CARBON ISOTOPE EFFECT ON THE ENZYMATIC DECARBOXYLATION OF PYRUVIC ACID

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The decarboxylation of pyruvic acid by the thiamine pyrophosphate dependent pyruvate decarboxylase from brewer's yeast is accompanied by a carboxyl carbon isotope effect $k^{12}/k^{13} = 1.0083 \pm 0.0003$ at 25°, pH 6.8. The small size of the isotope effect indicates that decarboxylation is not rate-determining in the overall reaction. The rate constant for decarboxylation of the enzyme-bound pyruvate-thiamine pyrophosphate complex is greater by about a factor of five than the rate constant for dissociation of this complex to form free pyruvate and the enzyme-thiamine pyrophosphate complex.

INTRODUCTION

Carboxyl carbon isotope effects on enzymatic decarboxylations can be used to estimate the rate of decarboxylation of the appropriate enzyme-substrate complex relative to the rate of dissociation of the substrate from this complex to regenerate free enzyme and free substrate (1,2). For the simple mechanism

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} EP + CO_2 \longrightarrow E + P + CO_2 \qquad (1)$$

in which k_1 and k_2 are rate constants for the formation and dissociation of the enzyme-substrate complex immediately preceding the decarboxylation step and k_3 is the rate constant for decarboxylation, the observed carboxyl carbon isotope effect is given by

$$\text{observed } \frac{k^{12}}{k^{13}} = \frac{k_3^{12}/k_3^{13} + k_3/k_2}{1 + k_3/k_2} \qquad (2)$$

provided that decarboxylation is irreversible and that there are no carbon isotope effects on k_1 and k_2. The reaction represented by rate constant k_1 in eq. 1 may not be a single step. In such a case, eq. 2 is replaced by a

similar but more complex equation in which the ratio k_3/k_2 is replaced by a more complex function (1). A variety of enzymatic decarboxylations have been studied by this technique, and it is usually found that decarboxylation is not entirely rate-determining (2).

The thiamine pyrophosphate dependent pyruvate decarboxylase (EC 4.1.1.1) from yeast functions by the mechanism shown in Scheme I (3,4). We have used carbon isotope effects to study the fate of the covalent enzyme-pyruvate-thiamine pyrophosphate complex.

MATERIALS AND METHODS

All materials were of reagent grade and were shown not to contain impurities which would interfere with the isotope effect measurements. Enzyme assays were conducted on a Gilford Model 222 spectrophotometer at 25°. Isotope ratios were measured on a Nuclide Associates RMS 6-60 isotope-ratio mass spectrometer.

Pyruvate decarboxylase was purified to homogeneity from brewer's yeast by the method of Ullrich (5). During the course of purification the enzyme was assayed by observing the disappearance of NADH on reduction of acetaldehyde by alcohol dehydrogenase. For the isotope effect experiments the enzyme was assayed spectrophotometrically at 313 nm ($\Delta\varepsilon$ = 20).

Carbon isotope effects were determined by isotope ratio measurements of two parallel samples of carbon dioxide obtained from the decarboxylation of pyruvic acid (6). A solution of 0.03 M sodium pyruvate in 0.05 M phosphate buffer, pH 6.80, containing 1 mM Mg^{2+} and 10 μM thiamine pyrophosphate was divided into two parts and each was freed of CO_2 by purging with CO_2-free nitrogen for 1 hr. Following equilibration at 25°, known amounts of pyruvate decarboxylase which had been desalted in CO_2-free buffer were added to both solutions. One solution was allowed to decarboxylate to the extent of approximately 10% and then the reaction was stopped by addition of concentrated H_2SO_4. The other sample was allowed to decarboxylate completely before addition of acid. Completeness of decarboxylation in the latter sample was checked by neutralization of the solution and assay for pyruvate with lactic dehydrogenase after the CO_2 had been removed. The CO_2 liberated from both solutions on acidification was removed and repeatedly distilled on the vacuum line. The CO_2 samples following this procedure were free of H_2O and acetaldehyde. Isotope ratios and isotope effects were calculated as described previously (6).

RESULTS

Results of six independent measurements of the carboxyl carbon isotope effect on the enzymatic decarboxylation of pyruvic acid are shown in Table I. All experiments were conducted with the same lot of pyruvate, so the isotope ratios for all 100% decarboxylation samples are identical within experimental error, as expected. The carbon isotope effect is $k^{12}/k^{13} = 1.0083 \pm 0.0003$.

