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Mechanisms of Enzymatic Oxidative Decarboxylation

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Introduction

A number of important reactions in living cells involve oxidative decarboxylation of molecules containing a hydroxyl group β to a carboxyl, or two carbons further removed via a double bond, with a pyridine nucleotide (NAD or NADP) as the oxidizing agent (Table 1). In most cases the hydroxyl group is converted into a ketone, but with prephenate dehydrogenase it remains a hydroxyl group and the ring is aromatized. None of these reactions involve net proton uptake or release, and CO_2 is always the immediate product. Malic enzyme and isocitrate dehydrogenase require a metal ion activator, normally Mg^{2+}, but prephenate and 6-phosphogluconate dehydrogenases do not. (+)-Tartrate dehydrogenase requires Mn^{2+} and K^+.

In each case, a hydride ion is removed from the carbon bearing the hydroxyl group that is oxidized and is transferred directly to C-4 of the nicotinamide ring of the nucleotide. The transfer is to the *re* face (A side, *pro-R* position) with malic enzyme and isocitrate and (+)-tartrate dehydrogenases, and to the *si* face (B side, *pro-S* position) with prephenate and 6-phosphogluconate dehydrogenases.[1−3] With none of these enzymes is an intermediate ketone released during oxidation of the physiological substrate, so the question arises whether these reactions are concerted or occur via an enzyme-bound ketone intermediate that is not released from the enzyme. It turns out that some are concerted and some are stepwise, and by use of alternate substrates one can even induce several to change from stepwise to concerted, or vice versa.

All the compounds described herein are shown in Chart 1.

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Multiple Isotope Effect Method

These questions have been answered largely by use of the multiple isotope effect method, in which a ^{13}C isotope effect in the carbon released as CO_2 is determined with substrates containing either hydrogen or deuterium at the position transferred as a hydride ion.[4] If the mechanism is concerted, deuteration slows down hydride transfer and makes this step more rate limiting. The ^{13}C isotope effect is then more fully expressed and is larger than that with an undeuterated substrate (or the same, if the step was originally fully rate limiting). If the mechanism is stepwise, however, deuteration makes the ^{13}C-sensitive decarboxylation less rate limiting, and the observed ^{13}C isotope effect will be smaller than that with non-deuterated substrate.

If the mechanism is stepwise, moreover, the ^{13}C isotope effects with deuterated and non-deuterated substrates and the deuterium isotope effect itself are related by the following equations.[4]

In the direction where the deuterium-sensitive step comes first:

$$[^{13}(V/K)_H - 1]/[^{13}(V/K)_D - 1] = {}^{D}(V/K)/{}^{D}K_{eq} \quad (1)$$

In the opposite direction, where the ^{13}C-sensitive step comes first:

$$[^{13}(V/K)_H - {}^{13}K_{eq}]/[^{13}(V/K)_D - {}^{13}K_{eq}] = {}^{D}(V/K) \quad (2)$$

In the notation used in these equations, the leading superscript indicates the isotope effect (D for deuterium; 13 for ^{13}C). Thus, ${}^{D}(V/K) = (V/K)_H/(V/K)_D$ and is the kinetic isotope effect on V/K for the substrate containing the label. ${}^{D}K_{eq}$ and ${}^{13}K_{eq}$ are equilibrium isotope effects, where ${}^{D}K_{eq} = K_{eq\ H}/K_{eq\ D}$. In these equations, the isotope effects are those in the forward direction being considered. Thus, ${}^{D}(V/K)$ in eq 2 is not the same as ${}^{D}(V/K)$ in eq 1, and their ratio is the equilibrium isotope effect. As a result, ${}^{D}(V/K)_{forward}/{}^{D}(V/K)_{reverse} = {}^{D}K_{eq\ forward}$. A similar relationship applies for ^{13}C isotope effects. Equations 1 and 2 are thus the same equation, but expressed in terms of measured isotope effects in either forward or reverse directions. When the equilibrium isotope effects (particularly ${}^{D}K_{eq}$) are not unity, however, a given set of experimental isotope effects will fit one equation and not the other.

