X-ray Structure of Pyruvate Formate-Lyase in Complex with Pyruvate and CoA

HOW THE ENZYME USES THE CYS-418 THIYL RADICAL FOR PYRUVATE CLEAVAGE*

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The glycyl radical enzyme pyruvate formate-lyase (PFL) synthesizes acetyl-CoA and formate from pyruvate and CoA. With the crystal structure of the non-radical form of PFL in complex with its two substrates, we have trapped the moment prior to pyruvate cleavage. The structure reveals how the active site aligns the scissile bond of pyruvate for radical attack, prevents non-radical side reactions of the pyruvate, and confines radical migration. The structure shows CoA in a syn conformation awaiting pyruvate cleavage. By chemically modifying to an anti conformation, without affecting the adenine binding mode of CoA, the thiol of CoA could pick up the acetyl group resulting from pyruvate cleavage.

Proteins can domesticate amino acid radicals for enzymatic substrate conversions (Refs. 1 and 2, and for review, see Refs. 3–5). Among the best studied examples are ribonucleotide reductases and pyruvate formate-lyase (PFL). PFL and class III ribonucleotide reductase both depend on a glycyl radical (6, 7) and display extensive structural and mechanistic similarities (8–10), which suggests that these enzymes derive from ancestors dated in the anaerobic world before the first appearance of DNA (11).

Active PFL, a homodimer (2 × 759 residues), is generated from the non-radical form by the removal of the pro-S Ca hydrogens of Gly-734, which is catalyzed by PFL-activase (12). PFL catalyzes the reversible conversion of pyruvate and CoA into acetyl-CoA and formate, which has a central role in anaerobic glucose fermentation by Escherichia coli and other bacteria. The reaction divides into two half-reactions involving an acetyl-enzyme intermediate (E + pyruvate = acetyl-E + formate; acetyl-E + CoA = E + acetyl-CoA). The main participants in the reaction include Gly-734 for radical storage and a formate; acetyl-

form) in complex with oxamate, an isosteric and chemically inert analog of pyruvate, shows the close spatial proximity of the catalytic triad Gly-734, Cys-419, Cys-418 that could accommodate direct radical transfers from Gly-734 to Cys-418 via Cys-419. Oxamate fits into a compact pocket where its carboxamide C is juxtaposed with Cys-418 SY (3.3 Å). Based on the structure and biochemical information, along with theoretical data (13), a mechanism was proposed (10) that involves the following steps (Fig. 1a). First half-reaction: pyruvate binding triggers generation of the Cys-418 thyl radical by hydrogen transfer from Cys-418 S–H via Cys-419 to the glycyl radical; thyl addition to the carbonyl C of pyruvate produces the tetrahedral oxyradical intermediate, which collapses into the acetyl thioester of Cys-418 and the formyl radical; the formyl radical is quenched to formate by the SH of Cys-419, thus producing the 419 thyl. Second half-reaction: 419 thyl generates the thyl radical of CoA; the radical is replaced with the 418-linked acetyl resulting in acetyl-CoA.

An earlier mechanistic proposal (14) assumed the formation of a thiohemiketal between pyruvate and one of the active site cysteines. This possibility could not be ruled out by the available structure (10), as it contained the inert oxamate, which is principally unable to form a thiohemiketal-like compound. Moreover, the reported structures did not provide information on the binding site of the cosubstrate CoA/acetyl-CoA, since PFL shows no relationship to known folds of CoA binding proteins (15). We describe here the crystal structures of the non-radical form of PFL from E. coli in complex with pyruvate, with pyruvate and CoA, and for comparison, with oxamate and CoA. The new data support our view of the catalytic cycle (Fig. 1a) and show the binding site of the cosubstrate CoA for the first time.

EXPERIMENTAL PROCEDURES

Protein Preparation—PFL was isolated from overproducing E. coli cells using strain 234M1 transformed with the expression vector p153E1 as described (16).

