PARTIALLY DEAMINATED L-ASPARAGINASE

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Summary

Nitrous acid reacts with L-asparaginase to form a partially deaminated L-asparaginase in which the α-NH_2 group of the N-terminal leucine and one of the ε-NH_2 groups of the lysine residues are removed. The modified and crystallized enzyme retains full activity both *in vitro* and *in vivo*. It is more acidic than the native enzyme, which may explain its longer half-life in human serum.

L-asparaginase (EC 3.5.1.1), an enzyme which catalyses the hydrolysis of L-asparagine to L-aspartic acid and ammonia, is widely distributed in nature /1/. In 1961 BROOME /2/ demonstrated tumor inhibitory properties of guinea pig serum L-asparaginase and thereafter a number of other L-asparaginases /1/, particularly those of bacterial origin /3/, were found to be potent antineoplastic agents both *in vivo* and *in vitro*, and in some cases of human leukemia complete remissions were observed /4/. Meanwhile preparative methods have been worked out /5,6, 7,8/ and the L-asparaginases of some strains of *E.coli* are now available in larger quantities for extensive clinical and che-

mical investigations.

As was demonstrated /7,8/ the asparaginases from the different strains of E.coli are composed of several isoenzymes with different electrophoretic mobilities. Thus the group of isoenzymes from E.coli ATCC 11303 (asparaginase B) has an isoelectric point which is about 0.2 pH units lower than that from ATCC 9637 (asparaginase A). In vivo studies in humans surprisingly showed that asparaginase B has a longer half-life than asparaginase A. We therefore tried by selective and limited chemical modifications to render asparaginases A and B more acidic. This was accomplished by partial deamination and, as will be reported later, by partial acylation and other reactions.

Experimental

Asparaginases A and B have been prepared as described /8/. The enzymes were deaminated by mixing at $+4^{\circ}C$ a 1o % solution in 0.2 M Na acetate with solid $NaNO_2$ to a final concentration of 0.2 M and adjusting the pH to 4.8 by dropwise addition of 1o % CH_3COOH. The solution remained at constant pH and temperature for 24 hr. The protein was then precipitated by addition of one volume of precooled ethanol, dioxane or 2-methylpentandiol-(2.4). The precipitate was collected by centrifugation, washed twice with $-1o^{\circ}C$ cold acetone and vacuum-dried at room temperature /9/. This material could be crystallized in the same way as described for asparaginases A and B /8/. For analytical purposes the preparations were carefully dialyzed and lyophilized. Other technical details were as described /8/ or given in the text.

Results and Discussion

Fig. 1 shows the electrophoretic behaviour of the deami-

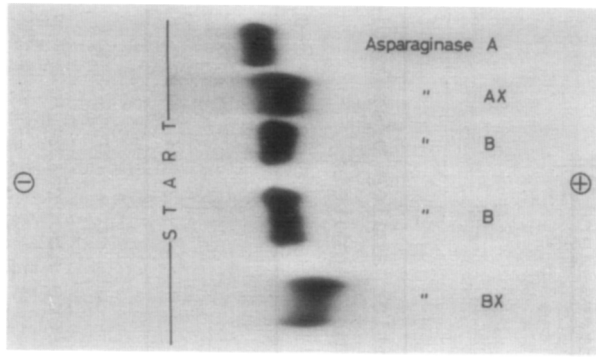

Fig. 1: Cellulose acetate electrophoresis of unmodified and partially deaminated asparaginases (2 µl of 2 % solutions) at pH 8.6. Veronal buffer I = 0.05, 140 V, 3 hrs. Staining with amido black 10 B.

nated enzymes (asparaginase AX and asparaginase BX, resp.) compared with that of the unmodified enzymes (asparaginases A and B, resp.). Clearly there is an increase in the net negative charge of the deaminated products; asparaginase BX migrates further towards the anode than asparaginase B and asparaginase AX further than asparaginase A, whereas asparaginase AX has about the same net charge as asparaginase B.

This increase of the total net charge is explained by the loss of NH_2-groups. As shown in Table 1, asparaginase AX has a lower ninhydrin value and also a deficit of 2.1 moles of Van Slyke nitrogen as compared with asparaginase A. Since there are 13 lysine residues and one α-NH_2 group per mole of asparaginase,

_____ Asparaginase A

⊕-⊕-⊕-⊕-⊕ Asparaginase A-deamination reaction mixture (without addition of nucleophilic acceptors; No. of nitroso-groups / 21.000 g protein = 2.7

△-△-△-△-△ Asparaginase AX (2 x crystallized); No. of nitroso-groups / Mol = 0.7

x-x-x-x-x Asparaginase AX (2 x crystallized); No. of nitroso-groups / Mol = <0.05

Table 1: Van Slyke amino nitrogen and "ninhydrin" determinations of asparaginases A and AX

