STUDIES ON CONFORMATION AND ANTIGENICITY OF REDUCED *S*-METHYLATED ASPARAGINASE IN COMPARISON WITH ASPARAGINASE

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1. Introduction

The discovery that L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) acts as an antitumor agent in children with lymphoblastic leukemia led to the extensive studies on the physicochemical properties of the enzyme as well as on clinical effect. In our laboratory, states of amino acid residues in the asparaginase molecule were classified by applying chemical modification techniques and spectrophotometric method and the amino acid residues participating in the enzymic activity were assumed to be amino groups, tyrosine and histidine residues in the molecule [1–3]. The antigenic reactivity of asparaginases modified by chemicals or by photooxidation towards anti-asparaginase serum was measured and the results were discussed in relation to amino acid residues and to the higher order structure of the protein molecule in order to clarify the antigenic determining site [4,5]. Asparaginase obtained from *Escherichia coli* has the mol. wt. of 136 000 and consists of four identical subunits [6,7], and its amino acid sequence was determined [8], in which only one disulfide bonding between the residue 77 and the residue 105 exists in the subunit molecule.

In the present study, it was found the physicochemical, immunochemical and enzymic characteristics of SM-asparaginase, obtained by substitution of reduced half cysteine residues with methyl-*p*-nitrobenzenesulfonate, are almost the same as those of native asparaginase.

Abbreviation: SM-asparaginase: *S*-methylated asparaginase obtained by reduction of the disulfide bonds followed by methylation.

2. Materials and methods

A crystalline asparaginase obtained from *E. coli* was donated from Kyowa Hakko Kogyo Co. and its specific activity was 220 I.U./mg of protein. The molar extinction coefficient of asparaginase was 8.53×10^4 M^{-1} cm^{-1} at 278 nm. Methyl-*p*-nitrobenzenesulfonate was purchased from Eastman Kodak Co. SM-asparaginase was prepared by the method of Heinrikson [9] as follows; 50 μl of 10% 2-mercaptoethanol was added to 4 ml of asparaginase solution (50 mg) in 8 M urea buffered with 0.5 M Tris buffer (pH 8.6) containing 0.1% EDTA. After keeping the mixed solution standing for 2 h at room temperature, 150 μl of methyl-*p*-nitrobenzenesulfonate (43.6 mg) in dioxane was added to the mixed solution. After incubation for 1 h at 40°C, the reaction mixture was charged to a column of Sephadex G-15 and eluted with 8 M urea. The protein thus eluted was dialyzed completely against distilled water. Amino acid composition was determined with a JEOL amino acid analyzer model JLC-5AH. The enzymic activity of asparaginase was estimated by Nessler reagent. Disc electrophoresis was performed with 7.5% acrylamide gel. Thin-layer chromatography was carried out with Sephadex G-200(superfine). The α-helix content was determined from Moffitt-Yang equation with a Jasco optical rotatory dispersion recorder model ORD/UV-5 Modification of amino groups in native and SM-asparaginases by fluorescamine was carried out by the

method of Tamaura et al. [10] Anti-asparaginase serum was obtained from white rabbits by the method described previously [4]. Anti-asparaginase immunoglobulin G was separated from antiserum by precipitants as antigen–antibody complex. The complex was dissociated in 3 M sodium thiocyanate [11] and passed through Sephadex G-200 column equilibrated with phosphate buffer saline containing 3 M sodium thiocyanate, and anti-asparaginase immunoglobulin G was obtained in a pure form.

3. Results and discussion

The amino acid composition of asparaginase and SM-asparaginase are shown in table 1, in which each value represents the number of each amino acid residue per one subunit in the molecule. Judging from the table, the all of cystine residues in the molecule was completely converted to *S*-methyl cysteine and no methylation of amino acid residues except cystine residues takes place.

Table 1
Amino acid composition of asparaginase and SM-asparaginase

Amino acid	Asparaginase	SM-Asparaginase
Lysine	21.6	21.7
Histidine	3.0	2.8
Arginine	8.1	7.6
Aspartic acid	52.8	53.3
Threonine	34.1	34.9
Serine	14.2	14.6
Glutamic acid	21.4	21.1
proline	14.2	15.2
S-Methylcysteine[a]	0.0	1.9
Glycine	27.7	27.5
Alanine	33.0	33.0
1/2 Cystine	2.0	0.0
Valine	27.8	28.3
Methionine	6.0	5.7
Isoleucine	12.3	12.6
Leucine	22.9	19.8
Tyrosine	12.5	11.9
Phenylalanine	8.2	7.7

The samples were hydrolyzed with 6 N HCl for 22 h at 110°C. Amino acid composition was expressed as the number of amino acid residues in one subunit (mol. wt. 34 000) of the asparaginase molecule.

