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# Demonstration of the Acyl-Enzyme Mechanism for the Hydrolysis of Peptides and Anilides by Chymotrypsin†

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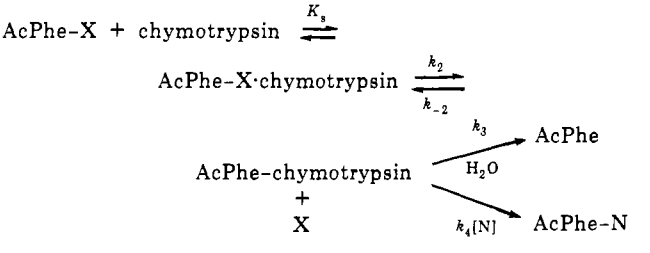
ABSTRACT: The acyl-enzyme mechanism for chymotrypsin was tested by determining the product ratios on the hydrolysis of substrates in the presence of added acceptor nucleophiles which compete effectively with water in the reaction. The product analysis was facilitated by the use of new substrates which could be separated easily from the products by ionophoresis. Direct determination of the ratio of AcPhe (*N*-acetyl-L-phenylalanine) to AcPhe-$AlaNH_2$ produced on the hydrolysis of AcPhe-OMe and AcPhe anilides in the presence of $AlaNH_2$ showed that 1 M $AlaNH_2$ is 44 times more reactive than 55 M water for both substrates. The decrease in $V_{max}/K_M$ for the hydrolysis of AcPhe-$AlaNH_2$ in the presence of $AlaNH_2$ is also accounted for by $AlaNH_2$ being 44 times more reactive. This suggests a common intermediate in the hydrolysis of ester, anilide and peptide substrates. $k_{cat}/K_M$ for the hydrolysis of AcPhe-$AlaNH_2$ was calculated using the above partition ratio, the values of the formation constants of AcPhe-OMe and AcPhe-$AlaNH_2$, the relative reactivities of methanol and water towards the acyl-enzyme derived from ester substrates and the free energy of hydrolysis of AcPhe-OMe. This agrees well with the directly measured value. This is *proof* of the acyl-enzyme mechanism in peptide hydrolysis. Previous attempts to demonstrate a common intermediate have always failed due to an artifact inherent in the approach used. The partitioning of an amide has always been determined indirectly from the decrease in $V_{max}$ on the addition of the leaving group amine (*e.g.*, benzoyltyrosylglycinamide hydrolysis in the presence of added glycinamide). It is pointed out that the addition of certain organic solvents and amines increases $V_{max}$ (and $K_M$) by a nonspecific effect. This partially compensates the decrease and underestimates the partitioning. In the present study the products from the hydrolysis of tritium-labeled compounds were separated by ionophoresis and assayed directly.

Esters are hydrolyzed by chymotrypsin in a two step process. The first step is the acylation of Ser-195 of the enzyme and the second is the subsequent deacylation (Hartley and Kilby, 1954). The rate-determining step for activated esters of nonspecific substrates and for most esters of specific substrates is deacylation. The acyl-enzyme accumulates in solution and may easily be detected by kinetic or spectral observation. Earlier evidence for the acylation of Ser-195 is summarized by Bender and Kézdy (1964) and Bruice and Benkovic (1966). The ultimate proof for nonspecific substrates is that the structures of the crystalline indolylacryloyl-chymotrypsin (Henderson, 1970) and carbamyl-chymotrypsin (Robillard *et al.*, 1972) have been solved by X-ray diffraction methods. Ser-195 is seen to be acylated.

The hydrolysis of amides is too slow to involve rate-determining deacylation and so acylation of the enzyme is assumed to be the slow step. The acyl-enzyme, if it occurs, is a high-energy intermediate present in low concentration and not easily detectable.

SCHEME I




It should be possible to detect the acyl-enzyme in amide hydrolysis by chemical trapping (see Scheme I). The addition of an acceptor nucleophile, N, will give mixed products, *e.g.*, AcPhe and AcPhe-N. The product ratio AcPhe-N:AcPhe should then be the same for both esters and amides if there is a common intermediate. Experiments along these lines have produced evidence *against* the acyl-enzyme mechanism. For example, Epand (1969) showed that benzoyltyrosine ethyl

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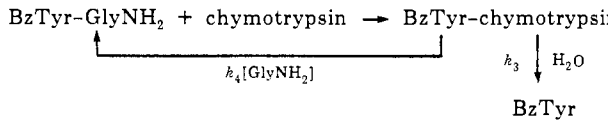
SCHEME II




ester and benzoyltyrosylglycinamide apparently do not partition in the same way with $GlyNH_2$. In these experiments the products formed on the hydrolysis of the amide in the presence of the nucleophile were not directly assayed. The partition was found from the inhibition of the hydrolysis of benzoyltyrosylglycinamide by $GlyNH_2$ (Scheme II).

Evidence consistent with the acyl-enzyme mechanism is that the rate of release of *p*-nitroaniline from acetyl-DL-tyrosine-*p*-nitroanilide is unaffected by the addition of 1.6 M hydroxylamine although 50% acetyltyrosinehydroxamic acid is formed (Inagami and Sturtevant, 1964). This indicates that the hydroxylamine is involved after the rate-determining step and that 50% of the reaction must pass through an intermediate subsequent to this step.

We have attempted (a) to devise a system for directly analyzing the products on hydrolysis of substrates by chymotrypsin in the presence of nucleophiles in order to determine the product ratios accurately; (b) to find a sufficiently reactive nucleophile to show that in the hydrolysis of anilides an intermediate occurs after the rate-determining step and more than, say, 90% of the reaction goes through the intermediate; and (c) to show that the rate constants for amide hydrolysis give the correct equilibrium constant for the overall hydrolysis reaction when calculated on the acyl-enzyme scheme.

The following experiments are described. (1) [$^3$H]AcPhe-OMe was hydrolyzed by δ-chymotrypsin in the presence of various nucleophiles ($AlaNH_2$, $GlyNH_2$, and $NH_2NH_2$). When the AcPhe-OMe had completely reacted, subsequent reactions were quenched and the hydrolysis products, [$^3$H]-AcPhe and [$^3$H]AcPhe-nucleophile, were separated by ionophoresis and assayed. (2) [$^3$H]AcPhe-(*p*-trimethylammonium)-anilide and the (*p*-dimethylamino)anilide were hydrolyzed by δ-chymotrypsin in the presence of various nucleophiles. After about 5% of the substrate had reacted, further reaction was quenched. The products were separated from the substrate by ionophoresis and the [$^3$H]AcPhe and [$^3$H]AcPhe-nucleophile were assayed. In expt 1 and 2 the yields of reaction products and hence the partition ratios were *directly* determined. Rate measurements were not involved. (3) The partition of AcPhe-$AlaNH_2$ between Ala-$NH_2$ and water was determined *indirectly* by the inhibition of $k_{cat}/K_M$[1] for the δ-chymotrypsin catalyzed hydrolysis of AcPhe-$AlaNH_2$ by $AlaNH_2$. (4) AcPhe-δ-chymotrypsin was generated *in situ* in the stopped-flow spectrophotometer and its deacylation rate constants were determined in the absence and presence of the various nucleophiles used in the product ratio experiments. The partition of the acyl-enzyme between nucleophiles and water was obtained kinetically and compared with the product ratio expt, 1 and 2. (5) The effect of $AlaNH_2$, $GlyNH_2$, and $NH_2NH_2$ on the $k_{cat}$, $k_{cat}/K_M$, and $K_M$ for the δ-chymotrypsin-catalyzed release of aniline from AcPhe- and AcTyr-anilides was determined. (6) Formation constants for AcPhe-OMe from AcPhe and MeOH and for AcPhe-$AlaNH_2$ from AcPhe and $AlaNH_2$ were measured. From these values and the rate constants for the hydrolysis of AcPhe-OMe and the partition of AcPhe-chymotrypsin between nucleophiles and water $k_{cat}/K_M$ for the hydrolysis of AcPhe-$AlaNH_2$ by δ-chymotrypsin was calculated on the acyl-enzyme scheme and compared with the experimentally measured value.

[1] The parameters $k_{cat}$, $K_M$, and $V$ are defined by $V = k_{cat}[E]/(K_M + [S])$, where [E] and [S] are the enzyme and substrate concentrations; $\mu$ = ionic strength.

