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How Specific is the Effect of Penicillins on the Conformation of Penicillinase? An Experimental Model

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SUMMARY

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The specificity of enzymes may ultimately depend on the conformational adaptability of the active site. Evidence supporting this view is provided by unusually sensitive tests based on the hysteretic kinetics of the penicillinase reaction. Parameters derived from such kinetics show that a unique conformation is induced in the enzyme by each of several closely related substrates. Similar differences in conformation can be deduced from rates of inactivation of penicillinase at saturating levels of such substrates.

INTRODUCTION

Substrate-induced changes in the conformation of an enzyme (1) have been amply documented in numerous systems (2, 3). Yet the role of such changes in the function of enzymes remains to be clarified. We know that the altered conformation is not merely incidental to the binding of the substrate: it certainly does modify the parameters of the enzyme-substrate interaction (1-4). But does the conformational change contribute to the specificity of the catalytic reaction? The answer depends on a better delineation of the specificity of the conformational response than has been hitherto available. We must know whether the response is specific enough to account for differences in, say, the rates of reaction observed with closely related substrates. The difficulty in comparing the catalytic specificity of an enzyme and the specificity of the conformational response of that enzyme to a series of substrates is obvious. The assay of a catalytic reaction usually can be carried out with the precision needed for measuring the effect of subtle modifications in the substrate on the catalytic parameters. By contrast, the available methodology provides no direct tools which are likely to even detect corresponding differences in the conformation of the active site. In this report we describe an approach which makes use of catalytic data for the derivation of parameters reflecting conformational transitions induced by closely related substrates. We also show that such parameters are as distinctive as the corresponding catalytic constants and conclude that the unique characteristics of an enzymic reaction may be traced to the effect of the substrate on the conformation of the enzyme.

The experimental work described here is based on our previous observations on the interaction of penicillinase (β-lactamase I, E.C. 3.5.2.6, from *Bacillus cereus* strain 569/H) with one class of substrates, namely *A-type* penicillins. Members of that class, like all other (*S-type*) penicillins, are N-acyl derivatives of 6-aminopenicillanic acid (6-APA). But, unlike those of the *S* class, *A-type* penicillins are more resistant to en-

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zymic hydrolysis than the unsubstituted parent compound, 6-APA. They were also found to have a distorting effect on the conformation of the active site of the enzyme (5, 6). It thus became apparent that the side-chains (i.e., the N-acyl substituents) of *A-type* penicillins induce in the enzyme a conformational transition which is unfavorable to the catalytic reaction. The transition, which is fully reversible, is slow enough to result in biphasic kinetics of the reaction with *A-type* substrates (4, 7). Remarkably, the initial phase shows a considerable decrease in both k_{cat} and K_m values. This "decelerating" phase is followed by a second ("linear") phase, where both values are constant. The linear phase is maintained indefinitely, i.e., until the substrate is exhausted or displaced by an *S-type* penicillin. The displacement causes a slow conformational transition which is also reflected in biphasic kinetics: the hydrolysis of the *A-type* substrate stops immediately, whereas the hydrolysis of the displacing, *S-type* penicillin, accelerates slowly before reaching the constant rate normally observed with that substrate (4).

It occurred to us that the kinetics of deceleration of hydrolysis of an *A-type* penicillin, and the kinetics of acceleration (upon its displacement), provide a very sensitive gauge of the effect of that penicillin on the conformation of the enzyme. We asked, thus, whether the respective kinetics will reveal differences in the conformation induced by closely related substrates.

MATERIALS AND METHODS

Penicillins. Crystalline preparations of benzylpenicillin were obtained from Rafa Laboratories, Israel; methicillin, flucloxacillin and cloxacillin were from Beecham Research Laboratories, England; oxacillin was obtained from Bristol Laboratories, USA; 6-aminopenicillanic acid (6-APA) was from Aldrich Chem. Co., USA; dicloxacillin and nafcillin were from Wyeth Labs, Inc., USA; pyrazocillin was kindly provided by Dr. V. Csanyi (University Medical School, Budapest).

Other Reagents. All chemicals were CP grade commercial preparations. Fresh substrate solutions were prepared daily, with distilled water. In colorimetric experiments, phosphate buffer 0.003 M pH 7.3 was used. In all other cases, the buffer used was 0.1 M phosphate pH 7.

