AN INHIBITOR OF SOLUBLE D-ALANINE CARBOXYPEPTIDASE FROM ESCHERICHIA COLI B.

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Summary: A thermostable protein that strongly inhibits the soluble E. coli D-alanine carboxypeptidase was isolated from a cell-free extract of E. coli B. The inhibitor was purified 140-fold by heat treatment, selective precipitation at pH 4.5, ion exchange chromatography on DEAE-cellulose and gel chromatography on Sephadex G-100. Inhibition of soluble D-alanine carboxypeptidase by this inhibitor is reversed by cations such as Mg^{++} or Na^{+} and abolished by digestion of the inhibitor with proteolytic enzymes. The inhibitor does not affect either the particulate D-alanine carboxypeptidase of E. coli or the growth of the bacteria.

D-Alanine carboxypeptidase cleaves the terminal D-alanine residue from UDP-MurNAc-pentapeptide, the precursor of bacterial peptidoglycan (1-5). The enzymes from various bacterial strains have attracted considerable attention, since they are inhibited by very low concentrations of penicillin. E. coli D-alanine carboxypeptidase occurs in both a particulate fraction, bound to bacterial membranes and in a soluble cytoplasmic fraction (2, 6). Recently we purified this soluble D-alanine carboxypeptidase by affinity chromatography on a p-aminobenzylpenicillin-Sepharose column (6). In the course of purification we observed a marked increase in the total absolute activity of the purified enzyme as compared with that of a crude extract. This led us to postulate that an inhibitor of soluble D-alanine carboxypeptidase had been removed during the purification procedure. This paper describes the partial purification and some properties of an inhibitor of soluble D-alanine carboxypeptidase from E. coli.

Materials : DNase and RNase were purchased from Sigma. Chymotrypsin, trypsin and lipase were from Worthington Biochemical Co.
Enzymes and substrates: The purification of soluble D-alanine carboxypeptidase

from E. coli by affinity chromatography on a p-aminobenzylpenicillin-Sepharose column and synthesis of the substrate UDP-MurNAc-L-Ala-D-Glu-mesoA$_2$pm-D-Ala-D-Ala and the same substrate labeled at terminal D-(^{14}C) alanine residue have already been described (6). The particulate, membrane bound D-alanine carboxypeptidase was prepared as follows: E. coli B cells (10g) were suspended in 25 ml of 0.05 M Tris-Cl, pH 7.6 and 25 g of glass beads were added. The mixture was agitated for 2 minutes in a Braun homogenizer. After removal of cell debris by centrifugation at 10,000 x g, the particulate material was collected by centrifugation at 100,000 x g, washed twice with 0.5 M Tris-Cl, pH 7.5 and finally resuspended in 1.5 ml of 0.05 M Tris-Cl, pH 8.2, containing 10^{-4} EDTA. The protein content was 10 mg per ml.

Protein determination: Protein was measured by the procedure of Lowry et al. (7) using crystalline bovine serum albumin as standard.

D-Alanine carboxypeptidase assay: The assay mixture contained in 25μl: 30mM Tris-HCl, pH 8.2, 0.1mM EDTA, 3mM UDP-MurNAc-L-Ala-D-Glu mesoA$_2$pm-D-Ala-D-(^{14}C) Ala (12,000 cpm) and enzyme 1 μg (2 units) of the purified soluble D-alanine carboxypeptidase or 50 μg (0.5 units) of the particulate enzyme in 5 μl. Assays were incubated for 30 min at 30°C, then chilled in ice and the reaction was terminated by the addition of 200μl of 0.2M citrate buffer, pH 2.2. Release of (^{14}C) alanine was analyzed as described (6). One activity unit was defined as the hydrolysis of 1mμmole of substrate per minute per mg protein.

Methods - Purification of the D-alanine carboxypeptidase inhibitor (DACI)

Extraction: Lyophilized E. coli B cells (100 g) were stirred into 4 liters of water for 10 minutes. The suspension was centrifuged at 10,000g in a Sharpless centrifuge. The supernatant (3.5 liters) was incubated with 2 mg of DNase and 2 mg of RNase for 3 hours at 23°C. The solution was chilled to 4°C and 1400 g of solid ammonium sulfate were added. The precipitate was collected by centrifugation using a Sharpless centrifuge, redissolved in 500 ml of 0.05 M Tris-Cl, pH 8.2 and dialyzed at 4°C against the same buffer. The insoluble material was discarded and the solution dried by lyophilization.

