Pyruvate Decarboxylase: A Molecular Modeling Study of Pyruvate Decarboxylation and Acyloin Formation

Mario Lobell and David H. G. Crout*

Contribution from the Department of Chemistry, University of Warwick, Coventry CV4 7AL, U.K.

Received June 6, 1995[⊗]

Abstract: Using crystal structure data for the pyruvate decarboxylase from *Saccharomyces uvarum* (which is nearly identical with the enzyme from *Saccharomyces cerevisiae*), molecular modeling studies have been carried out to investigate the mode of action of the enzyme. Each step of the decarboxylation mechanism can be explained by assuming that the 4′-amino group of thiamin diphosphate (TDP) acts as a general acid and, in its deprotonated form, as a general base. The carboxyl group of Glu 477 plays a key role in both pyruvate decarboxylation and acyloin formation. In the first case it interacts with the carboxylate group of pyruvate to stabilize the incipient dianion formed by attack of the thiazolium carbanion on pyruvate. In the second case, it interacts with the developing alkoxide anion arising from attack on acetaldehyde or benzaldehyde by the carbanion−enamine intermediate. These studies have permitted the assignment of configuration to all of the key intermediates in the catalytic process. Thus the carbanion−enamine intermediate **5** is found to have the *E*-configuration. The *S*-configuration is imposed on the 2-(2-hydroxypropionyl)ethyl)thiamin diphosphate intermediate **4** by the chiral conformation induced in the achiral cofactor through its interactions with the protein. The *R*-configuration is assigned to the 2-(1-hydroxyethyl)thiamin diphosphate intermediate **6** arising through protonation of the carbanion−enamine intermediate **5**. The tight stereochemical control observed in acyloin formation from aromatic aldehydes and pyruvate is explained, as is the relaxed stereocontrol in acyloin formation from acetaldehyde and pyruvate.

Introduction

Pyruvate decarboxylase (PDC) from brewers' yeast is a key enzyme in ethanolic fermentation. It decarboxylates pyruvate to acetaldehyde, which in the brewing process is reduced to ethanol by yeast alcohol dehydrogenase (YADH) (Figure 1a). However, in the presence of acetaldehyde or benzaldehyde, yeast pyruvate decarboxylase (YPDC) catalyzes the formation of (*R*)-acetoin [(*R*)-3-hydroxybutan-2-one, about 50% ee[1] (Figure 1b)] or (*R*)-phenylacetylcarbinol [(*R*)-1-hydroxy-1-phenylpropan-2-one, 99% ee[2] (Figure 1c)]. We were able to confirm[3] the surprising observation[4,5] that the wheat germ enzyme consistently and reproducibly produces scalemic acetoin of constant enantiomeric excess and with predominantly the *R*-configuration. More recently[2] we have obtained a similar result with the enzymes from *Sacccharomyces sp.* and *Zymomonas mobilis*, although, remarkably, the products are of predominantly the opposite configuration (*R* and *S*, respectively). The yeast enzyme catalyzes formation of acyloins from a range of heterocyclic[6] and aromatic[2] aldehydes. However, with these substrates, the acyloin products are consistently of high optical purity and of the *R*-configuration.[7] The catalysis of carbon−carbon bond formation by PDC is an anomalous reaction. However, it becomes understandable in the light of the discovery that PDC is homologous with acetolacetate synthase,[8] a TDP-dependent enzyme that catalyzes a critical carbon−carbon bond forming step in the pathway of valine and isoleucine biosynthesis.[9] (*R*)-Phenylacetylcarbinol prepared from benzaldehyde using brewers' yeast as the biocatalyst[10] is used for the manufacture of L-ephedrine.[11] The wide substrate range of the enzyme[2,6] points to its potential as a biocatalyst for the production of acyloins of high optical purity. The continuing interest in biotransformations as a tool in organic synthesis requires an understanding of the mechanistic features of the biocatalysts used. The recent publication of an X-ray crystal structure of PDC[12] has provided an opportunity to interpret the experimental data obtained on acyloin formation[1−7] in terms of the molecular architecture of the active site of the enzyme. At the same time it has been possible to relate the picture of the

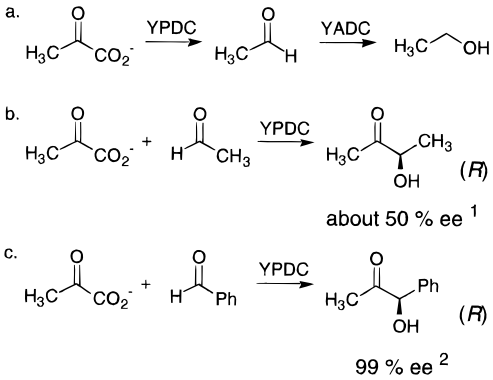


Figure 1. Reactions catalyzed by yeast pyruvate decarboxylase (YPDC).

[⊗] Abstract published in *Advance ACS Abstracts,* February 1, 1996.

(1) Bornemann, S.; Crout, D. H. G.; Dalton, H.; Hutchinson, D. W.; Dean, G.; Thomson, N.; Turner, M. M. *J. Chem. Soc., Perkin Trans. 1* **1993**, 309−311.

(2) Kren, V.; Crout, D. H. G.; Dalton, H.; Hutchinson, D. W.; König, W.; Turner, M. M.; Dean, G.; Thomson, N. *J. Chem. Soc., Chem. Commun.* **1993**, 341−343.

(3) Crout, D. H. G.; Littlechild, J.; Morrey, S. M. *J. Chem. Soc., Perkin Trans. 1* **1986**, 105−108.

(4) Singer, T. P.; Pensky, J. *J. Biol. Chem.* **1952**, *196*, 375−388.

(5) Singer, T. P. *Biochim. Biophys. Acta* **1952**, *9*, 316−327.

(6) Crout, D. H. G.; Dalton, H.; Hutchinson, D. W.; Miyagoshi, M. *J. Chem. Soc., Perkin Trans. 1* **1991**, 1329−1334.

(7) Bringer-Meyer, S.; Sahm, H. *Biocatalysis* **1988**, *1*, 321−331.

(8) Green, J. B. A. *FEBS Lett.* **1989**, *246*, 1−5.

