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INACTIVATION OF β-LACTAMASE I FROM *B. CEREUS* 569/H WITH PHENYLGLYOXAL, AN ARGININE-SELECTIVE REAGENT

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SUMMARY. β-Lactamase (EC 3.5.2.6: penicillin amido-β-lactam hydrolase) I from *B. cereus* 569/H is inactivated by treatment with phenylglyoxal. Inactivation depends on the pH and the presence of bicarbonate in a manner which suggests that it is due to the modification of arginyl residues. Total inactivation correlates with the loss of 8 arginines per β-lactamase, and a competitive inhibitor provides full protection from inactivation and protects *ca.* 2 arginines from modification.

As a consequence of intensive investigation, the chemical basis for the catalytic function of β-lactamases is starting to unfold. The most successful recent developments (1-4) have used penicillin and cephalosporin analogs which are mechanism-based inhibitors and/or poor substrates to identify serine-70 (5) as an essential component of the active site of class A (6) enzymes. But modification with group-specific reagents has given little information on other important residues (7). β-Lactamase I from *B. cereus* 569/H is inactivated by water-soluble carbodiimides (8,9), but further work is needed before it can definitely be stated that carboxyl groups are essential for activity. Tyrosine-105 can be selectively nitrated in four different class A enzymes, but a recent elegant study (10) has shown that this residue is not essential. It is generally conceded that neither histidine nor tryptophan is essential for activity, while there are conflicting data on the involvement of lysine at β-lactamase active sites (7,9).

It has recently become apparent that numerous enzymes use positively charged arginyl residues in the binding of anionic substrates and cofactors (11,12). One can even use one arginine-specific reagent, phenylglyoxal (13), to selectively in-

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activate the membrane anion transport protein of intact erythrocytes (14). Since all known substrates for β-lactamases contain a carboxylate moiety in the C-3 or C-4 position, it is conceivable that an arginine may be involved in substrate binding. We report herein our findings which suggest that this is the case for β-lactamase I from *B. cereus* 569/H. A preliminary report of this work has been given (15).

MATERIALS AND METHODS

β-Lactamase I from the constitutive mutant strain 569/H of *Bacillus cereus* was purified to homogeneity as previously described (16). Penicillin G (K^+ salt) and all buffer salts were obtained from Sigma, and phenylglyoxal monohydrate was from Aldrich. Plastic labware was used whenever possible in handling enzyme solutions to minimize problems associated with its observed tendency to adhere to glass surfaces. β-Lactamase activity was measured at pH 7.00 and 25°C by a titrimetric assay after the method of Hou and Poole (17), using a Radiometer ABU 12/TTT60/PHM62 recording pH stat assembly with a GK2320C electrode and a 0.250 ml burette. A standard assay (3.0 ml) contained: 5 mM penicillin G (K^+ salt), 100 mM KCl, 0.1 mM EDTA and 0.1% bovine serum albumin. After an aliquot of enzyme (∿0.4 - 1 μg) was added, the pH was maintained at 7.00 by automatic addition of 25.0 mM NaOH. The electrode was washed with 10 mM HCl between assays to minimize the accumulation of enzyme on the glass bulb of the electrode. D-Benzylpenicilloamide (K^+ salt) (*I*) was synthesized as described elsewhere (18). Infrared and proton magnetic resonance spectra and the elemental analysis (C,H,N) were consistent with the proposed structure *I*.

Chemical modifications were carried out at 25°C under conditions given in the figure legends. Modification of amino acids was determined by analysis on a Durrum D-500 amino acid analyzer after workup analogous to published procedures (19). Thioglycolic acid (1%) was included in the 6 M HCl used for protein hydrolysis to minimize the chances of arginine regeneration (20,21). A value of 18 leucines per β-lactamase (5,22) was used to normalize the analyses. A typical set of analyses for the control gave 23.38 lys, 4.08 his, and 11.78 arg, as compared to values of 23, 4, and 11, respectively, based on available sequence data (5,22).

