Transition-State Structures in Enzyme-Catalyzed Decarboxylations

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Since the discovery of the stable isotopes of the elements heavier than hydrogen, chemists have been interested in measuring effects of isotopic substitution on rates and equilibria.[1,2] Although for the elements heavier than hydrogen such effects never exceed a few percent, they have been measured accurately for many years, principally by isotope-ratio mass spectrometry. Early work by physical chemists[2] was concerned with simple reactions in solution and in the gas phase, with the objective of defining transition-state structure. Later work by organic chemists was concerned with mechanism and transition-state structure in multistep organic reactions.[3]

Application of similar methods to enzyme-catalyzed reactions has come more recently.[4–6] Studies with enzymes awaited technical improvements that allowed accurate isotope-ratio measurement on small samples of sensitive, unstable materials that are not amenable to classical treatments. At the same time, studies of enzymatic reactions have required development of new theoretical approaches that acknowledge the complexity of these systems. Enzymatic reactions involve a large number of steps, and in many cases, the actual number of steps is not known. Fortunately, steps that are fast and steps that are subsequent to the first irreversible step can generally be neglected, and only relatively slow steps need be considered. Although early application of heavy-atom isotope effects concentrated on identification of the "rate-determining step", more recent studies have approached questions of transition-state structure as well.

Mechanisms of enzymatic reactions can be approached at several levels. First is the order of binding of substrates and dissociation of products (i.e., the kinetic mechanism). Second is the description of the chemical events in the overall transformation (the chemical mechanism). Third is the nature of the catalytic action provided by the enzyme (the catalytic mechanism). Our purpose here is to show how heavy-atom isotope effects can contribute to our understanding of enzymatic reaction mechanisms at all three levels. Of particular concern is the question of transition-state structure in these reactions.

## A Simple Example: Formate Dehydrogenase

A minimal mechanism for an enzyme-catalyzed reaction involves three steps: substrate binding, a chemical step, and product dissociation. Unfortunately, few

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Scheme I
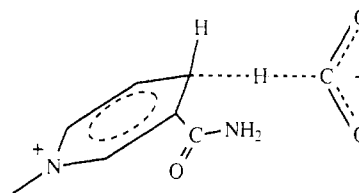
enzymatic reactions involve only a single chemical step. One such reaction is the oxidation of formic acid by an enzyme from yeast. The reaction catalyzed is
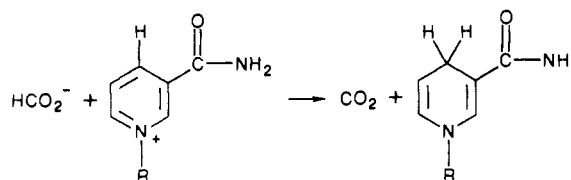
in which the hydrogen acceptor is NAD^+, nicotinamide adenine dinucleotide. In this reaction, there is the transfer of a formal hydride from formate to NAD^+, forming CO_2 and NADH.

Both steady-state kinetics and isotope effects indicate that the kinetic mechanism is "ordered", with NAD^+ binding first and formate second.[7] The order of product release steps has not been determined, but CO_2 is probably released first, followed by NADH. Substrate binding and dissociation are rapid, so these steps do not influence the isotope effects observed (this is not always true in other cases).

Some isotope effects for formate dehydrogenase are summarized in Table I.[8] Blanchard and Cleland[7] showed that for formate dehydrogenase at pH 7.8, 25 °C, the carbon isotope effect is $k^{12}/k^{13} = 1.042$, where k^{12} and k^{13} are the corresponding rate constants. The hydrogen isotope effect for the hydrogen being transferred during the formate dehydrogenase reaction under the same conditions is $k^H/k^D = 2.2$. The carbon isotope effect is the same whether formate or deuterated formate is used as substrate. This indicates that the re-

