Rate-determining steps in penicillopepsin-catalysed reactions

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Received 26 September 1990

The hydrolysis of Ac-$(Ala)_2$-Lys-Nph-$(Ala)_2$-amide (**II**) by penicillopepsin is characterized by a solvent isotope effect of 2.11, whereas the hydrolysis of Ac-Lys-Nph-amide (**I**) shows no solvent isotope effect. The dependence of the isotope effect on the concentration of D_2O in H_2O for substrate **II** is not linear and suggests that two or more protons are involved in its rate-determining step. We propose that for substrate **I** the rate-determining step is the distortion of the scissile bond towards a tetrahedral configuration, and for substrate **II** a conformational change induced by the occupation of the S_3 pocket in the enzyme.

Penicillopepsin; Aspartic proteinase; Enzyme kinetics; Enzyme mechanism

1. INTRODUCTION

Although several high resolution structures of aspartic proteinases and their complexes with substrate analogue inhibitors are known (see Gilliland et al. for a recent list [2]) and several proposals for their mechanism of action have been made [3–7], many aspects, including the nature of the rate-determing step, are still poorly understood. Information on rate-determining steps in hydrolytic reactions can be obtained from a study of solvent isotope effects. Three such studies were carried out earlier with pig pepsin, but they led to conflicting conclusions. No solvent isotope effects were observed with the substrates Ac-Phe-Tyr-OMe [8] and methyl phenyl sulfite [9], whereas Holland and Fruton [10] observed a solvent isotope effect of about 2 with Gly_3-Nph-Phe-OMe. The latter authors could not find a satisfactory explanation for this discrepancy and felt that further work was needed to resolve this problem. However, to the best of our knowledge no such additional study with any of the aspartic proteinases has been carried out. In the present study we looked at the solvent isotope effect on the hydrolysis by penicillopepsin of the -Lys-Nph- bond of two substrates of the series Ac-Ala_m-Lys-Nph-Ala_n-amide, one with $m = n = 0$, the other with $m = n = 2$. This substrate series was used previously to determine the effect of different subsites on k_{cat} of the scissile bond [11]. We showed that subsites S_3 and S_2' were primarily responsible for the large increases in k_{cat} with increasing chain length. In a study of the effect of temperature on the reactions [2] we further showed that hydrolysis of Ac-Lys-Nph-amide was not accompanied by any demonstrable conformational changes, whereas hydrolysis of Ac-Ala_2-Lys-Nph-Ala_2-amide was. Both of these earlier studies suggested the possibility that rate-determining steps could be different for the short and the long substrates.

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Abbreviations: Ac-, acetyl; FAB, fast atom bombardment; HPLC, high-pressure liquid chromatography; Nph, *p*-nitrophenylalanyl; OMe, *O*-methyl ester; TFA, trifluoroacetic acid; the definition of Schechter and Berger [1] for denoting amino acid residues in peptide substrates as P_1 to P_n and P_1' to P_n' and subsites in the enzyme to which the side chains of these residues bind as S_1 to S_n and S_1' to S_n', respectively, is used throughout

2. MATERIALS AND METHODS

2.1. *Materials*

Penicillopepsin was prepared as described [12]. The synthesis of Ac-Lys-Nph-amide (**I**) and Ca-Ala_2-Lys-Nph-Ala_2-amide (**II**) was described by Hofmann and Hodges [13]. Chemicals used for buffers were of the highest quality available. [^{18}O]water (97 atom% ^{18}O) and D_2O (99.9 atom% D) were obtained from MSD Isotopes (Merck Frosst Canada Inc., Montreal).

2.2. *Enzyme kinetics*

All enzyme assays were carried out at 25°C in 20 mM formate (pH 3.5, pD 3.9), or acetate (pH 4–5.5, pD 4.4–5.9), adjusted to an ionic strength of 20 mM with NaCl, in a Uvikon 820 spectrophotometer (Kontron AG, Zurich, Switzerland) at a wavelength of 335 nm. (This wavelength, rather than the optimal wavelength of 296 nm [13], was chosen because of the high absorbance at the substrate concentrations used.) The buffers contained between 0% and 93% D_2O. The enzyme concentrations were 6.3 μM for peptide **I** and 0.052 μM for peptide **II**.

