THE ACTION OF ELASTASE ON p-NITROANILIDE SUBSTRATES

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Received May 3, 1973

Summary : The action of elastase has been studied on four p-nitroanilides : BOC-$(Ala)_2$-NA, $(Ala)_3$-NA, Ac-$(Ala)_3$-NA and BOC-$(Ala)_3$-NA. The second order rate constant kcat/Km increases considerably with the chain length of these substrates. With $(Ala)_3$-NA, activation by excess substrate was observed. DMF and DMSO inhibit strongly the elastase catalyzed hydrolysis of Ac- and BOC-$(Ala)_3$-NA. The later substrate may be used to assess rapidly elastase activity : concentrations as low as 0.2 µg/ml may be determined accurately.

Pancreatic elastase has aroused more and more interest in recent years (1). Investigations with synthetic model substrates have indicated that this enzyme hydrolyzes links involving the carboxyl group of non-aromatic uncharged aminoacids like L-alanine and that its active center may be composed of several subsites (2-7).

Most of the compounds tested were esters of N-acylated aminoacids or peptides although studies have been done on amides or peptides (6, 7) which better mimic the physiological substrate elastin. Investigations involving the later substrates may however be greatly facilitaded by the use of a coloured leaving group like p-nitroaniline. The successful introduction of p-nitroanilides for the study of proteases (8) led us to design

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Abbreviations : BOC-$(Ala)_2$-NA : N-α-ter-butyloxycarbonyl-L-alanine-L-alanine-p-nitroanilide ; BOC-$(Ala)_3$-NA : N-α-ter-butyloxycarbonyl-$(L\text{-alanine})_3$-p-nitroanilide ; Ac-$(Ala)_3$-NA : N-α-acetyl-$(L\text{-alanine})_3$-p-nitroanilide ; $(Ala)_3$-NA : $(L\text{-alanine})_3$-p-nitroanilide hydrochloride. DMF : dimethylformamide ; DMSO : dimethylsulfoxyde.

such substrates for exploring the active center of elastase. A recent report (9) prompts us to disclose, at this time, the current status of our investigations.

EXPERIMENTAL

1° Preparation of the substrates :

a) BOC-$(Ala)_2$-NA was obtained by condensation of p-nitrophenyl BOC-L-alaninate with L-alanine-p-nitroanilide in methylene chloride for 24 h at room temperature. Tan cristals ; m. p. 198° (i-Pr OH) ; yield 81 %. Anal. Calcd. for $C_{17}H_{24}O_6N_4$: C 53.67, H 6.36, N 14.73 ; found : C 53.53, H 6.36, N 14.60.

b) (L-alanine)$_2$-p-nitroanilide, hydrochloride : the protective BOC group of compound a) was removed by a 30 minutes contact with dry-HCl-Ac-OH. Shining needles (MeOH-Et_2O) which decompose on melting ; yield 86 % ; $[\alpha]_D^{20}$ = - 58.2° (c = 1 in H_2O). Anal. Calcd. for $C_{12}H_{17}O_4N_4Cl$: C 45.50, H 5.41, N 17.69 ; found C 45.62, H 5.41, N 17.58.

c) BOC-$(Ala)_3$-NA : the base of compound b) was liberated by treatement with triethylamine in CH_2Cl_2 and acylated with p-nitrophenyl BOC-L-alaninate as described for compound a). Colorless cristals ; m. p. 210° (MeOH) ; yield 62 %. Anal. Calcd for $C_{20}H_{29}O_7N_5$: C 53.20, H 6.47, N 15.51 ; found C 53.04, H 6.37, N 15.69.

d) $(Ala)_3$-NA : compound c) was treated with HCl/AcOH as indicated for b). Tan cristals, m. p. 206° (abs. EtOH) yield 59 % ; $[\alpha]_D^{20}$ = - 147.1° (c = 1 in H_2O). Anal. Calcd. for $C_{15}H_{22}O_5N_5Cl$, H_2O : C 44.39, H 5.96, N 17.26 ; found : C 44.81, H 5.99, N 17.00.

e) Ac-$(Ala)_3$-NA : the base of compound d) was liberated as described for c) and acetylated with Ac_2O in chloroform. Colorless cristals ; m. p. > 280° (AcOH) ; yield 67 %. Anal. Calcd. for $C_{17}H_{23}O_6N_5$: C 51.90, H 5.89, N 17.80 ; found : C 52.04, H 5.82, N 17.65.

A detailed account of these preparative procedures will be published elsewhere. The compounds have absorbance maxima at 223 nm (peptide bonds) and 315-317 nm. The molar extinction coefficients at 315 nm (in 0.1 M Tris-buffer pH 8.0 and 0.37 % dimethylformamide) are 13,400 for BOC-$(Ala)_2$-NA, 14,700 for BOC-$(Ala)_3$-NA, 14,500 for Ac-$(Ala)_3$-NA and 12,050 for $(Ala)_3$-NA.