## Materials

*L-Alaninamide.* L-Alanine methyl ester was prepared by dissolving 30 g of L-alanine (0.33 mol) in 250 ml of dry methanol containing 42.8 g of thionyl chloride (0.36 mol) which had been prepared in a Dry Ice–isopropyl alcohol bath. After stirring for 15 min, the cold solution stood at room temperature for 1 hr. The solvent was removed below 40°. The ester hydrochloride was recrystallized from methanol after storage under vacuum over sodium hydroxide pellets: mp 108–111° (lit. (Zaoral *et al.*, 1967) mp 109–111°). The ester (30 g) was added at 0° to 250 ml of methanol saturated with ammonia in a pressure bottle. After standing for 2 hr at 0° and 24 hr at room temperature, the solvent was removed by evaporation. The amide was recrystallized from methanol–ether: mp 222–224° and $\alpha_D$ +10.6° (lit. (Beilstein) $\alpha_D$ +10.5°).

*Cbz-L-Phe-AlaNH$_2$* was prepared by adding 2.1 g of Cbz-Phe-*p*-ONph to a mixture of 0.65 g of L-$AlaNH_2 \cdot HCl$ and 0.55 g of triethylamine in 7 ml of dimethylformamide at 0° and leaving overnight in the cold. After precipitation by the addition of 50 ml of $H_2O$, the compound was washed with 2.5% $K_2CO_3$, dried, and crystallized from methanol: mp 194–200° (1.5 g).

*Acetyl-L-AlaNH$_2$.* The Cbz derivative (1.5 g) was debenzylated by dissolving in 10 ml of $HBr \cdot CH_3CO_2H$. After 30 min the Phe-$AlaNH_2 \cdot HBr$ was precipitated by ether. The hydrobromide was dissolved in chloroform by the addition of triethylamine and then acetylated with 1 g of acetic anhydride. The white precipitate was recrystallized from methanol (mp 242–244°).

*[$^3$H]Acetyl-L-Phe-AlaNH$_2$.* Cbz-L-$AlaNH_2$ (0.6 g) was debenzylated and the hydrobromide was collected as above. The free base was obtained by dissolving the hydrobromide in 5 ml of $H_2O$ and adding 2 N NaOH until pH 10. After extraction with ethyl acetate the product crystallized on the addition of petroleum ether (bp 40–60°). Phe-$AlaNH_2$ (0.2 g) was dissolved in 2 ml of chloroform containing 25 mCi of [$^3$H]acetic anhydride (500 mCi/mol). After 30 min 0.1 ml of (cold) acetic anhydride was added. The precipitate was recrystallized from methanol: mp 242–244° as above.

*[$^3$H]Ac-L-Phe-OMe.* Phe-OMe · HCl (2.3 g) was dissolved in 15 ml of water and the free base was extracted with 10 ml of ethyl acetate after the addition of 2.5 g of potassium carbonate. Nonradioactive material was prepared by adding 1 g of acetic anhydride to 5 ml of the ethyl acetate solution which had been dried over $CaCl_2$. On addition of petroleum ether at 50° and cooling, the ester crystallized (mp 89–90.5°, lit. (Huang *et al.*, 1952) mp 90–91°). The tritiated material was prepared by adding 25 mCi (25 mCi/mmol) of [$^3$H]acetic anhydride to 2 ml of the dried ethyl acetate extract and after 30 min 0.5 ml of acetic anhydride was added. The ester was crystallized as above: mp 89–90.5°.

*Cbz-L-Phe-(p-dimethylamino)anilide.* *p-N,N*-Dimethylaminoaniline was prepared from the dihydrochloride salt (recrystallized from ethanol–acetone) (2.75 g) by the addition of 27.5 ml of 1 N KOH followed by 30 ml of ethyl acetate. The organic layer was separated, the solvent was removed by evaporation, and the base was dried by the addition

of 30 ml of benzene followed by rotary evaporation. Cbz-L-phenylalanine *p*-nitrophenyl ester (3.3 g), dissolved in 50 ml of purified dimethylformamide (previously stirred under vacuum to remove dimethylamine) cooled to 0°, was added to the cooled dimethylaminoaniline and left overnight. Ethyl acetate (125 ml) was added, the solution was filtered, the precipitate was retained, and the solution was extracted several times with potassium carbonate solution when further product precipitated. The combined precipitates were washed with ethyl acetate and potassium carbonate, dried, and then recrystallized from acetone–petroleum ether (mp 187–189°). Further product was obtained by concentrating the ethyl acetate solution and extracting with 1 N HCl. The neutral compound was then precipitated from the aqueous solution by the addition of base to pH 6. The product was recrystallized as above.

L-*Phe-(p-dimethylamino)anilide* was prepared by debenzylation of the above in $HBr-CH_3CO_2H$. The ether precipitate was dissolved in water and the free base was liberated by the addition of base to pH 9.5 (mp 92–95°).

*Ac-L-Phe-(p-dimethylamino)anilide.* The above compound was dissolved in 15 ml of acetonitrile and 1.5 g of acetic anhydride and 1.5 ml of triethylamine were added. The product crystallized and after washing with water was recrystallized from ethyl acetate (mp 239–241°).

*AcPhe-(p-trimethylammonium)anilide Iodide.* The above (1.3 g) was dissolved in 13 ml of dimethylformamide (previously subjected to evacuation) and 1.5 g of methyl iodide was added. After 3 hr the product was precipitated by the addition of 90 ml of ether, then triturated with hot acetone, and crystallized from ethanol (mp 169–173°).

[$^3H$]*Ac-L-Phe-(p-trimethylammonium)anilide iodide* was prepared as above except 25 ml (25 Ci/mol) of [$^3$H]acetic anhydride was first added to 120 mg of L-Phe-(*p*-dimethylamino)-anilide and the acetic anhydride and triethylamine after a further 30 min. Methylation was as above.

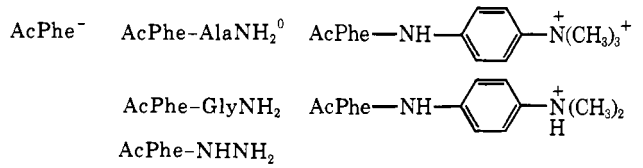
*Ac-L-Phe-p-ONph* was synthesized according to Ingles and Knowles (1968).

Satisfactory nuclear magnetic resonance (nmr) spectra and elemental analyses were obtained for the substrates. Optical purity was in all cases >98% as determined by assaying the product release on hydrolysis with chymotrypsin by the kinetic techniques described. Other materials were purified commercial products. The δ-chymotrypsin was obtained from Sigma (lot 20C-0150).

## Methods

### *Product Distribution on the Hydrolysis of Substrates by δ-Chymotrypsin in the Presence of Added Acceptor Nucleophiles*

*Preparation of Anilide Substrates.* Two new anilide substrates were prepared. One was positively charged, and the other could be protonated at low pH so that they could be separated from the negatively charged and neutral products by high-voltage electrophoresis.




HYDROLYSIS OF [$^3$H]AcPhe-OMe. To 0.96 ml of a solution containing carbonate buffer, nucleophile ($AlaNH_2$, $GlyNH_2$, or $H_2NNH_2$) at 25° and ionic strength 1.0 (added KCl) was added 10 μl of 1 mM δ-chymotrypsin, followed by 50 μl of 50 mM [$^3$H]AcPheOMe in $Me_2SO$. The pH was 9.30 ± 0.02. After 30 sec, sufficient time for the substrate to be completely hydrolyzed, the reaction was quenched by the addition of 200 μl of 50% trichloroacetic acid. The solution (10 μl) was spotted on to Whatman No. 3MM paper and the products, AcPhe and AcPhe-nucleophile, were separated by high-voltage electrophoresis. The systems used were either a 1% ammonium carbonate buffer (pH 8.9) or, more generally, pyridine acetate (pH 3.5) at 3 kV with white spirit as coolant or, as a check, pyridine acetate (pH 6.5) at 3 kV using the flat-bed method.

