Permeable Membrane/Mass Spectrometric Measurement of Solvent $^1H/^2H$, $^{12}C/^{13}C$, and $^{16}O/^{18}O$ Kinetic Isotope Effects Associated with α-Chymotrypsin Deacylation: Evidence for Reaction Mechanism Plasticity†

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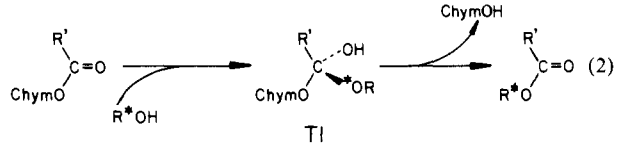
ABSTRACT: We have measured, by permeable membrane/mass spectrometry, the $^{16}O/^{18}O$, $^{12}C/^{13}C$, and solvent H_2O/D_2O kinetic isotope effects (kie) associated with acyl-α-chymotrypsin hydrolysis and transesterification. The hydrolysis of α-chymotrypsinyl 2-furoate has a $^{12}C/^{13}C$ kie of approximately 1.06. Transesterification of the same acyl enzyme shows $^{16}O/^{18}O$, $^{12}C/^{13}C$, and solvent H_2O/D_2O kinetic isotope effects of 1.015 (0.003), 1.01–1.02, and 2.226 (0.007), respectively. From the temperature independence of the $^{16}O/^{18}O$ transesterification kinetic isotope effect and kinetic data reported elsewhere [Wang, C.-L. A., Calvo, K. C., & Klapper, M. H. (1981) *Biochemistry 20*, 1401–1408], we conclude that there are two active forms of acylchymotrypsin. We also propose that formation of the tetrahedral intermediate is the rate-limiting step in both hydrolysis and transesterification and that the position of the transition state in the transesterification is closer to the starting enzyme ester while that for the hydrolytic reaction is closer to the tetrahedral intermediate. These results are discussed in terms of reaction mechanism plasticity.

Arrhenius plots associated with the α-chymotrypsin-catalyzed hydrolysis and transesterification of *p*-nitrophenyl 5-*n*-alkyl-2-furoates, eq 1, are nonlinear (Baggott & Klapper, 1976; Wang et al., 1981), results similar to those found with other chymotrypsin-catalyzed ester hydrolyses (Kaplan & Laidler, 1967; Glick, 1971; Wedler et al., 1975). On the basis of the analysis of kinetic data obtained for the enzyme-catalyzed hydrolysis of *p*-nitrophenyl 2-furoate (NF)[1] and *p*-nitrophenyl 5-*n*-propyl-2-furoate (NPF), we concluded previously that a temperature-sensitive equilibrium between two active forms of the furoyl–enzyme is an appropriate explanation for the observed temperature dependence of the rate-limiting catalytic step, chymotrypsin deacylation. This proposal is consistent with reported spectroscopic evidence for two acyl enzyme species (MacClement et al., 1981; Shah et al., 1984; McWhirter et al., 1985). However, we could not with kinetic data alone exclude a second possible explanation, a temperature-dependent change of rate-limiting steps in a multistep deacylation. The commonly accepted mechanism for the deacylation reaction, in fact, involves the famous tetrahedral intermediate, the TI of reaction 2.



(2)

To discriminate between these mechanistic possibilities, we turned to the measurement of kinetic isotope effects (kie), the changes in reaction rates that may occur when we replace an atom at the reaction locus with its heavier isotope. Contained within the kie is information on both transition-state structure and reaction pathway [e.g., Maggiora and Schowen (1977), Klinman (1978), and Melander and Saunders (1980)]. We reasoned that the substitution of $R^{18}OH$ for $R^{16}OH$ in reaction 2 would result in a kie only over that part of the temperature range where formation of the tetrahedral intermediate was rate-limiting, should the Arrhenius nonlinearity be due to a temperature-dependent change in rate-limiting steps.

However, because the kie from replacement of ^{16}O by ^{18}O are small, the direct determination of rate changes is difficult, although not impossible (Mitton & Schowen, 1968; Gorenstein, 1972; Rosenberg & Kirsch, 1979a,b). The errors that arise from the sequential measurements of separate ^{16}O and ^{18}O reactions are minimized by mixing the isotopic reagents together so as to run the two reactions simultaneously. The kie is then obtained by the mass spectrometric determination of the difference between reactant and product isotopic ratios. Isotopic ratios of high precision can be determined for enzyme-catalyzed reactions with an isotope ratio mass spectrometer (O'Leary, 1980). However, this technique is useful primarily with small and volatile compounds. Otherwise, reactants and/or products can be introduced into a mass spectrometer after either bulk (Sawyer & Kirsch, 1973) or gas chromatographic (Wang et al., 1980) purification. In all these techniques sample preparation precludes the rapid measurement of many isotope ratios. Mass spectral measurements without prior species separation have been done in kie studies of gas-phase reactions (Kwart, 1982). Cleland (1980) has described an equilibrium perturbation technique in which there is no analyte purification but which requires a reasonably reversible reaction.

We have developed a still unique approach to kie measurements, permeable membrane/mass spectrometry (PM/

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[1] Abbreviations: NF, *p*-nitrophenyl 2-furoate; NPF, *p*-nitrophenyl 5-*n*-propyl-2-furoate; TI, tetrahedral intermediate; kie, kinetic isotope effect; PM/MS, permeable membrane/mass spectrometry.

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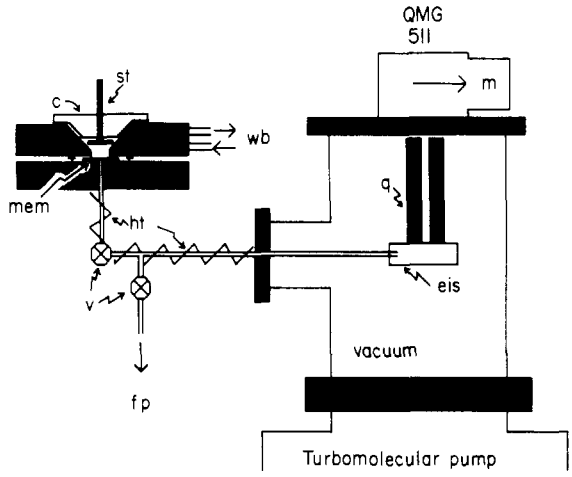


FIGURE 1: Schematic diagram of PM/MS reaction cell and spectrometer: st, stirrer; c, cell cover; mem, membrane and stainless steel support; ht, heating tape; v, Nupro bellow valves; fp, forepump; m, 90° off-axis secondary electron multiplier; eis, gas-tight electron-impact ion source; q, quadrupole ion filter.

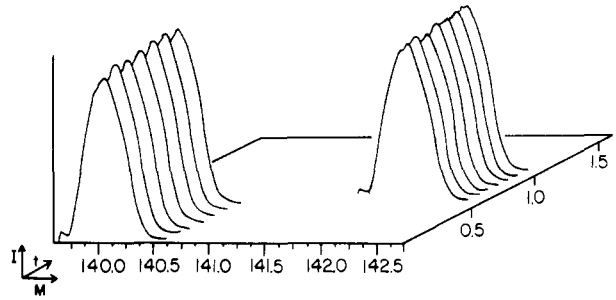


FIGURE 2: MS scans of [*ethoxy*-^{16}O]- and [*ethoxy*-^{18}O]ethyl 2-furoate parent ions (*m/e* 140 and 142). The ester ion peak is scanned several times (seven shown here only as an example) followed by a similar set of scans for the ester containing the heavier isotope. The results are presented as signal intensity (in arbitrary units) vs. *m/e* as a function of time. For data analysis, the peaks are individually integrated and then averaged. The scan time for one peak is 60 ms; hence, the total time required to obtain the two sets of peaks was approximately 0.8 s.

