Modification of Cys-418 of pyruvate formate-lyase by methacrylic acid, based on its radical mechanism

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Abstract The recently determined crystal structure of pyruvate formate-lyase (PFL) suggested a new view of the mechanism of this glycyl radical enzyme, namely that intermediary thiyl radicals of Cys-418 and Cys-419 participate in different ways [Becker, A. et al. (1999) Nat. Struct. Biol. 6, 969–975]. We report here a suicide reaction of PFL that occurs with the substrate-analog methacrylate with retention of the protein radical ($K_I = 0.42$ mM, $k_i = 0.14$ min^{-1}). Using [1-^{14}C]methacrylate (synthesized via acetone cyanhydrin), the reaction end-product was identified by peptide mapping and cocrystallization experiments as *S*-(2-carboxy-(2*S*)-propyl) substituted Cys-418. The stereoselectivity of the observed Michael addition reaction is compatible with a radical mechanism that involves Cys-418 thiyl as nucleophile and Cys-419 as H-atom donor, thus supporting the functional assignments of these catalytic amino acid residues derived from the protein structure.

Key words: Pyruvate formate-lyase; [1-^{14}C]Methacrylic acid; *S*-(2-Carboxy-(2*S*)-propyl)-L-cysteine; Enzyme inactivation; Radical enzyme

1. Introduction

Pyruvate formate-lyase (PFL), catalyzing the CoA-dependent reversible cleavage of pyruvate into acetyl-CoA and formate, is the key enzyme of the anaerobic glucose fermentation route that is typical for *Escherichia coli* and various other microorganisms [1]. A glycyl radical (Gly-734) is present in the active form of this enzyme cooperating with a pair of cysteinyl residues (Cys-418, Cys-419) in performing substrate processing by a homolytic radical mechanism [2]. Biochemical studies, including those of mutants and substrate-analogs, indicated that the Cys-Cys site functions as covalent acetyl-carrier in the two half-reactions comprising the catalytic cycle and, moreover, involves an intermediary thiyl radical for promoting the homolytic substrate cleavage ([3,4] and review in [5]). Exact function assignments of the two Cys residues, however, remained elusive until the recent x-ray crystallographic structure determination of PFL (non-radical form), together with that of the binary complex with the substrate-analogous oxamate [6]. This showed the geometric positioning of the catalytic amino acid residues Gly-734, Cys-418 and Cys-419 relative to the bound substrate molecule, leading to a new mechanistic proposal where both Cys residues participate as thiyl radicals. While Cys-419 functions as relay for H-atom transfers, Cys-418 thiyl attacks covalently the carbonyl of pyruvate or, in the reverse direction of the catalytic cycle, the thioester carboxyl of acetyl-CoA (see Section 4).

We report here the interaction of PFL with methacrylate, an isosteric analog of the pyruvate substrate. Irreversible enzyme inhibition by this compound is shown to reside on covalent modification of Cys-418 by regio- and stereospecific addition of the SH-group to methacrylate's C2 = C3 double bond. The glycyl radical in PFL is absolutely required for this reaction but remains intact. These results give experimental support of the roles of the Cys residues suggested by the protein structure, in particular the function assigned to Cys-418 thiyl as the critical nucleophile.

2. Materials and methods

2.1. Chemicals

Methacrylic acid was obtained from Serva (Heidelberg, Germany) and K^{14}CN from Amersham-Buchler (Braunschweig, Germany). Methyl esters of *R*(+)- and *S*(−)-3-bromo-2-methylpropionate (Aldrich, Steinheim, Germany) were hydrolyzed with HBr (in 90% acetic acid) and the carboxylic acids purified by distillation (120–121°C/15 mm Hg). 2-Bromo-isobutyryl amide was prepared from the acyl bromide (Aldrich) and purified by crystallization from ethanol (mp 147°C).

