Oxidative Degradation of Pyruvate Formate-Lyase

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Abstract: The reaction mechanisms of oxidative degradation of the anaerobic radical enzyme pyruvate formate-lyase (PFL) have been examined at the theoretical hybrid Hartree−Fock/density functional theory level B3-LYP. It is concluded that the most likely scenario involves O_2 addition at the glycyl radical site, followed by $H^\bullet$ abstraction/transfer from C419 and subsequent rearrangements to generate an α-hydroxyglycine and the sulfinyl radical R−$SO^\bullet$, observed by EPR spectroscopy. In addition, the present model accounts for alternative fragmentations observed in wild-type PFL, and the formation and degradation of Gly−O-$O^\bullet$ in the absence of C419 (as observed in C419A and C419AC418A mutants).

Introduction

An increasing number of enzymes have been found to contain a radical amino acid residue, and such radical centers play key roles in the enzymes' catalytic function.[1−3] In many radical-containing enzymes the radical residue is situated within the enzyme, for example, class I ribonucleotide reductases.[4] When required, the "radical reactivity" is transferred from the radical residue to the active site of the enzyme. However, in some radical-containing enzymes, for example, anaerobic pyruvate formate-lyase (PFL), the radical residue is situated at the active site.

PFL plays a central role in the metabolic pathway of glucose in *Escherichia coli* and belongs to a growing class of radical-containing enzymes that have been found to contain a glycyl amino acid radical.[5] Consequently, PFL has been the subject of a number of experimental[6−14] and theoretical[15] studies, and it has been found that the glycyl radical (G734) and two cysteine residues (C418 and C419) are essential for the catalytic function of PFL. It has been postulated[7,15] that the glycyl radical acts as the storage site of the radical reactivity with the initial step in the PFL mechanism proposed to involve the generation of a reactive thiyl radical by transfer of a hydrogen from C419 to G734. These two residues are thought to lie spatially close to each other.[12,14] The energy required for a direct hydrogen transfer from C419 to G734 has been calculated to be approximately 9.9 kcal mol^{-1}.[15]

The glycyl radical is remarkably stable.[9,16] However, exposure of PFL to an oxygenated solution results in cleavage of the PFL peptide backbone at the C_α−N bond of the glycyl radical.[9,14] Indeed, the oxidative degradation and fragmentation of PFL by this method was used to identify the glycyl radical as the G734 residue.[9]

Recently, Reddy et al.[14] reported a detailed experimental investigation of the oxidative degradation of PFL. Electron spin resonance (ESR) measurements and mass spectrometry were employed to identify intermediates and products in the oxidative degradation of wild-type PFL and mutant PFL enzymes in which either or both of C418 and C419 were replaced by an alanine amino acid. In addition to observing the previously[9] noted fragmentation of the PFL backbone at the C_α−N bond of G734, ESR evidence for the formation of a long-lived sulfinyl radical (R−$SO^\bullet$) at C419 was also obtained. Furthermore, mass spectrometric characterization of the resulting cleavage products suggested that fragmentation may also occur at the C_α−C_1 bond of G734. However, it was unclear if such a cleavage did indeed occur or if the products observed were a result of further oxidation of the enzyme fragments after cleavage at the C_α−N bond. In the alanine-substituted mutants lacking C419, ESR evidence for formation of a peroxyl radical at G734 was obtained and was also thought to be transiently observed in wild-type PFL. Exposure of all mutants to O_2 also resulted in cleavage of the PFL backbone at G734, indicating that the cysteine residues were not essential for oxidative degradation and fragmentation of PFL. On the basis of their observations, they proposed three possible mechanisms, which are shown in Scheme 1. They were unable to unambiguously determine, however, which mechanism, if any, was most plausible. Their preferred choice is that shown in Scheme 1a.