MS), with which we can measure isotopic ratios in seconds (Calvo et al., 1981). Briefly, the aqueous reaction mixture is in contact with a semipermeable membrane that permits passage of apolar but not polar or charged molecules. The other side of that membrane leads to the mass spectrometer inlet (Figure 1). In the transesterification reaction 1, only the product furoyl ester is readily permeable, and during that reaction the newly formed product appears in the mass spectrometer with its formation rate determined from the increase in parent or base peak. When the isotope-enriched reactant is also present, we see peaks for both "heavy" and "light" product (e.g., Figure 2), and we obtain an apparent kie either by comparison of product formation rates or from the ratio of the light and heavy product peaks (Calvo et al., 1983).

We have previously reported (Calvo et al., 1983) the PM/MS measurement of the $^{16}O/^{18}O$ kie associated with the chymotrypsin-catalyzed transesterification of NPF to the product [$^{16}O/^{18}O$]ethyl 5-*n*-propyl-2-furoate. Because of improvements in both instrumentation and data analysis, we have repeated and extended these earlier PM/MS experiments with the homologous substrate NF. These new $^{16}O/^{18}O$ results demonstrate the significant technical gains made since our last paper. We have also extended the PM/MS methodology to the measurements of a $^{12}C/^{13}C$ kie and the effects of solvent H_2O/D_2O replacement on both oxygen and carbon kie. We interpret the reported results with respect to the nonlinear Arrhenius plot and to the mechanism of furoylchymotrypsin deacylation. We also discuss the results with respect to the mechanism specificity of α-chymotrypsin.

Materials and Methods

The experimental apparatus, represented with the block diagram of Figure 1, is a modification of that described earlier (Calvo et al., 1981). The reaction cell is constructed with a dimethylsilicon membrane, nominally 25 μm thick (General Electric, Schenectady, NY), supported on a fine stainless steel screen and clamped with O rings between two stainless steel blocks. A conical hole machined through the upper block forms the cell with the semipermeable membrane as its bottom. The cell temperature is maintained by water circulating through the upper block. The bottom of the membrane is connected to the gas-tight electron-impact ion source of a Balzers (Nashua, NH) QMG-511 quadrupole mass spectrometer via a 7 mm i.d. hole bored through the lower block, a Nupro bellows valve and 4 mm i.d. stainless steel tubing. The inlet system, heated to 140 °C below the cell, is approximately 6 in. long from membrane to ion source. The vacuum in the chamber around the ion source, the quadrupole mass filter, and the 90° off-axis secondary electron multiplier that served as the current detector are maintained below 10^{-6} bar with a Balzers turbomolecular pump. The signal from the detecter is fed through an electrometer amplifier to a 12-bit digital voltmeter (Balzers Model VM512). A MINC-DECLAB 11/23 (Digital Equipment Corp., Maynard, MA) controls the mass spectrometer and collects the digitized signals. The unprocessed binary data are stored on hard disk while collected from the mass spectrometer and processed after the experiment has ended. In this way, we can obtain up to 64 ion current readings in an approximately 10-ms scan of a single peak. However, to enhance signal to noise (S/N) we generally scanned one *m/e* peak 26 times over approximately 1.5 s. After discarding the first scan, we averaged the peak areas of the remaining 25. In a two-ion experiment, the 26 scans of the first peak are immediately followed by 26 scans of the second, and then again immediately of the first, etc. The results of an example experiment in which there are seven, for illustrative purposes only, scans of each of two ion peaks are presented in Figure 2.

In a typical transesterification $^{16}O/^{18}O$ kie experiment we added 60 μL of 20 mg/mL α-chymotrypsin (Sigma, lot 102F-8050) at pH 3 to 520 μL of 0.1 M K_2HPO_4 buffer, pH 8.5. After equilibration at 25 °C in the reaction cell for 10–12 min, we added 15 μL of an approximately 1:1 ^{16}O:^{18}O ethanol mixture, waited for 3 min, took a 1–2-min base-line reading, and then started the reaction by addition of 25 μL of 12.5 mM NF dissolved in *N*,*N*-dimethylformamide. (At higher and lower temperatures the enzyme concentration was adjusted to obtain approximately the same reaction rate as that at 25 °C.) During the reaction the instrument sequentially scanned the parent peak of the ^{16}O product at *m/e* 140 for approximately 1.5 s and then the parent peak of the ^{18}O product at *m/e* 142 for the same time. Thus, we obtained a new $^{16}O/^{18}O$ peak area pair approximately every 3 s.

We adjusted for the time difference between the two sequentially scanned peaks with a two-point interpolation before calculating peak area ratios. A linear interpolation was appropriate once steady state had been reached but was clearly inadequate early in the reaction. While a three-point quadratic interpolation gives a better description of the $^{16}O/^{18}O$ peak area ratio in the first minute or so of the experiment, there

is little difference between the two interpolation results once the reaction reaches steady state. Because our primary concern in these experiments was with the apparent steady-state kie and the computational time is shorter, we commonly used linear interpolation only to obtain peak area ratios during the steady-state portion of the reaction.

We computed the apparent $^{16}O/^{18}O$ kie from the steady-state 140/142 peak ratio averaged over approximately 5 min and normalized by division with a "calibration" factor R required to account for the $^{16}O/^{18}O$ mass ratio in the starting ethanol and for the dependence of the instrument response on m/e (Calvo et al., 1983). To obtain the normalization factor we first converted the original [$^{16}O/^{18}O$]ethanol mixture to ethyl furoate with excess furoyl chloride under conditions we had established previously for the complete conversion. We then added an aliquot of the ester/excess furoyl chloride solution into the reaction cell under conditions identical with those for the enzyme-catalyzed reaction, but for the absence of substrate and enzyme. The calibration factor R is then the ratio of the peak areas, $area_{140}/area_{142}$, obtained once the two peaks had reached their maximum values.

We determined the $^{12}C/^{13}C$ kie associated with furoyl-chymotrypsin deacylation by a single turnover (Baggott & Klapper, 1976) as opposed to a steady-state procedure. We added 0.6 mL of a previously equilibrated solution of α-chymotrypsin, [^{16}O]ethanol (0.1–2.5 M), and 0.1 M phosphate, pH 8.0, to the empty PM/MS cell and after approximately 2 min added a mixture of [$^{12}C/^{13}C$]NF in N,N-dimethylformamide (5 μL) to initiate the reaction and followed the time dependencies of both 140 and 141 peak areas. Enzyme was in slight excess over substrate in order to minimize multiple enzyme turnover.

