Correction. In the article “Acylation of α-Chymotrypsin by Oxygen and Sulfur Esters of Specific Substrates: Kinetic Evidence for a Tetrahedral Intermediate” by Hideo Hirohara, Myron L. Bender, and Richard S. Stark, which appeared in the May 1974 issue of the Proc. Nat. Acad. Sci. USA, 71, 1643–1647, an error was made on page 1645 in Table 2. In the third column, k4, the sixth entry should be 2250 ± 240, not 225 ± 240.

Correction. In the article “Triplet-Triplet Energy Transfer in α-Trypsin” by C. A. Ghiron, J. W. Longworth, and N. Ramachandran, which appeared in Part II of the December 1973 issue of the Proc. Nat. Acad. Sci. USA 70, 3703–3706, the authors request that the following changes be made. On p. 3705, in the left-hand column the fifth sentence of the second paragraph should read, “The time dependence of the phosphorescence emission at 408 nm is resolved into two exponential components, 74% with a lifetime of 1.4 sec, and 26% with a lifetime of 5.9 sec (see Table 1).” In Table 1, on the same page, lines 5 and 6 of the column headed “Lifetime (sec)” should read, Tyr = 1.4(0.74) and Trp = 5.9(0.26).

Correction. In “The Isolation of Simian Virus 40 Variants with Specifically Altered Genomes” by William W. Brockman and Daniel Nathans, appearing in the March 1974 issue of Proc. Nat. Acad. Sci. USA, 71, 942–946, there is a misprint on p. 942 in the paragraph entitled Complementation Plaquing for the Cloning of Possible Deletion Mutants. Beginning at the end of line 11 the sentence reading “After incubation at 37° for 3–6 hr, approximately 10⁴ freshly suspended ...” should read “After incubation at 37° for 3–6 hr, approximately 10⁶ freshly suspended BSC-40 cells in 1.5 ml of medium were added to each dish and immediately dispersed.”
Acylation of α-Chymotrypsin by Oxygen and Sulfur Esters of Specific Substrates: Kinetic Evidence for a Tetrahedral Intermediate

(enzyme/presteady state/rate constant/binding constant/leaving-group effect)

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Contributed by Myron L. Bender, February 4, 1974

ABSTRACT

The acylation step of the α-chymotrypsin-catalyzed hydrolysis of N-acetyl-L-(or DL)-tryptophan p-nitrophenyl, p-nitrothiophenyl, ethyl, and thiolethyl esters has been studied by the stopped-flow technique at 25°C. The acylation rate constant, k2, and the enzyme substrate dissociation constant, Kd, were directly determined at pH 4, 5, and 8. Steady-state kinetics were studied at pH 7. The k2 values are nearly identical for oxygen esters and their sulfur counterparts, whereas the Kd value of the ethyl ester is larger by an order of magnitude than those of the other three. The results strongly suggest that oxygen and thiol esters of these specific substrates are hydrolyzed via the same pathway, and furthermore that acylation consists of more than one step; the formation and breakdown of a tetrahedral intermediate, the former being rate-determining. Effects of leaving-group hydrophobicity on k2 and Kd are also discussed.

Whether or not a tetrahedral intermediate is formed in the reaction pathway of α-chymotrypsin-catalyzed hydrolysis has been a subject of great interest. Some of the first evidence for such intermediates in nonenzymatic acyl-transfer reactions came from the study of the alkaline hydrolysis of esters labeled specifically in the carbonyl group with 18O (1). The occurrence of a similar intermediate in the α-chymotrypsin-catalyzed hydrolysis of some anilides and hydrazides has been recently proposed from kinetic studies by several research groups (2–9). An important contribution to this problem on (non-specific) ester substrates has been made by Frankfater and Keddy (10), who obtained identical rate constants for the α-chymotrypsin-catalyzed hydrolysis of p-nitrophenyl acetate and thiocetate. Since an —SR group was expected to be a much better leaving group than an —OR group, it was argued that the bond-breaking process could not occur in the rate-determining step. Studies of the breakdown of similar tetrahedral complexes as hemithioacetals and acetaldheyde hydrate (29) and ketene O,S acetals (43) have demonstrated the leaving superiority of —SR over —OR. Because there is no observable accumulation of a tetrahedral intermediate, a rapid decomposition step must follow a slower addition step. Such a reaction scheme is identical to that postulated for the alkaline hydrolysis of oxygen and thiol esters (10).