Heat treatment: The lyophilized material was redissolved in 500 ml water and heated in a boiling water bath for 10 minutes. The precipitate was removed by centrifugation at 30,000 x g in a Sorvall centrifuge.

Selective precipitation: The supernatant solution was cooled to room temperature and brought to pH 4.5 with 2 M HCl and left for 30 minutes. The precipitate formed was collected by centrifugation and dissolved in 50 ml of 0.05 M Tris-Cl, pH 8.2. The solution was clarified by centrifugation and concentrated to 5 ml by ultrafiltration on a Diaflo XM-50 membrane using an Amicon apparatus. The ultrafiltration step was repeated twice after diluting the inhibitor to 50 ml with the same buffer. This process removed all low molecular weight material absorbing at 280 nm.

Ion exchange chromatography: The DACI solution was applied to a DEAE cellulose column (10 x 0.4 cm) previously equilibrated with 0.05 M Tris-HCl, pH 8.2. The column was washed with the same buffer supplemented with

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Abbreviations: MurNAc, N-acetylmuramic acid, mesoA$_2$pm, α, ξ - mesodiaminopimelic acid DACI, D-alanine carboxypeptidase inhibitor

0.25 M NaCl until the effluent was free of material absorbing at 280 nm. The inhibitor was then eluted from the column with 0.05 M Tris-HCl, pH 8.2 containing 1 M NaCl and the pooled fractions were concentrated to 1.0 ml by ultrafiltration.

Gel filtration: The inhibitor (1 ml) was applied to a Sephadex G-100 column (120 x 1.4 cm) equilibrated with 0.05 M Tris-Cl buffer (pH 8.2) and eluted with the same buffer. The first fractions emerging after the void volume (80 cc) and containing inhibitor activity were pooled and concentrated by ultrafiltration.

Results and Discussion:

Purification and molecular properties of the inhibitor: Addition of a cell-free extract of E. coli entirely prevented hydrolysis of UDP-MurNAc-pentapeptide by D-alanine carboxypeptidase. The inhibitory factor was purified as summarized in Table 1. Purification resulted 140-fold increase in specific activity of the inhibitor and took advantage of its unusual heat stability and of the fact that it precipitates at a pH of about 4.5. The latter step indicated that the inhibitor is an acidic molecule. This was further

TABLE I

Purification of the Inhibitor of D-alanine carboxypeptidase

Step	Volume (ml)	Total protein (mg)	Total inhibition units*	Yield %	Purification
Extraction	470	2820	9400	100	1
Heat treatment	430	645	3160	55	2.4
Selective precipitation	50	80	2700	27	16.2
Ion exchange chromatography	10	12.5	2000	21	48
Gel filtration	3	3.1	1480	15	140

*Inhibition of 25% of the activity of D-alanine carboxypeptidase is defined as one inhibition unit. The assay conditions were specified in Materials and Methods. Inhibitor (1-5μl) was added in the beginning of assay, substituting buffer, and the reaction mixture was incubated and the products analyzed as described.

supported by tenacious binding to DEAE cellulose, the inhibitor being eluted from the column only by 1 M NaCl.

Ultrafiltration, using an XM-50 Amicon membrane and the exclusion of the inhibitory activity on Sephadex G-100, indicated that the molecular weight of the inhibitor is at least 50,000. This minimal molecular weight estimation was corroborated by the fact that about 75% of the inhibitor was retained upon ultrafiltration through an XM-100 Amicon membrane.

The protein nature of the inhibitor was shown by digestion with various proteolytic enzymes. Digestion of inhibitor with either chymotrypsin or trypsin (1:100 ratio of protease to inhibitor) at 30°, for 16 hours, destroyed about 80% of the inhibitory activity. Incubation with pronase under the same conditions led to complete destruction of the inhibitory activity.

Incubation of DACI with RNase, DNase or with lipase had no effect on inhibitory activity.

Inhibition of the soluble D-alanine carboxypeptidase by the partially purified inhibitor - Inhibition of soluble D-alanine carboxypeptidase by the partially purified inhibitor is shown in Fig. 1. The inhibition was linear over the range of inhibitor concentration up to 10 μg/ml. Complete inhibition was reached at about 60 μg/ml of DACI. Conversion of this plot to its semi-reciprocal, Dixon form resulted in linear relation of $\frac{1}{v}$ vs (DACI) From this plot the maximal apparent inhibition constant $K_I \simeq 20\mu g/ml$ was obtained. Using the estimated minimal molecular weight value for DACI as 50,000 the K_I was calculated to be $\leq 4.10^{-7}$M. This low value for the inhibition constant indicates very strong interaction, of the same order of magnitude as reported for the inhibition of D-alanine carboxypeptidase by penicillin (2, 6).