The m/e peak area time dependencies for a typical single turnover experiment are shown in Figure 7. These results were fit individually to

$$I(t) = a_1(\exp[-a_2(t + a_3)] - \exp[-a_4(t + a_3)]) \quad (3)$$

by a modification of the CURFIT algorithm of Bevington (1969). $I(t)$ represents the peak area at the arbitrary time t. The constants a_1 to a_4 represent respectively an arbitrary intensity factor, an apparent pseudo-first-order constant associated with permeation of the ester through the membrane and into the mass spectrometer, a correction factor to account for our omission of early points during the initial lag, and finally the apparent enzyme deacylation rate constant.

The [^{13}C]carbonyl-enriched ethyl furoate $m + 1$ peak is contaminated with material that contains natural abundance ^{13}C in any one of the carbons other than the carbonyl. Thus, if there is a kie, then the observed time dependence of the $m + 1$ peak area will depend on the kinetics of both carbonyl-enriched and contaminating carbonyl-unenriched product. To minimize possible error, we estimated this $m + 1$ contamination from the 140/141 peak area ratio obtained when the reaction was run under the same conditions but without ^{13}C-enriched substrate. This "background" was then suitably subtracted from the 141 peak area before we fit the ^{13}C-enriched results to eq 3. The errors this simple correction might involve were not significant since the starting substrate $^{12}C/^{13}C$ ratio in our experiments was approximately 1/5; if we assume approximately 7% natural abundance ^{13}C contamination in the [^{12}C]ethyl furoate product and an error of 20% in the estimation of this natural abundance, we can estimate a maximum possible error of approximately 0.3% in the corrected m/e 141 peak area. This conservatively assigned maximum error is less than the estimated standard deviation in the computation of the apparent deacylation rate constant.

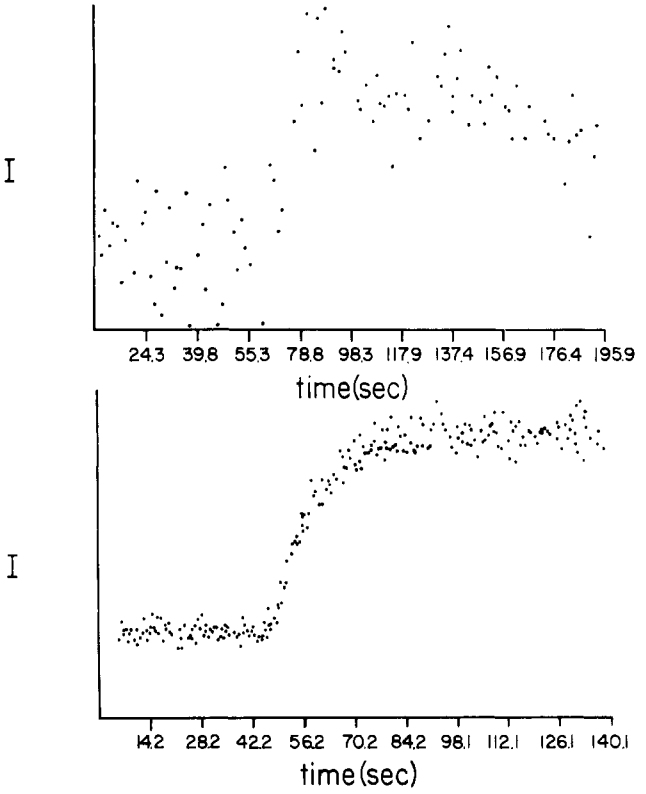


FIGURE 3: MS response to ethyl 2-furoate addition into the permeable membrane reaction cell. Parent peak area (m/e 140: each point is the average of 20 scans) in arbitrary units vs. time upon addition of 0.4 (top) and 100 nM (bottom) ethyl 2-furoate to the aqueous buffer solution, pH 8.5, 25 °C, in the reaction cell. Analyte addition is at the point of the jump in signal intensity.

To prepare 2-furoyl chloride, we heated furoic acid, that had been recrystallized from hot ligroin, in excess thionyl chloride. After distillation of the excess thionyl chloride, we purified the furoyl chloride by vacuum distillation (bp ~110 °C at 30 mmHg). The [^{13}C]carbonyl-enriched NF was synthesized as described elsewhere (McWhirter, et al., 1985) and the ^{18}O-enriched ethanol by the method of Sawyer (1972). N,N-Dimethylformamide was treated with P_2O_5 for 3 days, with KOH for 3 additional days, and then distilled under vacuum. All other reagents were purchased commercially and used without further purification.

RESULTS

The validity of the PM/MS assay, as of any assay, requires a usable correlation between instrument response and analyte, in this case ethyl 2-furoate concentration. This correlation was our first concern. After addition of the furoate ester to the reaction cell, the area of the parent peak at m/e 140 increases with a rise time of approximately 10 s, reaches a maximum plateau (Figure 3), and then declines more slowly (Figure 4) because of ester loss through the membrane and into the mass spectrometer. The maximum response correlates linearly ($r = 0.995$) with the initial ester solution concentration over the range 34–402 μM (results not shown). We assumed a similar linear dependence for the ^{13}C- and ^{18}O-enriched parent peaks at m/e 141 and 142, respectively. The estimated sensitivity, assigned as the solution concentration at which the signal to noise (S/N) is 2, was 0.3 nM when the m/e 140 peak was measured. Examples of the analyte measurement at low concentrations are given in Figure 3. We believe that the currently available sensitivity could be increased by as much as 2 orders of magnitude with only minor changes in cell design

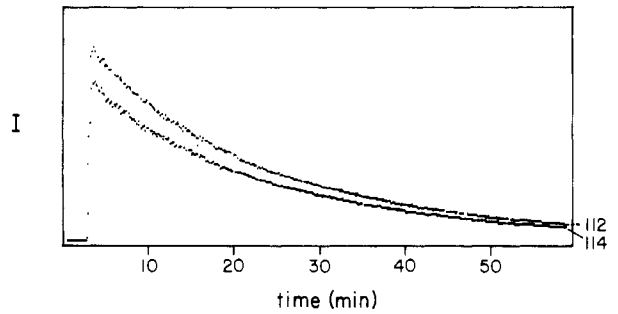


FIGURE 4: Solute permeation loss of [*ethoxy*-^{16}O]- and [*ethoxy*-^{18}O]ethyl 2-furoate. Signal intensity (20-scan average over base peak at *m/e* 112 and 114 in arbitrary units) vs. time after addition of 5 μM ester (^{16}O:^{18}O approximately 1:1) into reaction cell, pH 8.5, 25 °C.

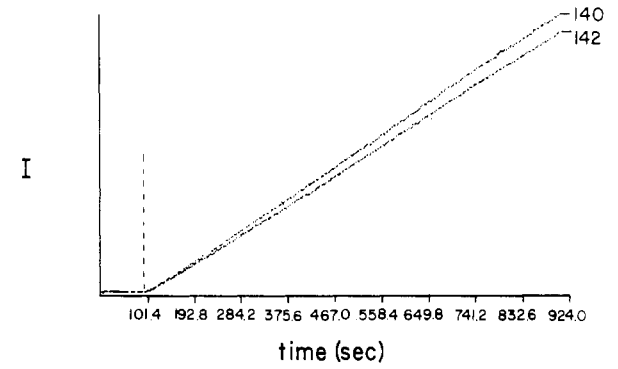


FIGURE 5: Chymotrypsin-catalyzed formation of [*ethoxy*-^{16}O]- and [*ethoxy*-^{18}O]ethyl 2-furoate. The reaction solution contained initially 0.5 M ethanol (^{16}O:^{18}O approximately 1:1) and ca. 30 μM α-chymostrypsin in 0.1 M phosphate buffer, pH 8.5, 25 °C. The reaction was started (vertical dashed line) with the addition of *p*-nitrophenyl 2-furoate in *N,N*-dimethylformamide to a final concentration of ca. 0.5 mM and 1.6% (v/v), respectively. Peak intensities are presented in arbitrary units.

and data collection protocol. PM/MS clearly has a potential use, not yet pursued by us, for analyzing dilute aqueous solutions of apolar compounds.

