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Kinetic mechanism of pyruvate decarboxylase

Evidence for a specific protonation of the enzymic intermediate

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Decarboxylation of pyruvate by pyruvate decarboxylase (EC 4.1.1.1) was performed in a reaction mixture containing 50% deuterium. The isolated product, acetaldehyde, was investigated directly by ^1H NMR and by mass spectrometry after conversion to the 2,4-dinitrophenyl hydrazone. The protium content of 56% at acetaldehyde C1 demonstrates a specific protonation of the corresponding intermediate by the enzyme. Proton inventory studies and enzyme modification indicate the 4′ amino group of the coenzyme, thiamine pyrophosphate, in an immonium structure being a possible proton donor. A 'partially concerted' mechanism is suggested for the reaction steps following the decarboxylation.

Pyruvate decarboxylase mechanism; Thiamine pyrophosphate; Proton inventory

1. INTRODUCTION

Pyruvate decarboxylase (PDC; EC 4.1.1.1) catalyzes the decarboxylation of 2-oxoacids to the corresponding aldehydes using thiamine pyrophosphate (TPP) and Mg^{2+} as cofactors. In addition to the C2 atom of the thiazolium ring reacting with the carbonyl group of the substrate to form 2-α-lactyl-TPP [1], both the N1′ atom and the 4′ amino group on the pyrimidine ring [2,3] play an essential role in the catalytic mechanism. Decarboxylation of 2-α-lactyl-TPP leads to an optically active enamine/α-carbanion intermediate [4,5].

In this paper, the protonation was proved to be specific, performed by the enzyme using product analysis of acetaldehyde formed in a reaction mixture of 50% ^2H_2O. Results from proton inventory studies of the maximum velocity indicate the 4′ amino group of TPP as the proton donor.

2. MATERIALS AND METHODS

PDC (40 U/mg) was extracted from yeast from the brewery Wernesgrün [6]. Sodium pyruvate (98%) was from Merck. Deuterium oxide (99,86%) was purchased from Sigma and d_6-benzene (99.5%) from Aldrich. The six reactive thiol groups of PDC were modified with 3-bromopyruvamide [7].

2.1. *Enzymic synthesis of acetaldehyde*

The enzymic reaction was performed in a distillation apparatus by mixing appropriate volumes of sodium citrate buffer solutions and ^2H_2O or ^1H_2O. The receiver flask contained 1 ml d_6-benzene. After a reaction time of 20 min the enzymic solution was heated and the acetaldehyde was trapped in the ice-cooled d_6-benzene. Care was taken to avoid the distillation of isotopic water.

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2.2. *^1H NMR measurements*

^1H NMR spectra was recorded on a Bruker AM 500 Series spectrometer equipped with an Aspect 3000 computer.

2.3. *Mass spectra*

After reaction of the acetaldehyde with 2,4-dinitrophenylhydrazine, the resulting hydrazones were measured on a Joel JMS-D100 mass spectrometer.

2.4. *Kinetic measurements*

Decarboxylation of pyruvate was measured on a Specord M40 spectrophotometer (Carl Zeiss Jena) at 25°C using the optical assay described by Holzer et al. [9].

3. RESULTS

In the recorded spectra of acetaldehyde formed in ^1H_2O quartet appearing 1.9 ppm downfield from the aromatic proton signal (7.27 ppm) and a doublet appearing 5.8 ppm upfield were attributed to the carbonyl proton and the methyl protons, respectively (Fig. 1C and D). The integrated peak areas are given in Table I. Their ratio is close to the theoretical value of 3. In the spectra of acetaldehyde formed in 50% ^2H_2O the peak positions were unchanged, but instead of a doublet at 1.4 ppm three peaks were obtained (Fig. 1A and B). This can be explained by an overlapping of the doublet of the methyl protons of C1-^{1}H-acetaldehyde and a singlet for C1-^{2}H-acetaldehyde. From the ratio of the integrated peak heights a protonation extent of 56% for acetaldehyde formed in 50% ^2H_2O can be calculated. No concentration dependence (corresponding to the isola-