(1) Melander, L.; Saunders, W. H. *Reaction Rates of Isotopic Molecules*; Wiley: New York, 1980.
(2) Bigeleisen, J.; Wolfsberg, M. *Adv. Chem. Phys.* **1958**, *1*, 15.
(3) Fry, A. In *Isotope Effects in Chemical Reactions*; Collins, C. J., Bowman, N. S., Eds.; Van Nostrand Reinhold: New York, 1970, p 364.
(4) O'Leary, M. H. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; p 285.
(5) Cleland, W. W. *Bioorganic Chemistry* **1987**, *15*, 283.
(6) Cleland, W. W. *CRC Crit. Rev. Biochem.* **1982**, *13*, 385.
(7) Blanchard, J. S.; Cleland, W. W. *Biochemistry* **1980**, *19*, 3543.
(8) Hermes, J. D.; Morrical, S. W.; O'Leary, M. H.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5479.
(9) Cook, P. F.; Blanchard, J. S.; Cleland, W. W. *Biochemistry* **1980**, *19*, 4853.

Table I
Isotope Effects on the Oxidation of Formate by Formate Dehydrogenase at 25 °C, pH 7.8[a]

substituent on pyridine ring	k^{12}/k^{13}	k^{12}/k^{13} with formate-d	k^{16}/k^{18} per oxygen	k^H/k^D primary	k^H/k^D secondary
CONH$_2$	1.042	1.044	1.005	2.17	1.23
CSNH$_2$	1.038	1.036	1.007	2.60	1.18
COCH$_3$	1.036		1.008	3.32	1.06
CHO	1.041	1.041	0.995	2.85	0.99

[a] Reference 8.

action occurs in a single rate-determining step (Scheme I), and substrate binding and dissociation steps are rapid compared to catalysis. The size of the hydrogen isotope effect indicates that the transition state is asymmetric, but it is not possible to tell whether the hydrogen being transferred is closer to CO$_2$ or to NAD$^+$.

More detail concerning transition-state structure can be obtained by looking at the secondary deuterium isotope effect at the 4-position of NAD$^+$. Carbon-4 of the pyridine ring of NAD$^+$ undergoes a hybridization change during the hydride transfer. Such hybridization changes give rise to significant secondary isotope effects, with the isotope effect intermediate between unity for an early transition state and the equilibrium value (0.899) for a late transition state. A relatively large isotope effect of 1.23 is obtained at this position. The fact that the isotope effect is larger than unity suggests that either the transition state has an unusual structure, with a significant degree of C–H bond weakening, or else the motion of this hydrogen is a significant component of the reaction-coordinate motion for the reaction. The latter seems to be the case.

Surprisingly, the secondary hydrogen isotope effect at carbon-4 of NAD$^+$ is reduced from 1.23 to 1.07 when the isotope effect is measured with deuterated formate as the substrate. Calculations suggest that this occurs because the enzyme compresses formate against NAD$^+$, causing the energy barrier for hydrogen transfer to be narrower (though no less high) than it would ordinarily be. These conditions allow a substantial degree of hydrogen tunneling. Motion of the secondary hydrogen at C-4 of the coenzyme must be a significant component of the reaction-coordinate motion, and this component becomes less significant with deuterated substrate. A similar conclusion has been reached for model hydride transfers by Powell and Bruice[10] and for enzymatic hydride transfer by Klinman et al. (J. Klinman, personal communication). A similar role for tunneling has been suggested in the case of enzyme-catalyzed S$_N$2 reactions by Schowen, et al.[11]

Formate dehydrogenase is active (at varying rates) with a variety of NAD$^+$ analogues having various substituents at the 3-position of the pyridine ring, and isotope effects have been measured for several of these (Table I). As the redox potential of the analogue becomes more positive, the carbon isotope effect decreases, the primary hydrogen isotope effect increases, and the secondary hydrogen isotope effect decreases. The principal contributor to the carbon isotope effect is the loss of the carbon–hydrogen bond. For this reason, we expect that the carbon isotope effect should increase as the transition state becomes more product-like, although no direct demonstration of this exists.

If this is true, then the small primary hydrogen isotope effect is due to a product-like transition state (i.e., we are on the descending side of the Westheimer curve[12]) and a more positive redox potential results in a more symmetrical transition state.

