Carbon isotope effects on the decarboxylation of carboxylic acids

Comparison of the lactate oxidase reaction and the degradation of pyruvate by H_2O_2

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The isotope effect at C-1 on the H_2O_2 -catalysed decarboxylation of pyruvate (used as a model reaction for the enzymic reaction) increases between pH 3 and 10 from 1.0007 ± 0.0004 to 1.0283 ± 0.0014 (25 °C). This result indicates a change in the rate-determining step from formation of the tetrahedral intermediate to decarboxylation of this intermediate. Practically no isotope fractionation at C-1 (1.0011 ± 0.0002 , pH 6.0, 25 °C) is found in the lactate oxidase-catalysed decarboxylation of lactate, which is indicative for the existence of an irreversible O_2 -dependent step prior to the enzyme-catalysed decarboxylation. In addition, the result provides further evidence that dissociation of pyruvate and H_2O_2 from the enzyme can be excluded. The isotope effect at C-2 of lactate in the enzymic reaction (1.0048 ± 0.0004) is attributed to the hydrogen transfer step from lactate to the coenzyme.

INTRODUCTION

Oxidative decarboxylations of 2-oxocarboxylic acids play a major role in biochemical substrate metabolism. Enzymes involved in these reactions are commonly TPP-dependent, and the mechanism and kinetics of the corresponding reactions have been the subject of various studies (cf. Walsh, 1979). While these reactions imply a two electron transfer, the oxidative decarboxylation of 2-hydroxycarboxylic acids, yielding the same products, demands a four electron transfer. Lactate oxidase (EC 1.13.12.4), a flavoprotein belonging to the 'internal mono-oxygenases', catalyses the corresponding oxidative decarboxylation of lactate:

According to Lockridge *et al.* (1972), after substrate binding enzyme-bound pyruvate and FMNH_2 are produced. It is supposed that upon reaction of the reduced coenzyme with O_2 , H_2O_2 is formed, which then decarboxylates the enzyme-bound pyruvate, finally leading to the products acetic acid and CO_2 . A principal step of the enzyme-catalysed reaction seems therefore to be analogous to or even identical with the non-enzymic oxidative decarboxylation of pyruvate by H_2O_2 , a reaction already described by Holleman (1904) and well-known in its mechanism.

An approved means for the study of enzyme mechanisms is the determination of kinetic isotope effects. A comparison of the isotope fractionation in an enzymic reaction and a corresponding chemical reaction can be especially useful. In general, carbon isotope effects on non-enzymic, one-step decarboxylation reactions show k_{12}/k_{13} values between 1.03 and 1.06 at the CO_2 (O'Leary, 1977). Therefore, a similar isotope fractionation can be expected for the intrinsic carbon isotope effect on enzyme-catalysed decarboxylations. For example, for the pyruvate dehydrogenase reaction we determined (Melzer & Schmidt, 1987) an isotope effect at the

carboxyl group of pyruvate of 1.009 and 1.024 (depending on the enzyme source), while for a corresponding model reaction, the spontaneous decarboxylation of 2-(1-carboxy-1-hydroxyethyl)-3,4-dimethylthiazolium chloride, an isotope effect of 1.05 at the carboxyl group has been reported (Jordan *et al.*, 1978). This difference between intrinsic isotope effect (as measurable in the chemical reaction) and observed isotope effect in enzyme catalysis is due to the multistep character of enzymic reactions and can be quantitatively interpreted in relation to the enzyme mechanism. Accordingly, this was our approach in the present study, where we used the H_2O_2 -catalysed decarboxylation of pyruvate as a model reaction and compared the carbon isotope effects in connection with the proposed mechanism of the two reactions.

MATERIALS AND METHODS

Materials

Lactate oxidase from *Mycobacterium smegmatis* was purchased from Boehringer Mannheim. All chemical compounds were of analytical grade.

Carbon isotope effects on the pyruvate decarboxylation

For the determination of R_0 (eqn. 2; see below) the reaction vessel contained 1 ml of 85 mM-sodium pyruvate solution in the main chamber and in two side arms 0.2 ml of 30% H_2O_2 and 0.2 ml of 5 M- H_2SO_4 , respectively. The solutions were made CO_2 -free by sparging with N_2 . The reaction was started by addition of the H_2O_2 solution. After complete decarboxylation (controlled in parallel experiments by enzymic pyruvate determination) the H_2SO_4 was added, and the CO_2 was isolated by two freeze-thaw cycles on a vacuum line and transferred into a sample tube. Isotope ratio analysis was performed on a VG 903 Micromass mass spectrometer. For experiments with partial turnover, 220 mM solutions of sodium pyruvate in CO_2 -free water (final pH 6.4) or buffers of a given pH [0.05 M-sodium borate, pH 10.0,

Abbreviations used: TPP, thiamin pyrophosphate; 1 unit of enzyme activity is defined as 1 μmol of L-lactate/min at 25 °C.