2.1.1. Synthesis of [1-^{14}C]methacrylic acid. The synthetic procedure via the cyanohydrin of acetone [7] was carried out in a septum-closed 1 ml Reacti-vial (Pierce), containing the mixture of 200 μmol K^{14}CN (435 μCi or 16 MBq), dissolved in 0.14 ml 0.4 M KOH, and 0.14 ml acetone. After thermostatting to 10°C, 115 μl 2 M H_2SO_4 was gradually added over a period of 1 h using a syringe and the mixture subsequently stirred at room temperature for further 2 h. The solution was extracted with diethyl ether (5×0.1 ml) and the combined extracts, containing the acetone cyanohydrin, were put in a new vial. After solvent removal by a nitrogen stream and finally a short vacuum-evaporation (5 min; 20 mm Hg), 50 μl 98% H_2SO_4 and copper powder (20 mg) were added and the mixture heated with stirring in the closed vial to 130°C (aluminum block) for 20 min, yielding methacrylamide sulfate as principal product. After cooling, 0.2 ml 20 mM hydroquinone (in water) was added and the mixture stirred at 100°C for 1 h. The methacrylic acid produced was extracted into diethyl ether (5×0.3 ml) and carried through preparative TLC (silica gel 60 F_{254}, Merck; thickness 1 mm; width 16 cm) using dichloromethane/2-propanol/acetic acid (93:3:4, by vol.). After the solvents were largely evaporated off (room temperature; 30 min), the methacrylate zone ($R_F \approx 0.5$) was eluted with water (5×3 ml), the solution brought to pH 8.5 (NaOH) and concentrated by lyophilization. This stock solution (5 ml), stabilized by 4-methoxyphenol (finally 10 μM), contained 17 mM [1-^{14}C]methacrylate at a specific radioactivity of 4800 dpm/nmol.

Prior to use in enzyme labeling experiments, a sample of 0.5 ml was incubated with bovine serum albumin (5 mg) for 30 min at 30°C and

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carried then through gel-filtration (Sephadex G-25) in 50 mM Tris/HCl pH 7.4, collecting the low molecular weight fraction. This removed a ^{14}C-contaminant (about 1%) that adsorbed unspecifically to proteins.

*2.1.2. Synthesis of R(+)- and S(−)-S-(2-carboxypropyl)-L-cysteine (**1a** and **1b**).* These diastereomeric compounds (obtained previously [8] via fractional crystallization, without configurational assignment) were prepared individually by reacting L-cysteine (20 mmol) with the (*R*)(+) or (*S*)(−) enantiomer of 3-bromo-2-methylpropionic acid (13 mmol). The mixtures, contained in 30 ml 30% ethanol, were purged with nitrogen, adjusted continuously to pH 8 (NaOH) and left at room temperature for 30 h. Purification of the products comprised chromatography on Dowex-1 (acetate form; gradient up to 1 M HOAc) and crystallizations; yields, about 9 mmol. **1a**: Prisms from H_2O; mp 199°C; $[\alpha]_D^{20} = +35.8$ ($c = 1$, H_2O) or +38.2 ($c = 1$, 3 M HCl). **1b**: Needles from H_2O/acetone; mp 197°C; $[\alpha]_D^{20} = -64.1$ ($c = 1$, H_2O) or −44.4 ($c = 1$, 3 M HCl).

*2.1.3. Synthesis of S-(2-carboxyisopropyl)-L-cysteine (**2**).* 2-Bromo-isobutyryl amide (65 mmol), dissolved in 0.5 l H_2O, was added to a solution of L-cysteine hydrochloride (40 mmol) in 0.2 l 0.36 M $NaHCO_3$ and the mixture stirred under nitrogen at 40°C for 6 days. After acidification (7 ml HOAc) and concentration in vacuo to 100 ml, excessive acyl amide educt was extracted into chloroform (3×50 ml). The aqueous phase was evaporated to dryness, the solid material (amide of **2**) taken into 300 ml 2 M HCl and then heated at reflux under nitrogen for 7 h. Purification used chromatography on Dowex-1 (chloride form; gradient up to 0.1 M HCl) and crystallization; yield, 10 mmol. Needles from 65% ethanol; mp 195°C; $[\alpha]_D^{20} = -24.2$ ($c = 0.5$, H_2O) or +5.1 ($c = 1$, 3 M HCl). The structure was authenticated by elemental analysis and NMR spectroscopy.

2.2. Enzyme studies

Recombinant *E. coli* pyruvate formate-lyase (non-radical form) was converted to the active radical form as described in [9] (using oxamate as effector) and the sample (200 U/mg) subsequently gel-filtered (Sephadex G-25) into anaerobic standard buffer (0.1 M Tris/HCl pH 7.4 containing 8 mM dithiothreitol and 0.4 mM $(NH_4)_2Fe(SO_4)_2$). Catalytic activity was measured with the coupled optical assay [9]; 35 U corresponds to 1 nmol (0.17 mg) of the dimeric enzyme with one glycyl radical. The glycyl radical content of methacrylate-modified PFL was measured EPR spectroscopically or via the polypeptide scission (formation of 82 kDa fragment) on enzyme exposure to oxygen [9]. Protein-bound radioactivity was determined as described [10].

