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THE INTERACTION OF PENICILLINASE WITH PENICILLINS VI. COMPARISON OF FREE AND ANTIBODY-BOUND ENZYME

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SUMMARY

1. Antibodies to penicillinase (penicillin amidohydrolase, EC 3.5.2.6) increase the stability of the enzyme to heat and to variations of pH. The stabilizing effect of the antibodies was correlated with their effect on the activity of the enzyme.

2. Stabilization was observed with antibodies which inhibit the enzyme activity as well as with antibodies which stimulate the hydrolysis of methicillin, oxacillin and cloxacillin.

3. A catalytically active enzyme–antibody complex has been prepared and purified by selective removal of the free enzyme. Several properties of the free and antibody-bound enzyme have been compared.

4. The difference in the response of antibody-bound and free enzyme to variations in pH and temperature appears to be eliminated by the substrate. This, and other observations reported here, suggests that the antibodies exert their effect on both the stability and the catalytic behavior of penicillinase by imposing a constraint on the conformation of the enzyme molecule.

INTRODUCTION

The effect of specific antibodies on the activity of an enzyme has been widely investigated in a variety of systems[1]. Although in some cases no effect was observed, the activity of most enzymes examined was inhibited by specific antisera[2,3]. Inhibition of the activity of the extracellular penicillinase (penicillin amidohydrolase, EC 3.5.2.6) of *Bacillus cereus* by the homologous antiserum was first reported by HOUSEWRIGHT AND HENRY[4]. A careful analysis of this observation and of the inhibition pattern of related penicillinase preparations has been included in several studies by POLLOCK and his school[5–8]. It laid the ground for the surprising discovery, that antisera to penicillinase may stimulate, as well as inhibit, the enzymic activity[9]. The stimulatory effect was attributed to a distinct class of antibodies and was clearly demonstrable when methicillin, rather than benzylpenicillin served as the substrate[10].

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In previous studies in this series[11–14] evidence was presented that the conformation of penicillinase in the presence of methicillin (or other poorly hydrolyzed penicillins) is very different from that observed in the presence of benzylpenicillin. It was suggested that the difference in conformation was sufficient to account for the difference in the rate of hydrolysis of these compounds[14,15]. The implication was that a constraint imposed on the conformational flexibility of penicillinase would reduce the difference in the rate of hydrolysis of benzylpenicillin and methicillin. Evidence consistent with this proposition has been recently described[16]. It was thus conceivable that the antibodies modify the activity of penicillinase by imposing a constraint on the flexibility of the enzyme molecule. In the present communication we examine the effect of specific antibodies on stability in relation to their effect on activity of penicillinase. In an accompanying report[17] we illustrate the use of antibodies as a specific tool for constraining the conformation of the enzyme.

MATERIALS AND METHODS

Substrates

Benzylpenicillin and phenoxymethylpenicillin were purchased from Rafa Laboratories. Methicillin and cloxacillin were obtained from Beecham Laboratories. Oxacillin and ampicillin were gifts from Bristol Laboratories. Cephalosporin C and cephalothin were provided by Eli Lilly and Co. Benzylcephalosporin and cephaloridine were gifts from Glaxo Laboratories, Ltd.

Penicillinase

The enzyme was prepared from the culture supernatant of *Bacillus cereus* strain 569/H. The cultivation and the early stages of purification were as previously described[18]. Further purification was obtained by CM-cellulose column chromatography. The procedure was similar to that described for the purification of a related penicillinase preparation[16].

Assay of penicillinase

Penicillinase activity was determined manometrically[20,21] or by the timed iodometric procedure[22].

Immune sera

Antisera to 569/H penicillinase were prepared by injecting 3–4 mg of the purified enzyme, homogenized with Freund's adjuvant, into the subscapular region of a rabbit[19]. The injection was repeated 4 times at 10-day intervals. The rabbits were bled from the ear 10 days after the last injection. The blood was allowed to clot overnight and the sera were separated and stored at $-20°$. One month after the last injection, the rabbits were given a booster injection of 3–4 mg of the enzyme preparation, and the sera were collected and stored as before. All sera were pooled before use.