Oxygen isotope effects for the oxygens of formate can also be used in studies of transition-state structure. Experimentally, measurement of such oxygen isotope effects is difficult because when formate is converted to CO$_2$, the isotopic integrity of the oxygens is rapidly lost due to exchange with the solvent. A number of years ago, we designed a multiple-label method (now called the "remote label" method) for looking at such cases.[13] The method works as follows: We synthesize formate containing nearly 100% of both ^{13}C and ^{18}O. Separately, we synthesize formate containing only ^{12}C (^{12}CO$_2$ from which nearly all the ^{13}C has been removed is a byproduct of the production of ^{13}CO$_2$ and is very inexpensive). We then mix these two formates in the ratio of 1:90. The resulting formate contains the usual "natural abundance" of ^{13}C, but every formate that contains ^{13}C also contains two ^{18}O. Measurement of the apparent "^{13}C isotope effect" on this material actually gives the product of the ^{18}O and ^{13}C effects. Since the latter isotope effect is already known, we can calculate the oxygen isotope effect.

The oxygen isotope effects so obtained (Table I) are small and vary with nucleotide. Two phenomena contribute to the oxygen isotope effects: First, formate is desolvated on binding to the enzyme, and this produces an isotope effect of perhaps 1.01–1.02. Second, decarboxylation increases the C–O bond order and thus tends to produce an inverse isotope effect. The observed isotope effect is the product of these two factors and varies with transition-state structure.

Thus the dehydrogenation of formic acid proceeds in a single step. The transition state is product-like, and its structure varies as the redox potential of the nucleotide varies. Tunneling is an important factor in the overall reaction. Desolvation of the carboxyl group and steric compression of the transition state probably contribute to the catalytic power of the enzyme.

Measurement and Precision

Heavy-atom isotope effects on enzymatic reactions, like those on organic reactions, are generally measured by the competitive method, which involves measurement of changes in the isotope ratio (e.g., ^{13}C/^{12}C) of a starting material or product over the course of the reaction.[2,14] The isotope ratio is ordinarily measured with an isotope-ratio mass spectrometer, which provides a very precise measurement of the ^{13}C/^{12}C ratio in simple substances such as CO$_2$. Ordinary mass spec-

(10) Powell, M. F.; Bruice, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 7139.
(11) Gray, C. H.; Coward, J. K.; Schowen, K. B.; Schowen, R. L. *J. Am. Chem. Soc.* **1979**, *101*, 4351.

(12) Westheimer, F. H. *Chem. Rev.* **1961**, *61*, 265.
(13) O'Leary, M. H.; Marlier, J. F. *J. Am. Chem. Soc.* **1979**, *101*, 3300.
(14) O'Leary, M. H. *Methods Enzymol.* **1980**, *64*, 83.

trometers cannot be used because they do not provide the necessary precision. Substances more complex than CO_2 are not used because of interferences from minor isotopic species other than the one of interest and because of potential memory effects in the inlet system of the mass spectrometer. Although it would seem that increased precision could be achieved by use of isotopically enriched materials, this is in fact not the case. All samples are contaminated to some extent (however slight) by atmospheric contaminants (e.g., CO_2), and the error caused by this contamination increases as the isotopic difference between sample and contaminant increases. Thus, this error is minimized by use of natural-abundance materials.

Current isotope-ratio mass spectrometers are sufficiently precise that the mass spectrometer does not contribute to the total experimental error in studies with enzymes. In carefully conducted investigations, isotope ratios of samples remain constant over periods of months or years. Most experimental error arises from problems in sample handling and sample purity. The latter is of particular importance in biochemical investigations, where the substrate may be present at low concentration in a solution containing many components. Purity of commercial biochemicals is often inadequate for accurate isotope-effect measurements.

Current mass spectrometers are much more sensitive than earlier models. Samples of 1–10 μmol are handled routinely, and accurate measurement is possible on samples an order of magnitude smaller. In practice, pure samples smaller than 10 μmol are difficult to prepare.

The carbon isotope effects discussed above in connection with formate dehydrogenase were measured by isotopic analysis of the CO_2 produced in the reaction. Materials other than CO_2 must be converted into CO_2 before analysis. This conversion must be carried out with high isotopic fidelity in order to avoid introducing spurious isotopic differences. Although combustion analysis has sometimes been used in connection with enzymatic studies, this is generally not an adequate method.[15] Development of an adequate conversion method and demonstration of its isotopic fidelity may take more time than the actual isotope effect measurements.

