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THE INTERACTION OF PENICILLINASE WITH PENICILLINS

VII. EFFECT OF SPECIFIC ANTIBODIES ON CONFORMATIVE RESPONSE

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SUMMARY

1. The conformative response (*i.e.* the substrate-induced change in the conformation of the enzyme) of penicillinase (penicillin amidohydrolase, EC 3.5.2.6) to methicillin and oxacillin is reflected in decreased stability to heat, urea and proteolysis. It is also reflected in increased susceptibility to changes in pH, to inactivation by iodine and by *p*-hydroxymercuribenzoate. These criteria were used to compare the conformative response of the antibody-bound and free enzyme.

2. Homologous antibodies inhibit the conformative response in penicillinase of *Bacillus cereus* strain 569/H. The inhibition of the conformative response shows significant correlation with the inhibition of activity, although it requires shorter incubation and a lower antibody to enzyme ratio. Identical patterns of inhibition are obtained with the anti-569/H serum and the penicillinase of the parental strain 569. The partial inhibition of the activity of the cross-reacting penicillinase of *B. cereus* strain 5/B is paralleled by the partial inhibition of the conformative response.

3. The kinetic anomaly which characterizes the interaction of penicillinase with benzylpenicillin, and methicillin or oxacillin, is not observed in the antibody-bound enzyme. The anomaly has been attributed to the persistence of the conformative response, and its elimination is interpreted as the result of the inhibition of the conformative response. The observed stimulation of the hydrolysis of 'resistant' penicillins by antibodies to the enzyme is consistent with this interpretation.

INTRODUCTION

The remarkable flexibility of the extracellular penicillinase (penicillin amidohydrolase, EC 3.5.2.6) formed by several strains of *Bacillus cereus* and *Bacillus licheniformis* is probably related to the fact that the tertiary structure of this enzyme is not stabilized by covalent bonds[1]. Thus, a subtle change, associated with the binding of a substrate by this enzyme, may lead to a readily recognizable conformational

Abbreviation: PCMB, *p*-hydroxymercuribenzoate.

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transition which would be prevented in a more rigidly constructed enzyme. Indeed, extracellular bacillary penicillinases have been found particularly suitable for the study of the conformational transition which accompanies the interaction of the enzyme with its substrates. The transition, which is specific and fully reversible (reversal follows the dissociation of the enzyme–substrate complex) has been termed "conformative response"[2]. The nature of the conformative response of penicillinase depends on the structure of the side-chain of the substrate (penicillin). Similarly, the rate of hydrolysis of the substrate by penicillinase is determined by the structure of the side-chain. It has been suggested therefore, that the side-chain determines the catalytic function of the enzyme by modifying the conformative response of the enzyme[3,4]. Evidence consistent with such causal relationship has been reported in the preceding papers in this series[5,6]. Furthermore, the observation has been made that the kinetics of hydrolysis of two substrates, differing in the conformative response elicited, is incompatible with the kinetics of the hydrolysis of each substrate alone. Thus, the apparent dissociation constants obtained from such mixed-substrate determinations, were found to differ very markedly from the corresponding constants obtained from single-substrate determinations[7]. This anomaly was attributed to the effect of the conformative response to one substrate on the affinity of the enzyme for the other substrate[7].

It was expected that the role of the conformative response in the regulation of the catalytic activity could be tested directly, if specific means were available for constraining the conformation, and consequently the conformative response of the enzyme. The observations presented in the preceding report[8] indicate that homologous antibodies impose a constraint on the conformation of the enzyme, and, on the conformative response to benzylpenicillin. In this communication we apply the antibody molecule as a specific tool for constraining the conformative response to three substrates, and examine the catalytic consequences of the suppression of the response.

MATERIALS AND METHODS

Penicillins and cephalosporins were the same as in the preceding report[8]. Other materials were: Potassium iodide (C.P.) and iodine (C.P.) both purchased from Agan Chemical Manufacturers. Methylene blue (B.D.H. Standard Strain) obtained from the British Drug Houses, Ltd.; *p*-hydroxymercuribenzoate (PCMB) (Lot No. 32202), obtained from Calbiochem Co., Ltd.; pronase (a protease from *Streptomyces griseus*, Type V, purified) purchased from Sigma Chemical Co.; urea (Analytical Reagent Grade) purchased from the British Drug Houses, Ltd.

Penicillinase preparations

Preparations were derived from culture supernatants of the bacterial strains listed below. The details of the purification procedures have been previously published[8,9,10].

B. cereus strain 569/H

This mutant strain, which produces penicillinase constitutively is derived from the inducible penicillinase-forming *B. cereus* 569. The enzyme preparation was obtained and purified as in the preceding report[8]. Unless otherwise stated, this penicillinase preparation was used as the source of the enzyme.

