Chemical synthesis and papain-catalysed hydrolysis of \( N\alpha \)-benzyloxy carbonyl-L-lysine \( p \)-nitroanilide

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1. The chemical synthesis of \( N\alpha \)-benzyloxy carbonyl-L-lysine \( p \)-nitroanilide (Z-Lys-pNA) is described in detail. 2. The pH-dependence of the catalytic parameters \( k_{cat} \), \( K_m \) and \( k_{cat}/K_m \) for the papain-catalysed hydrolysis of Z-Lys-pNA are determined. \( k_{cat} \) and \( K_m \) are pH-independent between pH 5 and pH 7.42, but the pH-dependence of \( k_{cat}/K_m \) is bell-shaped, decreasing at high and low pH values with \( pK_a \) values of 7.97 and 4.40 respectively. 3. The catalytic parameters and their pH-dependence are shown to be similar to those reported for other anilide substrates and it is concluded that the \( K_m \) value of 0.01 mM previously reported [Angelides & Fink (1979) Biochemistry 18, 2355–2369] is incorrect. The possibility of accumulating a tetrahedral intermediate during the papain-catalysed hydrolysis of Z-Lys-pNA is discussed.

The cysteine proteinase papain (EC 3.4.22.2) has an extended active site containing binding sites for at least seven amino acids \( (H_4N^+−S_4−S_3−S_2−S_1−S_1′−S_2′−S_3′−CO_2−) \) (Schechter & Berger, 1967; Berger & Schechter, 1970). Peptide \( (H_4N^+−P_4−P_3−P_2−P_1−P_1′−P_2′−P_3′−CO_2−) \) hydrolysis occurs between \( P_1 \) and \( P_1′ \) of the substrate.

The \( S_2 \) subsite shows a strong preference for hydrophobic residues such as L-phenylalanine and is the primary specificity site for peptide hydrolysis between \( P_1 \) and \( P_1′ \). Substrates with lysine or arginine at \( S_1 \) are also good substrates but the positively charged side chains are not essential for hydrolysis (Bender & Brubacher, 1966; Gray et al., 1984).

\( p \)-Nitroanilide substrates provide a convenient spectrophotometric assay for proteolytic enzymes (Erlanger et al., 1961; Gray et al., 1984). Moreover, interest in these substrates has been greatly promoted by recent claims that they provided spectrophotometric evidence for the accumulation of tetrahedral intermediates in reactions catalysed by elastase (Hunkapiller et al., 1976), trypsin (Petkov, 1978; Compton & Fink, 1980) and papain (Angelides & Fink, 1979a,b). However, subsequent studies of the trypsin- and elastase-catalysed hydrolysis of \( p \)-nitroanilide substrates led to the claim that these results were not reproducible and could be ascribed to experimental artifacts and substrate isomerization (Markley et al., 1981). Earlier claims (Compton & Fink, 1980) of direct observation of a tetrahedral intermediate in the trypsin catalysed hydrolysis of Z-Lys-pNA have been retracted and the spectral changes assigned to enzyme isomerization (Coll et al., 1982; Compton & Fink, 1984).

Attempts to detect a tetrahedral intermediate during the papain-catalysed hydrolysis of anilide substrates under ambient conditions had been unsuccessful (Lowe & Yuthavong, 1971b), but cryoenzymological studies of the papain-catalysed hydrolysis of Z-Lys-pNA have purported to show direct evidence for the accumulation of a tetrahedral intermediate (Angelides & Fink, 1979a,b).

These authors (Angelides & Fink, 1979a,b) report that in fully aqueous media at 25°C the apparent Michaelis constant \( (K_m) \) for the papain-catalysed hydrolysis of Z-Lys-pNA is 10 \( \mu \)M. This \( K_m \) value is at least two orders of magnitude lower than the \( K_m \) values obtained for any other anilide substrate of papain (see Table 1). Since the accumulation of an intermediate during catalysis would be expected to lower both \( K_m \) and \( k_{cat} \) (Fersht & Renard, 1974), a low \( K_m \) value would appear to support their claim that a tetrahedral intermediate accumulates. However, Z-Lys-pNA
is not at present commercially available and the anomalously low $K_m$ has not been confirmed.

In the present investigation we describe the chemical synthesis of Z-Lys-pNA and determine the catalytic parameters for its hydrolysis by papain. The catalytic parameters obtained are compared with those obtained by using other anilide substrates.

