Structure-Activity Relationship in the Urokinase Hydrolysis of α-N-Acetyl-L-lysine Anilides

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(Received April 30/October 19, 1974)

The absence of both nonproductive binding and substrate activation and also the good solubility of the substrates make the urokinase-catalysed hydrolysis of specific anilides a very suitable reaction for substrate structure—enzyme activity studies.

Derivatives of α-N-acetyl-L-lysine anilide with high σ⁺-value substituents in the aniline ring were synthesized. Rate constants $k_{cat.}$ and apparent Michaelis-Menten constants $K_m^{(app.)}$ are presented. From the substituent dependence of $k_{cat.}$ and from the fact that $k_{cat.}$ is 13 to 37 times smaller than the deacylation rate constant it is concluded that the rate-limiting step proceeds prior to deacylation.

The catalytic rate constant $k_{cat.}$ obeys a linear free-energy relationship of the Hammett type with $\rho = +0.72$. Two different mechanisms implied by the results obtained from the model reaction (specific base and general acid-base catalysed hydrolysis of N-acetylglycine anilides under extreme conditions) are proposed in order to account for this positive and low $\rho$-value. In the first mechanism the breakdown of an enzyme tetrahedral intermediate is rate-limiting, while in the second one its formation controls the overall rate. The discrimination between the two mechanisms, however, could not be found.

The investigation of the electronic effects of the substituents in the substrate molecule on its reactivity in enzyme-catalysed reactions provides very valuable information for the mechanism of enzyme action. The responses to changes in the charge density at the reaction site have been exploited successfully in the study of the chymotrypsin-catalysed hydrolysis of nonspecific [1] and specific [2, 3] O-acylphenols and specific anilides [4–7]. These investigations have shown that, in the chymotrypsin hydrolysis of specific anilides substituted in the aniline ring, the catalytic rate is decreased with increase in the electron withdrawal ($\rho < 0$). These results, however, were obtained in studies that cover the low σ⁻-value region ($\sigma < 0.4$). There are some indications [5, 8] that in the high σ⁺-value region the substituent dependence is the reverse of that observed in the low σ⁻-value region.

The specificity of chymotrypsin arises from a hydrophobic interaction between enzyme and substrate [9]. For this reason chymotrypsin-specific anilides have poor solubility in buffer solutions. In order to increase the substrate solubility, organic solvents were used up to 22% (v/v). Organic solvents, however, perturb the enzymatic reaction [5, 8]. Moreover, in model reactions the effects of organic solvents are opposite in the low and high $\sigma$-value regions [10]. This makes the rate measurements of the chymotrypsin hydrolysis very difficult and the separation of $k_{cat.}$ and $K_m$ somewhat imprecise [11]. On the other hand, chymotrypsin substrates with a hydrophobic anilide portion bind predominantly in a nonproductive mode and then the variation in the Michaelis-Menten parameters are due to the substituent effects on the hydrophobic nature of the aniline ring [11]. These complications could be avoided by studying structure-activity relationship in the hydrolysis of specific anilides catalysed by trypsin or trypsin-like enzymes, whose specificity arises from a ionic enzyme-substrate interaction. Apparent substrate activation of trypsin [12, 13], however, prevents any quantitative interpretation of the results. This is not the case with the trypsin-like enzyme urokinase, which does not display substrate activation [13].

Urokinase, a trypsin-like enzyme found in urine [14], is considered to be a powerful thrombolytic
agent in vivo and its efficiency is now being evaluated in many national and international research groups [15]. Urokinase is a physiological activator of the precursor (plasminogen) of the blood proteolytic enzyme plasmin [14]. Modeling the plasminogen re-active site (Arg-Val bond [16]) by \( \alpha-N\)-acetyl-L-lysine \( p\)-nitroanilide, we have recently demonstrated the amidase (aminidase) activity of urokinase [17]. In order to elucidate the mechanism of this catalytic action, we have synthesized other \( p\)-electron-acceptor anilides of \( \alpha-N\)-acetyl-L-lysine and studied the substrate structure–urokinase activity relationship. In the present paper we describe the results of this research.