(9) Chipman, D. M.; Gollop, N.; Damri, B.; Barak, Z. In *Biosynthesis of Branched Chain Amino Acids*; Barak, Z., Chipman, D. M., Schloss, J. V., Eds.; VCH Publishers: Weinheim, Basel, Cambridge, New York, 1990; pp 243−267.

(10) Neuberg, C.; Hirsch, J. *Biochem. Z.* **1921**, *115*, 282−310.

(11) German Patent 548 459, 1930.

0002-7863/96/1518-1867$12.00/0

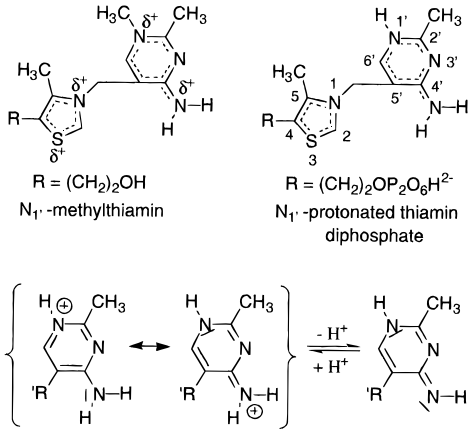


Figure 2. Structures of $N_{1'}$-methylthiamin and $N_{1'}$-protonated TDP.

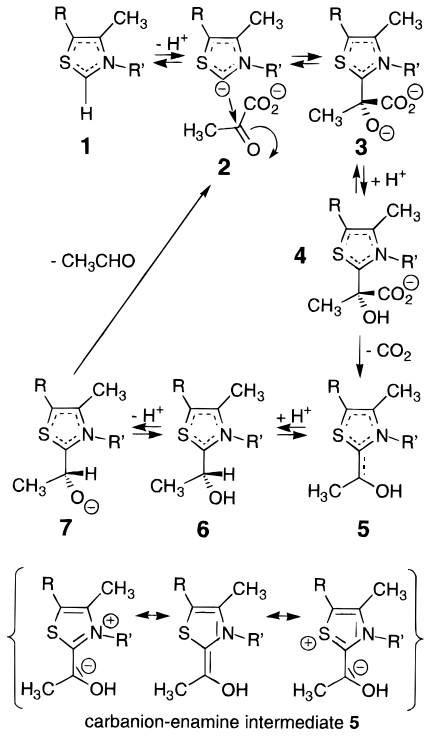


Figure 3. Mechanism of the PDC-catalyzed decarboxylation of pyruvate; mesomeric structures of the carbanion−enamine intermediate **5**.

enzymatic mechanism, as revealed by molecular modeling, to the accumulated mass of experimental data relating to the solution chemistry of free thiamin diphosphate.

The cofactor thiamin diphosphate (TDP, Figure 2) plays a key role in the enzymatic mechanism (Figure 3). Although the importance of the pyrimidine moiety of TDP has always been a major point of interest, its detailed participation in the catalytic mechanism has never been fully understood. Schellenberger *et al.*[13] proved that the pyridyl nitrogen $N_{1'}$ and the 4′-amino group of TDP are essential for the catalytic function of the enzyme−TDP complex. From three synthesized analogs of TDP, $N_{1'}$-pyridyl-TDP causes nearly full catalytic activity whereas $N_{3'}$-pyridyl-TDP and 4′-deamino-TDP are generally inactive.

Apo-PDC recombined with analogs of TDP in which the 4′-amino group had been substituted by H, $NH(CH_3)$, $N(CH_3)_2$, OH, or SH also did not show any catalytic activity.[14] Schellenberger *et al.*[15] also measured the nitrogen isotope effect of the 4′-amino group of TDP. PDC containing the ^{15}N-labeled TDP showed a 0.6% higher decarboxylation rate than PDC of normal isotopic composition, which is direct evidence for participation of the 4′-amino group in the catalytic process.

Jordan and Mariam[16] showed that $N_{1'}$-methylthiamin (Figure 2) is a superior catalyst to thiamin in the nonenzymatic decarboxylation of pyruvate. The methylation establishes a positive charge in the pyrimidine moiety and makes it comparable to an $N_{1'}$-protonated thiamin, which has a pK_a of 5[16−18] in water. However, in a less polar solvent like methanol, with triethylamine as a proton acceptor, the pK_a rises to 7.3[19] [Hopmann and Brugnoni[19] assigned their first pK_a of thiamin (4.8 in water, 7.3 in methanol) to protonation of the 4′-amino group. However, comparison to more recent literature shows that the ring nitrogen $N_{1'}$ is protonated first (pK_a = 5[16−18]) and then the 4′-amino group (pK_a < 0[20,21])]. All three so far known crystal structures of TDP-dependent enzymes (transketolase,[22] pyruvate oxidase,[23] and PDC[12]) contain a glutamic acid residue forming a hydrogen bond to the ring nitrogen $N_{1'}$. This interaction is probably all that is necessary to maintain the $N_{1'}$ atom in the protonated state. The positive charge is delocalized to the 4′-amino group, making it possible for it to act as a proton donor (Figure 2).

The conformation of TDP in terms of the relative orientation of its two aromatic rings (thiazolium and pyrimidine) can be described by the torsion angles Φ_T (C(2)−N(3)−C(6)−C(5′)) and Φ_P (N(3)−C(6)−C(5′)−C(4′)).[24] In all three crystal structures of TDP-dependent enzymes so far determined, the conformations of the TDP cofactors are remarkably similar. TDP is bound in the "V" conformation with torsion angles of $\Phi_T \approx 95°$ and $\Phi_P \approx -70°$.[25] This conformation brings the 4′-amino group within interaction distance of the C(2) reaction center.

These experimental results strongly support Schellenberger's suggestion[13,26] that the 4′-amino group of the $N_{1'}$-protonated TDP catalyzes several of the various protonation and deprotonation steps during the PDC-catalyzed decarboxylation of pyruvate.

Washabaugh and Jencks[27] have shown that the aminopyrimidinyl group does not provide significant intramolecular catalysis of nonenzymic C(2) proton removal in thiamin in aqueous solution. This, however, does not neccessarily mean that the same applies to enzyme-bound TDP. Recent studies

(12) Dyda, F.; Furey, W.; Swaminathan, S.; Sax, M.; Farrenkopf, B.; Jordan, F. *Biochemistry* **1993**, *32*, 6165−6170. The crystallographic coordinates have been deposited with the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973, under the file name 1pyd.