Other reagents

The chemicals were of reagent grade. Celite 535 (diatomaceous silica filter-aid) was obtained from Johns-Manville Products Corp.

Heat treatment and assay of residual activity

The treatment was carried out in test tubes immersed in a water bath, with temperature regulated by a Thermomix II Immersion Thermostat within 0.1°. At the end of incubation, the tubes were immersed in an icebath for 1 min. The assay reagent mixture consisted of 1.0 ml of 0.1 M phosphate buffer (pH 7), 3.0 ml of 0.5% gelatin, 0.5 ml of 0.025 M I_2 in 0.125 M KI, and 3 mg benzylpenicillin. The assay of residual activity was started by adding the assay reagent mixture, prewarmed to 30°, to the tubes containing the treated preparations and transferring the tubes to a 30° water bath. The activity was determined as in the timed iodometric assay[22].

RESULTS

Effect of the antiserum on the thermostability of the enzyme

Antiserum to penicillinase was found to increase the stability of the enzyme to

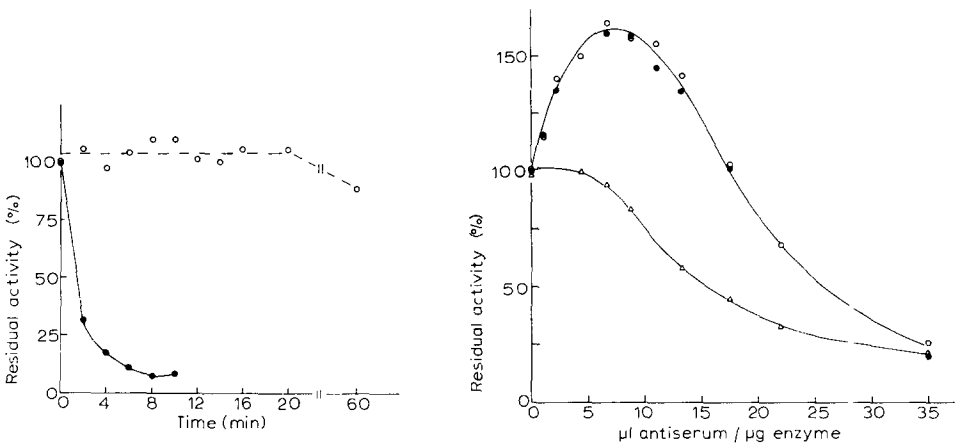

Fig. 1. Effect of antiserum on the rate of thermal inactivation of penicillinase. Penicillinase (88 units in 0.2 ml of 0.5% gelatin) was incubated with (○) and without (●) 0.1 ml of the antiserum (diluted 1:80) for 10 min at 30° in the presence of 0.2 M phosphate buffer (pH 7.3). The volume (0.5 ml) was made up with normal rabbit serum (diluted 1:80). The samples were subsequently incubated at 58° for various times (as indicated). The residual activity (for assay see METHODS) is expressed as % of the activity of unheated samples.

Fig. 2. Hydrolysis of methicillin, oxacillin, and benzylpenicillin in the presence of varying amounts of antiserum. The hydrolysis of methicillin (●), oxacillin (○) (by 0.5 µg of penicillinase) and of benzylpenicillin (△) (by 0.05 µg of penicillinase) was measured in the presence of varying amounts of the antiserum. Normal rabbit serum was used to make up the final serum concentrations (50 µl/mg). The activity was assayed manometrically and presented as % of the activity in the absence of antiserum.

heat. The stabilizing effect of the antiserum is illustrated in Fig. 1 where the rate of inactivation of penicillinase at 58° in the presence of the antiserum is compared with the rate of inactivation of the enzyme in the presence of normal serum.

