BIPHASIC KINETICS INDUCED BY MODIFIED SUBSTRATES OF PENICILLINASE

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Summary: The stopped-flow technique was used to examine the initial kinetics of the reaction of penicillinase with benzylpenicillin and with 3 synthetic derivatives of 6-aminopenicillanic acid. While no deviation from linear kinetics was detected with the natural substrate, the slow constant rate of hydrolysis of the derivatives was in each case preceded by an initial, faster rate. The time-dependent transition to the constant rate is reversible and is not due to inhomogeneity of enzyme or substrate preparations. Preincubation with any one derivative eliminates the first phase in the biphasic kinetics of the other 2 derivatives. Since all 3 derivatives are known to distort the active site of penicillinase, the present results demonstrate the capacity of the enzyme to adjust to unfavourable modifications in the substrate.

It has been postulated that the structure of the substrate may determine the rate of the catalytic reaction by inducing a conformational change in the active site of the enzyme (For review of the literature see Refs. 1 and 2). In principle, the substrate induced change could be either favourable or unfavourable to the catalytic reaction. When favourable, the change can be readily recognized in hysteretic (3), i.e. slowly responding, enzymes. It is reflected in the time-dependent increase in activity which is sometimes observed in the course of the catalytic reaction (4, 5). When factors other than the interaction of a single substrate with a single site are ruled out, such observation constitutes clear evidence for substrate-induced activation.

In contrast to the phenomenon of activation by substrates, which is now well established, the converse situation has not been investigated before. This may be due in part to a semantic difficulty. Thus it may have appeared that the occasionally observed inactivation induced by substrates represents the complementary aspect of activation. However, a distinction should be made between progressive inactivation and the transition to a lower, constant level of activity, which is the mirror-image of activation. Evidence for this latter, unnamed phenomenon is presented below.

Methods and Materials

All chemicals were CP grade commercial preparations. Methicillin and cloxacillin were provided by Beecham Research Laboratories, England; oxacillin by Bristol Laboratories, U.S.A., and benzylpenicillin by Rafa Laboratories, Israel. Fresh substrate solutions were prepared daily in 0.1 M phosphate buffer, pH 7.0. Penicillinase (penicillin amidohydrolase E.C. 3.5.2.6) was derived from the culture supernatant of Bacillus cereus strain 569/H and purified as previously described (6). The enzyme activity was assayed colorimetrically (7) or in a Varian Techtron 635 recording spectrophotometer by following the change in absorption caused by cleavage of the β-lactam ring in the substrate (7).

Initial kinetics of the catalytic reaction was examined by spectrophotometric monitoring with the aid of an Aminco stopped-flow apparatus. The rates of hydrolysis of benzylpenicillin and methicillin were followed at 240 nm and 305 nm, respectively (8), and the corresponding difference extinction coefficients taken (8) were 500 M^{-1} cm^{-1} and 41 M^{-1} cm^{-1}.

Results

The hydrolysis of methicillin, a semi-synthetic penicillin, by the extracellular penicillinase of Bacillus cereus was followed spectrophotometrically in a stopped-flow apparatus. The results (Fig. 1) show that the reaction proceeds at an initial rapid rate before a slower rate of hydrolysis becomes established. This final rate remains constant throughout the period of observation (cf. Fig. 2), that is until the substrate concentration falls below saturation.

Similar biphasic kinetics have been previously noticed with pyrazocillin (9) and can be observed with oxacillin and cloxacillin (Fig. 3). All these penicillins, like methicillin, carry side-chains which are known to labilize penicillinase to heat and to proteolysis (9, 10). By contrast, the kinetics of the reaction with benzylpenicillin, the unmodified, rapidly hydrolyzed substrate, shows no detectable deviation from linearity.

The time-scale of the transition to the lower, constant rate was found to be independent of the concentration of the enzyme, and under otherwise identical