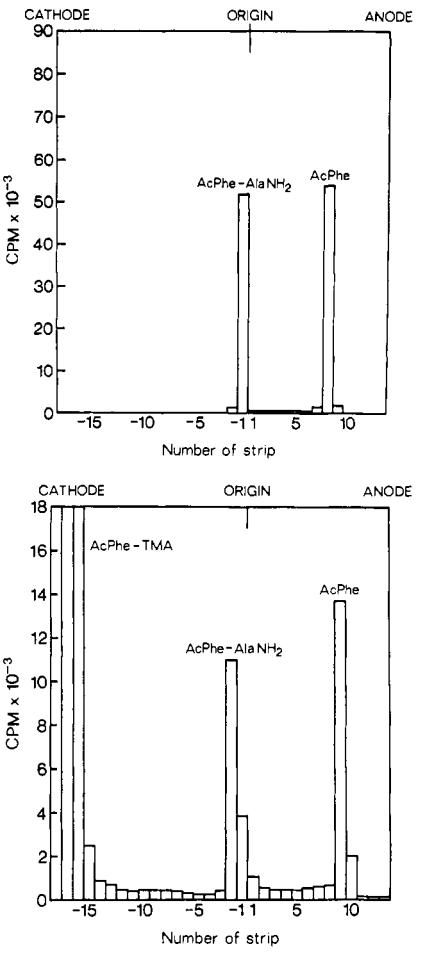



FIGURE 1: Separation of reagents and products by ionophoresis at pH 3.5. (a) Hydrolysis of [$^3$H]AcPhe-OMe in presence of $AlaNH_2$. (b) Hydrolysis of [$^3$H]AcPhe-(*p*-trimethylammonium)anilide in presence of $AlaNH_2$.

The paper was then cut into about 25 or 30 strips (1 × 3 cm) which were then soaked overnight in vials containing 4 ml of Bray's (1960) solution. The $^3$H content was then counted on a Nuclear-Chicago Unilux scintillation counter. The histogram thus obtained gave the yield of free acid and acylated nucleophile (see Figure 1). The recovery of products was quantitative.

HYDROLYSIS OF (*p*-TRIMETHYLAMMONIUM)ANILIDE. To 0.99 ml of a solution containing 50 μl of $Me_2SO$, 5 mM substrate, carbonate buffer, nucleophile, and KCl to give $\mu = 0.95$ and pH 9.30 at 25° was added 10 μl of 1 mM δ-chymotrypsin. After 3.0 min (time for ~5% of the substrate to be hydrolyzed), the reaction was quenched with 200 μl of 50% trichloroacetic acid. The initial products, [$^3$H]AcPhe$CO_2^-$ and [$^3$H]AcPhe-nucleophile$^0$, were separated from the positively charged sub-

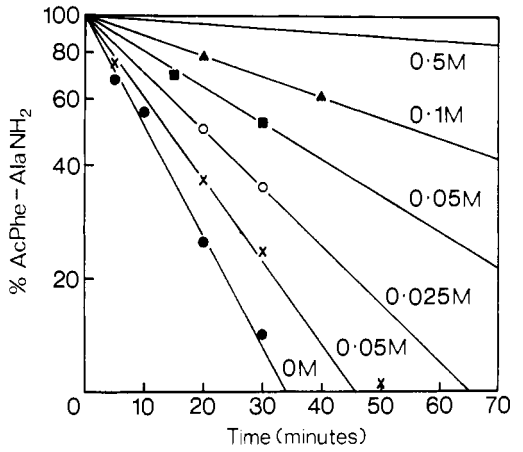



FIGURE 2: Inhibition by $AlaNH_2$ of the hydrolysis of AcPhe-$AlaNH_2$ by δ-chymotrypsin at 25°, $\mu$ = 0.95, pH 9.30. AcPhe-$AlaNH_2$ (2.5 mM) is at a concentration well below $K_M$. The concentration of $AlaNH_2$ is given in M.

strate by high-voltage electrophoresis as above using the pH 3.5 pyridine acetate system. The Whatman No. 3MM paper was cut into strips and counted as before (see Figure 1).

Some experiments were performed with [$^3$H]AcPhe-(*p*-dimethylamino)anilide as substrate. This is protonated under the conditions of the high-voltage electrophoresis and is also easily separated. The results are presented in Table I.

*Inhibition of the Hydrolysis of AcPhe-AlaNH₂ by AlaNH₂.*

$$\text{AcPhe-AlaNH}_2 + \text{chymotrypsin} \underset{k_4[\text{AlaNH}_2]}{\overset{k_2/K_S}{\rightleftharpoons}} \text{AcPhe-chymotrypsin} \xrightarrow{k_3} \text{AcPhe}$$

The hydrolysis of [$^3$H]AcPhe-$AlaNH_2$ by δ-chymotrypsin in the presence of varying concentrations of $AlaNH_2$ was determined for [S] $\ll K_M$. Under these conditions the disappearance of substrate should be pseudo first order in [S].

A solution containing 2.5 mM [$^3$H]AcPhe-$AlaNH_2$, pH 9.3 carbonate buffer, and various amounts of $AlaNH_2$ (made up to $\mu$ = 1.0) was incubated at 25° and 10 μl of $10^{-3}$ M δ-chymotrypsin was added. Aliquots of about 200 μl were periodically removed and added to 50 μl of 50% trichloroacetic acid; 10 μl of each quenched mixture was subjected to high-voltage electrophoresis as before and the ratio of [$^3$H]AcPhe:[$^3$H]AcPhe-Ala-$NH_2$ was determined. The initial slope of the semilogarithmic plot of substrate against time gave $V_{max}/K_M$. Enzyme autolysis ($t_{1/2} \sim 2$ hr) caused curvature in the plots for the slower reactions. The results are presented in Table II and Figure 2.

$AlaNH_2$ competes with water for the acyl-enzyme (or any other intermediate covalent or otherwise) and causes noncompetitive inhibition. The partition ratios may be calculated from the inhibition.

If $V_{max}/K_M$ in the presence of nucleophile is designated by the subscript "obsd" and in the absence of nucleophile by subscript "0," then

$$1 - \frac{[V_{max}/K_M]_{obsd}}{[V_{max}/K_M]_0} = \frac{k_4[\text{AlaNH}_2]}{k_3 + k_4[\text{AlaNH}_2]}$$

The results are presented in Tables I and II. (Note: these calculations assume that there is no effective competitive inhibition by the $AlaNH_2$. This is justified later.)

TABLE I: Hydrolysis of AcPhe-OMe, AcPhe-$AlaNH_2$, AcPhe-(*p*-trimethylammonium)anilide (AcPhe-TMA), and AcPhe-(*p*-dimethylamino)anilide (AcPhe-DMA) by δ-Chymotrypsin in the Presence of Acceptor Nucleophiles.[a]

| Nucleophile and Concn (M)[b] | % AcPhe-nucleophile Produced on Hydrolysis of | | | | |
|---|---|---|---|---|---|
| | AcPhe-OMe[c] | AcPhe-TMA[c] | AcPhe-Ala-$NH_2$[d] | AcPhe-DMA[c] | Calcd[e] |
| $AlaNH_2$ | | | | | |
| 0.010 | 24.3 | 28.4 | 26.3 | | 28.8 |
| 0.025 | 48.1 | 47.9 | 48.4 | | 50.4 |
| 0.05 | 69.0 | 67.0 | 67.3 | 63.2 | 66.9 |
| 0.10 | 81.8 | 80.3 | 81.6 | | 80.14 |
| 0.50 | | | 97.3 | | 95.3 |
| 0.84 | | >94 | | | 97.1 |
| $GlyNH_2$ | | | | | |
| 0.010 | | 9.0 | | | 9.6 |
| 0.025 | | 15.8 | | | 20.9 |
| 0.10 | 51.8 | 47.7 | | 46.6 | 51.4 |
| 0.25 | 75.5 | 73.9 | | | 72.6 |
| 0.50 | 86.0 | 83.9 | | | 84.1 |
| $H_2NNH_2$ | | | | | |
| 0.5 | 51.0 | 46.3 | | | 48.6 |
| 0.94 | 63.8 | | | | 64.0 |
| $AlaNH_2$[f] | | | | | |
| 0.05 (+α-chymotrypsin) | 71.3 | | | | |

[a] 25°, 5% $Me_2SO$, $\mu$ = 0.95. [b] Total ionic forms. [c] From direct radioactivity assays. [d] From inhibition of hydrolysis ($k_{cat}/K_M$) by $AlaNH_2$. No $Me_2SO$ present. [e] Calculated from mean values of partition (for free base) $AlaNH_2$–$H_2O$, 44:1; $GlyNH_2$–$H_2O$, 11.5:1; $H_2NNH_2$–$H_2O$, 2.04:1 $M^{-1}$. [f] Using α-chymotrypsin rather than δ-chymotrypsin.