Because we obtain apparent ^{16}O/^{18}O kie from ^{16}O/^{18}O isotope ratios measured *after* the analyte has passed through the membrane, we were concerned with a possible permeation isotope effect that might "distort" the reaction kie we hoped to measure. Isotopic discrimination by the membrane should be displayed in the rates at which ^{18}O-enriched and unenriched esters are lost from the cell. The decays of both *m/e* 140 and 142 peak areas (Figure 4) fit well to first-order equations with decay half-lives of approximately 15 min at room temperature. The permeation isotope effect is the ratio of these two half-lives, 1.0033 (0.0003). Because this permeation effect is small and the ^{16}O/^{18}O kie experiments to be described were usually completed in less than 10 min, we concluded that permeation will not introduce significant error into the measured mass ratios. (In theory, the ^{12}C/^{13}C permeation isotope effect should be similar or smaller in magnitude and, hence, also have negligible significance.)

We are now ready to consider the kie associated with the substrate NF in reaction 1. After NF addition to a solution of enzyme and excess [^{16}O/^{18}O]ethanol (ca. 1:1) mixture, we monitored the parent peaks (*m/e* 140 and 142) of both [*ethoxy*-^{16}O]- and [*ethoxy*-^{18}O]ethyl furoate (Figure 5). Because of the large ethanol excess, the reactant ^{16}O/^{18}O mass ratio remained effectively constant throughout the reaction. The 140 to 142 peak area ratio calculated from the data of Figure 5 declined initially but was constant after the reaction reached steady state (Figure 6).[2] The steady-state kie for the deacylation of furoylchymotrypsin is this constant peak area ratio normalized by the calibration factor *R* (see Materials and Methods; Calvo et al., 1983). The apparent ^{16}O/^{18}O kie associated with the ethanol transesterification of NF under a variety of conditions are presented in Table I. The kie at pH 8.5 is temperature-independent within the limit of our present precision. There is a small change of unknown sig-

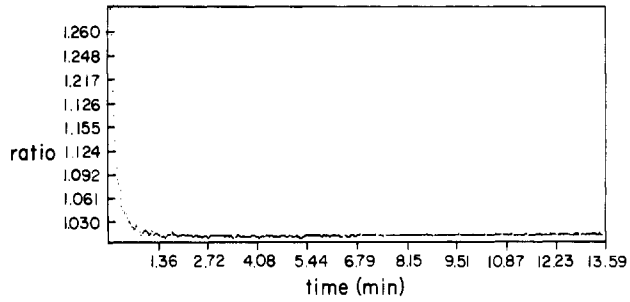


FIGURE 6: ^{16}O/^{18}O kie for α-chymotrypsin-catalyzed transesterification of NF. The reaction conditions are those of Figure 5. The ratio computed from the data of Figure 5 is plotted against time, with 0 the time of substrate addition.

Table I: Steady-State ^{16}O/^{18}O kie in a Chymotrypsin-Catalyzed Transesterification

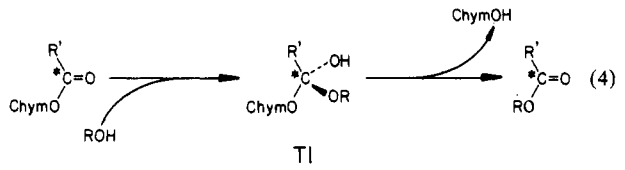
pH	temp (°C)	kie in H_2O	expt no.	kie in D_2O	expt no.
6.0	25	1.009 (0.001)[a]	2		
6.5		1.008 (0.001)	3		
7.0		1.013 (0.002)	3		
8.5[b]		1.015 (0.003)	24	1.022 (0.004)	12
8.5	10	1.017 (0.007)	1	1.027 (0.004)	3
	14	1.015 (0.008)	1		
	20	1.013 (0.003)	2		
	25	1.015 (0.003)	24	1.022 (0.004)	12
	30	1.014 (0.005)	4		
	40	1.016 (0.002)	4	1.025 (0.001)	3
	av[c]	1.015 (0.003)	36	1.025 (0.004)	18

[a] Numbers in parentheses are estimated standard deviations. [b] The same pH 8.5, 25 °C data are repeated in the table twice simply to emphasize trends. [c] The averages are taken from all the pH 8.5 data, each individual kie weighted with its estimated variance.

[2] We had previously reported a qualitatively similar initial ratio decline in the chymotrypsin-catalyzed transesterification of PNF and had argued for its mechanistic significance (Calvo et al., 1983). We know now that much of that decline and the inverse kie we had also reported at that time were artifactual because of a confluence of problems we have since identified. (i) The two peak areas are measured sequentially and not at the same time (see Materials and Methods). To correct for this time shift, we calculate peak area ratios by using one observed and one interpolated value. The linear interpolation we had been using works well when product concentrations increase linearly or approximately linearly, as during steady state. However, we have found that linear interpolation is not valid near the start of the reaction, presumably because the acceleration in product appearance is greatest in the early stages of the reaction. When we replace linear with quadratic interpolation, the time for apparent peak area ratio decay to a constant value is shortened considerably, although not eliminated. Presently, we do not know whether the ratio decay that still exists (e.g., Figure 5) is significant or due to errors that the quadratic interpolation has not eliminated. Because of the enhanced sensitivity and S/N with which we now work, we have also found a second problem. (ii) The *N,N*-dimethylformamide in which we dissolved the NPF for later addition to the reaction system appears to have contained a still unidentified contaminant, since both the time to reach a steady product ratio and the value of that ratio changed upon further *N,N*-dimethylformamide purification. Finally, (iii) there is a poorly characterized noise problem associated with the inlet system to the MS source. We have eliminated that instability in the case of ethyl furoate by decreasing dead space below the membrane and heating the feed line inlet into the ion MS source. That these measures reduce the signal instability when ethyl 2-furoate, but not yet ethyl 5-*n*-alkyl-2-furoate, is the analyte suggests that adsorption of the analyte to the inlet system walls is the cause.

nificance in the kie between pH 6.0 and pH 8.5 at 25 °C. We also observe a small but significant increase in the apparent $^{16}O/^{18}O$ kie when D_2O replaces H_2O as solvent. With respect to this last result, we can also obtain a solvent H_2O/D_2O kie of 2.226 (0.007) from the comparison of the steady-state rate of unenriched product formation in H_2O vs. D_2O.