(13) Schellenberger, A.; Golbik, R.; Neef, H.; Hübner, G.; König, S.; Seliger, B.; Meshalkina, L.; Kochetov, G. A. *Bioorg. Chem.* **1991**, *19*, 10−17.

(14) Schellenberger, A. *Angew. Chem., Int. Ed. Engl.* **1967**, *6*, 1024−1035.

(15) Schellenberger, A.; Hübner, G.; Neef, H.; Fischer, G. *Z. Chem.* **1975**, *15*, 221.

(16) Jordan, F.; Mariam, Y. H. *J. Am. Chem. Soc.* **1978**, *100*, 2534−2541.

(17) Gallo, A. A.; Sable, H. Z. *J. Biol. Chem.* **1975**, *250*, 4986−4991.

(18) Suchy, J.; Mieyal, J. J.; Bantle, G.; Sable, H. Z. *J. Biol. Chem.* **1972**, *247*, 5905−5912.

(19) Hopmann, R. F. W.; Brugoni, G. P. *Nature (London), New Biol.* **1973**, *246*, 157.

(20) Zoltewicz, J. A.; Baugh, T. D.; King, R. W. *J. Org. Chem.* **1978**, *43*, 4670−4672.

(21) Stewart, R.; Harris, M. G. *J. Org. Chem.* **1978**, *43*, 3123−3126.

(22) Lindqvist, Y.; Schneider, G.; Ermler, U.; Sundström, M. *EMBO J.* **1992**, *11*, 2373−2379.

(23) Muller, Y. A.; Schulz, G. E. *Science* **1993**, *259*, 965−967.

(24) Pletcher, J.; Sax, M.; Blank, G.; Wood, M. *J. Am. Chem. Soc.* **1977**, *99*, 1396−1403.

(25) Muller, Y. A.; Lindqvist, Y.; Furey, W.; Schulz, G. E.; Jordan, F.; Schneider, G. *Structure (London)* **1993**, *1*, 95−103.

(26) Schellenberger, A. *Chem. Ber.* **1990**, *123*, 1489−1494.

(27) Washabaugh, M. W.; Jencks, W. P. *Biochemistry* **1988**, *27*, 5044−5053.

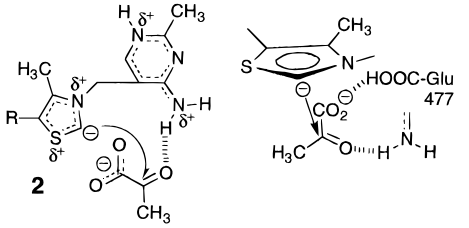


Figure 4. Nucleophilic attack of the TDP carbanion **2** on the carbonyl carbon atom of pyruvate.

by Shin *et al.*[28] have shown that the catalytically active V conformer of enzyme-bound TDP does not significantly occur in aqueous solution. There the F and S conformers dominate, in which the 4′-imino group is far from the C(2) proton and thus cannot provide intramolecular catalysis. For the same reasons, an involvement of the 4′-amino group in protonation of the carbanion−enamine intermediate in TDP-dependent enzymes must be considered, although it is not likely to be observed in aqueous solution.[29] It will always be difficult to judge if results obtained from experiments and theoretical calculations with intermediates in free solution are significant for the enzyme-catalyzed reaction mechanism. Therefore, molecular modeling studies in the enzymes active site are essential to verify proposed mechanistic pathways. Those studies really put proposed mechanisms to the test since it can be easily verified if the steric requirements for bond formation and electron/proton transfers are satisfied.

As noted above, the crystal structure of the PDC from *S. uvarum* has been determined to 2.4 Å resolution.[12] The amino acid sequence of this enzyme is nearly identical with the sequence of the enzyme from *S. cerevisiae*. Two identical monomers combine to give the active dimer in which the catalytic sites are situated at the interface between the A and B chains. On the basis of the X-ray structural data, it has been possible to explain convincingly each step in the process of pyruvate decarboxylation and acyloin formation.

Step 1: Formation of 2-(2-Hydroxypropionyl)thiamin diphosphate and Decarboxylation

The TDP carbanion **2** (Figure 4) can be assumed to add to the carbonyl group through an anticlinal conformation with respect to the C═O axis in a plane perpendicular to that of the carbonyl group.[30,31] This establishes an axis of maximal probability that still accommodates complete rotation of the plane of the thiazolium ring, disregarding constraints imposed by the architecture of the active site. In the absence of some form of stabilization, nucleophilic attack would lead to a very unfavorable double negatively charged intermediate **3** (Figure 3). Modeling of intermediate **3** from the crystal structure coordinates and subsequent energy minimization showed that it is stabilized by the formation of two hydrogen bonds. One hydrogen bond is formed between the carboxylate group and the γ-carboxyl group of Glu 477. The other is formed between the alkoxide ion and the amino group of the protonated pyrimidine moiety of TDP (Figures 5 and 6). Finally the 4′-amino group transfers a proton to the former carbonyl oxygen and the 2-(2-hydroxypropionyl)TDP intermediate **4** relaxes to a conformation in which the carboxylate group is almost perpendicular to the plane of the thiazolium ring (torsion angle = 85°, Figure 7).

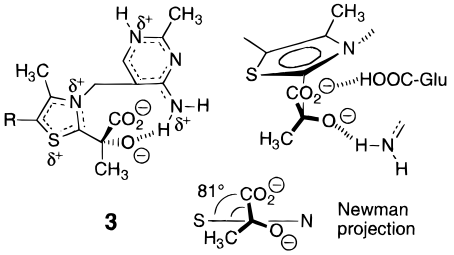


Figure 5. Three different two-dimensional projections of the energy-minimized conformation of intermediate **3**.