*Kinetic Determination of Partition of AcPhe-Chymotrypsin between Nucleophiles and Water.* δ-Chymotrypsin and AcPhe-*p*-ONph were mixed in the stopped-flow spectrophotometer (Gilford 2400 spectrophotometer equipped with rapid mixer, dead time = 4 msec) under conditions that ensured complete acylation of the enzyme and *p*-nitrophenol release in the dead time of the mixer. The subsequent first-order deacylation of the enzyme was followed by monitoring proflavine binding at 465 nm (Bernhard *et al.*, 1966).

One syringe of the mixer contained $5 \times 10^{-5}$ M proflavine, $5 \times 10^{-6}$ M δ-chymotrypsin, pH 9.30 carbonate buffer, and nucleophile, at $\mu$ = 1.0. The other syringe contained $5 \times 10^{-5}$ M proflavine, $5 \times 10^{-5}$ M AcPhe-*p*-ONph, and 4% acetonitrile at pH 4.5. After mixing there was a first-order increase in absorbance at 465 nm as the enzyme deacylated. The results are presented in Table III.

## Calculation and Measurement of Equilibrium Constants

*Partition of AcPhe-Chymotrypsin between Methanol and Water From Michaelis–Menten Plots.* The values of $k_{cat}$ and $K_M$ for the release of *p*-nitrophenolate from AcPhe-*p*-ONph as it is hydrolyzed by δ-chymotrypsin (at pH 9.30, 25°, 5% $Me_2SO$) in varying concentrations of methanol were found from

TABLE II: Inhibition of AcPhe-$AlaNH_2$ Hydrolysis by $AlaNH_2$.[a]

| $AlaNH_2$ (M)[b] | $k_{obsd}$[c] ($sec^{-1} \times 10^4$) | $k_{cat}/K_M$[d] ($sec^{-1}\ M^{-1}$) |
|---|---|---|
| 0 | 11.2 | 132 |
| 0.01 | 8.25 | 97 |
| 0.025 | 5.78 | 68 |
| 0.05 | 3.66 | 43 |
| 0.10 | 2.06 | 24.2 |
| 0.50 | 0.304 | 3.6 |

[a] 25°, $\mu$ = 1.0, pH 9.30, $8.5 \times 10^{-6}$ M $\delta$-chymotrypsin, 2.5 mM substrate. [b] Total ionic forms. [c] First-order rate constant for hydrolysis. [d] $k_{obsd}$/[$\delta$-chymotrypsin].

stopped-flow experiments monitored at 400 nm. One syringe of the mixer contained enzyme (2.4 $\mu$M) in 5% $Me_2SO$, pH 9.30 carbonate buffer, and added KCl at $\mu$ = 0.95. The other held 0.95 M KCl, 5% $Me_2SO$, 110 $\mu$M AcPhe-*p*-ONph, and varying concentrations of methanol. Tangents were taken at various points along the curve of product against time and a reciprocal plot was obtained to give $V_{max}$ and $K_M$. In most cases $V_{max}$ only was obtained, by a small extrapolation.

In Scheme I, where X = *p*-nitrophenol and N = MeOH, $k_2 \gg k_3 + k_4$[MeOH], it is easily shown that

$$\frac{d[\text{p-nitrophenol}]}{dt} = \frac{k_3 + k_4[\text{MeOH}][E_0S_0]}{[S_0] + K_s(K_3 + k_4[\text{MeOH}])/k_2}$$

where $[S_0]$ = the initial substrate concentration and $[E_0]$ the total enzyme concentration.

A plot of $V_{max}$ against [MeOH] should give a slope of $k_4[E_0]$ and intercept $k_3[E_0]$ (*cf.* Bender *et al.*, 1964a).

*From Pseudo-First-Order Kinetics.* These experiments were similar to those for the kinetic determination of the partition of AcPhe-chymotrypsin between nucleophiles and water by the proflavine displacement method. The stopped-flow spectrophotometer for these experiments had a path length of 2 mM and dead time of 0.9 msec.

One syringe of the mixer contained $1.45 \times 10^{-4}$ M $\delta$-chymotrypsin, $10^{-4}$ M proflavine, pH 9.30 carbonate, 5% $Me_2SO$, and KCl to give $\mu$ = 0.95. The other syringe contained $3.5 \times 10^{-5}$ M AcPhe-*p*-ONph, 5% $Me_2SO$, 0.95 M KCl, and varying amounts of methanol; 4 msec after mixing there was a good first-order decrease in transmission at 465 nm as the enzyme deacylated. (Reacylation of the enzyme by the methyl ester produced was insignificant.) The results along with those from the zero-order rates are presented in Table IV.

*Formation Constants of AcPhe-$AlaNH_2$, AcPhe-$GlyNH_2$, and AcPhe-OMe*

*Equilibration between AcPhe and AcPhe-$AlaNH_2$ or AcPhe-$GlyNH_2$* at 25° and ionic strength 1.0 was catalyzed by chymotrypsin and the mixtures were assayed as follows.

HYDROLYSIS. To 1 ml of 1 M $AlaNH_2$ (or $GlyNH_2$) at pH 6.80 was added 10 $\mu$l of 0.5 M [$^3$H]AcPheOMe in acetonitrile followed by 10 $\mu$l of 1 mM $\alpha$-chymotrypsin. (The partition experiments show that an appreciable fraction of the ester is converted rapidly to the AcPhe-$AlaNH_2$ (or -$GlyNH_2$) which may then hydrolyze.) The mixture was then left for 36 hr to hydrolyze to equilibrium.

SYNTHESIS. A solution (0.5 ml) containing 10 $\mu$l of 1 mM $\delta$-chymotrypsin and 10 $\mu$l of [$^3$H]AcPhe-OMe (0.5 M in acetonitrile) was incubated for several minutes until hydrolysis was complete; then 0.5 ml of 2 M $AlaNH_2$ (or $GlyNH_2$) at pH 6.8 was added. The mixture was left for 36 hr to synthesize the peptide and equilibrate.

TABLE III: Deacylation of AcPhe-$\delta$-Chymotrypsin in the Presence of Nucleophiles.[a]

| Nucleophile | Concn[b] (M) | $k_{obsd}$[c] ($sec^{-1}$) | Partition[d] ($M^{-1}$) (Nucleophile:$H_2O$) | Partition[e] from Product Ratios ($M^{-1}$) |
|---|---|---|---|---|
| | | 142 ± 3 | | |
| $AlaNH_2$ | 0.02 | 232 ± 11 | 34 ± 4 | 44 |
| $GlyNH_2$ | 0.05 | 215 ± 7 | 11.1 ± 1 | |
| | 0.083 | 255 ± 11 | 10.4 ± 1 | 11.5 |
| $H_2NNH_2$ | 0.2 | 202 ± 4 | 2.3 ± 0.2 | 2.0 |

[a] Determined directly in stopped-flow spectrophotometer by proflavine displacement, 25°, $\mu$ = 1.0. [b] Total ionic forms. [c] Observed first-order rate constant. [d] $(k_{obsd} - 142)/([\text{nucleophile}] \times 142)$. [e] From the experiments involving $^3$H-labeled reagents.

The products were separated by high-voltage electrophoresis and the products were assayed in the scintillation counter.

*AcPhe-OMe.* Synthesis of AcPhe-OMe from AcPhe (initially 9.8 mM) and hydrolysis of AcPhe-OMe (initially 10.15 mM) catalyzed by 1 N HCl in 4.95 M methanol was followed to equilibrium ($t_{1/2}$ = 4.0 hr) by the alkaline hydroxamate assay. Samples (0.5 ml) were added to 1.0 ml of a freshly prepared mixture of 1.33 M hydroxylamine hydrochloride and 2.67 M NaOH. After 5.0 min 1.5 ml of 20% $FeCl_3 \cdot 6H_2O$ in 4 N HCl was added and the absorbance at 540 nm was measured after 12.0 min. A calibration curve was made using

TABLE IV: Hydrolysis and Methanolysis of AcPhe-*p*-nitrophenyl Ester.[a]

| [MeOH], M | Michaelis–Menten[b] Kinetics: $k_{cat}$ ($sec^{-1}$) | Michaelis–Menten[b] Kinetics: $K_M$ ($\mu$M) | First-Order Kinetics[c] of Deacylation: $k_3$ ($sec^{-1}$) |
|---|---|---|---|
| 0 | 144 | 3.16 | 144 |
| 0.062 | | | 148 |
| 0.123 | | | 164 |
| 0.25 | 231 | | 249 |
| 0.37 | | | 260 |
| 0.5 | | | 264 |
| 0.625 | 330 | | 329 |
| 0.75 | 368 | | |
| 0.94 | 416 | | |
| 1.25 | 482 | 12.6 | |

[a] pH 9.30, $\mu$ = 0.95, 5% $Me_2SO$, 25°. [b] From $1/V$ *vs.* 1/[S] plots, $[S_0]$ = 55 $\mu$M, $\delta$-chymotrypsin = 1.1 $\mu$M. [c] $\delta$-chymotrypsin = $9.8 \times 10^{-4}$ M.