The isotope effect is obtained by measuring the isotopic content of a substrate or product as a function of the extent of reaction. For studies of the product, samples are isolated at about 10% conversion and at 100% conversion, and the isotope effect is calculated from the difference. For studies of substrate, isotopic compositions are compared at 0% reaction and at greater than 50% reaction. In the few cases where both methods have been applied, the two give the same results. The reproducibility of isotope effect measurements on enzymes is typically near ±0.0005, but precision several-fold higher than this is possible in well-controlled experiments.

Mathematics

Enzymatic reactions proceed by multistep mechanisms; in many cases, we know only a lower limit on the total number of steps in the reaction. The virtue of working with heavy-atom isotope effects is that to a

good approximation, only the step or steps in which there is a change in bonding to the isotopic atom will be subject to an isotope effect, and usually there are only one or two such steps. Steps that are relatively rapid make no difference to the observed isotope effect. Other relatively slow steps diminish the observed isotope effect, as shown below.

Because heavy-atom isotope effects are measured by a competitive method in which both isotopic substrates are present simultaneously, these isotope effects are always the ratio of values of V_{max}/K_m for the two isotopic substrates. Heavy-atom isotope effects on V_{max} would be of interest, but they are only available by direct comparison of rates for labeled and unlabeled substrates, and the precision of measurement of such effects is low.
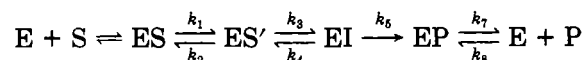
For reaction of a single-substrate enzyme

$$E + S \rightleftharpoons ES \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES' \overset{k_3}{\longrightarrow} EP \rightleftharpoons E + P$$
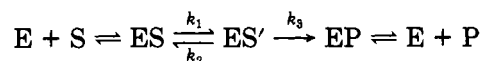
in which the first step is substrate binding, the second and third are the chemical steps, and the last is product dissociation, if k_3 is irreversible and is the only step that shows an isotope effect, the observed isotope effect is given by

$$(k^{12}/k^{13})_{obsd} = \frac{k_3^{12}/k_3^{13} + R}{1 + R}$$

where R is the partition ratio for the reaction (see below). The isotope effect k_3^{12}/k_3^{13} is the "intrinsic isotope effect"; it is the isotope effect for the isotope-sensitive step in the absence of contributions from all other steps. Intrinsic isotope effects reflect only the difference in structure between an intermediate and the adjacent transition state and are independent of the rate of the step in question relative to other rates in the mechanism. Intrinsic isotope effects do not usually vary with pH, and variations with temperature, substrate structure, and other factors are usually small. A first approximation to intrinsic isotope effects can often be obtained from model reactions. However, current practice sometimes permits direct calculation of intrinsic isotope effects from enzymatic data,[16] and comparison of these values with predictions from model reactions can give insight into the catalytic power of enzymes.

The isotope-independent ratio R is called the partitioning factor (Cleland[5,6] calls the R factors "commitments"). This factor reflects the rate of the isotope-sensitive step relative to other steps in the sequence. Mathematically, the partitioning factor is a collection of terms of the form k_n/k_{n-1}, in which all rate constants refer to the lighter isotope. For the two-step mechanism shown above, $R = k_3/k_2$. For a three-step mechanism involving two intermediates
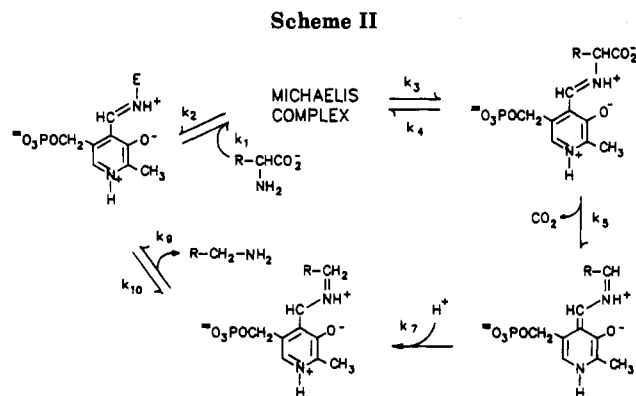
$$E + S \rightleftharpoons ES \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES' \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EI \overset{k_5}{\longrightarrow} EP \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + P$$

