Flavo Pyruvate Decarboxylase: A Semisynthetic Enzyme Model for Pyruvate Oxidase and Acetolactate Synthetase

Nikoi Annan and Frank Jordan*

Department of Chemistry, Rutgers The State University of New Jersey Newark, New Jersey 07102

Received December 4, 1989

One current method for biocatalyst design involves alteration of the catalytic environment of an enzyme to change its reaction specificity.[1] Brewer's yeast pyruvate decarboxylase (PDC, EC 4.1.1.1) catalyzes the nonoxidative decarboxylation of pyruvic acid to acetaldehyde employing thiamin diphosphate (TDP) as an essential cofactor.[2] Covalent incorporation of an oxidative cofactor on this enzyme in the vicinity of TDP may convert this nonoxidative to an oxidative function and provide insight concerning the oxidative fate of the central enamine (2α-carbanion) intermediate[3] in an intramolecular environment. Oxidative pathways currently documented are (1) the pyruvate dehydrogenase multienzyme complex[4] (oxidation by lipoamide); (2) pyruvate oxidase (POX)[5] (oxidation by flavin adenine dinucleotide, FAD); and (3) pyruvate-ferredoxin oxidoreductase[6] (oxidation by the Fe_4S_4 cluster).

We have successfully incorporated a flavin analogue at the active center of PDC, found it to be competent in redox processes, and found it to perform the reaction anticipated for POX. Four 10-methylisoalloxazine analogues were synthesized according to the synthesis outlined by Kaiser's group.[7] Chemical models for such a reaction had been reported.[8] This is the first demonstration of the diversion of the fate of a common intermediate by covalent incorporation of an oxidative cofactor.

At a concentration of 1 mM, 8α-(bromoacetyl)-10-methylisoalloxazine (**1**) was found to totally and irreversibly inactivate purified PDC[9] with respect to acetaldehyde formation at pH 6.0 with a $t_{1/2}$ of 2 min. There is no X-ray structure yet available for PDC, but there is ample evidence[3f,11] for a highly reactive Cys near the active center, the likely target for **1**. Under identical conditions, the 6α-bromoacetyl analogue was found to be less effective ($t_{1/2}$ = 4 min, but only ca. 80% inactivation resulted), while the 6-acetyl and 8-acetyl derivatives gave no inhibition of any kind. The active-center-directed behavior of **1** could be adduced from the fact that when **1** and (*E*)-4-(α-bromo-*p*-tolyl)-2-oxo-3-butenoic acid (**2**), a mechanism-based inhibitor of PDC,[3g] were added to the enzyme together, **1** competitively inhibited bromide release from **2**, a process ordinarily catalyzed by PDC (Figure 1), while pyruvate provided weak protection and pyruvamide (a known allosteric regulator[12]) none.

The stoichiometry of bound **1** (without bromide) in 8-acetyl-10-methylisoalloxazine-modified PDC (8-AcI-PDC), after extensive dialysis of the inactive PDC to remove unbound **1**, was established by UV-vis spectrophotometry ($\epsilon_{440} = 1.03 \times 10^4$ M^{-1} cm^{-1} for **1**,[13] and $\epsilon_{280} = 2.81 \times 10^5$ M^{-1} cm^{-1} for PDC,[14] corrected for the contribution from the isoalloxazine $A_{280}/A_{435} = 1.58$) to

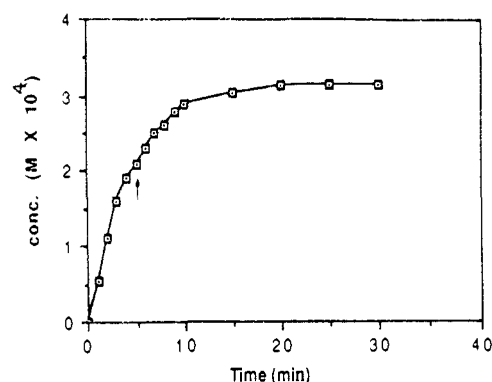


Figure 1. 8-(Bromoacetyl)-10-methylisoalloxazine (**1**) competes for the active center with the mechanism-based inactivator (*E*)-4-(α-bromo-*p*-tolyl)-2-oxo-3-butenoic acid (**2**). On the ordinate, the concentration of bromide ion released from **2** is plotted against time. The reaction mixture contained 1 mM each of TDP, $MgCl_2$, and EDTA as well as 20 units of pyruvate decarboxylase and 0.8 mM compound **2** in 0.2 M citrate, pH 6.0, at 23 °C. At the time indicated by the arrow, 0.5 mM 8-(bromoacetyl)-10-methylisoalloxazine was added. Bromide release was monitored by a bromide electrode.[3g]