RESULTS

The time course for the inactivation of β-lactamase I by phenylglyoxal in 50 mM Bicine, 100 mM $NaHCO_3$, pH 8.3, is shown in Fig. 1. Semi-log plots of residual activity vs. time are linear at all phenylglyoxal concentrations employed, suggesting that the inactivation is first-order in enzyme and likely due to a single chemical event. A plot of the log of the pseudo first-order rate constant for inactivation vs. the log of phenylglyoxal concentration gives a straight line with a slope of 1.01 (Fig. 1, insert), indicating that inactivation is also first-order in phenylglyoxal. The overall second-order rate constant for inactivation under these conditions is 1.43 $M^{-1}min^{-1}$. Modification of arginine by phenylglyoxal is often enhanced by the presence of bicarbonate (23,12). When $NaHCO_3$ is omitted from the medium outlined in Fig. 1, the rate of inactivation is only about half that observed in its presence. If, however, 100 mM $NaHCO_3$ is replaced by 100 mM NaCl, the rate of inactivation is identical to that in the absence of $NaHCO_3$, indicating that the effect is specific for bicarbonate and not due to a change in ionic strength. Thus, the known selectivity of phenylglyoxal for the modification of arginine (12-14), as well as the observed enhancement of inactivation by bicarbonate, strongly suggest that an arginyl residue may be critical for the activity of β-lactamase I.

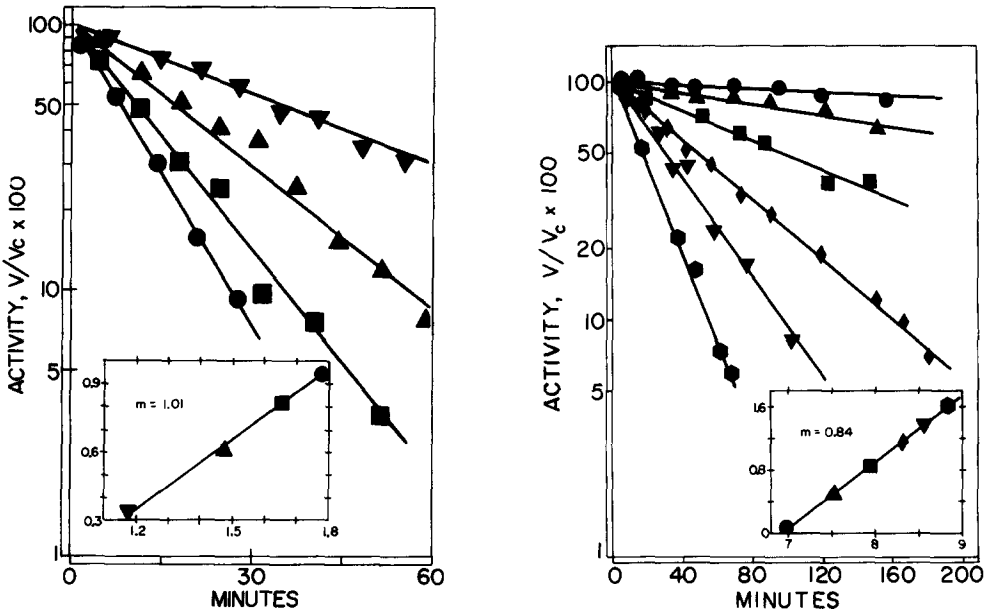
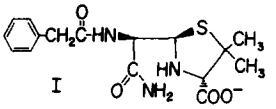


FIGURE 1: Inactivation of β-lactamase I by phenylglyoxal. The enzyme, 1.5 μM, in 50 mM Bicine, 100 mM $NaHCO_3$, pH 8.3, was modified with (▼) 15 mM, (▲) 30 mM, (■) 45 mM, and (●) 60 mM phenylglyoxal. The control retains full activity over this period of time. Insert: A plot of log(k') + 2 (ordinate, k' = the observed pseudo first-order rate constant for inactivation in min^{-1}) vs. log [phenylglyoxal] (abscissa, concentration in mM) gives a straight line for which the slope, 1.01, gives the order of the reaction in phenylglyoxal.