Materials and methods

Materials

Papaya latex was obtained from Powell and Scholefield Ltd., Liverpool L7 3JG, U.K. 2,2′-Dipyridyl disulphide was purchased from Aldrich Chemical Co., and $p$-nitrophenylisocyanate (lot no. 062883) was purchased from Alfa Products, Danvers, MA, U.S.A.

Methods

Although the enzymic hydrolysis of Z-Lys-pNA has been described extensively (Angelides & Fink, 1979a,b; Coll et al., 1982; Compton & Fink, 1984), there is scant spectral or physical data [a melting point of 86–88°C was reported by Angelides & Fink (1979a,b) and Compton & Fink (1984)] and no synthetic procedures were given to support either the purity or the identity of this compound. To remedy this situation, we herein report a detailed synthesis with corroborating spectral and physical evidence.

$^{13}$C n.m.r. spectra were recorded by a Bruker WM300 wide-bore spectrometer at 75.47 MHz for $^{13}$C nuclei. Chemical shifts (δ) are given in p.p.m. relative to tetramethylsilane at 0 p.p.m. Mass spectra were recorded by a Kratos MS50 spectrometer operating in the FAB (fast atom bombardment) mode. Melting points are uncorrected.

The bis-protected lysine derivative Z-Lys(Boc) (2, Scheme 1) was prepared from lysine mono-hydrochloride (I, Scheme 1) in an overall 44% yield, following the synthesis of Schwyzer & Rittel (1961). Conversion of 2 to Z-Lys-pNA (4, Scheme 1) paralleled the route of Nishi et al. (1970) who prepared $N$-substituted L-arginine $p$-nitroanilide derivatives.

Synthesis of Z-Lys(Boc)-pNA (3)

To a stirred solution of Z-Lys(Boc) (2) (0.34 g, 0.5 mmol) in dry acetonitrile (3 ml), freshly prepared 3M HCl in diethyl ether (20 ml) was added with stirring at room temperature. The stopped reaction mixture was then left for 3 h during which time a colourless precipitate formed. The latter was filtered, washed with dry acetonitrile and dried. Recrystallization from acetonitrile gave the desired product as a colourless microcrystalline material, m.p. 169–172°C (literature m.p. 86–88°C; Angelides & Fink (1979a,b); Compton & Fink (1984)] (0.19 g, 87%). (Found: C, 54.7; H, 5.6; N, 12.8. C$_{30}$H$_{27}$ClIN$_2$O$_5$ requires C, 55.0; H, 5.7; N, 12.8%). m/z (%), negative ion mode: 435(M−Cl)$^-$ (50), 400(M−H−Cl)$^-$ (29), 291 (100), 285 (30), 249 (10), 214 (45); positive ion mode: 401(M+Cl)$^+$ (100). $^{13}$C n.m.r. δ (p.p.m.) (C$_2$H$_5$SO) 22.34 (C-4), 26.23 (C-5), 30.68 (C-3), 38.22 (C-6), 55.46 (C-6), 65.40 (C-8), 118.85, 124.77, 128.22 (C-10), 11.12, 13.14, 16.17, 19, 20), 135.93 (C-9), 143.51 (C-15), 143.80 (C-18), 156.58 (C-7), 169.98 (C-21), 171.05 (C-1).

Preparation of Z-Lys(Boc)-pNA monohydrochloride (4)

To a solution of Z-Lys(Boc)-pNA (3) (0.25 g, 0.5 mmol) in dry acetonitrile (3 ml), freshly prepared 3M HCl in diethyl ether (20 ml) was added with stirring at room temperature. The stopped reaction mixture was then left for 3 h during which time a colourless precipitate formed. The latter was filtered, washed with dry acetonitrile and dried. Recrystallization from acetonitrile gave the desired product as a colourless microcrystalline material, m.p. 169–172°C (literature m.p. 86–88°C; Angelides & Fink (1979a,b); Compton & Fink (1984)] (0.19 g, 87%). (Found: C, 54.7; H, 5.6; N, 12.8. C$_{30}$H$_{27}$ClIN$_2$O$_5$ requires C, 55.0; H, 5.7; N, 12.8%). m/z (%), negative ion mode: 435(M−H)$^-$ (50), 400(M−H−Cl)$^-$ (29), 291 (100), 285 (30), 249 (10), 214 (45); positive ion mode: 401(M+Cl)$^+$ (100). $^{13}$C n.m.r. δ (p.p.m.) (C$_2$H$_5$SO) 22.34 (C-4), 26.23 (C-5), 30.68 (C-3), 38.22 (C-6), 55.46 (C-6), 65.40 (C-8), 118.85, 124.77, 128.22 (C-10), 11.12, 13.14, 16.17, 19, 20), 135.93 (C-9), 143.51 (C-15), 143.80 (C-18), 156.58 (C-7), 169.98 (C-21), 171.05 (C-1).