We have also measured a $^{12}C/^{13}C$ kie with ^{13}C substitution into the carbony carbon of NF. Since ethanol is involved only in enzyme deacylation, the rate-determining step in α-chymotrypsin-catalyzed transesterification (Wang et al., 1981), we wanted the comparable deacylation $^{12}C/^{13}C$ kie of eq 4 to



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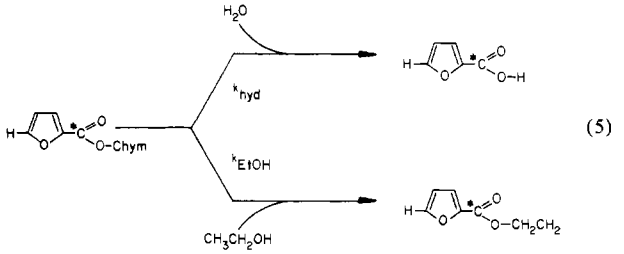
complement the $^{16}O/^{18}O$ results. However, unlike the ethoxy oxygen the carbonyl carbon also participates in formation of the acyl enzyme, which reaction(s) may well contribute to and complicate the interpretation of the steady-state $^{12}C/^{13}C$ kie. The single turnover protocol, in which acylation and deacylation are separated temporally (Baggott & Klapper, 1976; Wang et al., 1981), is for this reason a more appropriate protocol. On mixing excess chymotrypsin with the substrate NF, there is rapid enzyme acylation and simultaneous NF depletion. After the NF is consumed, deacylation continues with first-order appearance of ethyl 2-furoate; the $^{12}C/^{13}C$ kie observed in this first-order process is associated with the deacylation reaction 4.

But while we can obtain the sought for kie in theory, there remain three technical problems. The first of these, the $m + 1$ problem, is due to the relatively high (ca 1%) ^{13}C natural abundance. To obtain a $^{12}C/^{13}C$ kie, we must compare unenriched with [^{13}C]carbonyl-enriched product, for example, by comparison of the ethyl 2-furoate parent peak at m/e 140 with the [*carbonyl*-^{13}C]ethyl 2-furoate parent peak at m/e 141. Since this ester contains six other non-carbonyl carbon atoms, the $m + 1$ peak will be contaminated (ca. 5%) with ions that contain one ^{13}C atom somewhere other than at the carbonyl carbon. To avoid potential errors due to this contamination, we must subtract the contribution of the incorrectly substituted material to the $m + 1$ peak magnitude.[3] Since correction by subtraction involves absolute not relative quantities and since the absolute contamination of the $m + 1$ peak must decrease as the ratio of [^{12}C]- to [^{13}C]carbonyl substrate decreases, we alleviated the $m + 1$ problem by using a 1/5 [$^{12}C/^{13}C$]carbonyl substrate mixture. The correction procedure based on this strategy and made possible by the PM/MS sensitivity is described under Materials and Methods. The single turnover ^{13}C and ^{12}C reaction profiles from a typical experiment are shown in Figure 7. After an initial lag, because of the time required to reach both reaction and permeation steady state, there is a first-order increase in peak area due to furoylchymotrypsin breakdown to ethyl 2-furoate and free chymotrypsin. The subsequent slower decline in the peak area is due to analyte loss into the mass spectrometer.

The second of the three problems to which we alluded earlier is the computation of an apparent kie from reaction profile pairs such as those of Figure 7. Over the course of the $^{16}O/^{18}O$ steady-state experiment, the ethanol isotopic ratio remains essentially constant, since ethanol is in large excess. In the single turnover experiment the substrate, with a concentration less than that of the enzyme, is consumed, and its $^{12}C/^{13}C$ mass ratio could change over the course of the reaction because of the very kie that we are attempting to measure. Hence, we cannot obtain a time-independent product mass ratio, as done in the $^{16}O/^{18}O$ steady-state experiment. Another commonly used approach is also not appropriate in this case. Because of mass conservation, the final product isotopic ratio at infinite time in an irreversible reaction must be identical with the substrate isotopic ratio before the start of the reaction. By contrast, the initial product isotopic ratio reflects the reaction mass discrimination. Thus, it is common to obtain the reaction kie from the comparison of initial and final product isotopic ratios. However, since the formation of the acyl enzyme, the reactant of these experiments, is also subject to a possible kie and since we cannot completely separate enzyme acylation and deacylation, we cannot assign the infinite product $^{12}C/^{13}C$ ratio to the initial substrate $^{12}C/^{13}C$ ratio. This assignment would not be possible even with complete separation of acylation and deacylation, because hydrolysis to furoic acid competes with transesterification to ethyl 2-furoate (eq 5; Wang et al., 1981). Were hydrolysis and transesterification to have different kie, then the $^{12}C/^{13}C$ ratio of the ester product at reaction end would not be identical with the $^{12}C/^{13}C$ ratio of the substrate before reaction initiation.

Fortunately, the PM/MS methodology permits us a different strategy. Since overall deacylation and subsequent ester permeation into the MS are pseudo-first-order processes, we individually fit both reaction traces of Figure 7 to the biexponential eq 3 to obtain ^{12}C and ^{13}C deacylation rate constants and then from their ratio an apparent kie. Unfortunately, the $^{12}C/^{13}C$ kie computed this way is less precise than the $^{16}O/^{18}O$ kie obtained directly from product isotopic ratios. However, there are two advantages. For one, a first-order process is independent of initial reactant concentration so that we do not have to account for the initial substrate $^{12}C/^{13}C$ ratio. Additionally, we have access to information that is unobtainable from the steady-state experiment, as we shall see shortly.

A third procedural problem arises because hydrolysis and transesterification of furoylchymotrypsin are competitive under our experimental conditions (eq 5). Because the two reactions



are coupled, the apparent deacylation rate constant contains both k_{hyd} and k_{EtOH} *even though we detect only the ethyl ester product*:[4]

$$k_{app} = k_{hyd} + k_{EtOH}[CH_3CH_2OH] \quad (6)$$

$$kie_{app} = \frac{^{12}k_{app}}{^{13}k_{app}} = \frac{^{12}k_{hyd} + ^{12}k_{EtOH}[CH_3CH_2OH]}{^{13}k_{hyd} + ^{13}k_{EtOH}[CH_3CH_2OH]} \quad (7)$$

[3] Enrichment of the $m + 1$ peak with carbonyl-unlabeled material requires an identical depletion of the m peak; but since natural abundance contamination is responsible for the identical percentage shift of the $m + 1$ peak to the $m + 2$ position, no correction is required. For example, assume that the relative amounts of m and $m + 1$ peaks should be 20 and 100, respectively. If 5% of the m peak is lost because of a natural abundance shift, then the m peak becomes 19. But 5% of the $m + 1$ peak must also be shifted to $m + 2$, or (after correction for contamination) the $m + 1$ peak becomes 95, and the $m/(m + 1)$ ratio remains unchanged.

In theory, therefore, the apparent kie depends upon the ethanol concentration, provided $kie_{hyd} \neq kie_{EtOH}$ (eq 7). We have, in fact, found that the apparent deacylation kie is ethanol-dependent, decreasing with increasing ethanol concentration (Figure 8), presumably because $kie_{hyd} > kie_{EtOH}$. To extract kie_{hyd} and kie_{EtOH}, we would fit the results of Figure 8 to eq 7; in this case, however, the precision, which is poorest at high and low ethanol concentrations, is not sufficient for a reliable fit. Qualitatively, the results suggest that the kie of hydrolysis is near 1.06, while that of transesterification is closer to 1.02. As seen with the $^{16}O/^{18}O$ kie, there is a small but consistent increase in the apparent $^{12}C/^{13}C$ kie when D_2O replaces H_2O (Figure 8); the hydrolysis kie in D_2O may be as high as 1.07–1.08.