Variations in pH have been shown to modify the conformation and, consequently, the thermostability of penicillinase[11,12]. We found that the pH-dependent variations in thermostability are also largely prevented by the antiserum. The pro-

tective effect of specific antibodies is particularly clear in the alkaline region, where the enzyme is most susceptible to heat inactivation (ref. 12 and Fig. 4, below).

Effect of the antiserum on activity

Antisera to penicillinase are known to inhibit the activity of the enzyme[4–6]. However, POLLOCK[10] has shown that when methicillin is the substrate, low concentrations of anti-penicillinase serum may stimulate, rather than inhibit, the activity. The "biphasic" pattern obtained with such serum is illustrated in Fig. 2, where the effects of the present antiserum on the hydrolysis of three substrates are summarized. The antiserum has a purely inhibitory effect on the hydrolysis of benzylpenicillin, but it is "biphasic" (*i.e.* stimulatory at lower concentrations and inhibitory at the higher concentrations) with respect to methicillin. Significantly, a virtually identical "biphasic" pattern is obtained when oxacillin, rather than methicillin, is the substrate (Fig. 2).

Fractionation of the antiserum

The "biphasic" pattern indicated the presence of two types of antibody, which could be separated by the procedure suggested by POLLOCK[10]. The procedure is based on the assumption that the stimulation which is observed at the higher dilutions of the antiserum is due to an excess of stimulatory antibodies over inhibiting antibodies. Indeed, absorption of "biphasic" antisera by appropriate amounts of the antigen was found to completely eliminate the inhibitory activity and yield a "monophasic" stimulatory preparation[10]. In the present study 15 μg of enzyme were incubated with 0.6 ml of antiserum for 30 min at 37° and left overnight at 4°. The precipitate was spun off and washed in borate saline.

The supernatant, which retained the stimulating activity alone, was tested and found to have the following properties.

1. The preparation stimulated the hydrolysis of all the resistant penicillins

TABLE I

RELATIVE RATES OF HYDROLYSIS OF VARIOUS SUBSTRATES IN THE PRESENCE OF NORMAL AND IMMUNE SERUM PREPARATIONS

The rate of hydrolysis of the compounds listed below was measured under the conditions of the manometric assay. The enzyme (0.5 μg, *i.e.* 155 units) was assayed in the presence of A, 7.5 μl normal rabbit serum; B, 7.5 μl whole antiserum; C, 15 μl stimulating antibody preparation. All substrates were used in 50 μmole amounts in a final volume of 3 ml. The rates are expressed relative to the rate of hydrolysis of benzylpenicillin (= 100).

	Substrate	*A*	*B*	*C*
1	Benzylpenicillin	100	100	100
2	Phenoxymethylpenicillin	—	110	108
3	Ampicillin	120	94	99
4	Methicillin	3	6	9
5	Oxacillin	5	10	11
6	Cloxacillin	0.7	2	3
7	Cephalosporin C	<0.1	<0.1	<0.1
8	Benzylcephalosporin	<0.1	<0.1	<0.1
9	Cephalothin	<0.1	<0.1	<0.1
10	Cephaloridine	3	3	3

tested but had no effect on the hydrolysis of non-resistant penicillins and of cephalosporins (Table I).

2. The stimulating antibodies appeared not to compete with the inhibiting antibodies for the same determinant groups. This is illustrated by the following experiment. 180 Units of enzyme were preincubated with the stimulating antibody preparation. After 10 min at 30°, 5 μl of unadsorbed antiserum were added and, after 10 min at 30°, the residual activity was tested. The stimulating antibody did not affect the extent of inhibition by the whole antiserum.