[a] The value of *S*-methylcysteine was calculated by the method of Heinrikson [9].

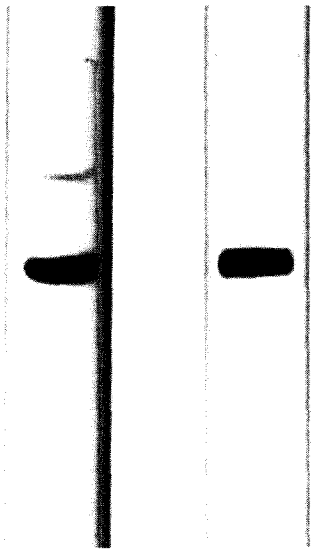

Fig.1. Disc gel electrophoretic patterns of SM-asparaginase (right side) and asparaginase (left). Electrophoresis was carried out with 7.5% polyacrylamide gel in Tris–glycine buffer (pH 8.3), using 30 μg protein. The small band present at the upper portion of the band of asparaginase is a polymerized form of asparaginase [16]. No polymerized form was observed for SM-asparaginase.

Disc electrophoretic pattern of SM-asparaginase and asparaginase is shown in fig.1, in which a single sharp band obtained for SM-asparaginase agrees in mobility with that for asparaginase. From the thin-layer chromatographic patterns of SM-asparaginase and asparaginase, their mol. wts were estimated to be 136 000. Optical rotatory dispersion analysis of SM-asparaginase gives the b_0 value of −166, which also agrees with the value of −163 obtained for asparaginase. The α-helix contents of asparaginase and SM-asparaginase were calculated to be 26.1% and 26.5%, respectively, SM-asparaginase has the enzymic activity of 187 I.U./mg of protein, which is slightly lower than 220 I.U./mg of protein for asparaginase. These results are summarized in table 2.

The quantitative precipitin reaction curves obtained for asparaginase (curve A) and SM-asparaginase (curve B) are shown in fig.2. The pattern of the reaction curve of SM-asparaginase is in good agreement in shape and in equivalent point with that of asparaginase, indicating that methylation of reduced half cystine

Table 2
Characteristics of SM-asparaginase and asparaginase

	Asparaginase	SM-asparaginase
Number of cystine residues in the molecule	4	0
Number of *S*-methylcysteine residues in the molecule	0	8
Number of subunits in the molecule	4	4
Optical rotatory dispersion parameter, b_0. (α-helix content)	−163 (26.1%)	−166 (26.5%)
Disc electrophoresis mobility[a]	0.37	0.37
Enzymic activity (%)	220 I.U./mg (100%)	187 I.U./mg (85%)
Number of amino groups reactive with fluorescamine in the molecule, *n*.	20	30
Relative amount of antigen-antibody complex at the equivalent point of precipitin reaction.	100%	97%

[a] Electrophoresis was carried out with 0.22 M Tris-glycine buffer (pH 8.3), by using bromophenol blue as a marker.

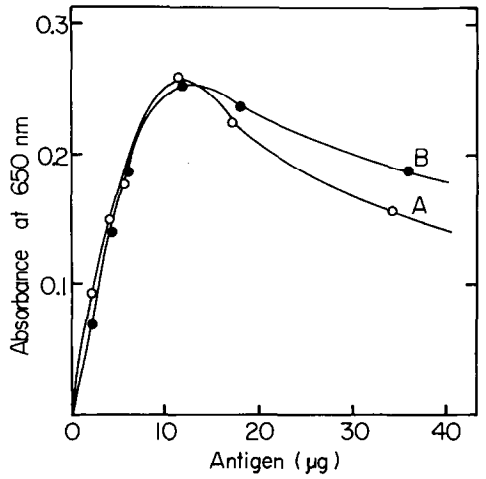

Fig.2. Quantitative precipitin curve obtained for plotting the amount of precipitants of the complex of SM-asparaginase (curve B) or asparaginase (curve A) with anti-asparaginase immunoglobulin G against antigen concentration. The amount of precipitin was determined by Lowry method [17].

residues in the asparaginase molecule does not cause any loss of the antigenic reactivity towards anti-asparaginase immunoglobulin G. Attassi and his co-workers [12] demonstrated that *S*-methylated and *S*-carboxymethylated lysozymes show almost complete loss of the enzymic activity and the antigenic reactivity towards anti-lysozyme serum.