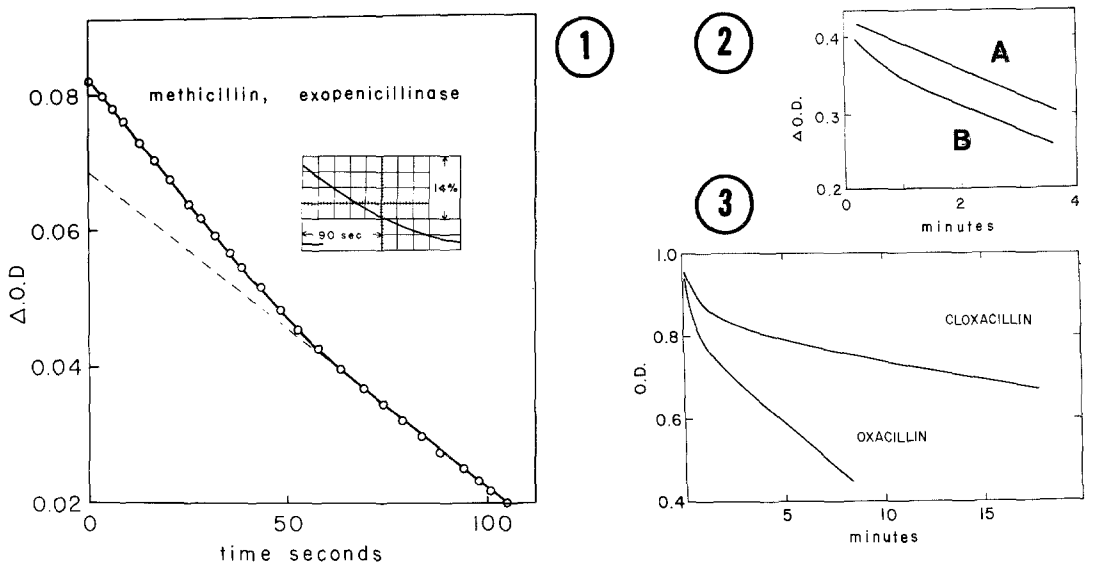

Fig. 1. Initial kinetics of the hydrolysis of methicillin measured spectrophotometrically in 0.1 M phosphate buffer, pH 7, at 37°, by means of stopped-flow apparatus. Inset: oscilloscope tracing showing the disappearance of the substrate. Penicillinase 2500 units/ml, methicillin 10 mM. Total light intensity: 1.43 volt. Ordinate: 0.05 volt/div. Abscissa: 18 sec/div. Measured at 305 nm. Optical path: 0.2 cm.

Fig. 2. Elimination of the initial phase by preincubation. The hydrolysis of methicillin (10 mM) by penicillinase (1300 units/ml) was followed spectrophotometrically (8) at 305 nm. The enzyme was preincubated for 3 minutes (a) with or (b) without methicillin (5 mM). Preincubation and assay were at 37° in 0.1 M phosphate buffer, pH 7.0. Optical path: 1 cm.

Fig. 3. Kinetics of hydrolysis of oxacillin and cloxacillin measured colorimetrically (7) in 0.003 M phosphate buffer, pH 7.3, at 37°.

(a) Substrate - cloxacillin (14 mM)
penicillinase - 300 units/ml.

(b) Substrate - oxacillin (10 mM)
penicillinase - 30 units/ml.

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conditions of assay reflects the structural differences between the modified substrates (cf. Figs. 2 and 3).

The biphasic kinetics is clearly not due to product inhibition or time-dependent inactivation, since in either case the final rate of the reaction would be constant. Two possible artifacts must be, however, considered. These could arise from inhomogeneity of the enzyme or of the substrate preparation.

In the first case it could be assumed that the enzyme preparation consists

of two fractions one of which is inactivated before the final rate of the reaction is established. Such possibility is ruled out by the simple observation that when the reaction is allowed to proceed until all substrate has been exhausted, the pattern of biphasic kinetics is repeated upon the addition of fresh substrate.

The second possibility, namely the presence in the substrate preparation of a rapidly hydrolyzed contaminant is quite remote, when the assay technique is considered. Each substrate has unique absorption characteristics (8) and in each case the contaminant would have to absorb at the wavelength at which the substrate is read. Nevertheless, the possibility remained to be tested, and the result is shown in Fig. 2. The enzyme was preincubated with half the amount of methicillin used for the assay. That amount, which is over 10 times the Km concentration, was reduced by 50 % within 3 minutes. At that point the assay level of methicillin was made up with fresh substrate and the rate of hydrolysis recorded. In the control (Fig. 2, b), the total amount of methicillin from the same batch was used for the assay of enzyme which was preincubated in the absence of methicillin. Clearly, the initial rapid rate observed in the control was completely eliminated by the preincubation (Fig. 2, a).

Identical results were obtained with oxacillin and cloxacillin. Of particular interest is the further observation that all three substrates are interchangeable in their effect on the kinetics of the reaction. In other words, preincubation with any such substrate was found to eliminate the initial, rapid phase in the hydrolysis of the other two substrates.

Discussion

Penicillinase is a single-site monomeric enzyme, which has been very useful in the study of substrate-induced changes, since its conformation is not stabilized by covalent bonds (11). Previous work, reviewed in Refs. 2 and 10, has shown that the side-chain of the substrate (penicillin) determines the conformation of the active site of the enzyme. It also determines the rate at which the substrate is hydrolyzed. A causal relationship between the effect on conformation and on the rate of the catalytic reaction has been assumed, and the present results support that assumption.

More generally, the present observations indicate a mechanism which allows an enzyme to cope with an unfavourable modification in the structure of the substrate. The biphasic step-down kinetics described here is the result of replacing the side-chain of benzylpenicillin with synthetic substituents which are known to distort the active site and labilize the enzyme (11). The instability induced by such substrate analogs might be expected to lead to progressive inactivation (2). Instead, the flexibility of the enzyme allows it to make the kinetically observable transition to a lower, yet constant, level of activity.

It is conceivable that conformational adjustment at the expense of catalytic efficiency will turn out to be a fairly common phenomenon. It certainly provides a physiologically preferable alternative to progressive loss of function.

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