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INHIBITION OF *BACILLUS SUBTILIS* AMINOPEPTIDASE D BY β-LACTAM ANTIBIOTICS

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Summary

Penicillins and cephalosporins inhibit the hydrolysis of D-alanyl-β-naphthylamide by aminopeptidase D (EC 3.4.11.—) from *Bacillus subtilis*. The inhibition is predominantly non-competitive, although the more effective inhibitors, i.e., those with the lower K_i values, seem to exhibit a tendency toward competitive kinetics, while penicillin V, the antibiotic with the largest K_i, exhibits a strong tendency toward uncompetitive kinetics. The removal of antibiotic from the enzyme by gel filtration on Sephadex G-25 restores 100% activity to the enzyme, and suggests that the inhibition does not derive from a covalent antibiotic-enzyme complex. The antibiotics which have been studied, with their respective K_i values (mM) and inhibition type are: methicillin (1.01 ± 0.34, mixed non-competitive-competitive); cephaloridine (2.43 ± 0.17, non-competitive); cloxacillin (3.19 ± 1.29, mixed non-competitive-competitive); oxacillin (6.88 ± 0.77, non-competitive); cephalothin (8.12 ± 0.99, non-competitive); penicillin G (20.0 ± 3.21, non-competitive) and penicillin V (32.1 ± 4.90, mixed non-competitive-uncompetitive). An empirical correlation exists between the K_i value and the freedom of rotation about the bond between the phenyl ring and the atom alpha to the ring in the variable side chain portion of the penicillin molecule.

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Introduction

Penicillins inhibit bacterial growth at concentrations at which penicillins also react reversibly and irreversibly with certain peptidoglycan-synthesizing enzymes, involved in bacterial cell wall synthesis and with other proteins of unknown function (for reviews, see Refs. 1 and 2). At least five penicillin-binding components have been identified in the cell walls of *Bacillus subtilis* [1,3] and of these, at least one is the enzyme, DD-carboxypeptidase [1,4—6]. In addition to a carboxypeptidase with similar specificity, a penicillin-sensitive transpeptidase has been identified and studied to varying degrees in *Escherichia coli* [1,7,8], *Bacillus stearothermophilus* [1,6,9], *Bacillus megaterium* [1,01] and *Streptomyces* R 61, R 39 and K 11 [1,11—16]. A third penicillin-sensitive enzyme, endopeptidase, and at least six penicillin-binding components have been identified in various wild type and thermolabile strains of *E. coli* [17—22]. Apparently, the purified carboxypeptidases of *E. coli*, *B. stearothermophilus* and Streptomyces also exhibit some endopeptidase activity [1]. The carboxypeptidase activity has been studied in *Micrococcus lysodeikticus* [23], *Proteus mirabilis* [24] and *Streptococcus faecalis* [25]. A penicillin-sensitive transpeptidation reaction has been identified in *Staphylococcus aureus*, but this organism seems to lack the carboxypeptidase activity [1,26—28].

A number of other studies of the effects of β-lactam antibiotics on peptidoglycan synthesis in microorganisms such as *Bacillus licheniformis* [29,30], *Micrococcus luteus* [31,32], *Pseudomonas aeruginosa* [33] and in *Neisseria gonorrhoeae* [34] indicate that the mechanism of inhibition of crosslinking may be common. However, in the latter two cases (*P. aeruginosa* and *N. gonorrhoeae*) this inhibition is not always correlated with the minimal concentrations inhibiting growth of the organism. There has been a suggestion that, at least in the L-forms of *Proteus mirabilis* [35], the crosslinking reaction may not be inhibited at growth-inhibiting concentrations of penicillin. None of these enzymatic activities has been unequivocally identified as the primary site at which penicillin exercises its bacteriocidal action, although good evidence suggests, at least in *S. aureus*, that the transpeptidation reaction(s) is implicated [26—28,36]. In general, the activity of cephalosporins is parallel to the penicillins, although cephalosporins usually require higher concentrations than penicillins to exhibit the same effects in vivo.