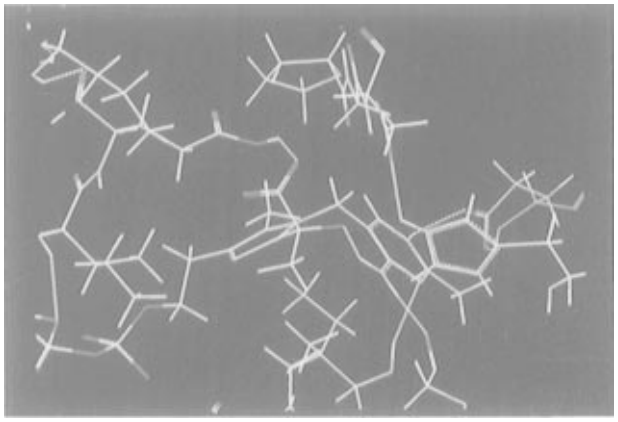


Figure 6. Energy-minimized conformation of intermediate **3**. Calculated hydrogen bonds are drawn as white, dashed lines.

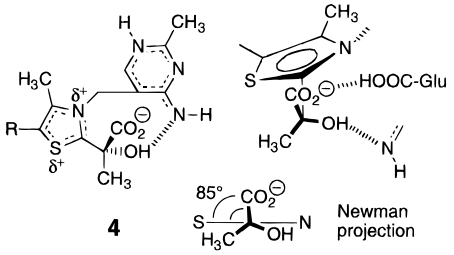
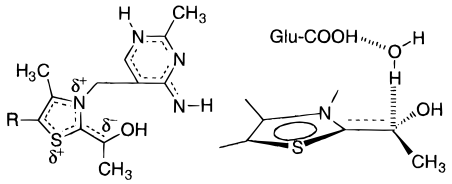


Figure 7. Three different two-dimensional projections of the energy-minimized conformation of intermediate **4**.

Conversion of the carboxylate group of the 2-(2-hydroxypropionyl)TDP intermediate **4** to carbon dioxide results in the transfer of a pair of electrons to the α-carbon. Stabilization of the carbanion can be achieved by overlap of the orbital containing the pair of electrons with the π-system of the thiazolium ring.[31,32] This orbital and therefore the carbon−carboxylate group bond must be in a plane perpendicular to the plane of the thiazolium ring. This requirement is readily attained in the relaxed conformation of the 2-(2-hydroxypropionyl)TDP intermediate **4** (Figure 7) so that decarboxylation can easily occur. The illustrated pathway is the only possible and accessible low-energy one. It leads inevtiably to the 2-(2-hydroxypropionyl)TDP intermediate **4** having the *S*-configuration and finally to the carbanion−enamine intermediate **5** (Figures 8 and 9) having the *E*-configuration. The key features that make these configurations imperative are the alkoxide−amino group interaction and the carboxylate−Glu 477 interaction (Figures 4−6).

(28) Shin, W.; Oh, D.-G.; Chae, C.-H.; Yoon, T.-S. *J. Am. Chem. Soc.* **1993**, *115*, 12238−12250.
(29) Stivers, J. T.; Washabaugh, M. W. *Bioorg. Chem.* **1992**, *20*, 155−172.
(30) Bürgi, H. B.; Dunitz, J. D.; Lehn, J. M.; Wipff, G. *Tetrahedron* **1974**, *30*, 1563−1572.
(31) Kluger, R. *Chem. Rev.* **1987**, *87*, 863−876.
(32) Kluger, R.; Turano, A.; Furey, W.; Pletcher, J.; Sax, M.; Pike, D. *J. Am. Chem. Soc.* **1982**, *104*, 3089−3095.



5

Figure 8. Two different two-dimensional projections of the energy-minimized conformation of the carbanion−enamine intermediate **5** including a docked water molecule.

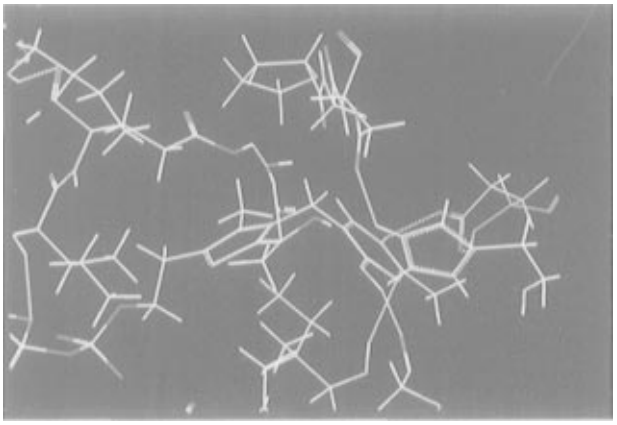
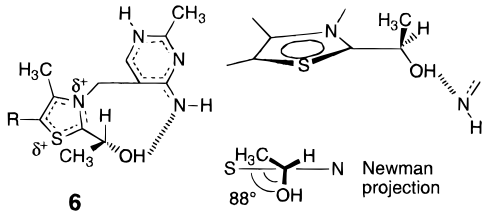


Figure 9. Energy-minimized conformation of the carbanion−enamine intermediate **5** including a docked water molecule.



6

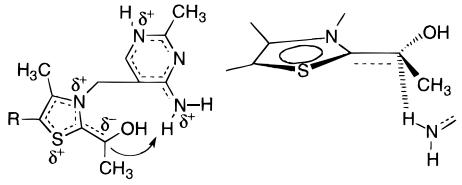
Figure 10. Three different two-dimensional projections of the energy-minimized conformation of intermediate **6**.

Step 2: Formation of 2-(1-Hydroxyethyl)thiamin diphosphate and Release of Acetaldehyde

By product analysis of acetaldehyde formed by PDC in 50% D_2O Schellenberger *et al.*[33] demonstrated a specific protonation of the carbanion−enamine intermediate **5** by the enzyme. The present modeling studies show in fact that the carbanion−enamine intermediate **5** can be easily protonated by Glu 477 aided by a bridging water molecule (Figures 8 and 9). The 2-(1-hydroxyethyl)TDP intermediate **6** having the *R*-configuration is formed (Figure 10). Energy minimization of intermediate **6** brings the hydroxyl group into close contact to the deprotonated 4′-imino group. The 4′-imino group can form a strong hydrogen bond to the hydroxyl proton. This proton is transferred to the 4′-imino group and acetaldehyde is eliminated.

