KINETIC DETECTION OF INTERMEDIATES DURING THE ELASTASE-CATALYZED HYDROLYSIS OF SUCCINYL-TRIALANINE-*p*-NITROANILIDE AT SUBZERO TEMPERATURES

Claude BALNY* and Joseph Gabriel BIETH**

**INSERM, U 128, BP 5051, 34033 Montpellier Cedex, France* and ***Laboratoire d'Enzymologie, Université Louis Pasteur, 67083 Strasbourg Cedex, France*

Received 10 June 1977

1. Introduction

In recent years, a number of enzyme-catalyzed reactions have been studied in mixed solvents at subzero temperatures to stabilize and analyze enzyme-substrate intermediates [1–6] including those of anilide substrate-chymotrypsin [6].

Elastase shares many properties of this latter enzyme. Both proteases have nearly identical tertiary structures and catalytic centers (i.e. the 'charge relay system' including the Asp–His–Ser triade). They differ in their specificity sites, elastase being specific for non-bulky amino acid residues [7]. Fink and Ahmed [8] studied the experimental conditions required for preparing stable crystals of acyl-elastase derivative prepared by reacting the enzyme with the substrate carbobenzoxy-alanine-*p*-nitrophenyl ester. The crystal structure of this acyl-enzyme was then determined by X-ray diffraction, the data being collected at −55°C [9]. As pointed out by Fink and Petsko in their respective papers, low temperature crystallography of elastase-substrate complexes would yield much more refined mechanistic information if the substrate were not an activated ester like the one they employed but a *p*-nitroanilide for which acylation is rate-limiting. Hence, one could expect to get crystallographic data on a preacyl-enzyme intermediate. Such a substrate, succinyl-trialanine-*p*-nitroanilide has recently been synthesized by Bieth et al. [10]. In addition to being a stable anilide (compared to the unstable *p*-nitrophenyl esters) it has the advantage over monomeric substrates to occupy fully the extended active center of elastase [11] and hence to bind probably in an exclusively productive way to the enzyme.

The work reported herein was therefore undertaken in order to see whether soluble preacyl-enzyme intermediates can be spectroscopically detected and isolated in fluid medium at subzero temperatures with this enzyme-substrate system.

2. Materials and methods

Porcine pancreatic elastase was isolated as described by Shotton [12]. Succinyl-trialanine-*p*-nitroanilide, the specific substrate, was prepared according to Bieth et al. [10]. Ethylene glycol was distilled under vacuum before use. The mixed solvent used was a mixture of equal volumes of ethylene glycol and 20 mM phosphate buffer pH 7.0. The protonic activity (paH) of this medium was respectively 7.5 and 8.0 at +20 and −40°C [13,14]. A 1 mM stock solution of elastase was prepared in water and diluted at 0°C with the above mixed solvent (to a final volume ratio of ethylene glycol of 50%) up to a final concentration of 34 μM. A 16.3 mM stock solution of substrate was prepared directly in the mixed solvent. The paH of this solution was readjusted to 7.5 with NaOH. Both solutions were kept at 0°C until used.

Low temperature kinetic measurements were performed on an Aminco DW 2 spectrophotometer equipped with the previously described device [15]. Differential spectra were recorded using double compartment quartz cells. Each compartment had an

North-Holland Publishing Company – Amsterdam

optical pathlength of 45 mm and was filled with 1.2 ml of solution. For the base-line recording, one compartment of each of the two cells was filled with the 34 μM solution of enzyme and the two other compartments were filled with 1.2 ml of the mixed solvent and 5 μl of the 16.3 mM solution of substrate. The base-line was carefully adjusted after temperature equilibration. The substrate solution was then removed from the sample cell and replaced by mixed solvent. After the temperature had reached the desired value, 5 μl of stock substrate solution were pipetted into the enzyme compartment of the sample cell and mixing was performed with a glass rod which had previously been cooled to the working temperature. Mixing took about 30 sec and control experiments showed it to be very efficient. Five μl of mixed solvent were also added to the second compartment of the reference cell. Whatever the working temperature, the stock substrate solution was always pipetted at 0°C in order to keep its density constant [16] and hence to deliver equal quantities of substrate for each experiment. Preliminary results showed this precaution to be a prerequisite for getting reliable results. The other kinetic measurements were performed on an Unican SP 800 spectrophotometer thermostated at +15°C.