Substance	Ninhydrin-values (µmoles leucine equiv. per µmole protein)	Van Slyke deamination (µmoles nitrogen per µmole protein)		Deamination analog preparation (µmoles nitrogen per µmole protein)	
		o°C	loo°C, 2 min.	o°C	loo°C, 2 min.
Asparaginase A	12.64	1.44	2o.2	o	4,78
Asparaginase AX	11.42	o.57	18.1	o	o.65

For protein determinations the biuret method was used /8/; ninhydrin estimations according to /lo/. The molecular weight for the subunits of asparaginase is assumed to be 2looo /7/.

one should expect 14 moles of amino nitrogen if complete deamination occurs. Deamination at $o^{o}C$, however, liberates only 1.44 moles whereas more vigorous conditions yield 2o.2 moles. This larger amount is nevertheless not unusual considering the conditions for Van Slyke deamination and arises probably from secondary reactions /11,12/. Analytical deamination under the conditions used for preparation obviously releases the N_2 too slowly for accurate volumetric measurements. A short heating (2 min. at $loo^{o}C$), however, liberates significantly more N_2 from the native enzyme than from the already partially deaminated protein. Considering the difference of the ninhydrin values (1.22 moles of leucine equivalents), the N_2 released from asparaginase A during Van Slyke deamination at $o^{o}C$ and the difference of the total amino nitrogen content, we assume that about two amino groups are absent in the partially deaminated asparaginase.

This suggestion is supported by the results of the amino acid analysis. As is apparent from Table 2, asparaginase AX has approximately one mole of leucine and one mole of lysine less than the native enzyme. Little or no variation occurred in the content of the other amino acids. Since the analyses were done to compare the two proteins, the hydrolysis time was kept constant (22 hr) and the data were not corrected for loss of labile amino acids or for incomplete hydrolysis.

Further characterisation of asparaginase AX was achieved by quantitative N-terminal amino acid analyses according to the EDMAN procedure as outlined by ARENS et al. /8/. As found by these authors the only N-terminal amino acid of asparaginase is leucine. The results in Table 3 clearly demonstrate that the α-NH_2 group of leucine is almost completely (97 %) removed

Table 2: Comparison of the amino acid analysis of the asparaginases A and AX after acid hydrolysis

	Moles amino acid per 21.000 g of protein	
Amino acid	Asparaginase AX	Asparaginase A
Lysine	11.95	12.92
Histidine	1.77	1.79
Ammonia	2o.15	19.33
Arginine	5.o6	5.o4
Aspartic Acid	31.41	31.69
Threonine	18.91	18.87
Serine	8.71	9.oo
Glutamic Acid	11.7o	11.94
Proline	8.17	8.o8
Glycine	16.99	17.14
Alanine	2o.o2	2o.oo
Half Cystine[a)]	1.22	b)
Valine	17.82	18.13
Methionine	3.34	3.35
Isoleucine	6.76	6.78
Leucine	12.91	13.85
Tyrosine	6.99	6.9o
Phenylalanine	4.95	5.o2
Tryptophane	b)	b)

a) determined as cysteic acid after performic acid oxidation

b) not determined

during the deamination procedure. Though the mechanism of the deamination reaction is rather complex /13/, it is most likely that the N-terminal leucine is converted to its hydroxy derivative, the α-hydroxyisocapronic acid. Whether the ε-NH_2 group of a particular lysine is deaminated or whether random deamination occurs remains an open question. One of the current difficulties is that both asparaginase A and asparaginase B are composed of a series of isoenzymes whose isoelectric points

differ only slightly and hence individual separation has not yet been achieved.

Partial deamination of ribonuclease has permitted the isolation of two still active homogenous products, 1-α, x-ε, y-ε, trihydroxy and 1-α, x-ε dihydroxy derivatives /11/. In trypsin the N-terminal isoleucine is deaminated with loss of activity /13,15/, while the N-terminal amino acid of chymotrypsin is not available for deamination /5/ and that of elastase only below pH 4 with gradual loss of activity /14,15/. The α-NH_2 group of asparaginase can be rather selectively removed under relatively mild conditions without a significant change of the enzymatic activity. However, under more vigorous conditions (higher temperature, lower pH) more lysine residues are affected and this leads to a gradual inactivation, possibly by alteration of the tertiary structure.

Nitrous acid shows both nucleophilic (diazotation of amines) and electrophilic (nitrosation of phenoles) properties.

Table 3: Quantitative determination of the N-terminal leucine of asparaginases A and AX.