Schellenberger *et al.*[33] have suggested that the 4′-amino group acts as the proton donor for formation of intermediate **6**. However, this is impossible because the 4′-amino group remains deprotonated as a 4′-imino group after formation of the 2-(2-hydroxypropionyl)TDP intermediate **4** (Figure 7). The 4′-imino group is completely inaccessible and isolated from the solvent or any other possible proton donor so that reprotonation is impossible. However, the situation might be different for other TDP-dependent enzymes.

(33) Schellenberger, A.; Ermer, J.; Hübner, G. *FEBS Lett.* **1992**, *299*, 163−165.



5

Figure 11. Two different two-dimensional projections of the energy-minimized conformation of the carbanion−enamine intermediate **5** with a protonated 4′-imino group.

Conclusions for the Mode of Action of Pyruvate Dehydrogenase and Other TDP Dependent Enzymes

The pyruvate dehydrogenase complex catalyzes the formation of acetyl-coenzyme A from pyruvate and coenzyme A. The initial steps of catalysis involve the TDP-dependent decarboxylation of pyruvate catalyzed by the pyruvate dehydrogenase component E_1 (PDHE1) to form the same carbanion−enamine intermediate **5** as is found in the PDC-catalyzed decarboxylation.[34] It is unclear whether this carbanion−enamine becomes protonated during catalysis, but the 2-(1-hydroxyethyl)TDP intermediate **6** has been isolated from pig heart pyruvate dehydrogenase[35] and is known to have the *S*-configuration.[36] This observation is striking in view of the fact that the present modeling studies indicate an *R*-configuration for the corresponding intermediate **6** in PDC. Two different explanations can be given for this observation:

(1) It is important to note that TDP is a dissymmetric molecule and that the "V" conformation is represented by two centrosymmetrically related chiral conformers [($\Phi_T \approx +90°$ and $\Phi_P \approx -90°$) or ($\Phi_T \approx -90°$ and $\Phi_P \approx +90°$)], that are readily interconverted in solution. Since the amino acid sequences of PDHE1 and PDC show no significant homology,[8,37] these enzymes may represent an example of convergent evolution and it would have been a matter of chance whether both enzymes had developed to support the same "V" conformer of TDP or different ones. Assuming the same mechanistic features in both enzymes leading to the formation of intermediate **6**, support of "V" conformers with opposite configurations would explain the finding of opposite configurations of intermediate **6** in PDHE1 and PDC. However, there is another and more likely explanation which assumes that both enzymes support the same "V" conformer.

(2) As outlined above, in PDC, the 4′-imino group of TDP is completely masked from any possible proton donor so that its reprotonation is impossible and therefore it cannot act as the proton donor for formation of intermediate **6**. In PDHE1, however, this reprotonation of the 4′-imino group might be possible before intermediate **6** can be formed. Modeling studies with the 4′-imino group protonated in the carbanion−enamine intermediate **5** (Figure 11) show that the α-carbon atom could make a close approach (2.3 Å in the energy-minimized complex) to the 4′-amino group so that a proton transfer could easily be accomplished. The 2-(1-hydroxyethyl)TDP intermediate **6** having the *S*-configuration would be formed. Intermediate **6** should maintain a hydrogen bond between the 4′-imino group and the proton just transferred, preventing reprotonation of the 4′-imino group so that the hydrogen transfer could be easily reversed, allowing the pyruvate dehydrogenase complex to proceed with its usual catalytic action.

(34) Flournoy, D. S.; Frey, P. A. *Biochemistry* **1986**, *25*, 6036−6043.
(35) Ullrich, J.; Mannschreck, A. *Eur. J. Biochem.* **1967**, *1*, 110−116.
(36) Kluger, R.; Karimian, K.; Gish, G.; Pangborn, W. A.; DeTitta, G. T. *J. Am. Chem. Soc.* **1987**, *109*, 618−620.
(37) Reynen, M.; Sahm, H. *J. Bacteriol.* **1988**, *170*, 3310−3313.

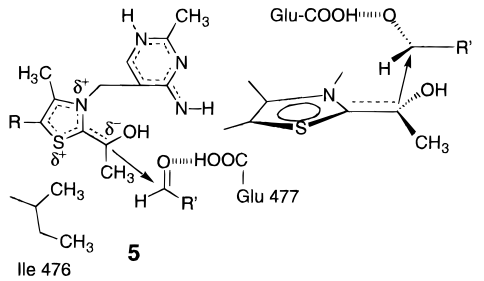


Figure 12. Nucleophilic attack of the carbanion−enamine intermediate **5** on the carbonyl carbon atom of an aldehyde (acetaldehyde or benzaldehyde) creating a new chiral center with the *R*-configuration; two different two-dimensional projections of the energy-minimized structures.

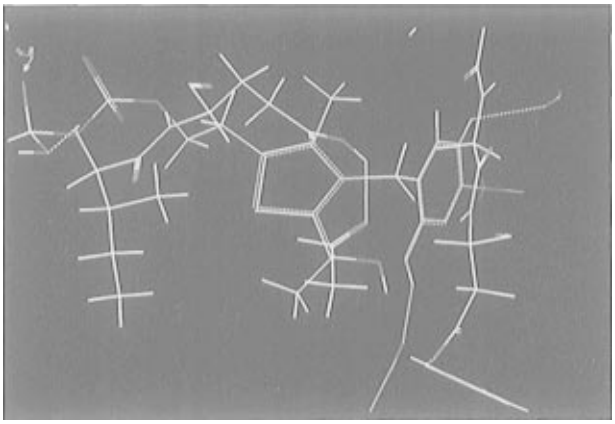


Figure 13. Energy-minimized structures of PDC with acetaldehyde bound near to the α-carbon of the carbanion−enamine intermediate **5** leading to (*R*)-acetoin.

The pathway for PDHE1 described in explanation 2 would have the striking advantage that it prevents the enzyme from catalyzing the elemination of acetaldehyde as an unwanted side reaction. In fact, all TDP-dependent enzymes except PDC face the problem of how to prevent protonation of their carbanion−enamine intermediate with subsequent elimination. That reprotonation of the 4′-imino group before the α-carbon can be protonated from the *si* face provides a possible answer.