FIGURE 2: Effect of pH on the inactivation of β-lactamase I by phenylglyoxal. The enzyme, 3.1 μM, was treated with 26.4 mM phenylglyoxal in 30 mM Bicine, 30 mM HEPES, at the following pH values: (●) 6.97, (▲) 7.54, (■) 7.95, (◆) 8.32, (▼) 8.56, and (⬢) 8.84. Sodium bicarbonate was omitted from the modification medium because of problems associated with the loss of CO_2 below pH 8.3. The observed rate of inactivation at the phenylglyoxal concentration and pH approximating those of Fig. 1 are thus considerably slower. Insert: A plot of the log (k') + 3 (ordinate, min^{-1}, see Fig. 1) vs. pH (abscissa) gives a straight line with a slope of 0.84.

Phenylglyoxal reacts with the deprotonated free base of the arginine side chain (14,23), so the rate of modification of an essential arginine should be strongly dependent on the pH. The rate of inactivation of β-lactamase I by 26.4 mM phenylglyoxa indeed varies greatly with pH (Fig. 2). The $t_{½}$ for inactivation decreases from 615 min at pH 6.97 to 17.0 min at pH 8.84. A plot of the log of the pseudo first-order rate constant for inactivation vs. pH gives a straight line (Fig.2, insert). The slope of 0.84 and the non-deviation from linearity, even at the highest pH used, strongly suggest that inactivation is due to the modification of the free base form of a residue with a pK_a well above 9.

D-Benzylpenicilloamide (*I*) is a competitive inhibitor of β-lactamase I from *B. cereus* 569/H, with a K_i of 19 mM (18) when N-(2-furylacryloyl)penicillin (24) is used as substrate. The rate of inactivation by 30 mM phenylglyoxal is progressively decreased by progressively increasing amounts of *I* in the medium (Fig. 3). If the



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binding of *I* to β-lactamase provides complete protection against inactivation by phenylglyoxal, a plot of the reciprocal of the first-order rate constant for inactivation under otherwise fixed conditions vs. the concentration of *I* should give a straight line with the x-intercept equal to $-K_d$, the dissociation constant for the E·*I* complex. Such a plot (Fig. 3, insert) gives a straight line, with an extrapolated value for K_d of 18 mM. Thus, *I* presumably protects the enzyme against inactivation by phenylglyoxal by binding at the active site and preventing access to an arginyl residue involved in the catalytic mechanism.

The inactivation of β-lactamase by phenylglyoxal is coincident with the loss of arginyl residues (Fig. 4). It appears that initially one residue is rapidly modified with no loss of activity; then activity decreases linearly with further modification of arginine. Extrapolation to complete inactivation indicates that total loss of activity correlates with the modification of 8 arginyl residues per enzyme molecule. In a separate experiment, the enzyme was modified with phenylglyoxal as in Fig. 4 in the absence and presence of *I*. After 65 min modification, the former sample had lost 53% activity and 3.9 arginines, while the sample modified in the presence of 125 mM *I* had lost only 12% activity and 2.9 arginines. These data suggest that full protection against inactivation by *I* correlates with the protection of *ca.* 2 arginines from modification.