B. cereus strain 569

This inducible penicillinase-forming strain was grown as previously described[10]. Penicillinase formation was induced by the addition of methicillin to the growth medium after 1 h (0.1 μg/ml) and 5 h (1.0 μg/ml). The purification procedure was as for the mutant (569/H) strain[8].

B. cereus strain 5/B

This constitutive mutant was isolated from a penicillinase-negative strain (strain 5) of *B. cereus*[11]. A crystalline preparation of this enzyme[12] was kindly provided by Professor M. R. POLLOCK.

Immune sera

Antisera to penicillinase of *B. cereus* strain 569/H were the same as in the preceding report[8].

Antibody-bound enzyme

Penicillinase preparations were incubated with antiserum to the 569/H enzyme, and the free enzyme removed by the procedure described in the previous communication[8]. The "bound" enzyme corresponds to Fraction 2 obtained in that procedure.

Assay of penicillinase

Penicillinase activity was determined manometrically[13,14] or by the timed iodometric procedure[15]. In some experiments a modified procedure of the alkalimetric assay[5] was used. The instrumentation and procedure was as follows:

pH-stat assembly. The pH-stat assembly consisted of Radiometer Type TTA31 titration assembly equipped with type ABU1 Auto Burette and SBR2/SBU1 Titrigraph.

Solutions. Buffer—0.01 M phosphate buffer (pH 7.1); substrate solution—36 mg/ml of the substrate dissolved in the buffer; gelatin solution—0.5% solution in the buffer; enzyme solution—10–20 units in the gelatin solution; titrant—0.005–0.01 M NaOH (as indicated).

Radiometer setting. Proportional band 1.0; end-point 7.1.

Procedure. The reaction mixture consisting of 1.0 ml of the phosphate buffer and 1.25 ml of the enzyme solution was placed in the jacketed titration vessel adjusted to 30°. Five min were allowed for temperature equilibration with the automatic titration on. The recording was started, and 1 min later the reaction was initiated by injecting 0.25 ml of the substrate solution, prewarmed to 30°.

The automatically recorded delivery of the titrant was taken as a measure of the rate of the reaction.

Iodination and assay of residual activity

Standard conditions of iodination consisted of exposing penicillinase for 5 min at 0° to a reaction mixture (total volume—1.0 ml) containing 45 μmoles of phosphate buffer (pH 7.3), 0.15 ml of the iodinating reagent (0.025 M I_2 in 0.125 M KI) and 100 μg of the analog. All reagents were cooled to 0° before use.

The residual activity was assayed by transferring the assay reagent mixture (kept for 5 min at 30°) into the tube containing the treated enzyme preparation. The reagent

mixture consisted of 0.35 ml of 0.025 M I_2 in 0.125 M KI, 1.0 ml of 8.5 mM benzylpenicillin in 0.1 M phosphate buffer (pH 7.0), and 3.0 ml of 0.5% gelatin.

PCMB treatment

Test tubes containing penicillinase, 20 μmoles of phosphate buffer (pH 7.3), 1000 μmoles of PCMB, and 100 μg of analog were incubated for 10 min at 37°. The total volume was 0.7 ml. The reaction was terminated by transferring the test tubes to an ice bath for 1 min. The residual activity was assayed by adding the prewarmed (30°) reagents of the timed iodometric method[15] to the reaction mixture.

Proteolysis

The enzyme used for proteolysis was pronase, a broad spectrum protease derived from *Streptomyces griseus*. Stock solutions were prepared by dissolving 10 mg of pronase in 1 ml of 0.01 M $CaCl_2$, stored at −20° and used within one week. Proteolysis was carried out in test tubes containing penicillinase in 0.025 M phosphate buffer (pH 8.0), pronase (250 μg), and analogs (100 μg). After 10 min at 37°, the test tubes were immersed in a freezing bath for 1 min. The residual activity was assayed by the timed iodometric method[15].

Photooxidation

Samples of penicillinase were illuminated (1200 Lux) for 90 min at 30° in the presence of 0.01% methylene blue and 0.1 M phosphate buffer (pH 8.0), as previously described[5]. The final volume was made up to 0.65 ml. The residual activity was assayed by the timed iodometric method[15].

Urea treatment

Penicillinase was delivered to a solution containing urea (5 M), gelatin (0.25%), phosphate buffer 0.01 M (pH 7) and 150 μg of methicillin, and incubated at 0° for 1 min. All the reagents were precooled before use. The final volume was 3 ml. The treatment was terminated by adding 0.5 ml of iodine (0.025 M I_2 in 0.125 M KI) and 1 ml of substrate (3 mg of benzylpenicillin) in 0.1 M phosphate buffer (pH 7) which had been prewarmed to 30°. The residual activity was assayed by the timed iodometric method[15].