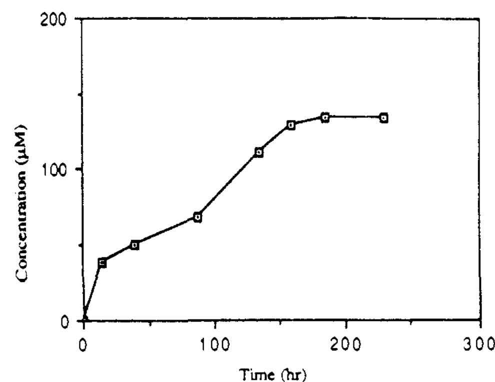


Figure 2. Time-dependent production of acetate by pyruvate decarboxylase that was totally inhibited with respect to acetaldehyde production by 8-(bromoacetyl)-10-methylisoalloxazine. A 5-mL reaction mixture containing 0.1 M pyruvate and 5.0 mg of enzyme (inactivated with 8-(bromoacetyl)-10-methylisoalloxazine and then purified on Sephadex G-25 M ion-exchange resin, subsequent to extensive dialysis to remove unbound isoalloxazines) in 0.2 M citrate, pH 6.0, was incubated at ca. 22 °C. The enzyme concentration is ca. 4 μM for a molecular weight of 260 kDa. At the times indicated, 0.2-mL aliquots were removed and added to a 1.0-mL mixture containing 50 mM of Tris·HCl, pH 7.4, 10 mM each of ATP and $MgCl_2$, 750 mM of hydroxylamine adjusted to pH 7.4, and 15 units of acetate kinase. These mixtures were incubated for 1 h at 30 °C, then 4.0 mL of $FeCl_3$ (1.25% by weight in 1.0 N HCl) and 1.0 mL of 10% v/v trichloroacetic acid were added, and the resulting absorbance was read at 540 nm.[15]

(1) For a review by the originator of flavin-modified active centers, see: Kaiser, E. T. *Angew. Chem., Int. Ed. Engl.* **1988**, *27*, 913. We acknowledge our indebtedness to the late Professor Kaiser for inspiring the concept here developed.

(2) Various authors. In *Thiamine Pyrophosphate Biochemistry*; Schellenberger, A., Schowen, R. L., Eds.; CRC Press: Boca Raton, FL, 1988; Vol. 1.

(3) On PDC: (a) Kuo, D. J.; Jordan, F. *Biochemistry* **1983**, *22*, 3735. (b) Kuo, D. J.; Jordan, F. *J. Biol. Chem.* **1983**, *258*, 13415. (c) Jordan, F.; Adams, J.; Farzami, B.; Kudzin, Z. H. *J. Enzyme Inhib.* **1986**, *1*, 139. (d) Kuo, D. J.; Dikdan, G.; Jordan, F. *J. Biol. Chem.* **1986**, *261*, 3316. (e) Jordan, F.; Kudzin, Z. H.; Kuo, D. J. *Ann. N.Y. Acad. Sci.* **1986**, *471*, 308. (f) Jordan, F.; Akinoyosoye, O.; Dikdan, G.; Kudzin, Z.; Kuo, D. J. In *Thiamine Pyrophosphate Biochemistry*; Schellenberger, A., Schowen, R. L., Eds.; CRC Press: Boca Raton, FL, 1988; Vol. 1, pp 79. (g) Annan, N.; Paris, R.; Jordan, F. *J. Am. Chem. Soc.* **1989**, *111*, 8895. In models: (h) Jordan, F.; Kudzin, Z. H.; Rios, C. B. *Stud. Org. Chem.* **1987**, *31*, 461. (i) Jordan, F.; Kudzin, Z. H.; Rios, C. B. *J. Am. Chem. Soc.* **1987**, *109*, 4415. (j) Bordwell, F. G.; Satish, A. V.; Rios, C. B.; Chung, A. C.; Jordan, F. *J. Am. Chem. Soc.* **1990**, *112*, 792.

(4) Reed, L. J. *Acc. Chem. Res.* **1974**, *7*, 40.

(5) Hager, L. P. *J. Biol. Chem.* **1957**, *226*, 251.

(6) (a) Uyeda, K.; Rabinowitz, J. C. *J. Biol. Chem.* **1971**, *246*, 3120. (b) Kerscher, L.; Oesterhelt, D. *Eur. J. Biochem.* **1981**, *116*, 595.

(7) Slama, J. T.; Radziejewski, C.; Oruganti, S.; Kaiser, E. T. *J. Am. Chem. Soc.* **1984**, *106*, 6778.

(8) (a) Shinkai, S.; Yamashita, T.; Kusano, Y.; Manabe, O. *J. Org. Chem.* **1980**, *45*, 4947. (b) Shinkai, S.; Yamashita, T.; Manabe, O. *Chem. Lett.* **1981**, 961. (c) Yano, Y.; Kimura, M.; Shimaoka, K.; Iwasaki, H. *J. Chem. Soc., Chem. Commun.* **1986**, 160.