The reason that the kinetic isotope effects are shown as ones on V/K is that the internal competition method used to determine ^{13}C isotope effects measures only isotope effects on V/K and not those on the maximum velocity. Carbon in nature contains 1.1% ^{13}C, and the $^{13}C/^{12}C$ ratio in CO_2 after partial reaction (R_p) compared to that after 100% reaction (R_o) can be combined with the fraction of reaction (f) to give the isotope effect on V/K:

$$^{13}(V/K) = [\log(1 - f)]/[\log(1 - fR_p/R_o)] \quad (3)$$

Mass ratios can be measured in CO_2 with a precision of 0.0002 with an isotope ratio mass spectrometer. Deuterium isotope effects are normally big enough to be

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Table 1. Enzymatic Oxidative Decarboxylations

reaction	enzyme
L-malate^{2-} + NAD(P)$^+$ $\xrightarrow{Mg^{2+}}$ pyruvate$^-$ + CO_2 + NAD(P)H	malic enzyme
threo-D$_S$-isocitrate^{3-} + NAD(P)$^+$ $\xrightarrow{Mg^{2+}}$ α-ketoglutarate^{2-} + CO_2 + NAD(P)H	isocitrate dehydrogenase
prephenate^{2-} + NAD$^+$ → *p*-hydroxyphenyl pyruvate$^-$ + CO_2 + NADH	prephenate dehydrogenase
6-phospho-D-gluconate^{3-} + NADP$^+$ → D-ribulose-5-phosphate^{2-} + CO_2 + NADPH	6-phosphogluconate dehydrogenase
D-malate^{2-} + NAD$^+$ $\xrightarrow{Mn^{2+},\ K^+}$ pyruvate$^-$ + CO_2 + NADH	(+)-tartrate dehydrogenase

determined by direct comparison of reciprocal plots with deuterated or unlabeled substrate, with the ratio of the slopes being $^D(V/K)$.

Malic Enzyme

The multiple isotope effect method was first applied to chicken liver malic enzyme,[4] where $^DK_{eq}$ in the forward direction is 1.18 (that is, hydrogen at C-2 of malate is more stiffly bonded than that at C-4 of the dihydronicotinamide ring of NADPH). The ^{13}C isotope effects at C-4 of malate, which becomes CO_2 during the reaction, were 1.031 with unlabeled malate and 1.025 with malate deuterated at C-2, while the deuterium isotope effect on V/K at C-2 was 1.47. The reaction thus appears to occur stepwise, and when these numbers were inserted into eq 1, they gave $1.21 \pm 0.05 = 1.25 \pm 0.03$, while when inserted into eq 2 they gave (using the more recently determined[5] $^{13}K_{eq}$ value of 0.988) $1.14 \pm 0.05 \neq 1.47 \pm 0.03$.[4] Thus, to no one's great surprise, the deuterium-sensitive dehydrogenation precedes the ^{13}C-sensitive decarboxylation. Figure 1 shows the geometry changes that accompany dehydrogenation of malate. As C-2 becomes trigonal, the C-2, C-3, C-4 bond angle is narrowed, which helps catalyze the subsequent decarboxylation.

While oxaloacetate is not released from malic enzyme during the reaction, it can be added to enzyme along with NADPH, and it binds and partitions between reduction to malate and decarboxylation to pyruvate. The partition ratio of pyruvate/malate was 0.47 with unlabeled NADPH, but with NADPH deuterated at the *pro-R* position of C-4 of the dihydronicotinamide ring, the ratio was 0.81.[6] These ratios were determined spectrophotometrically by simultaneously observing NADPH oxidation at 340 nm and oxaloacetate disappearance at 281.5 nm, the isosbestic point of NADP−NADPH. By combining the partition ratio (r) with the $^{13}(V/K)$ value, one can calculate the intrinsic ^{13}C isotope effect on the decarboxylation step:[6]