in which substrate binding and dissociation are rapid and the isotope-sensitive step (k_5) is irreversible

$$R = (k_5/k_4)(1 + k_3/k_2)$$

Partitioning factors are often sensitive to pH, temper-

(15) Roeske, C. A.; O'Leary, M. H. *Biochemistry* **1985**, *24*, 1603.

(16) Hermes, J. D.; Roeske, C. A.; O'Leary, M. H.; Cleland, W. W. *Biochemistry* **1982**, *21*, 5106.

Scheme II

Scheme III

ature, and other variables. Indeed, the ability to understand and manipulate partitioning factors by changing reaction conditions is one of the keys to interpretation of isotope effects.

Isotope effects are distinguished as much by their insensitivity to certain factors as by their sensitivity. For example, in an irreversible reaction, the isotope effect is insensitive to rates of steps subsequent to the first irreversible step (often dissociation of the first product). For multisubstrate enzymes, the isotope effect is insensitive to rates of steps prior to the binding of the isotopic substrate. For reversible reactions, the isotope effect may become small if the overall rate is limited by the rate of dissociation of the isotopic product from the enzyme. Thus, the term "rate-determining step" acquires a very restricted meaning when applied to isotope effects.

Decarboxylations Involving Pyridoxal 5′-Phosphate

The decarboxylation of amino acids by enzymes that require pyridoxal 5′-phosphate (PLP) serves a variety of different functions in different organisms.[17] An investigation of isotope effects, in combination with a number of other studies, provides a detailed view of the reaction mechanism.

The chemical mechanism of decarboxylation is well-known (Scheme II) due to model studies of Bruice, Snell, and others.[19] PLP is initially bound to the enzyme as a Schiff base between an ε-amino group of a lysyl residue and the carbonyl group of PLP. Following binding of the substrate to the enzyme, the amino group of the substrate reacts with this Schiff base, ultimately forming a new Schiff base between PLP and the amino acid substrate and simultaneously liberating the lysyl amino group. The o-hydroxyl group of the coenzyme plays an important catalytic role in this step.[18] The next step is decarboxylation of this Schiff base, forming the well-known quinonoid intermediate, which, in its various manifestations, characterizes nearly all PLP-dependent enzymes. Following protonation of the quinonoid intermediate, another Schiff-base interchange liberates the amine product and returns the enzyme to its original state.

Interestingly, nonenzymatic decarboxylation of amino acids by PLP or its analogues is not well-known. If PLP and an amino acid are mixed in the absence of an enzyme, a Schiff base is rapidly formed, and trans-
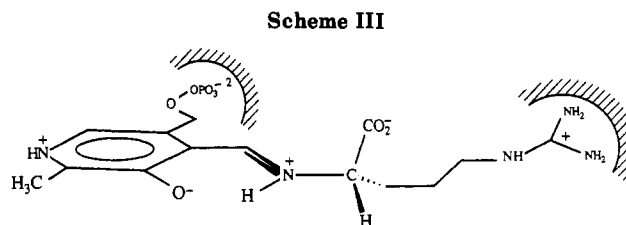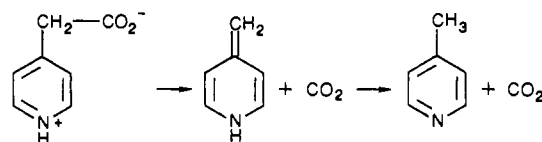
Table II
Isotope Effects on PLP-Dependent Decarboxylases

substrate	pH	temp, °C	k^{12}/k^{13}	ref
glutamate decarboxylase				
glutamate	4.70	37	1.018	23
glutamate (in D_2O)	4.70	37	1.009	27
arginine decarboxylase				
arginine	5.25	25	1.015	26
homoarginine	5.25	25	1.057	26
norarginine	5.25	25	1.043	26
histidine decarboxylase				
histidine	6.30	37	1.031	34
aspartate β-decarboxylase				
aspartate	5.00	37	1.010	34

amination, rather than decarboxylation, occurs.[19] If an α-methyl amino acid is used, so that transamination is precluded, decarboxylation occurs only slowly at temperatures in excess of 100 °C.[19] These results suggest that for the enzymatic reaction, Schiff base interchange is likely to be much faster than decarboxylation.