3. Results and discussion

3.1. *Activity of elastase in mixed solvents at room temperature*

We have previously shown that the activity of elastase on *p*-nitroanilide substrates is strongly inhibited by organic solvents [17]. It was therefore mandatory to test the influence of high solvent concentrations before undertaking experiments at subzero temperatures. Table 1 shows that the enzyme has still an appreciable activity in the 50 : 50 mixed solvents. In all cases, the release of product was pseudo-zero order indicating that the enzyme did not denature during the assay. Moreover, these solvent effects are totally reversible by dilution with pure aqueous buffer. The inhibitory effect of dimethylsulfoxide is due to a 7-fold increase in K_m. On the other hand, k_{cat} is slightly increased. Similar effects (i.e., large increases in K_m and small increases of k_{cat}) have been experienced with small concentrations of dimethylformamide and *N*-methypyrrolidone at 25°C [18]. The qualitative effect of solvents on the elastase catalyzed hydrolysis of succinyl-trialanine-*p*-nitroanilide appears thus to be independent of the solvent's nature. For subzero temperature studies, the phosphate–ethylene glycol mixture was used because the protonic activity of this buffer does not appreciably change with temperature [13,14].

3.2. *Subzero temperature studies under turnover conditions*

Between −1°C and −27°C with $(E^\circ) \simeq (S^\circ) \ll K_m$, the addition of substrate to the enzyme resulted in a steady-state release of *p*-nitroaniline which could be monitored at 400 nm (fig.1). The product formation was sufficiently slow (except at −1°C) to allow the determination of its initial rate v which is given by the following equation:

$$v = \frac{k_{cat}}{K_m} (E^\circ)(S^\circ)$$

Table 1
The elastase-catalyzed hydrolysis of succinyl-trialanine-*p*-nitroanilide de 15°C in the presence of high concentration of organic solvents. The enzyme reactions were started by adding 5 μl of an aqueous enzyme solution to 1 ml of substrate dissolved in the appropriate mixed-solvent

Buffer composition	pH or paH	$\frac{v}{(E^\circ)}$ (s^{-1})[a]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (M^{-1} s^{-1})
99.2% 0.2 M tris + 0.8% *N*-methyl-pyrrolidone	8.0	2.7	8.5	2.1	4050
50% 0.02 M tris + 50% dimethylsulfoxide	8.5	0.83 (30%)	13.3	15	890
50% 0.02 M tris + 50% ethylene-glycol	8	0.48 (18%)			
50% 0.02 M phosphate + 50% dimethylsulfoxide	8.6	0.43 (16%)			
50% 0.02 M phosphate + 50% ethylene-glycol	8	0.34 (13%)			

[a]For (S°) = 1 mM

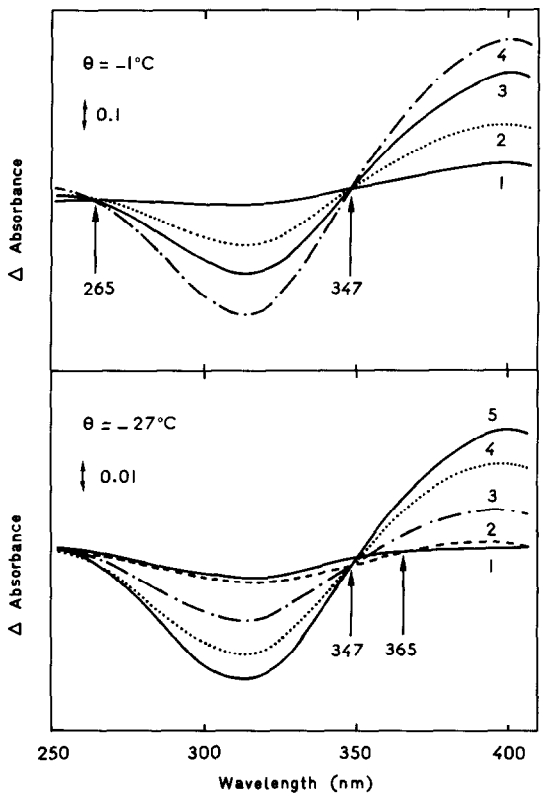

Fig.1. Differential spectra recorded during the elastase catalyzed hydrolysis of succinyl-trialanine-*p*-nitroanilide at −1°C (upper curves) and at −27°C (lower curve). (E°) = 34 μM, (S°) = 68 μM. For the curves at −1°C, the records were performed respectively, 1 = 2 min; 2 = 5 min; 3 = 10 min; 4 = 20 min after injection of the substrate into the enzyme solution. For the curves at −27°C; 1 = 14 min; 2 = 18 min; 3 = 30 min; 4 = 44 min; 5 = 1 h.