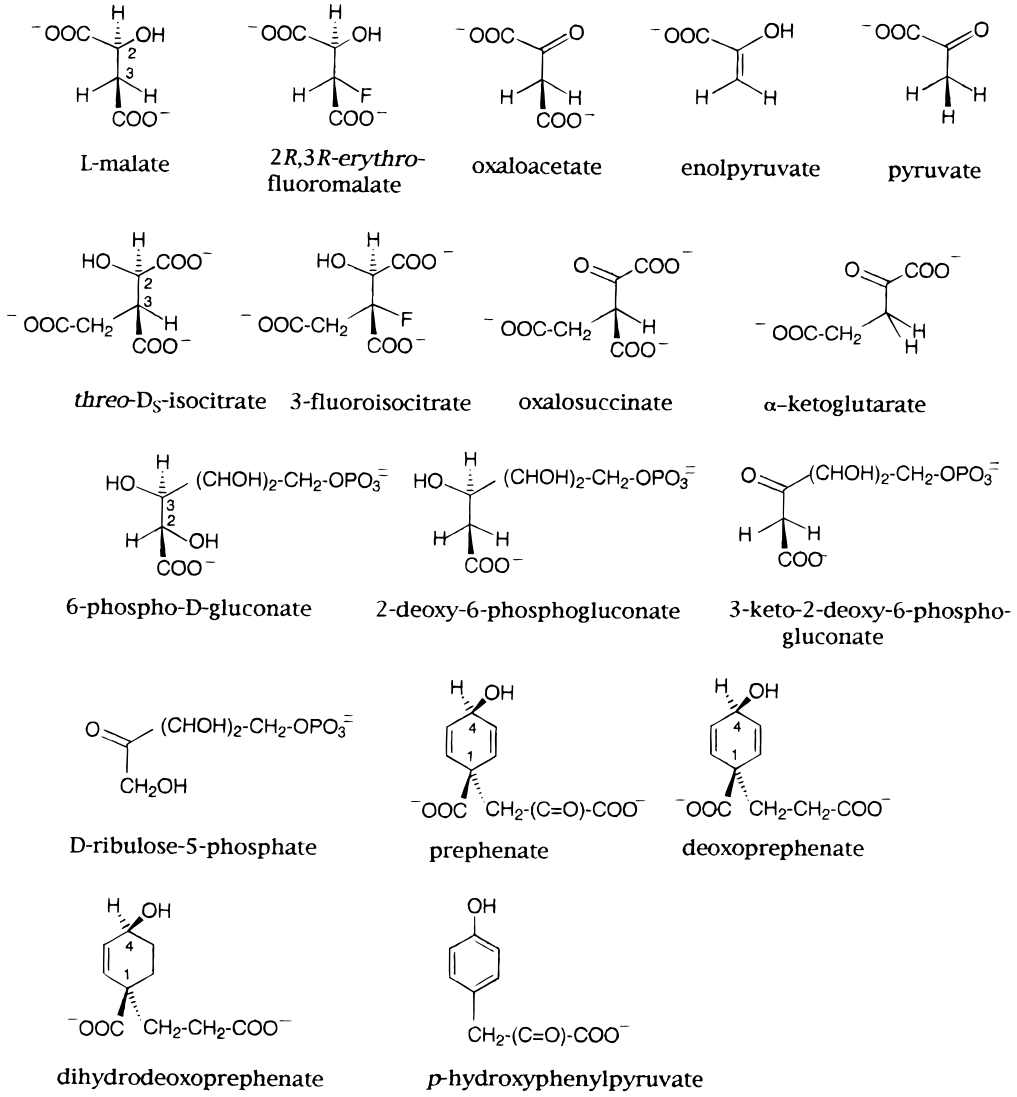
$$^{13}k = {}^{13}(V/K) + r[{}^{13}(V/K) - 1] \qquad (4)$$

The values with non-deuterated reactants gave 1.044, and the values with deuterated reactants gave 1.045, in excellent agreement. By using the tritium isotope effect of 2.02[7] (again, an isotope effect on V/K) along with the others, it was possible to calculate that the intrinsic deuterium isotope effect on the hydride transfer step was 5.7 and that reverse hydride transfer was 10 times faster than decarboxylation.[6]

These experiments were done with the physiological nucleotide NADP, which has a redox potential of −0.32 V at pH 7. When thio-NADP and acetylpyridine-NADP, which have higher redox potentials (−0.285 and −0.258 V, respectively) were used as substrates, however, the ^{13}C isotope effects were *larger* with deuterated than with non-deuterated malate, rather than smaller (1.0147 vs 1.0090 for thio-NADP and 1.0084 vs 1.0051 for the acetylpyridine analogue).[8] Similar results were obtained for the NAD-malic enzyme from *Ascaris*, with the mechanism appearing to be stepwise with NAD, NADP, and ϵ-NAD, but concerted with thio-NAD, acetylpyridine-NAD, and pyridinealdehyde-NAD (redox potential = −0.262 V).[8−10]