Crystallization—Tetragonal crystals of PFL in complex with pyruvate were obtained as described (10) with oxamate replaced by pyruvate and shock frozen. Monoclinic cocrystals of PFL with CoA and either pyruvate or oxamate were obtained by the hanging drop method and seeding (4 μl of protein solution with 10 mg ml−1 PFL, 25 mM MOPS/NaOH, pH 7.3, 50 mM sodium oxamate or sodium pyruvate, 3 mM CoA:Li3, 1 mM dithiothreitol, 1 mM EDTA, 3 mM NaN3) and stored according to Ref. 10.

Data Collection—Each diffraction data set (Table I) was collected from one crystal at 100 K using synchrotron radiation or x-rays generated by a GX-18 rotating anode (Elliot/Enraf-Nonius, Delft). PFL + pyr + CoA: EMBL outstation at DESY (Hamburg, Germany), 40036

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beamline X13, λ = 0.8045 Å, MARCCD detector; PFL+oxa+CoA-GX-18 rotating anode, CuKa, MAR345 detector; PFL+pyr: high resolution data at the European Synchrotron Radiation Facility (Grenoble, France), beamline ID29, λ = 0.915 Å, ADSC detector, low resolution data at rotating anode. Data were processed by the program package XDS (17). The quality of the data sets and the derived structures is shown in Table I.

Structure Determination—The structure of PFL+pyr+CoA was solved by molecular replacement using the published structure PFL+oxa (Protein Data Bank code 3pfl) (10) as the starting model. By comparison with PFL+pyr+CoA, refined at 1.53 Å, a minor error in the starting model was discovered and corrected by a peptide flip of residue Cys-418, which is less than the van der Waals distance. All atoms of the structures, except for the four N-terminal residues and very few side chains, were clearly defined by the electron densities. No significant differences in the protein atomic coordinates between these new and the previously reported (10) structures of PFL were detected. Crystallographic data and refinement statistics are compiled in Table I. The structure of PFL+pyr+CoA is depicted in Fig. 1b.

Pyruvate Binding Site—Unique positions for the pyruvate atoms were obtained from the 2Fo-Fc electron density map of PFL+pyr+CoA computed without the pyruvate atoms. Pyruvate binds exclusively in the active site of each protomer (Fig. 2a) with temperature factors of the same magnitude as those of the surrounding protein atoms. Details of the binding mode of pyruvate are summarized in Fig. 2c. The carboxyl C of pyruvate lies 2.6 Å from Cys-418 S, which is less than the van der Waals distance and indicates the formation of a weak bond. However, no significant deviations from planarity at the carboxyl C could be detected. Apparently, pyruvate does not form a thiohemiketal with PFL in the crystal structure.

Analysis of the PFL+oxa+CoA data set (Table I) revealed that oxamate binds in the active site of PFL in a mode similar to that of pyruvate (Fig. 2, b and c). Oxamate is an isosteric and chemically inert analog of pyruvate (NH₂ replaces the CH₃ of pyruvate) and was used in a previous study (10) to mimic the pyruvate substrate. Not unexpectedly, we found in our new highly isomorphic structures, PFL+pyr+CoA and PFL+oxa+CoA, that the differences between equivalent pyruvate and oxamate atomic coordinates are small and amount to a root mean square of 0.46 Å. Allowing for a rigid body rotation and translation (26), oxamate and pyruvate could be superimposed with a root mean square of 0.11 Å. Oxamate is slightly rotated (6°) with respect to pyruvate and translated by 0.4 Å away from Cys-418 so that its carboxamide C atom is found at

