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Additivity of Isotope Effects for Successive Deuteration in the Deacetylation of Acetyl-α-Chymotrypsin[1]

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α-Chymotrypsin, converted to the acetyl enzyme by the *p*-nitrophenyl esters of CH_3COOH, CH_2DCOOH, CHD_2COOH, and CD_3COOH, undergoes deacetylation at pH 7.6 (phosphate buffer) and 25°C with secondary isotope effects of $k(CH_3)/k(CH_2D) = 0.985 \pm 0.006$, $k(CH_3)/k(CHD_2) = 0.971 \pm 0.010$, and $k(CH_3)/k(CD_3) = 0.956 \pm 0.008$. These isotope effects obey the simple additivity rule ("Rule of the Geometric Mean") to within 20 J/mol, corresponding to about 5–6% of the maximum isotope effect for carbonyl addition. Thus, to this level, the three hydrogenic sites of the acetyl group are not rendered distinct in their contributions to the overall isotope effect even in the chiral environment of the chymotrypsin active site.

In the natural action of chymotrypsin, its amide substrates are hydrolyzed in a well-known two-step process (*1*). First, the amino fragment is expelled by nucleophilic attack of an active-site serine residue on the substrate carbonyl, leading to an intermediate acyl enzyme. The acyl enzyme then hydrolyzes to produce the acyl fragment of the substrate and regenerate the active enzyme. This deacylation reaction can be studied in isolation by several techniques, among them the use of very reactive artificial substrates such as *p*-nitrophenyl esters; this renders the acylation reaction so fast that the deacylation is wholly rate-limiting (*2*).

p-Nitrophenyl acetate as substrate is a special case, since the acetyl group represents a degenerate form of substrate acyl group; the enzyme is completely deprived of all interactions with substrate structural units (such as the Cα side chain, the Cα-N acyl group, and the succeeding units along the "polypeptide tail" of a natural substrate) except for the acyl center at which chemical change is taking place (*1, 3*). Nevertheless, the deacetylation of α-chymotrypsin proceeds with a first-order rate constant of around 10^{-2} sec^{-1}, corresponding to a substantial catalytic acceleration.[3] This implies that the enzyme interacts with the substrate in the hydrolytic transition state with a certain intimacy. The question

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[3] The uncatalyzed hydrolysis of O-acetyl-N-acetylserinamide has a first-order rate constant around 10^{-9} s^{-1} (B. M. Anderson, E. H. Cordes, and W. P. Jencks, *J. Biol. Chem.* **236**, 455 [1961]), suggesting a catalytic acceleration factor of 10^7.

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then arises whether the interaction is of such strength and subtlety that the hydrogenic centers of the methyl group of the acetyl moiety are made distinct by the chiral environment of the active site.

We have investigated this problem through the use of the additivity property of kinetic isotope effects. If a single deuterium atom is introduced into the acetyl methyl group, a "1D" kinetic isotope effect, $k(CH_3)/k(CH_2D)$, can be measured, the magnitude of which is expected to reflect the degree of nucleophilic interaction and related rehybridization which has occurred at the transition state (*4*). This is because the isotope effect arises from expulsion of electron density out of the carbonyl π orbital into the adjacent CH(D) bonds as the nucleophile attacks; this strengthens the CH(D) bonds and produces an inverse isotope effect (*5*). Calibration experiments with, for example, the hydration of ketones, where an equilibrium isotope effect can be measured which corresponds to a completely tetrahedral product, suggest (*4*) that complete "tetrahedrality" at the carbonyl center will produce a "1D" isotope effect, $k(CH_3)/k(CH_2D)$, of 0.955.

If a second deuterium atom is now introduced, a "2D" isotope effect, $k(CH_3)/k(CHD_2)$, can be measured. If the two deuterated centers are chemically equivalent to each other in both the reactant and transition states, the Rule of the Geometric Mean [RGM (*6*)] holds that the "2D" isotope effect should be simply the square of the "1D" isotope effect, i.e., that the free energy effects of the two isotopic substitutions be additive and identical. In similar fashion, a "3D" effect, $k(CH_3)/k(CD_3)$, should be the third power of the "1D" effect according to the RGM.

