Catalytic Mechanism of Pyruvate Formate-Lyase (PFL). A Theoretical Study

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Received June 16, 1998. Revised Manuscript Received August 27, 1998

Abstract: Pyruvate formate-lyase (PFL) is a glycyl radical containing enzyme that catalyzes the reversible CoA-dependent conversion of pyruvate into acetyl-CoA and formate. We have studied the catalytic mechanism of this enzyme by means of accurate quantum chemical methods. It is shown that an overall homolytic radical mechanism is very feasible. In particular, the formation of a tetrahedral radical intermediate, by addition of thiyl radical to pyruvate, is supported by the calculated reaction energies and barriers. Furthermore, we propose that the thioester exchange between active site cysteine and CoA proceeds via a radical mechanism. This is made possible by the quenching of the formate radical by Cys418, and not Gly734, as previously proposed.

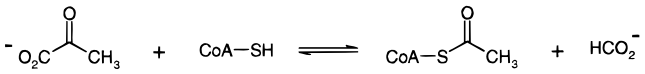
I. Introduction

Pyruvate formate-lyase (PFL) from *Escherichia coli* catalyzes the reversible conversion of pyruvate and CoA into acetyl-CoA and formate (Scheme 1). It is essential for the anaerobic glucose metabolism in *Escherichia coli* and several other prokaryotes.[1] The PFL reaction is fully reversible with very high turnover numbers in both directions (k_{cat} = 770 and 260 s^{-1} for the forward and backward directions, respectively). The enzyme exhibits two-step ping-pong kinetics with acetylated enzyme intermediate.

Three amino acid residues have emerged as essential for the overall catalysis, namely the glycyl radical at position 734 and two cysteines at positions 418 and 419. The glycyl radical is the site of fragmentation of the activated protein upon exposure to oxygen,[2] and it has been shown by means of electron paramagnetic resonance (EPR) measurements that it exchanges its α-hydrogen with the solvent, a process for which active site cysteine 419 is required.[3] The two cysteines are both required for overall catalysis.[4] However, there is no consensus about their specific roles in the catalytic process. Both Cys418 and Cys419 are, for instance, proposed to be the site for transient creation of thiyl radical and, moreover, both are suggested to be the site for acetylation.[1]

By studying dioxygen inactivation of PFL, Reddy et al. showed in a recent paper that the radical storage site, Gly734, and the substrate binding site, Cys418 and Cys419, are in close proximity to each other.[5] It was concluded that the protein-based radicals generated after oxygen exposure are a sulfinyl ($RSO^{\bullet}$) and a peroxyl radical ($ROO^{\bullet}$), shown to be located at C419 and G734, respectively. Another indication for the proximity was obtained earlier, in that Cys419 catalyzes the hydrogen exchange of the glycyl radical as mentioned above.[3]

Scheme 1. Reaction Catalyzed by Pyruvate Formate-Lyase



On the basis of numerous biochemical studies on engineered enzymes, isotope labeling experiments, and mechanism-based inhibition, two different mechanisms have been proposed. Although a heterolytic mechanism has not completely been ruled out, both the proposed mechanisms are of homolytic nature. This is based on several experimental observations. For instance, there is a paramagnetic species observed in the active form of the enzyme, but not in the inactive form. There is also a correlation between the increase of the EPR signal of the organic radical and the increase in catalytic activity during activation, indicating participation of the radical in the catalysis. And third, primary deuterium kinetic isotope effects are observed on the rates of pyruvate formation with deuterioformate.[1b]

In the first mechanism, proposed by Knappe and co-workers (Scheme 2), the protein radical is transferred from glycyl to Cys418 after pyruvate has added to the Cys419, building a thiohemiketal moiety. The thiyl radical of Cys418 then forms an adduct to the carboxyl of the thiohemiketal. An intramolecular hydrogen atom transfer follows, yielding the alkoxy radical intermediate that undergoes the homolytic C−C bond cleavage. Another intramolecular hydrogen atom transfer occurs in the Cys418−formate radical adduct, resulting in an oxy radical that dissociates to form free formate and Cys418 radical. The thiyl radical at Cys418 is finally quenched by Gly734, completing the first half reaction. The acetyl group transfer from Cys419 to CoA is proposed to use Cys418 as a nucleophilic relay.

The second mechanism was first proposed[6] and later modified by Kozarich and co-workers[3] (Scheme 3). A transient formation of a thiyl radical (formed by abstraction of a hydrogen atom

(1) For recent reviews see: (a) Knappe, J.; Wagner, A. F. V. *Methods Enzymol.* **1995**, *258*, 343. (b) Wong, K. K.; Kozarich, J. W. In *Metal Ions in Biological Systems*; Vol. 30, *Metalloenzymes Involving Amino Acid-Residue and Related Radicals*; Sigel, H., Sigel A., Eds.; Marcel Dekker: New York, 1994; p 279.

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(3) Parast, C. V.; Wong, K. K.; Lewisch, S. A.; Kozarich, J. W.; Peisach, J.; Magliozzo, R. S. *Biochemistry* **1995**, *34* (8), 2393.