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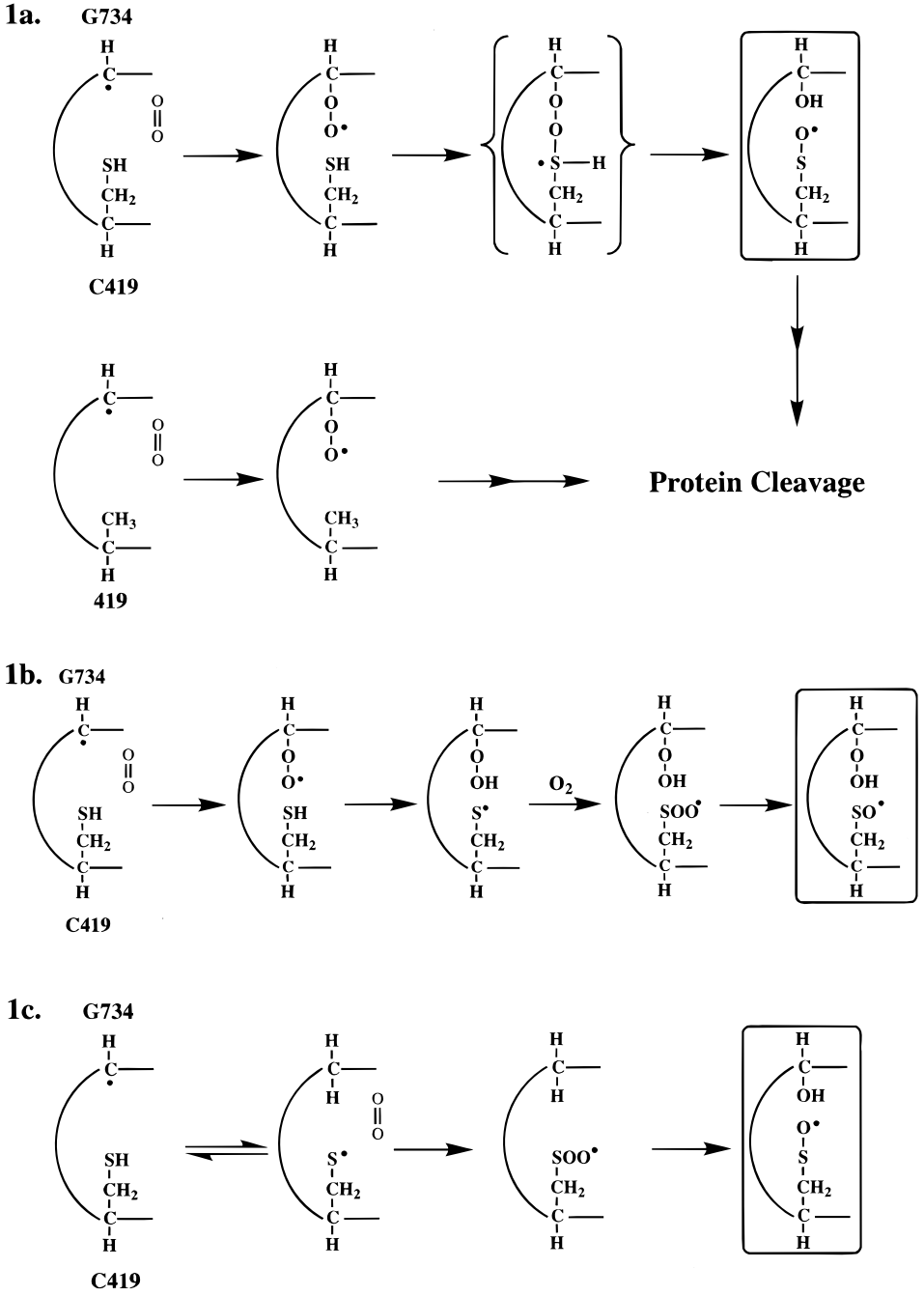
Scheme 1. Three Possible Mechanisms for Oxidative Degradation of PFL as Proposed by Reddy et al.[14,a]



[a] Also included in 1a is a schematic representation of oxidative degradation of PFL mutants in which C419 was replaced by an alanine. Adapted from ref 14.

The oxidative degradation of radical-containing enzymes is often employed to assist in identifying the position of the radical amino acid residue. Hence, a more complete understanding of the mechanisms involved in the oxidative degradation of PFL may also enable greater insight into the mechanisms by which other radical-containing enzymes and biological radicals in general may react with O_2.