TABLE V: Formation Constants for AcPhe-X at 25°, $\mu$ = 1.0.

| AcPhe-X | pH | Equilibrium Concn (Total Ionic Forms), M | | | Direction to Equilibrium of AcPhe-X | [AcPhe-X]/[AcPhe]$_{total}$-[XH]$_{total}$ (Total Ionic Forms) | [AcPhe-X]/[AcPhe][XH] (Un-ionized) |
|---|---|---|---|---|---|---|---|
| | | AcPhe | AcPhe-X | XH | | | |
| AcPhe-AlaNH$_2$ | 6.80 | $4.51 \times 10^{-3}$ | $4.85 \times 10^{-4}$ | 0.98 | Hydrolysis | 0.108 | $8.81 \times 10^{3}$ |
| | 6.80 | $4.50 \times 10^{-3}$ | $5.025 \times 10^{-4}$ | 0.98 | Synthesis | 0.114 | $9.25 \times 10^{3}$ |
| AcPhe-GlyNH$_2$ | 6.80 | $3.99 \times 10^{-3}$ | $1.01 \times 10^{-3}$ | 0.98 | Hydrolysis | 0.258 | $2.00 \times 10^{4}$ |
| | 6.80 | $3.00 \times 10^{-3}$ | $9.95 \times 10^{-4}$ | 0.98 | Synthesis | 0.254 | $1.97 \times 10^{4}$ |
| AcPhe-OMe | 0 | $5.38 \times 10^{-3}$ | $4.42 \times 10^{-3}$ | 4.95 | Hydrolysis | | 0.166 |
| | 0 | $5.66 \times 10^{-3}$ | $4.39 \times 10^{-3}$ | 4.95 | Synthesis | | 0.157 |

standard solutions of AcPhe-OMe. The *N*-acetyl group also slowly hydrolyzed. The end points were extrapolated back to zero time to give a small correction. The results are presented in Table V.

THE p$K_a$ VALUES of AlaNH$_2$, GlyNH$_2$, and AcPhe at 25° and $\mu$ = 1.0 were found by titrating 20 mM solutions of the amines (10 ml) and 10 mM of the acid (10 ml) in 1 M KCl with 1 N NaOH from a Burroughs Wellcome Agla syringe. Corrections were made for [H$^+$] in the case of the acid. The p$K_a$ values were as follows: for AlaNH$_2$, 8.24 ± 0.01; for GlyNH$_2$, 8.22 ± 0.01; and for AcPhe, 3.33 ± 0.02.

HYDROLYSIS OF AcPhe-OMe AND AcPhe-AlaNH$_2$. These reactions were carried out in 10-ml volumes in a Radiometer TTT II autotitrator in 0.95 M KCl and 5% Me$_2$SO, using $8 \times 10^{-9}$ M δ-chymotrypsin with 0.5–3.5 mM AcPhe-OMe, $4 \times 10^{-6}$ M δ-chymotrypsin and 1.0–8.0 mM AcPhe-AlaNH$_2$.

KINETICS OF ANILIDE HYDROLYSIS IN PRESENCE OF NUCLEOPHILES. The hydrolysis of AcPhe-(*p*-trimethylammonium)-anilide and AcTyr-*p*-chloroanilide were followed using a Gilford 2400 spectrophotometer. The reactions were at 25°, pH 9.30 (carbonate buffer), 5% Me$_2$SO and $\mu$ = 0.95, and various concentrations of AlaNH$_2$, GlyNH$_2$, and H$_2$NNH$_2$.

## Results

We have used δ-chymotrypsin in preference to α-chymotrypsin since (a) $K_M$ values do not increase as much at alkaline pH because more δ-chymotrypsin remains in the active conformation, (b) the fraction of inactive conformation is lower and this avoids artifacts in presteady-state studies (Fersht, 1972), and (c) we have found that for many substrates $K_M$ is smaller.

The titration of δ-chymotrypsin with Cbz-L-tyrosine *p*-nitrophenyl ester (Kézdy *et al.*, 1964) underestimated the active-site content. A value of 65% was found compared with 88% by the *trans*-cinnamoylimidazole method (Schonbaum *et al.*, 1961). (The δ-chymotrypsin in the paper of Fersht (1972) was also 88% active and not 65% as quoted.) A similar error was found with α-chymotrypsin which had been blocked on the surface carboxyls (Fersht and Sperling, 1973). Again, *trans*-cinnamoylimidazole and *p*-nitrophenyl acetate gave the correct values. The Cbz-tyrosine *p*-nitrophenyl ester method is reliable for α-chymotrypsin.

*Product Ratios.* We have introduced two new substrates which may be separated easily from their hydrolysis products in order to be able to directly analyze the products of the enzymatic hydrolysis reactions. It is seen in Table I that the product ratios on the simultaneous hydrolysis and acyltransfer reactions of AcPhe-OMe, AcPhe-(*p*-trimethylammonium)anilide and AcPhe-(*p*-dimethylamino)anilide are identical, within the limits of experimental error, for each set of experimental conditions. Furthermore, the effect of AlaNH$_2$ on the $k_{cat}/K_M$ for the hydrolysis of AcPhe-AlaNH$_2$ is also consistent with the partition ratios. AlaNH$_2$ is 43.6 ± 2.4 times more reactive than water in the reaction of δ-chymotrypsin with AcPhe-OMe, 44.1 ± 1.4 times more reactive for AcPhe-(*p*-trimethylammonium)anilide, and 43.1 ± 2.1 times more reactive for the inhibition of $k_{cat}/K_M$ for AcPhe-AlaNH$_2$ hydrolysis.

The mean values of all the partitioning experiments show that AlaNH$_2$ is 44 times, GlyNH$_2$ 11.5 times and hydrazine 2.0 times more reactive than water toward the acyl-enzyme, AcPhe-chymotrypsin. There is no systematic deviation from the calculated product ratios (Table I, last column) based on these values. There appears to be no saturation of a nucleophile binding site on the *acyl-enzyme*.

Direct measurements, although of somewhat lower accuracy, of the attack of the nucleophiles on the acyl-enzyme generated *in situ* by the acylation of the enzyme by AcPhe-*p*-nitrophenyl ester are consistent with the above (Table III).

It is seen in Table VI that the values of $k_{cat}$ for the release of aniline from AcPhe-*p*-(trimethylammonium)anilide and AcTyr-*p*-chloroanilide increase for increasing concentrations of AlaNH$_2$. GlyNH$_2$ and H$_2$NNH$_2$ also increase $k_{cat}$. Dimethylformamide, a secondary amide which is not a nucleophile, also increases $k_{cat}$ for several substrates of chymotrypsin and trypsin (Table VII). AlaNH$_2$ does not cause serious changes in $k_{cat}/K_M$ (Table VI) and it was felt justified to use this parameter in the calculations for the inhibition of AcPhe-AlaNH$_2$ hydrolysis by AlaNH$_2$.

The increase in $k_{cat}$ does not increase linearly with AlaNH$_2$ concentration. This could be due to binding to the *enzyme–substrate complex* with a dissociation constant of about 20 mM.