2.3. *Analysis of ^{18}O incorporation*

The reaction conditions for the analysis of the incorporation of ^{18}O into the amino moieties of the substrates were the same as those described above, except that the buffer contained 92.4% [^{18}O]H_2O. The pH was 5.5, the substrate concentrations were about 2 mM, and the enzyme concentrations were as given above. The reactions were allowed to reach over 90% hydrolysis and were stopped by the addition of 50 μl of 1 N ammonia. The products Ac-Lys and Ac-Ala_2-Lys from peptides **I** and **II** respectively, were isolated by HPLC on a C_{18}

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

Table I

Solvent isotope effect for penicillopepsin catalysed hydrolyses.

pH (pD)	Rate constant k_{obs} (s^{-1})		K_m (mM)		
	H_2O	D_2O	H_2O	D_2O	k^H/k^D
I = Ac-Lys-Nph-amide					
5.5 (5.9)	0.013[a]	0.012[a]	0.2	0.25	1.08
5.0–5.5 (5.4–5.9)	0.0123 ± 0.007[b]	0.0113 ± 0.006[b]			1.09 ± 0.04
II = Ac-Ala_2-Lys-Nph-Ala_2-amide					
4.5 (4.9)	46.4[a]	23.6[a]	0.08	0.06	1.97
3.5 (3.9)	34.5[a]	19.7[a]			1.75
5.5 (5.9)	35.6[c]	15[c]			2.33
4.5 (4.9)	40[c]	18[c]			2.22
3.5 (3.9)	28.2[c]	12.9[c]			2.18
5.25 (5.65)					2.11 ± 0.16[d]

The conditions were as described in section 2.
[a] k_{cat}
[b] Substrate concentration = 10 × K_m, average of 6 experiments
[c] Substrate concentration = 23 × K_m, average of 2–3 experiments
[d] from the experiments shown in Fig. 1

reverse-phase Vydac 218TP104 column (250 × 4.6 mm) in 0.1% TFA, run at room temperature with a linear gradient from 0% to 50% acetonitrile over 25 min. The peptides were analysed by FAB mass spectrometry on a VG Analytical ZAB SE mass spectrometer (V.G. Analytical, Manchester, UK). The bombarding xenon atom (8 kV, 1.2 mA anode current) was generated with the 'Ion Tech' gun supplied with the instrument; glycerol or *m*-nitrobenzyl alcohol was used as the matrix. Spectra were recorded with a VG 11-250 data system in a multichannel analysis mode in a downfield exponential magnetic scan.

2.4. *Analysis of dependence of the rate constant on the D_2O/H_2O ratio*

The shape of the plot of the rate constants k_{obs} versus the ratio (n) of D_2O/H_2O was analysed in an attempt to determine the number of protons involved in the rate-determining step [14]. Three different hypotheses, corresponding to the following equations, were tested.

one-proton mechanism: $k_n = k_0 - a\,n$ (1)
two-proton mechanism: $(k_n/k_0)^{0.5} = b - a\,n$ (2)
'infinite' proton mechanism: $\ln(k_n/k_0) = -a\,n + \ln k_0$ (3)

Statistical tests were carried out to determine how well the data fit these 3 linear equations. For each run the data were normalized as follows. First, the mean value for each run was calculated, then the value of each point was divided by the mean value for that run. These normalized data were then combined to produce a single data set that was used in the statistics. The linearity of the curves was tested with the REG procedure from the SAS package [15].

3. RESULTS AND DISCUSSION

Table I shows the kinetic parameters for the hydolysis of the -Lys-Nph- bond in the two substrates. In the range of pH 5–5.5 (pD 5.4–5.9) the rate constants for peptide **I**, measured at a substrate concentration about 10 × K_m, are independent of pH and show no significant solvent isotope effect. The K_ms, determined at pH 5.5 (pD 5.9) in separate experiments, were 0.2 mM in H_2O and 0.25 mM in D_2O and not considered significantly different. They agree well with the previous value in H_2O of 0.22 ± 0.01 mM [16]. The absence of a solvent isotope effect shows that the rate-determining step does not involve the bond breaking step. According to the mechanistic proposals at present generally accepted, the bond breaking is catalysed by a general base mechanism leading to a tetrahedral intermediate, the decay of which involves a proton transfer to the leaving carboxyl moiety [3,4,17]. Both steps involve a proton transfer and hence are likely to show a solvent isotope effect.

If we assume the following scheme as a minimal scheme for the action of penicillopepsin [11].

$$E + S \leftrightarrow ES \leftrightarrow E^*S \leftrightarrow E^*T \leftrightarrow E^*P \leftrightarrow EP \leftrightarrow E + P$$

where the steps E*S to E*P represent the bond breaking steps via the tetrahedral intermediate E*T, the rate-determining step for peptide **I** lies either before or after these steps. We can eliminate the release of products as the rate-determining step on the following grounds. We show elsewhere (Blum, M., Cunningham, A., Pang, H. and Hofmann, T. submitted for publication) that during transpeptidation reactions release of some products is the rate-determining step. The evidence for this comes from a high degree of incorporation of ^{18}O into peptide carbonyl and α-carboxyl groups of the products when the reactions are carried out in $[^{18}O]H_2O$ buffers. Since the rate constants for these reactions are similar to those observed with peptide **I** product release could also be the rate-determining step for this peptide. We therefore isolated Ac-Lys from a reaction carried out in $[^{18}O]H_2O$ and found that only one atom of ^{18}O had been incorporated into the the terminal carboxyl group. This shows that the peptide bond cleavage is irreversible, unlike that observed in the transpeptidation reactions and suggests that product release is not the rate-determining step.