In this paper, density functional theory methods have been employed to investigate possible mechanisms by which PFL may be degraded upon exposure to O_2. At such levels of theory it is not computationally feasible to include the complete enzyme. Thus, for the PFL enzyme, we have considered only the crucial glycyl radical (G734) and cysteine (C419) residues and have modeled these by use of glycyl radical ($H_2NC^{\bullet}HCOOH$) and CH_3CH_2SH, respectively.

Computational Methods

Density functional theory (DFT) calculations were performed using the GAUSSIAN 94[17] and GAUSSIAN 98[18] suite of programs. The hybrid Becke exchange functional (B3),[19,20] as implemented[21] in GAUSSIAN 94 and 98,[17,18] in combination with the correlation functional of Lee, Yang, and Parr (LYP),[22] was employed. Optimized geometries, harmonic vibrational frequencies, and zero-point vibrational

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energies (ZPVEs) were obtained using the 6-31G(d,p) basis set in conjunction with the above B3-LYP method. Relative energies were calculated by performing single-point energy calculations at the B3-LYP/6-311+G(2df,p) level using the above optimized geometries and with inclusion of the appropriate ZPVE, i.e., B3-LYP/6-311+G(2df,p)//B3-LYP/6-31G(d,p) + ZPVE.

Initial calculations were performed using two models for the G734 radical residue, the glycine radical ($H_2NC^{\bullet}HCOOH$) and the "extended" glycine radical[15] ($OHCNHC^{\bullet}HCONH_2$), and two models for the C419 residue, methylthiol (CH_3SH) and ethylthiol (CH_3CH_2SH). On the basis of these initial findings it was concluded that employing $H_2NC^{\bullet}HCOOH$ and CH_3CH_2SH as models of G734 and C419, respectively, represented a satisfactory compromise between obtaining reliable energetics for the types of reactions encountered in this study and computational size. All relative energies in this study refer to these preferred model systems and are in kcal mol^{-1}, unless otherwise stated.

The calculation of relative energies by use of large basis set single-point calculations using optimized geometries obtained at lower levels of theory is a standard technique of computational chemistry. Reliable optimized geometries are often obtained at relatively modest levels of theory, e.g., B3-LYP/6-31G(d,p). However, in general, more reliable and accurate relative energies are obtained using larger basis sets, e.g., 6-311+G(2df,p). In the present study, slightly lower reaction barriers were obtained with the larger single-point calculations compared to those calculated at the B3-LYP/6-31G(d,p) level. Total energies (obtained at both levels of theory), ZPVEs, and optimized geometries (in the form of GAUSSIAN archive files) are presented in Tables S1 and S2, respectively, of the Supporting Information.

The unrestricted (U) and restricted (R) B3-LYP procedures were used for open- and closed-shell species, respectively. The symbols U and R are hereafter omitted for simplicity.

Results and Discussion

Initial Reactions of the Glycyl Radical. In the proposed mechanisms of Reddy et al.[14] (Scheme 1), the initial reaction of the glycyl radical consists of one of two possibilities: direct addition of O_2 to the carbon radical center of the glycyl radical to form the corresponding carbon-peroxyl radical (see Scheme 1a and 1b), or a hydrogen transfer from the cysteine C419 to the glycyl radical, thus forming a thiyl radical and glycine (see Scheme 1c).

Reddy et al.,[14] however, discounted the latter possibility on the basis of several experimental observations. First, the glycyl/thiyl radical equilibrium favors the glycyl radical; α-amino carbon-centered radicals are thermodynamically favored over thiyl radicals. Indeed, recent theoretical calculations[15] at levels of theory similar to those employed in this present study estimated the glycyl radical/thiol to be favored over the glycine/thiyl radical by approximately 3.4 kcal mol^{-1}. In addition, as noted previously, they also estimated that direct transfer of hydrogen from the thiol group of cysteine to the glycyl radical involved a barrier of approximately 9.9 kcal mol^{-1}. Second, thiyl and carbon-centered radicals have comparable rates of reaction for addition of O_2. Thus, the relative rates for addition of O_2 to the glycyl or thiyl radicals will be determined mainly by their relative concentration, which, as noted above, favors the glycyl radical.

