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THE INTERACTION OF PENICILLINASE WITH PENICILLINS

V. CONFORMATIVE RESPONSE CONSTANTS

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SUMMARY

1. The combination of penicillinase (EC 3.5.2.6) with certain substrate analogs is accompanied by a specific change in the conformation of the active site of the enzyme. The change is termed conformative response.

2. The kinetics of the conformative response (as reflected in analog-induced inactivation) has been studied in eight enzyme–analog systems, comprising four different molecular species of penicillinase and two substrate analogs, methicillin and oxacillin.

3. The concentration of the analog which causes half-maximal rate of inactivation has been determined for, and found distinctively characteristic of, each enzyme–analog system. This value (K_{cr}) is significantly related to the corresponding K_m value, and appears to be a necessary parameter for correlating conformative response and catalysis.

INTRODUCTION

In earlier reports in this series[1–4], evidence was presented that the interaction of penicillinase (EC 3.5.2.6) with penicillins is accompanied by changes in the conformation of the active site of the enzyme. The nature of the conformational change was found to depend on the structure of the side-chain of the penicillin.

Several penicillins (*e.g.* methicillin or oxacillin) carry side-chains which cause unmasking of reactive groups involved in the activity of penicillinase. Such penicillins are relatively resistant to hydrolysis by this enzyme and have been referred to as substrate analogs.

One of the consequences of the unmasking effect of the analogs is sensitization of penicillinase to iodination. The expected correlation between the appearance of

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sensitivity to iodination and other expressions of a conformational shift has been previously established[3].

Since the proportion of penicillinase which acquires the iodine-sensitive conformation can be readily determined, this criterion was adopted for the kinetic studies of the analog-induced conformational changes to be reported here.

According to the terminology suggested elsewhere[5], the limited and reversible conformational transition induced by the analogs through specific interaction at the active site will be referred to as *conformative response.*

The term *conformative response constant* (K_{cr}) will be used, according to the operational definition[5,6], as a value numerically equal to the concentration (M) of the analog which causes half-maximal change in the property taken as a measure of the conformative response.

In the present report we describe the kinetics of conformative response and the derivation of conformative response constants in a comparative study of four penicillinases and two substrate analogs, methicillin and oxacillin. A preliminary account of this work has been included in ref. 5.

MATERIALS AND METHODS

Reagents

Sodium salt of benzylpenicillin was obtained from Merck and Co., Inc. Methicillin (Celbenin Batch No. F 209/1) was obtained from Beecham Research Laboratories. Oxacillin (Prostaphlin Lot 63 F 2411) was a gift from Bristol Laboratories.

Iodine, KI and sodium thiosulfate (all C.P. grade) were purchased from Agan Chemicals Ltd.

Water redistilled in glass was used in all experiments.

Penicillinase preparations

Preparations were derived from culture supernatants of the following strains: *Bacillus cereus,* strain 569, an inducible penicillinase producer[7]; *B. cereus,* strain 569/H, a constitutive mutant of strain 569 (ref. 8); *B. cereus,* strain 5B, an independently isolated constitutive strain[9]; *Bacillus licheniformis,* strain 749 (previously classified as *B. subtilis*)[10] ,an inducible penicillinase producer[11].

The crystalline preparation of penicillinase of *B. cereus* 5/B (ref. 12) used in this work was kindly provided by Dr. M. R. POLLOCK. The procedures for preparation and purification of other penicillinases were as previously described[3,6].

Iodination and assay of residual activity

The treatment consisted of exposing the enzyme at 0° to iodine in the presence of varying amounts of substrate analogs. Test tubes placed in an ice-bath contained 0.1 ml of an analog solution in 0.25 M phosphate buffer (pH 7.3), and 0.15 ml of the iodinating reagent (0.025 M I_2 and 0.125 M KI). For the *B. licheniformis* enzyme 0.05 ml of the reagent was used and the volume made up to 0.25 ml with twice distilled water.