3. The preparation protected the enzyme against heat inactivation as illustrated by the following experiment. Samples of 170 units of the enzyme in 0.5 ml of 0.04 M phosphate buffer (pH 7.3) were heated at 60° in the presence of 15 μl of the stimulating antibody preparation or of normal rabbit serum. At the end of 2 min the residual activity of the antibody-bound enzyme was 91% of the initial (*vs.* 24% in the normal serum control). At the end of 4 min treatment the residual activities were 89% and 9%, respectively.

The precipitate obtained in this fractionation was suspended in borate–saline. The activity of the suspension, which represents the so-called residual activity of the maximally inhibited enzyme in the enzyme–antibody complex was measured with benzylpenicillin and methicillin as substrates. There was no indication of the presence of stimulating antibodies in the preparation. The stabilizing effect of the antibodies present in the precipitate was tested as follows. The precipitate suspension was diluted in borate–saline to have an activity of 76 units per ml. Untreated enzyme preparation, appropriately diluted in normal rabbit serum and borate–saline to the same activity was used as control. Aliquots (0.2 ml) of the two preparations were exposed to 60° for various times. At the end of 5 min, the initial activity of the precipitate fraction was fully retained, whereas the control samples were completely inactivated. It is obvious that antibodies carried down with the precipitate, in common with the antibodies remaining in the supernatant, stabilize the enzyme.

Fractionation of enzyme–antiserum preparations

Since the stabilizing effect of the antiserum is not uniquely related to a single kind of antibody, it can be readily investigated in the zone of low antiserum to enzyme ratio (*cf.* Fig. 2). The main advantage of working in that range was that the catalytic activity of the enzyme–antiserum preparations was largely retained. We found, however, that in such preparations a considerable proportion of the enzyme was not bound to antibody. A procedure which would selectively remove the free enzyme from an enzyme–antiserum preparation, was consequently devised. The procedure was suggested by the observation[18,23] that at pH 5.0 and low ionic strength penicillinase is quantitatively adsorbed to charged surfaces (*e.g.* glass, diatomaceous earth) Although foreign proteins (including serum proteins) were known to interfere with the adsorption (ref. 23 and unpublished observations), it was expected that antibody molecules specifically directed to the enzyme would be much more effective. This would permit substantial elimination of free enzyme from the enzyme–antiserum preparation. The fractionation procedure employed was as follows: Penicillinase of *B. cereus* 569/H (100 μg in 1.0 ml of 0.001 M phosphate buffer, pH 7.0) was incubated with 0.6 ml of the homologous antiserum for 10 min at 30°. The enzyme–antiserum reaction mixture was then adjusted to pH 5.0 with 0.1 M HCl and 100 mg of a diatomaceous earth

preparation (Celite 535) was added. The Celite suspension was transferred to a sintered glass filter funnel; the volume was made up to 10 ml with twice-distilled water and suction was applied to speed up filtration. The Celite cake was then washed with 3 ml of distilled water followed by 3 ml of 1 M NaCl in 0.1 M sodium citrate (pH 8.5) to elute the adsorbed enzyme. All fractions were collected separately and stored at 4°. The enzymic activity and the thermostability of the enzyme in each fraction was tested.

The results of a typical fractionation are presented in Table II and compared

TABLE II

FRACTIONATION OF ENZYME–ANTISERUM PREPARATION

The fractionation procedure was as described in the text. Aliquots of the fractions (0.2 ml), containing 90 units of penicillinase, were heated to 60° for 2 min in the presence of 0.2 M phosphate buffer (pH 7.3), and the residual activity assayed (see METHODS) and expressed as % of the initial activity.