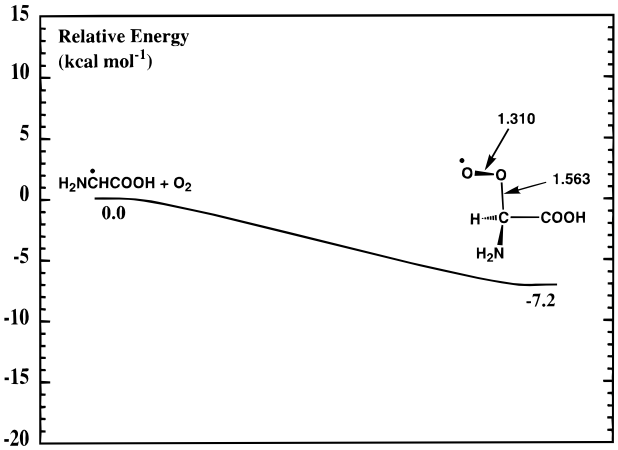

Figure 1. Schematic energy profile for the addition of molecular oxygen to the glycyl radical.

O_2 in its triplet ground state is found to add to the glycyl radical without a barrier to form the glycyl−peroxyl radical (Gly−O-O$^{\bullet}$), which lies approximately 7.2 kcal mol^{-1} lower in energy than the initial reactants, $O_2 + H_2NC^{\bullet}HCOOH$ (Figure 1). The present level of theory is known, however, to yield triplet O_2 too stable by approximately 5 kcal mol^{-1}.[23] Hence, the intial formation of Gly−O-O$^{\bullet}$ is likely to be exothermic by at least 10 kcal mol^{-1}.

For the remainder of this paper only those mechanisms that may occur after initial formation of Gly−O-O$^{\bullet}$ are considered.

Glycyl−Peroxyl Radical (Gly−O-O$^{\bullet}$). Following the formation of Gly−O-O$^{\bullet}$, Reddy et al.[14] proposed that it may then react directly with C419 to give an α-hydroxyglycine and the sulfinyl radical (Scheme 1a) or, alternatively, that it may abstract a hydrogen from the thiol group of C419 to give the corresponding glycyl-hydroperoxide (Gly−O-OH) and thiyl radical (Scheme 1b).

At the B3-LYP/6-31G(d,p) level, no complex or transition structure corresponding to that proposed to partake in the direct reaction of Gly−O-O$^{\bullet}$ with the thiol (see Scheme 1a) was located. Rather, interaction of Gly−O-O$^{\bullet}$ with the thiol (R−SH) was found to lead directly to the formation (not shown) of the hydrogen-bonded complex **1** (Gly−O-O$^{\bullet}\cdots$HS−R). The thiol moiety is then able to transfer a hydrogen to the peroxyl group via TS**1**, at a cost of 10.4 kcal mol^{-1}, to form hydrogen-bonded complex **2** (Gly−O-OH$\cdots$S$^{\bullet}$−R), as shown in Figure 2a. It is interesting to note that this latter mechanism is analogous to the hydrogen-transfer step from C419 to G734, which is postulated to be the initial step in the catalytic mechanism of PFL.[12,15] Furthermore, it is calculated to require approximately the same amount of energy.[15]

A possible alternative mechanism involves the transfer of an oxygen from the peroxyl radical to the thiol group, i.e., rearrangement of **1** via TS**2** to **3**, as shown in Figure 2b. The resulting hydrogen-bonded complex **3** (Gly−O$^{\bullet}\cdots$H−SO−R) is calculated to lie considerably lower in energy than **2** by approximately 18.5 kcal mol^{-1}. The R−SHO moiety would then be able to quite easily transfer a hydrogen to the Gly−O$^{\bullet}$ moiety to form the corresponding sulfinyl radical (R−SO$^{\bullet}$) and α-hydroxyglycine (Gly−OH). The Gly−OH moiety could then react