The purpose of this investigation is to determine whether such results are applicable to the acylation of α-chymotrypsin by specific ester substrates. The acylation rate constant, k2, and the enzyme-substrate complex dissociation constant, Kd, [k2 = k−1/kd, Eq. 4], for N-acetyl-tryptophan esters were directly measured at pH 4.0, 5.0, and 8.0 and 25°C in this investigation. These constants for specific ester substrates have not previously been determined under conditions close to their physiological environment, although some data for alkyl esters at acidic pH levels has been reported by Hess et al. (11, 12). Therefore, it is expected that the kinetic parameters themselves obtained here will provide further knowledge about the individual steps in the α-chymotrypsin-catalyzed reaction.

MATERIALS AND METHODS

α-Chymotrypsin (three times crystallized) was a Worthington Biochemical Corp. product. Its concentration was determined by active site titration using p-nitrophenyl p′-guanidinobenzoate (13).

N-Acetyl-L-tryptophan p-nitrophenyl ester (AcTrpONp) was obtained from Cyclo Chemical Co., mp 131–132°C.

N-Acetyl-L-tryptophan ethyl ester (Cyclo) (AcTrpOEt) was recrystallized from dry ethyl acetate-ether as described previously (14), mp 105–106°C.

N-Acetyl-DL-tryptophan p-nitrothiophenyl ester (AcTrpSNp) was prepared from N-acetyl-L-tryptophan (Sigma) and p-nitrothiophenol with dicyclohexylcarbodiimide (DCC) in acetonitrile at room temperature. The urea was filtered and the solvent removed in vacuo at room temperature. The residue was repeatedly recrystallized from acetone and anhydrous ether at 4°C, mp 195–197°C. The product was determined to be 61% racemate (with the L form predominating) from its behavior with α-chymotrypsin.

N-Acetyl-L-tryptophan thiolethyl ester (AcTrpSEt) was also prepared with DCC in acetonitrile according to the method described previously (15). A small amount of acetanilide was, however, added in order to facilitate the miscibility of N-acetyl-L-tryptophan with ethanethiol. The crude material was purified from ethyl acetate-ether to give white crystals, mp 176.5–177.5°C. The product is almost solely the L form as judged by its behavior with α-chymotrypsin.

Proflavin hemisulfate (Aldrich) was recrystallized from water in the dark. All buffers were reagent grade from Mallinckrodt and Sigma. Acetonitrile and N,N-diethyl-
formamide used for stock solutions of substrates were Eastman Kodak Co. spectro grade products.

Steady-state kinetics were determined at pH 7.0 using a Cary 14 PM recording spectrophotometer with a thermostated cell holder. The hydrolyses of AcTrpONp and AcTrpSNp were followed at 400 nm (ε = 8,500 M⁻¹ cm⁻¹) and 410 nm (ε = 13,400 M⁻¹ cm⁻¹), respectively. The hydrolyses of AcTrpOEt and AcTrpSEt were followed at 300 nm (Δε = 240 M⁻¹ cm⁻¹) described previously (14).

Acylation reactions were measured by a Durrum stopped flow spectrometer (model D-110) with a Durrum Temperature jump accessory, according to methods described previously (16). Volumes of both components were 300 μl per measurement at pH 4.0 and 5.0, and 450 μl at pH 8.0 so as to observe approximately the last 100 μl which is the freshest after mixing. The monitored wavelengths were 340 nm for p-nitrophenol at pH 4.0 and 5.0, 402 nm for p-nitrophenolate ion at pH 8.0, 340 nm for p-nitrothiophenol at pH 4.0, 410 nm for p-nitrothiophenolate ion at pH 5.0 and 8.0, and 465 nm for the enzyme-proflavin complex for both ethyl and thiolethyl esters at pH 5.0 and 8.0. The values of k₂ and Kₐ were calculated by means of a CDC6400 computer program written for the least-squares analysis of an Eadie plot (17). The experimental conditions along with the results are summarized in Table 2.