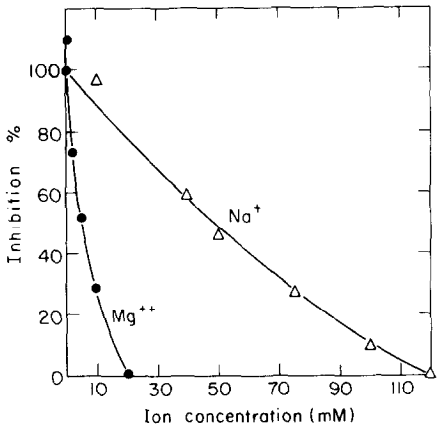

Fig. 1. Inhibition of D-alanine carboxypeptidase by the inhibitor. Inhibition of the enzymic activity of D-alanine carboxypeptidase was tested by adding by adding DACI to the assay mixture.

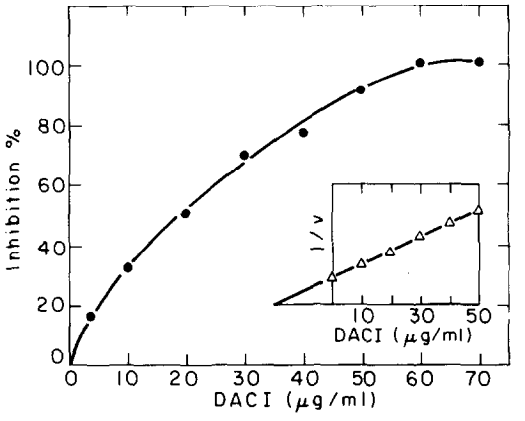

Fig. 2. Effect of cations on the inhibition of D-alanine carboxypeptidase by the inhibitor. The inhibition of D-alanine carboxypeptidase by DACI was tested in assay mixtures containing $MgCl_2$ (●) or NaCl (△).

Effect of cations on D-alanine carboxypeptidase inhibition: Inhibition of D-alanine carboxypeptidase by DACI was tested in the presence of $MgCl_2$ and NaCl (Fig. 2). The inhibition was sensitive to increasing concentrations of these cations, complete reversal being reached at 15mM $MgCl_2$. Sodium ions had a much weaker effect on the D-alanine carboxypeptidase inhibition; total reactivation occurred only in 120mM NaCl. This effect can be

TABLE II

Effect of various metal ions on inhibition of soluble D-ananine carboxypeptidase by the inhibitor

Assays were carried out as described under Materials and Methods and in Table 1. The final concentration of bivalent cations was 20mM and of monovalent cations was 120mM. The concentration of DACI was 100 μg/ml.

Ion added	Addition or omission of DACI	Relative activity (%)
none	-	100
	+	2
Mg^{++}	-	130
	+	155
Ca^{++}	-	93
	+	102
Mn^{++}	-	212
	+	183
Zn^{++}	-	61
	+	61
K^{+}	-	51
	+	74
Na^{+}	-	147
	+	92
Li^{+}	-	75
	+	61
NH_4^{+}	-	89
	+	77

undoubtedly attributed to general salt effect, as various cations reversed the inhibition to the same extent (Table II). Moreover, the concentration of monovalent ions necessary to obtain the same percent of reactivation was much higher than that of divalent metals.

It is noteworthy that the addition of cations over a limited concentration range enhanced D-alanine carboxypeptidase activity (ref. 6 and Table II). However, the enzyme purified by affinity chromatography showed no strict

requirement for a monovalent or divalent cations. This contrasts with the data of Izaki and Strominger which demonstrated an absolute requirement for a divalent cation for E. coli D-alanine carboxypeptidase (2). Their data can be explained by the fact that the enzyme used for kinetic studies was only partially purified and still contained bound inhibitor. Therefore, the absolute metal ion requirement may be prerequisite to dissociate the existing enzyme-inhibitor complex.

Effect of the inhibitor of particulate D-alanine carboxypeptidase - D-Alanine carboxypeptidase activity occurs in both the particulate and soluble fractions of E. coli although the two enzymes have not been shown to be identical. The particulate enzyme can be detached readily from the membrane under variety of lytic conditions (2, 6).