2.2.1. Enzyme modification and peptide mapping. PFL (900 U, 26 nmol) contained in 1.6 ml anaerobic standard buffer was reacted under argon at 30°C with 1.8 μmol of [^{14}C]methacrylate for 65 min. After addition of EDTA (2.5 μmol), the enzyme was gel-filtered into 0.2 M Tris/HCl pH 8.5 containing 1 mM EDTA, denatured with guanidine hydrochloride (7 M) and then *S*-carboxymethylated (25 mM iodoacetate, 20 min, 37°C). The modified protein, containing 24 nmol ^{14}C-label, was precipitated with trichloroacetic acid, washed with 80% acetone, dried in vacuo and digested with chymotrypsin (3 mg in 2 ml 0.1 M NH_4HCO_3; 30 min, 37°C). The solution was adjusted to pH 2 with TFA, filtered through SepPak C_{18} (Waters) and the adsorbed peptide mixture then eluted with 80% CH_3CN in 0.1% TFA.

Fractionation of the peptide mixture by HPLC used successive runs with the following systems: (a) C_{18} RadialPak (Waters), with 0–60% CH_3CN in 0.1% TFA; (b) DEAE-TSK (Beckman/Toyo Soda), with 5–500 mM potassium phosphate pH 6.8; (c) C_{18} (Nucleosil, Macherey-Nagel), with 0–60% CH_3CN in 0.1% TFA; (d) C_{18} column as in (c) with 0–60% methanol in 10 mM potassium phosphate pH 7.0. With run (a), two distinctive ^{14}C-fractions (at 27% CH_3CN (peptide CM-1) and 31% CH_3CN (peptide CM-2)) at a ratio of about 1:1 (total ^{14}C-recovery, 73%) were obtained. Purifications to homogeneity yielded 3 nmol of CM-1 (via (b), (c) and (d)) and 4 nmol of CM-2 (via (b) and (c)). Radiosequencing of these peptides was performed as in [10].

2.2.2. Structure assignment of the Cys-418 derivative. [1-^{14}C]Methacrylate labeled enzyme (25 nmol, prepared as described above) was precipitated with trichloroacetic acid, washed with 80% acetone, vacuum-dried and hydrolyzed in 2 ml 6 M HCl (24 h, 105°C). The vacuum-dried hydrolysate was dissolved in 0.05% TFA (1 ml) and carried (in 100 μl aliquots) through HPLC with a C_{18} column (Macherey-Nagel ET 250/8/4 Nucleosil 300-5) operated isocratically with 0.05% TFA at 1 ml/min. The radioactivity (recovery about 98%) appeared in three fractions that eluted after 4.5 min (9% ^{14}C, unidentified), 9 min (85% ^{14}C, **1a** or **1b** by cochromatography) and 14 min (3% ^{14}C, **2** by cochromatography).

Table 1
Structure assignment by cocrystallization

	Specific radioactivity (dpm/mg)		
	1a	**1b**	**2**
Initial sample (calc.)	127	127	255
3rd crystallization	26	99	16
5th crystallization	25	99	4

Aliquots (each 25 500 dpm, 5.3 nmol) of the prepurified ^{14}C-fraction as obtained from the total hydrolysate of labeled enzyme were mixed with **1a** (200 mg), **1b** (200 mg) or **2** (100 mg). The mixtures were carried through repeated crystallizations, from which radioactivity values were determined.

For the cocrystallization experiments (see Table 1), aliquots of the collected main fraction (9 min) were separately mixed with 100–200 mg of compounds **1a**, **1b** or **2**. Crystallizations (five times) were from water (**1a**), 65% acetone (**1b**) or 65% ethanol (**2**).

2.2.3. Partial racemization. The synthetic cysteine derivatives were subjected to protein hydrolysis conditions (6 M HCl, 24 h, 105°C) and the $[\alpha]_D^{20}$ values ($c = 1$, 3 M HCl) redetermined to be as follows: +23.7 (formerly +38.2) for **1a**, −27.8 (formerly −44.4) for **1b**, and +5.1 (unchanged) for **2**. These data indicated **1a** conversion into 18% **1b** and **1b** conversion into 20% **1a**; while the chiral C_α of the cysteine moiety remains stable.