The iodination was started by injecting the precooled penicillinase samples (in 0.2 ml of 0.5% gelatin) into the reaction mixtures. Iodination was terminated in one of the following ways: (1) By reduction of the iodine by the equivalent of sodium

thiosulfate, added in 2 ml of 0.5% gelatin. The samples were subsequently assayed by the manometric procedure of HENRY AND HOUSEWRIGHT[13] as described by POLLOCK[6]. (2) By dilution and assay according to the procedure previously described[3].

The activity was expressed in units as defined by POLLOCK AND TORRIANI[14].

RESULTS AND DISCUSSION

Kinetics of analog-induced inactivation

The exposure of penicillinase *(E)* to iodine *(I)* in the presence of a substrate analog *(A)* results in inactivation of the enzyme in a way consistent with the following scheme (ref. 5):

$$E + A \overset{a}{\rightarrow} E'A \overset{b}{\rightleftharpoons} E' + A \overset{c}{\rightarrow} E + A \qquad (1)$$

$$(E'A + E') + I \rightarrow EI + A \text{ (or } EI + A + EIA) \qquad (2)$$

where E' is the enzyme in the iodine-sensitive conformation, and EI (or EIA) is the iodinated and inactive derivative.

It will be noted that product formation from A is neglected in this scheme. This appears justified since the treatment was carried out with methicillin and oxacillin at 0° and pH 7.3. We found that under these conditions no detectable product accumulates within 60 sec. Thus the concentration of A, as well as that of I, both used in great excess over the molar concentration of the enzyme, can be considered constant throughout the treatment.

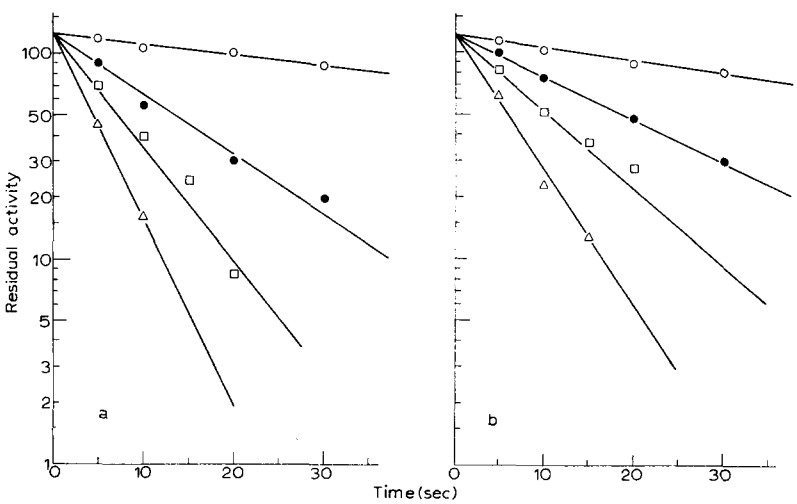

Fig. 1. Effect of analog concentration on the rate of inactivation of penicillinase of *B. licheniformis*, strain 749. Samples of the purified enzyme were exposed to (a) methicillin and (b) oxacillin and to 0.05 ml of the iodinating reagent. The concentrations used: a. ○, 0.0; ●, 5.5; □, 13.8; △, 27.5 μM methicillin. b. ○, 0.0; ●, 2.75; □, 5.50; △, 11.00 μM oxacillin. Iodination was terminated at the indicated time intervals and the residual activity assayed and expressed as units per sample. For other details see MATERIALS AND METHODS.

Reaction 1 consists of three steps, namely, (a) complex formation accompanied by a conformative response, (b) reversible dissociation, and (c) relaxation (*i.e.* termination of the conformative response).

We shall make no assumptions as to the relative rates of the various steps. We wish to point out, however, that, unless the relaxation step is extremely rapid, the proportion of the enzyme which persists in the E' form cannot be neglected.

Reaction 2 consists of the single, irreversible step of iodination of the enzyme in the E' (or $E'A$) state. The rate of this reaction can be determined from the rate of inactivation of the treated enzyme preparation.