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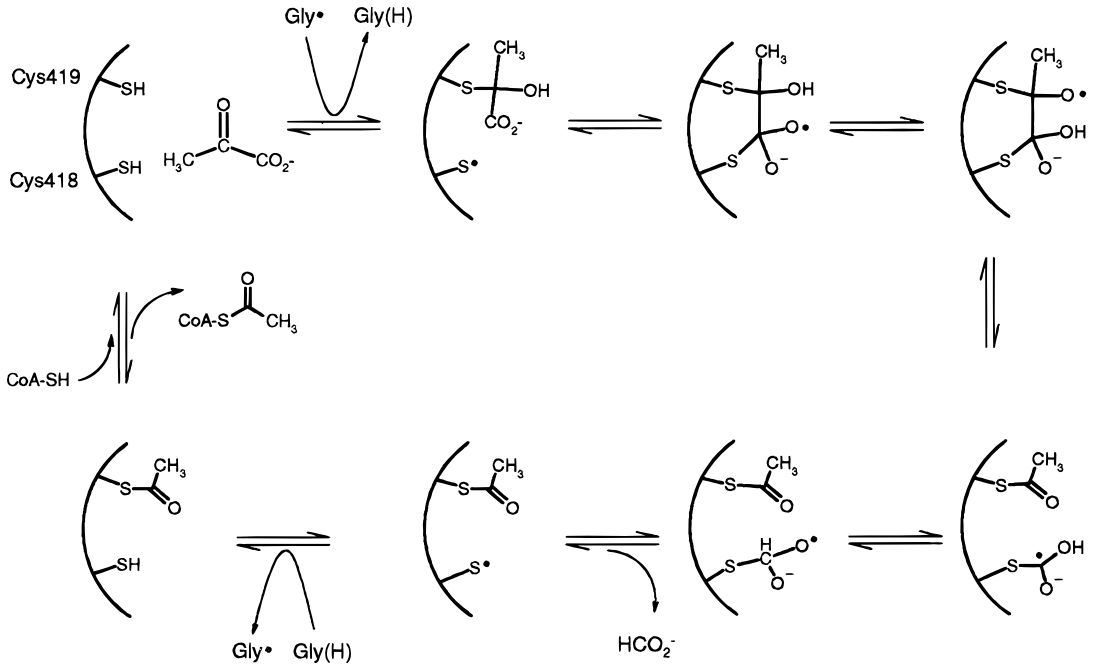
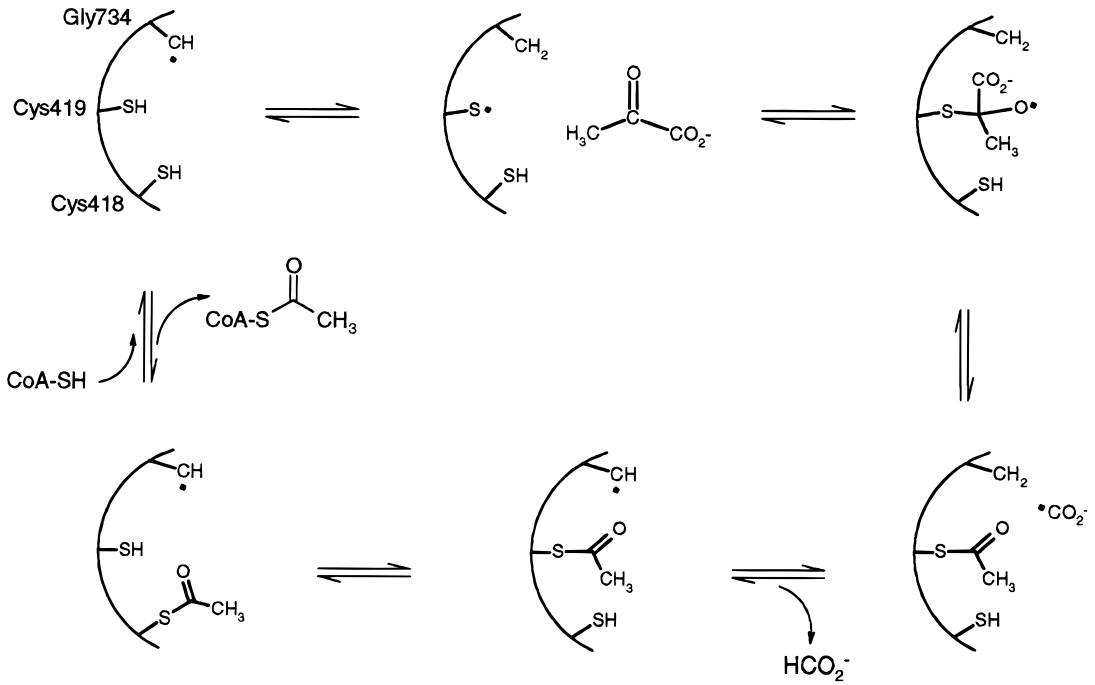
10.1021/ja9820947 CCC: $15.00
Published on Web 10/22/1998

Scheme 2. Reaction Mechanism Proposed by Knappe and Co-workers



Scheme 3. Reaction Mechanism Proposed by Kozarich and Co-workers



from a cysteine residue to the glycyl radical) has been proposed as the initial step in the catalytical reaction. This step is followed by the formation of a tetrahedral oxy-radical intermediate from the addition of the thiyl radical to the keto group of the pyruvate. This intermediate collapses into an acetylated cysteine and a formyl radical, which subsequently abstracts a hydrogen atom from the quenched glycine radical. A step of the transesterification between C419 and C418 takes place before the reaction is completed by the CoA-dependent thioester exchange. This reaction mechanism was modeled after the Minisci reaction for the cleavage of α-ketoesters by Fenton's reagent.

In the present work we focus mainly on the Kozarich mechanism. The first three steps of this model, i.e., the hydrogen atom transfer from Cys419 to the glycyl radical, the formation of a tetrahedral radical intermediate, and its collapse, are supported by favorable reaction energies and barriers. For the transesterification step, we will propose a radical mechanism initiated by the quenching of the formate radical by Cys418, and not Gly734 as previously proposed. We will also propose a radical mechanism for the acetyl transfer to CoA.