On the other hand, if the three hydrogenic centers of the methyl group are not chemically equivalent in both the reactant and transition states, then the RGM becomes inapplicable and should not hold. The general circumstance can be formulated as follows. Equation [1] gives the isotope effect on the free energy of activation expected for the introduction of N isotopic substitutions.

$$(\Delta\Delta G^*)_N = N \cdot (\Delta\Delta G^*)_1 + \sum_{i=2}^{N} \varepsilon_i. \qquad [1]$$

Here the term $N \cdot (\Delta\Delta G^*)_1$, the number of centers substituted times the free-energy isotope effect for the first substitution $[(\Delta\Delta G^*)_1]$, expresses the contribution to the isotope effect which obeys the RGM. The other term expresses the sum of the defects from the RGM for each of the successive stages of isotopic substitution, and thus the magnitude of the violation of the RGM which will be observed at any particular stage. Let us simplify the expression by assuming that the total defect from the RGM at any stage of isotopic substitution may be considered, as in Eq. [2], just the degree of substitution N times an average defect, $\bar{\varepsilon}$.

$$\sum_{i=2}^{N} \varepsilon_i = N \cdot \bar{\varepsilon}. \qquad [2]$$

It is then possible, as Eq. [3] shows, to solve these equations for the average defect, $\bar{\varepsilon}$. Furthermore, $\bar{\varepsilon}$ may be evaluated as

$$\bar{\varepsilon} = (\Delta\Delta G^*)_N/N - (\Delta\Delta G^*)_1 \qquad [3]$$

from experimental data if one or more successive stages of substitution can be achieved and the corresponding isotope effects measured.

This has been done for the deacetylation of α-chymotrypsin, and we report the results in this paper.

RESULTS

The deacetylation rate of acetyl-α-chymotrypsin was measured by determination of the rate of absorbance increase at 400 nm in solutions containing the enzyme and *p*-nitrophenyl acetate, in any of its *methyl*-deuterated forms, with the substrate in 15- to 35-fold excess over its K_m value of 2–5 μM (*2, 7*). The resulting rates, isotope effects, and their free-energy equivalents are displayed in Table 1.

DISCUSSION

From the free-energy equivalents of the isotope effects presented in Table 1, the magnitude of the defect from the RGM, $\bar{\varepsilon}$, may readily be calculated. One-third of the free-energy equivalent of the "3D" isotope effect is 37 ± 7 J/mol, while one-half of the free-energy equivalent of the "2D" isotope effect is 37 ± 13 J/mol. Each of these is to be compared with the free-energy equivalent of the "1D" isotope effect, which is 37 ± 15 J/mol. Thus: $\bar{\varepsilon} = (37 \pm 7) - (37 \pm 15) = 0 \pm 17$ J/mol, $N = 3$; and, $\bar{\varepsilon} = (37 \pm 13) - (37 \pm 15) = 0 \pm 20$ J/mol, $N = 2$.

TABLE 1

RATES,[a] ISOTOPE EFFECTS, AND FREE-ENERGY EQUIVALENTS IN THE DEACETYLATION OF ACETYL-α-CHYMOTRYPSIN AT pH 7.59, 25.02 ± 0.02°C

$10^7 V_{3H}$ (A s^{-1})	$10^7 V_{ND}$ (A s^{-1})	$k(CH_3)/k(CH_{3-N}D_N)$ (±SD)	$(\Delta\Delta G^*)_N$ ± SD (J/mol)
$N = 3$: p-$NO_2C_6H_4OOCCD_3$			
4275, 4267	4465, 4490		
4310, 4295	4526, 4460		
Mean 4287 ± 19	4485 ± 30	0.956 ± 0.008	112 ± 21
$N = 2$: p-$NO_2C_6H_4OOCCCHD_2$			
4326, 4332	4463, 4432		
4261, 4282	4391, 4403		
4274			
Mean 4295 ± 32	4422 ± 32	0.971 ± 0.010	73 ± 26
$N = 1$: p-NO2$C_6H_4OOCCCH_2D$			
4274, 4263	4314, 4347		
4267, 4297	4359, 4337		
Mean 4275 ± 15	4339 ± 19	0.984 ± 0.006	37 ± 15

[a] Increase in absorbance at 400 mm. Enzyme concentration, 7×10^{-6} M; substrate concentration, 7.5×10^{-5} M; 0.05 M phosphate buffer; 1.61% (v/v) acetonitrile.