MATERIALS AND METHODS

Urokinase

Human urokinase was either a standard preparation (4800 units/vial, where the units are those defined by the Committee on Thrombolytic Agents, National Heart Institute, U.S.A.) or preparations with amidase activity ranging from 1000 to 1500 units/mg protein, obtained in our laboratory [17]. Urokinase stock solutions were made in 0.5 M NaCl. These solutions could be kept at 4 °C for one month without any change in activity.

The functional molarity of the urokinase solutions was determined by active-site titration with \( \alpha-N\)-nitrophenyl-\( \alpha\) -guanidinobenzoate, a titrant for trypsin-like enzymes [18]. Titrations were carried out in the VSU 2-P (VEB Carl Zeiss, Jena, Germany) spectrophotometer, equipped with recorder, by a procedure similar to that of Chase and Shaw for titrating trypsin active site [19].

Assay of urokinase amidase activity was performed as previously described [17].

Synthesis of Substrates

Derivatives of \( \alpha-N\)-acetyl-L-lysine anilide with \(-SO_2CH_3, -CN, -COCH_3\) and \(-COOC_2H_5\) groups in the para position of the aniline moiety were prepared from \( \alpha-N\)-acetyl-\( \alpha\) -carbobenzoxy-L-lysine amide by the two-step synthesis used by us for the preparation of \( \alpha-N\)-acetyl-L-lysine \( p\)-nitroanilide [17] as described below.

\( \alpha-N\)-Acetyl-\( \alpha\) -carbobenzoxy-L-lysine anilides were prepared via acylation of the appropriate aniline with \( \alpha-N\)-acetyl-\( \alpha\) -carbobenzoxy-L-lysine amide [17] by the method of Galat and Elion [20]. All these compounds are colourless crystalline solids. Melting points, specific rotations and elemental analysis are given in Table 1.

\( \alpha-N\)-Acetyl-L-lysine anilides were prepared by hydrogenobromidolysis [21] of the corresponding \( \alpha-N\)-acetyl-\( \alpha\) -carbobenzoxy-L-lysine anilides. Unfortunately, the attempts to synthesize \( \alpha-N\)-acetyl-L-lysine \( p\)-cyanoanilide were not successful due to the instability of \(-CN\) group in HBr/CH_3COOH. The urokinase substrates obtained are colourless, slightly hydroscopic crystalline solids, soluble in water. Their physical constants and elemental analysis are given in Table 2. Standard urokinase hydrolyses more than 99% of each substrate indicating that racemization during the synthesis is less than 1%. Our preliminary results show that urokinase hydrolyses 50% of \( \alpha-N\)-acetyl-DL-lysine \( p\)-nitroanilide and does not hydrolyses \( \alpha-N\)-acetyl-D-lysine \( p\)-nitroanilide.

L-lysine and various anilines used to synthesize the substrates were obtained from commercial sources (Fluka, Switzerland) except \( p\)-methylsulfonyl aniline, which was prepared by the method of Fuller et al. [22].

The active-site titrant \( p\)-nitrophenyl-\( \alpha\) -guanidinobenzoate was synthesized as described by Chase and Shaw [19]. As indicated by alkaline hydrolysis in 0.1 M NaOH the compound obtained was 94% pure, but as pointed out by the authors it was suitable for use as a titrant with this degree of purity.

Kinetic Measurements

The time course of the urokinase-catalysed hydrolysis of \( \alpha-N\)-acetyl-L-lysine anilides was followed spectrophotometrically by continuous measurement of the increase in absorbance due to the \( p\)-substituted anilines released. The wavelengths chosen (\( \lambda_m \)) for the kinetic studies and the relevant absorption coefficients (\( \epsilon_m \)) of the anilides and anilines are given in Table 3.

Substrates and urokinase (0.2–0.6 \( \mu\)M) were reacted at pH 8.50 (pH optimum [17]) and at 37 ± 0.1 °C in thermostated cells of the VSU 2-P spectrophotometer, equipped with recorder. The maximum concentrations of the substrates used in the experiments were equal or near to the values of the substrate \( K_m \). The initial rates (less than 3% reaction) were used and two or three determinations were made for each Lineweaver-Burk plot. At least seven points were used for each Lineweaver-Burk plot. Michaelis-Menten parameters \( k_{cat} \) and \( K_m \) (app.) were determined from these plots and the least-squares method.