3. Results

3.1. Enzyme inactivation and protein labeling by [1-^{14}C]methacrylate

Incubations (at 30°C in anaerobic buffer pH 7.4) of pyruvate formate-lyase (radical form) with excess methacrylate (up to 10 mM) showed time-dependent losses of catalytic activity that followed first-order kinetics. Inactivation rates showed saturation characteristics for the inhibitor and were suppressed by the pyruvate substrate. The inhibitor compound itself was stable for at least 1 h in the buffer medium, which routinely contained 8 mM dithiothreitol. Our experimental data (not shown) were consistent with an active site-directed inhibition pattern according to

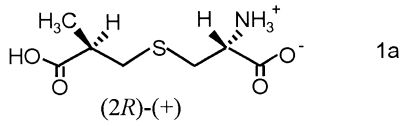
$$E + I \overset{K_I}{\leftrightarrow} EI \xrightarrow{k_i} E{-}I$$

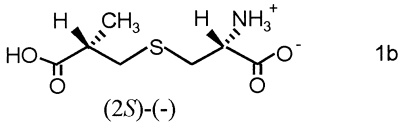
with $K_I = 0.42$ mM and $k_i = 0.14$ min^{-1}.

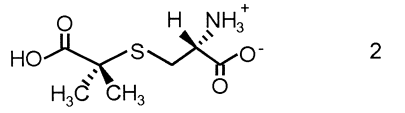
To analyze the putative covalent modification, ^{14}C-labeled methacrylate was used which we synthesized to a specific radioactivity of 4800 dpm/nmol (see Section 2). 1.1 nmol of inhibitor per nmol enzyme dimer was incorporated into a TCA-stable protein linkage concomitant with loss of catalytic activity (Fig. 1). This stoichiometry agrees with the known half of the sites behavior of in vitro activated pyruvate formate-lyase (one spin per dimer). The glycyl radical was still contained (= 90%) in the modified protein as shown by EPR spectroscopy (characteristic doublet signal, $g = 2.0037$) and by the polypeptide fragmentation by oxygen (monitored via SDS–PAGE) that is typical for the Gly-734 radical in PFL [2]. On the other hand, no protein labeling was detected when [^{14}C]methacrylate was incubated with the non-radical form of PFL.

3.2. Peptide mapping of the modification site and structure assignment

A larger batch of [^{14}C]methacrylate-modified enzyme (26







Scheme 1.

nmol) was subjected to *S*-carboxymethylation (in 7 M guanidine hydrochloride) and then digested with chymotrypsin. By using standard HPLC techniques, two radioactive peptides (CM-1 and CM-2) were detected and purified to homogeneity, with an overall isolation yield of 30%. Edman sequencing identified these peptides as overlapping fragments covering the Cys-418/Cys-419 region of the polypeptide chain (Fig. 2). The radiolabel was consistently found in the position corresponding to Cys-418, thus demonstrating that methacrylate reacts specifically with this particular Cys residue of the active site, apparently forming a thioether product.

For the structural characterization of the adduct, a further batch of ^{14}C-labeled enzyme was totally hydrolyzed with 6 M HCl (105°C) and the released radioactive cysteine derivative then identified by comparisons with authentic synthetic compounds which comprised the L-cysteine adducts to C3 (**1a** and **1b**) or C2 (**2**) of methacrylic acid (Scheme 1). Chromatographic properties excluded readily compound **2**. The distinction between **1a** and **1b**, not possible with conventional HPLC techniques, was then accomplished by cocrystallization experiments (Table 1). The immediate data obtained indicated a mixture of about 20% **1a** and 80% **1b**, suggesting an imperfect

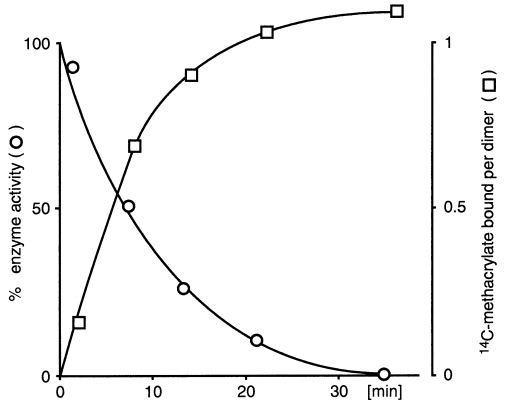


Fig. 1. Enzyme modification by [1-^{14}C]methacrylate. Pyruvate formate-lyase (radical form; 100 U or 2.9 nmol per ml) was incubated with [1-^{14}C]methacrylate (1 mM; 4800 dpm/nmol) in anaerobic standard buffer (pH 7.4) at 30°C. Residual catalytic activity was assayed by an optical assay and protein-bound radioactivity determined on TCA-precipitated samples.