States of amino groups in the SM-asparaginase molecule were discriminated by fluorescamine, which is explored by Tamaura et al. [10] Fig.3 shows the plotting of the number, *n*, of amino groups reacted with fluorescamine in the asparaginase molecule (curve A) or in the SM-asparaginase molecule (curve B) against fluorescamine concentration. Each curve rises gradually and tends to approach a constant level, $n = 20$ for asparaginase and $n = 30$ for SM-asparaginase. The reactivity of amino groups in SM-asparaginase with the reagent become higher than that of amino groups in asparaginase, suggesting that the higher order structure of asparaginase was slightly

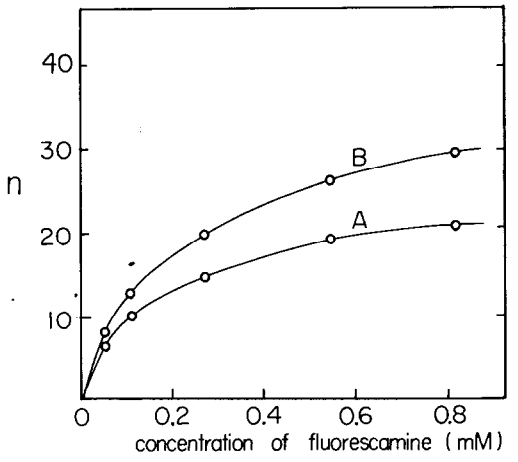

Fig.3. Reactivity of amino groups in asparaginase (curve A) and SM-asparaginase (curve B) with fluorescamine. Plotting of the number, n, of amino groups reacted with fluorescamine in the protein molecule against fluorescamine concentration. The total number of amino groups in the asparaginase molecule was 92 ([8]). The reaction was carried out at pH 9.0 and room temperature.

changed by methylation and some of amino groups become accessible with the reagent. This slight change may induce directly or indirectly the minor loss of the enzymic activity of SM-asparaginase compared with that of asparaginase.

When all of disulfide bonds in the enzyme molecule is reduced and modified with chemicals such as carboxymethylating and methylating agents, there occur generally a complete loss of the enzymic activity and the antigenic reactivity due to the profound conformational change of the enzyme molecule [13–15]. This indicates that disulfide bonds in protein play a crucial role in maintaining a protein conformation. In the present study, however, SM-asparaginase, in which all four disulfide bonds in the molecule were reduced and methylated, has the same physicochemical characteristics in mol. wt, acid-base properties and α-helix content, and the same immunochemical characteristics in the antigenic reactivity towards anti-asparaginase immunoglobulin G as those of asparaginase.

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References

[1] Nishimura, Y., Makino, H., Takenaka, O. and Inada, Y. (1971) Biochim. Biophys. Acta 227, 171–179.
[2] Makino, H., Takenaka, O. and Inada, Y. (1972) Biochim. Biophys. Acta 263, 477–481.
[3] Makino, H. and Inada, Y. (1973) Biochim. Biophys. Acta 295, 543–548.
[4] Makino, H., Satoh, H., Kuroiwa, Y., Yamazaki, S., Tamaura, Y. and Inada, Y. (1975) Immunochemistry 12, 183–185.
[5] Tamaura, Y., Satoh, H., Makino, H., Todokoro, K., Ikebe, M., Yamazaki, S., Kuroiwa, Y. and Inada, Y. (1975) Immunochemistry, in the press.
[6] Frank, B. H., Pekar, A. H., Veros, A. J. and Ho, P. P. K. (1970) J. Biol. Chem. 245, 3716–3724.
[7] Greenquist, A. C. and Wriston, Jr. J. C. (1972) Arch. Biochem. Biophys. 152, 280–286.
[8] Maita, T., Morokuma, K. and Matsuda, G. (1974) J. Biochem. 76, 1351–1354.
[9] Heinrikson, R. L. (1971) J. Biol. Chem. 246, 4090–4096.
[10] Tamaura, Y., Todokoro, K., Ikebe, M., Makino, H., Hirose, S. and Inada, Y. (1975) FEBS Lett. 50, 70–73.
[11] de Saussure, V. A. and Dandliker, W. B. (1969) Immunochemistry 6, 77–83.
[12] Lee, C. L. and Atassi, M. Z. (1973) Biochemistry 12, 2690–2695.
[13] Neumann, H., Goldberger, R. F. and Sela, M. (1964) J. Biol. Chem. 239, 1536–1540.
[14] Kress, L. J., Wilson, K. A. and Laskowski, Sr. M. (1968) J. Biol. Chem. 243, 1758–1762.
[15] Lie, W. K., Meienhofer, J. (1968) Biochem. Biophys. Res. Comm. 31, 467–473.
[16] Shifrin, S. and Solis, B. G. (1972) J. Biol. Chem. 247, 4121–4125.
[17] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.