RESULTS

Conformative response of free and bound enzyme

The experiments described below are grouped according to the criteria used for the determination of the conformative response of penicillinase to the 'resistant' penicillins[16,17], represented here by methicillin and oxacillin. The conformative response to cephalosporins[5] is illustrated by the effect of cephalosporin C on photooxidation. The term "bound" (as opposed to "free") enzyme refers to a preparation of penicillinase, in which the enzyme is combined with the homologous antibody. The procedure of preparation and purification has been described in the preceding report[8]. The bound enzyme used here corresponds to Fraction 2 obtained by that procedure.

Heat inactivation. The thermostability of the free enzyme shows a characteristic pH-dependence, and it decreases progressively as the pH is raised above 6.0 (ref. 4). The thermostability of the antibody-bound enzyme is considerably less affected by

variations of pH (ref. 8). When one of the penicillin analogs (oxacillin or methicillin) is present during the heat treatment, the difference in the stability of the two enzyme preparations is much more pronounced. These observations are illustrated in Fig. 1, where the effect of exposure to 58° for 2 min at pH 4.5–8.0 is summarized. Under these conditions the characteristic response pattern of the free enzyme is hardly recognizable in the bound enzyme. In the presence of oxacillin the free enzyme is completely inactivated throughout the range of pH tested. In contrast, the thermostability of the bound enzyme is fully preserved in the presence of the analog except for a slight decrease at pH 5.0 and below. Identical results were obtained when oxacillin was replaced with methicillin in these experiments.

The analog-induced inactivation illustrated in Fig. 1 is one of several[5,16] manifestations of the conformative response to oxacillin. Its absence in the bound enzyme is taken as indication that the antibodies may interfere with the conformative response.

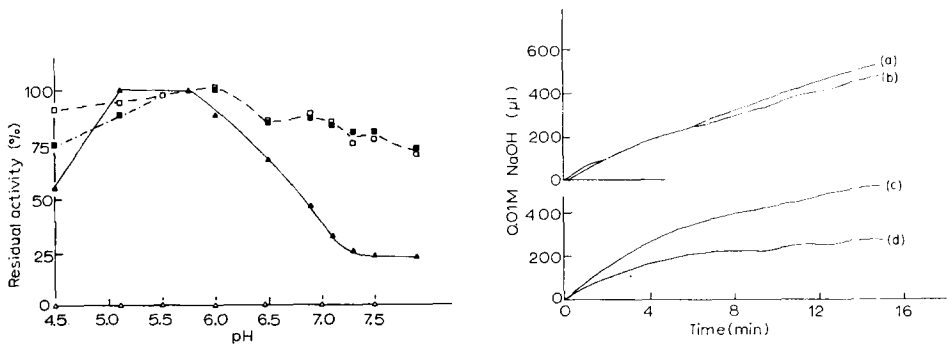

Fig. 1. Effect of a substrate analog on the thermostability of free and bound enzyme. Both enzyme preparations were diluted in 0.5% gelatin to approximately 250 units/ml. Samples (0.2 ml) were added to tubes containing 0.25 ml of normal rabbit serum (1:80) and 0.05 ml of the appropriate buffer solution (see below). Oxacillin (100 μg) was included in the buffer solution as indicated. The tubes were immersed for 2 min in a 58° water bath, cooled (1 min at 0°) and the residual activity assayed (see METHODS). The results are presented as % of the maximum activity of each preparation. Buffers used: 0.5 M phthalate–NaOH (pH 4.5–5.5); 1.0 M phosphate (pH 6.1–8.0). Broken line, bound enzyme with (■) and without (□) oxacillin; solid line, free enzyme with (△) and without (▲) oxacillin.

Fig. 2. Proteolytic inactivation of free and bound enzyme in the presence of oxacillin. Samples of bound (a, b) and free (c, d) enzyme (280 units in 2.0 ml of 5 mM phosphate buffer, pH 7.5) were assayed with oxacillin (9.0 mg) as substrate. The assay was carried out at 30° and pH 7.5, and product formation was followed by the alkalimetric procedure (see METHODS). Pronase (1 mg in 0.1 ml of 0.01 M $CaCl_2$) was present in samples (b) and (d). Tracings of actual recordings are presented.

Note that in this and the following experiments the antibodies do not prevent the recognition of the analogs by the enzyme, as these analogs are in fact hydrolyzed more rapidly by the antibody-bound enzyme preparation[8].

Inactivation by proteolysis. Native penicillinase is highly resistant to proteolytic attack. Attempts to inactivate the enzyme by incubation with various proteolytic enzymes (*e.g.* trypsin, chymotrypsin, pepsin, subtilisin, amino- or carboxypeptidase) have failed. We found, however, that treatment with pronase results in partial inactivation of the enzyme. The action of pronase on penicillinase is remarkably enhanced by the presence of penicillin analogs[5] and thus can serve as a test of conformative response.

