FRAGMENTATION OF PENICILLIN CATALYSED BY THE EXOCELLULAR DD-CARBOXYPEPTIDASE-TRANSPEPTIDASE OF *STREPTOMYCES* STRAIN R61

Isotopic study of hydrogen fixation on carbon 6

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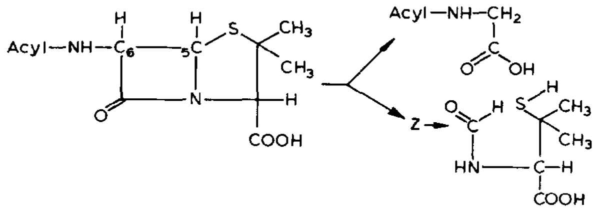
1. Introduction

The exocellular DD-carboxypeptidase-transpeptidase (EC 3.4.12.6) excreted by *Streptomyces* strain R61 (in short the R61 enzyme) (E) reacts with penicillin (I) according to the equation:

$$E + I \underset{}{\overset{K}{\rightleftharpoons}} EI \xrightarrow{k_3} EI^* \xrightarrow{k_4} E + \text{antibiotic metabolites}$$ [1,2].

The slow degradation of complex EI* (the first-order rate constant k_4 is of the order of $10^{-4}\ s^{-1}$) results in enzyme reactivation and in the fragmentation of the penicillin molecule. The fragments released are *N*-acylglycine and an unstable intermediate (Z) which in turn gives rise to *N*-formyl-D-penicillamine [3–5]. Formation of *N*-acylglycine requires a double hydrolysis within the β-lactam ring, i.e., rupture of both the amide linkage and the C_5–C_6 bond. This latter reaction results in the formation of a $-CH_2$-methylene group at C_6. This report describes isotopic studies on the mechanism of hydrogen fixation on C_6. The approach rested upon the effects of D_2O on the fragmentation reaction; it made use of the fact that the protons of the methylene groups are not exchangeable.



2. Materials and methods

The R61 enzyme was 95% pure [6]. Its activity was estimated by measuring the amount of terminal D-Ala released from Ac_2–L-Lys–D-Ala–D-Ala [6]. Penicillinase Riker (EC 3.5.2.6) was purchased from Serva. Free SH-groups were titrated with 5,5′-dithiobis-(2-nitrobenzoic acid) (Sigma) in 0.1 M sodium phosphate pH 7.0 [5]. Deuterium oxide (99.8% pure) was purchased from IRE, Fleurus, Belgium.

Phenylacetylglycine (formed from benzylpenicillin) and phenoxyacetylglycine (formed from phenoxymethylpenicillin) were extracted from the reaction mixtures and methylated as follows: freeze-dried samples were dissolved in 200 μl 6 N HCl and the solutions extracted 3 times with 200 μl ethylacetate; the pooled extracts were evaporated and the residues supplemented with 1 ml solution diazomethane in ether [7]. After at least 20 min at 22°C, the solutions were evaporated and the residues dissolved in methanol.

Mass fragmentometric determinations were carried out on a LKB 9000 S gas–liquid chromatograph–mass spectrometer unit. The glass column (200X0.6 cm) was filled with 1% OV1 on Gas-Chrom P (AW-DMCS) (100–200 mesh). The retention time of phenylacetylglycine methyl ester was 4 min at 200°C and that of phenoxyacetylglycine methyl ester 5 min at 190°C. The energy of the incident electrons was 20 eV. The mass spectrometer was focused on the following ions: *m/e* 207, 208 and 209, corresponding to M^+, $(M+1)^+$ and $(M+2)^+$, respectively, of phenylacetylglycine methyl ester, or *m/e* 223, 224 and 225, corresponding to M^+, $(M+1)^+$ and $(M+2)^+$, respectively, of phenoxyacetylglycine methyl ester. The molecular ions $M^+ = 207$ and $M^+ = 223$ represented 37% and 85%, respectively, of the most abundant ones (*m/e* 91 in the case of phenylacetylglycine methyl ester and *m/e* 77 in the case of phenoxyacetylglycine methyl ester). The ions M^+_{207} and M^+_{223} were selected because they were highly characteristic of the products studied and because signals were not detected in control samples at these *m/e* values.

3. Results and discussion

3.1. *Effect of D_2O on the mass of the phenylacetylglycine fragment formed from benzylpenicillin*

The R61 enzyme (3.4 nmol) and an equimolar amount of benzylpenicillin were incubated together in 3 mM sodium phosphate pH 7.5 for 5 min at 22°C (formation of complex EI*) and then, for 300 min at 37°C (breakdown of complex EI*; half-life of the complex in H_2O, 80 min). Depending upon the cases, both formation and breakdown of complex EI* were carried out in H_2O (exp. 1); both formation and breakdown of complex EI* were carried out in 82% D_2O (exp. 2); formation of complex EI* was carried out in 82% D_2O and breakdown in 20% D_2O (exp. 3); finally, formation of complex EI* was carried out in H_2O and breakdown in 75% D_2O (exp. 4). The released phenylacetylglycine was then extracted, methylated and analyzed by mass fragmentometry (table 1).