We may conclude that, to the extent of at least 20 J/mol (corresponding to an isotope effect of about 0.8%), the RGM is precise for the conversion of reactant to transition states in the deacetylation of acetyl-α-chymotrypsin. The "3D" isotope effect for complete conversion of a trigonal carbonyl to a tetrahedral structure (*4*) is 0.87, with a free-energy equivalent of 345 J/mol. The defect in the RGM is thus less than around 6% of this value. The differential interaction of the chiral active site with the minimal acetyl structure is insufficient to differentiate the hydrogenic sites to a greater extent than this.

EXPERIMENTAL SECTION

Materials and solutions. All salts and organic reagents were obtained as analytical- or reagent-grade materials. Zinc dust, bromacetic and dibromacetic acids, *p*-nitrophenol, *N,N*-dicyclohexylcarbodiimide, and deuterium oxide (99.8%) were obtained from Aldrich Chemical Company. Acetic acid-*methyl*-d_3 was obtained from Diaprep. Acetonitrile was Fisher Certified ACS grade, distilled before use. Water was distilled from a copper-bottom still, passed through a Barstead mixed-bed ion-exchange column, and distilled in glass. Sodium monohydrogen phosphate and potassium dihydrogen phosphate were obtained from Fisher Scientific Company. Bovine pancreatic α-chymotrypsin (EC 3.4.21.1) was obtained from Sigma Chemical Company as a salt-free, lyophilized, three-times recrystallized powder with an activity of 52 BTEE units/mg.

Labeled p-nitrophenyl acetates. Each of the esters were prepared from *p*-nitrophenol and the labeled acetic acid by use of a carbodiimide coupling reaction. For example, a solution of acetic acid-*methyl*-d_1 (7 mmol) and *p*-nitrophenol (7 mmol) in 140 ml methylene chloride was cooled with stirring in an ice bath. *N,N*-Dicyclohexylcarbodiimide (7.7 mmol) was added and the reaction mixture was stirred at 0°C for 1 hr and overnight at room temperature. The precipitated urea was filtered and the solvent removed at reduced pressure, the residue was taken up again in methylene chloride, and the solution was filtered a second time, washed with 0.5 M hydrochloric acid and saturated sodium bicarbonate, and finally dried over anhydrous magnesium sulfate. The pale yellow solid recovered from evaporation of the solvent was four times recrystallized from hexane to give white needles, mp 78.5°C (lit. [*8*] 77.5–78.0 for the protiated compound). Deuterium contents for the labeled esters were estimated by MS and NMR as *p*-nitrophenyl acetate-*methyl*-d_3, M^+ 184(*m/e*), >99%; *p*-nitrophenyl acetate-*methyl*-d_2, M^+ 183(*m/e*), >96%; *p*-nitrophenyl acetate-*methyl*-d_1, M^+ 182(*m/e*), >92%.

Acetic acid-*methyl*-d_1 and acetic acid-*methyl*-d_2 were obtained by zinc-dust debromination of bromacetic acid and dibromacetic acid, respectively, in deuterium oxide. For example, bromacetic acid was stirred with an excess of zinc dust in deuterium oxide for 24 hr at room temperature. The insoluble zinc salts were filtered, the acidified solution was extracted with ether, and the ether extract was dried with magnesium sulfate. Distillation afforded acetic acid-*methyl*-d_1, bp 100–101°C. Deuterium content was determined only after conversion to the esters.

Kinetics. Reaction rates were determined as the increase in absorbance at 400 nm. Phosphate buffer containing α-chymotrypsin (3 ml) was allowed to equilibrate thermally in a thermostated cuvet in the Cary-118 spectrophotometer, interfaced to a Heath-DEC H-11A computer for data collection and storage. The reaction was initiated by injection of 50 μl of substrate stock solution in acetonitrile. Data were collected only early in the reaction, while good zero-order behavior was observed. Rates for isotopic substrates were measured in alternation.

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