(9) Purified according to ref 3d and assayed according to ref 10.

(10) Schellenberger, A.; Hubner, G.; Lehmann, H. *Angew. Chem., Int. Ed. Engl.* **1968**, *11*, 886.

(11) Schellenberger, A.; Hubner, G.; Sieber, M. In *Thiamine Pyrophosphate Biochemistry*; Schellenberger, A., Schowen, R. L., Eds.; CRC Press: Boca Raton, FL, 1988; Vol. 1, pp 113.

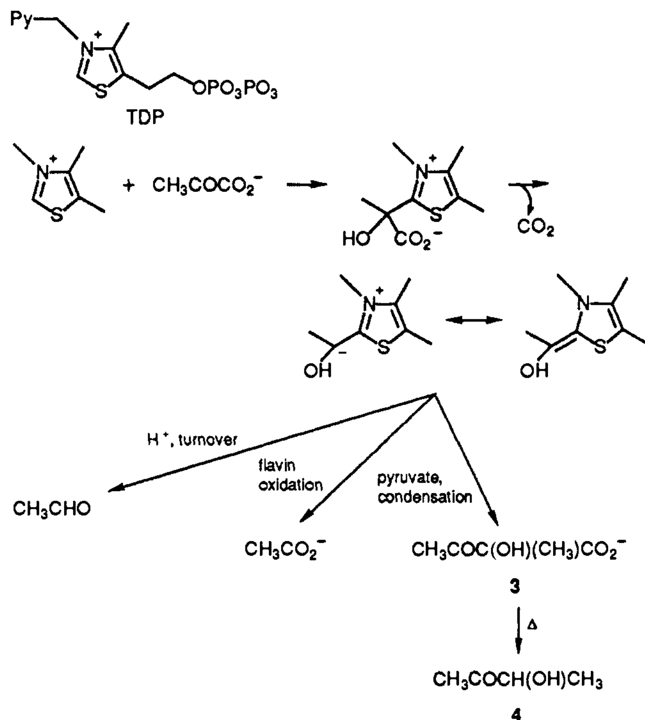
(12) Hubner, G.; Weidhase, R.; Schellenberger, A. *Eur. J. Biochem.* **1978**, *92*, 175.

(13) Slama, J. T.; Oruganti, S.; Kaiser, E. T. *J. Am. Chem. Soc.* **1981**, *103*, 6211.

(14) Lehmann, H.; Fisher, G.; Hubner, G.; Kohnert, K. D.; Schellenberger, A. *Eur. J. Biochem.* **1973**, *32*, 83.

0002-7863/90/1512-3222$02.50/0 © 1990 American Chemical Society

Scheme I[a]



[a] Py = 5-(4-amino-2-methylpyrimidinyl)

be four molecules of **1** per holo-PDC (M_r = 260 kDa) or one per subunit.

The isoalloxazine at the active center was chemically competent according to the following criteria: (1) under aerobic conditions, the A_{340} due to *N*-propyl-1,4-dihydronicotinamide or NADH was reduced by 8-AcI-PDC in a time-dependent reaction; (2) when a mixture of NADH and 8-AcI-PDC was deoxygenated in an anaerobic cell, there was no time-dependent decrease (the small amount of 8-AcI-PDC being used did not allow detection of a single turnover) in A_{340}; (3) in an anaerobic cell and in the presence of excess catalase and superoxide dismutase, pyruvate diminished the isoalloxazine absorbance at 450 nm; (4) under aerobic conditions, 8-AcI-PDC produced acetate[15] in a time-dependent fashion (Figure 2), not enhanced in rate or quantity by the presence of electron acceptors, such as 0.5 mM 3-(4,5-dimethylthiazol-2-yl)-2,4-diphenyltetrazolium bromide, 1 mM NAD^+, and 0.2 mM ubiquinone, and suggesting that dioxygen is responsible for the reoxidation of the reduced isoalloxazine; (5) interestingly, the 8-AcI-PDC also produced acetolactate, **3** (detected as acetoin, **4**).[16] Control reactions proved that all observations were due to the reactivity of the isoalloxazine covalently linked to PDC, not to model TDP reactions.[17] The ratio of acetate to acetoin produced at pH 6 is 300.