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0.1 M-sodium citrate, pH 5.9 and 0.1 M-sodium citrate, pH 3.0 (final pH of the incubation 3.1)] were used; 2 ml of these solutions and 0.2 ml of 250 mM-H₂O₂ solution were allowed to react for a given time under N₂ at 25 °C. After acidification the CO₂ was isolated and the fraction of reaction (3–11 %, relative to the pyruvate concentration) was determined volumetrically.

Carbon isotope effects on the lactate oxidase reaction

From orientating experiments the conditions for the quantitative turnover of lactate by lactate oxidase in O₂-saturated solutions (amperometric control of the O₂ consumption by means of a Clark oxygen electrode and enzymic lactate determination) were known. In a 50 ml round-bottom flask with two side arms (containing 20 units of lactate oxidase in 0.5 ml of 0.25 M-sodium citrate buffer, pH 5.9, and 0.2 ml of 5 M-H₂SO₄, respectively) 2 ml of 42 mM-lithium L-lactate solution was freed from CO₂ and saturated with O₂. Under an O₂ atmosphere the reaction was started by addition of the enzyme. The solution was stirred at 25 °C. After complete decarboxylation of the substrate the H₂SO₄ was added, and the CO₂ was isolated as described above (determination of R₀; see eqn. 2 below). Isolation of the acetic acid by lyophilization and its subsequent combustion (as sodium salt) for isotopic analysis (R₀ of C-2 and C-3 of lactate) was performed as described previously (Melzer & Schmidt, 1987).

For assays with partial turnover the substrate solution was 350 mM. After 6–17 % of the lactate had been consumed, the reaction was stopped (H₂SO₄). First, the CO₂ was isolated. Lyophilization of the medium yields the acetic acid, quantitatively contaminated with about 10 % of the lactic acid. Therefore, the lyophilizate was trapped in 1 ml of 0.7 M-Tris, pH 9.1, and 100 µmol of (NH₄)₂SO₄, 45 µmol of NAD⁺, 56 units of lactate dehydrogenase and 3.6 units of alanine dehydrogenase

Table 1. Carbon isotope effects on the non-enzymic decarboxylation of pyruvate and the lactate oxidase-catalysed decarboxylation of L-lactate

In the chemical reaction with partial turnover 200 mM-sodium pyruvate and 23 mM-H₂O₂ in 0.1 M-sodium citrate buffer, pH 3.0 or pH 5.9, or in 0.05 M-sodium borate buffer, pH 10.0, or in buffer-free aqueous solution (pH 6.4) were allowed to react at 25 °C. In the enzymic reaction with partial turnover 20 units of lactate oxidase in 0.5 ml of 0.25 M-sodium citrate buffer, pH 6.0, were added to 2 ml of oxygen-saturated 42 mM-lithium L-lactate solution (25 °C). (For reaction conditions for complete turnover in both reactions, see the Materials and methods section.) The isotope effect k_{12}/k_{13} was calculated according to eqn. (2). The single values for R₀ and R_i are not shown. In parentheses is the number of determinations. Abbreviation: LOD, lactate oxidase.

Substrate, reaction	pH	Carbon position	k_{12}/k_{13}	<i>n</i>
Pyruvate, H ₂ O ₂	3.1	C-1	1.0007 ± 0.0004	(5)
	5.9	C-1	1.0044 ± 0.0016	(5)
	6.4	C-1	1.0069 ± 0.0020	(5)
	10.0	C-1	1.0283 ± 0.0014	(5)
Lactate, O ₂ , LOD	6.0	C-1	1.0011 ± 0.0002	(6)
	6.0	C-2	1.0048 ± 0.0004	(6)

RESULTS AND DISCUSSION

The reaction of 2-oxocarboxylic acids with hydrogen peroxide proceeds via the reversible formation of a tetrahedral intermediate and its subsequent irreversible decarboxylation (Hamilton, 1971; Siegel & Lanphear, 1979):

were added. After 2 h at 36 °C, 0.5 ml of 5 M-H₂SO₄ was added, and the lyophilization was repeated (amount of lactic acid < 0.5 %). The acetic acid was neutralized, combusted and analysed for its isotopic composition.

Calculation of the kinetic isotope effects

Isotope measurements were performed relative to a standard and the values were corrected for ¹⁷O.

The isotope effects were calculated from the ¹³C/¹²C ratio of CO₂ of the carboxyl group after complete (R₀) and partial (R_i) turnover and the corresponding fraction of reaction *f* according to eqn. 2 (O'Leary, 1980):

$$k_{12}/k_{13} = \frac{\log(1-f)}{\log[1-f(R_i/R_0)]} \quad (2)$$

As a significant isotope fractionation at C-3 of lactate can be excluded, the isotope effect at C-2 was calculated by using the isotope ratios R₀ and R_i for the acetate formed (C-2 and C-3 of lactate). The value for k_{12}/k_{13} thus calculated from eqn. 2 has to be doubled (i.e. [$k_{12}/k_{13} - 1$] · 2 + 1) to yield the actual isotope effect at C-2.