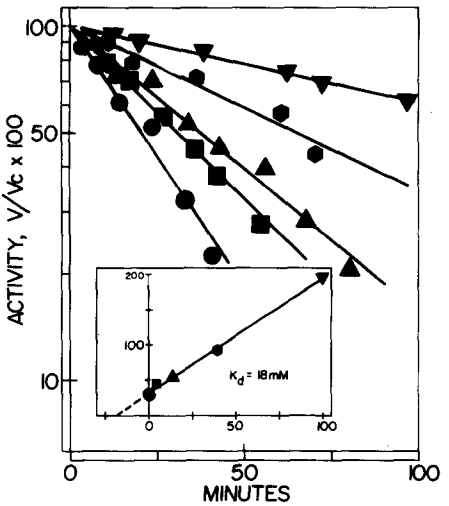

FIGURE 3: β-Lactamase I, 1 μM, was treated with 30 mM phenylglyoxal in 50 mM Bicine, 100 mM $NaHCO_3$, pH 8.3 in the presence of the following concentrations of D-benzylpenicilloamide (*I*): (●) none, (■) 5 mM, (▲) 15 mM, (⬢) 40 mM, and (▼) 100 mM. Insert: A plot of $(k')^{-1}$ (ordinate, where k' is given in min^{-1}, see Fig. 1) vs. [*I*] (abscissa, mM) gives a straight line, with the y-intercept giving the maximum rate of inactivation under these conditions (no *I* present) and the x-intercept giving $-K_d$, the dissociation constant for the E·*I* complex. The value of K_d determined from this plot is 18 mM.

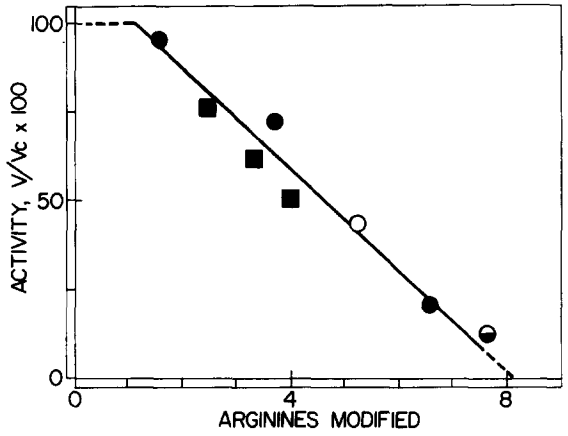

FIGURE 4: Correlation of inactivation of β-lactamase I with arginine modification by phenylglyoxal. The enzyme, 28 μM, was incubated with 20 mM phenylglyoxal in 50 mM Bicine, 100 mM $NaHCO_3$, pH 8.3, and aliquots were periodically withdrawn and subjected to gel filtration and subsequent analyses as described in the text. The circles and squares represent data from two separate experiments. Enzyme assays on each control and modified sample were performed in triplicate, then averaged. The number of arginines in the control was 11.78 (average of six analyses), and the values of the modified samples represent the average of duplicate or triplicate analyses as compared to the control.

The only other residue found to be slowly modified by phenylglyoxal is lysine, but modification does not correlate with inactivation. Amino acid analysis of a sample which was 55% inactivated by phenylglyoxal (denoted by ○ in Fig. 4) showed that 5.2 arginines were modified, but only 0.1 lysine was lost. A sample which was 88% inactivated (◒ in Fig. 4) had lost 7.7 arginines and 1.2 lysines. It is likely that the N-terminal lysine of the β-lactamase I exo-enzyme (5) is deaminated by phenylglyoxal, but this is not likely the reason for the loss of activity (*vide infra*).

DISCUSSION

In recent years, phenylglyoxal (13) and 2,3-butanedione (25) have been used for the selective modification of essential arginyl residues in proteins. Observations that the rate of reaction of the guanidino function with phenylglyoxal and butanedione is enhanced in the presence of bicarbonate (23) and borate (25), respectively, have been exploited to demonstrate a vital role for arginyl residues in the catalytic mechanisms of many enzymes (11,12,19,20). Previous reports on the modification of β-lactamases with α-dicarbonyl reagents have failed to provide a clear insight into the role of arginine in the catalytic function of these enzymes. Reaction of *B. cereus* 569/H β-lactamase I with the trimer of butanedione at pH 6.0 was shown to result in the modification of nearly half the arginines present in the protein without any loss of enzymatic activity (9). In contrast, Pain and Virden (7) and Abraham and Waley (26) have reported preliminary results which suggest that β-lactamases from *S. aureus* PC1 and *B. cereus*, respectively, are inactivated by phenylglyoxal. Covalent

modification of arginine(s) has been suggested as a possible explanation for the inactivation of β-lactamases by phenylpropynal (27).