Preparation of fully active papain

Papain was isolated from papaya latex essentially as described by Baines & Brocklehurst (1979) except that 12.5 g of latex/250 ml of solution were used. Chromatography using SP-Sephadex-50 (Baines & Brocklehurst, 1979) showed that only a single protein was isolated in this way. Fully active papain was then prepared by covalent chromatography (Brocklehurst et al., 1973). Papain prepared in this way was incubated for 20 min at pH 8.0 in the presence of 20mm-L-cysteine before removal of low-$M$ compounds by chromatography on Sephadex G-25. Papain was shown to be free of

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any contaminating chymopapains by its equal thiol titration at pH 4 and pH 8 (Baines & Brocklehurst, 1978, 1979) and its identity was confirmed by stopped-flow analysis (Malthouse & Brocklehurst, 1976) of its reactivity towards 2,2’-dipyridyl disulphide (Brocklehurst & Little, 1972; Baines & Brocklehurst, 1978, 1979).

Spectrophotometric and enzymic analysis of Z-Lys-pNA

In fully aqueous media, $\lambda_{\text{max}} = 314 \text{nm}$ and $\varepsilon_{314} = 13900 \text{M}^{-1} \text{cm}^{-1}$. These values obtained for Z-Lys-pNA are very similar to the values ($\lambda_{\text{max}} = 315 \text{nm, } \varepsilon_{315} = 13000 \text{M}^{-1} \text{cm}^{-1}$) obtained for Bz-Arg-pNA (‘BAPA’) (Erlanger et al., 1961).

Spectrophotometric analysis after hydrolysis by trypsin at pH 6.5, 25°C showed that $\geq 90\%$ of Z-Lys-pNA was hydrolysed, whereas with Z-Lys-pNA prepared using DL- or D-lysine either approx. 50\% or 0\% hydrolysis was detected over the same period of time. Similar results were obtained by Erlanger et al., (1961) with DL-Bz-Arg-pNA. This therefore confirms that the synthesis of Z-Lys-pNA is stereospecific.

Kinetic studies

Steady-state kinetic parameters were determined at 25°C, I 0.1 M, 1 mM-EDTA, by using a thermostatted Varian 2200 spectrophotometer. Michaelis parameters were determined from initial rate data by computer fitting to the hyperbolic Michaelis–Menten equation (Wilkinson, 1961). The pH-dependent $k_{\text{cat}}/K_m$ data were computer fitted as described by Cleland (1979).

The concentration of fully active papain was determined by thiol titration with 2,2’-dipyridyl disulphide (Baines & Brocklehurst, 1978).

Results and discussion

The catalytic parameters obtained in the present study for the papain-catalysed hydrolysis of Z-Lys-pNA are presented in Table 1. The values of $k_{\text{cat}}/K_m$ are also shown in Fig. 1 along with the
Table 1. Catalytic parameters for the papain-catalysed hydrolysis of p-nitroanilide substrates
Hydrolysis of Z-Lys-pNA was studied at 25°C, 7.0 1M, [S]0 0.1–24 mM, [E]0 0.5–0.9 μM; all buffers contained 1 mM-EDTA.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>kcat. (s⁻¹)</th>
<th>Kₘ (mM)</th>
<th>kcat./Kₘ (M⁻¹s⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-Lys-pNA</td>
<td>4.28</td>
<td>0.122 ± 0.004</td>
<td>6.04 ± 0.39</td>
<td>20.1</td>
<td>Present work</td>
</tr>
<tr>
<td>Z-Lys-pNA</td>
<td>4.98</td>
<td>0.240 ± 0.020</td>
<td>7.45 ± 1.27</td>
<td>32.2</td>
<td>Present work</td>
</tr>
<tr>
<td>Z-Lys-pNA</td>
<td>5.88</td>
<td>0.211 ± 0.052</td>
<td>5.90 ± 2.31</td>
<td>35.8</td>
<td>Present work</td>
</tr>
<tr>
<td>Z-Lys-pNA</td>
<td>7.42</td>
<td>0.174 ± 0.027</td>
<td>6.36 ± 1.55</td>
<td>27.3</td>
<td>Present work</td>
</tr>
<tr>
<td>Z-Lys-pNA</td>
<td>6.10</td>
<td>0.25</td>
<td>0.01</td>
<td>25000</td>
<td></td>
</tr>
<tr>
<td>Z-Cit-pNA</td>
<td>7.0</td>
<td>0.35</td>
<td>14.3</td>
<td>24.5</td>
<td>Gray et al. (1984)</td>
</tr>
<tr>
<td>Z-Phe-Cit-pNA</td>
<td>7.0</td>
<td>42.56</td>
<td>2.23</td>
<td>191000</td>
<td>Gray et al. (1984)</td>
</tr>
<tr>
<td>Ac-Phe-Gly-pNA</td>
<td>6.0</td>
<td>1.3 ± 0.18</td>
<td>0.88 ± 0.13</td>
<td>1500</td>
<td>Lowe &amp; Yuthavong</td>
</tr>
<tr>
<td>Bz-Arg-pNA ('BAPA')</td>
<td>5.96</td>
<td>0.74 ± 0.03</td>
<td>2.86 ± 0.13</td>
<td>257.3</td>
<td>Mole &amp; Horton (1973)</td>
</tr>
</tbody>
</table>