Alternative Route Leading to the Formation of Acetoin or Phenylacetylcarbinol

In the presence of higher concentrations of acetaldehyde, the carbanion−enamine intermediate **5** can attack acetaldehyde (Figure 12) to form acetoin, which, remarkably, is consistently and reproducibly obtained as a scalemic product (about 50% ee) of predominantly the *R*-configuration.[1] Nucleophilic attack on the carbon atom of the carbonyl group should be favored if the oxygen atom with its developing negative charge could be stabilized by hydrogen bond formation leading eventually to proton transfer. In fact, the modeling studies show that the acetaldehyde molecule can be oriented in such a way that the carbonyl oxygen atom forms a strong hydrogen bond to the γ-carboxyl group of Glu 477 (Figures 12 and 13).

In the orientation of acetaldehyde leading to (*R*)-acetoin, the carbon atom of the carbonyl group approaches the α-carbon center very closely (with a separation of only 3.3 Å after energy minimization) so that a C-C bond can easily be formed. After formation of the C-C bond or in a concerted step, the proton is transferred from the glutamic acid residue to the former carbonyl oxygen atom. Finally, the deprotonated 4′-imino group abstracts the proton of the hydroxyl group attached to the α-carbon center and acetoin is released.

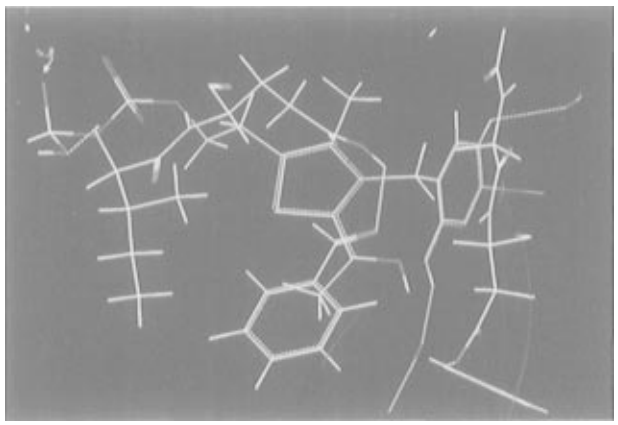


Figure 14. Energy-minimized system showing the bound benzaldehyde in relation to the α-carbon atom of the carbanion−enamine intermediate **5** leading to (*R*)-phenylacetylcarbinol.

The stereoselectivity of the transformation can be explained as follows. The position of the carbonyl group is always fixed and is determined by the position of the glutamic acid residue 477 relative to the α-carbon of the carbanion−enamine intermediate **5** (Figures 12 and 13). The positions of the formyl hydrogen atom and methyl group of acetaldehyde are in principle exchangeable, but positioning of the methyl group of acetaldehyde close to the methyl group at the α-carbon of the carbanion−enamine intermediate **5**, leading to (*S*)-acetoin, causes greater steric hindrance. Energy minimization of intermediate **5** including bound acetaldehyde leading to (*S*)-acetoin resulted in drifting of the acetaldehyde out of the correct docking position. Therefore, this structure was modeled by exchanging the positions of the formyl hydrogen atom and methyl group in the minimized docking position leading to (*R*)-acetoin (Figure 13). Subsequently the structure was minimized while fixing all atoms except the methyl group in their positions. The potential energy of the resulting structure is only 13.8 kJ/mol higher if compared to the energy of the structure leading to (*R*)-acetoin. This energy difference can be seen as an upper limit, and the real energy difference between the *re* and *si* face attack to acetaldehyde is certainly much smaller.

The situation is different if the carbanion−enamine intermediate **5** attacks benzaldehyde instead of acetaldehyde (Figure 14). Now the positions of the formyl hydrogen atom and phenyl group of benzaldehyde are not exchangeable because the isoleucine residue 476 forces the phenyl group into the position leading to (*R*)-phenylacetylcarbinol. In fact, the pyruvate decarboxylase-catalyzed formation of phenylacetylcarbinol is highly stereospecific, leading exclusively to the product with the *R*-configuration (99% ee).[2]

The mechanism of action of PDC proposed here requires the γ-carboxyl group of Glu 477 to be protonated. Therefore, its pK_a is of importance. The pK_a's of ionizable groups in proteins strongly depend on their microenvironment. From data on small proteins, glutamic and aspartic acid residues are predicted to have average pK_a's around 4.6.[38] However, it is clear that very large perturbations of pK_a can arise. Thus the γ-COOH of Glu 35 in egg white lysozyme exhibits a pK_a of 5.9 at 25 °C.[38] A pK_a of about 6.4 is observed for Glu 35 in the lysozyme−tri-*N*-acetylglucosamine complex.[38] These high pK_a values appear to be due to the low polarity of their microenvironment. Visual inspection of the position of Glu 477 in PDC also shows a very nonpolar microenvironment. Glu 477 is surrounded by the lipophilic residues Ile 476, Ile 480 and Pro 26. The next charged

(38) *The Proteins*, 3rd ed.; Neurath, H., Hill, R. L., Eds.; Academic Press: New York, San Francisco, London, 1976; Vol. 2, pp 4−7.

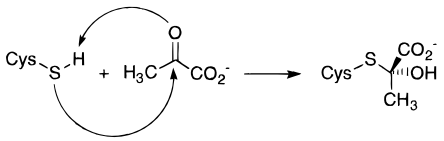


Figure 15. Thioketal formation.

residue apart from the TDP cofactor is the negatively charged Asp 28, which is kept deprotonated by hydrogen bonding with the positively charged His 115. This relative proximity to the negatively charged Asp 28 should furthermore increase the pK_a value of Glu 477. Therefore, it seems to be reasonable to expect a significant amount of Glu 477 to be protonated at pH 6.

