-based inhibitors and during solvent exchange of the α-hydrogen of the glycyl radical (*6*, *17*, *24*). These data also argued for the involvement of C419 in the normal catalytic mechanism via the transient formation of a thiyl radical (*13*).

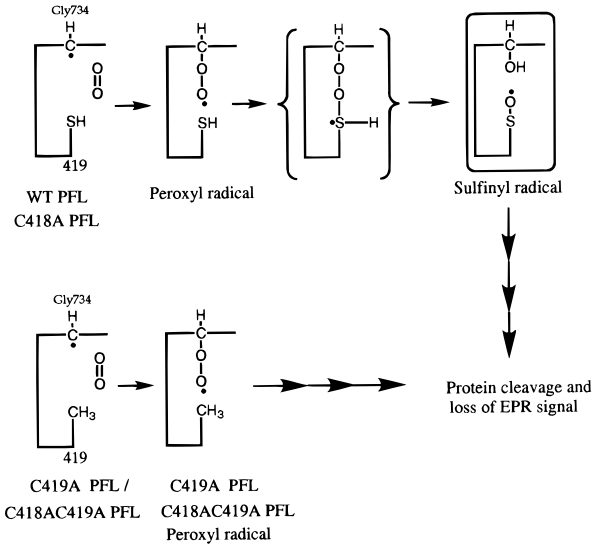
We reasoned that an analysis of the well-established inactivation of PFL by dioxygen might also provide a fruitful approach for assessing a glycyl/thiyl radical interaction at the active site. The reaction of dioxygen with carbon- and sulfur-based radicals to generate oxygen-based radicals has been extensively studied, and many of these have been characterized by EPR. Thus, wild-type PFL and three mutants, C418A, C419A, and the double mutant C418A-C419A, were examined for the formation of new radical species when the enzyme was exposed to dioxygen.

The data reported here demonstrate that at least two distinct protein-based radical species can be detected by EPR upon exposure of the glycyl radical to dioxygen, a peroxyl radical, and a sulfinyl radical. The sulfinyl radical appeared in wild-type PFL and C418A but not in C419A and the double mutant C418A/C419A, where only a peroxyl radical was found, strongly implicating C419 as the site of sulfinyl radical formation observed in wild-type PFL and the C418A mutant. It was shown previously that the reaction of active PFL with dioxygen ultimately leads to the quenching of the glycyl radical preceeding peptide cleavage at the glycine 734 (*4*). All of the mutants examined here exhibited the same cleavage pattern as that of the wild type enzyme consistent with cleavage occuring at G734 for each protein.[3]

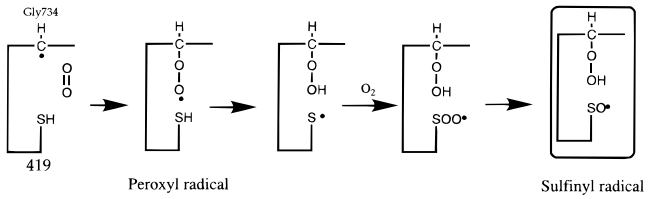
What is the relationship between the sulfinyl radical and the peroxyl radical in the mechanism of inactivation? Scheme 1 outlines a mechanism that we favor. The first step involves oxygen addition to the glycyl radical to form a peroxyl radical at the α-carbon of G734. Addition of dioxygen to carbon-based radicals, in general, and glycyl radicals, in particular, is well documented (*26*). We propose that the fate of the peroxyl radical differs for the wild-type enzyme and C418A mutant compared to mutants altered at C419. The proximity of C419 to the peroxyl radical at G734 would facilitate addition of the C419 thiol to the peroxyl radical followed by decomposition of the intermediate (bracketed in Scheme 1) to yield a sulfinyl radical at C419

[3] Oxygen-inactivated samples of wild-type PFL, C418A, C419A, and C418A/C419A mutants were run on PAGE gels to identify low mass fragmentation products. All four samples were analyzed by HPLC-electrospray ionization mass spectrometry and afforded molecular ions (isotope-averaged) of 3064.3 ± 0.5 and 3020.6 ± 0.3. The molecular ion of 3064 is consistent with fragmentation at G734 yielding a oxalylamide N-terminus as has been previously observed (*4*); the molecular ion of 3020 suggests that an *N*-formyl terminus may also be generated during G734 fragmentation. The mechanism of cleavage leading to these products awaits further elucidation and is complicated by the potential for side reactions from the complex redox buffer and oxidation of an initially formed glyoxalyl N-terminus during workup as previously noted (*4*). Recent studies on the structure of cleavage products derived from the C225S mutant of the R1 subunit of the aerobic ribonucleotide reductase from *E. coli* have provided a biochemical identification of the active site region; however, no distinct radical intermediates were reported (*25*).

Scheme 1: Proposed Mechanism of PFL Inactivation by Oxygen



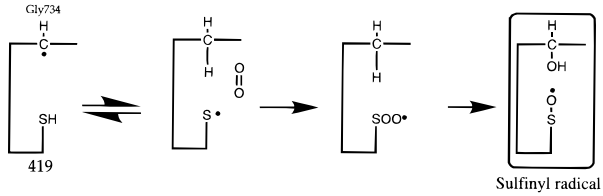
Scheme 2: Alternate Pathway for Sulfinyl Radical Formation



that builds up sufficiently to permit its detection by EPR. This type of mechanism has been proposed for the formation of sulfinyl radicals from thiols and carbon-based peroxyl radicals prepared by irradiation of organic matrices in the presence of dioxygen at 77 K (*18*). The enzyme inactivation process also yields an α-hydroxyglycine at G734 that can readily fragment into the observed 82 kDa fragment and into the 3 kDa fragment containing an oxalyl group at its N-terminus (*4*). In the mutants lacking C419, the initially formed peroxyl radical at G734 is long-lived and becomes the predominantly observed species. The peroxyl radical is ultimately susceptible to decay via other reductive processes that also lead to the formation of α-hydroxyglycine. Thus, cleavage of the enzyme occurs in all cases examined here yielding the 3 kda fragment[3] consistent with G734 as the site of cleavage. Glycyl radicals formed in peptides by radiolysis have been shown to undergo similar fragmentation via initial formation of peroxyl radicals (*26*). These results also demonstrate that formation of the sulfinyl radical at C419 observed in wild-type PFL and C418A is not a requirement for cleavage at G734.