In the example presented in Fig. 2, the rate of hydrolysis of oxacillin by free and bound enzyme is recorded. The activity of the free enzyme decays gradually in the course of the reaction; the decay is accelerated by pronase. In contrast, the bound enzyme retains full activity in the course of the reaction; and even in the presence of pronase the activity is preserved. The results were similar when methicillin, rather than oxacillin, was used as the substrate.

Thus, in the present system, the conformative response of the free enzyme to oxacillin and to methicillin is reflected in instability of the enzyme in the course of the reaction and in increased susceptibility to proteolysis. In contrast, the bound enzyme appears to maintain a stable conformation in the course of its interaction with these analogs.

Susceptibility to photooxidation. Photooxidation provides another means of studying the stabilizing effect of antibodies on the enzyme. We found that the activity of the enzyme is sensitive to light in the presence of methylene blue[5]. The survival of activity of free and bound enzyme is compared in Table I. The antibodies clearly protect the enzyme against the consequences of this treatment.

Comparison of the conformative response in this system involved the use of another substrate analog, cephalosporin C, instead of methicillin and oxacillin, both of which have little effect on the susceptibility of the free enzyme to photooxidation[5]. In contrast to the class of penicillin analogs, represented here by methicillin and

TABLE I

EFFECT OF ANALOGS ON THE STABILITY OF FREE AND BOUND ENZYME

The sensitivity of free and bound enzyme to urea, PCMB and photo-oxidation was tested as described in METHODS. The residual activity is expressed as % of the activity of untreated controls. Concentrations in urea treatment (per 3.6 ml final volume): penicillinase, 85 units; methicillin, 150 μg; urea, 5 M. Concentrations in PCMB treatment (per 0.7 ml final volume): penicillinase, 85 units; analogs (methicillin and oxacillin) 100 μg; PCMB, 330 μg. Concentrations in photooxidation (per 0.65 ml final volume): penicillinase 75 units; cephalosporin C, 500 μg.

Treatment	*Analog*	*Free enzyme*	*Bound enzyme*
Urea	—	100	100
	Methicillin	6.7	90.5
PCMB	—	100	100
	Methicillin	<1.0	44.5
	Oxacillin	4.8	100
Photooxidation	—	17.8	54.5
	Cephalosporin C	66.5	76.0

oxacillin, cephalosporins induce a stable conformation in the active site of penicillinase[5]. The conformative response of the free enzyme to cephalosporin C is indeed reflected in an increased resistance to photooxidation. The response of the bound enzyme to the stabilizing effect of this analog is much less pronounced (Table I).

Stability in urea. Methicillin is known to facilitate the disrupting affect of urea on penicillinase. Thus exposure to methicillin (60 μg/ml) in 5 M urea results in almost complete inactivation of the free enzyme although exposure to urea or methicillin alone at these concentrations causes no loss of activity[3,17]. This observation was used as a further criterion in comparing the effect of analogs on the conformation of the free

and bound enzyme. As is shown in Table I the effect of methicillin and urea on the enzyme is virtually eliminated by the antibody.

Inactivation by PCMB. The activity of penicillinase is not significantly affected by PCMB at concentrations below $2.7 \cdot 10^{-3}$ M. However, the analogs sensitize the enzyme to PCMB; this is reflected in loss of activity in the presence of less than $2 \cdot 10^{-3}$ M PCMB[5]. The sensitizing effect of the analogs is prevented by the antibody. Results of a typical experiment are summarized in Table I.