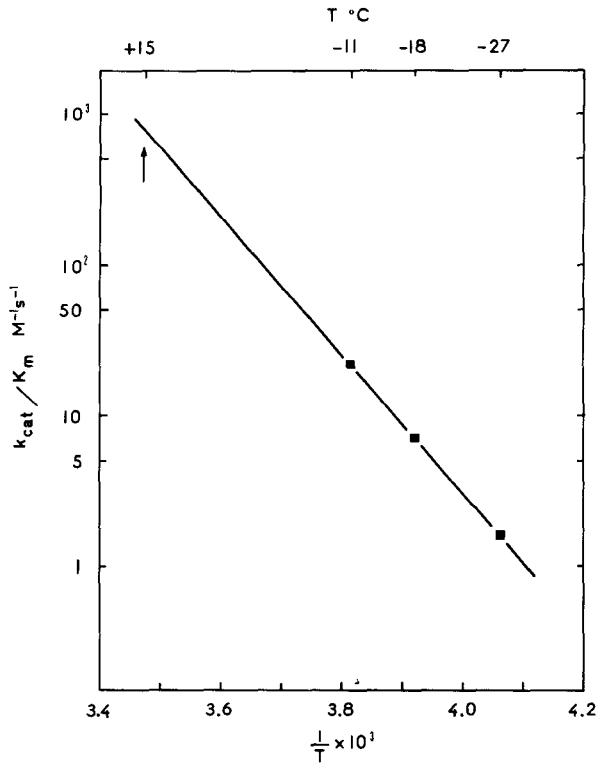

Fig.2. Arrhenius plot of k_{cat}/K_m for the elastase-catalyzed hydrolysis in 50/50 phosphate–ethylene glycol mixed solvent, paH = 8 at +20°C. Experimental conditions as in the legend to fig.1.

The second-order rate constant k_{cat}/K_m was calculated at −11°C, −18°C and −27°C. The activation energy derived from an Arrhenius plot (fig.2) was found to be 21 ± 2 kcal · mol^{-1}. This value is close to 15.6, the activation energy of the same enzyme-substrate system above 0°C in the absence of organic solvents [18]. On the other hand, the extrapolated value of k_{cat}/K_m at +15°C is 800 ± 100 M^{-1} s^{-1}, a result which is in accord with that depicted in table 1 for the tris-dimethylsulfoxide mixed solvent (890 M^{-1} s^{-1}). Taken together these results suggest strongly that elastase functions in a similar way above and below zero degree. Hence, conclusions concerning sequential reactions during catalytic cycle drawn from subzero temperature studies can be considered as valid for physiological temperatures.

Repetitive spectral scans during the turnover reaction led to the very interesting finding that the isobestic point between substrate and product changes during the substrate hydrolysis*. Figure 1 summarizes this observation. A −1°C, the isobestic point is constant and is identical to that obtained in the absence of enzyme, i.e., between pure substrate and pure product. Dramatic changes occur at −27°C. The first

*Preliminary experiments showed that the absorption spectra of substrate and product were only slightly different from those recorded in aqueous solution at room temperature (10) in agreement with the data of Fink (19) on acetyl-phenylalanine-*p*-nitroanilide. The isobestic point was 347 ± 1 nm.

recordable isobestic point, 15 min after the mixing of substrate in the enzyme solution is at 365 nm and tends to recover its normal position (347 nm) as the reaction proceeds, a value obtained one or two hr after the beginning of the reaction. At intermediate temperatures (−11°C, −18°C), this phenomenon is qualitatively the same, but more rapid. Since there is no reason to believe that the absorption spectrum of the reaction product (i.e., *p*-nitroaniline) does change in the course of the reaction, the variations of the isobestic point are most probably accounted for by small changes in the absorption spectrum of substrate due to its binding with the enzyme.

Our data suggest therefore that a series of metastable intermediates are visible during the turnover reaction of elastase provided the temperature be sufficiently low. These intermediates (I_n) of undetermined number follow the first enzyme-substrate complex (*ES*):

$$E + S \rightleftharpoons ES \rightleftharpoons I_1 \rightleftharpoons \ldots . I_n \rightarrow E + P$$

Their spectral characteristics must be very close to the substrate absorption spectrum, the difference between the optical density at the different isobestic points level being very small (see fig.1).

