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THE RATE-DETERMINING STEP IN THE OXIDATIVE DECARBOXYLATION OF ISOCITRIC ACID

MARION H. O'LEARY
Department of Chemistry, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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SUMMARY

The oxidative decarboxylation of isocitric acid by NADP dependent isocitrate dehydrogenase is accompanied by a β-carboxyl carbon isotope effect k^{12}/k^{13} of 0.996 at 25° pH 7.5. This indicates that decarboxylation is not the rate-determining step in this reaction. Some step prior to decarboxylation, probably dehydrogenation, must be at least tenfold slower than decarboxylation.

INTRODUCTION

Much useful information about the mechanism of action of the $NADP^+$-dependent isocitrate dehydrogenase (*threo*-D_s-isocitrate: $NADP^+$oxidoreductase (decarboxylating), EC 1.1.1.42) from pig heart has emerged from the studies of COLMAN[1,2,5] and COLMAN AND CHU[3,4,6], who have made a careful comparison of the four activities this enzyme demonstrates. From the varying sensitivities of these activities to protein modification[1–5], from solvent isotope effects[6] and from relative rates of various reactions[7,8] it has been inferred that the dehydrogenation step in the oxidative decarboxylation of isocitric acid is rate-determining.

Heavy-atom isotope effects are sensitive probes for determining the relative rates of various steps in enzyme reactions[9–13]. When used carefully, such effects can provide mechanistic information which is not available by any other method. We have now applied this technique to isocitrate dehydrogenase. Our results indicate clearly that the decarboxylation step is not rate-limiting, but is much faster than the rate-determining step.

MATERIALS AND METHODS

Isocitrate dehydrogenase, glutamate dehydrogenase, and $NADP^+$ were obtained from Sigma. Isocitric acid was obtained by hydrolysis of DL-isocitric acid lactone (Sigma) in aqueous NaOH[14]. Other chemicals were commercial materials of high purity.

The isotope effect procedure was patterned after that used in our previous stu-

dies[10–12]. The reaction mixture for the isotope effect studies contained the following components: 20 mM DL-isocitric acid, 0.1 M NaOH, 5 mM magnesium acetate, 20 mM ammonium acetate. The solution was adjusted to pH 7.50 with acetic acid. For each experiment two portions of this solution were thoroughly degassed by flushing with CO_2 free nitrogen and then were equilibrated at 25.0°. Enzymes were desalted on Sephadex G-25 with degassed buffer (0.05 M Tris–acetic acid, pH 7.5) before use. The reaction in one portion was initiated by the addition of measured amounts of isocitrate dehydrogenase, glutamate dehydrogenase, and $NADP^+$ (final concentration 0.02 mM). Sufficient isocitrate dehydrogenase was used to give a rate of approx. 0.02 mM $NADP^+$ reduced per min. An excess of glutamate dehydrogenase sufficient to keep the steady-state concentration of NADPH below 1 μM was used. The time needed for this solution to reach 10% reaction was calculated by spectrophotometric assay (340 nm) of a small sample of a reaction solution prepared as described above except for the omission of glutamate dehydrogenase and a 5-fold increase in the amount of $NADP^+$. The assay was linear until the $NADP^+$ was depleted, and the initial rate was used to calculate the approximate time required for 10% reaction. The exact time is not important because the correction for percent reaction is negligible[15] due to the very small isotope fractionation. After a sufficient time for 10% conversion, the reaction was stopped by addition of conc. H_2SO_4.

3-fold larger amounts of both enzymes and of $NADP^+$ were used and the reaction was continued for at least 12 h in the other solution in order to insure that the D-isocitric acid present was completely decarboxylated. Sulfuric acid was again added at the end in order to liberate CO_2. The absence of residual D-isocitric acid was confirmed by neutralization and assay of an aliquot of the solution with fresh isocitrate dehydrogenase and $NADP^+$.

Vacuum transfers, isotope ratio measurements, and calculations were accomplished as previously described[10].

RESULTS

The β-carboxyl carbon isotope effect on the oxidative decarboxylation of isocitric acid by $NADP^+$ dependent isocitrate dehydrogenase has been measured by comparison of the isotopic composition of the carbon dioxide evolved during the first 10% reaction with that of the carbon dioxide evolved upon complete decarboxylation of the substrate. This is the standard method for determination of carbon isotope effects on decarboxylations[6,9], but several special problems were encountered in this case.

For reactions of economy it was necessary to use DL-isocitric acid. It is not known at present whether the L-isomer acts as an inhibitor, but even if present such inhibition does not influence the isotope effect because the inhibition should be equally effective against both isotopic forms of the substrate.

The requirement for $NADP^+$ and the reversibility of this reaction also make this decarboxylation more complex than previous ones which have been studied. If the reverse reaction occurs in the isotope effect experiments it is no longer possible to interpret the results without ambiguity. It is impractical to use a stoichiometric amount of $NADP^+$. Instead, we added glutamate dehydrogenase and ammonia, which results not only in the regeneration of the $NADP^+$, but also in the removal of

ketoglutarate as it is formed, thus largely preventing the reductive carboxylation of ketoglutarate. A sufficient excess of glutamate dehydrogenase was always used to keep the steady-state level of NADPH (and thus that of ketoglutarate) below $1 \cdot 10^{-6}$ M.