*Reaction of AcPhe-Chymotrypsin with methanol.* The attack of methanol on AcPhe-δ-chymotrypsin was initially measured under pseudo-first-order conditions. An excess of enzyme was mixed with the substrate to give rapid acylation and then the deacylation rate constant was measured directly. The results became erratic at higher methanol concentrations due to mixing artifacts in the stopped-flow spectrophotometer. Higher concentrations of methanol were possible under zero-order con-

TABLE VI: Hydrolysis of AcTyr-*p*-chloroanilide and AcPhe-(*p*-trimethylammonium)anilide in the Presence of Nucleophiles.[a]

| Substrate | Nucleophile and Concn (M[b]) | $k_{cat}$ ($sec^{-1}$) | $K_M$ (mM) | $k_{cat}/K_M$ ($sec^{-1}$ $M^{-1}$) | % Increase in $k_{cat}$ |
|---|---|---|---|---|---|
| AcTyr-*p*-chloroanilide[c] | $AlaNH_2$, 0 | 0.0308 | 0.35 | 88 | 0 |
| | $AlaNH_2$, 0.033 | 0.0382 | 0.419 | 91.1 | 24 |
| | $AlaNH_2$, 0.10 | 0.0405 | 0.446 | 90.8 | 31 |
| | $AlaNH_2$, 0.5 | 0.0422 | 0.45 | 93.6 | 37 |
| AcPhe-TMA[d] | $AlaNH_2$, 0 | 0.403 | 4.3 | 94.2 | 0 |
| | $AlaNH_2$, 0.033 | 0.504 | 4.7 | 107 | 24 |
| | $AlaNH_2$, 0.10 | 0.523 | 5.37 | 97.4 | 29 |
| | $AlaNH_2$, 0.50 | 0.557 | 7.3 | 76.1 | 38 |
| | $GlyNH_2$, 0.5 | 0.642 | 7.7 | 81.9 | 58 |
| | $H_2NNH_2$, 0.5 | 0.543 | 4.72 | 115 | 35 |

[a] 25°, $\mu$ = 0.95, 5% $Me_2SO$, pH 9.30. [b] Total ionic forms. [c] Monitored at 300 nm, $\Delta\epsilon = 1.03 \times 10^3$. [d] Monitored at 295 nm, $\Delta\epsilon = 1.12 \times 10^3$.

ditions of excess substrate over enzyme and at concentrations much higher than $K_M$. The $V_{max}$ values were converted to $k_{cat}$ values by taking $V_{max}$ in the absence of methanol to be 144 $sec^{-1}$, as found from the first-order kinetics.

Total hydrolysis of the substrate occurred in less than 800 msec. This is fast compared with the conformational equilibration of the enzyme (Fersht, 1972). The percentage of the enzyme in the active conformation may be calculated from the ratio of the observed $V_{max}$/[chymotrypsin] (100 $sec^{-1}$) and the known $k_{cat}$ (144 $sec^{-1}$) (Fersht, 1972) and is 69% at pH 9.30, 25°, $\mu$ = 0.95, 5% $Me_2SO$.

Methanol is 1.9 ± 0.2 times more reactive than water towards AcPhe-chymotrypsin. A similar result was obtained by Bender *et al.* (1964a) for $\alpha$-chymotrypsin.

*Hydrolysis of AcPhe-OMe and AcPhe-AlaNH₂.* These hydrolyses were performed on the same day using the same stock solutions of enzyme to minimize errors in the ratios of the values of $k_{cat}/K_M$. AcPhe-$AlaNH_2$ was hydrolyzed at pH 9.30, 5% $Me_2SO$, 25°, and 0.95 M KCl with $k_{cat}/K_M$ = 126 $M^{-1}$ $sec^{-1}$. Approximate values for $k_{cat}$ and $K_M$ are 5 $sec^{-1}$ and 40 mM. Under the same conditions $k_{cat}$, $K_M$, and $k_{cat}/K_M$ for AcPhe-OMe are 135 $sec^{-1}$, 0.54 mM, and 2.5 × $10^5$ $M^{-1}$ $sec^{-1}$. These data may be tentatively analyzed, using the notation of Scheme I, and assuming $k_3$ to be 144 $sec^{-1}$, to give $k_2 \sim 2.2 \times 10^3$ $sec^{-1}$ and $K_S \sim 9$ mM.

*Free-Energy Diagrams.* Sufficient data have been derived to plot free energy against reaction coordinate diagrams (Figures 3 and 4).[2] The standard states are 1 M for all reagents except water which is assigned an activity of 1.0. The dissociation constant for AcPhe and chymotrypsin is estimated to be ~10 mM. The rate constants for the association of substrates with chymotrypsin is taken to be ~$10^7$ $sec^{-1}$ $M^{-1}$ (Hess *et al.*, 1970).

## Discussion

Acyl-chymotrypsins have been synthesized and shown to react with amines. Inward and Jencks (1965) isolated the relatively stable furoyl-chymotrypsin and determined its reactivity with a wide range of amines. We prepared AcPhe-chymotrypsin *in situ* and observed its reaction with $AlaNH_2$, $GlyNH_2$, and $H_2NNH_2$. It may be inferred from the principle of microscopic reversibility that the reverse reaction, the acylation of chymotrypsin by amides to give the acyl-enzyme, does occur. The question is, then, is acyl-enzyme formation the main route for amide hydrolysis or is it a side reaction?

A method of solving this problem is (a) to demonstrate the acyl-enzyme mechanism for one substrate where it accumulates and then to apply the following criteria to test whether it occurs in the other cases where it does not accumulate. These criteria are (b) "a common acyl portion should generate a common intermediate which should then partition in an identical manner in the presence of added acceptors"; (c) "the rate constants calculated on the acyl-enzyme mechanism must satisfy the overall equilibrium constant for the reaction"; and (d) "the acyl-enzyme occurs after the rate-determining step for the slower reactions and so the addition of nucleophiles should not affect the rate of disappearance of substrate."

In ideal situations, b, c, and d will hold. Criterion c is both

TABLE VII: Effect of Dimethylformamide on $k_{cat}$ for Some Reactions of Chymotrypsin and Trypsin.

| Enzyme | Substrate | [DMF], M | $k_{cat}$ ($sec^{-1}$ × $10^2$) |
|---|---|---|---|
| $\alpha$-Chymotrypsin | AcTyr-*p*-chloroanilide[a] | 0.32 | 1.19 |
| | | 0.65 | 1.37 |
| | AcTyr-*m*-chloroanilide[a] | 0.32 | 0.93 |
| | | 0.65 | 1.11 |
| | | 1.04 | 1.36 |
| | FormylPhe-semicarbazide[b] | 0 | 2.88 |
| | | 0.65 | 3.25 |
| Trypsin | CbzArg-*p*-toluidide[c] | 0.26 | 0.43 |
| | | 0.65 | 0.71 |
| | | 1.30 | 0.97 |

[a] Inagami *et al.* (1969). [b] Fastrez and Fersht (1973). [c] Inagami (1969).

[2] ADDED IN PROOF. The rate constant for the diffusion-controlled association of AcPhe derivatives with δ-chymotrypsin has now been measured (Renard and Fersht, unpublished results). The barrier height is 6.4 kcal $M^{-1}$, not the estimated value of 8.0 in the figures.

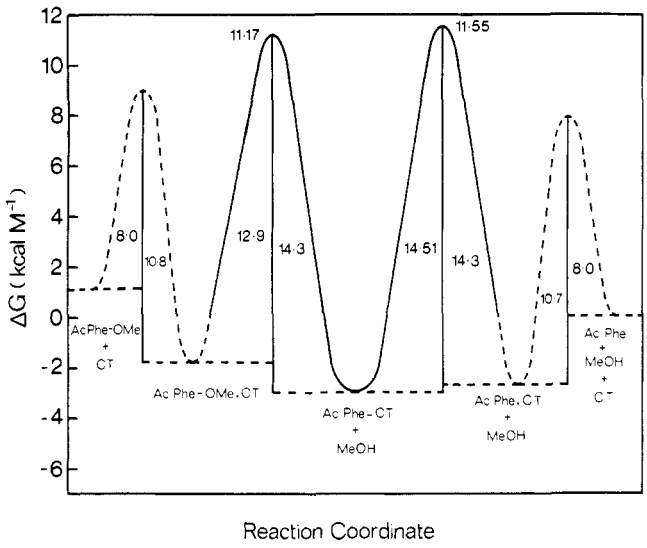



FIGURE 3: Free energy against reaction coordinate for the hydrolysis of AcPhe-OMe by δ-chymotrypsin at pH 9.30, 25°, ionic strength 0.95, 5% $Me_2SO$. Standard states are 1 M, activity of water is 1. AchPhe is un-ionized.[2]

necessary and sufficient. But artifacts could occur in the measurement of the required individual rate constants. The determination of $k_{-2}$ requires the presence of an added nucleophile and this could perturb the enzyme. Similarly, different enzyme concentrations may be required for each $k_2/K_S$. Criterion b is not essential. It is possible that the deacylation of the acyl-enzyme may occur while the leaving group is still bound to or slowly diffusing away from the acyl-enzyme and so causing steric or allosteric effects. Criterion d is also not essential. An added nucleophile could act as an allosteric effector or inhibitor and alter the $V_{max}$.

We shall now apply the above steps, a, b, c, and d.