The progress of inactivation of penicillinase iodinated in the presence of varying concentrations of analogs was studied in eight enzyme–analog systems. The systems represented each of the four molecular species of penicillinase described above (see MATERIALS AND METHODS), interacting with each of the two analogs, methicillin and oxacillin.

We found that in each case a semilogarithmic plot of the residual activity against time of iodination gives reasonably straight slopes which are proportional to the concentrations of the analog and extrapolate to a common point (initial activity). This is illustrated in Fig. 1. Thus, within the limits of our experimental conditions, the rate of inactivation appears to follow first-order kinetics, and, for each analog concentration, a first-order reaction rate constant can be postulated.

Determination of rate constants (k_a)

The first-order rate constants for analog-induced inactivation (k_a) can be calculated by substituting the E_o and E_t values in

$$k_a = \frac{1}{t} \ln \frac{E_0}{E_t} \tag{3}$$

where t is time of iodination (in seconds), E_0 is the initial enzyme activity and E_t the residual activity at time t.

The method is illustrated in Table I. It will be noted that the k_a values for each analog concentration derived from data obtained at different time intervals are, on the whole, in remarkably close agreement.

Alternatively, the rate constants can be derived from the slopes and arbitrary time points (t) in the semilogarithmic plots. Thus, for $t = 10$ sec, the equation in the decadic form becomes

$$k_a = 0.23 \log E_0/E_{10} \tag{4}$$

The k_a values derived from the slopes obtained as shown in Fig. 1 are listed in Tables II and III.

It is of significance that the effects of the two analogs on the kinetics of inactivation are very similar (*cf.* Tables II and III). This is in spite of the fact that the structural elements which induce the conformative response (*i.e.* the side-chains of the two analogs) are completely unrelated[4]. In contrast, the properties of the enzyme appear to be decisive as attested by the difference in the response of *B. cereus* and *B. licheniformis* enzymes to either analog.

TABLE I

FIRST-ORDER RATE CONSTANTS (k_a) FOR ANALOG-INDUCED INACTIVATION OF PENICILLINASE OF *B. cereus* 569

Samples of the purified enzyme (E_0 = 113 units) were exposed to varying concentrations of oxacillin and to 0.15 ml of the iodinating reagent. Iodination was terminated at the indicated time intervals (t) and the residual activity (E_t) assayed as described in MATERIALS AND METHODS For symbols see text.

Oxacillin concn. (μM)	*t (sec)*	*E_t (units)*	$k_a = \frac{2.3 \times \log E_0/E_t}{t}$ *(sec^{-1})*
0.0	5	113	0.000
	10	113	0.000
	30	110	0.001
21.6	5	99	0.025
	10	88	0.025
	20	73	0.022
	30	61	0.020
43.3	5	89	0.049
	10	71	0.047
	15	56	0.047
	20	43	0.048
86.6	5	72	0.090
	10	47	0.088
	15	37	0.080
	20	23	0.079

Derivation of conformative response constants (K_{cr})

Since the analog-induced inactivation is a consequence of the conformative response, the kinetics of the inactivation should be dependent on the concentration of ($E'A + E'$). Thus, according to our scheme (see Reactions 1 and 2 above) the rate constants (k_a) should approach a maximum value at analog concentrations approaching saturation of the enzyme (or at lower analog concentrations, if the ratio

TABLE II

FIRST-ORDER RATE CONSTANTS (k_a) AND K_{cr} VALUES FOR METHICILLIN-INDUCED INACTIVATION

For symbols see text. Data derived as illustrated in Figs. 1a and 3a.

Enzyme	*Analog (μM)*	*E_{10} (units)*	*$k_a = 0.23 \log E_0/E_{10}$ (sec^{-1})*	*K_{cr} (mM)*
B. cereus strain	27.5	48	0.097	
5/B	55.0	24	0.169	
E_0 = 127 units	110.0	9	0.263	0.15
B. cereus strain	27.5	81	0.055	
569/H	55.0	53	0.098	
E_0 = 141 units	110.0	29	0.158	0.17
B. cereus strain	27.5	72	0.049	
569	55.0	50	0.086	
E_0 = 118 units	110.0	22	0.167	0.17
B. licheniformis	5.5	63	0.069	
strain 749	13.8	35	0.127	
E_0 = 125 units	27.5	16	0.205	0.0023

TABLE III

FIRST-ORDER RATE CONSTANTS (k_a) AND K_{cr} VALUES FOR OXACILLIN-INDUCED INACTIVATION*

For symbols see text. Data derived as illustrated in Figs. 1b and 3b.