A recent report from this laboratory of four aminopeptidases found in *B. subtilis* 168 W.T., indicates that one of these aminopeptidases specifically hydrolyzes the peptide bonds of amino terminal D-aminoacyl residues [37]. This aminopeptidase D, as it has been designated, is found predominantly in the cell wall and/or periplasm of *B. subtilis* 168 W.T. and is produced throughout the growth cycle of this organism, although some variations in the specific activity of the enzyme are observed during growth.

The cellular site of the activity suggests a possible role in cell wall metabolism, and this along with its substrate preference suggests that the enzyme might interact with β-lactam antibiotics. Accordingly, the present report is a study of the inhibition of aminopeptidase D by several penicillins and cephalosporins.

Experimental procedure

Organisms. B. subtilis 168 W.T. was obtained from Dr. I.C. Felkner, Texas Tech University. This strain has been converted to nutritional prototrophy by transformation with DNA from *B. subtilis* W 23.

Chemicals. Aminoacyl-β-naphthylamides were obtained from Mann Research Laboratories (New York, NY) and from Fox Chemical Company (Los Angeles, CA). DEAE-cellulose, penicillin G, 6-aminopenicillanic acid and Sephadex G-50 were purchased from Sigma Chemical Co (St. Louis, MO). Cephaloridine and cephalothin were purchased from Eli Lilly and Company (Indianapolis, IN) and the other antibiotics were the gift of Bristol Laboratories (Syracuse, NY). Bio-Glas was purchased from Bio-Rad Laboratories (Richmond, CA).

Growth of cell and purification of aminopeptidase D. The cells were grown and the enzyme purified from them as described in the previous study [37].

Fluorimetric assay. Fluorimetric assays based on the appearance of β-naphthylamine from D-alanyl-β-naphthylamide were done essentially as reported elsewhere [38]. The concentration of D-alanyl-β-naphthylamide in the reaction mixture (0.1 M phosphate buffer, pH 7.0, 3 ml total volume) was 1 mM and enough enzyme was used to produce a convenient reaction rate. The reaction volume is determined here by instrumental limitations. The rate of change of fluorescence at 410 nm was followed and the excitation wavelength was 340 nm. Reaction rates are converted to μmol/min by comparison to the fluorescence of a 0.01 mM β-naphthylamine standard, which in all respects except enzyme is identical to the reaction mixture. All reactions with various penicillins were monitored for fluorescence quenching and corrected where necessary.

Colorimetric assay. A colorimetric assay for aminopeptidase D which has been described in detail elsewhere [37,38] has been modified somewhat. The assay is based on the same substrate at the same concentration as in the fluorimetric assay, but the free β-naphthylamine is measured colorimetrically after diazotization and coupling with *N*-(1-naphthyl)-ethylenediamine. The buffer has been changed, from the previous report, from Tris-maleate, pH 8.0, to 0.1 M phosphate, pH 7.0. This assay cannot be used in the presence of significant levels of penicillin.

Assay for β-lactamase activity. The hydroxylamine reaction with the β-lactam ring, with the subsequent formation of the ferric hydroxamate complex was utilized to determine the quantity of penicillin remaining at the end of a given reaction time. The assay mixture for the fluorimetric assay was used, except that no D-alanyl-β-naphthylamide was present. Penicillin G was present in concentrations varying up to 10 mM. For each level of penicillin G, a control was also incubated without enzyme for the same lengths of time, which ranged from 0.5 to 12 h. After incubation (37°C), a small aliquot was removed from the reaction mixture and an identical aliquot from the control, and each aliquot was placed into a test tube containing 1 ml of 800 mM hydroxylamine. After 5 min, 3 ml of a ferric chloride reagent (100 g $FeCl_3 \cdot 6\ H_2O$), 50 g trichloroacetic acid and 46.7 ml of 12 M HCl plus water was added to make a total volume of 1 l. The amount of penicillin in the reaction mixture and control were compared by colorimetric determination (540 nm) of the intensity of the ferric hydroxamate complex after 5 min.