Dunathan[20] was among the first to point out that geometric control within the Schiff base is probably a key factor in the catalytic mechanism. The quinonoid intermediate is planar, and the conformation of the preceding Schiff base must have the carboxyl group above the plane of the pyridine ring (Scheme III). A variety of stereochemical studies show that decarboxylases maintain very strict stereochemical control of their substrates,[21] as required by Dunathan's explanation.

Decarboxylation of amino acids was one of the earliest enzymatic reactions for which carbon isotope effects were measured.[22] Isotope effects on nonenzymatic decarboxylations[23] serve as models for the interpretation of the enzymatic isotope effects. The best chemical model for PLP-dependent decarboxylations is 4-pyridylacetic acid, which, like the enzymatic reaction, decarboxylates by way of a zwitterionic intermediate:

The carbon isotope effect[24] is $k^{12}/k^{13} = 1.05–1.06$.

Carbon isotope effects on PLP-dependent enzymatic decarboxylations are generally smaller than those observed in model reactions (Table II). Several lines of evidence suggest that the intrinsic isotope effect is 1.05–1.06, as expected from model studies, and decarboxylation is not totally rate determining. Observed

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(18) Bruice, T. C.; Benkovic, S. J. *Bioorganic Mechanisms*; Benjamin: New York, 1966; Vol. 2, p 181.
(19) Kalyanckar, G. D.; Snell, E. E. *Biochemistry* 1962, 1, 594.

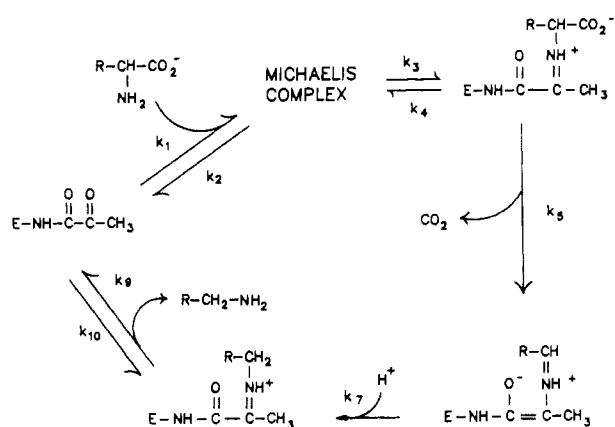(20) Dunathan, H. C. *Proc. Natl. Acad. Sci. U.S.A.* 1966, 55, 712.
(21) Floss, H. G.; Vederas, J. In *Stereochemistry*; Tamm, C., Ed.; Elsevier: New York, 1982; p 161.
(22) O'Leary, M. H.; Richards, D. T.; Hendrickson, D. W. *J. Am. Chem. Soc.* 1970, 92, 4435.
(23) Dunn, G. E. In *Isotopes in Organic Chemistry*; Buncel, E., Lee, C. C., Eds.; Elsevier: New York, 1977; Vol. 3, p 1.
(24) Marlier, J. F.; O'Leary, M. H. *J. Am. Chem. Soc.* 1986, 108, 4896.
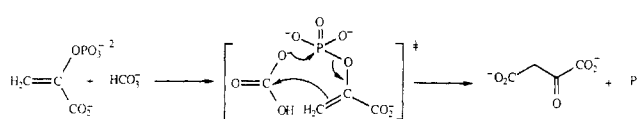
Scheme IV

Scheme V

Scheme VI

isotope effects get larger away from the pH optimum,[23] as well as when less reactive substrates are used.[25] Apparently, the rate constant ratio k_5/k_4 (Scheme IV) is near unity under optimum conditions, indicating that Schiff base interchange and decarboxylation are jointly rate determining.