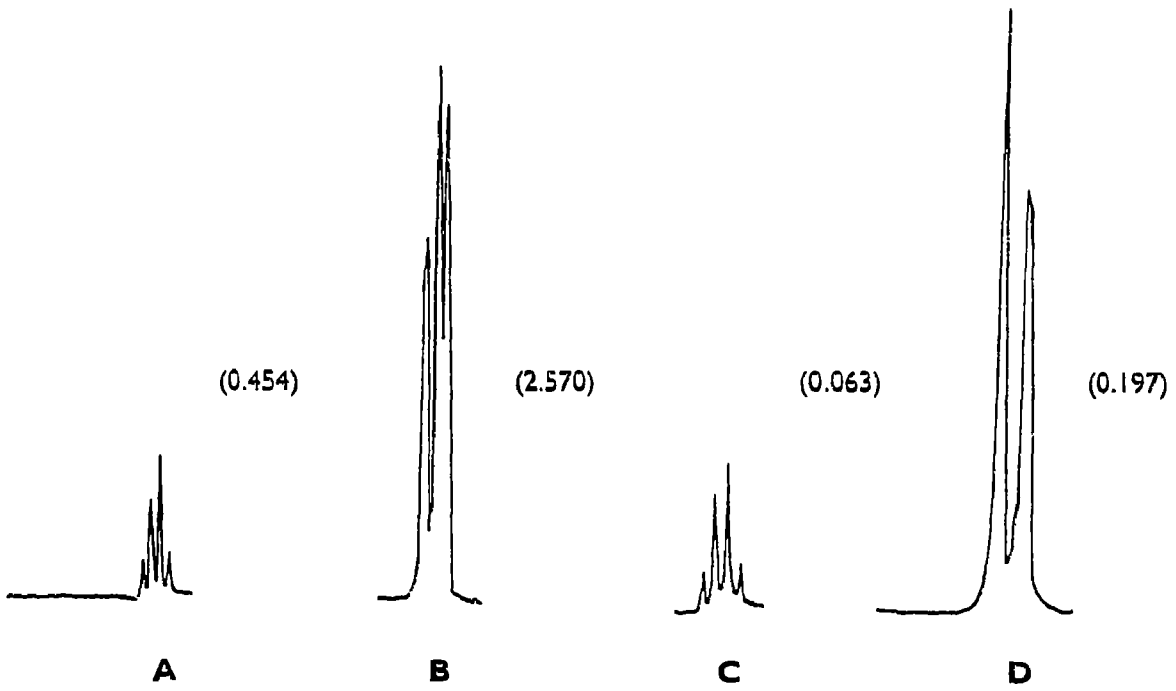

Fig. 1. ^1H NMR peaks of acetaldehyde formed by PDC in 50% ^2H_2O (A,B) and in ^1H_2O (C,D). The integrated peak areas are indicated beside the peaks.

tion efficiency) was observed. Furthermore, the protonation extent at acetaldehyde C1 was calculated from the ratio of the molecule peak (M, 224) to the (M+1)-peak (225) in the mass spectrum of acetaldehyde 2,4-dinitrophenylhydrazone mass spectra. A correction for the (M+1)-peak was made using acetaldehyde formed in ^1H_2O. The protonation extent of 55–56% was in good agreement with the NMR values.

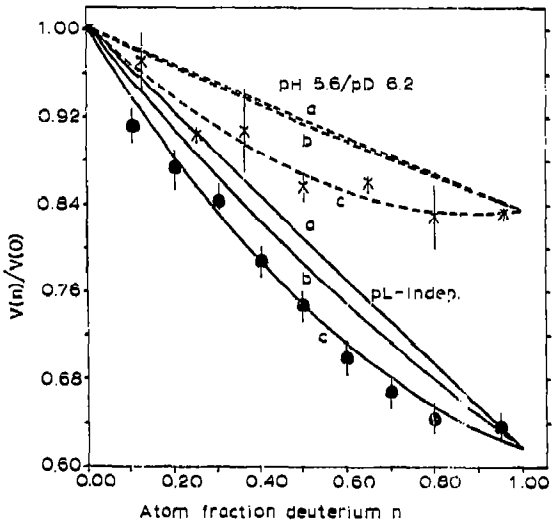

Fig. 2. Proton inventory graph for the maximum velocity (V) of the PDC reaction on 0.1 M sodium citrate buffer. The upper and lower sets of curves represent experimental values at pH 5.6/p^{2}H 6.2 (×) and calculated pL-independent values (●), respectively. The drawn graphs correspond to theoretical models of a one-proton mechanism (curves a), an infinite-proton mechanism (curves b), and a mechanism with reactant state contribution (curve c) [9,10].

Proton inventory investigations were performed by determining maximum velocities as a function of the atom fraction deuterium [9,10] at 7 different pH values. A pL difference of 0.6 units was taken for each series. For each atom fraction deuterium pL-independent velocities were calculated. Proton inventory curves are shown for experimental values at pH 5.6/p^{2}H 6.2 and for the calculated pL-independent parameters (Fig. 2, upper and lower curves, respectively). This hypercurvature remained after modification of the six reactive enzymic thiol groups with bromopyruvamide (data not shown).

Table 1

Integrated ^1H NMR peak areas for acetaldehyde[a] formed by PDC in ^1H_2O and in 50% ^2H_2O

Atom fraction of ^2H in enzymic reaction mixture[b]	Integrated peak area peak position at		Ratio methyl/carbonyl protons
	1.4 ppm	9.2 ppm	
0.00	0.197	0.063	3.13 ± 0.06
0.50	1.139	0.200	5.71
	2.570	0.454	5.66
	0.955	0.175	5.45
		average:	5.61 ± 0.11
		protonation extent at C1:	0.56 ± 0.02

[a] Acetaldehyde concentrations, 2–10 mM.
[b] 0.1 M sodium citrate pL 6.3, 50 mM pyruvate, 0.15 mg/ml PDC (40 U/mg), 25°C.