The inability, in earlier studies (9), to achieve inactivation of *B. cereus* 569/H β-lactamase I by butanedione trimer could conceivably be due to the low pH employed . Indeed, we have found that 2,3-butanedione inactivates the enzyme at pH 8.3 at a rate comparable to that observed when phenylglyoxal is used (data not shown). Inactivation is not enhanced by borate, however, but this could presumably be due to the ability of borate to serve as a competitive inhibitor of the enzyme (28). Phenylglyoxal has been shown to be highly selective for the modification of arginyl residues in proteins (12-14), the only reports to the contrary being the slow reaction with α-amino groups (13) and a highly reactive thiol in rhodanese (29). β-Lactamase I has no cysteinyl residues (5,22). In the work reported here, the following observations tend to eliminate the reaction of the α-amino group and a possible reaction of the ε-amino group of lysine as the cause for the inactivation of the enzyme: i) the increase in the rate of inactivation with increasing pH (Fig. 2), an observation inconsistent with the involvement of an α-amino group; and ii) the finding that treatment of β-lactamase I with 40 mM 2,4,6-trinitrobenzenesulfonate in Bicine buffer, pH 8.3, for 30 min causes less than 5% inactivation (data not shown), in agreement with an earlier report (9), suggesting that the enzyme does not depend on a reactive lysine residue for its catalytic function. These considerations, plus the observed enhancement of inactivation by bicarbonate, strongly suggest that inactivation of the enzyme by phenylglyoxal is a consequence of modification of arginine(s) in the protein.

Of the eight arginyl residues susceptible to modification by phenylglyoxal, *ca.* two are protected in the presence of *I*, a competitive inhibitor of the enzyme. Such protection also prevents the inactivation of the enzyme, suggesting that these two residues are at or near the vicinity of the active site or in a location of strategic importance to the maintenance of the active conformation of the protein. The well-documented involvement of arginyl residues at protein anion binding sites (11,12) supports the possibility that the protected arginine(s) may be intimately involved in the binding of the carboxylate moiety of substrates to the active site. Attractive as this possibility is, it is appropriate to point out that there may be alternative reasons for the inactivation of the enzyme by phenylglyoxal, as well as for the protection by *I*. The reaction of amides with aldehydes has been documented (30). Hence, the possibility, remote as it may be, of the inactivation of the enzyme being due to o the occurrence of such a reaction cannot be excluded.

A comparison of the sequences of class A β-lactamases from *B. cereus* 569/H, *B. licheniformis* 749/C, *S. aureus* PC1 and *E. coli* R_{TEM} shows that only three arginines (residues 65, 164 and 244) are conserved (5). If an arginyl residue is critical for catalysis, it seems reasonable that this residue would be conserved in the sequence of the class A enzymes. The observation that 8 arginines must be modified for com- m plete inactivation of β-lactamase I makes the identification of an essential arginine

a difficult task, but a careful study of the role of arginine in the *S. aureus* PC1 enzyme, which has only 4 arginines (5), might be more reasonable and productive.

β-lactamases have been reported (31) to be evolutionarily related to D-alanine carboxypeptidases, penicillin-sensitive enzymes which can be considered to be models for membrane-bound enzymes which are involved in the final stages of bacterial cell wall biosynthesis. The carboxypeptidases from *B. subtilis* and *B. stearothermophilus* share with β-lactamase a catalytically essential serine, and the sequences around the critical serines from both classes of enzymes are homologous (31). It is thus interesting to note a recent report (32) that the carboxypeptidase from *Streptomyces* R61, which also has an essential serine (33), is inactivated by the modification of a single arginine by phenylglyoxal. Thus, the work reported herein suggests the possibility of a second common feature between the β-lactamases and DD-carboxypeptidases

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