The following observations were made:

(i) None of the experiments gave rise to high $(M+2)^+/M^+$ ratio values
(ii) the $(M+1)^+/M^+$ ratio values were high in exp. 2, 4, and low in exp. 3
(iii) a $[D_2O]/[H_2O]$ ratio of about 4, as it was used in exp. 2, 4, yielded a $(M+1)^+/M^+$ ratio of about 2.

Table 1
Effect of D_2O on hydrogen fixation on C_6 of benzylpenicillin during interaction with the R61 enzyme

Exp.	Conditions during formation of complex EI*		Conditions during breakdown of complex EI*		$\frac{(M+1)^+}{M^+}$ $\frac{208}{207}$	$\frac{(M+2)^+}{M^+}$ $\frac{209}{207}$	$\frac{(M+2)^+}{(M+1)^+}$ $\frac{209}{208}$
	% D_2O[a] in H_2O	Vol. (μl)	% D_2O[a] in mixture	Vol. (μl)			
1	0	67	0	67	0.15 ± 0.01	0.018 ± 0.05	0.12 ± 0.03
2[b]	82	67	82	67	2.20 ± 0.20	0.32 ± 0.06[c]	0.15 ± 0.01
3[b]	82	67	20	267	0.32 ± 0.04	0.10 ± 0.02	0.31 ± 0.05
4	0	15	75	67	1.85 ± 0.05	0.27 ± 0.02[c]	0.15 ± 0.02
No enzyme (control)	0	57	0	57	no ion at *m/e* = 207		

[a] % in volume
[b] The same results were obtained when the enzyme alone was preincubated for 15 min at 22°C in 82% D_2O before formation of complex EI* in 82% D_2O
[c] The explanation of the slightly increased $(M+2)^+/M^+$ ratio values observed in these experiments when compared to the value observed in exp. 1 is that a high proportion of $(M+1)^+$ ions necessarily results in an increased proportion of $(M+2)^+$ ions

Each of these observations, respectively, supported the following conclusions:

1. Penicillin fragmentation resulted in the fixation of one single deuterium atom and hence, did not involve the transitory formation of an intermediate containing a double bond between C_5 and C_6 (in which case, two deuterium atoms would undergo attachment giving rise to high $(M+2)^+/M^+$ ratio values)

2. The fixation of one deuterium atom on C_6 occurred exclusively during breakdown of complex EI*, demonstrating that in complex EI* benzylpenicillin had an intact C_5–C_6 bond

3. The rate of fixation of one hydrogen atom on C_6 of benzylpenicillin was twice faster than that of one deuterium atom.

3.2. *Kinetics of fixation of hydrogen or deuterium on C_6 of phenoxymethylpenicillin*

In H_2O, the complex EI* formed between phenoxymethylpenicillin and the R61 enzyme is less stable than that formed with benzylpenicillin (half-lives: 40 min and 80 min, respectively) and for this reason, phenoxymethylpenicillin was selected for these studies. In one series of experiments (fig.1), the R61 enzyme (120 nmol) and phenoxymethylpenicillin (1 μmol), in final vol 4.38 ml, were incubated together for 5 min at 22°C in 4 mM sodium phosphate buffer pH 7.5 made in H_2O and containing 1 mM sodium ethylene-diaminetetraacetate (formation of complex EI*). The reaction mixture was supplemented with 20 IU penicillinase to destroy the excess of antibiotic and further incubated at 37°C (breakdown of complex EI*). After increasing times at 37°C (up to 200 min), various samples were removed:

(1) Samples, 5 μl, were used to estimate the extent of enzyme recovery
(2) Samples, 280 μl, to measure the amount of free SH-groups released (free SH-groups were protected against the possible effects of traces of heavy metals by the presence of EDTA in the reaction mixture)
(3) Samples, 100 μl, to estimate the extent of hydrogen fixation on C_6. For this purpose, these samples were supplemented with 1 ml 4 mM phosphate pH 7.5 made in D_2O and incubated at 37°C for a period of time such that altogether breakdown of complex EI* in H_2O and in the D_2O-enriched medium, lasted for 300 min in all cases.