The theoretical method employed is the Hartree−Fock/Density Functional Theory (HF/DFT) hybrid method B3LYP.[7] This method, developed by Becke, includes three parameters fitted to experiments and has recently been proven to be very

(7) (a) Becke, A. D. *Phys. Rev.* **1988**, *A38*, 3098. Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 1372. (b) Becke, A. D. *J. Chem. Phys.* **1993**, *98*, 5648.

suitable for these kinds of studies.[8−13] In particular, two studies are of importance for the present work. In a study on hydrogen atom transfer in the presence of amino acid radicals, Siegbahn et al. concluded that the B3LYP functional is adequate for studying these kinds of processes.[10] Siegbahn has also recently shown the B3LYP functional to be highly appropriate in a detailed study of the substrate mechanism on RNR.[11] Many of the reactions studied there resemble the reactions involved in the mechanism of PFL, especially those concerning thiol groups. A general feature of DFT is, though, that it underestimates reaction barriers by a couple of kcals/mol.[14] This should, however, not have any major implications on the present study, since we are not aiming at determining the exact energetics of the reactions studied, but rather testing whether a mechanism as a whole is energetically possible or not.

II. Computational Details

All geometries and energies presented in the present study are computed with the B3LYP[7] density functional theory method as implemented in the Gaussian94/DFT program package.[15]

The calculations were performed in two steps. Geometry optimizations were performed with the triple-ζ basis set 6-311G(d,p), followed by single point energy calculation with the larger basis set 6-311+G-(2d,2p). This basis set includes diffuse functions and double polarization functions on each atom.

Hessians were calculated at the B3LYP/6-311G(d,p) level of theory for all systems except the largest ones, containing the carboxyl moiety as catalytic mediator. For these systems, the Hessians were calculated at the HF/6-31G level of theory and then scaled with 0.9, as usual.

The reason for calculating Hessians is 2-fold. First, vibrational frequencies provide a control that the stationary points localized are correct, with no imaginary frequencies for minima and one imaginary frequency for transition states. Second, Hessians are used to estimate the zero-point vibrational effects on energy. All energies discussed below include zero-point energy (ZPE) effects.

Finally, the spin densities reported are calculated with Mulliken population analysis.

III. Results and Discussion

Before presenting the results, it is important to say a few words about the chemical models employed in the present work. To increase computational speed, it is important to use as small models as possible. Since the most critical property for our study is the X−H bond strength, it is hence crucial that the small models employed can reproduce these bond strengths accurately.

The smallest model of cysteine is, of course, H_2S. The calculated S−H bond strength for the full cysteine residue is 81.7 kcal/mol. For H_2S, it is 87.1 kcal/mol, an overestimation by 5.4 kcal/mol. Clearly H_2S is not an adequate model of cysteine. A larger, but still very small, model is methylthiol, $HSCH_3$. The calculated S−H bond strength for this species is 81.9 kcal/mol, i.e. only 0.2 kcal/mol higher than for the full residue. Addition of one more methyl group, yielding ethylthiol ($HSCH_2CH_3$), does not give any significant change to the S−H bond strength. The Mulliken spin densities on the sulfur atom for $^{\bullet}SH$, $^{\bullet}SCH_3$, $^{\bullet}SCH_2CH_3$, and the full cysteine radical are 1.03, 0.98, 0.98, and 0.98, respectively. Methylthiol is clearly sufficiently large to model the cysteine residue and is therefore used in the present work. For the same reasons, $HSCH_3$ is also chosen as a model for CoA, since the active part of CoA is its thiol tail.

For glycine, it is not possible to choose such a small model. Instead, in this case, one even has to include parts of the backbone of neighboring amino acids to achieve the correct (effective) π-electron delocalization known to be present in the radical. The model used, CHO−NH−CH_2−CO−NH_2 (Figure 1), is planar and has a C_α−H bond strength of 79.3 kcal/mol. The somewhat smaller model, NH_2−CH_2−CHO, was also tested, but was discarded on the basis of its too low C_α−H bond strength (70.3 kcal/mol).

As can be seen in Figure 1, the calculated spin density for glycyl is delocalized on basically all heavy atoms in the molecule. The spin density on the C_α center is 0.68, which agrees very well with other theoretical calculations,[16] but is a bit higher than the experimentally estimated spin density (0.55).[1a] The two carbonyl oxygens furthermore have relatively high concentration of spin (0.11 on each).

Another important point is that all our models are chosen to be neutral. Pyruvate, for instance, is modeled by pyruvic acid, and formate by formic acid (see Figure 1). This can be justified by noting that proteins in general have low dielectric constants ($\epsilon \approx 4$), which means that charge separation, if present, should be very small, unless the system is specifically set up to create and enhance charge separation, as in, e.g., photosystem II. This approach has furthermore been applied very successfully in previous work on, e.g., the Wacker process[17] and the substrate mechanism in ribonucleotide reductase.[11]

In the initial phase of this study, anionic models were tested. It turned out that Coulombic interactions were far too dominating, and we had major difficulties reaching convergence for these calculations.

It should be noted, though, that the approach chosen is not entirely without problems. Addition of a proton can result in isomers with slightly different energies. The added protons may also give rise to hydrogen bonds that do not exist in reality.

We should finally emphasize that we are dealing with models, and the agreement or disagreement with experimental observables, such as measured rates and energetics, is the only means to judge their adequacy.

a. Step 1. Creation of the Thiyl Radical. It is now well established, by means of mechanism-based inhibition experiments, that a sulfur-centered radical is involved in the PFL catalysis.[6] The initial step of the catalytic cycle proposed by Kozarich is the creation of a transient thiyl radical at the Cys419 position.