These data suggest that the reaction becomes concerted with nucleotides that are more powerful oxidants. The equilibrium constant for the oxidative decarboxylation is more favorable with thio-NADP or acetylpyridine-NADP by factors of 15 or 126, respectively. An alternate explanation for these results is that there is a secondary ^{13}C isotope effect at C-4 during the hydride transfer step that is more fully expressed when malate is deuterated at C-2. This secondary ^{13}C isotope effect would result from hyperconjugation into the keto group of the oxaloacetate intermediate (Figure 2). Carbon-4 must be held out of the plane of C-1, C-2, C-3 and the oxygen at C-2 in order for decarboxylation to take place (contrast malate dehydrogenase, where C-4 is held in plane to suppress decarboxylation of oxaloacetate). Polarization of the keto group of the oxaloacetate intermediate would be provided by metal ion coordination and/or hydrogen bonding to the group that removed the proton during hydride transfer, and such polarization would withdraw electron density from the C-3 to C-4 bond. This decreased bond order could not be made up by increased C−O bond order in the carboxyl group, because the trigonal geometry does not allow double bonds to both oxygens. Thus, the ^{13}C fractionation factor of C-4 would be expected to decrease in the enzyme-bound oxaloacetate intermediate, and a secondary ^{13}C isotope effect might be expected on the hydride-transfer step. Because the primary deuterium isotope

Chart 1



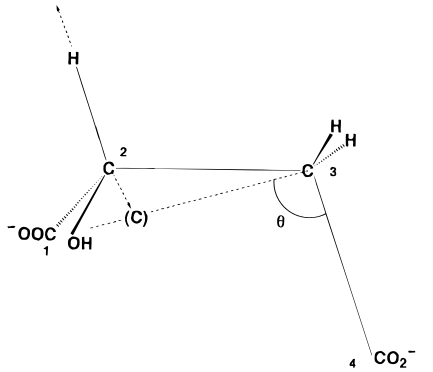


FIGURE 1. Hydride transfer from C-2 of malate narrows θ, the C-2, C-3, C-4 bond angle, as the result of the change from tetrahedral to trigonal geometry at C-2. This change facilitates decarboxylation of the resulting oxaloacetate.

effect is larger with the alternate nucleotides and the ^{13}C isotope effects are relatively small, one would see largely the ^{13}C isotope effect on hydride transfer, rather than that on decarboxylation.

This question was settled by measurement of the ^{13}C isotope effects at C-3 of malate.[5] Hyperconjugation of the

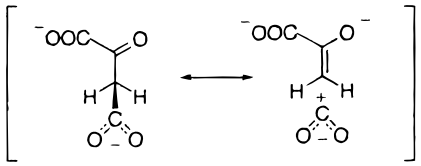


FIGURE 2. Hyperconjugation in the oxaloacetate intermediate in the malic enzyme-catalyzed reaction.

type proposed above should not affect the fractionation factor of C-3, since any bond order decrease between C-3 and C-4 would be accompanied by a corresponding increase between C-2 and C-3. Thus, there should only be a primary ^{13}C isotope effect on the decarboxylation step, and no isotope effect on hydride transfer. With acetylpyridine-NADP, the ^{13}C isotope effects at C-3 were 1.0067 with non-deuterated malate and 1.0125 with deuterated malate, while with NADP the values were 1.0210 and 1.0130, respectively. Thus, the reaction really has become concerted with acetylpyridine-NADP. The intrinsic primary deuterium isotope effect with acetylpyridine-NADP was 3.1 when calculated from isotope effects at C-4 and 3.7 when calculated from those at C-3. The intrinsic

^{13}C isotope effects at C-3 and C-4 were 1.018 and 1.012. The small ^{13}C isotope effects and values above 3 for the deuterium isotope effects show that the reaction is highly asynchronous, with C−H cleavage greatly preceding C−C cleavage, which has only just started in the transition state.

The secondary deuterium isotope effects at C-3 of malate reinforce these conclusions.[5] With NADP, the isotope effect was 1.17 with two deuteriums and 1.076 with deuteration only in the 3R position. These are effects on decarboxylation, since there should be no β-secondary effect on hydride transfer with these hydrogens held in plane by having C-4 fixed in the out-of-plane position. The equilibrium isotope effects for decarboxylation to enolpyruvate are 1.26 and 1.58 for mono- and dideuteration, and if one uses the commitments that reduce the intrinsic ^{13}C isotope effect to the observed one, one gets 1.22 for dideuteration. The observed value is 38% of this and suggests a transition state for decarboxylation with this degree of cleavage. With acetypyridine-NADP, however, the β-deuterium effect for dideuteration at C-3 is only 1.03−1.04. This value, after correction for commitments, is 10−14% of the equilibrium isotope effect and is consistent with an early transition state for C−C cleavage in the asynchronous concerted reaction.

