Specificity in enzymic decarboxylation

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Specificity in Enzymatic Decarboxylation

Sir:

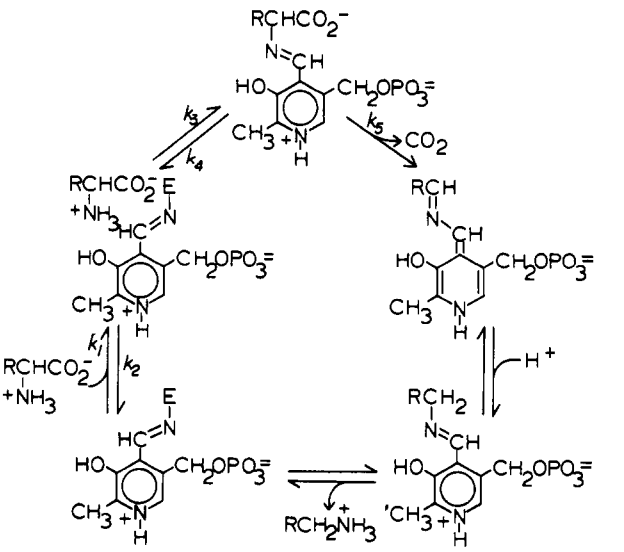
Specificity is a key feature of enzymatic reactions. At the first level of sophistication, specificity may be expressed either in binding or in catalysis. Discrimination between these two possibilities may often be accomplished by determining Michaelis constants and turnover rates for different substrates. If the specificity arises in catalysis, steady-state kinetics often does not permit assignment of the specificity to a single step or group of steps. For example, studies of chymotrypsin conducted by Niemann,[1] Neurath,[2] and their collaborators revealed examples of both types of specificity. To identify reaction steps responsible for specificity in catalysis, a number of people have measured acylation and deacylation rates for numerous tightly binding substrates.[3] Both acylation and deacylation rates parallel changes in catalytic rate. Thus, in the case of chymotrypsin specificity is not manifested in a single reaction step.

However, it is possible that in many cases specificity is manifested principally in a single reaction step. Few methods are available for dissecting enzymatic reaction mechanisms at the necessary level of sophistication. We show here that heavy-atom isotope effects are useful in probing the details of enzyme specificity. We are able by this method to identify the step responsible for specificity of the inducible form of the pyridoxal 5′-phosphate dependent arginine decarboxylase (E.C. 4.1.1.19) from *E. coli*.

Most amino acid decarboxylases require pyridoxal 5′-phosphate for activity. These enzymes have stringent specificity requirements.[4] Binding involves the α carboxyl and amino groups and usually a distal group (often charged) as well.[5] The chain which connects the α carbon to this distal group probably lies on the enzyme in its most extended conformation. For example, glutamate decarboxylase acts very slowly on aspartic acid or α-aminoadipic acid and inhibition of the enzyme by dicarboxylic acids is optimum for inhibitors whose carboxyl–carboxyl distance corresponds to that of the extended conformation of glutamic acid.[6]

Kinetic data summarized in Table I reveal that a similar pattern of specificity is observed with arginine decarboxylase. Arginine and canavanine are the proper size and are decarboxylated efficiently by the enzyme. Homoarginine and norarginine are proper except that the distal group is at the wrong distance from the α carbon. Michaelis constants for these substrates are similar to that of arginine, but the maximum velocities are down by about a hundredfold compared with arginine.

Scheme I



To establish that the Michaelis constants actually represent equilibrium constants for substrate binding, we measured inhibition constants for the inhibition of decarboxylation of arginine by canavanine, homoarginine, and norarginine. In each case (Table I) the observed inhibition constant is within experimental error of the corresponding Michaelis constant. Thus, binding of these substrates to the enzyme is an equilibrium process.[7]

Carboxyl carbon isotope effects for the decarboxylation of arginine, canavanine, homoarginine, and norarginine by arginine decarboxylase at pH 5.25 were measured by our usual procedure.[8] The isotope effects are summarized in Table I. The rapidly reacting substrates arginine and canavanine give isotope effects which are small but significantly different from unity, whereas the slowly reacting substrates homoarginine and norarginine give larger isotope effects.

The mechanism of the decarboxylation is shown in Scheme I. There are four steps following substrate binding, and one or more of these steps could control substrate specificity. However, the steps following decarboxylation are probably relatively rapid and have no role in specificity.[9] Decarboxylation is not reversible under our reaction conditions; so only reaction steps up through decarboxylation will be reflected in the carbon isotope effects.[10] Assuming that only the decarboxylation step shows a significant carbon isotope effect and that substrate binding is at equilibrium, the relation between the observed isotope effect and the mechanism shown in Scheme I is given by