TABLE I. Carboxyl carbon isotope effects on the enzymatic decarboxylation of pyruvic acid at 25° pH 6.8, in 0.05 M phosphate buffer containing 1.0 mM Mg^{2+} and 10 μM thiamine pyrophosphate.

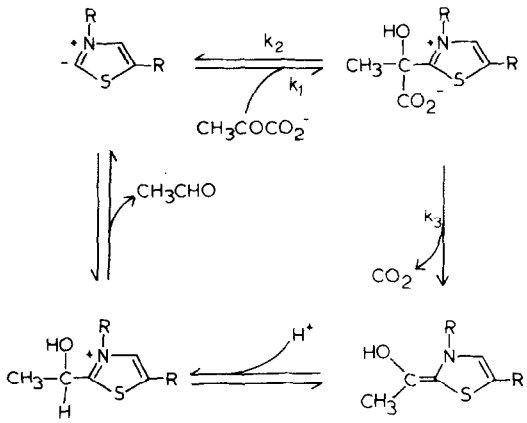
Percent reaction	Isotope ratios[a] x 10^6		k^{12}/k^{13}
	low conversion	100% conversion	
11	14432	14539	1.0083
11	14439	14546	1.0083
11	14437	14544	1.0083
5	14447	14562	1.0086
10	14445	14547	1.0078
11	14438	14549	1.0086
		Mean	1.0083
			±0.0003

[a]Mass spectrometer ratios m/e 46/44 corrected to a constant value of a CO_2 reference standard, but not corrected for the presence of oxygen-17.

DISCUSSION

A small but significant carbon isotope effect is observed in the enzymatic decarboxylation of pyruvic acid at pH 6.8. The effect is smaller than those observed in the enzymatic decarboxylation of glutamic acid (6), acetoacetic acid (7), and arginine*. The isotope effect is considerably smaller than those observed in nonenzymatic decarboxylations in which the decarboxylation step is entirely rate-determining (2). Small isotope effects such as this one are observed in decarboxylation reactions in which the decarboxylation step is somewhat faster than preceding steps in the reaction mechanism (2).

*A carbon isotope effect $k^{12}/k^{13} = 1.0144 \pm 0.0004$ has recently been obtained for the enzymatic decarboxylation of arginine by bacterial arginine decarboxylase at 25°, pH 5.25, by O'Leary and Piazza (unpublished results).



Scheme I. Mechanism of action of pyruvate decarboxylase.

The carbon isotope effect on the decarboxylation of pyruvic acid can be interpreted using the mechanism of Scheme I. The relationship between the observed isotope effect and the mechanism of Scheme I is given by eq. 2. Because decarboxylation is irreversible (8), no account is taken in eq. 2 of the rates of steps following the decarboxylation. Eq. 2 can be used to estimate k_3/k_2, the ratio of the rate of decarboxylation of the covalent pyruvate-thiamine pyrophosphate complex to the rate of its decomposition to free pyruvate and thiamine pyrophosphate, provided that an estimate can be made of the expected value of k_3^{12}/k_3^{13}, the carbon isotope effect on the decarboxylation step. Based on results of model studies, it has been argued that a carbon isotope effect in the range k^{12}/k^{13} = 1.04-1.06 is expected for this step (2). This estimate enables us to calculate a value of approximately 5-7 for k_3/k_2.

This rate constant ratio indicates that decarboxylation is not the rate-determining step in the decarboxylation of pyruvic acid. Instead, the decarboxylation step is slightly faster (that is, its transition state has a slightly lower energy) than the step in which the covalent pyruvate-thiamine pyrophosphate complex is formed. Stated another way, this covalent complex, once formed, usually undergoes decarboxylation, but perhaps 10-20% of the time the complex reverts to pyruvate and thiamine pyrophosphate. It should

be noted that this reversion must proceed all the way to free pyruvate to explain the observed isotope effect.

It has been suggested that the overall rate determining step in the enzymatic decarboxylation of pyruvic acid is the release of acetaldehyde from the enzyme-product complex (9). Because of the nature of the carbon isotope effect experiments it is not possible to evaluate the correctness of this proposal.

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