It has been shown that the enzyme acetolactate synthetase (ALS) is similar in sequence to pyruvate oxidase; it possesses FAD, which, however, has no redox function.[19] The two enzymes also bear considerable primary sequence homology to PDC isolated from yeast or *Zymomonas mobilis*.[20] The 8-AcI-PDC embodies functions attributed to both POX and ALS. The better inactivation achieved by the 8-bromoacetyl derivative compared to the 6-bromoacetyl analogue is probably due to steric factors. Steric factors probably also explain why the semisynthetic enzyme is more active as a POX model than as an ALS model since the latter would demand access of a second pyruvate to the enamine intermediate. The stoichiometry of bound 8-AcI confirms that there is a highly nucleophilic side chain near the active center of each subunit (probably Cys). The inactivity toward acetaldehyde release suggests that a group responsible for this release is no longer available (perhaps it is the Cys). Finally, the slow rate at which acetate is released may reflect slow nonenzymatic hydrolysis of the 2-acetyl-TDP, or hindered binding of pyruvate to the crowded active center, rather than an inefficient redox process. Resolution of this and other remaining questions will provide further significant details about the oxidative mechanisms involving thiamin-bound enamine intermediates, as envisioned in Scheme I.

Acknowledgment. This work was supported by the donors of the Petroleum Research Fund, administered by the American Chemical Society; the National Science Foundation (DMB 87-09758); the Rutgers University Busch Fund; and Hoffmann-La Roche, Nutley, NJ. We are grateful to Drs. Franz Scheidl and Wolfgang Benz of Hoffmann-La Roche, Nutley, NJ, for elemental and mass spectral analyses, respectively, and to the Anheuser-Busch Brewing Co., Newark, NJ, for their continued generosity in supplying the brewer's yeast.

(15) Lipmann, F.; Tuttle, L. C. *J. Biol. Chem.* **1945**, *159*, 21.
(16) Westerfield, W. W. *Proc. Soc. Exp. Biol. Med.* **1949**, *71*, 28.
(17) Control experiments all at pH 6.0, ca. 22 °C, included the following: sodium pyruvate per se; sodium pyruvate with 1 mM 8-AcI or 6-AcI; sodium pyruvate with 1 mM 6-BrAcI or **1**; sodium pyruvate with native PDC; sodium pyruvate with PDC and 1 mM 8-AcI or 1 mM 6-AcI. It is important to emphasize that in the 8-AcI-PDC experiments quoted the maximum PDC concentration was ca. 4 μM always at pH 6.0, and no free oxidizing agent was present. Hence the intermolecular oxidation of the enamine by a variety of reagents is irrelevant. The best intramolecular model for our semisynthetic enzyme is in ref 8b, incorporating the thiazolium ring and isoalloxazine in the same molecule, and producing acetate overnight at 5 mM concentration, in the presence of added micelle, and at pH 8.0. Furthermore, the model reactions reported to produce acetolactate were performed at pH 8.9, 40 °C;[18] the control experiments in our hands produced no acetolactate, detected as acetoin.
(18) Yatco-Manzo, E.; Roddy, F.; Yount, R. G.; Metzler, D. E. *J. Biol. Chem.* **1959**, *234*, 733.
(19) (a) LaRossa, R. A.; Schloss, J. V. *J. Biol. Chem.* **1984**, *259*, 8753. (b) Chang, Y.-Y.; Cronan, J. E. *J. Bacteriol.* **1988**, *170*, 3937.
(20) Green, J. B. A. *FEBS Lett.* **1989**, *246*, 1.

On the Behavior of Quantum Chemical Bond Order in the Vicinity of a Saddle Point on the Reaction Path

D. K. Maity and S. P. Bhattacharyya*

Department of Physical Chemistry
Indian Association for the Cultivation of Science
Jadavpur, Calcutta 700 032, India

Received August 1, 1989

We report that the computed profile of a generalized quantum chemical bond order index of the bond being broken or formed in a reaction may often reveal the existence of a point of inflection when viewed along the reaction path. It is conjectured that in many instances inflection point may be identified as the saddle point of the particular reaction. The relevance of this observation to bond energy–bond order (BEBO) methods is suggested.

The bond energy–bond order method (BEBO) was initially proposed by Johnston and Parr.[1,2] The method as such is capable of constructing narrow, curving, parabolic potential energy channels between the reactants and the products in a typical collinear-light atom-transfer reaction (A + HX → AH + X). The predicted minimun energy path has many desirable properties and predicts saddle-point characteristics well.[3]

The key of the BEBO method lies in the use of the Pauling rule[4] relating bond order (n_{AB}) and bond length (R_{AB}) for a singly bonded system [$R_{AB} = R_{AB}^e - 0.26$ (Å) ln n_{AB}], the postulate of the conservation of total bond order of the bonds being formed and broken[1,2] ($n_{HX} + n_{AH}$ = constant), and an empirical relation

(1) Johnston, H. S. *Adv. Chem. Phys.* **1960**, *3*, 131.
(2) Johnston, H. S.; Parr, A. C. *J. Am. Chem. Soc.* **1963**, *85*, 2544.
(3) Truhlar, D. G. *J. Am. Chem. Soc.* **1972**, *94*, 7548.
(4) Pauling, L. *J. Am. Chem. Soc.* **1947**, *69*, 542.

 © 1990 American Chemical Society