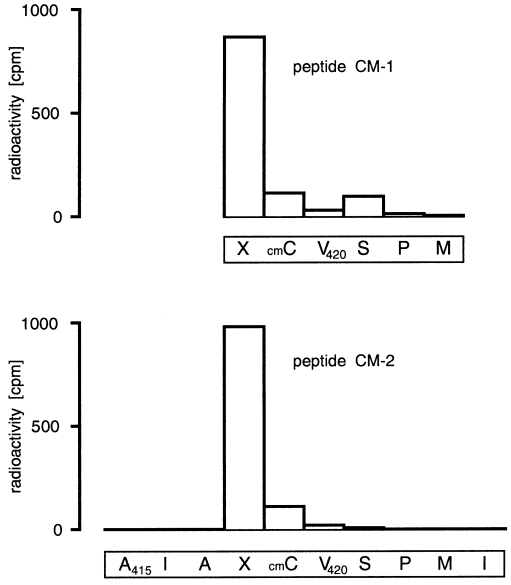


Fig. 2. Radiosequencing of isolated [^{14}C]peptides. Amino-terminal gas phase sequencing was performed through six (peptide CM-1) or ten (peptide CM-2) cycles. Identified amino acids are given in one-letter notation; cmC, carboxymethyl cysteine; X, unidentified. Radioactivity values of the anilinothiazolinones released at each cycle of the Edman degradation were measured on separately processed samples.

stereospecificity of the addition reaction. We found, however, that these data are biased by the fact that the protein hydrolysis step (6 M HCl, 105°C, 24 h) affords partial racemization at the chiral C2 center (while the (*R*)-chirality of the α-carbon of the L-cysteine moiety remains intact). Using the unlabeled synthetic compounds, we determined the extent of racemization to be 18–20% (see Section 2). When this is taken into account, the cocrystallization results (Table 1) are consistent with a unique (2*S*) configuration (**1b**) for the native [^{14}C]methacrylate adduct.

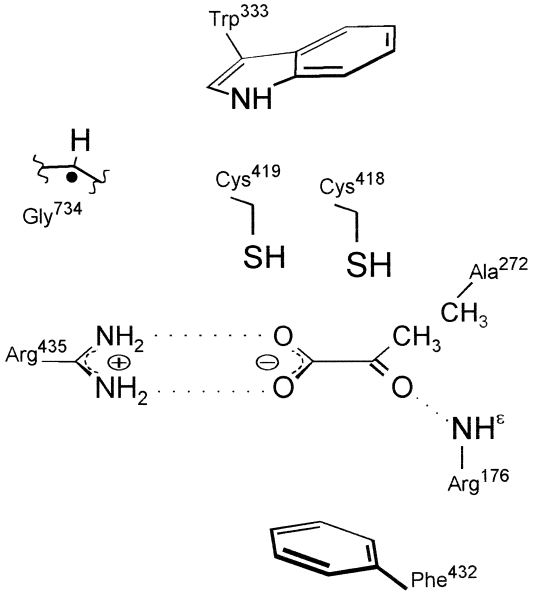


Fig. 3. Schematic drawing of the active site of PFL with bound pyruvate (after [6]).