The results of the isotope effect determinations are summarized in Table I. The observed isotope effect k^{12}/k^{13} is 0.996 ± 0.003. The reproducibility of the measurements in Table I is not as high as has been observed in other studies. Although the measured isotopic compositions of the 100% reaction samples are quite consistent, a larger than usual variation is observed in the 10% reaction samples. This variation is due to the slight amount of reductive carboxylation of ketoglutarate

TABLE I

CARBON ISOTOPE EFFECTS ON THE OXIDATIVE DECARBOXYLATION OF ISOCITRIC ACID AT pH 7.5, 25°
The ratios given have been corrected for the presence of oxygen-17.

Mass spectrometer decade settings for m/e 45/44 × 10⁶		k^{12}/k^{13}
10% reaction	*100% reaction*	
13 482	13 471	0.999
13 572	13 468	0.992
13 560	13 479	0.994
13 513	13 468	0.997
13 465	13 464	1.000
	Mean	0.996 ± 0.003

which occurs during the reaction. The decarboxylation of isocitric acid is significantly reversible[16], and the K_m values for the substrates in the reverse reaction are sufficiently small[16] that it is impossible to suppress this reaction completely even in the presence of an excess of glutamate dehydrogenase. The reverse reaction probably shows a substantial isotope effect, resulting in a slight variation in the isotopic composition of the CO_2 in the 10% sample from experiment. Unfortunately, the extent of this back reaction depends on the relative amounts of glutamate dehydrogenase and isocitrate dehydrogenase, the relative rate of hydration of CO_2, the $NADP^+$/NADPH level, and other factors. As a result the extent of the reverse reaction varies from experiment to experiment. The only effective means for minimizing this variation is the use of a large excess of glutamate dehydrogenase. We have estimated the rate of the reductive carboxylation of ketoglutarate by spectrophotometric measurement of NADPH which is present at the steady state, and it is clear that although the isotopic composition of the 10% reaction samples may vary slightly, the variations are not sufficient to bias the calculated isotope effects seriously.

DISCUSSION

Decarboxylation of organic compounds ordinarily results in a carbon isotope effect k^{12}/k^{13} of 1.03–1.06 if decarboxylation is rate-determining[17–20]. No isotope

effect is observed ($k^{12}/k^{13} = 1.00$) if decarboxylation does not contribute appreciably to the rate-determining step*.

A similar, if more complex, situation applies to enzymes, although it appears that the observed isotope effects may be somewhat smaller than those in organic reactions due to the kinetic importance of steps prior to decarboxylation. Appreciable carbon isotope effects are observed for the enzymatic decarboxylation of glutamic acid[10-12] and acetoacetic acid (R. L. BAUGHN, unpublished observations). Almost no isotope effect is observed in the enzymatic decarboxylation of oxalacetic acid[9] or isocitric acid.

The small isotope effect which is observed in the oxidative decarboxylation of isocitric acid is in the opposite direction from that expected for rate-determining decarboxylation. It is clear that not only is decarboxylation not rate-determining, but the lack of any isotope effect at all indicates that decarboxylation must be several times faster than the rate-determining step. This conclusion follows from the fact that for the simple kinetic scheme of Eqn. 1, assuming that k_1 and k_{-1} are rapid, the observed isotope effect is given[9] by Eqn. 2, where $R = k_3^{12}/k_{-2}$. The decarboxylation

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E.S' \overset{k_3}{\rightharpoonup} E \cdot P + CO_2 \qquad (1)$$

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

step is almost certainly subject to an isotope effect of the usual magnitude, so the absence of a measurable isotope effect indicates that R in Eqn. 2 is large; that is, decarboxylation is many times faster than the rate-determining step**.

It is also necessary to consider the possibility that a product release step is rate-determining in the overall enzymatic reaction. If product release is rate-determining and decarboxylation is readily reversible, then the carbon isotope effect observed will be the equilibrium isotope effect on the decarboxylation, which is quite small (ref. 21 and M. J. STERN, personal communication). However, this possibility is made unlikely by the observation that the decarboxylation of oxalosuccinate catalyzed by this enzyme is faster than the oxidative decarboxylation of isocitrate[7,8].

Thus we conclude that some step prior to decarboxylation in the oxidative decarboxylation of isocitric acid is rate-determining. This is consistent with the results of previous studies[6]. In addition, our results provide a limit on how slow the decarboxylation can be—it must be much faster than the slowest step in the reaction sequence.

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* The idea that no isotope effect is observed unless decarboxylation is rate-determining is a dangerous oversimplification both for organic reactions and for enzyme-catalyzed reactions. Lack of a carbon isotope effect indicates that the decarboxylation step is much faster (at least a factor of ten) than the rate-determining step. An isotope effect can be observed even if some previous step in the reaction sequence is somewhat slower than the decarboxylation step (see in particular Eqn. 2).

** The rate-determining step is defined to be the step whose transition state has the highest free energy; thus the decision as to whether the second or the third step in Eqn. 1 is rate-determining is properly decided by comparing k_3 with k_{-2}, not k_3 with k_2.

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