Ultraviolet Absorption Measurements

The ultraviolet spectra of anilines and \( \alpha-N\)-acetyl-L-lysine anilides were determined on a VSU 2-P

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Table 1. Physical constants of the p-substituted α-N-acetyl-ε-N-carbobenzyoxy-lysine anilides

<table>
<thead>
<tr>
<th>Substituents</th>
<th>Formula</th>
<th>Melting point °C</th>
<th>[α] \textsubscript{D} \textsuperscript{25} \textsuperscript{(c 0.5 MeOH)}</th>
<th>Analysis</th>
<th>C</th>
<th>H</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>-SO\textsubscript{2}CH\textsubscript{3}</td>
<td>C\textsubscript{23}H\textsubscript{29}N\textsubscript{3}O\textsubscript{5}S</td>
<td>158 - 159</td>
<td>-7.07</td>
<td>calc'd</td>
<td>58.10</td>
<td>6.10</td>
<td>8.66</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>found</td>
<td>58.41</td>
<td>6.21</td>
<td>8.21</td>
</tr>
<tr>
<td>-CN</td>
<td>C\textsubscript{23}H\textsubscript{26}N\textsubscript{2}O\textsubscript{4}</td>
<td>158 - 159</td>
<td>4.94</td>
<td>calc'd</td>
<td>65.38</td>
<td>6.20</td>
<td>13.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>found</td>
<td>64.90</td>
<td>6.28</td>
<td>13.01</td>
</tr>
<tr>
<td>-COCH\textsubscript{3}</td>
<td>C\textsubscript{24}H\textsubscript{25}N\textsubscript{3}O\textsubscript{6}S</td>
<td>166 - 167</td>
<td>-7.46</td>
<td>calc'd</td>
<td>65.58</td>
<td>6.65</td>
<td>9.56</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>found</td>
<td>65.70</td>
<td>6.80</td>
<td>9.45</td>
</tr>
<tr>
<td>-COOC\textsubscript{4}H\textsubscript{9}</td>
<td>C\textsubscript{25}H\textsubscript{34}N\textsubscript{3}O\textsubscript{6}S</td>
<td>183 - 185</td>
<td>-5.98</td>
<td>calc'd</td>
<td>65.17</td>
<td>7.09</td>
<td>8.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>found</td>
<td>65.80</td>
<td>7.29</td>
<td>8.57</td>
</tr>
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</table>

Table 2. Physical constants of p-substituted α-N-acetyl-lysine anilide hydrobromides

<table>
<thead>
<tr>
<th>Substituents</th>
<th>Formula</th>
<th>Melting point °C</th>
<th>[α] \textsubscript{D} \textsuperscript{25} \textsuperscript{(c 0.5 MeOH)}</th>
<th>Analysis</th>
<th>C</th>
<th>H</th>
<th>N</th>
<th>Br</th>
</tr>
</thead>
<tbody>
<tr>
<td>-SO\textsubscript{2}CH\textsubscript{3}</td>
<td>C\textsubscript{13}H\textsubscript{22}N\textsubscript{3}O\textsubscript{5}S Br · H\textsubscript{2}O</td>
<td>109 - 111</td>
<td>-16.9</td>
<td>calc'd</td>
<td>40.90</td>
<td>5.95</td>
<td>9.55</td>
<td>18.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>found</td>
<td>40.46</td>
<td>6.19</td>
<td>9.80</td>
<td>18.62</td>
</tr>
<tr>
<td>-COCH\textsubscript{3}</td>
<td>C\textsubscript{15}H\textsubscript{24}N\textsubscript{3}O\textsubscript{4} Br · H\textsubscript{2}O</td>
<td>177 - 178</td>
<td>-21.7</td>
<td>calc'd</td>
<td>47.50</td>
<td>6.43</td>
<td>10.40</td>
<td>19.80</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>found</td>
<td>47.20</td>
<td>6.37</td>
<td>10.49</td>
<td>20.80</td>
</tr>
<tr>
<td>-COOC\textsubscript{4}H\textsubscript{9}</td>
<td>C\textsubscript{19}H\textsubscript{30}N\textsubscript{3}O\textsubscript{4}Br</td>
<td>64 - 66</td>
<td>-17.7</td>
<td>calc'd</td>
<td>50.00</td>
<td>6.96</td>
<td>9.58</td>
<td>18.50</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>found</td>
<td>50.30</td>
<td>6.97</td>
<td>9.23</td>
<td>18.70</td>
</tr>
</tbody>
</table>

Table 3. Ultraviolet absorption of p-substituted anilines and α-N-acetyl-lysine anilides

Measurements were made at 37 ± 0.1 °C, in 0.06 M Tris, 0.09 M NaCl buffer pH 8.50. Solutions were prepared by weight and diluted to give optimum range of absorption for measurement.