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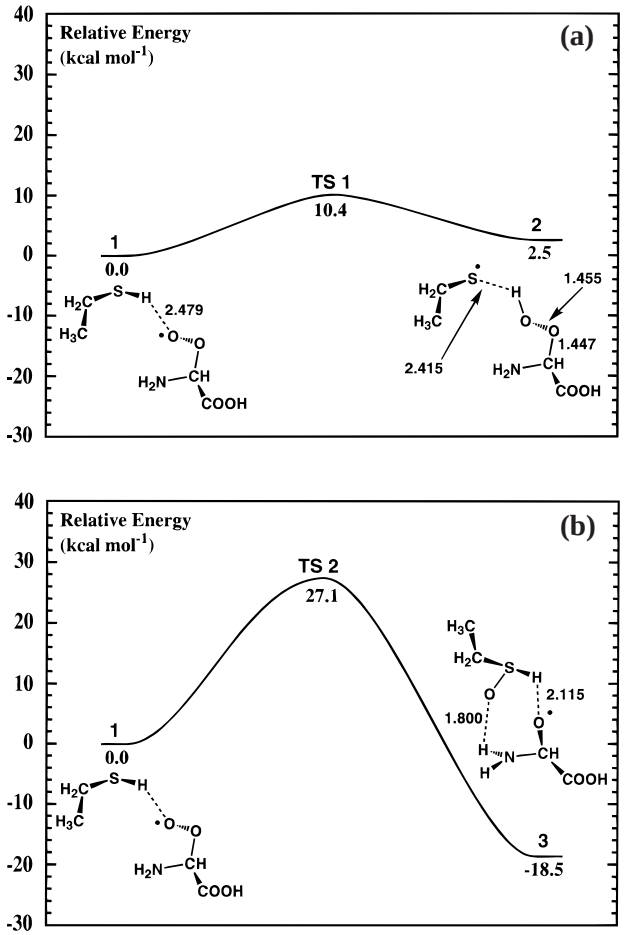

Figure 2. Schematic energy profile for reaction of the glycyl−peroxyl radical with the thiol moiety of **1** via (a) hydrogen transfer from the thiol to the glycyl−peroxyl radical or (b) oxygen transfer from the glycyl−peroxyl radical to the thiol.

further, resulting in fragmentation at the C_α−N bond. However, such an oxygen transfer mechanism via TS**2** is predicted to require a significant amount of energy, approximately 27.1 kcal mol^{-1}. This is almost three times more than that required for hydrogen transfer from the thiol to Gly−O-O$^\bullet$ (see above). Hence, it will not be a viable alternative mechanism.

One also needs to consider the possibility that Gly−O-O$^\bullet$ may react with other radicals that may be present in the oxygenated solution or that it may undergo intramolecular reactions. From experimental studies of radiation-induced oxidative degradation of monomeric amino acids and polypeptides,[24,25] the major reaction pathways of carbon-peroxyl radicals (Scheme 2) have been found to be an intramolecular hydrogen abstraction reaction (Scheme 2a) or a bimolecular reaction with HOO$^\bullet$ to give the corresponding carbon-peroxide + O_2 (Scheme 2b). It should be noted that in both cases, the resulting amino acid derivatives will continue to react further, leading to fragmentation of the amino acid backbone.

For the intramolecular hydrogen abstraction mechanism illustrated in Scheme 2a, the reaction products (HOO$^\bullet$ + HNCHCOOH) are calculated to lie 14.1 kcal mol^{-1} higher in energy than the Gly−O-O$^\bullet$ precursor. This is approximately 4 kcal mol^{-1} more energy than required for Gly−O-O$^\bullet$ to abstract a hydrogen from the thiol (see above). Hence, Gly−O-O$^\bullet$ will preferentially abstract a hydrogen from the thiol rather than undergo intramolecular hydrogen abstraction. This may help to explain why Gly−O-O$^\bullet$ was found to be long-lived enough to be observable in PFL mutants in which C419 was replaced by an alanine residue, whereas it was only transiently observed in wild-type PFL.[14] Furthermore, such PFL mutants would also be expected to undergo cleavage at the G734 residue upon exposure to O_2, as was observed experimentally.[14]

At the B3-LYP level, the reaction of $HO_2^\bullet$ with Gly−O-O$^\bullet$ to give O_2 + Gly−O-OH, which lie 32.5 kcal mol^{-1} lower in energy, is predicted to occur with little or no barrier. In experimental studies[24] of the effects of ionizing radiation on proteins in oxygenated solutions, $HO_2^\bullet$ is formed either by the reaction of O_2^- with H_2O or via the mechanism shown in Scheme 2a (see above). The O_2^- moiety, however, arises as a result of the fact that at sufficiently large relative concentrations, O_2 preferentially scavenges the aqueous electron (e_{aq}^- generated in the *radiation-induced decomposition* of H_2O. The experiments of Reddy et al,[14] however, and those under consideration here were performed in *nonirradiated* oxygenated solutions. As already noted above, the formation of $HO_2^\bullet$ via the above intramolecular hydrogen abstraction mechanism will not be significant, at least in the oxidative degradation of wild-type PFL. Thus, although the reaction of $HO_2^\bullet$ with Gly−O-O$^\bullet$ to give O_2 + Gly−O-OH is expected to occur rapidly, it is unlikely to be a major reaction pathway in the oxidative degradation of wild-type PFL in nonirradiated oxygenated solutions.