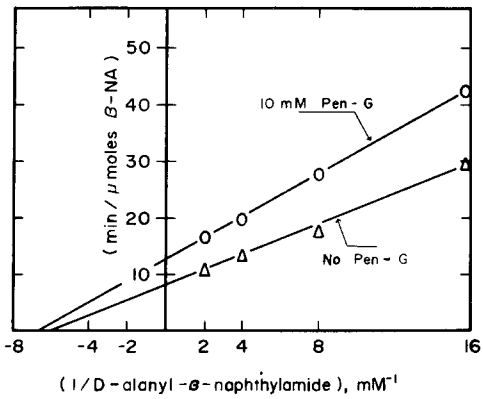
Assay for penicillin amidase activity. A cellulose TLC procedure was utilized to separate any deacylated product from penicillin G as substrate. The procedure is based on the appearance of new ninhydrin positive spots upon chromatographic analysis of a reaction mixture, prepared as for the fluorimetric assay above except that D-alanyl-β-naphthylamide was absent. The concentration of penicillin G and the time of incubation were as for the β-lactamase assay. The R_F value of the expected product of the amidase reaction, 6-aminopenicillanic acid, was the same as the control. This system was also used to determine the purity of the penicillin G solutions used in these experiments. Iodine vapor was used for visualization of organic material on the chromatographs, and ninhydrin reagent was used to detect compounds with free α-amino groups.

Estimation of maximum angle of free rotation, θ_α, about the bond between the variable side chain and the carbonyl carbon in the 6 position. The angle θ_α was obtained from space filling models of various penicillin molecules. The variable side chains were rotated through space and the maximum angles of free rotation were noted. The bond about which rotation was measured was the one between the variable side chain and the 6-carbon atom. Values assumed only steric hindrance and did not take into account any rotational restriction resulting from other factors such as π-orbital overlap.

Results

B. subtilis aminopeptidase D is clearly inhibited by concentrations of penicillin G, which are somewhat higher than concentrations of penicillin G that are normally bacteriocidal. A sigmoidal inhibition curve with 50% inhibition at 20 mM was obtained. Very low concentrations of penicillin G weakly stimulate the enzymatic hydrolysis of D-alanyl-β-naphthylamide. Penicillin does not appear to be a substrate for purified aminopeptidase D, since no products differing in R_F value from penicillin G have been observed. Neither β-lactamase activity nor penicillin amidase activity has been detected to date by the procedures used here. The sigmoid character of the inhibition curve is not pronounced and there is little influence of this characteristic on the kinetic experiments outlined in Figs. 1 and 2. To the extent of the sensitivity of TLC experiments, ion-exchange experiments and Sephadex G-50 chromatography experiments, the preparation of penicillin G utilized in these experiments is essentially pure. However, the appearance of benzyl penicillenic acid, after extended storage of penicillin G solutions, did require that all solutions be freshly prepared immediately before the experiments were performed.

In enzymatic assays done in the presence of penicillin the colorimetric assay is unsuitable. Therefore, all kinetic experiments reported in this paper have utilized the fluorimetric assay indicated in Experimental Procedure. Corrections have been made for fluorescence quenching at high concentrations of penicillin. Appropriate dose-response curves for substrate were obtained in the presence and in the absence of penicillins tested. Penicillin is a noncompetitive inhibitor of the D-aminopeptidase (Fig. 1). When assayed with D-alanyl-β-naphthylamide as substrate the K_m value for substrate is 0.18 mM. Since the Lineweaver-Burk plot is particularly sensitive to small variations at low substrate concentrations, a Hunter-Downs plot (Fig. 2) has been utilized to calculate the



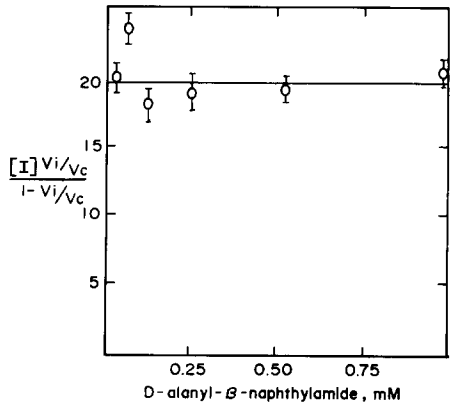


Fig. 1. Lineweaver-Burk plot for penicillin G (Pen-G) inhibition of aminopeptidase D. The lines are derived from an unweighted least-squares plot of the data. The intercepts on the abscissa are not significantly different. The fluorimetric assay was used here.