*Acyl-Enzyme Formation.* Bender and coworkers have amply demonstrated that AcPhe-OMe and other ester derivatives hydrolyze by acyl-enzyme formation (Bender and Kézdy, 1964). McConn *et al.* (1971) have shown the "burst" of acyl-enzyme formation from AcPhe-OEt and chymotrypsin and the subsequent deacylation by presteady-state kinetics. We have shown that AcPhe-chymotrypsin generated from AcPhe-*p*-ONph deacylates with a rate constant of 144 $sec^{-1}$, while AcPhe-OMe is hydrolyzed with a $k_{cat}$ value of 135 $sec^{-1}$. This is consistent with the acyl-enzyme being formed with $k_2 = 2.2 \times 10^3$ $sec^{-1}$ and $k_3$ 144 $sec^{-1}$ (Scheme I). (We shall later argue the uniqueness of the acyl-enzyme mechanism.)

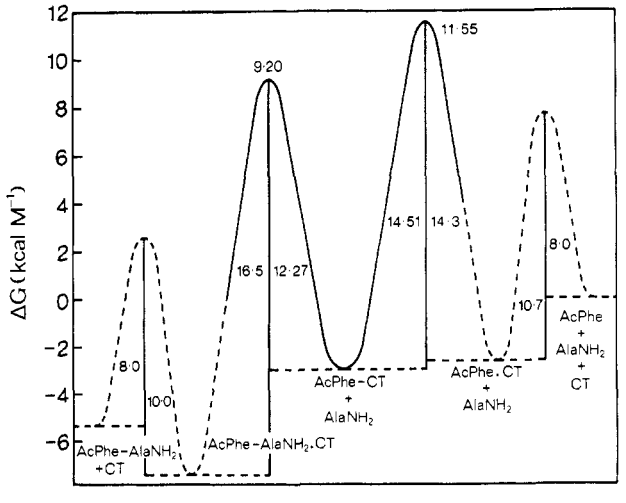



FIGURE 4: Free energy against reaction coordinate for the hydrolysis of $AcPhe\text{-}AlaNH_2$ by δ-chymotrypsin at pH 9.30, 25°, ionic strength 0.95, 5% $Me_2SO$. Standard states are 1 M, activity of water is 1. AcPhe and $AlaNH_2$ are taken as un-ionized. Under normal circumstances both are ionized and the free energy of the products is −6.7 kcal rather than zero as in the figure.[2]

*Product Distribution.* Direct analysis of the products from the hydrolysis of AcPhe-OMe, AcPhe-(*p*-trimethylammonium)-anilide and, where tested, AcPhe-(*p*-dimethylamino)anilide by δ-chymotrypsin in the presence of $AlaNH_2$, $GlyNH_2$, and hydrazine shows that the esters and anilides partition in an identical manner for each particular nucleophile. The inhibition of $k_{cat}/K_M$ for $AcPhe\text{-}AlaNH_2$ by $AlaNH_2$ is consistent with this substrate partitioning identically to the ester and anilides.

The product distribution is consistent with the acyl-enzyme mechanism for esters, anilides, and peptides.

$$\text{AcPhe-OMe} + \text{chymotrypsin} \overset{K_S}{\rightleftharpoons} \text{AcPhe-OMe·chymotrypsin} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{AcPhe-chymotrypsin} - \text{MeOH}$$

$$\text{AcPhe-chymotrypsin} \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} \text{AcPhe·chymotrypsin} \overset{K_P}{\rightleftharpoons} \text{AcPhe} + \text{chymotrypsin}$$

*Relationship between Equilibrium Constants and Rate Constants.* The Haldane (1930) relationship applied to Scheme III

SCHEME III

$$\text{AcPhe-AlaNH}_2 + \text{chymotrypsin} \overset{K_S}{\rightleftharpoons} \text{AcPhe-AlaNH}_2\text{·chymotrypsin} \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{AcPhe-chymotrypsin} + \text{AlaNH}_2 \overset{k_3}{\underset{k_{-3}}{\rightleftharpoons}} \text{AcPhe·chymotrypsin} \overset{K_P}{\rightleftharpoons} \text{AcPhe} + \text{chymotrypsin}$$

gives for the formation constants for AcPhe-OMe ($K_{OMe}$) and for $AcPhe\text{-}AlaNH_2$ ($K_{Ala}$)

$$K_{OMe} = \frac{k_{-3}}{K_P}\frac{k_{-2}}{k_3}\frac{K_S}{k_2} = \frac{[\text{AcPhe-OMe}]}{[\text{AcPhe}][\text{MeOH}]} \quad (1)$$

$$K_{Ala} = \frac{k_{-3}}{K_P}\frac{k_{-2}}{k_3}\frac{K_S}{k_2} = \frac{[\text{AcPhe-AlaNH}_2]}{[\text{AcPhe}][\text{AlaNH}_2]} \quad (2)$$

$K_{OMe}$, $k_3$, $k_{-2}/k_3$, and $k_2/K_S$ ($\equiv k_{cat}/K_M$) have been measured in this study at pH 9.30, etc. Using the un-ionized forms of AcPhe, $AlaNH_2$, etc., and the values in Table VIII we have for AcPhe-OMe (eq 1)

$$0.162 = \frac{k_{-3}}{K_P}\frac{274}{144}\frac{1}{2.5 \times 10^5}$$

hence $k_{-3}/K_P = 2.13 \times 10^4$ $M^{-1}$ $sec^{-1}$. From this we may calculate $K_{Ala}$ using the observed values of $K_S/k_2$ $k_{-2}$, $k_3$, and the calculated $k_{-3}/K_P$.

$$K_{Ala} = 2.13 \times 10^4 \times \frac{44 \times 144}{144} \frac{1}{126} M^{-1} = 7.4 \times 10^3 \text{ M}^{-1}$$

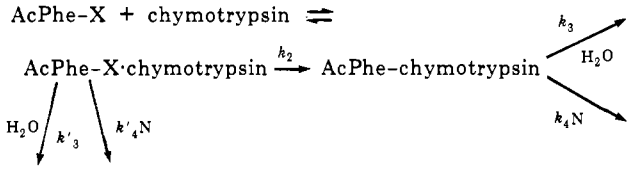
This is in excellent agreement with the observed value of $9.0 \times 10^3$ M$^{-1}$. This is proof of the acyl-enzyme mechanism being the major pathway for the reaction of chymotrypsin with amides. If the observed value of $k_2/K_S$ for the AcPhe-$AlaNH_2$ hydrolysis were due to another pathway, this would have to be larger than that for the acyl-enzyme mechanism and then the calculated value for $K_{Ala}$ would be much smaller than that observed.

Alternatively, as we know all the rate constants for the formation of AcPhe-$AlaNH_2$ by the acyl-enzyme route ($k_{-3}/K_P$ from the AcPhe-OMe reaction, $k_{-2}$ from the partition of AcPhe-CT between $AlaNH_2$ and $H_2O$; and $k_3$ from AcPhe-chymotrypsin deacylation), we can calculate $k_2/K_S$ for the acyl-enzyme route. This is found to be 152 M$^{-1}$ sec$^{-1}$.

This calculation involves five experimental terms each of an accuracy of ±5–10%. The calculated value is in the range of 152 ± 50 M$^{-1}$ sec$^{-1}$. This compares well with the observed value of 126 M$^{-1}$ sec$^{-1}$ for the $k_{cat}/K_M$ ($\equiv k_2/K_S$) for the hydrolysis of AcPhe-$AlaNH_2$.

We have shown so far that the acyl-enzyme pathway is the major route for amide hydrolysis. The next question is whether there is a significant contribution from a second mechanism of direct nucleophilic attack on the noncovalently bound enzyme–substrate complex (Scheme IV). It was hoped to put a

SCHEME IV




maximum limit on the contribution of a competing pathway by repetition of the Inagami and Sturtevant (1964) experiments using a more reactive nucleophile than hydroxylamine. Unfortunately, there are complications in that the values of $k_{cat}$ for the anilide reactions increase slightly on the addition of nucleophiles. The nucleophiles should not be involved in the reaction until after the rate-determining step.

*Effect of Nucleophiles on $k_{cat}$ for Anilide Hydrolysis.* $AlaNH_2$, $GlyNH_2$, and hydrazine increase $k_{cat}$ for the hydrolysis of anilide substrates (Table VI). We feel that this is not due to the direct chemical participation of the nucleophiles in the rate-determining step of the reaction but is a consequence of alterations in enzyme–substrate interactions. Dimethylformamide, a secondary amide which is not a nucleophile, also causes similar increases in $k_{cat}$ for the reaction of chymotrypsin and trypsin with amides (Table VII).

