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THE INTERACTION OF PENICILLINASE WITH PENICILLINS

II. TEMPERATURE-DEPENDENT CHANGES INDUCED IN PENICILLINASE BY COMPETITIVE INHIBITORS

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SUMMARY

The inactivation of penicillinase is accelerated in the presence of substrate analogs. The kinetics of the reaction with respect to time, temperature, pH and analog concentration indicate great similarity and slight but definite difference between the effect of substrate analogs and that of undiscriminating hydrogen-bond-breaking agents. The analog-dependent inactivation is partly reversed by the substrate or by pH changes. Both the inactivation and its reversal are temperature-dependent processes. The reported observations are discussed in terms of postulated differences between substrate and analog-induced conformation of penicillinase.

INTRODUCTION

In a previous communication[1] we have shown that the conformation of penicillinase is reversibly changed through interaction with a competitive inhibitor of the enzyme. The inhibitor used was DMP, a synthetic penicillin, characterized by its unusually high resistance to hydrolysis by penicillinase. Similar observations have been made with another recently synthesized competitive inhibitor (see ref. 2.) The changes observed in penicillinase exposed to either analog have been interpreted as due to a distortion of the active site of the enzyme. As a consequence, it was expected that the thermostability of the enzyme would be lowered by the substrate analogs. This indeed is the case; the kinetics of analog-induced labilization and its reversal are the subject of the present communication.

MATERIALS AND METHODS

Penicillinase: The enzyme was prepared from the culture supernatant of *Bacillus cereus* strain 569/H (see ref. 3). The preparation was purified as previously described[4] and its specific activity was similar to that reported for a crystalline penicillinase of *B. cereus*[5].

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Abbreviations: DMP, 6-(2,6-dimethoxybenzamido)penicillanic acid; P-12, 5-methyl-3-phenyl-4-isoxazolyl-penicillanic acid; BP, sodium benzylpenicillin.

Assay of penicillinase: Total penicillinase activity was assayed by the manometric method[6,7]. The iodine-resistant (α-type) activity was assayed directly by the iodometric procedure previously described[4].

Penicillins: BP was obtained from Merck and Co., Inc., DMP was supplied by Teva Ltd. and P-12 by Bristol Laboratories.

Heat treatment and assay of residual activity

Standard heat treatment consisted of exposing penicillinase in 0.05 *M* phosphate buffer (pH 7.3) and 0.25% gelatin to 48° for 2 min. (Variations in pH, temperature and duration of treatment will be specified in the respective experiments.)

The treatment was carried out in test tubes immersed in a water-bath, with temperature regulated by a Thermomix II Immersion Thermostat within $\pm$ 0.1°. At the end of the incubation the tubes were immersed in an ice-bath for 1 min and transferred to a 30° water-bath where the enzyme was assayed.

The assay was based on the iodometric procedure for the determination of iodine-resistant (α-type) activity[4]. The reagent mixture (0.5 ml of I_2, 0.025 *M* in 0.125 *M* KI, 1.0 ml of 0.1 *M* phosphate buffer, 3.0 ml of 0.5% gelatin and 3 mg of benzylpenicillin) was kept for 5 min at 30° before the assay. The assay was started by the transfer of the reagent mixture into the tube containing the treated preparation. The total volume of the assay was 5 ml.

RESULTS

Effect of DMP and P-12 on thermal inactivation of penicillinase

Reports on thermostability of penicillinase are "extremely variable and inconsistent"[8]. This may be due, in part, to the fact that only crude preparations of penicillinase were available for such studies. In fact POLLOCK and co-workers have shown[9,10] that "the enzyme can be strikingly protected from thermal inactivation by the presence of high-molecular-weight compounds which may be present in the preparation". Using pure penicillinase we have found that even a dilute solution of the enzyme (0.5 μg/ml) is quite stable in the presence of 0.25% gelatin at temperatures below 40°. Above that temperature, however, thermal inactivation is noticeable, and at 58° a 2-min exposure is sufficient to cause complete inactivation. The temperature-dependent inactivation of penicillinase at pH 7.3 is presented in Fig. 1 (Curve C).