Fig. 2. Hunter-Downs plot for penicillin G inhibition of aminopeptidase D. The units of the ordinate are mM. This plot has been done for several concentrations of penicillin G, but the one shown here is for 10 mM. The inhibited rate v_i, and the rate of control v_c, are measured for all such plots as in the experiment shown in Fig. 1. The line drawn is an unweighted least-squares plot of the data and is not significantly different over the several penicillin G concentrations used.

K_i, 20 mM, in these experiments. Fig. 4 also suggests that the inhibition is non-competitive since, in theory, the Hunter-Downs plot for non-competitive inhibition will produce a line which has zero slope and which intercepts the ordinate at the value of K_i. As indicated before, none of these kinetic data plots exhibit any strong influence of the sigmoid character of the inhibition originally observed. All kinetic parameters were estimated from Lineweaver-Burk (v^{-1} vs. s^{-1}), Hunter-Downs and Eadie-Hofstee (v vs. $v \cdot s^{-1}$, data not shown) plots. The parameters were derived by unweighted least-squares treatment of raw data. The values for K_i and the types of inhibition observed for the β-lactam antibiotics tested are presented in Table I.

Note that as the values obtained for K_i become smaller, and the penicillin

TABLE I

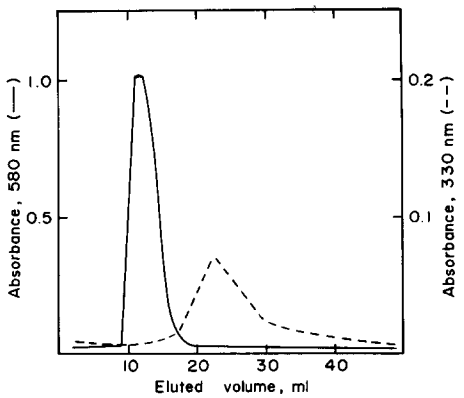
β-LACTAM ANTIBIOTIC INHIBITION OF AMINOPEPTIDASE D

Compound	K_i (mM) ± S.D.	n	Inhibition type	θ_α **
Methicillin	1.01 ± 0.34	10	N.C.-C. *	60
Cephaloridine	2.43 ± 0.17	4	N.C.-C.	—
Cloxacillin	3.19 ± 1.29	8	N.C.-C.	75
Oxacillin	6.88 ± 0.77	5	N.C.	90
Cephalothin	8.12 ± 0.99	3	N.C.	—
Penicillin G	20.0 ± 3.21	12	N.C.	150
Penicillin V	32.1 ± 4.90	3	N.C.-U.C.	180

N.C., non-competitive; C, competitive, U.C., uncompetitive.

* Use of two symbols indicates mixed inhibition kinetics.

** Maximum angle of free rotation between aromatic side chain and its alpha atom for the penicillins. θ_α is estimated to the nearest 5° from space filling models based on Van der Waals radii.



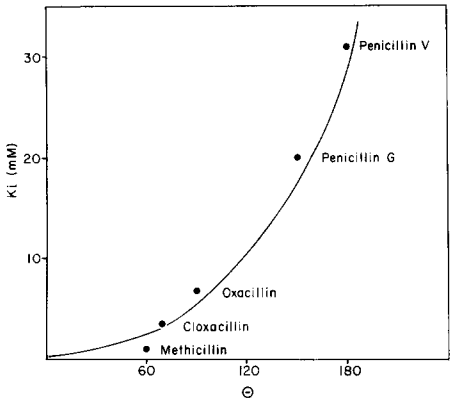


Fig. 3. Reversal of penicillin G inhibition of aminopeptidase D by gel filtration chromatography on Sephadex G-50. ———, aminopeptidase activity as measured in the colorimetric assay. 1.0 absorbance represents an activity of 0.1 μmol/min. - - - - - -, penicillin G measured as the isomeric benzyl penicillenic acid, which derives by rearrangement from penicillin G. This acid has an absorbance maximum at about 320 nm. Total activity recovered from the column was 106% of the activity assayed prior to a 10 min incubation with 50 mM penicillin G. Total inhibition prior to chromatography was 79% in this experiment.