<table>
<thead>
<tr>
<th>Substituents</th>
<th>λ\textsubscript{max} (ε\textsubscript{max})</th>
<th>λ\textsubscript{m} (ε\textsubscript{m})</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NO\textsubscript{2}</td>
<td>aniline</td>
<td>380 (13500)</td>
</tr>
<tr>
<td></td>
<td>anilide</td>
<td>315 (13000)</td>
</tr>
<tr>
<td>-SO\textsubscript{2}CH\textsubscript{3}</td>
<td>aniline</td>
<td>265 (18000)</td>
</tr>
<tr>
<td></td>
<td>anilide</td>
<td>262 (24000)</td>
</tr>
<tr>
<td>-COCH\textsubscript{3}</td>
<td>aniline</td>
<td>312 (19400)</td>
</tr>
<tr>
<td></td>
<td>anilide</td>
<td>283 (19000)</td>
</tr>
<tr>
<td>-COOC\textsubscript{4}H\textsubscript{9}</td>
<td>aniline</td>
<td>285 (18800)</td>
</tr>
<tr>
<td></td>
<td>anilide</td>
<td>269 (23400)</td>
</tr>
</tbody>
</table>

RESULTS

Urokinase Active-Site Titration

As has been shown by Landmann and Markward [23], urokinase is inhibited irreversibly by diisopropyl phosphofluoridate and therefore this enzyme is a serine proteinase. p-Nitrobenzyl-p' -guanidinobenzoate also inhibited urokinase irreversibly [23]. This suggests that the acyl part of this inhibitor binds strongly to the active-site serine hydroxyl. The formation of a stable acylenzyme compound is the most desirable property of an active-site titrant [19]. For this reason we investigated the p-nitrophenyl-p' -guanidinobenzoate [18, 19] as a titrant for urokinase. As may be seen in Fig. 1 the addition of this titrant to a solution of urokinase results in an initial burst formation of p-nitrophenol, followed by post-burst linear production. Extrapolation of the absorbance to the time zero gives the absorbance of the stoichiometric p-nitrophenol. This absorbance correlates with urokinase amidase activity (Fig. 1) and is independent of the titrant concentration.
Substituent Effects in the High \( \sigma^- \) Value Region

Fig. 1. Titration of urokinase with \( p \)-nitrophenyl-\( p' \)-guanidinobenzoate (0.1 mM). (I) 2240 and (II) 4480 units of urokinase; pH 8.30 (0.1 M barbital buffer), 25 ± 0.1 °C. The addition of the titrant (a) to a solution of urokinase results in an initial burst formation of \( p \)-nitrophenol (b), followed by post-burst linear production (c). Extrapolation of the absorbance to time zero (a) corresponds to enzyme concentration of 1.58 µM (I) and 3.20 µM (II) respectively.

Substrate and Product Ultraviolet Absorption

Besides the absorption bands pertinent to the benzene molecule, monosubstituted benzenes with electron-acceptor substituents (\( -\text{NO}_2 \), \( -\text{SO}_2\text{CH}_3 \), \( -\text{CN} \), \( -\text{COCH}_3 \), \( -\text{COOR} \)) show a new band (charge-transfer band) caused by charge-transfer interaction between the substituent group and benzene ring [24]. When electron-donating substituent groups (\( -\text{NH}_2 \), \( -\text{OH} \)) are introduced in the para position, the charge-transfer band shifts to the red and increases in intensity. The products of urokinase-catalysed hydrolysis of specific anilides studied are \( p \)-disubstituted benzenes having electron-accepting and electron-donating groups. The maxima locations \( (\lambda_{\text{max}}) \) and intensities \( (\epsilon_{\text{max}}) \) of the charge-transfer band of the products are summarized on Table 3. Acylation of the electron-donating group (\( -\text{NH}_2 \)) by \( \alpha\)-N-acetyl-L-lysine shifts the aniline charge-transfer band to the shorter wavelengths with little change in intensities (Table 3). These shifts allow the extent of substrate hydrolysis to be estimated spectrophotometrically.