Discussion

The impetus for these kie studies was the observed nonlinear Arrhenius plot associated with the hydrolysis and transesterification of the acyl enzyme chymotrypsinyl 5-*n*-alkyl-2-furoates (Baggott & Klapper, 1976; Wang et al., 1981). After we eliminated a number of plausible explanations for this observation, two nontrivial possibilities remained: a temperature-dependent switch between rate-determining steps in a multistep deacylation or a temperature-dependent equilibrium between two different active forms of the enzyme. Assuming that deacylation goes through the tetrahedral intermediate of eq 2, we reasoned as follows. Were TI formation rate determining over one temperature range and TI breakdown over another, then it would be reasonable to expect a primary $^{16}O/^{18}O$ kie in one but not the other temperature region. On the other hand, a temperature-dependent equilibrium between two active enzyme forms should be signaled by a finite kie over both temperature ranges. Because we cannot measure furoic acid by the PM/MS method (not to mention the expense of $H_2{}^{18}O$), we focused on transesterification, in which there is a membrane permeable ester product. Three interpretational ambiguities were foreseen: a very small kie that could be due either to the primary kie of interest or to a secondary ethoxy $^{16}O/^{18}O$ kie of the TI breakdown; no kie observed at any temperature; an ambiguous kie temperature dependence that

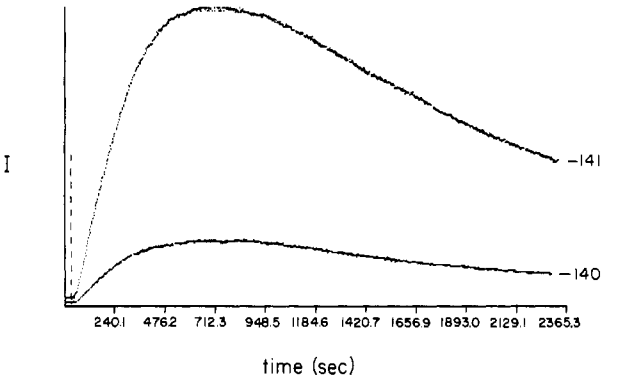


FIGURE 7: Chymostrypsin-catalyzed formation of [*carbonyl*-^{12}C]- and [*carbonyl*-^{13}C]ethyl 2-furoate. The reaction solution contained initially 1.34 M ethanol and ca. 20 μM α-chymostrypsin in 0.1 M phosphate buffer, pH 8.0, 25 °C. The reaction was started (vertical dashed line) with the addition of *p*-nitrophenyl 2-furoate in *N*,*N*-dimethylformamide to a final concentration of ca. 16 μM and 0.8% (v/v), respectively. The substrate was enriched to 80% with ^{13}C in the carbonyl position. This is a single turnover experiment in which the initial signal (peak area of the parent peak) increase is due to ethyl 2-furoate release from the acyl enzyme and the subsequent signal decay to the loss of product through the semipermeable membrane. At the $^{12}C/^{13}C$ ratio of 0.2, the estimated contamination of the $m + 1$ peak by noncarbonyl-enriched ester is less than 1%.

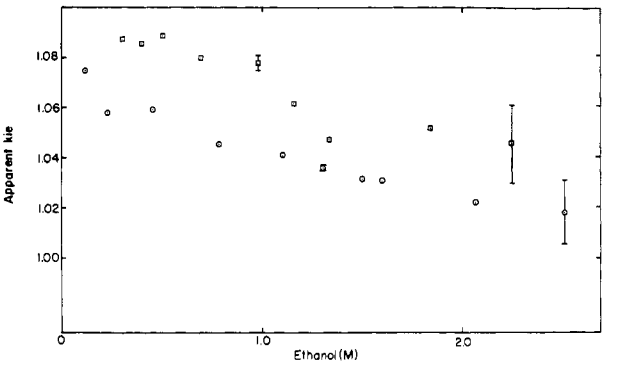
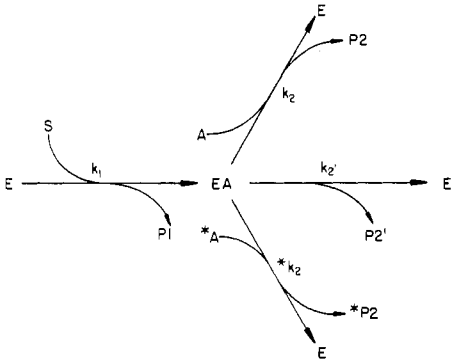


FIGURE 8: Dependence of apparent transesterification $^{12}C/^{13}C$ kie on alcohol concentration. Apparent kie were obtained as described in the text. The reaction conditions were those in the legend of Figure 7, except for the variable concentrations of ethanol. (○) Reaction in H_2O; (□) reactin in D_2O. The largest and smallest estimated standard deviations in both sets of data are presented as examples; the larger estimated standard deviations tended to be at the lower and higher ethanol concentrations.

[4] Equation 6 for competitive first-order reactions has been derived in a number of texts; for example, see Moore (1972). Hydrolysis and transesterification also occur simultaneously in the $^{16}O/^{18}O$ steady-state experiment, but here the apparent kie is independent of the absolute alcohol concentration because hydrolysis and transesterification are not coupled. This is easily shown for the simplified scheme



where the reactants and products are as follows: S, the substrate; P1, the nitrophenol product; A and *A, the ^{16}O- and ^{18}O-labeled alcohols, respectively; P2 and *P2, the ^{16}O- and ^{18}O-labeled product esters; P2′, the product acid. A convenient set of independent linear equations for the steady-state solution of this scheme is

$$[E] + [EA] = [E_0]$$

$$k_1[S_0][E] + v_S = 0$$

$$k_2[A_0][EA] - v_{P2} = 0$$

$$*k_2[*A_0][EA] - v_{*P2} = 0$$

$$k_1[S_0][E] - (k_2' + k_2[A_0] + *k_2[*A_0])[EA] = d[EA]/dt = 0$$

where v_S, $v_{P2'}$, v_{P2}, and v_{*P2} are the constant velocities for reactant and product formations (e.g., $v_S = d[S]/dt$ and $v_{*P2} = d[*P2]/dt$) and

$$-v_S = v_{P2'} + v_{P2} + v_{*P2}$$

The remaining symbols have their usual meanings with the zero subscript referring to concentrations at time zero. From the solution of this equation set we obtain

$$v_{P2} = k_1k_2[S_0][A_0][E_0]/(k_1[S_0] + k_2' + k_2[A_0] + *k_2[*A_0])$$

$$v_{*P2} = k_1*k_2[S_0][*A_0][E_0]/(k_1[S_0] + k_2' + k_2[A_0] + *k_2[*A_0])$$

$$kie_{app} = k_2/*k_2 = v_{P2}[*A_0]/(v_{*P2}[A_0])$$

We see that the apparent kie is dependent on the ratio $[A_0]/[*A_0]$, which is part of the correction factor R described under Materials and Methods but that it is not dependent on the absolute amount of alcohol present in solution, unlike the single turnover case, eq 7. The use of irreversible reactions in the above scheme is justified by the functional irreversibility of the chymotrypsin-catalyzed reaction. A more realistic scheme would contain additional enzyme intermediates. But since this would entail only additional first-order processes, the form of the solution and hence the conclusion would be unchanged.

would not discriminate between the two possibilities. Fortunately, the results can be interpreted. The ethanol transesterification of α-chymotrypsinyl 2-furoate has a temperature-independent $^{16}O/^{18}O$ kie of 1.015 (0.003), from which we conclude that the observed Arrhenius nonlinearity of the transesterification, if not hydrolysis as well, is consistent with a temperature-dependent equilibrium between two acyl enzyme forms that breakdown at different rates. There has been spectroscopic evidence for two forms of the acyl enzyme (MacClement et al., 1981; Shah et al., 1984; McWhirter et al., 1985), but it is not known whether the spectrally and kinetically observed heterogeneities are related.

