ON THE SUBSTRATE SPECIFITY OF L-ASPARAGINASE FROM *E. COLI*

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1. Introduction

Recently the question was discussed whether L-asparaginase (EC3.5.1.1) from *Escherichia coli*, and other asparaginases with anti-tumor activity may in vivo exert activities other than the hydrolysis of L-asparagine. For example the deamidation of fetuin [1] and L-asparaginyl-tRNA [2] by asparaginase was observed. Furthermore asparaginase from *Erwinia carotovora* was reported to enhance the velocity of deamidation of some peptides with COOH-terminal asparagine [3].

We therefore extended our studies on the substrate specifity and kinetics of *E.coli* asparaginase [4] on a series of N^2- and N^4-substituted asparagine derivatives and some other substrate analogues not yet tested with the *E.coli* enzyme.

2. Materials and methods

E.coli asparaginase A (260 U/mg) was a gift of Bayer AG., Wuppertal-Elberfeld [5]. Most of the substrate analogues used were prepared following published procedures. For references see table 1.

2.1. *Z-N^4-methoxyl-L-asparagine-α-benzyl ester (I)*

To a mixture of 4.25 g Z-L-asp-α-benzyl ester [6] in 100 ml of chloroform and 1.7 ml triethylamine, 1.63 ml of isobutyl chloroformate and a solution of 1 g 0-methyl hydroxylamine·HCl and 1.7 ml of triethylamine in 10 ml of chloroform were added dropwise at −5°C. The mixture was stirred overnight at room temperature, extracted with water, saturated $NaHCO_3$ solution and water. The organic phase was reduced in volume i.v. and the ester crystallized by addition of ethanol. Recrystallization from chloroform–ethanol.

Yield: 3.5 g, calcd. C 62.18%, H 5.74%, N 7.25%; m.p. 108–110°C, found C 62.11%, H 5.70%, N 7.23%.

2.2. *N^4-Methoxy-L-asparagine*

1.5 g of the ester (I) were dissolved in 150 ml of methanol and the solution treated with H_2/Pd. The catalyst was filtered off and the solution evaporated to dryness. The residue was crystallized from water–methanol.

Yield: 500 mg, calcd. C 37.04%, H 6.22%, N 17.28%; m.p. 212–214°C, found C 36.69%, H 6.18%, N 16.79%.

2.3. *N^4-Diethyl-L-asparagine*

It was prepared from Z-L-Asp-α-OBzl and diethylamine by a similar procedure. The final product was an oil which could not be crystallized. It was chromatographically pure.

2.4. *Enzymatic activities*

Enzymic activities were determined as described previously [4] by measuring the production of NH_3 and/or aspartic acid with coupled enzymatic assays. Usually substrate concentrations of 0.1–0.2 mM and 0.05 U (asparagine hydrolysis) of asparaginase in 50 mM Tris–HCl or phosphate buffer, pH 7.5 at 37°C were applied. If no activity could be detected under these conditions, substrate concentrations were raised to 1–2 mM and the activity to 5–10 U. Inhibition studies were conducted with L-asparagine or N^4-methoxy asparagine as substrates and a 5 to 20-fold excess of inhibitor. Incubation mixtures containing dipeptide substrates were analyzed on a Beckman Unichrom amino acid analyzer (0.9 × 69 cm column, 55°C, pH 3.28 buffer) after dilution of an aliquot with citrate–HCl buffer, pH = 2.2. The cleavage of L-amino succinimide was followed directly at 218 nm.

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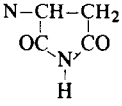
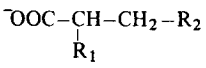
Table 1
Action of *E.coli* asparaginase on derivatives and analogues of *L*-asparagine.