In experiments with the p-nitrothiophenyl ester, the enzyme stock solution was purified with Sephadex G-25 (10).

**RESULTS**

Steady-state kinetic parameters obtained by conventional methods are shown in Table 1. Values of kₗ for all four substrates are identical within experimental error.

In the presteady state experiments with AcTrpONp and AcTrpSNp, the pseudo first-order rate constant, kₗ, was determined under conditions of [E₀] ≫ [S]. An advantage of these conditions is the elimination of the turnover portion of the enzymatic reaction. Furthermore, the results are not affected by the presence of the β-isomer. An oscilloscope trace is shown in Fig. 1. Good first-order plots were obtained from such traces. At pH 8.0, only the last part of the reaction is observable because of the “dead” time of the apparatus. The reliability of the data thus obtained was checked by the observation that data at the lower pHs fit well with the first-order plot up to greater than 90% re-

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**Table 1. Steady-state kinetic parameters of α-chymotrypsin-catalyzed hydrolysis of N-acetyl-tryptophan esters at pH 6.39**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kₗ (sec⁻¹) × 10</th>
<th>Kₐ (M) × 10⁶</th>
<th>kₗ/Kₐ (sec⁻¹) × 10⁻⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcTrpONp</td>
<td>3.1 ± 0.3</td>
<td>3.9 ± 0.4</td>
<td>7.9</td>
</tr>
<tr>
<td>AcTrpSNp</td>
<td>2.6 ± 0.4</td>
<td>4.5 ± 0.4</td>
<td>5.8</td>
</tr>
<tr>
<td>AcTrpOEt</td>
<td>2.9 ± 0.2</td>
<td>65 ± 2.5</td>
<td>0.45</td>
</tr>
<tr>
<td>AcTrpSEt</td>
<td>2.7 ± 0.2</td>
<td>6.4 ± 0.4</td>
<td>4.2</td>
</tr>
</tbody>
</table>


stals, \(k_b/K_c\), of the p-nitrothiophenyl and the p-nitrophenyl esters are negligibly identical whereas, the thioethyl ester has a larger \(k_b/K_c\) of the ethyl ester due to the lower \(K_c\) term, not to a difference in \(k_b\). (d) The observed \(k_b/K_c\) of the oxygen esters are in good agreement with published values of the algebraically equivalent \(k_{cat}/k_m\), considering effects of the organic solvent (26).

**DISCUSSION**

The foregoing results indicate that \(\alpha\)-chymotrypsin catalyzes the hydrolysis of specific thioesters with a pathway identical to that of the oxygen analogs and includes the same acyll enzyme as an intermediate. Thus, the reaction scheme for the enzymatic hydrolysis of the thiol substrates would be

\[
E + S \rightleftharpoons E \cdot S = ES' \rightarrow E + P_1
\]

In addition, the results in Table 2 indicate that the mechanism of the acylation part of the reaction is also identical for both the oxygen and sulfur esters.

Since an \(-SR\) group is a better leaving group than an \(-OR\) group by at least two or three orders of magnitude (ref. 27-29, 43, see introduction) the near identity of the \(k_b\) values for the thiol esters and their oxygen counterparts demonstrate that a one-step acyl-transfer reaction does not occur. The simplest way to explain the data is to postulate a metastable tetrahedral intermediate, the formation of which is the rate-determining step of the acylation reaction. The first slower bond making step is rate-determining and precedes the second faster bond breaking step. On the assumption that the stationary concentration of the intermediate, TI, is invariably very small, the observed overall acylation rate constants \(k_a\) can be expressed in terms of Eqs. 5 and 6. Since no tetrahedral intermediate is directly observed, \(k_a \gg k_{a-}\), and since \(k_a\) is the same for the thiol and oxygen esters, \(k_a \gg k_{a-}\). This is in agreement with the results obtained for the alkaline hydrolysis of oxygen and thiol esters (27, 29-31), and also for the \(\alpha\)-chymotrypsin-catalyzed hydrolysis of \(p\)-nitrophenyl acetate and thiolacetate (10).