The expected effects of hyperconjugation in the oxaloacetate intermediate were, however, seen with the slow alternate substrate (2*R*,3*R*)-*erythro*-fluoromalate.[11] This substrate has a turnover number decreased by a factor of 3300, so the chemistry is certainly rate limiting. Deuteration of fluoromalate at C-2 gave a V/K isotope effect of 1.39 with NADP and 3.32 with acetypyridine-NADP. With NADP, the ^{13}C isotope effects at C-4 were 1.0490 with nondeuterated and 1.0364 with deuterated fluoromalate, while with acetylpyridine-NADP they were 1.0138 and 1.0087, respectively. The smaller ^{13}C isotope effects with deuterated fluroromalate in both cases show that the reaction is a stepwise one for both nucleotide substrates.

With the data for acetylpyridine-NADP, however, eq 1 was not satisfied. With $^{D}K_{eq} = 1.09$,[12] this equation gave 1.59 for the left side and 2.94 for the right side.[11] To explain this result, it was necessary to assume a secondary ^{13}C isotope effect on the hydride-transfer step of ∼1.0064. This isotope effect is seen with acetypyridine-NADP because the hydride-transfer step is more rate limiting and decarboxylation is ∼2.6 times faster than reverse hydride transfer, compared to 0.1 times as fast with NADP. With NADP, eq 1 is satisfied, with values of 1.35 and 1.28 for the left and right sides, respectively, since decarboxylation is the major rate-limiting step and the secondary ^{13}C isotope effect on hydride transfer is not noticed. However, the "intrinsic" isotope effect calculated for decarboxylation is the product of the equilibrium ^{13}C isotope effect on hydride transfer and that on decarboxylation. Assuming 1.01 for the β-secondary ^{13}C equilibrium isotope effect on hydride transfer, the true intrinsic ^{13}C isotope effect on decarboxylation becomes 1.044 for fluoromalate. The values for the non-enzymatic decarboxylation of the dianion of oxalacetate are 1.0489 for the Mg^{2+}-catalyzed reaction and 1.052 for the non-catalyzed reaction.[13]

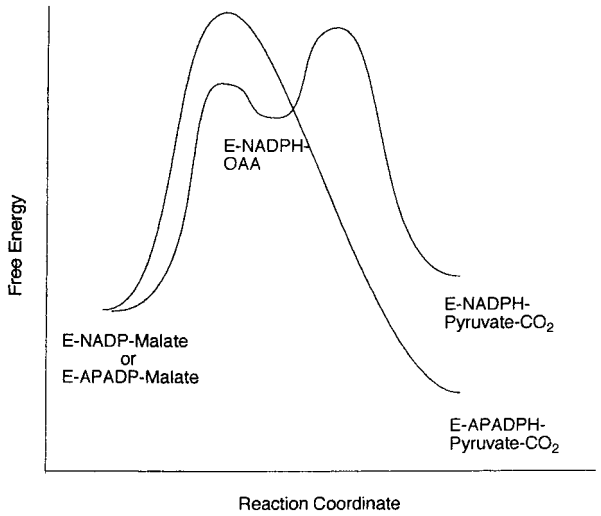


FIGURE 3. Free energy profiles for reactions catalyzed by malic enzyme. The nucleotide substrate is either NADP or acetylpyridine-NADP (APADP).

So why is the reaction of malate with acetylpyridine-NADP concerted, while reaction of NADP and malate, or reaction of fluoromalate with either nucleotide, is stepwise? The substitution with fluorine makes oxidation of malate more difficult by an order of magnitude, while replacement of NADP with acetylpyridine-NADP makes it more favorable by a factor of 126.[12] We believe it is the increased equilibrium constant of the overall reaction which converts a stepwise reaction with decarboxylation as the highest barrier to a concerted one. Lowering the energy level of E-NADPH-pyruvate relative to E-NADP-malate decreases the barrier to decarboxylation to the point where it disappears and the reaction becomes an asynchronous concerted one. The transition state is clearly one where C−C cleavage is lagging well behind C−H cleavage. Figure 3 shows free energy profiles for reaction of NADP and acetylpyridine-NADP with malate.