The direct attack of nucleophiles on the noncovalently bound enzyme–substrate as given in Scheme IV is unlikely for the following reasons. The inhibition of the hydrolysis of AcPhe-$AlaNH_2$ by $AlaNH_2$ can be only after the acyl-enzyme at the $k_3$ and $k_4$ steps unless the $AlaNH_2$ binds to the enzyme–substrate complex and sterically inhibits the attack of water ($k'_3$). If $AlaNH_2$ does not bind the $k'_3$ route occurs independently of the concentration of $AlaNH_2$. Yet at 0.5 M $AlaNH_2$ the hydrolysis is inhibited by 97.5% (see Table II). This limits $k'_3$ to being no more than ~2.5% of $k_3$. The alternative is that nucleophiles such as $AlaNH_2$ may bind to the noncovalent complex and increase the rate by the $k'_4$ route. This is

TABLE VIII: Rate and Equilibrium Constants Used for Application of (A) the Haldane Equation (Scheme III) and (B) Calculation of Free-Energy Diagrams.

| AcPhe-OMe | AcPhe-$AlaNH_2$ |
|---|---|
| Part A | |
| $k_2/K_S = 2.5 \times 10^5$ M$^{-1}$ sec$^{-1}$ | $k'_2/K'_S = 126$ M$^{-1}$ sec$^{-1}$ |
| $k_{-2} = 274$ M$^{-1}$ sec$^{-1}$ | $k'_{-2} = 6340$ M$^{-1}$ sec$^{-1}$ |
| $k_3 = 144$ sec$^{-1}$ | |
| $k_{-3}/K_P$[a] $= 2.13 \times 10^4$ M$^{-1}$ sec$^{-1}$ | |
| Part B | |
| $k_2$[b] $= 2.2 \times 10^3$ sec$^{-1}$ | $k'_2$[d] $= 5$ sec$^{-1}$ |
| $K_S$[b] $= 9 \times 10^{-3}$ M | $K'_S$[d] $= 4 \times 10^{-2}$ M |
| $k_{-3}$[c] $= 2 \times 10^2$ sec$^{-1}$ | |
| $K_P$[c] $= 1 \times 10^{-2}$ M | |

[a] Calculated from the overall equilibrium constant, etc. (see text). [b] Estimated from $k_{cat}$ and $k_3$. [c] Estimated by assuming $K_P \sim 10$ mM. [d] Approximate values.

inconsistent with the observed product distribution. If there is a second pathway for the formation of AcPhe-nucleophile then there will be an increase in the partition. For example, $k_{cat}$ for the disappearance of AcPhe-(*p*-trimethylammonium)anilide is increased by 58% in the presence of 0.5 M $GlyNH_2$. The acyl-enzyme route gives 84% AcPhe-$GlyNH_2$. The total percentage of AcPheGly$NH_2$ formed by both routes should then be (84 + 58)/158 × 100%, *i.e.*, 90%. Similarly $k_{cat}$ is increased by 35% in the presence of 0.5 M hydrazine. The product ratio should then increase from 48.6% for the acyl-enzyme mechanism to (48.6 + 35)/135 × 100%, *i.e.*, 62%. Similar calculations may be made for the partitioning with $AlaNH_2$. These increases would have been easily detected. This alternative is ruled out. (It is also unlikely that the noncovalent enzyme–*ester* complexes are susceptible to attack by external nucleophiles. The enzyme–substrate complex is at a much lower concentration than the acyl-enzyme and a significant rate of attack by water would increase the $K_M$. The $k_{cat}$ for the hydrolysis of AcPhe-OMe is 135 sec$^{-1}$ and is consistent with a $k_3$ of 144 sec$^{-1}$, as found directly from the deacylation of AcPhe-CT, and a $k_2$ of ~$2 \times 10^3$ sec$^{-1}$. An alternative route would increase $k_{cat}$. Similarly the direct attack of $AlaNH_2$, $AlaNH_2$, and $H_2NNH_2$ on AcPhe-CT is consistent with the product distribution on hydrolysis of AcPhe-OMe.)

The increase in $k_{cat}$ on the addition of nucleophiles does not appear to be due to an alternative pathway. This increase could come from the nucleophile binding to the enzyme–substrate complex and either decreasing nonproductive binding of the substrate or causing strain. Both effects would cause an increase in $k_{cat}$ *and* $K_M$ as found.

This stimulation of $k_{cat}$ invalidates the method of determining product ratios using the inhibition of $V_{max}$ on the addition of leaving group amine. For example, $V_{max}$ for the hydrolysis of AcPhe-$GlyNH_2$ in the presence of 0.5 M $GlyNH_2$ is decreased by 84% due to reaction of the acyl-enzyme with $GlyNH_2$ but is stimulated by 58% due to the nonspecific effect. The net result is a 75% decrease only. The reactivity of $GlyNH_2$ relative to water is then calculated to be 6:1 instead of 11.5:1. This is the probable cause of the anomaly in Epand's (1969) experiment; 0.10–0.20 M $GlyNH_2$ did not inhibit the hydrolysis of benzoyl-L-tyrosylglycinamide as much as ex-

pected from the product ratios measured directly from benzoyltyrosine ethyl ester hydrolysis in the presence of $GlyNH_2$. The failure to observe a leveling off of the rate of the enzymatic synthesis of acetyltyrosinehydroxamate from acetyltyrosine with increasing concentration of hydroxylamine (Caplow and Jencks, 1964) might also be accounted for by a similar effect. In this case the initial formation of AcTyr-$ONH_2$ followed by the hydroxylamine competing with the enzyme for this could also be a factor.

The observation that greater than 94% of the AcPhe-(*p*-trimethylammonium)anilide reacting with δ-chymotrypsin in the presence of 0.84 M $AlaNH_2$ is converted to AcPhe-$AlaNH_2$ in a step subsequent to the acylation implies that more than 94% of the reaction occurs by the acyl-enzyme mechanism.

In summary, the evidence for the acyl-enzyme mechanism for amides is: (a) the acyl-enzyme generated from ester substrates partitions in an identical manner between nucleophiles and water as the intermediate which occurs in amide hydrolysis; (b) this intermediate occurs after the rate-determining step and accounts for more than 94% of the total reaction; (c) the rate constant for $k_2/K_S$ for the hydrolysis of AcPhe-$AlaNH_2$ is, within experimental error, the same as that calculated on the acyl-enzyme hypothesis using the overall equilibrium constant of the reaction and the experimental values for the rate constants for the synthesis of AcPhe-$AlaNH_2$ from AcPhe and $AlaNH_2$ by the acyl-enzyme route.

The acyl-enzyme mechanism is expected to have an entropic advantage over a reaction which involves the direct attack of water on the enzyme-bound substrate. Jencks and Page (1972) suggest this is a significant effect.

A further advantage is that the nucleophilic –OH of Ser-195 is held rigidly as part of the "charge–relay system" (Blow *et al.*, 1969). The stereochemical orientation is possibly more easily controlled than that of a bound water molecule. Effects such as "strain" require rigid active sites.

*Free-Energy Diagrams.* The low reactivity of AcPhe-$AlaNH_2$ is due to its thermodynamic stability. The transition state for the acylation of the enzyme by AcPhe-$AlaNH_2$ has a free energy of 9.2 kcal, 2 kcal lower than that involving AcPhe-OMe. But the ground state of the peptide is 6.5 kcal more stable than the ester. The acyl-enzyme is about a kcal more stable than the noncovalent AcPhe-OMe·chymotrypsin complex but is some 4.6 kcal less stable than AcPhe-$AlaNH_2$·chymotrypsin. These results may be compared with those for acetyltryptophan ethyl ester, acetyltryptophanamide and acetyltyrosinehydroxamic acid (Bender *et al.*, 1964b; Rajender *et al.*, 1970; Epand and Wilson, 1964). In the diagram given for AcPhe-$AlaNH_2$ the apparent rate-determining step is deacylation. This is because the reaction conditions are defined for 1 M $AlaNH_2$. At low concentrations of $AlaNH_2$ the rate-determining step is, of course, acylation. Similarly, the equilibrium between the Michaelis complex and the acyl-enzyme is a function of concentration. The rate-determining step in the hydrolysis of AcPhe-OMe changes from deacylation to acylation as the substrate concentration decreases to below the $K_M$.

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