Kinetic Parameters

The Lineweaver-Burk representations of the initial rate data gave straight lines in all cases. Fig. 2 demonstrates a typical double-reciprocal plot of the data obtained for the urokinase-catalysed hydrolysis of \( \alpha\)-N-acetyl-L-lysine \( p \)-methylsulfonylanilide. These results indicate that the kinetics of the enzymatic reactions studied obey the Michaelis-Menten rate law:

\[
v_0 = \frac{V [S]}{[S] + K_m^{(\text{app})}}.
\]

Statistical analysis of these plots yielded the Michaelis-Menten parameters \( V \) and \( K_m^{(\text{app})} \) and their standard deviations. Using the determined urokinase functional molarity, the first-order rate constant \( k_{\text{cat}} \) were calculated. These parameters are summarized in Table 4. The dependence of \( \log k_{\text{cat}} \) on the substituent constant \( \sigma^- \) of the leaving group in the hydrolysis of the specific anilides studied are shown in Fig. 3.
Table 4. Steady-state data for the urokinase-catalysed hydrolysis of \(\alpha\)-N-acetyl-L-lysine derivatives

<table>
<thead>
<tr>
<th>Derivatives</th>
<th>(k_{\text{cat}})</th>
<th>(k_+2)</th>
<th>(K_m(\text{app}))</th>
<th>(K_+3)</th>
<th>(k_+3)</th>
<th>(\sigma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-COOCH(_3)</td>
<td>anilide</td>
<td>0.312</td>
<td>0.32 ± 0.06</td>
<td>4.07 ± 0.36</td>
<td>11.68 ± 1.47</td>
<td>0.68</td>
</tr>
<tr>
<td>p-COCH(_3)</td>
<td>anilide</td>
<td>0.505</td>
<td>0.53 ± 0.09</td>
<td>4.28 ± 0.28</td>
<td>11.68 ± 1.47</td>
<td>0.87</td>
</tr>
<tr>
<td>p-SO(_2)CH(_3)</td>
<td>anilide</td>
<td>0.658</td>
<td>0.70 ± 0.19</td>
<td>9.46 ± 1.04</td>
<td>10.0 ± 2.0</td>
<td>1.05</td>
</tr>
<tr>
<td>p-NO(_2)</td>
<td>anilide</td>
<td>0.833</td>
<td>0.90 ± 0.19</td>
<td>5.83 ± 0.38</td>
<td>6.3 ± 1.0</td>
<td>1.27</td>
</tr>
<tr>
<td>Methyl</td>
<td>ester</td>
<td>10.0</td>
<td>59.40 ± 9.00</td>
<td>0.84 ± 0.14</td>
<td>5.0 ± 0.6</td>
<td>11.68 ± 1.47</td>
</tr>
</tbody>
</table>

The straight line with a slope \(q\) equal to + 0.72 (regression coefficient 0.98) suggests that the catalytic rate constant obeys a linear free-energy relationship of a Hammett type. The \(\sigma\) -values were used in preference to \(\sigma\) because of strongly electronic interaction of the substrate reaction center with the strong electron-accepting substituents [25].

**DISCUSSION**

\[
E + S \xrightleftharpoons[k_{-1}\text{-acylation}]{k_{+1}\text{-deacylation}} E + P_1 + P_2 (2)
\]

This is the minimum kinetic scheme that serine proteinases require to explain the observed phenomena [26]. The initial \(p\)-nitrophenol burst occurring prior to the steady-state in the hydrolysis of \(p\)-nitrophenyl-\(p'\)-guanidinobenzoate (Fig. 1) catalysed by urokinase can not be explained by any scheme simpler than Eqn (2). Moreover, urokinase is irreversibly inhibited by diisopropyl phosphorofluoridate (acylation of an active-site serine hydroxyl [23]) and a basic group with a \(pK_a\) of approximately 7 is involved both in acylation and deacylation [27]. From Eqn (2) a steady-state derivation gives:

\[
k_{\text{cat}} = \frac{k_{+2}k_{+3}}{k_{+2} + k_{+3}} = k_{+2} \text{ if } k_{+3} \gg k_{+2} \quad (3)
\]

and

\[
K_m(\text{app}) = \frac{k_{+3}}{k_{+2} + k_{+3}} = K_m \text{ if } k_{+3} \gg k_{+2} \quad (4)
\]

where

\[
k_m = \frac{k_{-1} + k_{+2}}{k_{+1}} = k_{-1} \text{ if } k_{-1} \gg k_{+2} \quad (5)
\]