Substance	Protein (mg)	PTH-Leu[+] (μmoles)	Ratio (μmoles Leu/μmole protein)	%
Asparaginase A	18.64	0.434	0.490	100
Asparaginase A	13.77	0.350	0.535	
Asparaginase AX	16.88	0.0135	0.017	3.3

[+] Phenylthiohydantoin derivative of leucine

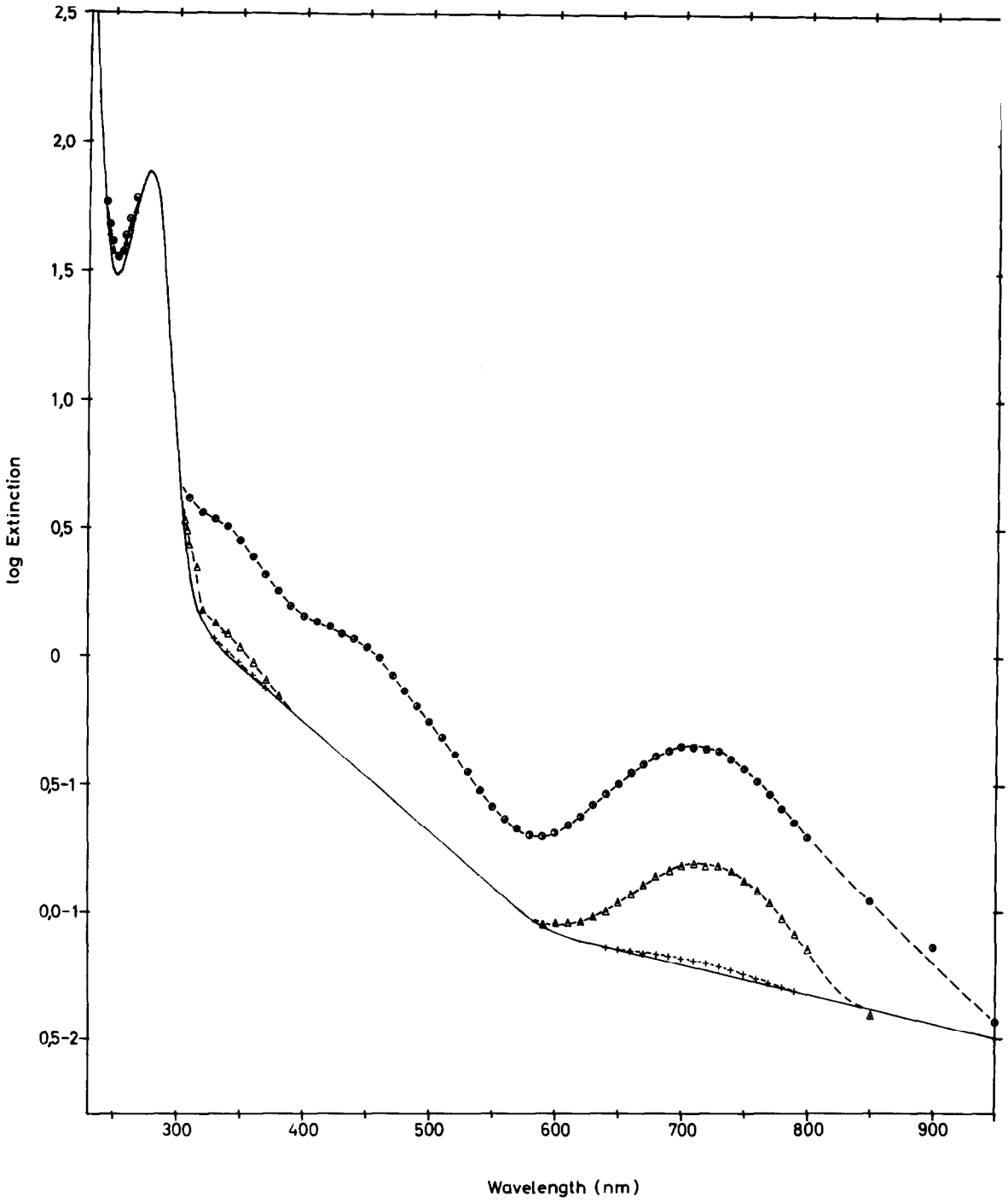

Fig. 2: UV-spectra of asparaginase A in comparison with deaminated asparaginases. 1oo mg of protein per ml; M/15 phosphate buffer, pH 7; light-path 1 cm; to calculate the number of nitroso-groups per 21.ooo g of protein an average molar extinction coefficient of 3o at 71o nm was assumed /16/.

With proteins the predominant reaction is the deamination. However nitrosation and nitration mainly on the aromatic rings of the amino acids tyrosine and tryptophane could also occur. We therefore checked and compared the UV-spectra of the deaminated and native enzymes. Fig. 2 shows that asparaginase AX has a typical peak at around 71o nm, indicating the presence of nitroso groups. Increased absorption is observed in the 28o-5oo nm range. Since the tyrosine contents of asparaginase A and AX are however not significantly different, the extent of the nitrosation reaction must be rather limited. Nevertheless for toxicological reasons we had to avoid any electrophilic reactions and this was finally accomplished by adding to the deamination mixture, phenol, salicylic acid or other water soluble compounds to act as nucleophilic acceptors /9/. Thus the nucleophilic deamination of the protein still occurs, while the electrophilic substitution is reduced to less than o.o5 nitroso-groups per mole (Fig. 2). During the protein precipitation these nucleophilic acceptors remain in solution and, as shown by gas chromatography, are not found to contaminate asparaginase AX.

In vivo experiments indicated that asparaginase AX is as active as the native enzymes /17/ and studies in humans showed that the elimination rate from the serum is markedly diminished /18/.

Acknowledgements

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