Assuming that the cysteines are in close spatial proximity to the glycyl radical (see the Introduction), we calculate a barrier

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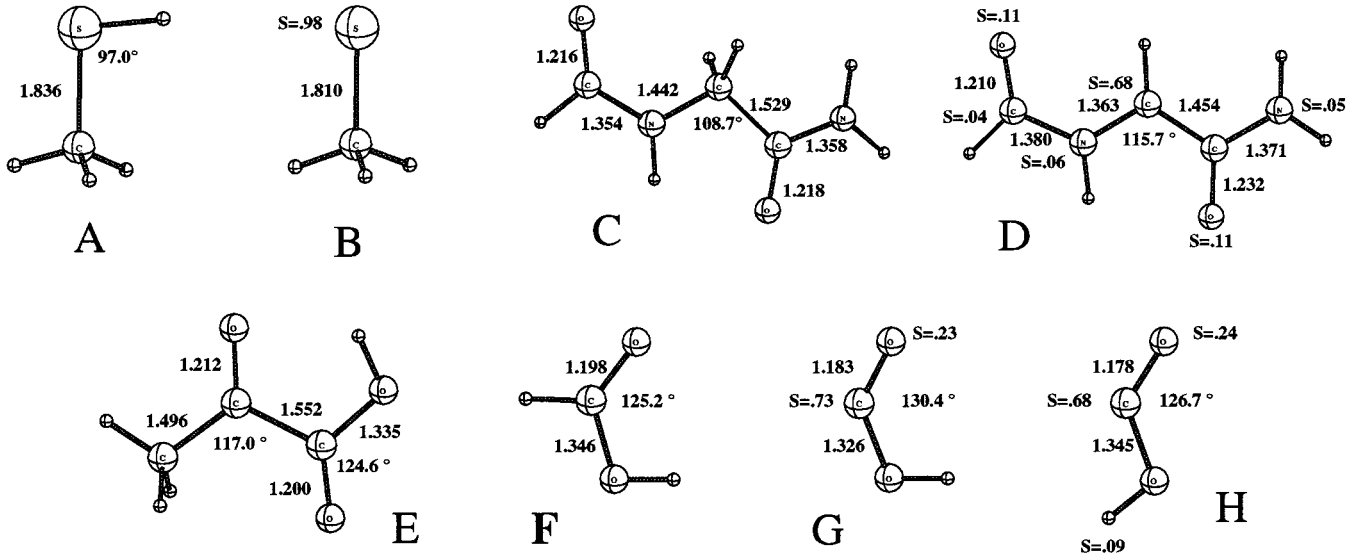

Figure 1. Geometry optimized structures and calculated Mulliken spin densities of models used in the present study: (A) methylthiol (model of cysteine and CoA); (B) methylthiyl radical; (C) extended glycine; (D) glycyl radical; (E) pyruvic acid; (F) formic acid; (G) formyl radical in the *cis*-conformer; (H) formyl radical in the *trans*-conformer. Distances, in this and the rest of the figures, are in Å.

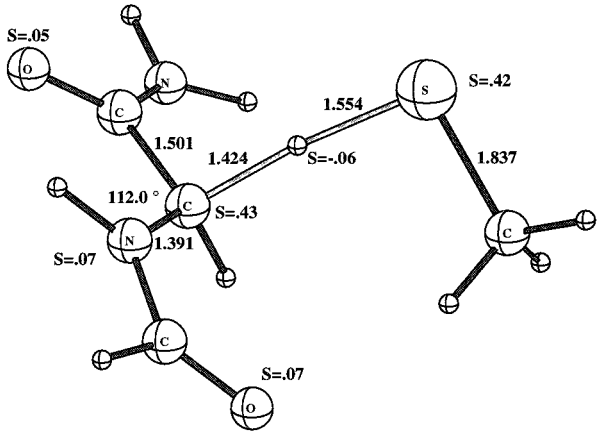

Figure 2. Optimized transition state structure for the hydrogen atom transfer from cysteine to glycyl radical (step 1).

of 9.9 kcal/mol for the direct hydrogen transfer reaction between glycyl and cysteine. The reaction is slightly endothermic (3.4 kcal/mol), i.e., the glycyl radical is more stable than the cysteinyl radical. This is supported by the fact that the stable radical observed in the EPR experiments is located on glycine and not cysteine. The spin population at the transition state is distributed mainly on the sulfur and the glycyl C_α center (0.42 and 0.43, respectively). Also here the carbonyl oxygens have some spin, 0.05−0.07 (see Figure 2).

The energetics calculated for this reaction clearly show that the radical transfer step, provided direct contact between glycyl and cysteine exists, is perfectly feasible. It has been speculated that the residues neighboring Gly734 play a certain role in stabilizing the radical at that position.[1] This would imply that both the barrier and the endothermicity calculated here would increase by a few kcal/mol. Even so, the reaction should be plausible.

b. Step 2. Formation and Collapse of the Tetrahedral Oxy Radical. For the first step we have shown that the radical transfer from glycyl to cysteine is perfectly feasible. The next step according to Kozarich's model is to add the thiyl radical to the C-2 (the carbonyl carbon) of pyruvate, building a tetrahedral oxy-radical intermediate.

We have in the present work succeeded in localizing this postulated radical intermediate. The calculated barrier for the addition was found to be 12.3 kcal/mol and the endothermicity 9.9 kcal/mol. This intermediate is highly unstable; the barrier for its dissociation into acetylated cysteine and formyl radical was calculated to be only 2.8 kcal/mol. The dissociation is exothermic by 3.9 kcal/mol, which makes the total reaction

$$\text{Cys419}^\bullet + \text{pyruvate} \rightarrow \text{Cys419-acetyl} + \text{formyl}^\bullet$$

endothermic by 6.0 kcal/mol.

