Chemical synthesis and papain-catalysed hydrolysis of *N*- α -benzyloxycarbonyl-L-lysine *p*-nitroanilide

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1. The chemical synthesis of *N*- α -benzyloxycarbonyl-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 ($\text{H}_3\text{N}^+-\text{S}_4-\text{S}_3-\text{S}_2-\text{S}_1-\text{S}_1'-\text{S}_2'-\text{S}_3'-\text{CO}_2^-$) (Schechter & Berger, 1967; Berger & Schechter, 1970). Peptide ($\text{H}_3\text{N}^+-\text{P}_4-\text{P}_3-\text{P}_2-\text{P}_1-\text{P}_1'-\text{P}_2'-\text{P}_3'-\text{CO}_2^-$) 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, 1979*a,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, 1971*b*), 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, 1979*a,b*).

These authors (Angelides & Fink, 1979*a,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 μM . 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

Abbreviations used: Z-, *N*- α -benzyloxycarbonyl; Bz-, *N*- α -benzoyl; Boc-, *t*-butoxycarbonyl; -pNA, *p*-nitroanilide; -pNP, *p*-nitrophenyl ester; Ac-, *N*- α -acetyl; Cit, citrulline.

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 monohydrochloride (1, 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, 0.9 mmol) in freshly distilled (from 1.3 nm molecular sieves) hexamethyl phosphoramide, dry freshly distilled (from NaOH pellets) triethylamine (0.128 ml, 0.9 mmol) was added at 20°C. Finely powdered *p*-nitrophenylisocyanate (0.30 g, 1.83 mmol) was then added to the reaction mixture which was then stoppered and left to stir at 20°C for 24 h. The precipitate which formed on the addition of 2% (w/v) NaHCO_3 (100 ml) was filtered and washed

successively with 2% (w/v) NaHCO_3 , water, 0.5 M-HCl and water. The filtrate was then extracted with hot dioxane (100 ml), cooled and the bis(*p*-nitrophenyl)urea, a by-product of this type of reaction (Nishi *et al.*, 1970), was filtered off. Water (220 ml) was added to the dioxane solution and the precipitate which formed was filtered and dried. Column chromatography on silica gel using a mobile phase gradient of chloroform to ethyl acetate/chloroform (3:17, v/v) gave a colourless, viscous oil which was crystallized from chloroform/diethyl ether to give Z-Lys(Boc)-pNA as colourless needles, m.p. 127–129°C (0.28 g, 62%). (Found: C, 59.7; H, 6.2; N, 11.5; $\text{C}_{25}\text{H}_{32}\text{N}_4\text{O}_7$ requires C, 60.0; H, 6.4; N, 11.2%). ^{13}C n.m.r.: δ (p.p.m.) (C_2HCl_3) 22.34 (C-4), 28.42 (C-23), 29.38 (C-5), 31.05 (C-3), 39.20 (C-6), 55.79 (C-2), 67.43 (C-8), 79.56 (C-22), 119.22, 124.83, 127.98, 128.32, 128.57 (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-L-Lys-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 3 M-HCl in diethyl ether (20 ml) was added with stirring at room temperature. The stoppered 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. $\text{C}_{20}\text{H}_{25}\text{ClN}_4\text{O}_5$ requires C, 55.0; H, 5.7; N, 12.8%). m/z (%), negative ion mode: 435($M-\text{H}$)[−] (50), 400($M-\text{H}-\text{Cl}$)[−] (29), 291 (100), 285 (30), 249 (10), 219 (45); positive ion mode: 401($M-\text{Cl}$)⁺ (100). ^{13}C n.m.r. δ (p.p.m.) ($\text{C}_2^2\text{H}_6\text{SO}$) 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, 127.64, 128.22 (C-10,11,12,13,14,16,17,19,20), 136.80 (C-9), 142.16 (C-15), 145.14 (C-18), 156.65 (C-7), 172.02 (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 20 mM-L-cysteine before removal of low- M_r compounds by chromatography on Sephadex G-25. Papain was shown to be free of

Scheme 1. *Synthesis of Z-Lys-pNA*
Further abbreviation used: HMPA, hexamethyl phosphoramide.

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 $\epsilon_{314} = 13900\text{M}^{-1}\cdot\text{cm}^{-1}$. These values obtained for Z-Lys-pNA are very similar to the values ($\lambda_{\text{max}} = 315\text{nm}$, $\epsilon_{315} = 13000\text{M}^{-1}\cdot\text{cm}^{-1}$) 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 0.1M, 1mM-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_{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_{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, I 0.1 M, $[S]_0$ 0.1–24 mM, $[E]_0$ 0.5–0.9 μ M; all buffers contained 1 mM-EDTA.