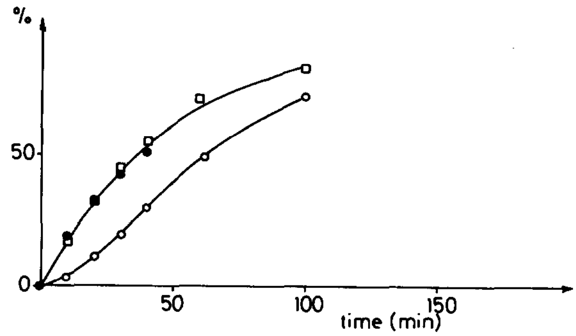


Fig.1. Time course of breakdown in H_2O of complex EI* formed between phenoxymethylpenicillin and the R61 enzyme. Enzyme reactivation (•–•), release of free SH-groups (○–○) and hydrogen fixation on C_6 of phenoxymethylpenicillin (□–□). Formation and breakdown of complex EI* were carried out in H_2O. For other conditions, see text. Results are expressed in % expected final values. Controls consisted of experiments carried out until complete reaction either in H_2O or in 87% D_2O. Half-life of complex EI* as measured on the basis of enzyme reactivation: 39 min. Half-life of the intermediate giving rise to the SH-group-containing compound: 15 min (calculated as in [5]).

From the measured $(M+1)^+/M^+$ ratio values (i.e., the M_{224}/M_{223} ratio values), the rate of hydrogen fixation on C_6 was determined and compared with those of enzyme reactivation and release of the free SH-groups.

A second series of experiments (fig.2) was carried out exactly under the same conditions as above except that (a) complex EI* was formed in 92% D_2O and (b) the 100 μl samples removed after increasing times of breakdown in 92% D_2O and at 37°C were supplemented with 1 ml 4 mM phosphate pH 7.5 in H_2O; after further incubation at 37°C, the rate of deuterium fixation on C_6 was estimated from the $(M+1)^+/M^+$ ratio values.

Figures 1 and 2 showed that the appearance of free SH-groups was a delayed phenomenon when compared with enzyme reactivation and hydrogen or deuterium fixation on C_6. Hence, as observed [5], the primary degradation product originating from the thiazolidine moiety of penicillin during breakdown of complex EI* had no detectable free SH-group but was further degraded into a SH-group-containing compound

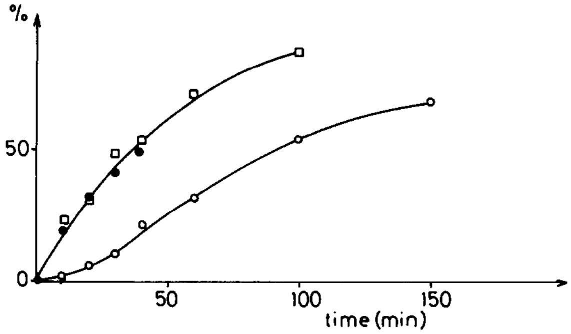


Fig.2. Time course of breakdown in D_2O of complex EI* formed between phenoxymethylpenicillin and the R61 enzyme. Enzyme reactivation (•–•), release of free SH-groups (○–○) and deuterium fixation on C_6 of phenoxymethylpenicillin (□–□). Formation and breakdown of complex EI* were carried out in 92% D_2O. For other conditions, see text and legend of fig.1. Half-life of complex EI*: 40.5 min. Half-life of the intermediate giving rise to the SH-group-containing compound: 37 min.

(identified as *N*-formyl-D-penicillamine). The postulated intermediate had a half-life of 15 min when breakdown occurred in H_2O (fig.1; a value of 10 min had been found [4]) and of 37 min when breakdown occurred in the D_2O-enriched medium (fig.2). D_2O was found not to have any effect on the rate of hydrolysis of D-5,5-dimethyl-Δ^2-thiazoline-4-carboxylic acid (half-life: 54 ± 8 min, at 37°C and in 3 mM phosphate, pH 7.5, made either in H_2O or in D_2O). This observation gave further support to previous findings [5] that this thiazoline derivative, at least in its free form, could not be the intermediate giving rise to *N*-formyl-D-penicillamine.

From fig.1 and 2 and within the limits of the sensitivity of the method used, enzyme reactivation and fixation of either one hydrogen atom or one deuterium atom on C_6 proceeded as if they were concomitant events, and although hydrogen was fixed twice as fast as deuterium on C_6, D_2O had no detectable retardation effect on the rate of enzyme reactivation. Thus, formation of the methylene group at C_6, i.e., the rupture of the C_5–C_6 bond, must be a very rapid reaction which follows the rate-limiting step involved in breakdown of complex EI*. As briefly discussed [8], once benzylpenicillin has been fixed on the R61 enzyme probably in the form of a penicilloyl derivative, the subsequent fragmentation of the β-lactam can be regarded as a process through which an activated phenylacetylglycyl moiety is formed and transferred either to water (with release of phenylacetylglycine) or to a proper acceptor such as the amino group of glycylglycine (with formation of phenylacetylglycylglycylglycine). This observation together with those described here, strongly suggest that breakdown of the complex EI* formed between penicillin and the R61 enzyme involves a rate-limiting reaction of unknown nature which is immediately followed by:

1. The rupture of the C_5–C_6 bond with formation of an activated *N*-acylglycyl moiety

2. The transfer of the *N*-acylglycyl fragment to a proper nucleophilic acceptor.

Whether regeneration of the free, active enzyme occurs during the first or the second of these processes is under current investigation.

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