Activation of PDC

Schellenberger *et al.*[39] have demonstrated activation of PDC by its substrate pyruvate, and also by pyruvamide and α-ketobutyramide. To a minor extent, even the decarboxylation product acetaldehyde can activate PDC. Introduction of an ethyl group into the amide (*N*-ethylpyruvamide) or elongation of the alkyl chain (α-ketovaleramide) prevents any activation capability. Kinetic studies of Kenworthy and Davies[40] suggested that PDC exists in an active and an inactive form, the latter being converted into the active form in the presence of pyruvate and by low pH. Printz and Gounaris[41] studied conformational changes in PDC measured by tritium−hydrogen exchange. Upon addition of pyruvate, a marked conformational change occurs in which 50% of the very slowly exchanging hydrogens become rapidly exchanging. Reaction of the enzyme with mercuric chloride caused a partial conformational change; subsequent addition of pyruvate then appeared to complete the unfolding as evidenced by a further increased exchange of formerly very slowly exchanging hydrogens. Since the effects of pyruvate and mercuric chloride are not additive, it seems likely that they are acting at common areas on the enzyme. Brauner and Ullrich[42] examined the reactivity of the cysteine residues in PDC. Two exposed mercapto groups per polypeptide chain were observed to react with *N*-ethylmaleimide. By using high concentrations of pyruvate (protected against rapid utilization by 1 M acetaldehyde), or α-ketobutyrate, a partial and temporary protection of one exposed mercapto group against *N*-ethylmaleimide was found. Glyoxylate bestowed full protection even in low concentrations. In summary, these experimental results suggest that PDC is transformed from its inactive into its active form by reaction of a cysteine residue with pyruvate (Figure 15). A thioketal is formed and a conformational change initiated.

In the yeast *S. cerevisiae*, PDC appears to be a mixture of the products of the genes *PDC1* (expressing the enzyme nearly identical with the enzyme from *S. uvarum* from which the crystal structure data have been obtained) and *PDC5*. The two isozymes are 88% identical, and both have four conserved cysteine residues per polypeptide chain (positions 69, 152, 221, and 222). A third structural gene in yeast is *PDC6*. This gene appears to be expressed at only very low levels, and thus, it does not contribute appreciably to the PDC activity.[43] Remarkably, the product of *PDC6* as deduced from the DNA sequence contains only one Cys at position 221, while the other three cysteine residues are replaced by aliphatic amino acids. Using

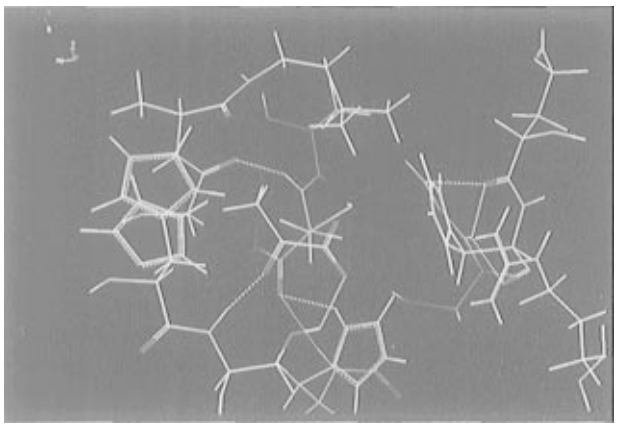


Figure 16. Energy-minimized conformation of the activation site around Cys 221 including a docked pyruvate molecule.

molecular genetic techniques and, in addition, taking advantage of a spontaneous recombination event between *PDC1* and *PDC6*, Hohmann[44] constructed a yeast strain which contains as its sole source for PDC a fusion gene consisting of the 45 N-terminal amino acids of *PDC1* and the 520 C-terminal amino acids of *PDC6*. The resulting fusion enzyme, which has only one Cys in position 221, is rather similar to the enzyme expressed from *PDC1* so far as steady-state kinetic parameters and substrate activation are considered, as tested for activation by pyruvamide.[45] Therefore, it was concluded that, if a cysteine is responsible for substrate activation, it must be Cys 221. Finally, Jordan *et al.*[46] proved that substrate activation of brewers' yeast pyruvate decarboxylase is abolished by mutation of cysteine 221 to serine.

Cys 221 is accessibly positioned (22.6 Å from the active center)[12] in a large cavity formed at the interface among all three chain domains. This cavity also lies precisely at the unoccupied "topological switch point" in the β domain and thus very likely encompasses the substrate activation site.[12] According to Jordan *et al.*,[46] addition of pyruvate as a thioketal to the sulfur of Cys 221 can easily bridge the gap between the β and α domains and therefore the regulation as expressed in substrate activation should be transmitted *via* this direct contact made by the two domains in the presence of the activator.

In fact, careful visual inspection of the crystal structure shows that only Cys 221 is easily accessible from the surrounding solvent. It is surrounded by four histidine residues (His 92, 225, 310, and 313) and some other charged or polar residues (Arg 161, Arg 224, Ser 311, Asp 312). The only very close nonpolar residue is Leu 288. This special environment of Cys 221 might cause the pH dependence of PDC activation. The correct docking of pyruvate in the activation site might, for example, depend on protonation of the four histidine residues.

Careful probing of the potential activation site with pyruvate resulted in a very favorable, energy-minimized docking position (Figure 16). The pyruvate forms two hydrogen bonds, the first from the carbonyl oxygen to the amide NH of Ser 311 and the second from the carboxylate oxygen, in *cis* position to the carbonyl oxygen, to the hydroxyl proton of Ser 311. These two hydrogen bonds hold the pyruvate molecule in position for an easy approach of the cysteine sulfur and carbonyl carbon atoms. In the energy-minimized docking position, the two bond-forming atoms are only 3.3 Å apart so that their van der Waals radii readily overlap. After formation of the *R*-thioketal and

(39) Schellenberger, A.; Hübner, G.; Fischer, G. *Z. Chem.* **1970**, *10*, 436−437.
(40) Kenworthy, P.; Davies, D. D. *Phytochemistry* **1976**, *15*, 279−282.
(41) Printz, M. P.; Gounaris, A. D. *J. Biol. Chem.* **1972**, *247*, 7109−7115.
(42) Brauner, T.; Ullrich, J. *Hoppe-Seyler's Z. Physiol. Chem.* **1972**, *353*, 825−831.
(43) Hohmann, S. *J. Bacteriol.* **1991**, *173*, 7963−7969.
(44) Hohmann, S. *Curr. Genet.* **1991**, *20*, 373−378.
(45) Zeng, X.; Farrenkopf, B.; Hohmann, S.; Dyda, F.; Furey, W.; Jordan, F. *Biochemistry* **1993**, *32*, 2704−2709.
(46) Baburina, I.; Gao, Y.; Hu, Z.; Jordan, F. *Biochemistry* **1994**, *33*, 5630−5635.