2° Porcine pancreatic elastase obtained from Worthington (code ESFF, lots 2 CB and 2 LA) was dissolved and stored in 0.1 M Tris-buffer pH 8.0. The molar concentration of enzyme solutions was determined using $E^{280}_{1\ \%} = 22.0$ and taking the molecular weight to be 25,000 (ref. 1).

3° Spectral absorption and kinetic measurements were carried out with a ZEISS PMQII spectrophotometer equipped with a thermostated cell compartment (25°) and a SERVOGOR RE 5 II/recorder.

a) Determination of kinetic constants. BOC-$(Ala)_2$-NA was dissolved in DMF. A mixture of 2.5 ml elastase and 0.2 ml substrate was incubated at 25°. After 15 minutes, 0.1 ml of 1 N HCl was added and the liberated p-nitroaniline was converted into a more coloured diazo-dye (10) by successive addition of 0.05 ml of each of the following aqueous reagents : 1.8 % sodium nitrate, 9 % ammonium sulfamate and 1.8 % N-naphtyl-1 N',N' diethylpropylene diamine, hydrochloride. The optical density was determined at 550 nm (10). BOC-$(Ala)_3$-NA and Ac-$(Ala)_3$-NA were dissolved either in DMF or in DMSO. Substrate solutions were added to elastase solutions in a 10 mm light-path cuvette (total volume 2.7 ml). The optical density at 410 nm was recorded up to more than 90 % completion of the reaction. The determination of the kinetic constants of $(Ala)_3$-NA will be reported elsewhere.

b) Assay of elastase activity (fig. 1) : BOC-$(Ala)_3$-NA was dissolved in DMF and $(Ala)_3$-NA in water (the later solution beeing adjusted to pH 8.0). 0.2 ml of BOC-$(Ala)_3$-NA or 0.5 ml of $(Ala)_3$-NA was added to 2.5 ml or 2.0 ml of elastase solution respectively in a 10 mm light-path cuvette and the optical density at 400 nm recorded for 2-3 minutes ($\varepsilon_{400} = 11{,}000$).

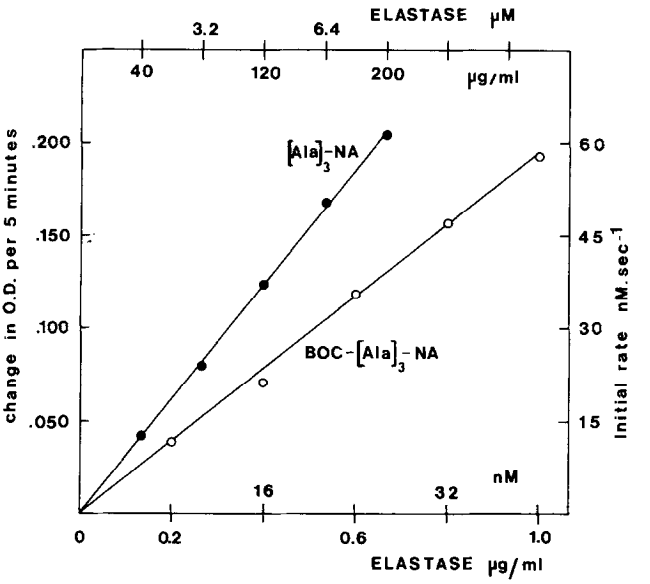

Fig. 1. Initial rate as a function of elastase concentration. $(Ala)_3$-NA (upper curve and upper scale) : 10 mM substrate, 80 mM Tris-buffer pH 8.0, 25°, reaction volume : 2.5 ml. BOC-$(Ala)_3$-NA (lower curve and lower scale) : 0.4 mM substrate, 92.6 mM Tris-buffer pH 8.0, 7.4 % (v/v) DMF, 25°, reaction volume : 2.7.

RESULTS

1° Kinetic properties of the four substrates : As expected, the N-acylated compounds were poorly soluble in water even in the presence of DMF or DMSO (so that no saturation kinetics could be observed. Assuming uncomplicated Michaelis-Menten behavior, $\frac{kcat}{Km}$ was estimated by making use of the simplified rate equation 1 $v = \frac{kcat}{Km}(E°)(S°)$ (1) which holds for $(S°) \ll Km$. In the case of BOC-$(Ala)_2$-NA which is a very poor substrate, v was measured at different substrate concentrations (0.1-0.4 mM) and a fixed elastase concentration (57 µM) and $\frac{kcat}{Km}$ was estimated from the linear plot relating v to (S°). For BOC-$(Ala)_3$-NA and Ac-$(Ala)_3$-NA which are more susceptible to elastase, use was made of the integrated form of equ. (1) :

$$\log \frac{(S)}{(S°)} = -\frac{K}{2.3} . t \quad (2)$$

where (S) is the substrate concentration remaining at any time t and $K = \frac{kcat}{Km}(E°)$. Measurements were made with different substrate, enzyme and solvent concentrations. The constant kcat/Km was calculated from a linear plot re-