Two alternate pathways to sulfinyl radical formation demand consideration. The mechanism outlined in Scheme 2 proposes a hydrogen abstraction by the peroxyl radical at G734 from the sulfhydryl of C419 yielding a thiyl radical, addition of a second molecule of dioxygen to the thiyl radical, and subsequent reductive cleavage of the thiol peroxyl radical to give a sulfinyl radical at C419. We cannot unambiguously rule out this alternate pathway at present. Indeed, this mechanism received serious consideration in the aforementioned study of the conversion of matrix (alkyl) peroxyl radicals to sulfinyl radicals (*18*). These workers, however,

Scheme 3: Alternate Pathway for Sulfinyl Radical Formation



favored a direct bimolecular reaction of the peroxyl radical and a thiol (Scheme 1) since, even at low concentrations of dioxygen, they observed a high conversion of peroxyl radicals to sulfinyl radicals. A unimolecular requirement for dioxygen as in Scheme 1 was interpreted as being more compatible with the efficiency of sulfinyl radical formation, although no kinetics were performed to resolve this point (*18*). We favor Scheme 1 for this reason as well and because it avoids the complexity of additional unspecified steps for reductive cleavage of the thiol peroxyl radical to afford the sulfinyl radical; for example, the possibility that C418 is required for efficient sulfinyl radical formation at C419 is excluded by the results obtained with the C418A mutant. Moreover, the direct reaction of peroxyl radical with thiol is exothermic (*18*).

Another pathway is considered in Scheme 3. This mechanism requires the initial reaction of dioxygen with a C419 thiyl radical that we have proposed to be in equilibrium with the glycyl radical (*6*). The resulting thiol peroxyl radical would regenerate the glycyl radical by α-hydrogen abstraction (required to account for protein cleavage) and ultimately decomposes to the sulfinyl radical at C419 and an α-hydroxyglycine (perhaps through the same carbon-based peroxyl intermediate proposed in Scheme 1). Issues of added complexity aside, we find this mechanism unlikely. First, the putative glycyl/thiyl radical equilibrium significantly favors the glycyl radical in the resting enzyme as evidenced by the facile detection and characterization of the glycyl radical by EPR and the corresponding inability to detect the distinctive thiyl radical under these conditions. That α-amino carbon-centered radicals are thermodynamically favored over thiyl radicals has also been noted in model systems (*27*). Second, the rates of addition of molecular oxygen to thiyl radicals are, at best, comparable to the rates for the corresponding carbon centered radicals. Carbon radicals react with dioxygen at close to the diffusion controlled limit ($\geq 10^9$ M^{-1} s^{-1}). Representative examples of rate constants for dioxygen addition to thiyl radicals are 2.2×10^9 M^{-1} s^{-1} for 2-mercaptoethanol (*28*) and 8×10^9 M^{-1} s^{-1} for cysteine (*29*). Thus, the relative rates of reaction of the active site glycyl and thiyl radicals with dioxygen will be governed largely by the relative concentrations of these radical species, which favors the glycyl radical and, thereby, formation of a carbon-peroxyl radical.

An important prediction based on Scheme 1 is that the generation of the G734 peroxyl radical precedes the formation of the sulfinyl radical in wild-type PFL. While a species consistent with the G734 peroxyl radical is detected in mutants lacking C419, its formation and decay in wild-type PFL remains to be established. Preliminary experiments with wild-type PFL has revealed that the low-field feature of this peroxyl radical signal ($g = 2.034$) was also transiently

observed prior to complete formation of the sulfinyl radical (data not shown). Rapid-freeze quench studies are underway to assess the kinetic competence of the peroxyl radical and should be useful in detecting other transient radical intermediates that are predicted by the mechanism.

The experiments reported here further strengthen the case for a close spatial and reactive proximity of G734 and C419 in PFL. The chemical interplay between these residues suggested by this work reflects, we believe, a potential glycyl/thiyl radical equilibrium that is an essential element of this unusual protein cofactor. Moreover, we speculate that a glycyl/thiyl radical equilibrium will be a general feature of other glycyl radical-containing enzymes, such as the anaerobic ribonucleotide reductase of *E. coli* (*30*). Thus far, no experiments that address the existence of such an equilibrium or the identity of a specific cysteine involved have been reported for the reductase. Although solvent hydrogen exchange at the α-carbon of the glycyl radical is not observed for the reductase (*30*), this process in PFL is postulated to rely on a small degree of stereoinfidelity during the putative glycyl/thiyl radical exchange process (*6*). Since a completely stereospecific glycyl/thiyl radical exchange would afford no solvent hydrogen exchange, the absence of hydrogen exchange does not exclude a glycyl/thiyl radical exchange in the reductase. Thus, the direct approach of EPR radical detection reported here for PFL may prove useful as a general method in establishing a glycyl/thiyl radical cofactor for the anaerobic ribonucleotide reductase and other systems.

ACKNOWLEDGMENT

We thank Tracey D. Klatt of Merck Research Laboratories for performing peptide analyses by HPLC electrospray mass spectrometry.

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BI972086N