Combining the experimental results that \(k_a \approx k_b \approx (1 - 5) \times 10^8\) sec\(^{-1}\) at pH 8.0 and that \(k_a \gg k_{a-}\), with the expectation that \(-SR\) ions are at least 2 to 3 orders of magnitude better leaving groups than \(-OR\) ions, the values of \(k_a\) for the thiol esters should be \(10^8\) to \(10^{11}\) sec\(^{-1}\). Such large rate constants were indeed reported recently for the breakdown of tetrahedral intermediates in the general base-catalyzed hydrolysis of thioesters (32). It is estimated that the rate constants of the breakdown of the intermediate are \(1.7 \times 10^7\) and \(5.8 \times 10^7\) sec\(^{-1}\) for ethyl trifluorothiolacetate and methyl \(S\)-trifluoroacetylmercaptoacetate, respectively. The value for the latter compound approaches that of a diffusion-controlled process (33).

Thus, the rate-determining step of the tetrahedral intermediate to acyl-enzyme does not involve further profavin displacement. In this case the observed rate constants by this method correspond to the formation of the tetrahedral intermediate and not to their breakdown. In terms of the Hammond postulate, some ambiguity may exist as to the magnitude of \(k_b\) of the ethyl ester as the pK\(_{a}\) of its leaving group (about 16) is probably larger than that of the serine hydroxyl nucleophile. Nevertheless, it should be emphasized that the conclusion regarding the tetrahedral intermediate in the previous paragraphs is applicable for all four esters since quasi-identical \(k_b\) values were obtained for the ethyl and thioethyl esters, also.

It is seen from the \(K_s\) values of the ethyl ester and its sulfur counterpart that the nature of the atom being displaced during acylation has a fairly large effect on binding to the enzyme, contrary to previous speculation. Since nonproductive binding should be a very minor factor, if any, in reactions

**Table 2.** Presteady-state kinetic constants for \(\alpha\)-chymotrypsin-catalyzed hydrolysis of 

\[N\text{-acetyl-p-tryptophan esters at 25.5 \pm 0.4^\circ C and } I = 0.1^\dagger\]

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>(k_a) (sec(^{-1}))</th>
<th>(K_s) (M) (\times 10^4)</th>
<th>(k_b/K_a) (M(^{-1})sec(^{-1})) (\times 10^{-4})</th>
<th>(k_{cat}/K_m) (M(^{-1})sec(^{-1})) (\times 10^{-4})</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcTrpONp</td>
<td>4.03</td>
<td>67.6 ± 0.6</td>
<td>2.53 ± 0.1</td>
<td>2.7</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>5.02</td>
<td>670 ± 50</td>
<td>1.5 ± 0.1</td>
<td>50</td>
<td>680 ±                     (25)</td>
</tr>
<tr>
<td></td>
<td>8.01</td>
<td>4380 ± 25</td>
<td>0.97 ± 0.2</td>
<td>450</td>
<td>24</td>
</tr>
<tr>
<td>AcTrpSNp$</td>
<td>4.03</td>
<td>35.6 ± 0.1</td>
<td>1.51 ± 0.02</td>
<td>2.4</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>5.02</td>
<td>450 ± 30</td>
<td>0.91 ± 0.14</td>
<td>50</td>
<td>7ystem (25)</td>
</tr>
<tr>
<td></td>
<td>8.01</td>
<td>225 ± 240</td>
<td>0.32 ± 0.08</td>
<td>700</td>
<td>600 $t$ (21)</td>
</tr>
<tr>
<td>AcTrpOEt</td>
<td>5.02</td>
<td>33 ± 4.5</td>
<td>21 ± 3</td>
<td>40 $t$ (21)</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>1090 ± 130</td>
<td>9.9 ± 1.2</td>
<td>10.8</td>
<td>600 $t$ (21)</td>
</tr>
<tr>
<td>AcTrpSEt</td>
<td>5.02</td>
<td>47 ± 8.2</td>
<td>4.4 ± 0.7</td>
<td>82</td>
<td>500 $t$ (21)</td>
</tr>
<tr>
<td></td>
<td>8.00</td>
<td>605 ± 55</td>
<td>0.85 ± 0.10</td>
<td>82</td>
<td>100 $t$ (21)</td>
</tr>
</tbody>
</table>

* Citrate, acetate, and Tris-HCl buffer for pH 4.0, 5.0, and 8.0, respectively. 0.5% (v/v) acetonitrile–water for AcTrpONp and AcTrpSNp. 0.33% (v/v) dimethylformamide–1.36% (v/v) acetonitrile–water for AcTrpOEt and AcTrpSEt.