Fraction	*Volume (ml)*	*Enzyme–antiserum preparation*				*Enzyme–normal rabbit serum preparation*			
		Units/ml	*Total activity*		*Residual activity (%)*	*Units/ml*	*Total activity*		*Residual activity)%)*
			Units	*%*			*Units*	*%*	
1 Celite suspension	10	1790	17 900	100	68.4	1940	19 400	100	12.7
2 Filtrate	10	1080	10 800	60	77.5	450	4 500	23	<0.5
3 Wash	3	210	630	4	—	190	570	3	—
4 Eluate	3	2680	8 040	49	36.1	4900	14 700	76	15.5

with the results obtained when the homologous antiserum was replaced with normal rabbit serum. The activity of the non-adsorbed fraction (Fraction 2) of the normal serum preparation reflects the non-specific effect of serum proteins on the adsorption. The considerably higher activity of the corresponding fraction of the enzyme–antiserum preparation is thus due to the presence of specific antibodies which effectively prevent the adsorption to Celite. This is further corroborated by the results of the heat stability test, included in Table II. The stabilizing effect of the antibodies is most clearly seen in Fraction 2 of the enzyme–antiserum preparation. We conclude that the free enzyme, originally present in that preparation, has been effectively removed by adsorption to Celite (it has been recovered in the eluate fraction, which is consequently much less stable to heat). Fraction 2 thus represents a purified preparation of the enzyme–antibody complex, and will be referred to as "bound" (as opposed to "free") enzyme.

Comparison of free and bound enzyme: effect of pH on activity and stability

Fig. 3 presents the pH/activity profiles of the free and bound enzyme. The characteristic curves obtained for the two enzyme preparations are identical and show a pH optimum between 6.0–7.0.

In contrast, there is a marked difference in the pH stability profiles of the free and bound enzyme, as shown in Fig. 4. The effect of pH on the thermostability of the free enzyme is consistent with the observation that the conformation of penicillinase

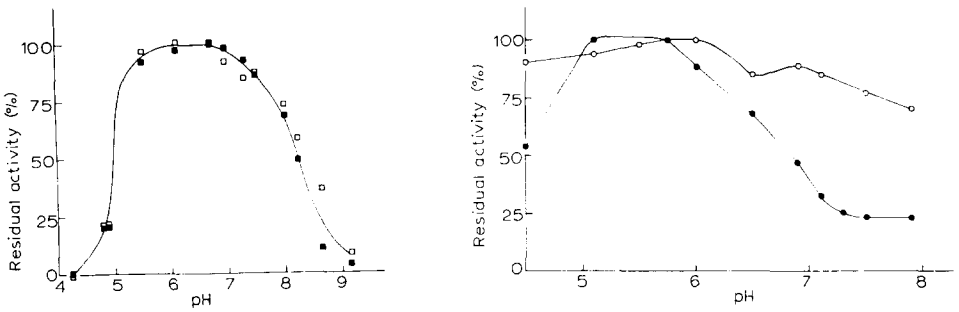

Fig. 3. Effect of pH on the activity of free and bound enzyme. Samples of free and bound penicillinase (90 units in 0.45 ml of 0.5% normal rabbit serum and 0.25% gelatin) were assayed at the indicated pH values. The timed iodometric method was used[22], except that the substrate solutions were added in the following buffers: 0.25 M phthalate–NaOH (pH 4.2–5.5); 0.5 M potassium phosphate (pH 5.5–8.0); 0.25 M Tris (pH 8.25–9.15). The residual activity is expressed as % of the maximum activity. (□) Bound enzyme; (■) free enzyme.

Fig. 4. Effect of pH on the stability of free and bound enzyme. Samples of free (●) and bound (○) penicillinase (58 units in 0.45 ml of 0.5% normal rabbit serum and 0.25% gelatin) were exposed to 58° for 2 min at the indicated pH values. Buffers used (0.05 ml): 0.5 M phthalate–NaOH (pH 4.2–5.5); 1.0 M phosphate (pH 6.1–8.0). The residual activity (for assay see METHODS) is expressed as % of the maximum activity of each preparation.

is strongly affected by the pH of the medium[11,12]. The bound enzyme is much less affected by variations in pH, and it retains most of its activity in the alkaline range where the free enzyme is largely inactivated (Fig. 4). The discrepancy between the effect of pH on stability and the effect on activity undoubtedly reflects the stabilizing effect of the substrate (see DISCUSSION).