Enzyme	*Analog* (μM)	E_{10} (*units*)	$k_a = 0.23 \log E_0/E_{10}$ (sec^{-1})	K_{cr} (*mM*)
B. cereus strain	21.6	72	0.055	
5/B	43.3	47	0.098	
$E_0 = 125$ units	86.6	24	0.165	0.13
B. cereus strain	50.0	56	0.073	
569/H	75.0	45	0.095	
$E_0 = 116$ units	100.0	31	0.132	0.35
B. cereus strain	21.6	88	0.025	
569	43.3	69	0.049	
$E_0 = 113$ units	86.6	50	0.082	0.30
B. licheniformis	2.75	76	0.047	
strain 749	5.50	52	0.085	
$E_0 = 122$ units	11.00	27	0.151	0.0033

$E'/E'A$ is not negligible). In any case there should be a concentration of the analog at which k_a has half the maximal value, and which, by definition, corresponds to the conformative response constant (K_{cr}).

This proposition can be examined by any treatment used for the determination of K_m. We have in fact used a graphic method analogous to the LINEWEAVER–BURK plot[15]. This is illustrated in Figs. 2 and 3, where the reciprocal values of k_a have been plotted against the reciprocal values of the analog concentrations.

In each case a straight line was obtained which extrapolated to intersect with the vertical and horizontal axis. It is evident that the vertical intercept gives the reciprocal value for the maximum rate constant of inactivation. Consequently, the intercept with the horizontal axis gives the negative reciprocal of the molar concentration of the analog for which the k_a value is half maximal.

In other words, the value given by the horizontal intercept in Figs. 2 and 3 is

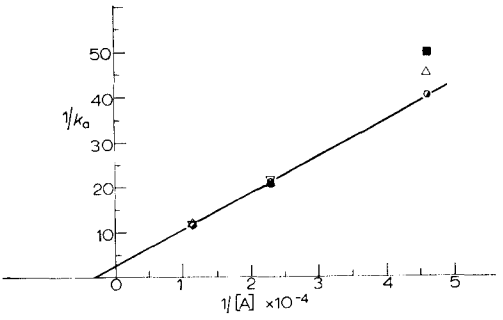

Fig. 2. Graphic determination of the conformative response constant for penicillinase of *B. cereus* strain 569 and oxacillin. Reciprocals of the inactivation rate constants (k_a) listed in Table I are plotted against reciprocals of the concentrations of oxacillin used in the experiment. The k_a values were derived from data obtained after 5 (●), 10 (○), 15 (▽), 20 (△), or 30 (■) sec of iodination (*cf.* Table I). The K_{cr} value derived from the intercept is $1.7 \cdot 10^{-4}$ M.

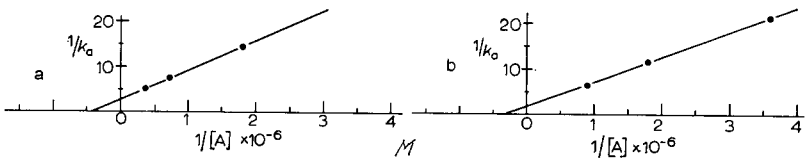

Fig. 3. Graphic determination of conformative response constants (K_{cr}) for penicillinase of *B. licheniformis*, strain 749 and (a) methicillin and (b) oxacillin. Reciprocals of inactivation rate constants (k_a) derived from the slopes in Fig. 1 are plotted against reciprocals of concentrations of the analogs (see also Tables II, III and text). The K_{cr} values derived from the respective intercepts are (a) $2.3 \cdot 10^{-6}$ M, and (b) $3.3 \cdot 10^{-6}$ M.

equal to $-1/K_{cr}$, where K_{cr} is the concentration of the analog at which the rate of analog-induced inactivation of the enzyme is half maximal.