Penicillinase. The extracellular preparation of penicillinase was derived from *Bacillus cereus* strain 569/H and purified as previously described (8). One penicillinase unit hydrolyzes 1 μmole benzylpenicillin in 1 hr at 30° and pH 7.0.

Kinetic measurements. To follow the progress of the enzymic hydrolysis of the penicillins, the spectrophotometric (9) and colorimetric (10) methods were employed. Among the A-type penicillins, only the hydrolysis of oxacillin, cloxacillin and methicillin could be followed spectrophotometrically (taking $\Delta\epsilon^{263}_{oxa} = -240\ M^{-1}\ cm^{-1}$; $\Delta\epsilon^{260}_{clox} = -120\ M^{-1}\ cm^{-1}$; $\Delta\epsilon^{305}_{meth} = 41\ M^{-1}\ cm^{-1}$). In the case of other *A-type* penicillins, the changes in absorbance accompanying the cleavage of the β-lactam ring were too small to enable the use of direct spectrophotometry. The measurements were carried out in thermostated cuvettes using Gilford model 2400 or Varian Techtron model 635 recording spectrophotometer.

In the colorimetric method of assay (10), the color shift of an acid-base indicator, bromthymol blue, was followed at 620 nm pH 7.3 0.003 M phosphate buffer, in Gilford model 300 N or model 2400 recording spectrophotometer. Since the change of absorbance of the indicator deviates from linearity as more and more of the basic form of the indicator converts into the acidic form, care was taken to monitor the reaction within a narrow range of pH. The recorded progress curves of the enzymatic reactions were digitilized with the aid of A → D converter (GP-2 Graf/Pen Sonic Digitizer) and the changes of substrate concentrations with time were determined.

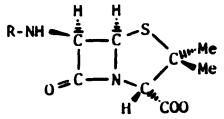
Iodination of Penicillinase. The iodination procedure of penicillinase was as previously described (6).

RESULTS AND DISCUSSION

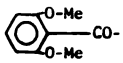
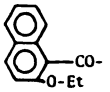
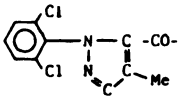
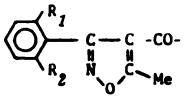
The penicillins selected for this study include several members of the isoxazolyl family of 6-APA derivatives as well as two other *A-type* penicillins with structurally unrelated side-chains (Table 1). Hydrol-

TABLE 1

Relative V_{max} values for 6-APA and A-type derivatives



General structure of penicillin

Name	Side-chain (R)	Relative V_{max}[a]
1. 6-Aminopenicillanic acid (6-APA)	H	100
2. Methicillin		24
3. Nafcillin		2.1
4. Pyrazocillin		0.9
5. Isoxazolyl-penicillins (General structure)		
6. Oxacillin	As in (5), $R_1 = R_2 = H$	25
7. Cloxacillin	As in (5), $R_1 = H$, $R_2 = Cl$	2.9
8. Dicloxacillin	As in (5), $R_1 = R_2 = Cl$	0.8
9. Flucloxacillin	As in (5), $R_1 = F$, $R_2 = Cl$	1.1

[a] Rate of hydrolysis in the linear phase of the biphasic reaction, relative to that of 6-APA (= 100). (The kinetics of 6-APA hydrolysis is linear throughout.)

ysis of 10 mM cloxacillin catalyzed by penicillinase (400 units/ml) and followed colorimetrically (10) in 3 mM phosphate buffer, is shown in Fig. 1 (top). The progress of the catalytic reaction (curve A) recorded as change in absorbance at 620 nm (left ordinate) shows biphasic kinetics at saturating substrate concentrations (4). Assuming first order kinetics (see below) for the transition from the initial ($v_{initial}$) to the final (v_{final}) rate of the reaction, the rate constant for the transition is obtained from the following expression:

$$\ln|v_t - v_{final}| = \ln|v_{initial} - v_{final}| - k_f \cdot t \quad 1$$

where v_t and k_f are, respectively, the catalytic rate at any moment t, and the first order rate constant of the forward conformational transition; t is the time elapsed since the substrate was added. The values for $\ln|v_t - v_{final}|$ were calculated from curve *A*. The linearity of the plot of $\ln|v_t - v_{final}|$ against t (curve *B* and right ordinate) confirms that the transition obeys first order kinetics. Evidence that the kinetic transition stems from a substrate-induced change in conformation has been presented previously (4). The deceleration rate constant, k_f, has been evaluated by a best fit of Eq. 1 using a computer regression program. Best estimates for the k_f values for cloxacillin and the other substrates tested (Table 1) are in good agreement with the corresponding values obtained by a similar analysis of direct spectrophotometric (9) or polarimetric (11) recordings of the progress of each catalytic reaction.