Comparison of free and bound enzyme: effect of temperature

The effect of temperature on activity of the free and bound enzyme is compared

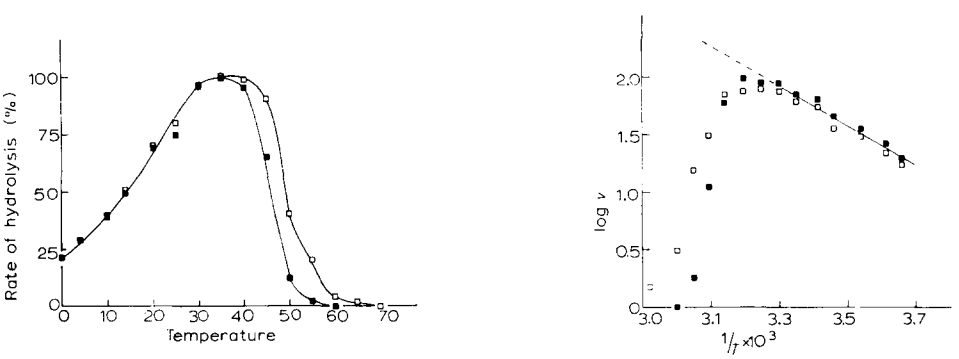

Fig. 5. Temperature optimum curve for free and bound enzyme. Samples of free (■) and bound (□) penicillinase (90 units in 0.45 ml of 0.5% normal rabbit serum and 0.25% gelatin) were assayed at the indicated temperatures, by the timed iodometric method [22]. All reagents were preincubated at the respective temperatures for 5 min before use.

Fig. 6. Arrhenius plot for the enzymic activity of the free and bound enzyme. Samples of free (■) and bound (□) penicillinase were prepared and assayed as in Fig. 5. The units of activity were converted to their log values (log v) and plotted against the reciprocal value of the absolute temperature ($1/T$).

in Fig. 5. The temperature/activity curves for both preparations overlap in the sub-optimal region and the optimum temperature is 35° for both the free and bound enzyme. Even at higher temperatures (45°–60°), where the bound enzyme is significantly more active, the shape of the temperature/activity curve retains its similarity to that of the free enzyme.

The similarity is further demonstrated by the comparison of the activation energies of the free and bound enzyme preparations. The respective Arrhenius plots are presented in Fig. 6. In the linear portion (temperatures 0°–30°) the plots coincide and the activation energy calculated from that slope (7850 calories/mole) is in agreement with the value published for a related penicillinase preparation[20]. Another significant similarity is in that the breaking point of the linear portion (30°) is common to the free and bound enzyme.

These observations are clearly in contrast with the striking differential effect of temperature on the stability of the free and bound enzyme, which has been demonstrated above (Fig. 1). As in the case of the pH effect, the discrepancy between the effect of temperature on stability and activity clearly reflects the stabilizing role of the substrate (see DISCUSSION).

DISCUSSION

The remarkable flexibility of the extracellular penicillinase, which has been noted in earlier studies[24,25], is probably due to the fact that its tertiary structure is not stabilized by disulphide bridges[26]. It was expected that the specific antibodies would have a marked stabilizing effect on this enzyme by imposing a rather rigid constraint on its conformation. This proved to be the case, as illustrated in Figs. 1 and 4. Thus, in the presence of the homologous antiserum, the stability of penicillinase to heat and to variation of pH is markedly increased. Further evidence of stabilization by specific antibodies (against inactivation by urea, proteolytic digestion and photooxidation) is included in the accompanying report[17].