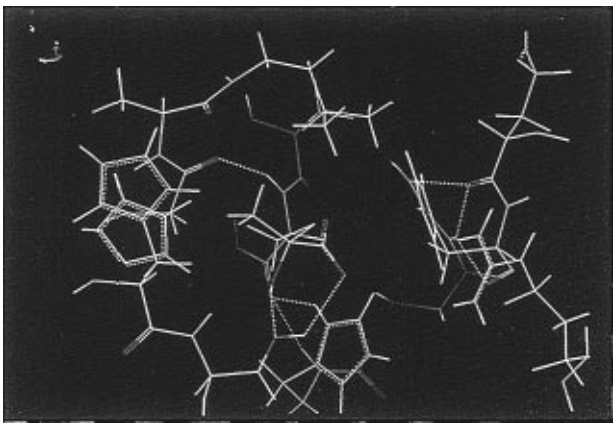


Figure 17. Energy-minimized conformation of the activation site including the *R*-thioketal formed by the sulfhydryl addition of pyruvate to Cys 221.

subsequent energy minimization (Figure 17) the hydroxyl oxygen atom of Ser 311 forms a hydrogen bond to the new formed OH group of the *R*-thioketal and the hydroxyl hydrogen atom of Ser 311 forms a hydrogen bond to the carboxylate oxygen *cis* to the OH group. Substitution of pyruvate for pyruvamide or α-ketobutyramide still allows the same docking and binding interactions. One of the carboxylate oxygen atoms is replaced by NH_2, which fills virtually the same space and has comparable hydrogen-bonding capacity.

Very recently Schowen *et al.*[47] studied in detail the kinetics and solvent isotope effects for the activation of PDC from brewers' yeast. On the basis of their results, they could reveal some new and very important aspects of the regulatory activation process. Apparently, the addition to and elimination of pyruvate from Cys 221 is coupled to opening and closing of the active site. Sulfhydryl addition drives the active site open to admit pyruvate. At some point following substrate entry but before addition of pyruvate to TDP, elimination drives closing of the active site to sequester the reactants during decarboxylation. Sulfhydryl addition would again drive the active site open to allow products to escape, with subsequent elimination closing the site before arrival of substrate for the next cycle. Between each elimination and subsequent addition, pyruvate could easily remain in the activation site by adopting the docking position described above (Figure 16).

It is important to emphazise that according to Schowen's findings, during the actual catalytic process, the pyruvate at the activation site is not added to Cys 221 in the form of a thioketal. Accordingly the crystal structure of PDC represents the catalytically active conformation for the decarboxylation process. Upon visual inspection of the crystal structure, there seems to be no sign of a lid closing the active site. This could be explained by the fact that three sequence segments 106−113, 292−301, and 557−563 (C-terminal end) had to be omitted from the crystal structure by Furey *et al.*[12] because their electron densities could not be interpreted. Residues 106−113 constitute an exposed loop, and residues 292−301 serve as a crossover connection between two parallel strands.[12] The residues at the borders of the three sequence gaps are situated at edges of the TDP binding cavity, and therefore, the omitted sequence segments could easily form the missing lid.

(47) Alvarez, F. J.; Ermer, J.; Hübner, G.; Schellenberger, A.; Schowen, R. L. *J. Am. Chem. Soc.* **1995**, *117*, 1678−1683.

Methods

All calculations were performed on a Silicon Graphics Iris Indigo workstation. QUANTA (version 4.0, Molecular Simulations Inc., Burlington, MA) was used for construction and analysis of all modeled structures. Energy minimizations were performed using CHARMm (subprogram used within QUANTA) and the Adopted Basis Newton−Raphson Minimization Algorithm. Electrostatic interactions were calculated by applying a distance-dependent dielectric term (value of dielectric constant: 4). Nonbonded interactions were calculated with a cutoff distance of 12 Å. The nonbonded atom list was updated every 25 minimization steps. A switching smoothing function was applied to the van der Waals and electrostatic terms between 8 and 10 Å.

Each yeast PDC monomer contains a single polypeptide chain. Two monomers are tightly associated to form a dimer with approximate 2-fold symmetry. A 2-fold rotation of the dimer about one axis generates another dimer, which contacts the first to form a complete PDC tetramer. The TDPs are situated at the interface between monomers within each tightly associated dimer, with two sites per dimer and four per tetramer. The computer model of PDC contains one dimer, which is represented by all carbon and heteroatoms of the two polypeptide chains (A and B chains) and the two bound TDP molecules. Predefined residue topology files were used by the HBUILD function of CHARMm to add all hydrogen atoms (polar and nonpolar). In general, aspartic acid and glutamic acid residues have not been protonated; the only exception is Glu 477. Protonation of histidine residues was decided according to hydrogen-bonding pattern and solvent accessibility: His 97 (uncharged, proton on N3), His 481 (uncharged, proton on N1), His 92, 114, 115, 126, 225, 310, 313, 495, and 510 (charged, both N's protonated). All arginine and lysine residues were protonated.

Some CHARMm parameters had to be corrected in the following cases: The 4′-amino group was held in the plane of the pyrimidine ring by applying an additional dihedral constraint [harmonic force constant: 50 kcal/mol (209.2 kJ/mol)]. The $CH_3C(\alpha)OH$ group of the carbanion−enamine intermediate **5** was held in the plane of the thiazolium ring by applying an additional dihedral constraint [harmonic force constant: 50 kcal/mol (209.2 kJ/mol)].

In all CHARMm calculations of minimum energy conformations, all enzyme atoms with the exception of some few residues were fixed in their positions as found in the crystal structure. In calculations dealing with the active site, the residues allowed to move were TDP (or a corresponding derivative) excluding the diphoshate group, Glu 477(A), Asp 28(B), His 115(B), and any docking substrate or water molecule. In calculations dealing with the activation site, the residues allowed to move were His 92, Arg 161, Ala 220, Cys 221, Cys 222, Arg 224, His 225, Gly 286, Ala 287, Leu 288, His 310, Ser 311, Asp 312, His 313 (all residues from the A chain), and any docking substrate. In general, starting conformations of intermediates have been modeled by modifying the minimized conformation of the preceding intermediate.

Acknowledgment. This investigation was supported by the Biotechnology and Biological Sciences Research Council (BBSRC).

JA951830T