Compound	Ref.	R_1	R_2	V_{rel}	
L-Asparagine		$-NH_3^+$	$-CO-NH_2$	100	K_M = 0.01 mM [4]
N^4-Hydroxyl-L-asparagine	see [4]	$-NH_3^+$	$-CO-NH-OH$	105	K_M = 0.8 mM [4]
N^4-Methoxy-L-asparagine		$-NH_3^+$	$-CO-NH-OCH_3$	20	K_M = 0.2 mM
N^4-Methyl-L-asparagine	see [4]	$-NH_3^+$	$-CO-NH-CH_3$	0.8	K_M = 0.03 mM [4]
N^4-Diethyl-L-asparagine		$-NH_3^+$	$-CO-N(C_2H_5)_2$	0	
N^4-Isopropyl-L-asparagine	[7]	$-NH_3^+$	$-CO-NH-CH(CH_3)_2$	0	
N^4-Benzyl-L-asparagine	[7]	$-NH_3^+$	$-CO-NH-CH_2-C_6H_5$	0	
Succinic acid monoamide	[8]	$-H$	$-CO-NH_2$	0	
N^2-Ethyl-DL-asparagine	see [4]	$-NH_2^+-CH_2-CH_3$	$-CO-NH_2$	2.5	K_M = 0.2 mM [4]
Glycyl-L-asparagine	[9]	$-NH-CO-CH_2COO^-$	$-CO-NH_2$	0.5	K_M = 16 mM
DL-Alanyl-DL-asparagine	[9]	$-NH-CO-CH(CH_3)COO^-$	$-CO-NH_2$	< 0.2	
L-2-Amino-4-oxo-5-chloro-valeric acid	[10]	$-NH_3^+$	$-CO-CH_2-Cl$	–	No inhibition
L-2-Amino-2-carboxy-ethane-sulfonamide	[11]	$-NH_3^+$	$-SO_2-NH_2$	0	Comp. inhibitor K_I = 0.095 mM
Maleic acid monoamide	[12]	$^-OOC-CH=CH-CO-NH_2$		0	No inhibition
L-Amino succinimide	[13]			0	No inhibition

Most of the compounds may be represented by the scheme



The structures of the residues R_1 and R_2 are indicated in these cases. The references given in brackets refer to the synthetic procedures. If no reference is specified see Materials and methods. The relative rates are maximal velocities and refer to the rate of asparagine hydrolysis taken as 100.

3. Results and discussion

The results of our specificity studies are summarized in table 1. Some important results published previously [4] are included in addition.

3.1. *N^4-Substituted asparagine derivatives*

Looking at the table one recognizes that derivatives of L-asparagine must not contain N^4-substituents greater than the methoxy group to be cleaved by *E.coli* asparaginase at a considerable rate. This stems mainly from steric hindrance of the substrate binding by bulky groups in this position. The small rate of hydrolysis observed with N^4-methyl-L-asparagine may be attributed to the greater stability of its methylamide bond whereas hydroxamates are more readily hydrolyzed than the corresponding amides.

In many cases *p*-nitroanilides have proved to be excellent amidase substrates. **N^4-*p*-nitrophenyl asparagine** however – bearing a very bulky N^4 substituent – is deamidated by *E.coli* asparaginase with only 0.006%

relative rate as shown by Takenaka et al. [14]. Therefore it does not seem very likely that aspartyl-β-peptides or asparaginyl-β-glycosides may act as true substrates of the *E.coli* enzyme in vivo.

As a new very good substrate we found N^4-methoxy-L-asparagine. This compound is deamidated at a relative maximal rate of about 20%. Surprisingly, its K_M is lower than that of the non-methylated hydroxamate. Methoxy-asparagine may become important in kinetic studies on the mechanism of the *E.coli* enzyme because it is easily obtained with ^{14}C-labelled O-methyl-hydroxylamine.