Glycyl−Hydroperoxide (Gly−O-OH). In the mechanism proposed by Reddy et al.[14] shown in Scheme 1b, the thiyl radical moiety of **2** (Gly−O-OH···S$^\bullet$−R) is proposed to react with a second oxygen molecule to form a sulfur−peroxyl radical. However, the thiyl radical moiety of **2** is also able to abstract the terminal hydroxyl group of the Gly−O-OH moiety via TS**3**, at a cost of 10.6 kcal mol^{-1}, to form the hydrogen bonded complex **4** (Gly−O$^\bullet$···HO-S−R), which lies considerably lower in energy than **2** by approximately 30.0 kcal mol^{-1} (see Figure 3). It is interesting to note that the calculated energy for such a rearrangement is approximately the same as that required for abstraction of the thiol hydrogen by the peroxyl radical, Gly−O-O$^\bullet$ (see above).

Glycyl−Alkoxy Radical (Gly−O$^\bullet$). The Gly−O$^\bullet$ moiety of **4** can readily abstract a hydrogen from the R−S-OH moiety with little or no barrier, via TS**4**, to form complex **5**, which lies 31.5 kcal mol^{-1} lower in energy than **4** (Figure 3). The vanishing barrier for TS**4** is a result of the ZPVE effects. Complex **5** (Gly−OH···O$^\bullet$-S−R) is a hydrogen-bonded complex between the resulting observed sulfinyl radical and α-hydroxyglycine. The α-hydroxyglycine moiety is then able to undergo hydrolysis, resulting in fragmentation of the glycine residue at the C_α−N bond.

In addition, one also needs to consider possible intramolecular rearrangements and dissociations of the glycyl−alkoxy radical (Gly-O$^\bullet$ or H_2N-CH(O$^\bullet$)-COOH) moiety of complex **4**. These are shown schematically in Figure 4.

The most stable isomer of H_2N-CH(O$^\bullet$)-COOH is the carbon-centered radical H_2N-C$^\bullet$OH-COOH, which lies approximately 31.6 kcal mol^{-1} lower in energy. However, the 1,2-hydrogen shift rearrangement of H_2N-CH(O$^\bullet$)-COOH to H_2N-C$^\bullet$OH-COOH via TS**5** has a barrier of approximately 17.6 kcal mol^{-1} (Figure 4). This is considerably higher than the negligible energy required for the Gly−O$^\bullet$ moiety to abstract a hydrogen from the R−S-OH moiety of complex **4**. Thus, H_2N−CH(O$^\bullet$)-COOH will not rearrange to its most stable isomer.

The C−N bond of H_2N-CH(O$^\bullet$)-COOH is relatively weak and dissociates via TS**6**, at a cost of 13.1 kcal mol^{-1}, to give

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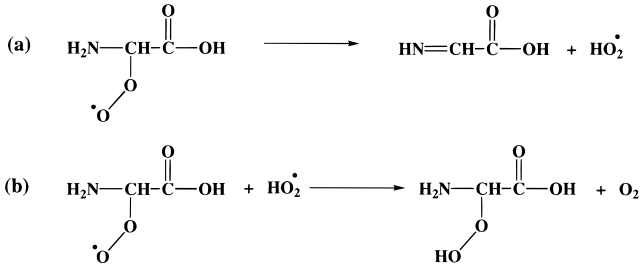
Scheme 2. Schematic Illustration of the Two Major Reaction Pathways of the Glycyl−Peroxyl Radical in Irradiated Oxygenated Solutions via (a) Intramolecular Hydrogen Abstraction and (b) Bimolecular Reaction with $HO_2^{\bullet}$[a]



[a] Adapted from ref 24.