Although the reaction of malate and acetylpyridine-NADP is concerted, the enzyme still partitions added oxaloacetate in the presence of acetylpyridine-NADPH to pyruvate and malate with a pyruvate/malate ratio of 9.88.[6] This value, when combined with a $^{13}(V/K)$ value of 1.0042, a $^{D}(V/K)$ value of 2.18, and a $^{T}(V/K)$ value of 3.58, gave intrinsic deuterium and ^{13}C isotope effects of 4.2 and 1.046, and a ratio of decarboxylation to reverse hydride transfer of 3.96.[14] It is not clear that this is a valid calculation, since the isotope effects are for the concerted reaction and the partition ratio is for a necessarily stepwise one, but the results look reasonable. The active site is clearly capable of catalyzing both halves of a stepwise mechanism, even when the overall forward reaction is concerted.

Prephenate Dehydrogenase

We thought that this enzyme, like malic enzyme, would have a stepwise mechanism in which C-4 of the ring would be oxidized to a ketone, which would subsequently decarboxylate at C-1 to give *p*-hydroxyphenyl pyruvate.

A concerted reaction violates the Woodward−Hoffmann rules and thus appears forbidden. For synthetic simplicity, the isotope effect experiments were done with deoxoprephenate, which lacks the keto group in the side chain. This molecule was an excellent substrate, having a maximum velocity 78% that of prephenate and a K_m value only 4.3 times higher.[2] The deuterium isotope effect on V/K at C-4 was 2.34, while the ^{13}C isotope effect at the C-1 carboxyl was 1.0033 with non-deuterated and 1.0103 with deuterated substrate. These numbers indicate a concerted, not stepwise reaction, and since the decarboxylation is certainly irreversible, the three isotope effects can be used to solve for the intrinsic deuterium isotope effect of 7.3 and a ^{13}C one of 1.0155. The large size of the deuterium isotope effect shows that C−H cleavage is well advanced in the transition state, while the small ^{13}C isotope effect shows that C−C cleavage lags far behind. Thus, although the concerted reaction violates the rules, the tremendous amount of energy released by aromatization causes the reaction to become concerted. In fact, efforts to make the 4-keto analogue of deoxoprephenate were unsuccessful. It was prepared as a diester, but all efforts to remove the ester function at the 1-carboxyl resulted in decarboxylation and aromatization to *p*-hydroxyphenyl propionate. Saponification gave ethyl carbonate, while reduction of the ketone with $NaBH_4$ gave some ethyl formate by reduction of the ester group. Clearly, the ring is the best leaving group from any tetrahedral structure at this carbon!

Further insight into this reaction was provided by using as a substrate deoxodihydroprephenate, which contains only one double bond in the ring.[2] This molecule is simply oxidized reversibly to a ketone with no decarboxylation. The resulting ketone, which cannot aromatize by decarboxylation, was stable in 1 N HCl at 80 °C and was only decarboxylated at 78 °C in 1 M morpholine, pH 9.5, with a half-life of 30 min. Prephenate dehydrogenase is thus really just a secondary alcohol dehydrogenase, and the decarboxylation occurs in concerted fashion because the expected ketone intermediate has no stability.

Isocitrate Dehydrogenase

The substrates for the pig heart enzyme are very sticky (that is, react to give products faster than they dissociate), and thus isotope effects are suppressed at neutral pH. The $^{13}(V/K)$ value for the CO_2 product goes from 1.0028 at neutral pH to 1.040 ± 0.004 at low pH, with an apparent pK of 4.46.[15] The low pH plateau value has sizable error, however, since the lowest pH used (4.1) gave a value of 1.0276. With deuterated isocitrate, the variation was from 1.0009 to 1.016 ± 0.002. It is clear, however, that deuteration decreases the ^{13}C isotope effect, as expected of a stepwise mechanism.

The application of eq 1 suggests that $^{D}(V/K)$ at low pH should be 2.9 ($^{D}K_{eq} = 1.17$). The highest $^{D}(V/K)$ value observed, however, was 1.008 at pH 4.5. Thus, deuteration of isocitrate appears to decrease $^{13}(V/K)$ more than predicted, and the reason for this is not known.