Fig. 1. pH-dependence of kcat/Km for the papain-catalysed hydrolysis of Z-Lys-pNA

The experimental data were fitted to the equation:

\[(k_{cat}/K_m)_{0.1} = \frac{k}{(1 + [H^+]/K_a + K_b/[H^+])}\]

as described in the Materials and Methods section. The fitted parameters obtained were: \(k = 38.11 ± 3.60\text{M}^{-1}\text{s}^{-1}\), \(K_a = 4.40 ± 0.11\) and \(K_b = 7.97 ± 0.1\). The solid line was calculated by using these parameters. Experimental details were: 70 1M, 25°C. Data obtained from Table 1; ○, data obtained when \(K_m \approx S_0\) ([S]₀ = 0.25 mM, [E]₀ = 1 μM); all buffers contained 1 mM-EDTA.

additional values when \(K_m \approx S_0\). The values of kcat./Km were maximal at pH 6, and decreased with decreasing or increasing pH according to \(K_a\) values of 4.4 and 8.0 respectively. A similar pH-dependence of kcat./Km is observed for Bz-Arg-pNA ('BAPA') (Mole & Horton, 1973), Ac-Phe-Gly-pNA (Lowe & Yuthavong, 1971b) and several other substrates (Lowe, 1976, and references therein). Both kcat. and Km were essentially pH-independent between pH 5.0 and pH 7.4 (Table 1), as is also observed for Ac-Phe-Gly-pNA (Lowe & Yuthavong, 1971b) and Bz-Arg-pNA (Mole & Horton, 1973). From values of kcat. and Km in Table 1, we estimate that their pH-independent values between pH 5 and pH 7.4 are kcat. = 0.208 ± 0.033 s⁻¹ and Km = 6.6 ± 0.8 mM. Using these values, kcat./Km is estimated to be 31.5 ± 3.8 M⁻¹ s⁻¹, which is in reasonable agreement with the value of 38.11 ± 3.60 M⁻¹ s⁻¹ obtained from the more extensive data in Fig. 1.

The larger value of kcat./Km for Bz-Arg-pNA compared with Z-Lys-pNA (Table 1) shows that papain is more specific for Bz-Arg-pNA. For N-substituted benzoyl- and Z-glycine methyl esters k+2 values are essentially the same, but Km is three times lower for the Z derivative (Yuthavong & Suttimool, 1978). This demonstrates that the Z group is bound three times more strongly in S2 than is the benzoyl group. The values of kcat./Km are 38.11 M⁻¹ s⁻¹ (present work) and 251 M⁻¹ s⁻¹ (Mole & Horton, 1973) for Z-Lys-pNA and Bz-Arg-pNA respectively. Allowing for the more efficient binding of the Z group, this shows that papain is approx. 20 times (251 × 3/38.11) more specific for arginine than for lysine at the S1 subsite.

Papain-catalysed hydrolyses are thought to proceed via a minimal three-step mechanism [eqn. (1); Lowe, 1976]:

\[
E + S \xrightarrow{k_{-1}} ES \xrightarrow{k_{+2}} ES' \underset{k_{-3}}{\xrightarrow{k_{+3}}} E + P_2
\]