Table I. Kinetic and Isotope Effect Data for Arginine Decarboxylase

Substrate	Formula	V_{max},[a] s^{-1}	K_m, mM	K_i, mM	k^{12}/k^{13} [b]
Arginine	$(NH_2)_2{}^+CNH(CH_2)_3CH(NH_2)CO_2H$	560	1.0 ± 0.5		1.0144 ± 0.0004
Homoarginine	$(NH_2)_2{}^+CNH(CH_2)_4CH(NH_2)CO_2H$	6	1.0 ± 0.3	6.0 ± 5.0	1.0535 ± 0.0002
Norarginine	$(NH_2)_2{}^+CNH(CH_2)_2CH(NH_2)CO_2H$	9	3.7 ± 0.3	4.0 ± 3.0	1.0438 ± 0.0004
Canavanine	$(NH_2)_2{}^+CNHO(CH_2)_2CH(NH_2)CO_2H$	140	2.0 ± 1.0	0.8 ± 0.2	1.0048 ± 0.0002

[a] Steady-state kinetic measurements were performed manometrically at pH 5.25, 37 °C, in 0.2 M sodium acetate buffer. The data were corrected using eq C of K. F. Gregory and H. C. Winter, *Anal. Biochem.*, **11**, 519 (1965). Velocities are given per enzyme active site, assuming that the specific activity of the pure enzyme is 410 μmol of CO_2 min^{-1} mg^{-1} of enzyme.[4] [b] Carbon isotope effects were measured using 0.2 M sodium acetate buffer, pH 5.25, 25 °C, by our published procedure.[8]

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$$\text{observed}\ \frac{k^{12}}{k^{13}} = \frac{(k_5{}^{12}/k_5{}^{13}) + (k_5/k_4)}{1 + (k_5/k_4)}$$

By analogy with model studies[11] the isotope effect on the decarboxylation step, $k_5{}^{12}/k_5{}^{13}$, is likely to be in the range of 1.04–1.06. Because of the similarity of the electronic structures of all substrates examined in this study, it is likely that this value will be similar or identical for all substrates. The variations in observed isotope effects are thus expected to appear primarily in the ratio k_5/k_4, which reflects the difference in free energy between the transition state for the Schiff base interchange step and that for the decarboxylation. Qualitatively, a carbon isotope effect near unity will be observed if Schiff base interchange is rate determining, whereas an isotope effect in the range of 1.04–1.06 will be observed if decarboxylation is rate determining.

The carbon isotope effects observed with the rapidly reacting substrates arginine and canavanine indicate that in these cases neither decarboxylation nor Schiff base interchange is entirely rate determining. This observation continues a pattern seen with other decarboxylases, whereby for the natural substrate no single step is entirely rate limiting.[12]

The carbon isotope effects observed with the slowly reacting substrates homoarginine and norarginine are in the range expected for rate-determining decarboxylation. Thus, the low rates of decarboxylation of these substrates reflect low rates of the decarboxylation step, and the specificity of arginine decarboxylase toward these substrates is primarily manifested in the decarboxylation step.[13]

Why is specificity manifested principally in the decarboxylation step? All substrates examined have the amino, carboxyl, and guanidino groups required for substrate binding. The lack of a correlation between Michaelis constant and substrate length indicates that flexibility in the initial fit between enzyme and substrate is sufficient to accommodate small changes in substrate size. The second step in the reaction, Schiff base interchange, may in fact show some change in rate with substrate structure, but this change must be considerably smaller than that shown by the decarboxylation step. Apparently the flexibility in the enzyme–substrate complex is to some extent maintained in the Schiff base interchange step, perhaps as a result of the fact that in this step some rotational freedom is maintained in the bonds between the 4 and 4′ carbons of the coenzyme and between the α carbon and the amino nitrogen of the substrate. This flexibility is lost in the decarboxylation step because the product is the highly conjugated quinoid intermediate. As first suggested by Dunathan,[14] the conformation required for decarboxylation places the carboxyl group substantially out of the plane of the conjugated system. The presence of substantial quinoid character in the transition state for decarboxylation places strong geometrical constraints on this step which were not present in earlier steps. Because of the extended conjugation, the system is no longer able to accommodate readily to changes in the distance between the distal binding group and the α carbon.

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Carbon Magnetic Resonance Spectroscopy on Carbon-13-Labeled Uracil in 5S Ribonucleic Acid[1]

Sir:

The carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra of the ^{13}C-enriched C-4 uridine carbons in 5S ribosomal ribonucleic acid (5S RNA) of *Salmonella typhimurium*, strain JL-1055,[2] has been obtained. The 5S RNA was isolated from a 5S RNA-tRNA mixture using gel permeation chromatography on Sephadex G-100 Superfine (Pharmacia Fine Chemicals). Gel permeation chromatography was also used to check the purity and the molecular size of the 5S RNA against suitable standards. The details of the separation along with the procedures for the incorporation of the ^{13}C label and the recovery of the low molecular weight RNA's are given elsewhere.[3,4] The ^{13}C-enrichment level was determined by mass spectroscopy to be 46%. The 5S RNA (34 mg) was dissolved in 370 μL of sterile 0.04 M $MgCl_2$, 5 mM dithiothreitol (DTT), 2mM EDTA solution. Proteinase K (0.2 mg) (EM Laboratories, Inc.) was added to destroy residual nuclease activity. The pH was adjusted to 7.4 with sterile 0.1 M NaOH. Dioxane (2%) was added for an internal reference and the sample placed in a 5-mm NMR sample tube. Deuterated

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