The effect of the competitive inhibitors of penicillinase on thermal inactivation is also shown in Fig. 1. It will be noted that in the presence of less than 0.2 μM of DMP (Curve A) or P-12 (Curve B) the thermostability of penicillinase has markedly decreased.

The effect of DMP on the stability of the enzyme at pH 7.3 was clearest at 48° where over 90% of the enzyme was inactivated as compared with 20% inactivation in the control.

The above conditions (*i.e.* 2 min at 48°) have been selected for further studies of analog-induced thermolability of penicillinase.

The precision of the present method of measuring the effect of an analog is evident from Fig. 2, where survival of enzyme activity is plotted against time of

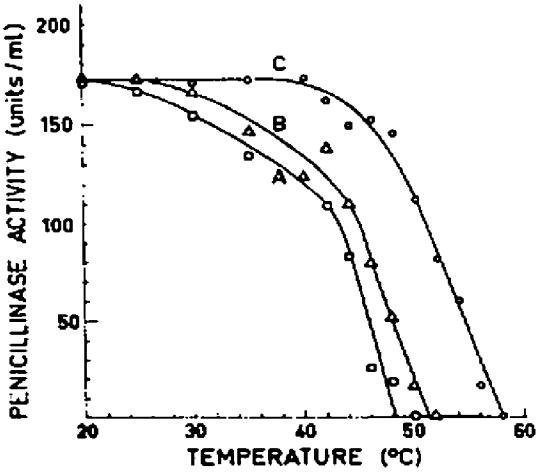

Fig. 1. Effect of DMP and P-12 on the thermostability of penicillinase. Reaction mixtures (final volume, 0.4 ml) consisted of: penicillinase, 0.5 μg; phosphate buffer (pH 7.3), 0.05 M; gelatin, 0.25% and respectively, DMP, 0.18 μM (Curve A), P-12, 0.18 μM (Curve B), no analog (Curve C)l At the end of 2 min exposure to the indicated temperatures the residual activity was determined as described in METHODS.

exposure to 48°. Readings at 10-sec intervals reveal considerable differences in the survival of penicillinase brought in contact with the analog (Fig. 2,A). Inactivation of the control preparations containing no DMP proceeds at a much slower rate (Fig. 2,B). Maximal DMP effect at 48° is reached after 120 sec exposure, *i.e.* under the conditions used throughout this work. Similar observations have been made at somewhat lower or higher temperatures. The results recorded at 46°, 48° and 50°

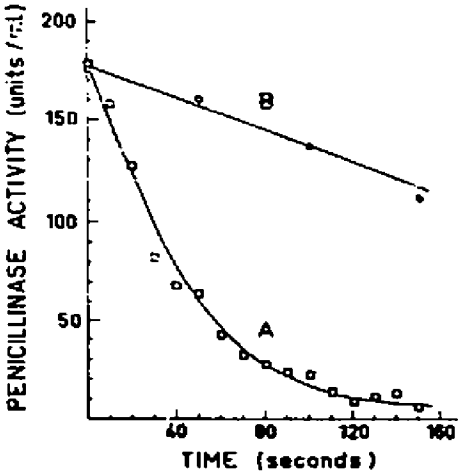

Fig. 2. Effect of DMP as a function of time. Reaction mixtures (final volume, 0.4 ml) consisted of: penicillinase, 0.5 μg; DMP, 0.18 μM; phosphate buffer (pH 7.3), 0.05 M; gelatin, 0.25%; (Curve A). Controls contained no DMP (Curve B). Treatment was started by introducing the enzyme to the reaction vessel, kept at 48°, and terminated by transferring the vessel to an ice-bath. Residual activity was determined as described in METHODS.

are summarized in Fig. 3. It will be seen that the logarithms of residual activities plotted against concentrations of the analog fall on straight lines. The slopes of the lines give a measure of the effect of increasing DMP concentrations on the stability of penicillinase at the respective temperatures. It follows clearly from Fig. 3 that decrease in stability as a function of analog concentration is accelerated by increasing the temperature.