where \(k_{cat} = k_{+2} \cdot k_{+3}/(k_{+2} + k_{+3})\), \(K_m = K_m^* \cdot k_{+3}/(k_{+2} + k_{+3})\) and \(K_m^* = (k_{-1} + k_{+2})/k_{+1}\). If \(k_{-1} \gg k_{+2}\) and acylation is rate limiting (\(k_{+3} \gg k_{+2}\)) then \(k_{cat} \approx k_{+2}\), \(K_m = k_{+1}/k_{+1} = K_a\), and \(k_{cat}/K_m = k_{+2}/K_a\). Using Z-Lys-pNP, Bender & Brubacher (1966) determined that \(k_{+3} = 45.9 ± 1.6 \text{s}^{-1}\). Thus
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for Z-Lys-pNA, \( k_{+3} \gg k_{+2} \) and therefore \( k_{\text{cat.}} = k_{+2} \) and \( K_m = K_c \). In a similar manner, Lowe & Yuthavong (1971b) showed that for Ac-Phe-Gly-pNA, \( k_{\text{cat.}} \approx k_{+2} \) and \( K_m \approx K_c \). For Bz-Arg-pNA (Mole & Horton, 1973) \( k_{+2} \) and \( k_{+3} \) are of a similar magnitude and \( K_c \) was estimated as 7.86 mM. This value is very similar to the value of \( K_m (K_m = K_c) \) obtained in the present study for Z-Lys-pNA.

Also the pH-dependence of the catalytic parameters is typical of those observed for other anilide substrates (Lowe & Yuthavong, 1971a,b; Mole & Horton, 1973). In general, \( K_m \) values for anilide substrates are approx. 1 mM or greater (Table 1) and are often a good approximation to \( K_c \) values (if \( k_{+3} \gg k_{+2} \), and \( k_{+3} \gg k_{+2} \)).

It is clear therefore that the \( K_m \) value of 10 \( \mu \text{M} \) (pH 6.1; Angelides & Fink, 1979a,b) for the papain-catalysed hydrolysis of Z-Lys-pNA is anomalous. Angelides & Fink (1979a,b) claimed to obtain spectrophotometric evidence for accumulation of a tetrahedral intermediate in fully aqueous media and under cryoenzymological conditions. When substrate binding involves charge neutralization, \( K_c \) values are expected to decrease in dimethyl sulphoxide cryosolvents (Malthouse & Scott, 1983) but when substrate binding does not involve charge neutralization, then \( K_c \) values should be increased in dimethyl sulphoxide cryosolvents. Therefore we would predict that \( K_m \) values (\( K_m = K_c \)) for the papain-catalysed hydrolysis of Z-Lys-pNA will be raised in dimethyl sulphoxide cryosolvents as substrate binding is due to hydrophobic enzyme–substrate interactions (Bender & Brubacher, 1966; see also Table 1). This prediction is supported by the fact that in the presence of 5% (v/v) dimethyl sulphoxide \( k_{\text{cat.}} \) values were essentially unchanged relative to 100% aqueous media but \( K_m \) increased by 24% for the papain-catalysed hydrolysis of Bz-Arg-pNA (Mole & Horton, 1973).

A \( K_m \) value less than the substrate concentration is essential for stoichiometric accumulation of an intermediate (Fersht & Renard, 1974; Malthouse & Scott, 1983). Therefore, claims that an intermediate can be detected at substrate concentrations of 1 mM in fully aqueous media and under cryoenzymological conditions (Angelides & Fink, 1979a,b) must be viewed with caution. As reported for Bz-L-Arg-pNA (Mole & Horton, 1973), solutions of Z-Lys-pNA became cloudy at high pH values and therefore accurate catalytic parameters could not be obtained at such values. However, at pH 9 in borate buffer, no significant deviation from second-order kinetics was observed for Z-Lys-pNA concentrations up to 3 mM and hence \( K_m \approx 3 \text{mM} \). We are unable to explain the low \( K_m \) values (\( K_m = 10 \mu \text{M} \) at pH 6.1; \( K_m = 35 \mu \text{M} \) at pH 9.16) reported by Angelides & Fink (1979a,b).

It should however, be pointed out that similar claims (Compton & Fink, 1980) that a tetrahedral intermediate was observed in the trypsin-catalysed hydrolysis of Z-Lys-pNA have been criticized and assigned to experimental artifacts and/or substrate isomerization (Markley et al., 1981). Subsequently, the experimental evidence previously cited (Compton & Fink, 1980) as providing direct evidence for the accumulation of a tetrahedral intermediate has been attributed to a cryosolvent-induced isomerization of trypsin (Coll et al., 1982; Compton & Fink, 1984).

Since in the present study the substrate and enzyme identity and purity have been exhaustively checked, we believe that the catalytic parameters presented here are the true catalytic parameters for the papain-catalysed hydrolysis of Z-Lys-pNA.

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References
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