If the hydrolysis of \(\alpha\)-N-acetyl-L-lysine anilides studied is consistent with the kinetic model outlined Eqn (2), the reaction should proceed through the formation of a common acylenzyme \(\alpha\)-N-acetyl-L-lysyl-urokinase. Owing to this elimination of the structural differences of the substrates, the deacylation rate constant \(k_{+3}\) should be identical for all substrates. Therefore, if deacylation is the rate-limiting step, \(k_{\text{cat}}\) for the different substrates should be identical within the experimental error. This is not the case in the urokinase hydrolysis of \(\alpha\)-N-acetyl-L-lysine anilides (Table 4). The fact that the overall rate constant \(k_{\text{cat}}\) varies depending on the leaving group, suggests that the rate-limiting step proceeds prior to deacylation. This conclusion is strongly supported by the results from the determination of the individual rate constants of the urokinase-catalysed hydrolysis of \(\alpha\)-N-acetyl-L-lysyl methyl ester with added nucleophilic agent, methanol [27]. The hydrolysis of this specific ester substrate proceeds also through the formation of the acylenzyme \(\alpha\)-N-acetyl-L-lysyl-urokinase. Using the determined value for its deacylation rate constant \(k_{+3}\), the rate constants of acylation \(k_{+2}\) of urokinase by the specific anilides studied, have been calculated according to Eqn (3). As may be seen from Table 4, the calculated acylation rate constants \(k_{+2}\) are 13 to 37 times smaller than the deacylation rate constant \(k_{+3}\) and practically equal to the observed catalytic rate constants \(k_{\text{cat}}\). Moreover, the value of the calculated equilibrium binding constant (substrate constant) \(K_m\) of Ac-Lys-OME is sufficiently close to the observed Michaelis-Menten constants \(K_m(\text{app})\) of the anilide substrates. This suggests that the observed \(K_m(\text{app})\) represents the substrate constant \(K_m\) and also that there is no unproductive binding due to the anilide portion of the substrates.

The Hamnett \(q\) value of + 0.72 (regression coefficient 0.98, Fig. 3) for \(k_{\text{cat}}\) shows that in the \(\sigma\) -value region studied (0.68 < \(\sigma\) < 1.27), the rate increases with increasing the electron-withdrawal power of the

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para substituents. The large positive deviation of p-nitroanilide from the correlation for $k_{cat}$, and the positive $q$ value between $m$- and $p$-nitroanilides have been observed also in chymotrypsin hydrolysis\[5,8]. These results cannot be explained without assuming the existence of a tetrahedral intermediate (ET) between enzyme-substrate complex ES and the acyl enzyme EA:

$$E + S \xrightleftharpoons[k_{-1}]{k_{+1}} ES \xrightarrow[k_{+2}]{k_{-2}} ET \xrightarrow[k_{+3}]{k_{-3}} EA + P_1 \quad \text{and} \quad P_2 \xrightarrow[k_{-4}]{k_{+4}} E + P_2 \quad (6)$$

A similar mechanism explained our results for the basic hydrolysis of N-acetylglycine p-nitroanilide and N-acetylglycine p-acetylanilide\[28]:

$$\text{OH}^- + S \xrightarrow[k_{-1}]{k_{+1}} \text{NT} \xrightarrow[k_{+2}]{k_{-2}} \text{EA} \quad \text{and} \quad \text{Products.} \quad (7)$$