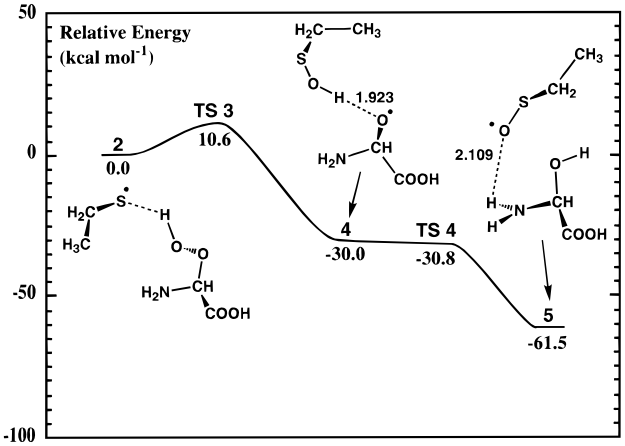


Figure 3. Schematic energy profile for $^{\bullet}OH$ transfer from the glycyl−hydroperoxide to the thiyl radical, rearrangement of **2** via TS**3** to **4**, followed by hydrogen abstraction by the glycyl−alkoxy radical to form the sulfinyl radical and α-hydroxyglycine, and rearrangement of **4** via TS**4** to **5**.

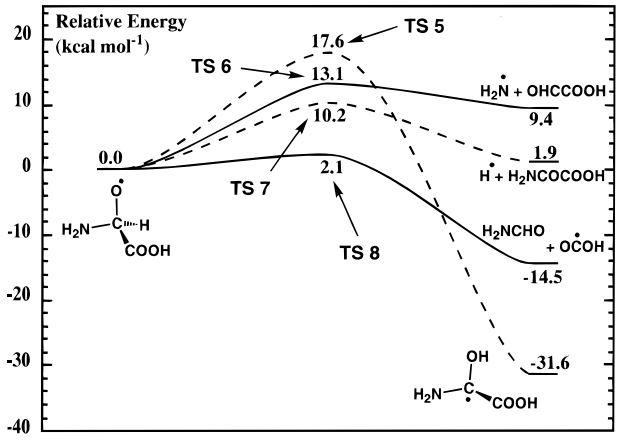


Figure 4. Schematic energy profiles for possible isomerization and dissociation reactions for the glycyl−alkoxy radical.

the fragmentation products $^{\bullet}NH_2$ + OHC-COOH. Similarly, the C−H bond of H_2N-CH($O^{\bullet}$)-COOH is also quite weak and dissociates via TS**7**, at a cost of 10.2 kcal mol^{-1}, to give the fragmentation products $H^{\bullet}$ + H_2N-CO-COOH (Figure 4). In both of these dissociations, the fragmentation products lie higher in energy than H_2N-CH($O^{\bullet}$)-COOH, by 9.4 and 0.9 kcal mol^{-1}, respectively. The energy required for both of these dissociation pathways is less than that required for H_2N-CH($O^{\bullet}$)-COOH to rearrange to its more stable isomer (see above). However, both of these dissociation mechanisms still require significantly more

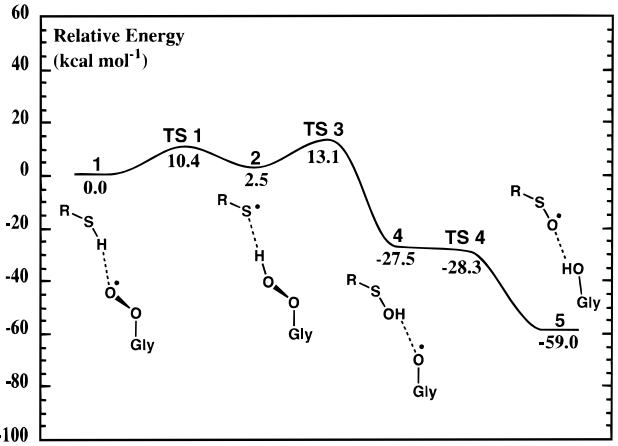


Figure 5. Summary of the main reaction pathway in the oxidative degradation of pyruvate formate-lyase. The alternative route leading to $C_α$−C_1 cleavage is shown in Figure 4. Note that the initial formation of Gly−O-$O^{\bullet}$ is exothermic by 7.2 kcal mol^{-1} at the present level of theory.

energy than that required for Gly−$O^{\bullet}$ to abstract a hydrogen from the R−S-OH moiety (i.e., rearrangement of **4** to **5** via TS**4**), which proceeds with little or no energy barrier (see above). Hence, it is unlikely that the Gly−$O^{\bullet}$ moiety will fragment via either of the above two dissociation pathways as they are unable to compete effectively with the preferred hydrogen abstraction mechanism.