An intermediate partitioning experiment with oxalosuccinate (the putative intermediate) added to enzyme and NADPH gave α-ketoglutarate/isocitrate ratios of 3.2 at pH 5 and 7.3 at pH 5.4.[15] Applying eq 4 gave intrinsic ^{13}C isotope effects of 1.046 ± 0.006 at pH 5 and 1.054 ± 0.008 at pH 5.4, which are not significantly different and are reasonable in size. Lower pH values gave problems with NADPH stability, while at higher pH values the increasing stickiness of isocitrate caused the α-ketoglutarate/isocitrate ratio to rise to above 15 so that accurate measurement was not possible. Still, the results are consistent with a stepwise mechanism via an oxalosuccinate intermediate that is not normally released from the enzyme.

3-Fluoroisocitrate is a substrate for pig heart isocitrate dehydrogenase with a V_{max} that is 3.4% that of isocitrate and a similar K_m.[15] It shows a $^{D}(V/K)$ value of 1.45 at pH 7 and $^{13}(V/K)$ values of 1.0129 at pH 7 and 1.0186 at pH 5.2. Unfortunately, the ^{13}C isotope effect was not determined with deuterated substrate. It is much less sticky than isocitrate, although still somewhat so, as the $^{13}(V/K)$ value is larger at pH 5.2 than at 7. Another alternate substrate, 3-hydroxyisocitrate (7% V_{max}), also gave a $^{D}(V/K)$ value of 1.53 at pH 7, but the product, 2-hydroxy-3-ketoglutarate, spontaneously decarboxylated so that extra CO_2 (total 2.8 per turnover) was produced, and measurement of the ^{13}C isotope effect on the initial enzymatic reaction was not possible.

6-Phosphogluconate Dehydrogenase

Unlike malic enzyme and isocitrate dehydrogenase, this enzyme does not require a metal ion activator. Presumably, the 2- and 4-hydroxyl groups inductively activate the putative 3-keto intermediate for decarboxylation. They are both needed, since when 2-deoxy-6-phosphogluconate was used as a substrate, the 3-keto-2-deoxy-6-phosphogluconate formed by dehydrogenation was released from the enzyme and only slowly decarboxylated on further incubation.[16] It was also reduced by NADPH back to 2-deoxy-6-phosphogluconate.

Enzymes from three sources, *Torula*[17] and *Candida*[18] yeasts and sheep liver,[18] have been studied by the isotope effect method, and all appear to have stepwise mechanisms. The *Torula* enzyme with NADP showed $^{13}(V/K)_H$ and $^{13}(V/K)_D$ values of 1.0096 and 1.0081, and a $^{D}(V/K)$ value of 1.54.[17] Equation 1 gave 1.19 for the left side and 1.31 for the right, with these values not significantly different. With eq 2, the two sides were 1.12 and 1.54. With a $^{T}(V/K)$ value of 2.05, possible intrinsic isotope effects were deduced, giving $^{13}k = 1.046$, $^{D}k = 3.2$, and a ratio of reverse hydride transfer to decarboxylation near unity.[17]

The *Candida* enzyme with NADP gave $^{13}(V/K)_H$ and $^{13}(V/K)_D$ values of 1.0209 and 1.0158, and a $^{D}(V/K)$ value of 1.63.[18] Equation 1 gave 1.34 = 1.33. The sheep enzyme with NADP gave similar values (1.0059, 1.0036, 1.87), and eq 1 gave 1.64 vs 1.58.[18] The intrinsic isotope effects appear very similar to those of the *Torula* enzyme.

When acetylpyridine-NADP was used as the substrate, however, a very interesting pattern was seen. $^{13}(V/K)_H$ and $^{13}(V/K)_D$ were 1.0106 and 0.9934 with the *Candida* enzyme,

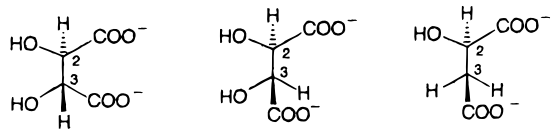


FIGURE 4. Presumed orientation of substrates bound to (+)-tartrate dehydrogenase.

and 1.0086 and 0.9950 with the sheep one, while $^{D}(V/K)$ values were 3.60 and 2.50 for the two enzymes.[18] The decrease in ^{13}C isotope effect upon deuteration implies a stepwise mechanism, but the inverse ^{13}C isotope effects with deuterated 6-phosphogluconate require that there be a step prior to decarboxylation where the fractionation factor of C-1 is increased by a factor of at least 1.005. This most likely is a binding isotope effect caused by raising the force constant for the torsional motion in which the carboxyl group rotates around the C-2 to C-3 axis. It appears that hydride transfer has become totally rate limiting with acetylpyridine-NADP when 6-phosphogluconate is deuterated, and thus the contribution of the ^{13}C isotope effect on decarboxylation is negligible. This phenomenon should occur with all enzymatic oxidative decarboxylations, since the carboxyl group that is to leave has to be held out of plane. While a binding isotope effect of 0.995 seems high, torsional modes have been shown to be very important for other heavy-atom isotope effects.[19,20]