In this model study designed no study the enzyme mechanism of Eqn (6) we observed general base catalysis by OH$^-$ and general acid-base catalysis by HCO$_3^{-}$. Applying the steady-state approximation to the tetrahedral intermediate (NT) in Eqn (7), the following equation for the observed rate, corrected for the ionisation of S, was derived\[28]:

$$v_{corr} = k_{+1} [\text{OH}^-] \frac{\Sigma k_{+3}^i [C_i]}{k_{-1} + \Sigma k_{+3}^i [C_i]} [S] \quad (8)$$

where $C_i$ is for the catalytic species OH$^-$, H$_2$O, HCO$_3^{-}$ and $k_{+3}^i$ the rate constants for the breakdown of NT, catalysed by $i$ species. At sufficiently low catalyst concentration $\Sigma k_{+3}^i [C_i]$ is much smaller than $k_{-1}$ and the breakdown of NT is rate-limiting:

$$v_{corr} = \Sigma k_{+3}^i [\text{OH}^-] [C_i] [S] \quad (9)$$

where

$$k_{+3}^i = k_{+1} \frac{k_{+3}^i}{k_{-1}} = k_{+3}^i K_{NT} \quad (10)$$

is the catalytic rate constant for the $i$ catalyst. Eqn (10) corresponds to the equation for the enzyme catalytic rate constant $k_{cat}$, derived under the same assumptions for the enzyme mechanism of Eqn (6).

The general derivation for $k_{cat}$ and $K_{m(app.)}$ for an enzymic reaction scheme similar to Eqn (6) has been given by Dixon and Webb\[29]. Assuming that, as in the monoenzymic mechanism of Eqn (7), the breakdown of the enzyme tetrahedral intermediate ET is rate-limiting, i.e. $k_{-1}, k_{+1}, k_{+1}', k_{+2}, k_{+2}' \gg k_{+3}$ and under the additional assumption $k_{+1}' \gg k_{+1}$, the Dixon and Webb expressions for the Michaelis-Menten parameters may be simplified\[30] to:

$$k_{cat} = k_{+1}' \frac{k_{+2}'}{k_{-1}'} = k_{+2}' K_{ET} \quad (11)$$

and

$$K_{m(app.)} = \frac{k_{-1}}{k_{+1}} = K_s. \quad (12)$$

The last expression suggests that the observed Michaelis-Menten constant $K_{m(app.)}$ should be equal to the equilibrium binding constant (substrate constant) $K_s$. As has been discussed above, and as may be seen from Table 4, this is observed. Being the case shows the validity of the assumption $k_{+1}' \gg k_{+1}$: the enzyme tetrahedral intermediate ET does not accumulate in substantial concentration along the reaction pathway.

The comparison of Eqns (10) and (11) for the catalytic rate constants in the enzymic and non-enzymic reactions shows that they are composed of identical quantities: the rate constants for the breakdown of the tetrahedral intermediates ET and NT and the equilibrium constants of their formation $K_{ET}$ and $K_{NT}$. Therefore, the comparison of the sign and magnitudes of the $q$-values calculated for $k_{cat}$ and $k_{+3}^i$ should give some implications for the mechanism of enzyme action on specific anilides.

The analysis of the pertinent transition-state structures in the model reactions\[28,31,32] reveals that the substituent effect on $k_{+3}^i$ should be slightly negative in the case of general acid catalysis and considerably, positive in the case of direct general or specific base catalysis of the breakdown of NT. In the former case proton transfer to anilide nitrogen in NT is important and should be observed in the high $\sigma$-value region, while in the second case the substituent effect reflects heavy-atom reorganization for the breakdown of NT and should be observed in the high $\sigma$-value region. When, however, the general catalyst acts as a general base and general acid at the same time (bifunctional general acid-base catalyst), the intermediate $q$-value should be observed\[28].

The $q$-values calculated for the kinetic constants of our model reaction are presented in Table 5. They are, of course, subject to considerable error since are based on only two points. Nevertheless, some conclusions can be drawn. While the $q$-value for $k_{+3}$ is in the usual range, the very large positive ($q = 5.87$) substituent effect for $k_{+3}^i$ is observed when OH$^-$, acting as a general base, catalyses the breakdown of NT. Notably, the $q$-value for $k_{+3}^i$ is reduced to 3.98 (the difference is more significant when the $q$-value for $k_{+3}/k_{-1}$ are compared) when the overall rate is governed by the breakdown of NT catalysed by HCO$_3^{-}$. HCO$_3^{-}$ has been found to be an extremely effective catalyst in the anilide hydrolysis, which has been attributed to its capacity to act as bifunctional general acid-base catalyst\[28,33]. This suggests that, with HCO$_3^{-}$ as catalyst, proton donation to anilide nitrogen

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of NT begins to play a role, bringing about the reduction of the substituent dependence of $k_i$. This implies the first alternative explanation of the small positive substituent dependence found in the urokinase-catalysed hydrolysis of specific anilides with electron-accepting $p$-substituents, i.e. enzyme active site acting as a bifunctional general acid-base catalyst in the rate-limiting breakdown of ET reduces the reaction constant to the observed value of +0.72.