† \(K_s\) used for the calculation of \(K_s\) are 11.2 and 3.3 \(\times 10^{-4}\) M at pH 5.0 and 8.0, respectively, see text.

‡ pH 7.9 aqueous solution.

§ 0.81% (v/v) acetonitrile–water.
Table 3. Comparison of kinetic parameters of α-chymotrypsin-catalyzed hydrolysis of several oxygen and sulfur esters at pH 7.8-8.0 and 25°C

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{cat}$ (sec$^{-1}$)</th>
<th>$K_m$ (M) $\times 10^3$</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AcGlyOEt* (15)</td>
<td>0.039</td>
<td>389</td>
<td>0.101</td>
</tr>
<tr>
<td>AcGlyEt (15)</td>
<td>0.23</td>
<td>41</td>
<td>5.61</td>
</tr>
<tr>
<td>ZGlyOEt (31)</td>
<td></td>
<td></td>
<td>3.65</td>
</tr>
<tr>
<td>ZGlyEt (31)</td>
<td>0.274</td>
<td>0.66</td>
<td>420</td>
</tr>
<tr>
<td>AcPheOEt (31)</td>
<td></td>
<td></td>
<td>54,000</td>
</tr>
<tr>
<td>AcPheEt (31)</td>
<td></td>
<td></td>
<td>1,000,000</td>
</tr>
<tr>
<td></td>
<td>$k_2$</td>
<td>$K_s$</td>
<td>$k_2/K_s$</td>
</tr>
<tr>
<td>AcPheEt (35)</td>
<td>265</td>
<td>7.14</td>
<td>37,200</td>
</tr>
<tr>
<td>NPA† (10)</td>
<td>4.55</td>
<td>1.47 (21)</td>
<td>3,940 (21)</td>
</tr>
<tr>
<td>NPTA‡ (10)</td>
<td>4.52</td>
<td>1.20</td>
<td>3,770</td>
</tr>
</tbody>
</table>

* According to Bender et al. (14), acylation is the rate-determining step ($k_2 = 2.29$ sec$^{-1}$). Therefore, the reported $k_{cat}$ and $K_m$ values can be regarded to be close to $k_2$ and $K_s$ values, respectively.
† NPA, p-nitrophenyl acetic.
‡ NPTA, p-nitrophenyl thiolacetic.

of N-acetyl-tryptophan esters, these data indicate that the hydrophobicity of the leaving group provides additional binding strength but that when the leaving group is strongly hydrophobic to begin with, additional hydrophobicity makes only an additional small effect on binding. For p-nitrophenyl acetate and thiolacetic, identical values of $K_s$ were observed (10, see Table 3), which were also similar to the value in the case of AcTrpOEt. It is likely that the binding modes of nonspecific substrates are entirely “nonproductive,” which, if so, would lower $k_2$ values drastically (8). Presumably, p-nitrophenyl acetate has good nonproductive binding to begin with so the additional hydrophobicity due to a sulfur atom showed only a small effect on binding. From this viewpoint, the experimental finding that the thiolester ester has a larger $k_2$ value at pH 5.0 than the ethyl ester, may be understandable; the relatively large $K_s$ value of the latter at this pH may imply that the substrate is not bound tight enough to give the optimum orientation. The additional binding due to the sulfur atom, therefore, allows a more favorable orientation for attack by the nucleophile, overcoming any slightly unfavorable reactivity of thiol substrates for the formation of the tetrahedral intermediate.