These results suggest that, in using eq 1 or 2, one should correct the experimental $^{13}(V/K)$ values for a possible binding isotope effect. If an effect of 0.995 is assumed, then these values should be multiplied by 1.005 before being used in eq 1 or 2. Clearly, more work is needed before the complete story here can be told.

D_2O solvent isotope effects have been measured on the *Candida* and sheep liver enzymes, as well as the effect of D_2O on the ^{13}C isotope effects.[21] Proton inventories were also obtained and showed the presence of significant medium effects on a step prior to hydride transfer, as well as a kinetic solvent isotope effect on hydride transfer. The ^{13}C isotope effect with the sheep enzyme was reduced from 1.0225 to 1.0185 in D_2O, and the conclusion was that all steps in the mechanism were altered by D_2O.

(+)-Tartrate Dehydrogenase

This enzyme shows a diferent reaction with each of the three substrates it acts on.[22] With (+)-(2*R*,3*R*)-tartrate the product is oxaloglycolate, while with *meso*-(2*R*,3*S*)-tartrate the products are CO_2 and D-glycerate. In the latter case, the opposite enantiomer of oxaloglycolate is presumably produced, but the carboxyl of the glycolyl portion is held out of plane and decarboxylates to CO_2 (Figure 4). NADH then reduces the hydroxypyruvate intermediate to D-glycerate. With (+)-tartrate, the glycolyl carboxyl must be held *in plane*, and thus cannot decarboxylate prior to release of oxaloglycolate (Figure 4).

This enzyme also acts on D-malate, producing pyruvate and CO_2 as products.[22] Without the steering effect of a 3-hydroxyl, the 4-carboxyl is apparently free to assume the out-of-plane position and decarboxylates (Figure 4). The partitioning of added oxaloacetate in the presence of NADH to either D-malate or pyruvate has been measured.[23] By determining deuterium and tritium isotope effects on the reaction, the equations for this mechanism were solved to give an intrinsic deuterium isotope effect of 5.1 ± 0.8, a forward commitment for hydride transfer of 6.3 ± 1.0, and a reverse commitment (presumably the partition ratio of oxaloacetate for reverse hydride transfer vs decarboxylation) of 2.0 ± 0.3.[23] By use of viscosity effects, the forward commitment was broken into an external part of 1.4 and an internal part of 2.6, but the errors in this derivation were very large. This analysis assumes that the reaction catalyzed by (+)-tartrate dehydrognase is stepwise, but in the absence of ^{13}C isotope effects with deuterated and non-deuterated D-malate, a concerted reaction cannot be ruled out.

Conclusion

This Account describes the mechanisms of five enzymes that catalyze oxidative decarboxylations, as determined from isotope effects and other studies. Prephenate dehydrogenase has a concerted although asynchronous mechanism as a result of the energy released by aromatization of the 1,4-cyclohexadiene ring. The other enzymes have stepwise mechanisms with physiological substrates, but the malic enzyme reaction becomes concerted when malate (but not fluoromalate) reacts with a high redox potential nucleotide such as acetylpyridine-NADP. Thus, a sufficiently high equilibrium constant for the oxidative decarboxylation eliminates stability of the intermediate ketone and leads to a concerted but asynchronous mechanism where C−H cleavage leads C−C cleavage.

When the mechanism is stepwise, the ketone intermediate is not normally released from the enzyme. However, with a 2-deoxy substrate, 6-phosphogluconate dehydrogenase releases the 2-deoxy-3-keto-6-phosphogluconate intermediate because the decarboxylation step is slowed by loss of the inductive effect of the 2-hydroxyl group. The ketone intermediate can, however, be added to these enzymes along with reduced nucleotide, and it partitions between reduction and decarboxylation. The non-release of the ketone intermediate results from a kinetic barrier and from the fact that decarboxylation, which is fostered by the geometry change accompanying hydride transfer, and reverse hydride transfer are much faster than release of the intermediate. Presumably, the steady-state level of the intermediate is quite small, although it has not been measured for any of these enzymes.

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