In the case of oxophenylacetic acid a steady increase of the overall reaction rate from pH 3 to 8 was observed and attributed to base catalysis (Siegel & Lanphear, 1979). Kinetic studies on the decarboxylation of pyruvic acid showed no pH-dependence of the reaction rate between pH 2 and 3.8, and a general and specific base catalysis above pH 3.8 (Darley, 1978). The author suggests a mechanism in which the attacking species is the H₂O₂ molecule below pH 3.8, and the HO₂⁻ anion above pH 3.8.

The isotope effect of the reaction at the carboxyl carbon as determined in this study (Table 1) shows a significant pH dependence, mainly above pH 5. This indicates a change in the rate-limiting step of the reaction with increasing pH. The experimental isotope effect k_{12}/k_{13} for reaction (3) can be expressed by:

$$k_{12}/k_{13} = \frac{k_3/k'_3 + k_3/k_2}{1 + k_3/k_2} \quad (4)$$

(*k'* indicates the rate constant of the molecule with the heavier isotope).

In eqn. 3 the carboxyl carbon isotope-sensitive step is obviously the decarboxylation step (rate constant k_3 , intrinsic isotope effect k_3/k'_3). However, as at pH 3 almost no isotope effect is observed ($k_{12}/k_{13} = 1.001$), the attack by H_2O_2 (or presumably the anion HO_2^-) must be rate limiting. Assuming an intrinsic isotope effect between 1.03 and 1.05, the partitioning factor k_3/k_2 (O'Leary, 1980) is about 40–70, indicating that under these conditions the first step is practically irreversible. On the other hand, at pH 10 the decarboxylation step becomes rate-limiting, as the observed isotope effect (1.028) is close to the expected intrinsic isotope effect. Accordingly, here k_3/k_2 is small (0.06–0.8), and the decay of the intermediate back to pyruvate is faster than its decarboxylation. The small but significant isotope effect at pH 6 indicates the transition in the rate-determining step. The slight increase of the isotope effect between pH 5.9 and 6.4 (1.004 and 1.007, respectively) is in line with the general increase in the isotope effect observed above pH 3. (The change in reaction conditions from buffered to non-buffered solutions should be without importance on the rate constants, as the pyruvate solution itself shows also some buffer capacity.)

In the **lactate oxidase-catalysed oxidative decarboxylation of lactate** the attack of pyruvate by H_2O_2 is one step in a complex reaction sequence (Massey & Hemmerich, 1975):

where E is enzyme, L is L-lactate, P is pyruvate and A is acetate. The corresponding equation for the observed isotope effect k_{12}/k_{13} at C-1 of lactate is:

$$k_{12}/k_{13} = \frac{k_7/k'_7 + R}{1 + R} = \frac{k_7/k'_7 + k_7/k_6[1 + k_5/k_4(1 + k_3/k_2)]}{1 + k_7/k_6[1 + k_5/k_4(1 + k_3/k_2)]} \quad (6)$$

where R is the partitioning factor.

From our results on the chemical model reaction we can estimate the lower limit for the intrinsic isotope effect on the decarboxylation (k_7/k'_7) to be near 1.03. With the observed isotope effect of 1.001 (Table 1), a value of $R = 30$ is calculated from eqn. (6). As this is a very high value for the ratios of forward to backward reaction steps, we postulate an additional irreversible step prior to decarboxylation, which has for consequence that the carbon isotope fractionation on the carbon-carbon bond scission does not take effect. Actually, Lockridge *et al.*

(1972) determined the rate constant for the reaction with O_2 (step k_5) to be one of the largest in the overall reaction, and Choong & Massey (1983) proposed a reaction sequence in which addition of O_2 and formation of the ternary enzyme-pyruvate- H_2O_2 complex (step k_5) is the first irreversible step. Our result, that essentially no primary isotope effect is observed, is in line with this mechanism, and also indicates that the reaction with O_2 and the decarboxylation are two subsequent reactions [a concerted step had been postulated by Lockridge *et al.* (1972)]. Additionally, our isotopic studies provide further evidence (cf. Lockridge *et al.*, 1972) that the decarboxylation proceeds solely at the active site, as an isotope effect similar to the one on the chemical reaction (at pH 6) should be observed, if pyruvate and H_2O_2 dissociated with a measurable rate from the enzyme and reacted in solution.

The isotope effect at C-2 of lactate is $k_{12}/k_{13} = 1.0048$ (Table 1). Based on the conclusion that O_2 addition is the first irreversible step, this isotope effect must be attributed to the dehydrogenation step (k_3 in eqn. 5). The value of the carbon isotope effect correlates well with the deuterium isotope effect for the same step ($k_{\text{H}}/k_{\text{D}} = 1.7$, pH 7.0, 25 °C), as determined by Walsh *et al.* (1973). Both isotope effects indicate that hydrogen transfer from lactate to the coenzyme is a partially rate-limiting step. As the isotope fractionation at C-1 of lactate cannot be related to the decarboxylation step, but is not exactly unity, this fractionation of 1‰ is most likely a secondary isotope effect on the dehydrogenation step.

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