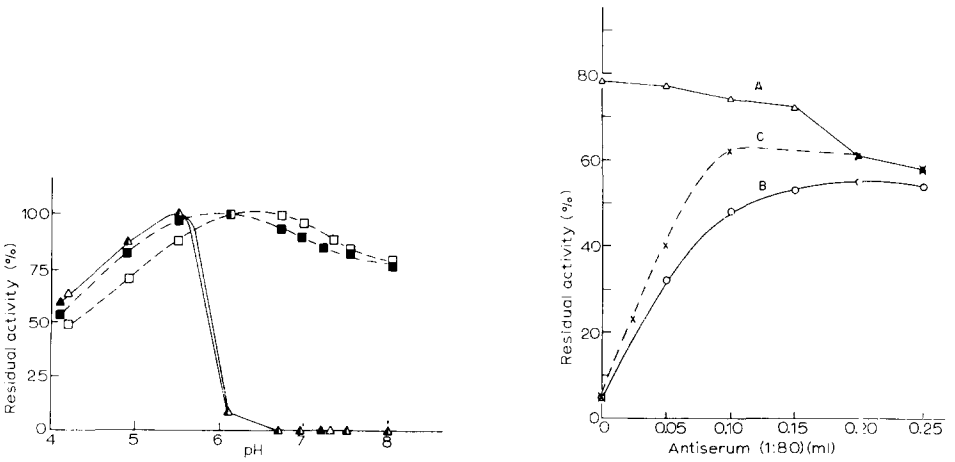

Fig. 3. Effect of substrate analogs on stability of free and bound enzyme: iodination at various pH values. Samples of free and bound penicillinase (47 and 65 units, respectively) were iodinated (for details see METHODS) in the presence of 100 μg of methicillin or oxacillin and 0.1 M buffer at various pH values. Iodination was performed in a total volume of 1.0 ml. Buffers used: 0.5 M phthalate–NaOH (pH 4.2–5.5); 1.0 M phosphate (pH 6.1–8.0). The residual activity was assayed iodometrically. The results are expressed as % of the maximum activity of each preparation. Broken line, bound enzyme with (□) methicillin or (■) oxacillin; solid line, free enzyme with (△) methicillin or (▲) oxacillin.

Fig. 4. Neutralization and protection as a function of antibody concentration. Samples of penicillinase were incubated (10 min at 30°) with the indicated amounts of the antiserum, in a total volume of 0.5 ml, made up with normal rabbit serum (1:80). The conditions of treatment were as described below. The residual activity (for assay see METHODS) is expressed as % of the initial activity of the untreated samples. *A. Neutralization.* The enzyme samples (80 units) were delivered in 0.2 ml of 0.5% gelatin. The incubation was terminated by the addition of 0.5 ml of precooled phosphate buffer (0.2 M, pH 7.3), and the transfer of the samples to an ice bath. The residual activity was assayed 5 min later without further treatment. *B. Protection against iodination.* The enzyme samples were delivered and incubated as above. The incubation was terminated by the addition of 0.5 ml of precooled phosphate buffer (0.2 M, pH 7.3) containing methicillin (100 μg) and the iodinating reagent (see METHODS). The residual activity was assayed after 5 min at 0°. *C. Protection against proteolysis.* The enzyme samples (100 units) were delivered in 0.2 ml of 0.1% gelatin. The incubation was carried out in the presence of pronase (250 μg) and methicillin (100 μg). The termination and assay of residual activity was as in *(A)*.

Inactivation by iodine. Susceptibility to iodination provides a very sensitive measure of the conformative response of penicillinase to the analogs[3,4]. In Fig. 3 we compare the effect of pH on the iodination of the enzyme in the presence of methicillin and oxacillin respectively. In both cases, the free enzyme is completely inactivated at pH values of 6.7 and above. The bound enzyme, however, shows no loss of activity at that pH, and retains most of the initial activity in the alkaline region.

Correlation between the effect of antibody on activity and on the conformative response

The effect of antibody on activity was tested by observing the inhibition of hydrolysis of benzylpenicillin ("neutralization"). The effect of antibody on the conformative response was tested by observing the prevention of proteolysis or iodination in the presence of penicillin analogs ("protection"). The correlation between neutralization and protection is illustrated in Fig. 4, where the two effects are presented as a function of the concentration of the antibody. Note that the protective effect is clearly visible at the lowest antibody concentrations, where neutralization is barely detectable. Furthermore, maximum protection is obtained with antibody concentrations which give less than half-maximal neutralization (Fig. 4 and ref. 8).

The correlation has been further examined by comparing the kinetics of neutralization and of protection. As shown in Fig. 5, there is an interesting discrepancy in the time dependence of the two effects of the antibody. The full protective effect is attained very rapidly (it is nearly maximal within 5 sec of incubation), whereas neutralization appears to require several minutes for completion. The discrepancy may be due to the effect of the substrate on the enzyme–antibody complex (see DISCUSSION).

An additional test of the correlation has been made possible by the availability of two other penicillinase preparations derived from related strains of *B. cereus*. It has been observed[18] that the penicillinase of strain 569 of *B. cereus* is serologically indistinguishable from that of the strain (569/H) used throughout this work. The peni-