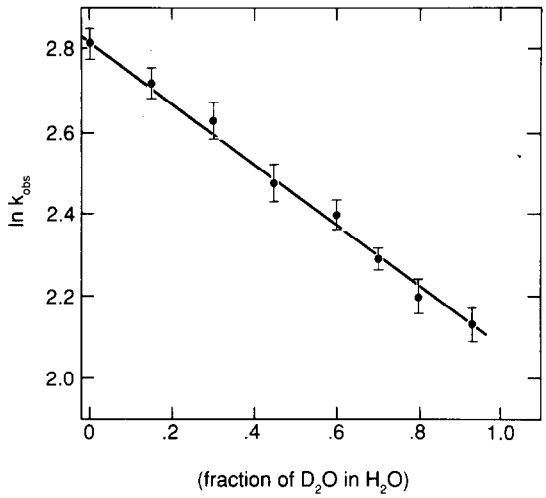

Fig. 1. Solvent isotope effects for penicillopepsin catalysed hydrolysis of Ac-$(Ala)_2$-Lys-Nph-$(Ala)_2$-amide as a function of D_2O concentration. The buffer used was 20 mM sodium acetate (pH 5.25; pD 5.65); penicillopepsin concentration was 32.5 nM, the substrate concentration was 1.17 mM.

In contrast, the rate constants for peptide **II** show a consistent solvent isotope effect of 2.11 ± 0.16 over the pH range 3.5–5.5 (pD 3.9–5.9) (Table I). In this case also the K_m values are unaffected. The results of a proton inventory, undertaken in order to find out the number of protons actively involved in the rate-determining step [18], are shown in Fig. 1 for the logarithmic relation (Eqn 3), the equation for which the best fit was obtained. We should stress, however, that the fit and the statistical analysis for the relation shown in Eqn 2 is such that it cannot be unambiguously excluded. This suggests that a process involving two or more protons controls the rate-determining step for peptide **II**. Although the interpretation of this finding in molecular terms is not straightforward, the rate-determining step can be placed later than that for substrate I and before the product releasing step. The experiments carried out in $[^{18}O]H_2O$ showed that, like substrate , substrate **II** did not incorporate excess ^{18}O. It is most likely that the rate-determining step is a conformational change that occurs only when a substrate or other ligand contains an amino acid residue in P_3. We showed previously that conformational changes are associated with the binding of ligands that contain amino acid residues in P_3 and in P_2' [19]. Furthermore, structural studies with inhibitor complexes of endothiapepsin indicate that occupation of the S_3 pocket induces a rotation of a domain comprising residues 190–302 (pig pepsin numbering) which has been described as a 'rigid body movement' [20,21]. The same type of change accounts for half the conformational differences among the aspartic proteinases [21]. On the basis of this the proposal has been made that this type of structural flexibility plays a major part in the function of these enzymes [21]. We also showed that the occupation of subsite S_3 involves the formation of a specific hydrogen bond between the NH of P_3 and the Oγ of Thr-219 (pepsin numbering) [22] and pointed out that an amino acid with a hydrogen bond accepting O atom in the side chain (Thr, Ser or Asn) is present in the analogous position in all 24 known sequences of aspartic proteinases [11]. There is also evidence that similar unique hydrogen bonds form when the S_2' subsite is occupied [11]. Hence we propose that it is the formation of these hydrogen bonds and possibly other protonic reorganisations involved in concurrent conformational changes [23] that are responsible for the solvent isotope effect and represent the rate-determining step in the hydrolysis of substrate **II**.

In retrospect the results obtained here also provide an explanation of the earlier experiments [8–10]. As in this study the short substrates, dipeptide and sulphite ester, showed no solvent isotope effect [8,9], whereas the longer substrate which had a glycine residue in P_3 showed an effect which was the same as that observed with peptide **II**.

Acknowledgements: We are grateful to Drs M. Blum, R. Kluger and A.J. Kresge for valuable discussions and to Dr Henrianna Pang for the ^{18}O analyses. This work was supported by the Medical Research Council of Canada (Grant MT-1982).

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