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**Mechanism of PFL**

**Table I**

| Compound | PFL+pyr+CoA | PFL+oxa+CoA | PFL+pyr | PFL+oxa* |
|----------|-------------|-------------|---------|----------|
| Space group | C222,5 | C222,5 | P4,2,2 | P4,2,2 |
| a (Å) | 54.90 | 54.94 | 158.90 | 158.21 |
| b (Å) | 153.05 | 153.17 | 158.90 | 158.21 |
| c (Å) | 205.95 | 205.91 | 190.92 | 190.27 |
| Outer shell (Å)* | 1.60–1.53 | 1.60–1.53 | 1.75–1.75 | 1.75–1.75 |
| r<10%> | 28.9 (7.5) | 28.9 (7.5) | 15.8 (4.8) | 15.8 (4.8) |
| Measured reflections | 1,104,993 | 1,104,993 | 968,602 | 968,602 |
| Unique reflections | 128,878 | 128,878 | 86,674 | 86,674 |
| Completeness(%) | 94.6 (89.6) | 94.6 (89.6) | 91.2 (92.1) | 91.2 (92.1) |
| R* | 4.2 (20.7) | 4.2 (20.7) | 12.2 (40.9) | 12.2 (40.9) |
| Rmerge(|%) | 3.7 (15.0) | 3.7 (15.0) | 7.5 (25.2) | 7.5 (25.2) |
| R(cut) (%) | 14.5 | 14.5 | 18.3 | 18.3 |
| Protein atoms* | 6,209 | 6,209 | 11,994 | 11,994 |
| Solvent molecules* | 1,267 | 1,267 | 1,208 | 1,208 |
| Ligands* | 1 CoA | 1 CoA | 1 Mg²⁺ | 1 Mg²⁺ |
| Ions* | 7 Na⁺ | 7 Na⁺ | 7 Na⁺ | 7 Na⁺ |

* This structure is based on previously published data (accession code 3pfl) and was corrected and re-refined in the light of the new high resolution data of this work (see “Experimental Procedures”).
* Values in this table given in brackets refer to the outer shell.
* Rmerge(|) and Rmerge(|) as defined by Diederichs and Karplus (30) are quality measures of the individual intensity observations and of the reduced structure factor amplitudes, respectively.
* R = 2<|Fo|>−<|Fc|>/2<|Fo|> where <|Fo|> and <|Fc|> are observed and atomic model structure factor amplitudes, respectively. Rmerge(|) is the R-factor calculated for 5% of randomly chosen reflections, which were excluded from the refinement.
* Coordinates— refers to atoms or molecules in the asymmetric unit, which contains either one or two PFL protomers for the monoclinic or tetragonal crystals, respectively.

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**RESULTS**

**Overview of PFL Structures**—We have determined by x-ray crystallography the structure of PFL in complex with both of its substrates CoA and pyruvate at 1.53 Å resolution (Rmerge(|) = 16.3%; coordinate error = 0.13 Å). In addition, we have solved the structure of PFL in complex with pyruvate at 2.3 Å resolution and of PFL with CoA and oxamate at 1.75 Å resolution. All atoms of the structures, except for the four N-terminal residues and very few side chains, were clearly defined by the electron densities. No significant differences in the protein atomic coordinates between these new and the previously reported (10) structures of PFL were detected. Crystallographic data and refinement statistics are compiled in Table I. The structure of PFL+pyr+CoA is depicted in Fig. 1b.
a distance of 3.0 Å from Cys-418 S. The small differences in the binding distances for pyruvate and oxamate are statistically significant and not artifacts caused by stereochemical restraints of the refinement procedure. This was confirmed by the \( F_{o}-F_{c} \) difference Fourier technique using the refined PFL/pyr/CoA structure without pyruvate for phase calculation.

The same small differences between the pyruvate and oxamate binding modes were found by comparison of the PFL/pyr structure with the re-refined PFL/oxa structures. Moreover these structures, when compared with their corresponding CoA-containing ones, displayed no significant differences. This excludes the possibility that the binding of CoA caused the observed differences between pyruvate and oxamate interactions with atoms in the active site.

**CoA Binding Site**—A unique location for each CoA atom was obtained from the \( 2F_{o}-F_{c} \) density map of the PFL/pyr/CoA structure computed without the CoA atoms (Fig. 3a). One CoA molecule binds to the surface of each protomer near the subunit-subunit interface of the PFL dimer (Figs. 1b and 3, a and b). The adenine moiety is located at a distance of 15 Å from the active site with the adenine plane normal pointing toward the protein center. CoA assumes a syn glycosidic torsion angle with the pantetheine chain extending away from the active site toward the opposing monomer where the thiol of CoA is found at a distance of ~30 Å from both active sites. The syn conformation of CoA seen in our structure is rather unusual and has been observed only in the structure of the acyl-CoA-responsive transcription factor FadR (27) (Protein Data Bank entry 1h9g).