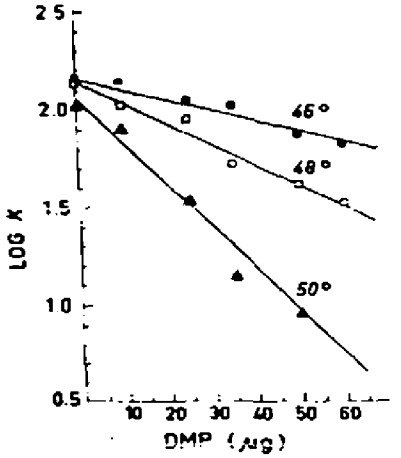

Fig. 3. Thermal inactivation of penicillinase as a function of DMP concentration. Reaction mixtures (final volume, 0.4 ml) consisted of: penicillinase, 0.5 μg; phosphate buffer (pH 7.3), 0.05 *M*; gelatin, 0.25% and varying amounts of DMP. At the end of 2 min exposure to 46° (●—●), 48° (□ ·□) and 50° (▲—▲) respectively, the residual activity was determined (as described in METHODS) and expressed as reaction velocity (*k*). Log *k* is plotted against concentration of DMP (in μg per 0.4 ml of reaction mixture).

Effect of pH on the thermostability of penicillinase in the presence and absence of analogs

The experiments reported so far have been carried out at pH 7.3. Variations of pH have a pronounced effect on the conformation of the enzyme. Consequently thermal inactivation of penicillinase is strongly affected by the pH of the medium. Thus a 2-min incubation at 58° causes complete loss of activity at pH values below 4.0 or above 7.4 whereas in the range of pH 4.8 to 5.3 the enzyme retains 80% of the initial activity. The pH-dependent changes in thermostability of penicillinase are plotted in Fig. 4

It will be noted that at 48° the pattern of thermostability is very different. Here the enzyme is fully stable over the range of pH 4.0 to 6.0, and retains most of its activity in the alkaline range.

The stability of penicillinase under these conditions is characteristically affected by interaction with the analogs. Exposure to DMP at 48° brings out the bell-shaped curve observed at 58°. Essentially identical results are obtained when DMP is replaced by P-12 at an equimolar concentration.

The effect of the analogs at 48° is strikingly similar to the effect of exposure to 58° in the absence of analog. Yet, whereas the thermostability curves obtained with the two analogs are superimposable, they are shifted to the right of the 58° curve (Fig. 4). This shift appears to reflect a specific effect of the analog on the conformation of the active site of penicillinase (see DISCUSSION).

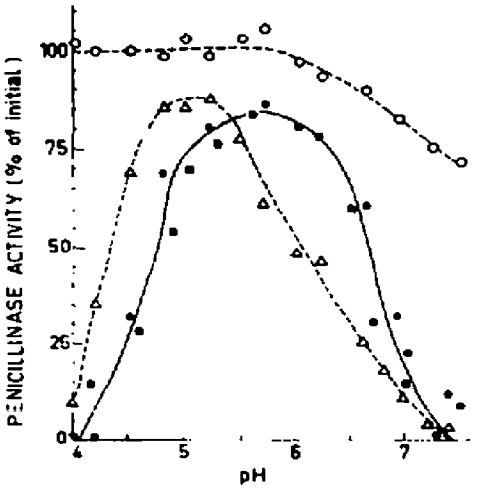

Fig. 4. Survival of penicillinase activity following heat treatment at varying pH. Aliquots of penicillinase (0.55 μg of enzyme protein) in 0.05 M buffer solution of varying pH, exposed for 2 min to the indicated temperatures with and without the analogs. Total volume, 0.4 ml. Buffers used: phthalate–NaOH (pH 4.0–6.0) and phosphate (pH 6.0–7.5). For assay of residual activity, see METHODS. ■ –■, 48°, 0.18 μM DMP; ● –●, 48°, 0.18 μM P-12; ○–○, 48°, no additions; △ –△, 58°, no additions.