The similar situation can be seen for acetylglucine ethyl ester (AcGlyOEt) and acetylglucine thiolethyl ester (AcGlySEt) in Table 3. The same argument might be applicable to benzoylcarbonylglucine ethyl ester (ZGlyOEt) and benzoylcarbonylglucine thiolethyl ester (ZGlySEt) and also to N-acetyl-L-phenylalanine ethyl ester (PheOEt) and N-acetyl-L-phenylalanine thiolethyl ester (AcPheSEt), to some extent (also see below). The observation that usually the oxygen ester has a slightly greater $k_2$ may be due to the fact either that the Michaelis complex of the sulfur ester is so tightly bound that a slightly larger activation free energy is required in order to “loosen” the binding in a transition state of the formation of the tetrahedral intermediate, or that within the Michaelis complex the carbonyl group of the oxygen esters are slightly more favorably oriented to attack by the nucleophile than the carbonyl group of the sulfur esters, or a combination of both.

Since the value of $K_s$ depends, more or less, on the nature of the leaving group, and since the difference in $k_2/K_s$ terms are mainly due to differences in $K_s$, it is obvious that $k_2/K_s$ ($= k_{cat}/K_m$) should not be used for studies of the acylation process without careful consideration. It can be easily said that the much larger $k_{cat}/K_m$ values of ZGlySEt and AcPheSEt than those of their oxygen counterparts (in Table 3) are mainly due to the stronger binding of the thiolethyl esters, although the larger value for $k_2$ of thiolethyl esters over their oxygen analogs is also possible, as was mentioned above.

The experimentally obtained $k_2$ values for AcTrpONp and AcTrpSNp at pH 8.0 are an order of magnitude smaller than that estimated for the oxygen ester from steady state studies in the past (14) and are at the usual upper limit of the rate constant for acid-base catalysis (10$^4$-10$^5$ sec$^{-1}$) (36), although faster than the rate constant of a simple proton transfer from water to imidazole (2300 sec$^{-1}$) (36). p-Nitrophenyl esters are usually two orders of magnitude more reactive toward alkaline hydrolysis than ethyl esters (37). The relative reactivity ratio of the specific ester substrates in enzymic hydrolysis is, however, only 4 to about 5 at pH 8.0. This may reflect that the $k_2$ value at this pH has approached the maximum rate constant for general base catalysis.

Some $k_2$ values for α-chymotrypsin-substrate complex formation are 6 × 10$^4$ M$^{-1}$ sec$^{-1}$ for N-trans (2-furyl) acryloyl-L-tryptophanamide at 15°C (38), 10$^5$ M$^{-1}$ sec$^{-1}$ for proflavin at 12°C (39), 2.1 × 10$^5$ M$^{-1}$ sec$^{-1}$ for biebrich scarlet at 20°C (40), and 1.5 × 10$^5$ M$^{-1}$ sec$^{-1}$ for trifluoracetyl-L-tryptophan at 32°C (41). Considering these values and the conditions that $k_2$ ≥ $k_2/K_s$ (42), and that $k_2$ is lower than diffusion controlled [about 10$^6$ sec$^{-1}$ (36)], the probable $k_2$ value for AcTrpONp and AcTrpSNp will be (6-10) × 10$^5$ M$^{-1}$ sec$^{-1}$. Taking a $K_s$ value of (0.5-1) × 10$^{-3}$ for both substrates, $k_{cat} = (3-10) × 10^5$ sec$^{-1}$. Therefore, the observed $k_2$ values for these substrates are at the most comparable to the $k_2$ values.

Finally, an interesting problem arises from the experimental data. Using the value of $k_2$ at pH 8.0 as the $k_2$ (lim) value, one can calculate an apparent pKa for the enzyme-substrate complex according to:

$$k_2 = k_2 (\text{lim})/(1 + [\text{H}]K_a).$$  [7]

The estimated pK; values are 5.8, 5.6, 6.5, and 6.2 for AcTrpONp, AcTrpSNp, AcTrpOEt, and AcTrpSEt, respectively. The value for the former two are abnormally low, even taking experimental error into account. Further study for this direction, including complete pH profiles of all four substrates, is now in progress, and details will be published elsewhere.

This research was supported by Grant HL-05726 of the National Institutes of Health.

Tetrahedral Intermediate in Chymotrypsin Acylation