The binding site for the adenine of CoA comprises Phe-149, Asn-145, and Gln-146 of a short \( \alpha \)-helix (Fig. 3, a). Remarkably, one side of the phenyl ring of Phe-149 is entirely exposed to solvent in all CoA-free structures. Upon CoA binding, the phenyl ring makes stacking interactions with the imidazole ring of adenine, while Asn-145 and Gln-146 fix the adenine amino group by
hydrogen bonds. The interactions of adenine with this site contribute significantly to the binding of CoA. This view is supported by our finding that the adenine base of 5' deoxyadenosine binds in exactly the same mode to this site, while the ribose remains flexible (structural data not shown).

The ribose of CoA adopts the 2'-endo conformation. It is fixed by salt bridges between the 3' and 5'-phosphates with Lys-161 and a weak hydrogen bond of the ring oxygen with the strictly conserved Arg-160. The pantetheine hydroxyl and the two amide nitrogens form hydrogen bonds to a strongly bound water molecule and the main chain carbonyl oxygens of Phe-149 and Asp-150. The thiol group is located in a predominantly hydrophobic pocket formed by the side chains of residues Phe-200, His-227, Leu-197, and Ala-224 of the opposing monomer.

Analysis of the PFL oxa + CoA data set by the $F_{o(1)} - F_{o(2)}$ difference Fourier technique revealed that CoA binds in exactly the same way, since no significant difference density near the CoA binding site was found. Replacing pyruvate with oxamate in the active site has no influence on CoA binding.

DISCUSSION
An earlier proposal for the first half-reaction of the PFL mechanism (14, 16) assumed the initial formation of a thiohemiketal between pyruvate and the active site Cys-419. The reaction was thought to proceed by attack of the Cys-418 thiol radical on the carboxylate C resulting in pyruvate cleavage. The formation of a thiohemiketal was inferred from gel filtration experiments at 4 °C which showed a [14C]pyruvate adduct to the non-radical form of PFL (14). Recent structural work on
PFL in complex with oxamate (10) has led to a new view of the PFL mechanism (Fig. 1a) that precludes thiohemiketal formation and assigns roles to the active site cysteines that differ from the earlier proposal. The new view was based on the assumption that pyruvate would display the same binding mode as the substrate analog oxamate. However, in contrast to pyruvate, oxamate is principally unable to form a thiohemiketal-like compound and for this reason the structural data were insufficient to rule out alternatives to the new view that might involve the formation of a thiohemiketal. The PFL+pyr and PFL+pyr+CoA structures described here are consistent with the new mechanistic proposal. The binding mode of pyruvate is very similar to that of oxamate. Moreover, pyruvate does not form a thiohemiketal, since the molecule does not display any significant deviations from planarity of its carbonyl group despite the close proximity of the carbonyl C to Cys-418 S (Fig. 2a). Apparently, pyruvate is tightly bound in the active site forming a kinetically stable complex with PFL, which could
well explain the outcome of the gel filtration experiments (14).