3.2. *Derivatives with a modified α-amino group*

As was shown previously [4] the α-amino group of asparagine plays an important role in substrate binding. Succinic acid monoamide, lacking such a group, is not deamidated at all. Alkylation of the α-NH_2 function, leaving the positive charge, does not abolish the enzymatic activity (see N^2-ethyl asparagine). Acylation of this group on the other hand has a more pronounced effect. Glycyl-L-asparagine, the most simple dipeptide containing COOH-terminal asparagine, is deamidated with a relative rate of less than 1%, its K_M being far beyond the physiological range of concentrations. Alanyl-asparagine is cleaved still more slowly. We thus could not confirm results of Allison et al. [15] who reported hydrolysis of L-asparagine and L-alanyl-L-asparagine at equal rates by *E.coli* asparaginase.

If there is any hydrolysis of asparagine-containing peptides in vivo it would be extremely slow. Furthermore no peptides of this kind with physiological significance are known as yet.

3.3. *2-Amino-4-oxo-5-chloro valeric acid*

The chloromethyl hetone analogue of asparagine was shown to be an 'active site-directed' irreversible inhibitor of glutamine synthetase [10]. The ineffectiveness of this compound against *E.coli* asparaginase – it does not even inhibit the enzyme competitively – is somewhat surprising since, according to Handschuhmacher et al. [16] 5-diazo-4-oxo norvaline – a nearly related analogue – not only inhibits the *E.coli* enzyme but is cleaved also.

The sulfonamide analogue on the other hand is a very efficient competitive inhibitor with an inhibition constant of K_I = 0.095 mM (see fig. 1). It is one of the few competitive inhibitors of the *E.coli* enzyme not acting as a substrate too. Therefore it may prove valuable as an agent protecting the active site during chemical modification of the enzyme.

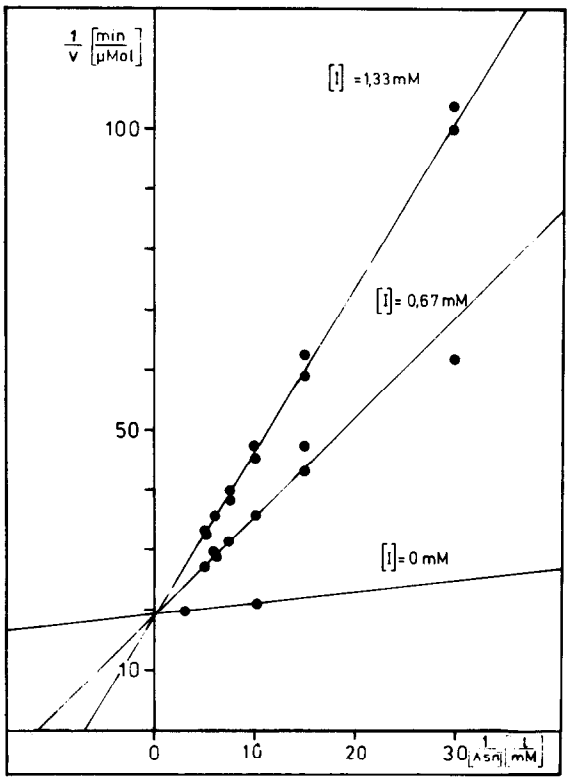


Fig. 1. Kinetics of inhibition of *E.coli* asparaginase by L-2-amino-2-carboxy sulfonamide. Lineweaver–Burk plots for the hydrolysis of L-asparagine without and in the presence of the inhibitor at pH 7.5 and 37°C.

3.4. *The amide bonds of maleic acid monoamide and L-amino-succinimide*

Both bonds undergo rapid nonemzymatic hydrolysis. This is due to intramolecular nucleophilic catalysis by the COOH-group in the case of maleic acid monoamide [17]. The lability of L-amino succinimide may be attributed to ring strain. The rate constant of the ring opening reaction was found to be $k = 2.4 \cdot 10^{-3}$ min^{-1} at pH 7.5 and 37°C.

Neither compound acts as a substrate of asparaginase however.

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