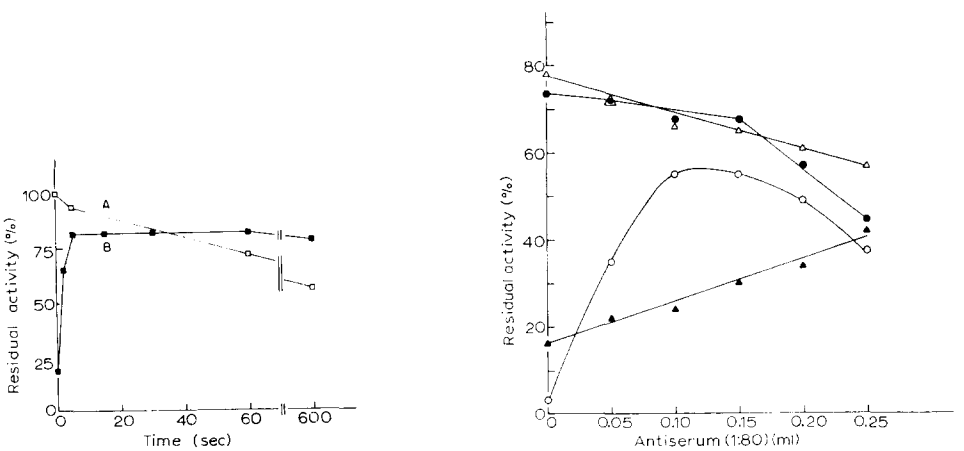

Fig. 5. Kinetics of neutralization and of protection. Samples of penicillinase (85 units in 0.2 ml of 0.5% gelatin) were incubated at 0° with 0.25 ml of antiserum (diluted 1:160 with normal rabbit serum). The incubation was terminated at the indicated time intervals, and its effect on the enzyme tested as described below. The residual activity (for assay see METHODS) is expressed as % of the initial activity of the untreated samples. *A. Neutralization.* The incubation mixture was diluted (× 10) with the prewarmed (30°) assay reagents and the assay of the residual activity started upon termination of the incubation (see METHODS). *B. Protection against iodination.* The incubation was terminated by injecting 0.65 ml of precooled phosphate buffer (0.1 M, pH 7.3) containing methicillin (100 μg) and the iodinating reagent (see METHODS). The residual activity was assayed after 5 min at 0°.

Fig. 6. Neutralization and protection in heterologous systems. Samples of penicillinase of *B. cereus* strain 569 (74 units in 0.2 ml of 0.5% gelatin) and of penicillinase of *B. cereus* strain 5/B (78 units in 0.2 ml of 0.5% gelatin) were incubated (10 min at 30°) with the indicated amounts of antiserum to 569/H penicillinase. The total volume (0.5 ml) was made up with normal rabbit serum (1:80). All other details were as in Fig. 4. 569 enzyme, neutralization (●) and protection (○); 5/B enzyme, neutralization (△) and protection (▲).

cillinase of the more distantly related strain (5/B) of *B. cereus* is also neutralized by antibodies to strain 569/H, though less effectively[19]. Fig. 6 gives a comparison of the neutralizing and protective effect of antibodies in heterologous systems comprising enzymes 569 and 5/B and antiserum to enzyme 569/H. Note that the neutralization and protection patterns in the 569 and anti-569/H system follow very closely the pattern of the homologous system (Fig. 4). The corresponding patterns in the 5/B and anti-569/H system also show a concentration-dependent inhibition by antibodies of the activity and of the conformative response of the enzyme. As expected the effect is considerably less pronounced than in the homologous system. Note however, that in this system too neutralization is very closely related to protection.

Comparison of dissociation constants of free and bound enzyme

In a preceding communication[7] we reported an anomalous relationship between the apparent dissociation constants obtained for penicillinase and benzylpenicillin, methicillin and oxacillin. The K_m values obtained for each substrate were considerably lower than the corresponding K_m values (or the relevant K_i values) derived from determinations made in the presence of a competing substrate.

We concluded that this anomaly was due to the interaction of the enzyme with the competing substrate, and suggested that the conformative response induced by the competing substrate modified the binding properties of the enzyme. Since antibodies have been shown to constrain the conformative response of the enzyme, we expected that the anomalous kinetics attributed to the extensive changes characteristically associated with the conformative response of the free penicillinase will

TABLE II

APPARENT DISSOCIATION CONSTANTS ($\times 10^{-3}$ M) FOR ANTIBODY-BOUND PENICILLINASE

A. Mixed substrate constants

The ratios of the Michaelis constants for benzylpenicillin (K_m^1) and for methicillin or oxacillin (K_m^2) were determined by the procedure of WHITTAKER AND ADAMS[25]. The K_i and K_m^1 values were obtained from competitive inhibition data, corrected for the hydrolysis of the inhibitor[21] and plotted according to DIXON[22]. The K_m^2 values were obtained by substitution of the K_m^1 values in the observed $K_m^1:K_m^2$ ratios. For other details see text.

