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Kinetic α-Deuterium Isotope Effects for Acylation of Chymotrypsin by 4-Methoxyphenyl Formate and for Deacylation of Formylchymotrypsin

Sir:

The kinetic α-deuterium isotope effect, $(V_{max}/K_m)_D/(V_{max}/K_m)_H$, for acylation of α-chymotrypsin by 4-methoxyphenyl formate in aqueous solution over the pH range 5.8–6.8 is 1.18 ± 0.02. For deacylation of the formylchymotrypsin generated in this reaction, the kinetic α-deuterium isotope effect, $(V_{max})_D/(V_{max})_H$, is 1.15 ± 0.03 over the pH range 6.2–6.8. These values strongly suggest that acylation of chymotrypsin by this reagent occurs at serine and that ≻C–O–Enz bond formation and cleavage in the transition states for acylation and deacylation reactions, respectively, are well advanced but not complete.

Kinetic α-deuterium isotope effects have proved to be useful criteria of mechanism for a growing number of enzymatic reactions.[2,3] We report here two such isotope effects, measured in an effort to obtain novel information concerning the transition state structure for α-chymotrypsin-catalyzed hydrolysis of esters. The nature of the measurements requires the utilization of formates, esters not previously established to be chymotrypsin substrates. 4-Methoxyphenyl formate, 4-methoxyphenyl [^2H]formate, 4-methoxyphenyl [^3H]formate (0.1 mCi/mmol), and 4-methoxyphenyl [^{14}C]formate (0.1 mCi/mmol) were synthesized from the appropriate substrates as described.[4] Titrimetric assay of reaction mixtures containing 4-methoxyphenyl formate and bovine pancreatic chymotrypsin (crystalline, Schwarz-Mann, 1220 U/mg) revealed that the rate of proton liberation increased with increasing enzyme concentration. Replacement of chymotrypsin by chymotrypsinogen abolished this effect. Employing a spectrophotometric assay, 4-nitrophenyl formate was also found to be a chymotrypsin substrate. At pH 5.5, this compound exhibited an "initial burst" of color at 320 nm, strongly suggesting rate-determining enzyme deformylation.[5] This conclusion was confirmed by the observation that the chymotrypsin-catalyzed hydrolysis of the 4-nitro- and 4-methoxyphenyl formates occurs at comparable rates under the same conditions. For isotope effect measurements, the 4-methoxyphenyl compound was chosen; the 18-min half-life of 4-nitrophenyl formate in water at 25 °C[6] results in a large nonenzymatic hydrolysis of this ester under the conditions of our measurements. The corresponding value for the methoxy ester is 630 minutes,[7] minimizing the contribution from nonenzymatic routes. A thorough spectrophotometric study (see eq 1) of the kinetics

$$E + S \underset{}{\overset{k_s}{\rightleftharpoons}} ES \xrightarrow{k_2} EP_2 \xrightarrow{k_3} E + EP_2 \qquad (1)$$
$$\downarrow$$
$$P_1$$

of chymotrypsin-catalyzed hydrolysis of isotopically normal 4-methoxyphenyl formate revealed a good fit to Michaelis-Menten kinetics: at pH 6.8, V_{max} is (11.3 mol of substrate/min)/mol of enzyme or $k_3 = 11.3\ min^{-1}$ and K_m is 4.3×10^{-4} M. The value of K_m is smaller than that for 4-nitrophenyl acetate and chymotrypsin at pH 7.8, 1.9×10^{-3} M, and the value of V_{max} (or k_3) is considerably greater than that for deacetylation of acetylchymotrypsin at pH 7.8, 0.32 min^{-1}[7]. Thus, 4-methoxyphenyl formate is at least as suitable a substrate for chymotrypsin as is the much-employed 4-nitrophenyl acetate.

The α-tritium isotope effect on V_{max}/K_m for chymotrypsin acylation was measured by a competitive method in which 0.001 M 4-methoxyphenyl [^3H]formate and 0.001 M 4-methoxyphenyl [^{14}C]formate were simultaneously incubated with 6×10^{-6} M chymotrypsin in dilute citrate-phosphate buffers. After an appropriate time interval, unreacted ester was extracted into ether, and an aliquot of the aqueous phase, containing the isotopically labeled formates, was removed, added to a small column of Dowex 1-X4 (chloride form), and washed with distilled water, followed by elution of the formate with 0.01 M HCl. Four aliquots of the formate eluant were counted in a Beckman LS-230 liquid scintillation spectrometer employing a modified Bray's solution. The isotope effect was calculated by comparing the ratio of ^{14}C to ^3H activity in reactant ester and product formate, as previously described.[8,9] Isotope effect measurements were made at three values of pH, 5.8, 6.3, and 6.8, and at several extents of ester consumption. The values are plotted as a function of extent of reaction in Figure 1; solid lines in this figure are theoretical plots for tritium isotope effects of 1.25, 1.27, and 1.29. We conclude that the isotope effect is 1.27 ± 0.02. The indicated error limit is the standard deviation from the mean of all measurements. This corresponds to an α-deuterium isotope effect of $(1.27)^{1/1.44}$[2] or 1.18 ± 0.02.[10,11] This isotope effect refers specifically to the acylation reaction, although enzyme deacylation is the rate-determining step, since isotopic discrimination can occur upon acylation only; the formyl enzyme is committed to yield products.