In addition to providing information about reaction sequence, the magnitude of a measured kie may also shed light on the nature of an elementary reaction's transition state. The observation of an $^{16}O/^{18}O$ kie in chymotrypsin deacylation suggests that formation of the TI (reaction 2) either partially or totally determines the rate of acyl enzyme transesterification. The absence of carbonyl oxygen exchange (Bender & Heck, 1967; Shain & Kirsch, 1968) in and the $^{16}O/^{18}O$ kie associated with the nonenzymatic base-catalyzed hydrolysis of benzoate esters (Sawyer & Kirsch, 1973; O'Leary & Marlier, 1979) suggest that TI formation is rate-determining in model systems as well. Furthermore, rate-determining TI formation is consistent with the conditions required to generate model tetrahedral intermediates (Capon et al., 1981; McClelland & Santry, 1983). The H_2O/D_2O solvent kie, 2.226, associated with chymotrypsin-catalyzed NF transesterification is similar to that for the hydrolysis of NPF—2.11 (.24) (C. M. Trout and M. H. Klapper, unpublished results)—and to the kie reported for the hydrolyses of other esters [e.g., Pollack et al. (1973) and Stein et al. (1983)]. More relevant to this discussion is the relatively small (and perhaps significant) increase in the $^{16}O/^{18}O$ kie caused by D_2O. This observation suggests that the C–O bond, and hence TI formation, remains rate determining even after deacylation is slowed by the solvent switch. Were TI formation fully rate determining in both solvents, then the D_2O perturbation of the measured $^{16}O/^{18}O$ kie is a secondary effect. On the other hand, were TI formation only partially rate determining in H_2O, then the D_2O perturbation is consistent with a more fully rate-determining TI formation in D_2O. While we cannot argue for one interpretation over the other, it is likely that the intrinsic $^{16}O/^{18}O$ kie is somewhere near 1.02.

There is a model reaction with which to compare our kie results. The $^{16}O/^{18}O$ kie associated with hydrazinolysis of methyl formate and methyl benzoate (^{18}O substitution at the ethoxy oxygen) are 1.06 (Sawyer & Kirsch, 1973) and 1.041 (O'Leary & Marlier, 1979). On the basis of the conclusion that TI breakdown is rate-determining in hydrazinolysis, it was suggested that the transition state for C–O bond cleavage is positioned closer to the free alcohol product than to the TI reactant. For chymotrypsin, we are dealing with the reverse process, rate determining C–O bond formation to the TI; hence, the smaller $^{16}O/^{18}O$ kie in chymotrypsin transesterification suggests a transition state closer to the reactant free alcohol than to the product TI. Thus, the hydrazinolysis of small esters and the transesterification of furoylchymotrypsin are satisfyingly complementary.

Because the carbonyl carbon of the α-chymotrypsinyl ester participates in both TI formation and breakdown, an observed $^{12}C/^{13}C$ kie cannot point to either step as rate determining. However, we have already concluded from the ^{18}O results that TI formation probably determines the acyl enzyme transesterification rate, and we have independent evidence to suggest that the same is true in acyl enzyme hydrolysis. We have previously reported the NMR spectrum of [*carbonyl*-^{13}C]-chymotrypsinyl 5-*n*-propyl-2-furoate obtained during the steady-state hydrolysis of NPF (McWhirter et al., 1985). If TI breakdown rather than formation were rate determining in this hydrolysis, then we would expect to detect the TI in the NMR spectrum. However, the only peak we observed has a chemical shift expected of an ester and not of a TI. We conclude that TI formation is most probably rate determining in the acyl enzyme hydrolysis and that the observed hydrolysis $^{12}C/^{13}C$ kie is also associated with the transition state leading to the TI. The remaining discussion of the $^{12}C/^{13}C$ kie is based on this premise.

Although novel, the ethanol dependence of the $^{12}C/^{13}C$ kie (Figure 8) is consistent with competitive hydrolysis and transesterification of enzyme deacylation under the conditions of our experiments. Because of this competition, the measured deacylation rate constant is the sum of two pseudo-first-order contributions (6), should be dependent on the ethanol/water ratio, and is measured identically whichever of the two products, ester or acid, is monitored.[4] The expected $^{12}C/^{13}C$ kie dependence on the alcohol concentration (7) is observed as a decrease with increasing ethanol so that the hydrolysis kie (ca. 1.06) is greater than the transesterification kie (ca 1.02). That this increase could be due either to ethanol-induced alteration of the reaction mechanism or to protein denaturation is unlikely. The apparent rate constant for the deacylation of chymotrypsinyl 5-*n*-propyl-2-furoate is linear up to 15% (v/v) ethanol (Wang et al., 1981); the highest ethanol concentration used in the experiments we described here was approximately 15%. Moreover, simple enzyme denaturation should lower the enzyme's specific activity but not alter the reaction kie. One might argue that the reaction trajectory is somehow altered by changing the solvent composition, but this is a more complex and hence less attractive proposal than the conclusion that hydrolysis and transesterification have different kie.

Ropp and Raaen (1954) reported carbonyl carbon $^{12}C/^{14}C$ kie of 1.07–1.09 for the basic hydrolysis of ethyl benzoates. Since the ratio ln ($^{12}C/^{14}C$)/ln ($^{12}C/^{13}C$) is approximately 1.9 (Melander & Saunders, 1980), these ^{14}C results correspond to $^{12}C/^{13}C$ kie in the range 1.04–1.05. The carbonyl carbon $^{12}C/^{13}C$ kie reported by O'Leary and Marlier (1979) for alkaline hydrolysis and hydrazinolysis of methyl benzoate are 1.043 and 1.041, respectively, consistent with the results of Ropp and Raaen. The largest reported $^{12}C/^{13}C$ kie of which we are aware is 1.08 (0.02) for an S_N2 transmethylation (Gray et al., 1979). Since the magnitude of a kie may be related to the extent of bond-order change between reactant and transition state, with larger kie reflecting greater nuclear movement, qualitative comparison between our results and those from the benzoate studies suggests that in the hydrolysis of furoylchymotrypsin the transition state is closer in structure to the product TI than to the reactant ester while the opposite holds for transesterification—the transition state is closer in structure to the reactant ester than to the product TI. That the $^{16}O/^{18}O$ kie is also consistent with an early transition state in transesterification is gratifying.