Reversal of the effect of analogs on the thermostability of penicillinase

Before considering the reversibility of the effects summarized in Fig. 4, it will be useful to examine the conditions of treatment and assay (see METHODS). The heat treatment was terminated by cooling the reaction vessel in an ice-bath. The contents were subsequently diluted by a factor of 12.5 with the assay reagent, pre-warmed to 30°. The buffering capacity of the assay reagents was sufficient to restore to neutrality variously treated samples. Thus the temperature and pH conditions during the assay of treated samples were identical. The assay was started by simultaneously exposing the enzyme to the substrate and to the iodine reagent. Under these conditions there was no reversal of the effect of pre-treatment with DMP at 48° and pH 8.0 (see Fig. 4).

It was conceivable, however, that such pre-treatment caused excessive sensitivity to iodine rather than irreversible inactivation. The enzyme is indeed sensitive to iodination in the presence of substrate analogs[1,2], but the iodine-sensitive state is reversed by the substrate[11]. Indeed, penicillinase pre-treated by DMP at 0° and pH 7.3 displayed no loss of activity when assayed in the presence of iodine, thus indicating that the reversal by the substrate was practically instantaneous[1]. Since the pre-treatment by DMP in the present experiments was carried out under different conditions (48°, pH 8.0) the kinetics of reversal may have been different.

The experiments concerned with this point are summarized in Fig. 5. In one series the pre-treated enzyme was brought in contact with the substrate immediately upon the termination of the heat treatment and 10–60 sec before the beginning of the iodometric assay (for details see legend to Fig. 5). The substrate was found to be effective in restoring up to 15% of the initial activity (Fig. 5,B).

It will be noted that this slight reversal by the substrate was induced at pH 8.0.

When the pH was lowered to 5.9 upon the termination of the heat treatment, and before the iodometric assay, the reversal was more rapid and complete. Indeed the presence of the substrate at this stage was not required for reversal. Spontaneous recovery of 13% was reached within 5 sec, and maximum reversal (30% of the initial activity) within 30 sec after lowering of pH and temperature (Fig. 5, C).

Effect of temperature on the reversibility of the analog-induced change

The analog-induced change described above was temperature dependent in the sense that it did not cause inactivation unless the temperature was raised. The change was partly reversed by the substrate (Fig. 5,B) or by lowering the pH (Fig. 5,C), but not by lowering the temperature (Fig. 5,A). The effect of temperature on the reversibility of the analog-induced change was further examined in the following experiment. As before, the enzyme was exposed to DMP at 48° and pH 8.0. Reversal was brought about by the addition of pH 5.5 buffer, and the assay of activity started 60 sec later, that is after maximum reversal was attained (*cf.* Fig. 5,C).

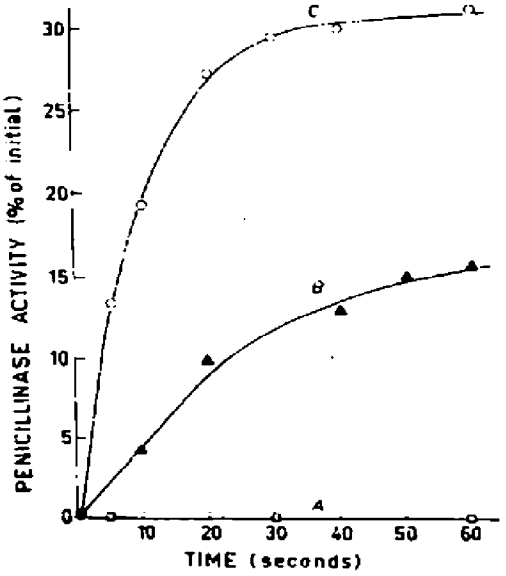

Fig. 5. Reversibility of the DMP-dependent thermal inactivation: effect of pH and substrate. Reaction mixtures (final volume, 0.4 ml) consisted of: penicillinase, 0.5 μg; phosphate buffer (pH 8.0), 0.05 *M*; gelatine, 0.25%; DMP, 100 μg. Treatment (2 min, 48°) was terminated by injecting into the reaction tube 0.5 ml of ice-cold phosphate buffer as follows: (A), 0.02 *M* phosphate buffer (pH 8.0); (B), 0.02 *M* phosphate buffer (pH 8.0) containing 3 mg of benzylpenicillin; (C), 0.2 *M* phosphate buffer (pH 5.5) (final pH of reaction mixture, 5.9). Residual activity (see METHODS) was assayed after termination of treatment, at the indicated time intervals. Correction has been made for the penicilloic acid formed in samples treated with benzylpenicillin (Curve B).