These results show that the proposed reaction mechanism involving the tetrahedral radical intermediate is energetically plausible. The structures of the first transition state (TS1), the tetrahedral intermediate, and the second transition state (TS2) are shown in Figure 3 along with the computed spin densities.

In TS1 (Figure 3A), the S−C2 bond length is 2.13 Å (cf. "normal" S−C distances 1.85−1.90 Å) and the C2−$C_{carboxyl}$ bond is elongated to 1.66 Å (1.55 Å in pyruvate). The carbonyl CO distance is still of doubly bonded nature (1.25 Å), only slightly longer than in free pyruvate (1.21 Å), and the spin is distributed on the sulfur atom (0.52), the carbonyl oxygen (0.24), and the carboxylic CO group (0.21).

The tetrahedral radical intermediate (Figure 3B) exhibits a different spin pattern. All the spin is mainly concentrated on two centers, the sulfur atom (0.49) and the carbonyl oxygen (0.55). The carbonyl CO bond length is now 1.34 Å, clearly of single bond nature. The S−C2 distance is 1.92 Å and the C2−$C_{carboxyl}$ is 1.56 Å.

At the second transition state (Figure 3C), when the formyl group leaves the tetrahedral radical intermediate, the carbonyl CO bond length is back to 1.25 Å, the S−C2 distance is 1.88 Å, and the critical C2−$C_{carboxyl}$ bond is 1.98 Å. The spin density is now moving over to the carboxylate group (0.58), although some of the radical character still remains on the other atoms, S (0.23) and carbonyl oxygen (0.20).

Another possible reaction at this step is the direct thiolate attack at the carbonyl carbon of pyruvate, yielding a nonradical tetrahedral intermediate (see Knappe's mechanism in Scheme 2). The energy of this intermediate is only 1.6 kcal/mol higher than the reactants. However, the barrier for the direct thiolate attack is calculated to the very high at 37.6 kcal/mol.

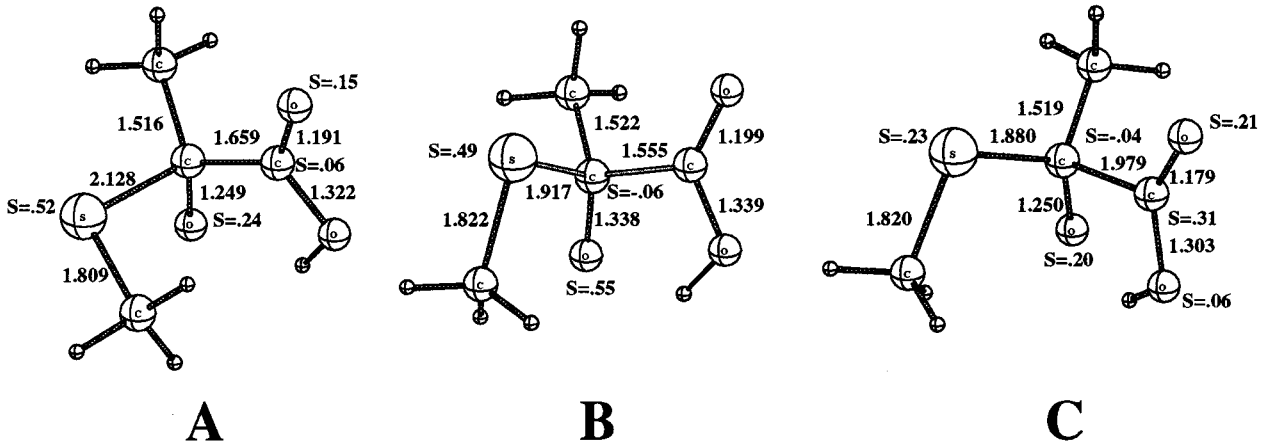

Figure 3. Formation and collapse of the tetrahedral oxy radical (step 2). Optimized structures for (A) the transition state of the thiyl radical addition to pyruvate (TS1), (B) the tetrahedral oxy-radical intermediate, and (C) the transition state of the dissociation of formyl radical (TS2). The energies of these species relative to (methylthiyl + pyruvic acid) are 12.3, 9.9, and 12.7 kcal/mol for A, B, and C, respectively.

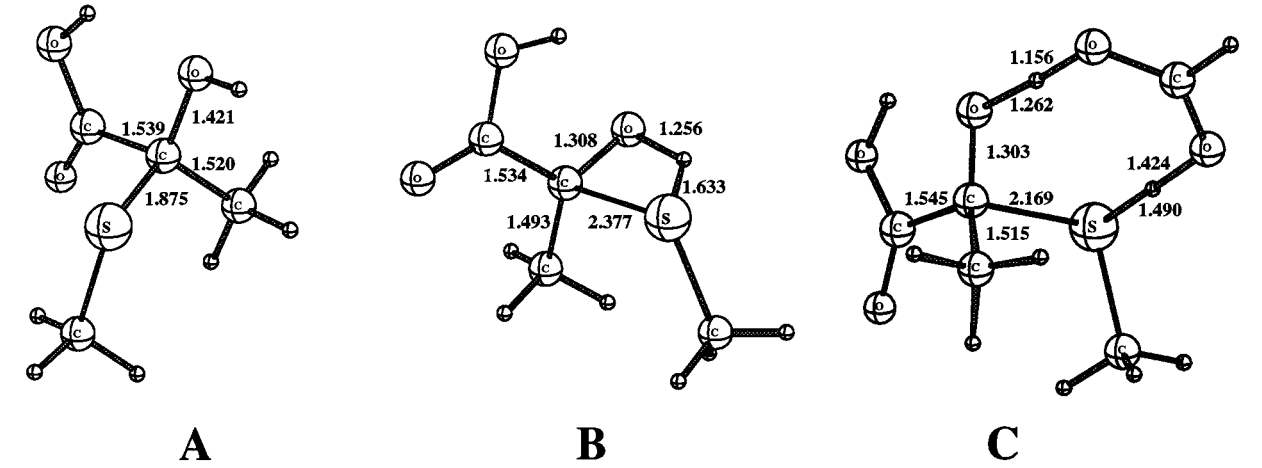

Figure 4. Optimized structures for (A) the nonradical tetrahedral intermediate formed upon addition of cysteine to pyruvate, (B) the transition state for direct thiolate attach on the carbonyl of pyruvate, and (C) the thiolate attack mediated by a carboxyl group.