Fig. 4. Maximum angle of free rotation θ_α, about the bond between the variable side chain of several penicillins and the carbonyl carbon in the 6-position vs. the respective K_i value for the penicillin.

becomes a more effective inhibitor, the inhibition type varies from mixed non-competitive/uncompetitive through non-competitive to mixed non-competitive/competitive. There also exists an observable correlation between the angle of free rotation of the variable side chain about the bond to the carbonyl 6-carbon and the K_i value. This correlation will be discussed later in this section. Fig. 3 shows the elution profile of aminopeptidase D activity and that of penicillin from the Sephadex G-50 column. The activity profile was obtained with the colorimetric assay, which in this case can be used because the penicillin has been separated from the aminopeptidase D activity. The activity recovered from the column, after the experiment, is the same as the activity placed into the incubation mixture prior to addition of penicillin G. The figure demonstrates rather clearly that the inhibition by penicillin G is reversible. No irreversible inactivation was observed even after 1 h of incubation with penicillin G prior to chromatography.

Fig. 4 illustrates the empirical relationship which exists for θ_α and the experimentally estimated K_i values for the penicillins used in this study. The data are from Table I. Note that the cephalosporins are not included in this plot, since they are not structurally homologous to the penicillins. The form of the curve drawn is based on two observations: (1) the K_i value would seem to be always greater than zero, and (2) the points appear to increase in some sort of geometric progression rather than an arithmetic progression.

Discussion

As indicated in the previous report concerning the properties of aminopeptidase D, this enzyme exhibits kinetic properties somewhat different from those

exhibited by the three L-aminopeptidases known to be synthesized by *B. subtilis* 168 W.T. [37]. Because the enzyme is primarily localized in the cell wall and/or periplasm of the organism, the physiological role of the enzyme may be in cell wall metabolism of D-amino acids or peptides containing amino terminal D-aminoacyl residues. No specific role has yet been established, but in searching for a role, the sensitivity of aminopeptidase D to inhibition by penicillin G (K_i 2.0 mM) was observed. This K_i value compares unfavorably for physiological significance with K_i values ranging from about 10^{-1}—10^{-6} mM, for penicillin G inhibition of carboxypeptidases involved in the synthesis of cell walls in *B. subtilis* [5], *B. megaterium* [10], *M. lysodeikticus* [24], *P. mirabilis* [25] and *E. coli* [7,8] and for penicillin G inhibition of transpeptidase activity from *S. aureus* [27—29,36,40], *B. megaterium* [41,42] and various strains of Streptomyces [43]. This difference in magnitude for the K_i values might reasonably be predicted for a different enzymatic activity. Since the other enzymes are more sensitive one might assume they are primary reaction sites for penicillin inhibition of growth for these oragnisms.

However, a K_i or 20 mM for *B. subtilis* aminopeptidase D compares rather well for physiological significance with a K_i of $1.3 \cdot 10^{-2}$ M for penicillin G inhibition of the carboxypeptidase activity of the exocellular DD-carboxypeptidase-transpeptidase of *Streptomyces* R61 [15]. The inhibition of the *Streptomyces* carboxypeptidase derives its importance from K_i value for other β-lactam antibiotics ranging from 10^{-4} M for carbenicillin inhibition up to the K_i for penicillin G.

In the case of many of the carboxypeptidases and transpeptidases mentioned in the previous paragraph, pencillin G associates irreversibly with the enzyme. Irreversible inactivation of aminopeptidase D has not been observed. Therefore, it is unlikely that aminopeptidase D is one of the penicillin-binding components of the cell walls of *Bacilli*. Furthermore, although the K_i value of aminopeptidase D measured in vitro may not be identical to the K_i value of the activity when measured in vivo, there seems little likelihood that aminopeptidase D is a so called 'kill site' of penicillin G for *B. subtilis* 168 W.T.

Arylamidase activity (aminoacyl-β-naphthylamide hydrolysing activity) has been, at least in one case [44], associated with β-lactamase activity specific for cephalosporins but not for penicillin. Penicillin amidase activity, according to Holt and Stewart [45], has not been found in Gram-positive bacteria. Amidase and β-lactamase activity are absent in our preparations of aminopeptidase D.