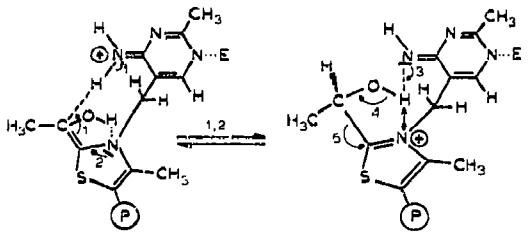

Fig. 3. Suggested 'partially concerted' mechanism for PDC reaction steps following decarboxylation.

4. DISCUSSION

The ^{1}H content of 56% for the C1 proton of acetaldehyde formed by PDC in 50% $^{2}H_2O$ demonstrates a specific protonation of the enamine/α-carbanion intermediate by the enzyme. This result is in agreement with observations of optically active α-hydroxyethyl-TPP isolated from enzymic reactions [4,5] and with the significant hypercurvature of the proton inventory graph for the maximum velocity. This kinetic parameter is related to the reactant state of the enzyme bound α-lactyl-TPP and the transition state of decarboxylation, and to the reactant state of the enamine/α-carbanion intermediate and all following transition states (including protonation and acetaldehyde release). This hypercurvature indicates the involvement of a functional group with low fractionation factor [9,10] in the reactant state (Fig. 2, curves c). Assuming such a group as the proton donor in the PDC reaction, a deuterium discrimination in the corresponding position will occur.

Low fractionation factors in enzymic reaction are commonly reported to thiol groups [9,10]. However, in our case after modification of the six reactive thiol groups of PDC with bromopyruvamide the hypercurvature in the proton inventory remained, excluding these groups as its cause.

Model investigations with *N*-1′-methylthiamine [11] as well as the influence of electronic alterations of the pyrimidine ring on the 4′ amino group [12] suggest an immonium structure in the enzyme bound cofactor. This positively charged group could have a low fractionation factor. The spatial proximity of the 4′ substituent to the α-C atom of the enamine/α-carbanion intermediate [2,3,13,14] could enable a direct proton transfer.

In accordance with these results, we suggest for PDC reaction steps following decarboxylation a 'partially concerted' mechanism (Fig. 3). First, protonation by the 4′ immonium group results in a high-energy 2-hydroxyethyl-4′-imino-TPP (Fig. 3, sequences 1,2). The second step, a basic attack of the 4′-imino group on the α-hydroxyl proton leads to the acetaldehyde elimination (Fig. 3, sequences 3–5), in accordance with the inverse ^{15}N isotope effect for 4′-$^{15}NH_2$-TPP [15]. Such an immonium–imine interconversion during the enzymic action of PDC would rule out the main objection of the low pK_a value of the 4′ amino group functioning as an acid–base catalyst.

REFERENCES

[1] Breslow, R. and McNelis, E. (1958) J. Am. Chem. Soc. 81, 3080–3082.
[2] Schellenberger, A., Müller, V., Winter, K. and Hübner, G. (1966) Hoppe Seyler's Z. Physiol. Chem. 344, 244–260.
[3] Schellenberger, A. (1982) Ann. N. Y. Acad. Sci. 378, 51–62.
[4] Holzer, H. and Beaucamp, K. (1961) Biochim. Biophys. Acta 46, 225–243.
[5] Ullrich, J. and Mannschreck, A. (1967) Eur. J. Biochem. 1, 110–116.
[6] Sieber, M., König, S., Hübner, G. and Schellenberger, A. (1983) Biomed. Biochim. Acta 42, 343–349.
[7] Hübner, G., König, S. and Schellenberger, A. (1988) Biomed. Biochim. Acta 47, 9–18.
[8] Holzer, H., Schulz, G., Villar-Palasi, C. and Juentgen-Sell (1956) J. Biochem. Z. 327, 331–344.
[9] Schowen, K.B. and Schowen, R.L. (1982) Methods Enzymol. 87C, 551–606.
[10] Schowen, K.B. (1978) in: Transition states of Biochemical Processes (R.D. Gandour and R.L. Schowen eds.) Plenum Press, New York, 225–283.
[11] Jordan, F. and Yitbarek, M. (1978) J. Am. Chem. Soc. 100, 2534–2541.
[12] Petzhold, D.R. and Storek, W. (1979) Studia Biophys. 75, 1–10.
[13] Shin, W. and Kim, Y.C. (1986) J. Am. Chem. Soc. 108, 7078–7082.
[14] Friedemann, R. and Uslar, W. (1991) in: Biochemistry and Physiology of Thiamin Diphosphate Enzymes (H. Bisswanger and J. Ullrich eds.) VCH Weinheim 1991, pp. 27–33.
[15] Hübner, G., Neef, H., Fisher, G. and Schellenberger, A. (1975) Z. Chem. 15, 221.