In the recent study by Siegbahn on the substrate mechanism of RNR,[11] a similar result was obtained for the addition of cysteine to a carbonyl carbon in a ribose ring. It was also shown that mediation of a glutamate group lowered the barrier considerably relative to the direct thiolate attack (41 vs 16 kcal/mol). In line with this result, we allowed a carboxylate group to mediate in this reaction. The barrier is thereby lowered to 12.0 kcal/mol. The structures are given in Figure 4.

This is clearly competitive with the radical step, provided a carboxylate group-containing residue (Glu or Asp) is present to do the catalysis. No such group is, however, known to participate in the catalytic reaction, and we, hereafter, consider only the radical reaction.

c. Step 3. Quenching of the Formyl Radical. The formyl radical, released in the previous step, is a very reactive species. In the mechanism proposed by Kozarich et al.,[3] it abstracts a hydrogen atom from Gly734, regenerating the stable glycyl radical. This step has a calculated barrier of 4.9 kcal/mol and is exothermic by 17.5 kcal/mol (Figure 5A). There is, however, no direct evidence for this specific reaction to occur. Another possibility is that the formyl radical abstracts a hydrogen atom from the cysteine at position 418 instead of Gly734. Our calculations show that this is more probable. The barrier for the reaction is only 1.1 kcal/mol, and the reaction is exothermic by 14.1 kcal/mol (Figure 5B). Both reactions are, thus, energetically very plausible. However, the hydrogen abstraction from cysteine, with its vanishing barrier, is a very attractive idea and there are several facts that speak in favor of this reaction. First, the very low barrier of 1.1 kcal/mol. Second, proximity; Cys418 is in the immediate vicinity of Cys419, the site from which the formyl radical is released. Finally, the creation of a radical at the Cys418 position will allow for the proposed transesterification step, between Cys419 and Cys418, to occur via a radical mechanism. This is much more plausible, owing to its lower barriers. The mechanism for this step is discussed in greater detail in the following subsections.

d. Step 4. Acetyl Transfer between Cys419 and Cys418. The acetyl group is now residing at Cys419. According to Kozarich's model, the acetyl group is transferred to Cys418, a proposal that is based on two facts. In acetylated PFL (the result of addition of pyruvate to activated PFL, in the absence of CoA), glycyl can still exchange its hydrogen, indicating that Cys418 is the site of acetylation, since Cys419 is needed for the hydrogen exchange reaction. From site-directed mutagenesis experiments it is also known that Cys418 is the primary residue participating in the thioester exchange with CoA, since thioester exchange to CoA is observed for C419S mutant, but not for C418S.[3]

We proposed in the previous step that Cys418 quenches the formyl radical, rather than Gly734. Once the cysteine radical is created at that position, the proposed acetyl group transfer from Cys419 to Cys418 is easily accomplished via a homolytic radical mechanism. In Figure 6A we present the transition state structure for this reaction. This thermoneutral reaction has a

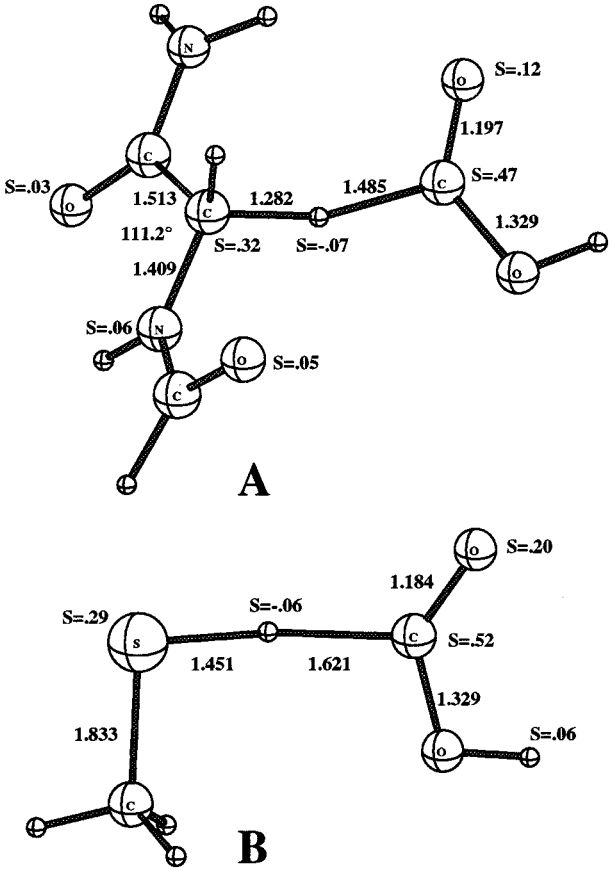

Figure 5. Optimized transition state structure for the hydrogen atom transfer to formyl radical from (A) glycine and (B) cysteine.