At this stage the treated samples were assayed in three series. In one series (A) the temperature was kept constant (at 48°) throughout, in another (B) the temperature was progressively decreasing, and in the third (C) increasing as samples were

acidified at 4-sec intervals. The experimental details and results are presented in Fig. 6.

The curves in Fig. 6 represent the activity of penicillinase recovered by acidifying samples at various stages of temperature change (Fig. 6, B and C) and at a constant high temperature (Fig. 6,A). It is clear from these data that the reversibility of the analog-induced change is temperature dependent. Maximal recovery is ob-

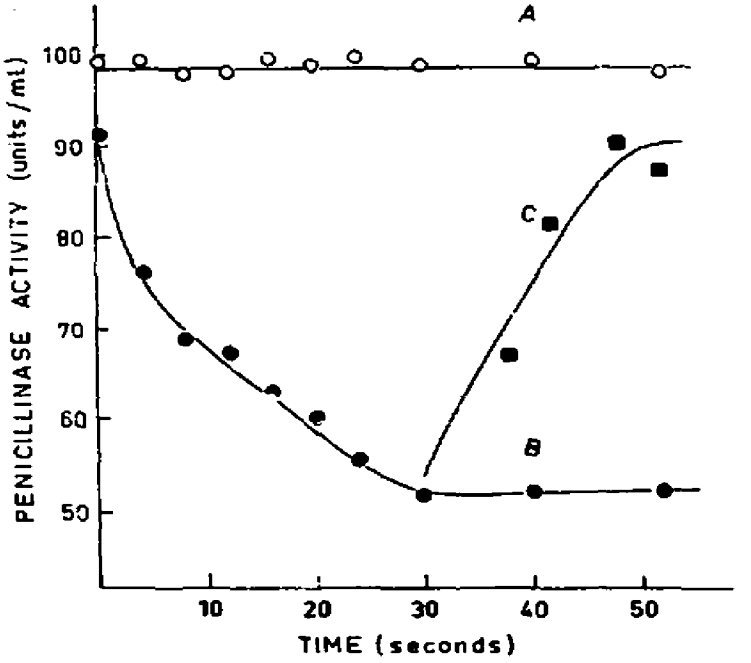

Fig. 6. Reversibility of the DMP-dependent thermal inactivation: effect of temperature. Reaction mixtures (final volume 0.4 ml) consisted of: penicillinase, 0.5 μg; phosphate buffer (pH 8.0), 0.05 M; gelatin, 0.25%; DMP, 100 μg. Standard heat treatment (2 min, 48°) was followed by acidification, which consisted of adding 0.4 ml of 1.0 M phosphate buffer (pH 5.5) to each reaction tube, at the indicated time intervals. (Final reaction, pH 5.9). The temperature at this step was controlled as follows: (A), pH 5.5 buffer pre-warmed to 48°; reaction tubes maintained at 48° throughout; (B), pH 5.5 buffer kept at 0°; reaction tubes transferred to ice-bath immediately upon termination of heat treatment; (C), Samples cooled as in (B) for 30 sec, then transferred to 48° along with 0.4-ml aliquots of pH 5.5 buffer. Penicillinase activity was assayed 60 sec after acidification as described in METHODS.

tained when the reversal is carried out at 48°, that is at the temperature used in the pre treatment which led to inactivation. As the samples are cooled the response to acidification becomes less effective. The recovery after 30 sec at 0° is about 50% of that of uncooled enzyme. If, however, at this stage the temperature is raised, the proportion of recovered enzyme is found to increase.

DISCUSSION

Previously published evidence has indicated that the conformation of penicillinase is affected by interaction with penicillins[8,11]. The penicillins tested for their effect

on the conformation of the enzyme fall into two groups, which have in common the penicillin nucleus (6-aminopenicillanic acid) but differ in side-chain structure. In one group the variously substituted side-chains enhance the enzymic action of penicillinase, *i.e.* the hydrolysis of the β-lactam ring in the penicillin nucleus[1]. In the other group the side-chains impart a high degree of resistance to the action of penicillinase[1,2].