This situation is in contrast to our prediction from model chemistry that decarboxylation should be the rate-determining step. The difference is probably in the conformational control of the catalytic mechanism, as suggested by Dunathan.

The role of conformational control in catalysis can be seen particularly clearly in studies of the arginine decarboxylase from *E. coli*. This enzyme is relatively specific for arginine, but it will also catalyze the decarboxylation of norarginine, the arginine analogue having one less carbon in the backbone, and homoarginine, the analogue having one more, at rates that are 1–2% of the rate for the natural substrate.[26] Michaelis constants for the three substrates are similar, and it does not appear that the rate variation is due to impaired binding. The carbon isotope effect for decarboxylation of arginine is 1.015 at pH 5.25, 25 °C, whereas the carbon isotope effects for the slow substrates are 1.04–1.06 under the same conditions. Decarboxylation has become entirely rate determining for the slow substrates, perhaps because of the stereochemical constraints imposed by the binding of the guanidino group of the substrate coupled with the need to keep the substrate carboxyl group above the plane of the conjugated system (Scheme III). As the length of the chain connecting the guanidino group to the α-carbon changes, the conformation of the Schiff base changes, and decarboxylation becomes more difficult.

Interesting details concerning the nature of the Schiff base interchange step can be obtained by measuring isotope effects in D_2O.[27] In the case of glutamate decarboxylase, the decarboxylation rate is reduced about fivefold in D_2O, and the carbon isotope effect decreases from 1.018 to 1.009. This change occurs because of a large solvent isotope effect on the Schiff base interchange step, which in turn changes the value of k_5/k_4. The solvent isotope effect occurs in part because of the fact that at least three protons must be transferred in the course of this step. In addition, X-ray studies of
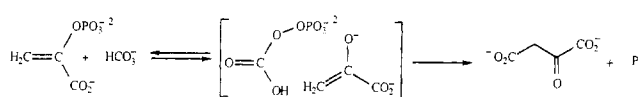
the PLP-dependent aspartate aminotransferase reveal that the Schiff base interchange is accompanied by a large conformation change involving rotation of the pyridine ring of the coenzyme.[28] This rotation, together with isotope effects associated with the proton transfers that accompany the Schiff base interchange, probably cause the large solvent isotope effect, which is in excess of 7 for this step.

The Schiff base interchange can also be studied by use of nitrogen isotope effects. In the case of glutamate decarboxylase,[29] a nitrogen isotope effect $k^{14}/k^{15} = 0.9855$ is obtained at pH 4.7, 37 °C. The initial deprotonation of the substrate gives rise to an isotope effect of approximately 0.984. Numerical modeling and comparison with a variety of model systems indicate that the Schiff base nitrogen is protonated in the amino acid–PLP Schiff base, and that the nitrogen isotope effect on k_3 is 1.02–1.03.

There is also a solvent isotope effect of about 2 associated with substrate binding. This probably occurs because of the amount of desolvation of polar groups of the substrate that accompanies binding to the active site. Studies of arginine decarboxylase indicate that a small carbon isotope effect may also be associated with this desolvation.[26]

Another aspect of the catalytic mechanism in enzymatic decarboxylation is control of the environment within which the decarboxylation occurs. The decarboxylation step is a charge neutralization (cf. Scheme III), and the enzyme appears to take advantage of this fact. The model decarboxylation of 4-pyridylacetic acid discussed above is also a charge neutralization, and the rate of this reaction is very sensitive to environment polarity: changing the solvent from water to 75% dioxane causes a 4000-fold rate acceleration. Isotope effect studies indicate that analogous, though smaller, changes in rates occur in enzymatic decarboxylations, but in this case the nonpolar medium is provided by the protein.[26]

Decarboxylations Involving Covalently Bound Pyruvate

Although most amino acid decarboxylases use PLP as a cofactor, there is a class that use covalently bound pyruvate instead.[30] These enzymes function by means of a Schiff base between the substrate and the pyruvate cofactor (Scheme IV). The mechanism is formally similar to the PLP mechanism, in the sense that the cofactor serves as an electron sink for catalysis of the decarboxylation process. However, the extent of electron delocalization is much less in the pyruvate case

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(27) O'Leary, M. H.; Yamada, H.; Yapp, C. J. *Biochemistry* **1981**, *20*, 1476.