The results we have presented here and in previous papers (McWhirter et al., 1985; Wang et al., 1981) permit us a more detailed, albeit still qualitative, picture of the α-chymotrypsin deacylation reaction mechanism. The temperature independence of the $^{16}O/^{18}O$ kie together with our earlier analysis of the nonlinear Arrhenius plots observed by us and others (Baggott & Klapper, 1976; Wang et al., 1981; Kaplan &

Laidler, 1967; Glick, 1971; Wedler et al., 1975) supports the proposal of at least two active acyl enzyme forms in a temperature-dependent equilibrium. We also propose here that the reaction mechanism is modulated by the nature of the attacking nucleophile; the transition state occurs "earlier" with alcohol than with water. This apparent "Hammond effect" has been postulated for a number of small molecule reactions; the general topic of shifts in transition-state position due to changes in reactant substituents has been recently reviewed by Jencks (1985) under the acronym (reluctantly offered by him and only reluctantly accepted by us) of the "Bema Hapothle". A substrate-dependent change in transition-state position has also been proposed for yeast formate (Hermes et al., 1984) and liver alcohol (Scharschmidt et al., 1984) dehydrogenases. Schowen and his co-workers [e.g., Stein et al. (1983)] have reported different but conceptually related observations from proton inventory studies; they have concluded that the hydrolysis of a minimal acyl group, such as CH_3-COO—, from α-chymotrypsin involves a one-proton catalysis mechanism but that the hydrolysis of a group, such as $C_6H_5CH_2CH_2COO$—, that more closely resembles a natural substrate involves a multiproton catalysis. They suggested that the larger substrates are somehow strapped into a more confined conformation that promotes a multiproton mechanism.

Our conclusions and those of Schowen and co-workers, while not necessarily inconsistent with, run counter to the impressions of many enzymologists. Not only are we proposing that there may be more than one reaction mechanism catalyzed by more than one enzyme form, but we are also proposing that even within one reaction scheme the transition state structure, as reflected in its position on the reaction potential energy surface, may depend on the substituent groups attached to the substrate. How different this view is from that of an enzyme reaction with only *one* mechanism and catalysis based in large part on stabilization of *the* transition-state structure by an exquisitely precise placement of active site groups around the reacting substrate. We can subsume both views under the concept of reaction mechanism specificity. (This term is related to though not identical with the better known concept of turnover specificity, in which enzyme turnover differences between substrates already bound to the enzyme have been commonly ascribed to the congruency of substrate–enzyme active site interactions.)

It is common for environmental conditions to influence the nonenzymatic reaction of a compound to different product sets or even to one particular reactant(s)/product(s) pair by different reaction mechanisms. An enzyme not only enhances the rate of substrate conversion to some product but also directs that substrate onto one of the many possible reaction pathways available in the enzyme's absence. The degree to which the enzyme enhances one pathway over all others is the reaction mechanism specificity, and that enhancement can be achieved, in theory, by increasing turnover through one chosen path and/or by decreasing turnover through the unchosen path(s). Perhaps the most spectacular example of mechanism specificity involves the heme group, which when free binds oxygen and catalyzes peroxidations, oxygen insertions, oxidations, and more. When that heme is attached to hemoglobin, it functions primarily as an oxygen binding moiety, to cytochrome *c* as an electron-transfer agent, to peroxidase as a catalyst for peroxidations, and so on. But the mechanistic specificity imposed by the protein need not be complete. As only one example, peroxidase catalyzes not only peroxidation but acts also as a catalase, an oxidase, and a mixed-function oxidase (Klapper & Hackett, 1963). Thus, just as enzymes display different degrees of substrate specificity so they may display different degrees of reaction mechanism specificity; i.e., enzymes are capable of both substrate and mechanism plasticity.

How general a phenomenon α-chymotrypsin or peroxidase reaction plasticity is or whether the phenomenon is peculiar to enzymes that display wide substrate specificity remains to be established. At the very least, the kie results we report here require us to reevaluate the importance of the transition-state stabilization theory. While the thermodynamic basis of that theory is correct [e.g., Pauling (1948), Wolfenden (1969), and Klapper (1973)], we should question its applicability to all enzyme catalytic mechanisms. If an enzyme reaction mechanism displays plasticity, then precise geometric alignment of enzyme substituents with transition-state state structure may not be of overriding importance in the catalysis.

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Prochiral Selectivity and Intramolecular Isotope Effects in the Cytochrome P-450 Catalyzed ω-Hydroxylation of Cumene†

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ABSTRACT: A kinetic model is presented from which steady-state equations are derived that describe the intramolecular competition for the enzymatically mediated hydroxylation of two like groupings of a prochiral substrate. The observed isotope effect in such a system if one of the groupings is isotopically labeled is shown to be a function of three parameters: (a) the equilibrium constant for the catalytically sensitive orientations of the two prochiral groupings at the active site, (b) the intrinsic isotope effect associated with the bond-breaking step, and (c) the relative rates of bond breaking vs. enzyme–substrate dissociation. The expected isotope effects associated with the ω-hydroxylation of racemic, (*R*)-, and (*S*)-2-phenylpropane-*1,1,1*-d_3 and the product stereoselectivity associated with the ω-hydroxylation of (*R*)- and (*S*)-[1-^{13}C]-2-phenylpropane were determined with microsomal preparations (cytochrome P-450) from untreated and phenobarbital- and β-naphthoflavone-pretreated male Sprague-Dawley rats. The data from these experiments allow the observed isotope effect to be evaluated in terms of its component parts, i.e., expected isotope effects, product stereoselectivity, and equilibrium constant. These data further suggest that (a) the intramolecular isotope effect is consistent with a hydrogen abstraction recombination mechanism and is largely dependent upon the chemical nature of the porphyrin–Fe–oxene complex but independent of specific apoprotein structure, (b) product stereoselectivity is primarily dependent upon apoprotein structure, and (c) product stereoselectivity is a good measure of the equilibrium constant and both parameters are dependent upon the chirality of the active site.

One of the major approaches to the elucidation of reaction mechanisms is via the measurement of isotope effects. The critical value to be determined in such experiments is the intrinsic isotope effect, i.e., the isotope effect associated exclusively with the bond-breaking step. The magnitude of the intrinsic isotope effect can then be directly related to transition-state structure, which describes the mechanism of the reaction in detail. The successful application of isotope effects to enzymatically mediated systems, however, has proven to be difficult because of the complexity of the multistep reaction sequences that describe many of these processes. In general, an observed intermolecular isotope effect provides a measure of the overall rate limitations for a given reaction and includes such factors as product release and substrate binding in addition to other steps that may lower (mask) the observed magnitude of the intrinsic isotope effect associated with the actual bond-breaking step (Northrop, 1975).

The problems associated with these complicated schemes and the attenuation or "masking" of an intrinsic isotope effect have been minimized by workers in the field through the application of intramolecular isotope effect experiments (Miwa et al., 1980; Gelb et al., 1982; Hjelmeland et al., 1977). In such an experiment a molecule that has two positions that are equivalent in all respects except for isotopic substitution is used as substrate, and the observed isotope effect reflects the intramolecular competition between the two otherwise equivalent sites. In most cases intramolecular isotope effects more nearly approximate the intrinsic isotope effect since they are primarily dependent upon the product-determining step rather than other rate-limiting steps (Lindsay Smith et al., 1984; Miwa et al., 1980).

A parameter that, at least theoretically, could have a large effect on the observed magnitude of an intramolecular isotope effect is stereoselectivity. If a substrate is chiral, the relationship between the two possible enantiomeric substrate–enzyme complexes must be diastereomeric. Thus, the equilibrium constant for their formation may be different and/or

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