It is highly significant that the two groups of penicillins (referred to as substrates and substrate analogs, respectively) also differ in their effect on the conformation of the active site of penicillinase. Thus the side-chain of a readily hydrolysed penicillin imparts a more ordered and more folded conformation. On the other hand interaction with a substrate analog has the opposite effect. In the presence of such analogs, penicillinase becomes completely inactivated by iodine or urea under conditions where the free enzyme is unaffected. Our interpretation was that the modification in the side-chain which makes a penicillin resistant to penicillinase appears to cause a marked distortion in the conformation of the enzyme.

The findings reported in the present paper are fully consistent with this interpretation. The basic finding was that the thermostability of penicillinase decreases in the presence of substrate analogs (Figs. 1 and 2). There is in fact a striking similarity between the effect of the analog and that of elevated temperature. Inactivation at constant temperature and increasing analog concentrations appears to follow the same kinetics as that observed when temperature is the variable (Fig. 3).

When the analog-induced thermolability was examined over a wide range of pH the similarity became even more apparent. Reference to Fig. 4 will serve to illustrate this point. It will be seen, for instance, that exposure to the analog at 48° appears to be essentially equivalent to increasing the temperature by 10°. This holds for the whole range of pH values at which the effect of analogs and temperature was compared.

There is, however, a significant shift in the pH-stability curve which is dependent on the interaction with the analog. Thus maximum stability is obtained in the presence of analogs at pH 5.6–5.8 rather than at pH 5.0–5.2 observed in their absence. The pH values for maximal stability with the analog present are in good agreement with the results observed in previous experiments[1] when the enzyme was tested for iodine sensitivity in the cold. The observations may also be relevant that the pH–activity curve obtained when the substrate (benzylpenicillin) was replaced by DMP displayed a shift in the same direction (CITRI[12]).

It follows that the conformation imparted by the analogs, although very similar, is not identical with that resulting from partial heat denaturation. This is hardly surprising since the analogs, unlike heat or other hydrogen-bond-breaking agents, exert their effect by specifically combining with the active site of the enzyme.

Indeed the analogs used in this work have been shown to act as competitive inhibitors of penicillinase[1,2]. It was therefore not unexpected that the thermal inactivation caused by analogs was partly reversed by the substrate under conditions which were not conducive to spontaneous reversal. Such reversal of analog-induced changes by interaction with substrates has been previously described[1,3,11]. Also in line with previously reported evidence[1,4,13] was the spontaneous reversal observed when pH was adjusted (Fig. 5, C).

In this connection we made the rather unexpected observation that lowering

the temperature did not promote reversal to the active conformation. Restoration to activity (by adjusting pH) was actually slowed down by cooling. It follows quite clearly from the data presented in Fig. 6 that the reversal of the DMP-induced change, like the change itself, is a temperature-dependent process.

Although the interactions described in this paper are undoubtedly relevant to the mechanism of enzyme action they are difficult to interpret with any degree of precision. The present methods have been useful in detecting very elusive conformational changes which go to completion or disappear in a matter of seconds. They yielded no direct information as to the bonds involved and very little is known about the chemical nature of the active site of penicillinase.

Nevertheless, an attempt to correlate the present observations with those previously reported leads to some distinctive conclusions. Analogs, in contrast to substrates, have been found to cause labilization as well as exposure of new reactive groups in the active site of the enzyme. The similarity between the effect of the analogs and that of hydrogen-bond-breaking treatment is probably the most striking characteristic of this interaction. We feel justified in concluding that the interaction between the enzyme and substrate analog involves breaking some specific secondary bonds, such as hydrogen bridges, in the vicinity of the active site.

We wish to emphasize, however, the difference between indiscriminate hydrogen-bond breaking and the specific interaction which is characterized by a slight shift in the pH-stability and pH–activity curves. We suggest that this shift may indicate the formation of an analog-directed secondary structure which is partly stabilized by newly formed bonds. The existence of such semi-stable conformation would also account for the effect of temperature on the reversal of the analog-induced change.

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