The generally non-competitive nature of the inhibition suggests that β-lactam antibiotics are likely to the binding at a site other than the substrate site on the enzyme (Table I). It is curious that an aminopeptidase which is specific for D-aminoacyl residues does not appear to bind penicillins (D-amino acid containing peptide analogs) in the substrate site. However, because aminopeptidases seem to have three or more subsites, which are distinguishable by substrate analog studies [46,47], it is possible for penicillins to bind in one of the specific subsites without interfering competitively with a substrate such as D-alanyl-β-naphthylamide. Binding at another subsite on the surface of the enzyme may either be a mixed or non-competitive type of binding and still be in a site which would fit a substrate with a more natural configuration such as tetra-D-alanine or tri-D-alanine, peptides which are found in bacterial cell walls.

Generally, this model would require that a larger side chain on the penicillin molecule would be a somewhat more competitive inhibitor. The data in Table I and the molecular structures of the penicillins used are generally congruent with this expectation. The aminopeptidase D is known to be active in hydrolyzing tri-D-alanine [37]. β-Lactam antibiotic inhibition of this reaction has not yet been studied.

A number of possible mechanisms for the inhibition not involving the catalytic site(s) of aminopeptidase D can be postulated. Many chemical mechanisms are known or have been suggested for penicillin interaction with protein molecules in somewhat nonspecific fashion (e.g., Ref. 48). However, column chromatography studies indicate that the binding of penicillin to aminopeptidase D is not covalent. This suggests that this enzyme has a site which is specific for reversible interaction with the penicillin nucleus.

The correlation exists for the mean free angle of rotation, θ_α, as defined in Experimental Procedure and Table I, and the K_i value is curious. This suggests that the penicillin site on the enzyme is restrictive for a specific configuration of the side chain, with respect to the common nucleus of the penicillin molecules. It should be noted that the mean free angle of rotation about the indicated bond will be a function of the bulk of the side chain, as well as its electronic properties, e.g., the degree of conjugation of the side chain with the carbonyl carbon. Neither the bulk of the side chain, nor the degree of conjugation correlated with the observed K_i value as well as the mean free angle of rotation. No rigorous theoretical model exists for the interpretation of this interesting result. However, this result does suggest that more effective penicillins could conceivably require both more bulk and more π-orbital overlap in the side chains.

On Fig. 4 we have chosen to draw a curved line through the data points, although a straight line could also be drawn. For the curved line there would be gradual increase in inhibitor effectiveness with decreasing values for θ_α. If one plots the natural logarithms of the K_i values vs. the natural logarithms of $(\theta_\alpha)^2$, one obtains a straight line whose intercept on the ln K_i axis is the estimated value of ln K_i for a compound with a θ_α value of zero. The value of K_i thus calculated is $1.3 \cdot 10^{-5}$ M. On the other hand, if one draws a straight line though the data points on Fig. 4, negative and nonsense values for K_i will be obtained upon extrapolation to the lower values for θ_α, unless one also postulates that minimum and constant positive values for K_i are obtained below a certain value for θ_α, which would also produce a very sharp inflection point.

The cell wall enzyme in *B. subtilis*, D-alanine carboxypeptidase, is several orders of magnitude more sensitive to penicillin G than aminopeptidase D, and no readily apparent rationale exists for assuming that aminopeptidase D is a penicillin-sensitive enzyme participating in cell wall synthesis. Although transpeptidase activity has not yet been identified as associated with the aminopeptidase D activity [37], the cell wall-synthesizing transpeptidase which is common to other *Bacilli* has not yet been studied in vitro in *B. subtilis*. Because of the structural similarity which exists among many of the peptidases and proteases, one might readily assume, (1) that this aminopeptidase D could possess penicillin-sensitive transpeptidase activity; (2) that the aminopeptidase D possesses structural similarity to an ancestral carboxypeptidase or trans-

peptidase and retains a site for penicillin binding, or (3) that this aminopeptidase D has a metabolic function which is unrelated to cell wall synthesis and that it is not descended from cell wall-synthesizing enzymes with the penicillin sensitivity being essentially fortuitous.

However, without regard to the solutions of these problems, the results reported here with respect to the penicillin inhibition and elsewhere with respect to the general character and substrate preference [37] of *B. subtilis* 168 W.T. aminopeptidase D, an enzyme located in the cell wall or periplasm of this organism, require that a role in cell wall metabolism must be considered likely.

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