The second alternative explanation for the low sensitivity of $k_{cat}$ to the leaving group structure arises from the mechanism of the model reaction of Eqn (7) at sufficiently high catalyst concentration $[C_i]$: under this extreme conditions $\Sigma k_{i+3} [C_i]$ is much larger than $k_{-1}$ and Eqn (8) can be simplified to:

\[
\nu_{corr} = k_4 [\text{OH}^-] [S] \quad (13)
\]

\[
k_4 = k_{+1} \quad (14)
\]

is the catalytic constant for the nonenzymic reaction under this condition. The observed rate is independent of the catalyst concentration and $k_4$ is independent of the nature of catalytic species. Therefore the rate of the nonenzymatic reaction should be controlled by the formation of NT. This was observed for the basic hydrolysis of N-acetylglycine $p$-nitroanilide and N-acetylglycine $p$-carboxyanilide [28].

Assuming as in the nonenzymic reaction that in the enzyme mechanism of Eqn (6) the formation of ET is rate-limiting, i.e. $k_{-1}$, $k_{+1}$, $k_{+2}$, $k_{+3} \gg k_{+11}$, the Dixon and Webb expressions for the Michaelis-Menten parameters are simplified to:

\[
k_{cat} = k_{+1} \frac{k_{+2}}{k_{-1} + k_{+2}} \quad (15)
\]

and

\[
K_m(\text{app}) = \frac{k_{-1}}{k_{+1}} = K_s. \quad (16)
\]

As in the case of a rate-limiting breakdown of ET, the apparent Michaelis-Menten constant $K_m(\text{app})$ should be equal to the substrate constant and therefore, substituent independent. This is observed (Table 4).

Eqn (15) for $k_{cat}$, may be further simplified assuming that a more effective concerted general acid-base catalysis of the breakdown of ET increases the rate of this step so that it exceeds the rate of the breakdown of ET to ES, i.e. $k_{+2} \gg k_{-1}$. Then Eqn (15) can be written as:

\[
k_{cat} = k_{+1} \quad (17)
\]

and the enzymic catalytic rate constant $k_{cat}$ should increase with increasing the electron withdrawal power of the $p$-substituents because of their favourable effect on $k_{+1}$. This is observed (Fig. 3). Moreover, a good agreement between the reaction constants for the nonenzymic ($q = +0.78$, Table 5) and enzymic ($q = +0.72$, Fig. 3) catalytic rate constants is observed.

Thus the small positive $q$-value for the catalytic rate constant $k_{cat}$ of the urokinase-catalysed hydrolysis of $\alpha$-N-acetyl-L-lysine high $\sigma^+$-value anilides, and similar results obtained for the hydrolysis of such anilides by other serine proteinases may be accounted for by two different mechanisms. The first one also explains the results obtained in the studies covering the low $\sigma^+$-value region. Compelling proof, however, for the discrimination between them is required.

We would like to thank Miss I. Stoineva for her skilful technical assistance.

**REFERENCES**


**Table 5. Hammett $q$-values associated with the rate constants for the basic hydrolysis of N-acetylglycine anilides**

The values are calculated based on only two points

<table>
<thead>
<tr>
<th>Constants</th>
<th>$q$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{+1}$</td>
<td>+0.78</td>
</tr>
<tr>
<td>$k_{+1}^{\text{OH}}/k_{-1}$</td>
<td>+0.59</td>
</tr>
<tr>
<td>$k_{+1}^{\text{O}}/k_{-1}$</td>
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<td>$k_{+1}^{\text{CO}}/k_{-1}$</td>
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<tr>
<td>$k_{+1}^{\text{OH}}$</td>
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<td>+5.23</td>
</tr>
<tr>
<td>$k_{+1}^{\text{CO}}$</td>
<td>+3.98</td>
</tr>
</tbody>
</table>

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32. Petkov, E. Christova, I. Pojarlieff, and N. Stambolieva: Substituent Effects in the High $\sigma^*$-Value Region


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