In several cases (not illustrated here) an alternative and somewhat simplified procedure was applied for the derivation of K_{cr}. In this procedure an arbitrary level of residual activity is selected; the time required for the enzyme to reach that level at each analog concentration is determined from the respective slopes in the semi-logarithmic plot; the time readings *(t)* are plotted against the reciprocals of the corresponding analog concentrations.

It will be evident from examining Eqn. 3 that for a constant ratio E_0/E_t, t is proportional to $1/k_a$ and the resulting plot is analogous to the double-reciprocal plots used above. The vertical intercept will give the time required for the enzyme to reach the selected level of activity at saturating analog concentrations. This intercept is inversely proportional to the level of residual activity selected. In contrast the horizontal intercept is independent of the ratio E_0/E_t and corresponds precisely to the value $-1/K_{cr}$ as previously derived.

The relationship between K_{cr} values and the apparent dissociation constants

The K_{cr} values derived for the eight enzyme–analog systems (as illustrated in Figs. 1 and 3) are listed in Table IV and compared with the K_m values obtained for the same systems by the conventional procedure[3].

The K_m values for the *B. licheniformis* enzyme were found to be considerably lower than the rest and could not be determined by the manometric assay method[3], although indirect estimates suggest an order of 10^{-6}–10^{-5} M.

A comparison of the constants for the enzyme–analog systems listed in Table IV

TABLE IV

COMPARISON OF K_{cr} VALUES WITH THE CORRESPONDING K_m VALUES

The K_{cr} data are as derived from data in Tables II and III; the K_m data are quoted from ref. 3.

	Methicillin			*Oxacillin*		
	K_{cr} *(mM)*	K_m *(mM)*	K_m/K_{cr}	K_{cr} *(mM)*	K_m *(mM)*	K_m/K_{cr}
B. cereus 5/B	0.15	0.23	1.5	0.13	0.33	2.5
B. cereus 569/H	0.17	0.46	2.7	0.35	0.57	1.6
B. cereus 569	0.17	0.41	2.4	0.30	0.55	1.8
B. licheniformis 749	0.0023	<0.1	—	0.0033	<0.1	—

shows certain significant correlations. The closely related *B. cereus* penicillinases have the closest K_{cr} values. The unrelated penicillinase of *B. licheniformis* has K_{cr} values which are lower by two orders of magnitude. This large difference appears to parallel the estimated difference in the corresponding K_m values. Within the group of *B. cereus* penicillinases the K_{cr} values of the generally indistinguishable 569 and 569/H enzymes[6] appear to be virtually identical; so are the ratios K_m/K_{cr} for both analogs.

It will be also noted that in every case the K_{cr} value is significantly lower than the corresponding K_m value. Similarly, in every case the K_{cr} value was found to be considerably lower than the corresponding K_i value, when the analogs were tested as competitive inhibitors of the hydrolysis of benzylpenicillin (unpublished observations).

The relation of K_{cr} to the catalytic constants is of the greatest interest in a study of the role of conformative response in the function of an enzyme. Obviously, more information will be required before a conclusion can be reached. At the present stage it appears that all available observations are consistent with the tentative proposition that the conformative response persists after the dissociation of the enzyme–analog complex. In other words, the rate of relaxation (Step *c* in Eqn. 1) is slow relative to the rate of dissociation, and thus the proportion of the enzyme which is susceptible to iodination is always larger than the proportion of the enzyme bound to the analog. Although the observed relation ($K_{cr} < K_i, K_m$) is predicted by this proposition, it is not offered as evidence for the persistence of the conformative response. It will be clear, however, that the postulated persistence of the conformative response is likely to be reflected in the catalytic behaviour of the system. This is indeed the case, and the catalytic consequences of the conformative response are presently being explored.

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