The enzymatic activity of particulate D-alanine carboxypeptidase was not affected by addition of DACI, even in 10-fold excess, relative to the amount of DACI sufficient to inhibit completely the soluble enzyme (Table IIIb).

Triton X-100 stimulates markedly the activity of the soluble enzyme (Table III[k]) and causes the solubilization of the particulate enzyme (Table III[c] and [f]). The solubilization of the particulate D-alanine carboxypeptidase by the non-ionic detergent renders it indistinguishable from the soluble enzyme in respect to its response to DACI (Table III[g]) and to cation induced reversal of inhibition (Table III[h]). Therefore the lack of inhibition of the particulate enzyme by DACI was probably due to inaccessibility of the high molecular weight inhibitor to the enzyme located within the insoluble carrier. The relatively small molecule of substrate can, however, penetrate inside the loosely shielded network of the membrane and can be easily hydrolyzed by the particulate enzyme. This assumption is further supported by the fact that

TABLE III

Effect of the inhibitor and Triton X-100 on the particulate and soluble D-alanine carboxypeptidase

Enzymes and inhibitor were assayed as described under Materials and Methods and in Table 1. The concentration of DACI in A was 0.5 mg/ml and in B 0.05 mg/ml.

Experiment	Addition or omission DACI	Triton X-100 (1%)	Mg^{++} (20mM)	Relative Activity (%)
A. Particulate D-alanine carboxypeptidase				
a	-	-	-	100
b	+	-	-	100
c	-	+	-	400
d	+	+	-	75
e	+	+	+	350
f*	-	+	-	375
g*	+	+	-	0
h*	+	+	+	325
B. Soluble D-alanine carboxypeptidase				
i	-	-	-	100
j	+	-	-	0
k	-	+	-	180
l	+	+	-	11
m	+	-	+	104
n	-	+	+	200
o	+	+	+	156

* In this experiment the membranes (10µl) were shaken for 1 min with Triton X-100 (2%, 10µl). The insoluble materials were removed by centrifugation (Eppendorf centrifuge) and 10µl of the supernatant were assayed for D-alanine carboxypeptidase activity as described.

both the soluble and particulate enzymes are highly sensitive to small penicillin molecules (2, 6 and unpublished observations).

Similar behavior had been demonstrated in the case of trypsin bound to the water insoluble carrier (8). The immobilized enzyme was sterically hindered and exhibited considerably lower proteolitic activity towards high molecular weight substrate (5-10%) than that to be expected from its

esteratic activity. Moreover, the inhibition of the esteratic activity of the insoluble enzyme by a high molecular weight soybean trypsin inhibitor was partial and reached only 40%, at 400-fold excess of an inhibitor, relative to the amount necessary to obtain 100% inhibition in solution.

Effect of the inhibitor on growth of E. coli - The assay for inhibition in vitro was performed using agar plates seeded with bacteria. No inhibition of growth was observed using filter discs wetted with 2 mg/ml solution of DACI. This result might be anticipated since it is rather unlikely that the high molecular weight inhibitor could be transported through the bacterial cell wall and exhibit activity inside the cell.

Conclusions:

In this paper the existence of a protein that inhibits in vitro D-alanine carboxypeptidase from E. coli has been demonstrated. The physiological role of the inhibitor so far remains obscure.

It is generally accepted that the physiological role of D-alanine carboxypeptidase is to regulate the incorporation of peptidoglycan into the cell wall by removal of a terminal D-alanine residue from newly synthesized peptidoglycan (2, 4). The inhibitor, found in the soluble fraction, may regulate the action of D-alanine carboxypeptidase. However, since in vitro the equilibrium between the active enzyme and inhibitor-enzyme complex is controlled by cations, the inhibitor may not act at the physiological ionic strength present in the cell.

The source of the inhibitor has not yet been ascertained. It will be of interest to find out if originally it is attached to the membrane and then secreted to the cytoplasm or if it is synthesized in the cytosol. Furthermore, the specificity of the inhibitor toward D-alanine carboxypeptidase should be

corroborated by studying its effect on other enzymes involved in biosynthesis of bacterial cell wall.

The ability of several trypsin proteolytic enzymes to function in the presence of D-alanine carboxypeptidase inhibitor demonstrates that this inhibitor is not a general protease inhibitor. Also, the possibility that the inhibitor acts as a chelating agent can be excluded, since D-alanine carboxypeptidase works in the presence of EDTA (6). Moreover, as was shown, the inhibition can be reversed by either bivalent and monovalent cations.

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