Substrate 1	*Substrate 2*	$K_m^1:K_m^2$	K_m^1	K_m^2	K_i
Benzylpenicillin	Methicillin	1:5.4	0.36	1.9	2.9
Benzylpenicillin	Oxacillin	1:5.7	0.33	1.9	1.9

B. Single-substrate constants

The K_m values were determined by the procedure of LINEWEAVER AND BURK[20]. The K_m^1 and K_m^2 values are the same as in Part A of this Table. The numbers in parentheses are the corresponding ratios for the free (rather than antibody-bound) 569/H penicillinase, and are based on data reported in a previous communication[7].

Substrate	K_m	*Ratio of single-substrate to mixed-substrate constants*
Benzylpenicillin	0.31	$K_m:K_m^1 = 0.86$–0.94 (0.03–0.14)
Methicillin	1.8	$K_m:K_m^2 = 0.95$ (0.26)
Oxacillin	1.7	$K_m:K_m^2 = 0.89$ (0.095)

not be observed in the antibody-bound enzyme. This indeed was found to be the case, as shown in Table II. The antibody-bound enzyme was prepared as previously described[8], and the K_m values of this preparation for benzylpenicillin, methicillin and oxacillin were determined by the standard procedure of LINEWEAVER AND BURK[20]. These values and the values similarly obtained for the free enzyme will be referred to as single-substrate constants.

Apparent dissociation constants derived from determinations carried out in the presence of two competing substrates will be referred to as mixed-substrate constants. The mixed-substrate constants for the free and antibody bound enzyme were determined by two independent procedures.

In the first procedure the analogs (methicillin and oxacillin) were considered as competitive inhibitors of hydrolysis of benzylpenicillin[3,4].

Initial reaction rates were determined manometrically[14] for mixtures containing 2, 4, 6 and 8 μmoles of benzylpenicillin, and 0, 15, 20, 30 and 40 μmoles of the inhibitor (methicillin or oxacillin) in a total volume of 3.0 ml. Since in this assay procedure no distinction is made between the products of the hydrolysis of the various substrates, the inhibited reaction rates were corrected for the hydrolysis of the inhibitor[21]. The reciprocal values of the corrected initial rates were plotted against the inhibitor concentrations to yield the K_i values directly[22]. As pointed out by DIXON[23] the horizontal intercept of each slope in this plot gives a value (A) equal to

$$-K_i\left(\frac{(S)}{K_m}+1\right).$$

It was thus possible to derive by this procedure mixed-substrate K_m values for benzylpenicillin as well as K_i values for each analog. The results obtained with the free and antibody-bound enzyme are compared in Table II.

In the second procedure used, each analog was considered as a substrate competing with benzylpenicillin for the active site of the enzyme. It has been suggested[24] that the ratio of the Michaelis constants of the enzyme with respect to two such competing substrates can be deduced from the variation of the rate of the two substrates in the reaction mixture. If the difference between the K_m values of the competing substrates is sufficiently large, as is the case here, the ratio of the Michaelis constants can be obtained from the following equation[25]

$$\frac{K_m^1}{K_m^2}=\frac{v_1-v_{12}}{v_{12}-v_2} \qquad (1)$$

where v_1, v_2 and v_{12} represent the maximum initial rates of the hydrolysis of Substrate 1 and Substrate 2 alone, and of the hydrolysis of an equimolar mixture of the substrates, respectively.

In a previous communication[7] we have shown that the K_m ratios obtained by this procedure were incompatible with the K_m values derived from single-substrate determinations. These ratios were, however, compatible with the apparent dissociation constants derived from mixed-substrate determinations. Thus, the results obtained by the equimolar procedure confirmed the observation that the presence of a competing substrate causes an apparent change in the binding properties of the free enzyme.

As pointed out above, we expected no such change in the antibody-bound enzyme. Consequently, the ratio of the Michaelis constants obtained by the equimolar

procedure was expected to agree with the ratio derived from single-substrate determinations. This was tested and confirmed as shown in Table II, where the single-substrate and mixed-substrate constants for free and bound enzyme are compared. It is clear that the anomaly which characterizes the interaction of the free penicillinase with penicillins is not observed when the enzyme is bound to antibody.

DISCUSSION

In the preceding report[8] we described differences in the properties of free and antibody-bound penicillinase, which indicated that the conformation of the bound enzyme is stabilized by the antibody. The effect of antibodies on the activity (hydrolysis of benzylpenicillin) suggested that the stabilization of the enzyme molecule imposed a constraint on the conformative response to the substrate. In the present report we examined the effect of antibodies on the conformative response to analogs of the substrate, methicillin and oxacillin. The conformative response to these analogs is reflected in a characteristic pH-dependent sensitization to heat and to iodination. It is also reflected in a highly increased susceptibility to inactivation by proteolysis, by urea and by PCMB. Judged by these criteria, the homologous antibody appears to prevent the conformative response. It will be obvious from the evidence presented above (Fig. 2) that under these conditions the interaction of the enzyme with the analog is not prevented.