Substrate	pH	$k_{cat.}$ (s^{-1})	K_m (mM)	$k_{cat.}/K_m$ ($M^{-1}\cdot s^{-1}$)	Reference
Z-Lys-pNA	4.28	0.122 ± 0.004	6.04 ± 0.39	20.1	Present work
Z-Lys-pNA	4.98	0.240 ± 0.020	7.45 ± 1.27	32.2	Present work
Z-Lys-pNA	5.88	0.211 ± 0.052	5.90 ± 2.31	35.8	Present work
Z-Lys-pNA	7.42	0.174 ± 0.027	6.36 ± 1.55	27.3	Present work
Z-Lys-pNA	6.10	0.25	0.01	25000	Angelides & Fink (1979a,b)
Z-Cit-pNA	7.0	0.35	14.3	24.5	Gray <i>et al.</i> (1984)
Z-Phe-Cit-pNA	7.0	42.56	2.23	191000	Gray <i>et al.</i> (1984)
Ac-Phe-Gly-pNA	6.0	1.3 ± 0.18	0.88 ± 0.13	1500	Lowe & Yuthavong (1971b)
Bz-Arg-pNA ('BAPA')	5.96	0.74 ± 0.03	2.86 ± 0.13	257.3	Mole & Horton (1973)

Fig. 1. *pH-dependence of $k_{cat.}/K_m$ for the papain-catalysed hydrolysis of Z-Lys-pNA*

The experimental data were fitted to the equation:

$$(k_{cat.}/K_m)_{obs.} = \tilde{k}/(1 + [H^+]/K_a + K_b/[H^+])$$

as described in the Materials and methods section.

The fitted parameters obtained were: $\tilde{k} = 38.11 \pm 3.60 M^{-1}\cdot s^{-1}$, $pK_a = 4.40 \pm 0.11$, $pK_b = 7.97 \pm 0.1$. The solid line was calculated by using these parameters. Experimental details were: I 0.1 M, 25°C. $\blacktriangle$, Data obtained from Table 1; $\bullet$, data obtained when $K_m \gg S_0$ ($[S]_0 = 0.25$ mM, $[E]_0 = 1 \mu$ M); all buffers contained 1 mM-EDTA.

additional values when $K_m \gg S_0$. The values of $k_{cat.}/K_m$ were maximal at pH 6, and decreased with decreasing or increasing pH according to pK_a values of 4.4 and 8.0 respectively. A similar pH-dependence of $k_{cat.}/K_m$ 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 $k_{cat.}$ and K_m 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 $k_{cat.}$ and K_m in Table 1, we estimate that their pH-independent values between pH 5 and pH 7.42 are $\tilde{k}_{cat.} = 0.208 \pm 0.033 s^{-1}$ and $\tilde{K}_m = 6.6 \pm 0.8$ mM. Using these values, $\tilde{k}_{cat.}/\tilde{K}_m$ is estimated to be $31.5 \pm 3.8 M^{-1}\cdot s^{-1}$, which is in reasonable agreement with the value of $38.11 \pm 3.60 M^{-1}\cdot s^{-1}$ obtained from the more extensive data in Fig. 1.

The larger value of $k_{cat.}/K_m$ 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 K_s is three times lower for the Z derivative (Yuthavong & Suttimool, 1978). This demonstrates that the Z group is bound three times more strongly in S_2 than is the benzoyl group. The values of $\tilde{k}_{cat.}/\tilde{K}_m$ are $38.11 M^{-1}\cdot s^{-1}$ (present work) and $251 M^{-1}\cdot s^{-1}$ (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 ($251 \times 3/38.11$) more specific for arginine than for lysine at the S_1 subsite.

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

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 $k_{cat.} = k_{+2}$, $K_m = k_{-1}/k_{+1} = K_s$, and $k_{cat.}/K_m = k_{+2}/K_s$. Using Z-Lys-pNP, Bender & Brubacher (1966) determined that $\tilde{k}_{+3} = 45.9 \pm 1.6 s^{-1}$. Thus

for Z-Lys-pNA, $\tilde{k}_{+3} \gg \tilde{k}_{+2}$ and therefore $k_{\text{cat.}} = k_{+2}$ and $K_m = K_s$. In a similar manner, Lowe & Yuthavong (1971b) showed that for Ac-Phe-Gly-pNA, $k_{\text{cat.}} \approx k_{+2}$ and $K_m \approx K_s$. For Bz-Arg-pNA (Mole & Horton, 1973) $\tilde{k}_{+2}$ and $\tilde{k}_{+3}$ are of a similar magnitude and K_s was estimated as 7.86 mM. This value is very similar to the value of K_m ($K_m = K_s$) 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_s values (if $k_{+3} \gg k_{+2}$, and $k_{-1} \gg k_{+2}$).

It is clear therefore that the K_m value of 10 μM (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_s values are expected to decrease in dimethyl sulphoxide cryosolvents (Malthouse & Scott, 1983) but when substrate binding does not involve charge neutralization, then K_s values should be increased in dimethyl sulphoxide cryosolvents. Therefore we would predict that K_m values ($K_m = K_s$) 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 $K_m \gg 3 \text{ mM}$. 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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