The C−C bond of H_2N-CH($O^{\bullet}$)-COOH, however, dissociates via TS**8** at a cost of just 2.1 kcal mol^{-1} to form NH_2CHO + $OC^{\bullet}OH$, which lie 14.5 kcal mol^{-1} lower in energy. The $OC^{\bullet}$-OH moiety can then readily abstract a hydrogen from the R−S-OH moiety of complex **4**. The remarkably low barrier for cleavage of the C−C bond may in fact allow this pathway to compete, to a minor extent, with abstraction of the R−S-OH hydrogen by Gly−$O^{\bullet}$. Although cleavage of the C−N bond via hydrolysis of the α-hydroxyglycine moiety, Gly−OH, is still predicted to be the favored pathway, the relative ease of cleavage of the C−C bond of H_2N-CH($O^{\bullet}$)-COOH may help to explain the observation by Reddy et al.[14] of fragmentation products that suggested cleavage at the C−C bond of G734, as well as cleavage at the C−N bond.

Conclusions

The mechanism proposed in this present paper (Figure 5) is similar, at least in the initial steps, to that proposed by Reddy et al.[14] and shown in Scheme 1b. In both mechanisms, O_2 initially adds to the glycyl radical ($Gly^{\bullet}$) to form a glycyl−peroxyl radical (Gly−O-$O^{\bullet}$). This addition reaction is calculated to occur with little or no barrier. The resulting glycyl−peroxyl radical is then able to abstract a hydrogen from a spatially close thiol moiety, with a barrier of approximately 10.4 kcal mol^{-1}, to form the corresponding glycyl−hydroperoxide (Gly−O-OH) and thiyl radical (R−$S^{\bullet}$).

In the mechanism proposed by Reddy et al,[14] a second O_2 then adds to the thiyl radical moiety. This necessarily implies that the O_2 concentration is indeed high enough and that it is physically possible for a second O_2 to enter into the active site. We propose that the thiyl radical may instead abstract the terminal −OH group of the glycyl-hydroperoxide to form the corresponding R−S-OH moiety and glycyl−alkoxy radical (Gly−$O^{\bullet}$). Such a mechanism is found to have a barrier of approximately 10.6 kcal mol^{-1}. The glycyl−alkoxy radical is then able to abstract a hydrogen from the R−S-OH moiety, with

little or no barrier, to give the corresponding sulfinyl radical (R−SO•) and the α-hydroxyglycine. The overall reaction scheme from the glycyl−peroxide radical + thiol to α-hydroxyglycine + sulfinyl radical is outlined in Figure 5. Once α-hydroxyglycine is formed, it will rapidly undergo hydrolysis and rupture at the C_α−N bond. Alternatively, the glycyl−alkoxy radical may fragment at the C−C bond with a barrier of just 2.1 kcal mol^{-1}. Thus, it is feasible that this alternative mechanism may compete to a minor extent with the former hydrogen abstraction mechanism, yielding the two fragmentation products observed in wild-type and C418A PFL.

It should be noted that the present model represents the most likely scenario in the absence of a well-defined protein structure. After the crystal structure of the enzyme has been determined, the present schemes may require modification to take into account stereochemical aspects or explicit interactions with additional amino acids not yet known to be crucial for the enzymatic reaction.

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Supporting Information Available: Total energies, obtained at the B3-LYP/6-31G(d,p) and B3-LYP/6-311+G(2df,p)//B3-LYP/6-31G(d,p) levels of theory, B3-LYP/6-31G(d,p) zero-point vibrational energies (Table S1), and archive entries for the B3-LYP/6-31G(d,p) optimized geometries (Table S2) (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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