To attempt to trap the first *ES* complex, we have studied the reaction under non-turnover conditions at a lower temperature.

3.3. *Subzero temperature studies under non-turnover conditions*

It was thought that under non-turnover conditions these intermediates would accumulate and hence would be spectrophotometrically detectable. The favorable activation energy of our enzyme-substrate system enabled us to stop the turnover at temperatures compatible with our mixed-solvent. Indeed, at −38°C, no product formation occurred. No significant spectral changes, in the visible and UV regions, could however be diagnosed indicating that if indeed intermediates accumulate, either their concentration must be very low or their spectra must be similar to the substrate spectrum. At this point we must emphasize that we arrived at this conclusion after a number of experiments where the mixing of the substrate solution in the enzyme solution were carefully controlled. If the mixing was not efficient at this temperature (−38°C) where the viscosity of the mixed solvent is important (≃ 100 cp) (16), erratic spectral changes in the 320 nm region (i.e. the wavelength of absorption of the substrate) were recordable.

Our results are different from those reported for the α-chymotrypsinacetyl–phenylalanine-*p*-nitroanilide system [6] where intermediates were spectrophotometrically detectable at low temperature. But our observations are not necessarily at variance with those of Fink since the enzyme-substrate system, the mixed-solvent and the temperature used by this author are quite different from those of this study.

To the best of our knowledge, this is the first report of a kinetic detection of enzyme-substrate intermediates at subzero temperatures through changes in the isobestic point.

Acknowledgements

We are grateful to Professor P. Douzou for his interest and support of this research and to Dr F. Travers for helpful discussion. This work was supported by grants from the D.G.R.T. (Conventions N° 71.7.3091 – 76.7.1857) and the Fondation pour la Recherche Médicale Française.

References

[1] Douzou, P. (1977) in: Cryobiochemistry: an Introduction. Academic Press, London.
[2] Douzou, P., Sireix, R. and Travers, F. (1970) Proc. Natl. Acad. Sci., USA 66, 787–792.
[3] Hastings, J. W., Balny, C., Le Peuch, C. and Douzou, P. (1973) Proc. Natl. Acad. Sci. USA 70, 3468–3472.
[4] Douzou, P., Hui Bon Hoa, G. and Petsko, G. A. (1974) J. Mol. Biol. 96, 367–380.
[5] Fink, A. L. (1973) Biochemistry 12, 1736–1742.
[6] Fink, A. L. (1976) Biochemistry 15, 1580–1586.
[7] Hartley, B. S. and Shotton, D. M. (1970) in: The Enzymes. (Boyer, P. D. ed) Vol. 3, 3rd ed., pp. 323–375, Academic Press, New York.
[8] Fink, A. L. and Ahmed, A. I. (1976) Nature 263, 294–297.
[9] Alber, T., Petsko, G. A. and Tsernoglou, D. (1976) Nature 263, 297–300.
[10] Bieth, J., Spiess, B. and Wermuth, C. G. (1974) Biochem. Med. 11, 350–357.
[11] Thompson, R. C. and Blout, E. R. (1970) Proc. Natl. Acad. Sci. USA 67, 1734–1740.

[12] Shotton, D. M. (1970) in: Methods in Enzymology (Perlmann, G. E. and Lorand, L. eds) Vol. 19, pp. 113–140, Academic Press, New York.
[13] Hui Bon Hoa, G. and Douzou, P. (1974) J. Biol. Chem. 248, 4649–4654.
[14] Larroque, C., Maurel, P., Balny, C. and Douzou, P. (1976) Anal. Biochem. 73, 9–19.
[15] Maurel, P., Travers, F. and Douzou, P. (1974) Anal. Biochem. 57, 555–563.
[16] Douzou, P., Hui Bon Hoa, G., Maurel, P. and Travers, F. (1976) in: Handbook of Biochem. and Mol. Biol. (Fasman, E. G., ed) 3rd ed., Vol. 1, pp. 520–539, CRC Press, Cleveland, USA.
[17] Bieth, J. and Wermuth, C. G. (1973) Biochem. Biophys. Res. Comm., 53, 383–390.
[18] Bieth, J. and Wermuth, C. G. (1974) Proceeding IXth FEBS Meeting, Budapest.