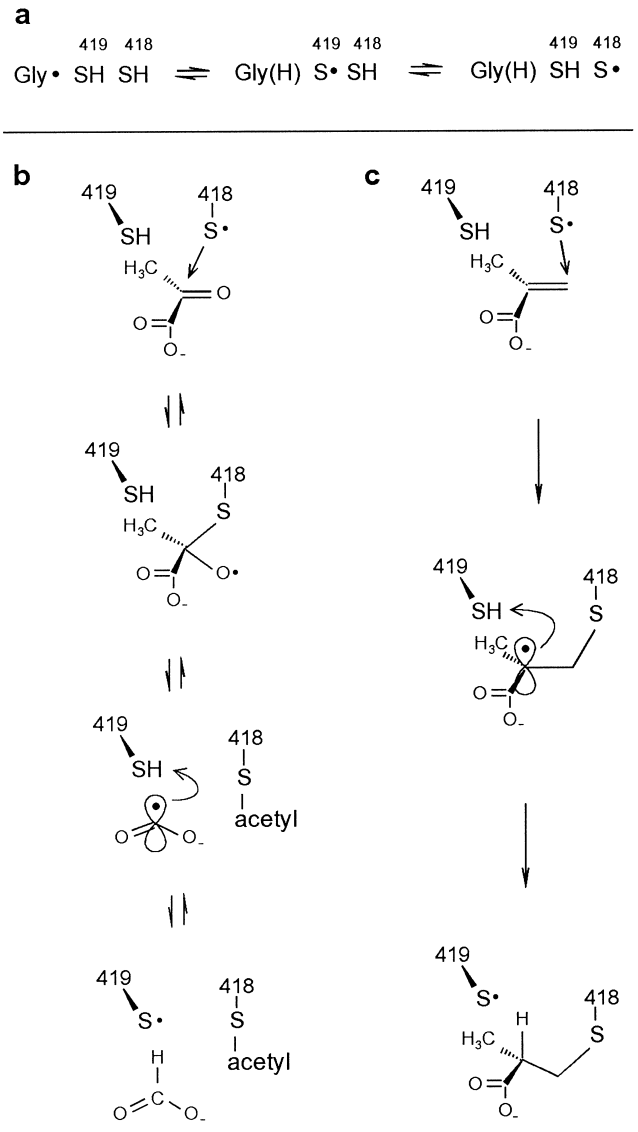


Fig. 4. Reaction mechanism of PFL. (a) Initial (and terminal) H-atom transfers involving the catalytic amino acid triad Gly/Cys/Cys; (b) first half-reaction of the catalytic cycle; (c) suicide inhibition by methacrylate.

4. Discussion

The recent X-ray crystal structure of the oxamate complex of (the non-radical form of) PFL suggests that the pyruvate substrate is positioned in the active site as shown in Fig. 3, which implied the new mechanistic proposal that the thiyl radical of Cys-418 (rather than the previously supposed Cys-419) attacks the carbonyl carbon of pyruvate in the crucial step leading to homolysis of the C1 = C2 bond, and the formyl radical produced is then quenched by the SH-group of the adjacent Cys-419 (see Fig. 4).

That alkylation of Cys-418 by methacrylate explicitly requires the radical form of PFL is strongly indicative of a radical mechanism for this process. The determined (2*S*) configuration of the 2-carboxy-propyl residue in the product is explained if methacrylate, bound analogously in the active site (i.e. $CH_2=$ in the O= subsite), is attacked by 418 thiyl on its C3 methylene carbon, and the H is added from the same side to C2 by the 419 thiol (Fig. 4c). The resulting 419 thiyl would finally regenerate the glycyl radical.

The rate of the modification reaction (half-time of 5 min at saturating methacrylate concentration) is very slow as compared to the catalysis rate of PFL ($k_{cat} = 760\ s^{-1}$). Clearly, the distance between the 418 sulphur atom and C3 of methacrylate and the angle for the nucleophilic attack (approximative values are 3.7 Å and 57°) appear far less favorable than those estimated – from the rigid crystal structure – for the reaction of 418 thiyl onto C2 of pyruvate (3.3 Å and 103°). Therefore, it appears feasible that 418 thiyl occasionally adds onto C2 of the methacrylate, forming the C3-centered adduct radical that corresponds to the tetrahedral oxyradical intermediate of the normal reaction route with pyruvate. However, the analogous fragmentation of the C1 = C2 bond, yielding an enol thioether of Cys-418, does not occur. Since the C3 radical remains inaccessible to the 419 thiol as H-donor, we expect that any occasional addition of 418 thiyl to C2 of methacrylate would be reversible. Intermediary radicals occurring in this system can perhaps be detected by stopped-flow or rapid-quench EPR techniques.

In summary, the identified modification of Cys-418 by the substrate-analogous methacrylate can be regarded as a mechanism-based inactivation of the glycyl radical enzyme PFL. The results provide experimental support for the proposal based on the crystal structure that it is this particular cysteinyl residue (in its thiyl radical form) that functions by covalent addition to the pyruvate carbonyl. In contrast, the adjacent Cys-419 operates by H-atom transfers, which is the usual chemical property of thiyl radicals, as exploited by other radical enzymes such as the ribonucleotide reductases (for a recent review, see [5]).

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