Figure 1 (bottom) illustrates the kinetics

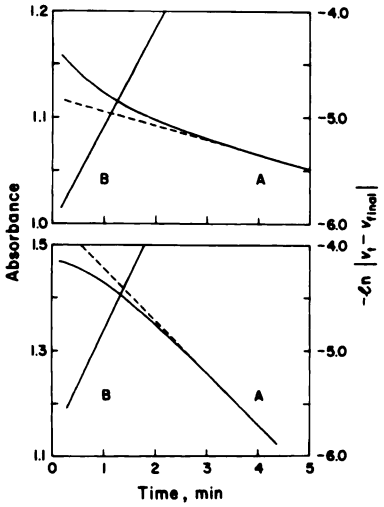


FIG. 1. *Derivation of rate constants for conformational transitions induced in penicillinase by substrates*

Top: Biphasic reaction progress of the hydrolysis of cloxacillin (10 mM) catalyzed by penicillinase (400 units/ml), and followed colorimetrically (9) at 620 nm (trace A, left ordinate). At 30°, phosphate buffer 3 mM, pH 7.3. Trace B: values of $\ln|v_t - v_{final}|$ vs. time calculated using Eq. 1, as described in the text, from Curve A.

Bottom: Kinetics of recovery of the catalytic recovery of penicillinase towards benzylpenicillin following 5 min preincubation (at 30°) with 10 mM dicloxacillin. Measured spectrophotometrically (9) in 0.1 M phosphate buffer, pH 7, 30°, after 1:300 dilution of the enzyme-dicloxacillin incubation mixture with 1.5 mM benzylpenicillin. For other details see legend to Fig. 1 (Top). A) change of absorbance with time at 240 nm (left ordinate). B) $-\ln|v_{final} - v_t|$ calculated from curve A as above (right ordinate).

of recovery of the catalytic activity of the penicillinase towards benzylpenicillin following 5 min preincubation at 30° with 10 mM dicloxacillin. The reaction has been measured spectrophotometrically (9) in 0.1 M phosphate buffer pH 7.0, 30°, after 1:300 dilution of the enzyme-dicloxacillin incubation mixture with 1.5 mM benzylpenicillin solution. Curve *A* shows the change of absorbance with time at 240 nm (left ordinate), while the dependence of $\ln|v_t - v_{final}|$ on time, calculated from curve *A* as above, is presented in curve *B* (right ordinate).

Such measurements have been repeated for the seven *A-type* penicillins (Table 1). Rate constants of the conformational transitions have been derived from the kinetics of the catalytic reaction: the rate constants for the forward transition k_f were obtained from the decelerating phase, and the constants, k_r, for the reverse transition, from the accelerating phase, as illustrated in Fig. 1. Both transitions showed apparent first order kinetics and the rate constants were independent of enzyme or substrate concentration and of the incubation time of the enzyme with the penicillin. The two sets of rate constants derived for the seven *A-type* penicillins are presented in Fig. 2 where k_f values are plotted against the respective k_r values. It will be obvious that although derived from enzyme kinetics, these values are nevertheless completely independent of the catalytic reaction. Thus, the value k_f obtained for a given substrate is uniquely related to the conformational transition induced (by that substrate) in the native enzyme. Similarly, the value of k_r is determined by the energy barrier between the conformational state induced by the displaced *A-type* substrate, and that induced

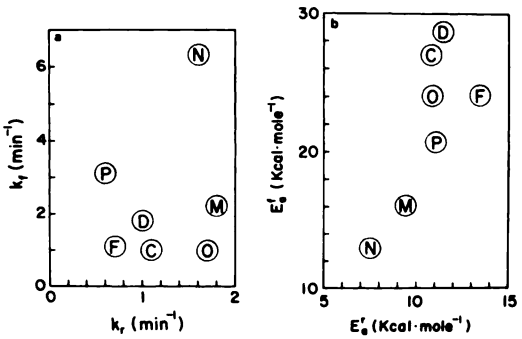


FIG. 2. *a) Comparison of transition rate constants (k_f and k_r) obtained for the individual A-type penicillins*