The PFL/pyr and PFL/pyr/CoA structures indicate a weak bond between the C2 of pyruvate and the S of Cys-418 thiol (bond length 2.6 Å), reminiscent of a launched nucleophilic attack on C2. In the radical form of PFL this attack would proceed upon hydrogen abstraction from Cys-418 thiol by Cys-419 thiyl. In the non-radical form the attack cannot proceed. The crystal structures are suggestive of a snapshot of a point on the reaction coordinate when the radical has moved from its storage location at Gly-734 to Cys-419, leaving Gly-734 a sp^3 carbon (Fig. 1a, boxed state). At this point the only difference between the radical and non-radical forms of PFL is the absence of a single hydrogen atom at Cys-419 S and in the enzyme. Thus, the PFL/pyr and PFL/pyr/CoA crystal structures provide excellent approximations to the corresponding radical enzyme-substrate complexes at the moment prior to generation of the Cys-418 thiol. The observed structure of the active site, which is identical in the PFL/pyr and PFL/pyr/CoA crystals, is consistent with the radical mechanism of the first half reaction: 1) pyruvate is bound in the active site displaying its planar C2 (sp^3) at sub-van der Waals distance to the S of Cys-418 (Fig. 2). The active site is designed to fix pyruvate in a position that prevents the formation of a thiohemiketal (implying a tetrahedral sp^3 configuration at C2), which would render hydrogen abstraction from Cys-418 thiol impossible (2). The thiol of CoA in the PFL/pyr/CoA structure is located outside of the active site. Thus, besides the catalytic residues, there is no radical quenching group present in the active site that could interfere with pyruvate cleavage or lead to a loss of the radical.

Consistent with biochemical data, our structures show that the presence of CoA is not required for pyruvate cleavage. If CoA is present during the first half-reaction, it binds in a waiting mode. Upon completion of the first half-reaction PFL must undergo some structural change that allows the thiol of CoA to reach the active site to pick up the acetyl from Cys-418. This requires a new binding mode for the ribose-pantetheine moiety of CoA that differs from that observed in the PFL/pyr/CoA structure.

An approximate model for the new CoA binding mode could
be obtained if one rotates the ribose-pantetheine moiety around the N-glycosidic bond. This could be visualized as a “fishing model” in which the angler (adenine) stands on a platform (Phe-149 and Asp-150) and moves the fishing rod (ribose-pantetheine moiety rotating from the syn to the anti conformation) through the air (solvent) into the water (protein) so that the hook (CoA thiol) catches the fish (acetyl at Cys-418). Indeed, as shown by model calculations, the ribose-pantetheine moiety can freely rotate through the solvent around the N-glycosidic bond from the unusual syn to the anti conformation so that the thiol reaches the active site (Figs. 1c and 3c). Since 5′-phosphorylated adenine nucleotides like CoA prefer the anti conformation in solution, the synanti transition is favored by a gain in free energy. Presumably, the strictly conserved Arg-160 supports this transition by forming a new salt bridge with the pyrophosphate of CoA in the anti conformation. Moreover, the modeled new binding mode for CoA involves predominantly conserved residues and requires only moderate conformational changes of PFL that do not disrupt secondary structure. We expect that the new CoA binding site formed upon completion of the first half-reaction also allows for binding of a free CoA molecule (anti conformation) that approaches from solution, by-passing the waiting position seen in the crystal structure.

The fixed binding site for the adenine of CoA throughout the reaction cycle proposed here is consistent with biochemical findings (28) that S-ethyl-(8-azido)-CoA and S-ethyl-(8-amino)-CoA, which prefer the syn conformation due to their bulky substituents at the C8 position, are competitive inhibitors (Ki values 0.035 and 0.2 mM, respectively) of acetyl-CoA (Km = 0.05 mM (29)). This means that the CoA binding site in the radical moiety required for the second half-reaction. Our finding that CoA may adopt a waiting position ready to get involved after pyruvate cleavage is consistent with the indirect group transfer of PFL (ping-pong) mechanism. This could contribute to optimizing the enzyme for the synthesis of acetyl-CoA, a key compound in numerous biochemical pathways.

Acknowledgments—We are grateful to Joachim Knappe for stimulating discussions and providing facilities for PFL production. We thank A. F. Volker Wagner for providing E. coli strain 234M1 transformed with the expression vector p153E1, Klaus Scheffzek for help with the synchrotron measurements at the European Synchrotron Radiation Facility, the staff at the synchrotron facilities at the European Synchrotron Radiation Facility and Deutsches Elektronen Synchrotron, Hans Wagner for maintenance of the x-ray facilities at the MPI Heidelberg, John Wray for reading the manuscript, and Kenneth C. Holmes for continuous support.

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