Although particularly striking in the case of a highly flexible enzyme, the stabilizing effect of antibodies may be a more general phenomenon. Thus, NAJJAR AND FISHER[27] mentioned that yeast alcohol dehydrogenase, which is ordinarily inactivated within several minutes at 4°, is stabilized by the homologous antibody and can be maintained in active form for months at that temperature. Pepsin has been found to be protected by anti-pepsinogen from inactivation and the interpretation was offered that "the conformation of the enzyme is "locked" by the antibody"[28]. KAPLAN AND WHITE[29] observed that the thermostability of the enzyme–antibody complex of lactic dehydrogenase depends on the amount of antibody present. The authors concluded that the "attachment of the antibody to the enzyme results in a more rigid structure whose folding characteristics are less sensitive to temperature"[29].

A most interesting, and particularly relevant observation, was reported by POLLOCK[30]. He found that antiserum against the wild-type penicillinase of *Bacillus licheniformis* 749/C partially restored the wild-type activity to the mutant penicillinase preparations with decreased specific activity ('muteins'). Furthermore, the greater the decrease in specific activity of a mutein, the greater was the proportional stimulation of activity by the antibody. POLLOCK suggested that this may be interpreted "on the

hypothesis that antibodies act by allowing or imposing on the mutein a configuration which more closely approximates to that of the wild type"[30].

Since antiserum to penicillinase modifies the activity as well as the stability of the enzyme it was of obvious interest to inquire whether the two effects are related. Fractionation of the antiserum, which resulted in the separation of the stimulating (non-precipitated) antibodies from the inhibitory (precipitated) antibodies did not provide a clear answer to this question. The observation that both fractions retained the stabilizing properties eliminates the possibility that stabilizing antibodies can be exclusively equated with either stimulating or inhibiting antibodies. It does not allow us to decide, whether stabilization is an attribute common to both kinds of antibody, or is due to other antibodies present in both fractions.

There is no indication, however, that the homologous antiserum contains antibodies which have no effect on the activity of this enzyme[9,10,17,19]. It will also be noted in this context that the inhibiting and stimulating antibodies do not appear to compete for the same determinant groups (see RESULTS).

The above observations are consistent with the following tentative conclusion. Penicillinase can bind, independently, each of the two kinds of antibody molecules present in the antiserum. Each molecule imposes a constraint on the conformation of the enzyme, and thus increases its stability. The site of the binding determines whether the constraint will inhibit the activity of the enzyme. The relation between the constraint and the stimulation of activity on several substrates (Table I) will be discussed in the accompanying report, which is concerned with the interaction of the antibody-bound enzyme with such substrates[17].

In the present report the substrate investigated was benzylpenicillin, which is known to stabilize the active conformation of the enzyme[31]. It was of considerable interest to examine the stabilizing effect of antibodies in the presence of benzylpenicillin. Since benzylpenicillin is readily hydrolyzed by the free and antibody-bound enzyme under most experimental conditions, the comparison is based on the experiments presented in Figs. 3–6. In these experiments the stability in the presence of the substrate was determined from pH/activity and temperature/activity curves obtained for the free and bound enzyme. The stability in the absence of the substrate was determined from the residual activity following exposure of free and bound enzyme to the corresponding conditions of pH or temperature.

There is a clear discrepancy between the two sets of results. The effect of pH and temperature on the residual activity is virtually eliminated by the antibody (Figs. 1 and 4). In contrast, the pH/activity and temperature/activity as well as the Arrhenius plots for the free and antibody-bound enzyme are very similar (Figs. 3, 5 and 6). The discrepancy is obviously due to the presence of the substrate in the second set of experiments. It may be concluded that the stabilizing effect of the substrate masks the effect of antibodies. Alternatively, it may be postulated, that the substrate promotes partial dissociation of the enzyme–antibody complex.

A third, and most interesting, possibility is that the stabilizing effect of benzylpenicillin is largely prevented by the antibody. It implies that antibodies can modify the specific effect of substrates on the conformation of the active site of the enzyme. Evidence which is consistent with this last proposition and rules out the alternatives will be presented in the accompanying report[17].

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