An alternative to be considered is that the conformative response does take place but it is masked by the antibody. It is indeed conceivable that in the bound enzyme, as in the free enzyme, the relevant region is unfolded in the presence of the analog; the antibody would then be said to hinder the access (*e.g.* of iodine or pronase) to the exposed region, and the conformative response might not be observed. However, steric hindrance will not account for the lack of measurable response by other criteria (*e.g.* thermostability). We suggest, therefore, that the conformative response to these analogs is indeed largely inhibited by the constraint imposed by the antibody. The results obtained with benzylpenicillin[8] and with cephalosporin C (Table I) are consistent with the expectation that the conformative response to other classes of compounds interacting specifically with the active site of the enzyme is similarly inhibited by the antibody. In view of that, we suggest that the antibody be considered as a specific tool for constraining the conformative response of the enzyme. This is to say, that by comparing the catalytic properties of the free and antibody-bound enzyme we may obtain relevant information on the role of conformative response in the function of the enzyme.

To illustrate this possibility we shall first consider the difference in the rate of hydrolysis of the various substrates (penicillins), which are *N*-acyl derivatives of 6-aminopenicillanic acid. The substrates can be grouped in two classes, according to their susceptibility to enzymic hydrolysis, relative to the unsubstituted 6-aminopenicillanic acid[5]. The conformative response to the relatively resistant penicillins (represented here by methicillin and oxacillin) is entirely different from the conformative response to the relatively susceptible penicillins (represented here by benzylpenicillin)[3,4]. The consistent correlation between the effect of the substituent ('side-chain') on the susceptibility to hydrolysis and the conformative response suggested a causal relationship. Specifically, we proposed that the conformative response elicited

by the side-chain determines the catalytic efficiency of the enzyme. The corrollary was that a constraint of the conformative response would narrow down the difference in the rate of hydrolysis of the susceptible and resistant substrates. In other words, the hydrolysis of susceptible substrates would be inhibited, while the hydrolysis of resistant substrate would appear to be stimulated.

The correlation between the inhibition of activity (on benzylpenicillin) and the inhibition of the conformative response, which has been illustrated in Fig. 4 (and in Fig. 6 for heterologous systems), is highly suggestive of a possible essential function of the conformative response in catalysis. The discrepancy observed in the kinetics of inhibition of the conformative response and of the activity suggests that the enzyme–antibody complex which is formed very rapidly (see Fig. 5) tends to dissociate in the presence of excess benzylpenicillin (*i.e.* when tested for activity) before it acquires maximal stability.

An interesting consequence of the assumed functional correlation would be that the inhibition of enzymic activity by neutralizing antibody is the result of extensive "paralysis" of the conformative response. The present evidence, which is limited to a single system, does not exclude, of course, other mechanisms (*e.g.* steric hindrance[26] which may determine, or modify, the extent of inhibition in different systems.

The stimulating effect of antibodies on the hydrolysis of methicillin[27] and other resistant penicillns[8] is, however, extremely difficult to reconcile with other mechanisms of interaction of antibodies with enzyme.

In contrast, the observations summarized above make this phenomenon an obvious and necessary consequence of the constraint of the conformative response. It would be of considerable interest to see whether this conclusion can be extended to other systems, but as yet nothing is known about the conformative response patterns of enzymes other than penicillinase, where stimulation by antibody has been observed[28,29].

A mechanism remarkably related to our conclusions has, in fact, been anticipated by CINADER[29] in the just-published comprehensive discussion of the subject of inhibition and activation by antibodies. The only important difference is that, according to CINADER, the stimulating antibody imposes a conformation which is favorable to catalysis, whereas we conclude that the antibody prevents a change which is unfavorable to catalysis.

Another aspect of the catalytic behavior of penicillinase, which has been attributed to the conformative response, is the kinetic anomaly reflected in a discrepancy between the dissociation constants determined under two sets of experimental conditions[7]. The details of experimental procedures are given in the last section of RESULTS. The essence of this observation was that two competing substrates, which differ in their effect on the conformation of the enzyme, appear to have a reciprocal effect on the dissociation constants for each substrate[7]. Our tentative interpretation of this observation is based on a previous study[6] which indicated that the conformative response may persist after the dissociation of the enzyme–substrate complex and thus affect the affinity of the enzyme for the competing substrate molecule. It was expected, that a constraint imposed on the conformative response will eliminate the kinetic anomaly. Our present results (Table II) are fully consistent with that expectation.

In conclusion, the seemingly unrelated differences in the patterns of stability, activity and affinity, observed in the free and antibody-bound penicillinase can be

attributed to the constraint of the conformative response by the antibody. Conversely, specific antibodies may serve as a useful tool in the study of the role of the conformative response in the function of the enzyme.

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