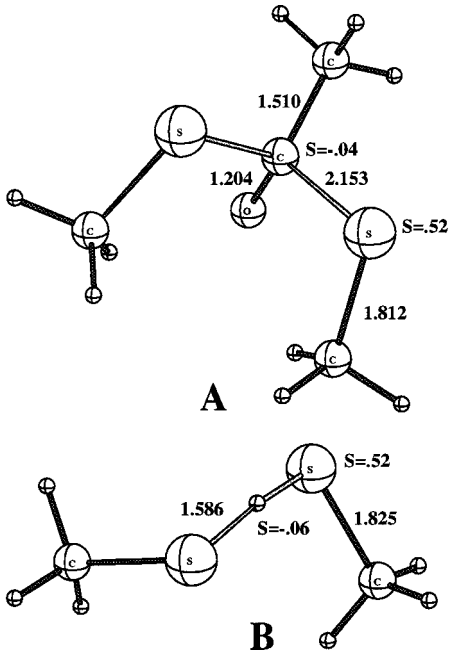

Figure 6. Optimized transition state structure for (A) homolytic radical acetyl group transfer between two cysteines (step 4) or a cysteine and CoA (step 5) and (B) hydrogen atom transfer between a cysteine radical and CoA (step 5).

calculated barrier of 11.6 kcal/mol, which is quite low considering that homolytic substitution reactions at carbon are known to be very rare.[18] Several attempts to localize a tetrahedral oxy-radical intermediate, similar to the one in step 2, were undertaken without any success; the reaction simply occurs in one step, without any intermediate.

Alternatively, direct nucleophilic attack by the sulfur of nonradical cysteine on the carbonyl carbon of acetylated cysteine, yielding a tetrahedral intermediate, may be considered. As in the case of nucleophilic attack of cysteine on pyruvate (see step 1), the intermediate lies relatively low in energy (9.0 kcal/mol). The calculated barrier, however, is very high (41.2 kcal/mol). Even when letting a carboxyl group mediate in the reaction, the barrier is considerable (20.0 kcal/mol). A nucleophilic attack can thus be ruled out for this step.

e. Step 5. Acetylation of CoA. The first half-reaction is completed by the acetyl group transfer to Cys418. The second half consists of two parts, the transfer of the acetyl group to CoA and the regeneration of the stable glycyl radical. The transfer of the acetyl group to CoA is similar to the acetyl group transfer between the two cysteines (step 4). In fact, in our model these two steps are identical, since both cysteine and CoA are modeled by methylthiol. A radical reaction is thus appropriate even here. Therefore, we propose that the transesterification occurs in two steps:

$$\text{CoA-SH} + \text{Cys419}^{\bullet} \rightarrow \text{CoA-S}^{\bullet} + \text{Cys419} \quad (1)$$

$$\text{CoA-S}^{\bullet} + \text{Cys418-acetyl} \rightarrow \text{CoA-acetyl} + \text{Cys418}^{\bullet} \quad (2)$$

Reaction 1 is a simple hydrogen atom transfer, where CoA-SH donates its hydrogen atom to the Cys419 radical. The calculated barrier for this thermoneutral step is very low, 2.4 kcal/mol. The structure of the transition state for this reaction is shown in Figure 6B. Reaction 2 is identical to the acetyl group transfer step discussed in the previous subsection, with a barrier of 11.6 kcal/mol. One experimental fact that supports the hypothesis of the radical reaction at this step is that the reaction rate for the thioester exchange of CoA with the radical acetylated enzyme is enhanced 10^5-fold compared to the nonradical form, which also can be separated.[1b]

f. Step 6. Regeneration of the Glycyl Radical. The final step in this mechanism is the regeneration of the glycyl radical. The radical is now located at Cys418, which makes this step the reverse of step 1. The reaction has now, in the backward direction, a barrier of 6.5 kcal/mol and an exothermicity of 3.4 kcal/mol. This assumes of course that the two moieties are in close enough proximity that direct H-transfer can occur. If not, the radical can first be transferred to Cys419 via a hydrogen atom transfer (barrier = 2.4 kcal/mol), and then over to Gly734.

IV. Summary and Conclusions

In Table 1 we summarize the calculated energetics for the various reaction steps of the PFL mechanism, and in Scheme 4 the energetics are displayed.

In general, the calculations support the homolytic reaction mechanism proposed by Kozarich and co-workers. In particular, the tetrahedral radical intermediate, proposed to be created upon addition of the thiyl radical of the cysteine residue to pyruvate, is shown to have reasonable energy (+9.9 kcal/mol) and activation barrier (+12.3 kcal/mol). It was also shown that an alternative nonradical direct nucleophilic attack, although having feasible overall energy (+1.6 kcal/mol), has a very high activation barrier (37.6 kcal/mol). Addition of a carboxylate group as a mediator in the reaction lowers the activation barrier considerably (to 12.0 kcal/mol), which clearly can compete with the radical reaction, provided there is a carboxyl-containing group in the vicinity.

(18) Perkins, M. J. Radical Chemistry. In *Ellis Horwood Series in Organic Chemistry*; Ellis Horwood: London, 1994.