TABLE I

Second order rate constants for the elastase catalyzed hydrolysis of p-nitroanilides at pH 8.0, 25°

SUBSTRATES	[Tris] mM	% DMF (v/v)	% DMSO (v/v)	kcat/Km (M^{-1}. sec^{-1})
BOC-$(Ala)_2$-NA(I)	92.6	7.4	0	0.34
$(Ala)_3$-NA(II)	100	0	0	3.4(a)
Ac-$(Ala)_3$-NA(III)	100	0.74	0	1,760
	92.6	7.4	0	745
	100	0	0.74	2,160
	92.6	0	7.4	1,240
BOC-$(Ala)_3$-NA(IV)	100	0.74	0	14,150
	92.6	7.4	0	4,300
	100	0	0.74	15,300
	92.6	0	7.4	7,500

(a) kcat = 7.9×10^{-3} sec^{-1} Km = 2.3 mM : from an Eadie plot (11) at low substrate concentration (no substrate activation)

lating K to (E°). Besides the considerable difference in reactivity between the di- and trialanine substrates, table I shows the inhibitory effect of DMSO and DMF (the later solvent beeing the most active). It could not be experienced whether these solvents act as competitive inhibitors like acetonitrile (3).

The elastase catalyzed hydrolysis of $(Ala)_3$-NA exhibited unusual saturation kinetics : an Eadie plot (11) of the effect of substrate concentration (0.55-97.5 mM) on initial velocity afforded a concaved line diagnosing activation by excess substrate. This phenomenon is now beeing studied in more details. The cons-

tants recorded in table I correspond to the uncomplicated kinetics observed at low substrate concentrations.

2° The usefulness of $(Ala)_3$-NA and BOC-$(Ala)_3$-NA for measuring elastase activity is illustrated in fig. 1 which shows the linear relationship between enzyme concentration and activity. No substrate autolysis occured. Very accurate activity measurements are practicable since the release of p-nitroaniline is zero-order during the assay time. BOC-$(Ala)_3$-NA is obviously the most convenient elastase substrate reported thus far. Ac-$(Ala)_3$-NA is about 5 times less effective.

If organic solvents are to be avoided in tests, the water-soluble but less sensitive substrate $(Ala)_3$-NA may be used as shown in fig. 1.

DISCUSSION

This paper describes some properties of p-nitroanilides, a new class of model substrates for elastase. In comparing the rate constants listed in table I to those found for other substrates, we shall assume that the elastase catalyzed hydrolyzis proceeds via an acylenzyme intermediate (12) so that kcat/Km is a second order acylation rate constant whatever may be the substrate (ester, amide, peptide or anilide) or its binding mode (13). For the sake of clarity, we shall also adopt the subsite nomenclature introduced by Schechter et al (7, 14).

As expected for nucleophilic catalyzis, the efficiency of Ac-$(Ala)_3$-NA lies between that of its corresponding amide and methyl-ester derivative (kcat/Km = 15 and 300,000 M^{-1} sec^{-1} respectively ref. 6).

Increasing the chain length of the anilides from three units (compounds I and II) to four units (compounds III and IV) increases kcat/Km by a factor ranging from 2×10^2 to 4×10^4. This agrees with what has been found for esters (5, 6) amides (6, 15) and peptides (7) and demonstrates again the importance of the

P_4-S_4 interaction. BOC-$(Ala)_3$-NA is 6 to 8 times more active than the corresponding acetyl derivative (table I). It is interesting to note that replacement of the acetyl group of Ac-Ala-Pro-Ala-NH_2 by a trimethylacetyl substituant (which is structurally related to the BOC group) increases kcat/Km by a factor of 8 as shown in 1973 by Thompson and Blout (16). Both these findings emphasize the effect of methyl groups on the P_4-S_4 interaction.

Of considerable interest is the substrate activation noticed with $(Ala)_3$-NA, a substrate which does probably not occupy subsite S_4. It is attractive to postulate that this activation is due to the binding of a second substrate molecule (i.e. in S_{654}) which favours the breakdown of the substrate bound in S_{321} by bringing its scissile bond nearer to the catalytic site. Such a model supports strongly the hypothesis of a P_4-S_4 induced conformational change in the elastase active center (15). It need however not be added that other interpretations may be devised to account for the phenomenon of substrate activation.

While this work was in progress, Feinstein et al (9) published the properties of Ac-$(Ala)_3$-NA and reported a value of kcat/Km which is about 10 times higher than that reported in this paper. We have no explanation for this discrepancy.

Acknowledgments : We wish to thank Professor Métais for his kind interest and Miss J. Barth for her skilful technical assistance. One of us (J. B.) is Chargé de Recherche à l'I. N. S. E. R. M.

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