The position of each penicillin was specified by the average of about 10 determinations of k_f and k_r, as illustrated in Fig. 1. Relative standard deviations were generally below 10%. Symbols: (O) oxacillin; (C) cloxacillin; (F) flucloxacillin; (D) dicloxacillin; (M) methicillin; (N) nafcillin; (P) pyrazocillin.

b) Comparison of activation energies of the forward (E_a^f) and the reverse (E_a^r) transitions induced by A-type penicillins. Rate constants k_f and k_r were derived as illustrated in Fig. 1, from runs at temperatures ranging from 9° to 37°. The activation energies were evaluated from Arrhenius plots constructed from 4–6 replicate determinations of each constant. Symbols are as in Fig. 2a. Relative standard deviations ranged between 4% and 7%.

by the displacing *S-type* substrate. And, since the k_r values are all based on data obtained with the same displacing substrate (benzylpenicillin) under presumably identical conditions, differences in these values can be traced to the effect of structural differences among the displaced substrates.

The range of variation observed with replicate determinations of k_f and k_r (shown in Fig. 2a) allows us to conclude that the plotted position of each substrate is uniquely specified by these parameters. As expected, the largest differences are observed with structurally unrelated substituents of 6-APA. We wish, however, to emphasize the important differences caused by the relatively minor modifications within the isoxazolyl family of substrates.

The significance of these results was further tested by measuring the respective k_f and k_r values at temperatures ranging from 9° to 37° in order to obtain the activation energies for the forward (E_a^f) and reverse (E_a^r) transitions. The plot of E_a^f vs. E_a^r shown in Fig. 2b confirms that each substrate induces a unique change in the conformation of the enzyme.

Independent evidence in support of this conclusion is presented in Fig. 3, in which we compare the rates of inactivation of penicillinase in the presence of saturating concentrations of the various *A-type* penicillins. In each case, the conformational change induced by the penicillin leads to exposure of an essential tyrosine residue (Ref. 12 and unpublished results); iodination of the exposed residue causes loss of catalytic activity (13). Thus, the rate of inactivation under the conditions described in Fig. 3 is in direct proportion to the extent of exposure of the essential tyrosine in the enzyme-substrate complex.

The different inactivation rates (Fig. 3) indicate that the position of the displaced tyrosine in such complexes varies with the different substrates. Similar results were obtained when the thermostability of the same enzyme-substrate complexes was compared. Although all A-type penicillins labilize the enzyme to heat (3), the rate of inactivation at 48° was specific for each penicillin tested at saturating concentrations (not shown). Taken together, these

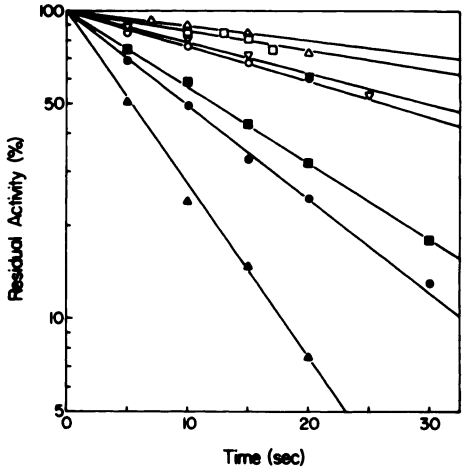


FIG. 3. *Rates of inactivation induced by A-type penicillins*

Enzyme samples were iodinated at 0° in the presence of substrates, and their activity determined as previously described (13). Residual activity is expressed as percent of the initial activity of each sample. No inactivation was observed in the absence of added substrate. The substrate concentrations (>3 mM) used are considered saturating since further excess did not affect the rates of inactivation. (△) oxacillin; (□) methicillin; (▽) cloxacillin; (○) nafcillin; (■) flucloxacillin; (●) pyrazocillin; (▲) dicloxacillin.

results provide further evidence that the subtle structural differences in the substrates are clearly reflected in the conformational state of the enzyme.

We conclude that the exquisite specificity of catalytic parameters is matched by, and thus may largely derive from, the uniqueness of the conformational response of the enzyme to the substrate.

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