Scheme 4. Calculated Overall Energetics for the Various Steps in the Catalytic Mechanism of PFL

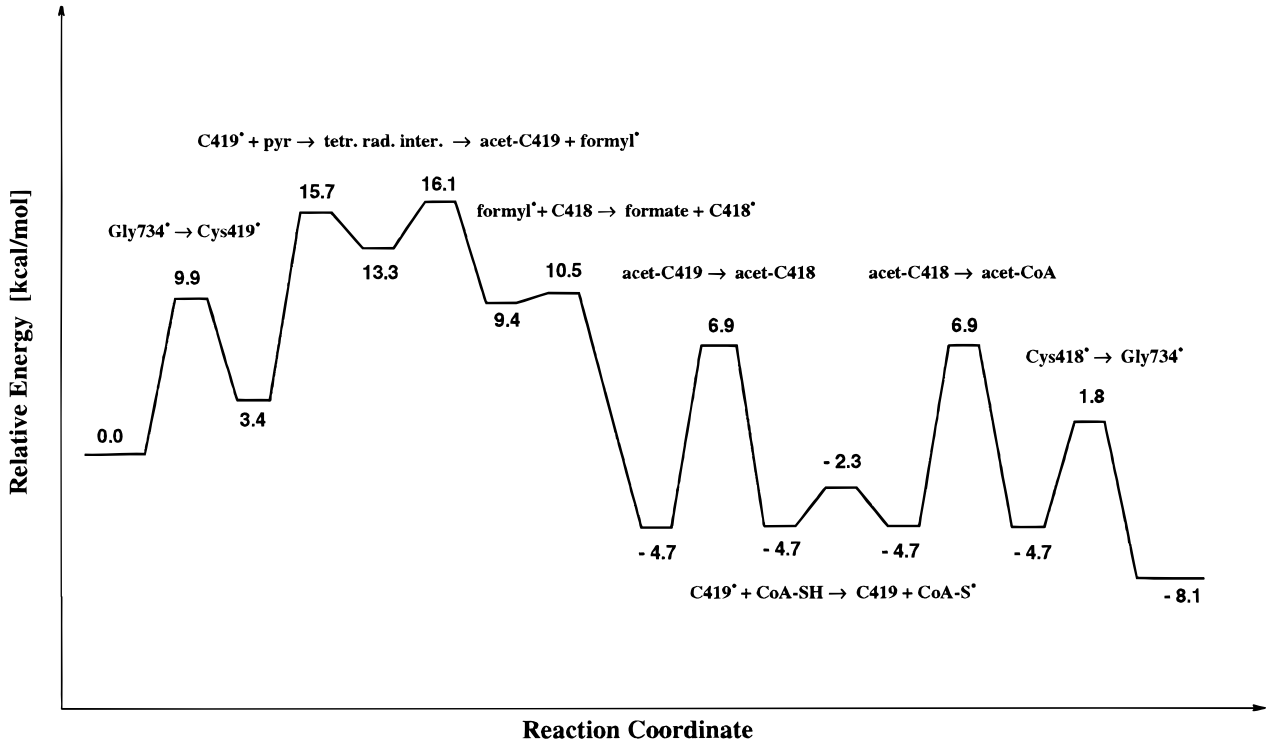

Table 1. Summary of the Reaction Energies and Barriers Calculated in the Present Work (kcal/mol)

reaction	barrier	energy
$Gly^{\bullet} + Cys \rightarrow Gly + Cys^{\bullet}$	9.9	3.4
Cys + pyruvate → tetrahedral intermediate	37.6	1.6
Cys + pyruvate $\xrightarrow{\text{carboxyl}}$ tetrahedral intermediate	12.0	1.6
$Cys^{\bullet}$ + pyruvate → tetrahedral radical intermediate	12.3	9.9
tetrahedral radical intermediate → acetylated Cys + $formyl^{\bullet}$	2.8	−3.9
$Cys^{\bullet}$ + pyruvate → acetylated Cys + $formyl^{\bullet}$		6.0
$formyl^{\bullet} + Gly \rightarrow formate + Gly^{\bullet}$	4.9	−17.5
$formyl^{\bullet} + Cys \rightarrow formate + Cys^{\bullet}$	1.1	−14.1
acetyl-Cys + $Cys^{\bullet} \rightarrow Cys^{\bullet}$ + acetyl-Cys	11.6	0.0
acetyl-Cys + $CoA\text{-}S^{\bullet} \rightarrow Cys^{\bullet}$ + acetyl-CoA	11.6	0.0
acetyl-Cys + Cys (or CoA) → tetrahedral intermediate	41.2	9.0
acetyl-Cys + Cys (or CoA) $\xrightarrow{\text{carboxyl}}$ tetrahedral intermediate	20.0	9.0
$Cys + Cys^{\bullet} \rightarrow Cys^{\bullet} + Cys$	2.4	0.0
$CoA\text{-}SH + Cys^{\bullet} \rightarrow CoA\text{-}S^{\bullet} + Cys$	2.4	0.0
$Cys^{\bullet} + Gly \rightarrow Cys + Gly^{\bullet}$	6.5	−3.4
net reaction: pyruvate + CoA → formate + acetyl-CoA		−8.1

The tetrahedral radical intermediate, once dissociating, will release the formyl radical. This radical was previously proposed to abstract a hydrogen atom from Gly734, regenerating the stable enzyme radical at that site. In the present work, we propose that the quencher of the formyl radical is Cys418 rather than Gly734. This would have a lower barrier (1.1 vs 4.9 kcal/mol), and most importantly, the thiyl radical created will allow for radical mechanisms in the following steps.

For the two transesterification steps, first between the two cysteines and later from cysteine to CoA, we show that a radical reaction is again energetically favorable over the corresponding nonradical reaction. The barrier for the radical reaction is 11.6 kcal/mol, whereas for the nonradical reaction, the barrier is 41.2 kcal/mol.

The highest barrier was found for the addition of the cysteine radical to pyruvate, indicating that this could be the rate-limiting step of the reaction. The net gain in energy for the entire reaction was calculated as 8.1 kcal/mol.

Acknowledgment. The authors wish to thank Prof. Per Siegbahn and Dr. Curtis Hoganson for valuable discussions and comments. The Swedish Natural Science Research Council (NFR) is gratefully acknowledged for financial support.

JA9820947