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(29) Abell, L. M.; O'Leary, M. H. *Biochemistry* **1988**, *27*, 3325.
(30) Recsei, P.; Snell, E. E. *Annu. Rev. Biochem.* **1984**, *53*, 357.

Table III
Isotope Effects on Histidine Decarboxylases from Two
Sources at the pH Optimum, 37 °C[a]

isotope effect	PLP enzyme	pyruvoyl enzyme
carboxyl carbon	1.0308	1.0334
carboxyl carbon with [α-²H]His	1.0331	1.0347
carboxyl carbon in D_2O		1.0313
amino nitrogen	0.993	0.980
α-deuterium isotope effect	1.20	1.10

[a] Reference 35.

than in the PLP case, and we had expected that the pyruvoyl enzyme would not be an efficient catalyst. This turned out not to be the case, as outlined below.

The pyruvate-dependent histidine decarboxylase from *Lactobacillus*[30] has been studied in particular detail by Snell and collaborators,[32] and an X-ray structure of the enzyme has recently been published.[31] For comparison, Snell's group has also purified and studied a PLP-dependent histidine decarboxylase from *Morganella morganii*.[32]

We were interested in using isotope effects to determine how the efficiencies and catalytic strategies of the two enzymes compare. Our initial expectation was that the pyruvoyl enzyme was a less functional, less efficient catalyst than the PLP enzyme, and generations of textbook writers in bioorganic chemistry apparently felt the same way. A number of carbon, hydrogen, and nitrogen isotope effects on these two histidine decarboxylases are given in Table III.[33] Carbon isotope effects for the two enzymes are similar but are larger than have been seen with previous PLP-dependent enzymes. In both cases, comparison of observed and expected isotope effects indicates that the partition factor k_5/k_4 must be near unity, indicating that Schiff base interchange and decarboxylation are about equally rate determining. Nitrogen isotope effects, though superficially different, are consistent with this conclusion and indicate that in both cases the Schiff base intermediate must be protonated in order for reaction to occur. What is most surprising is that both enzymes have the *same turnover number*;[32] neither has a significant catalytic advantage in terms of either V_{max} or V_{max}/K_m. Thus, to our surprise, both enzymes operate

at similar rates by similar mechanisms having two rate-determining steps, and we fail to find any catalytic advantage for the PLP system whatsoever.

The only difference we found is a very subtle one: For further definition of the transition-state structure in these systems, we prepared L-[α-²H]histidine and measured carbon isotope effects with this material. As expected for a stepwise reaction with two partially rate-determining steps, the carbon isotope effect is slightly larger for the deuterated substrate than for the protonated substrate (deuteration slows the decarboxylation step, thereby increasing the carbon isotope effect). From the available data, we were also able to calculate secondary deuterium isotope effects for the decarboxylation step. For the pyruvate-dependent enzyme, this value is about 1.10, whereas for the PLP enzyme, it is about 1.20. Thus, the transition-state structures are significantly different for the two enzymes, with the PLP enzyme having a more product-like transition state than the pyruvoyl enzyme. Surprisingly, this does not translate into significantly different carbon isotope effects or different decarboxylation rates.

Thus nature has evolved two independent, equally efficient mechanisms for catalysis of decarboxylation. Perhaps the "specialness" of PLP lies in its ability to catalyze a number of additional reactions of amino acids.

Conclusion

Methods have been developed that permit us to analyze isotope effects on individual steps in complex enzymatic reaction mechanisms. These isotope effects can be used to elucidate details of transition-state structure and thus to compare transition-state structures in enzymatic reactions with corresponding transition-state structures for model reactions. Evidence from isotope effects confirms that enzymes operate by use of precise structural and geometric control, including in at least some cases a significant degree of steric compression. Studies with substrate analogues and with mutant enzymes promise to provide further details regarding transition-state structures.

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