The α-deuterium secondary isotope effect on V_{max} (and therefore on the hydrolysis of formylchymotrypsin) was measured by a direct spectrophotometric assay. Reactions were monitored using an HP2100A minicomputer interfaced to a Cary spectrophotometer by following formation of 4-methoxyphenol with time at 295 nm. Reactions were initiated at values of $[S]_0$ of four-five times the K_m values of the isotopic substrates and were followed until substrate was depleted.

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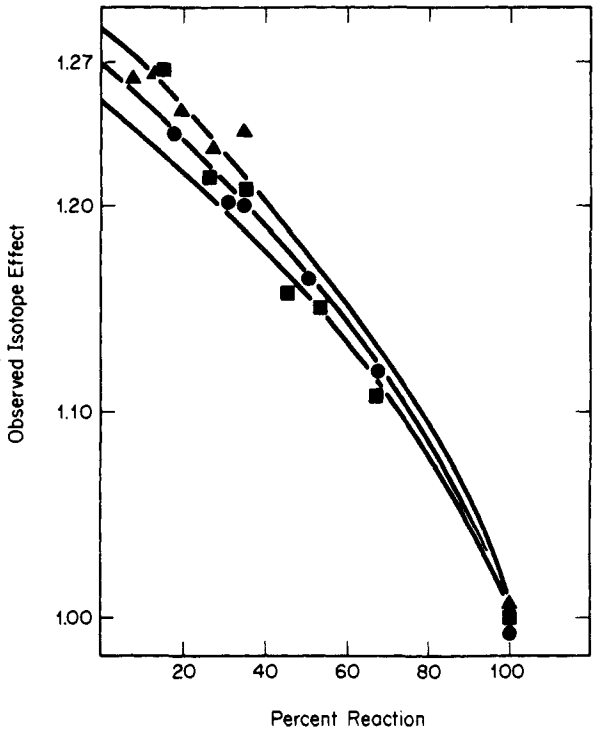


Figure 1. Observed secondary titrium isotope effect, $(V_{max}/K_m)_H/(V_{max}/K_m)_T$, for acylation of bovine pancreatic chymotrypsin by 4-methoxyphenyl formate at pH 6.3 and 25 °C plotted as a function of extent of substrate conversion into products. Solid lines are theoretical ones for isotope effects of 1.25, 1.27, and 1.29. The experimental points represented by squares, circles, and triangles refer to measurements made at values of pH 5.8, 6.3, and 6.8, respectively.

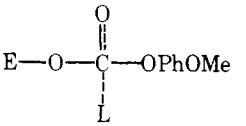
Absorbances were measured and recorded over each time course in 1000 equal time intervals. The first derivatives of the resultant absorbance-time array were calculated according to the methods of Savitzky and Golay,[12] thereby producing an array of instantaneous velocities [in units d (absorbance)/dt] vs. absorbance (which, along with the requisite extinction coefficients, is a measure of instantaneous substrate concentration). The velocities were corrected for contributions from the background reaction, since

$$v_{i,\,total} = \frac{V_{max}[S]_i}{K_m + [S]_i} + v_{i,background} = v_{i,enzymatic} + v_{i,background} \quad (2)$$

Values for V_{max} and K_m were then calculated from each time course by hyperbolic least-squares analysis, as described by Cleland.[13] A value of $(V_{max})_D/(V_{max})_H = 1.15 \pm 0.03$ was obtained.

The secondary deuterium isotope effect on V_{max}/K_m for acylation of chymotrypsin by 4-methoxyphenyl formate, 1.18 ± 0.02, suggests that acylation occurs at serine and not at histidine. Corresponding isotope effects for nonenzymatic reactions of oxy anions with the same substrate are near 1.20,[6] a value consistent with that found for the enzymatic reaction. In contrast the nonenzymatic reactions of imidazoles with this substrate have isotope effects much smaller: ~1.07.[14] Moreover, the magnitude of the isotope effect suggests that bond formation between the serine hydroxyl and the acyl carbon atom, or bond cleavage involving the acyl carbon atom and the leaving 4-methoxyphenyl group depending on the nature of the rate-determining step, is not complete in the transition state. Were the transition state to have sp^3 geometry, one would expect a limiting secondary deuterium isotope effect; the value of this isotope effect is not known with certainty but probably falls in the range 1.25–1.30.[6] The measured isotope effect is less than the limiting value; hence hybridization at acyl carbon must be somewhere between sp^2 and sp^3. If the relationship between bond order and isotope effect is linear or nearly so, the isotope effect suggests that transition-state geometry is nearer tetrahedral than trigonal. Were the transition state to closely resemble the acyl enzyme, we would expect an equilibrium isotope effect, which should be near unity, a value not in accord with experimental observation.

Since departure of the 4-methoxyphenyl function is probably easier than that for the serine hydroxyl group, tetrahedral intermediate formation is likely to be rate determining. Thus, the isotope effect requires little C–O bond cleavage in the transition state (**1**).



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The secondary deuterium isotope effect for chymotrypsin deformylation, 1.15 ± 0.03,[15] is consistent with the conclusions indicated above. The kinetic isotope effect for pH-independent nonenzymatic hydrolysis of phenyl formates, which may resemble chymotryptic deformylation in significant ways, is near 1.25.[6] The limiting isotope effect must be at least this large. It follows that geometry at the formyl carbon atom in enzyme deacylation is intermediate between tetrahedral and trigonal. Note that this isotope effect, and therefore the mechanistic interpretation, is free from the uncertainty introduced by a ^{14}C isotope effect.[10]

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