MOLECULAR PHARMACOLOGY, 8, 59–64

Modification of L-Asparaginase by Subunit Cross-linking with Dimethylsuberimidate

R. E. HANDSCHUMACHER[1] AND C. GAUMOND

Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut 06510

(Received October 12, 1971)

SUMMARY

Cross-linkage of L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1), a tetrameric enzyme from *Escherichia coli*, has been accomplished with dimethylsuberimidate. Approximately 60% of the enzyme is converted to dimers and higher oligomers. Associated with cross-linkage is a loss of enzymatic activity to about 17% of that found in the native enzyme. Using [1,8-^{14}C]dimethylsuberimidate, 5 molecules of the suberimidate were shown to bind per monomer, a value corresponding to 25% of the lysine residues. The relative catalytic activity of the modified enzyme toward the alternative substrates L-glutamine, β-cyano-L-alanine, and 5-diazo-4-oxo-L-norvaline was essentially the same as that for the native enzyme.

INTRODUCTION

The therapeutic activity of L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) against lymphoblastic leukemia in humans (1, 2) may be limited by its distribution after intravenous injection. The enzyme (mol wt 133,000) has been shown to be composed of four identical subunits (3, 4), with one catalytic site on each monomer (3). Since the degree of dissociation of the enzyme into monomers may control the passage of activity from the plasma through capillaries into the interstitial space, molecular modifications of the enzyme appear to be a profitable approach to the improvement of therapy. As part of this study, it was considered important to prepare enzyme that could not dissociate. The reagent dimethylsuberimidate has been shown by Davies and Stark (5) to cross-link subunits of oligomeric enzymes by reaction with lysine ε-amino groups. The present study examines the reaction of L-asparaginase with dimethylsuberimidate, quantifies the degree of cross-linkage with this reagent, and compares the relative enzymatic activity toward alternative substrates with that of the native enzyme.

This work was supported by Grant T112 from the American Cancer Society and Grant CA10748 from the National Cancer Institute.

[1] Career Professor of the American Cancer Society.

MATERIALS AND METHODS

L-Asparaginase from *Escherichia coli* (Lyovac; Merck Sharp & Dohme, lot C-7941) was dialyzed against distilled water to remove mannitol added as a preservative. After lyophilization the material had a specific activity of 300–310 units/mg of protein and was stored at −10°. Acrylamide electrophoresis and ultracentrifugal analysis has indicated at least 95% purity on a protein basis in this preparation. Dimethylsuberimidate was prepared as described by Davies and Stark (5) from suberonitrile

Copyright © 1972 by Academic Press, Inc.

(Aldrich Chemical Company). [1,8-^{14}C]-Dimethylsuberimidate was kindly prepared by Dr. P. K. Chang from 1,6-diiodohexane by condensation with [^{14}C]KCN (0.5 mCi/mmole) and subsequent conversion of the suberonitrile as described. Radioactivity was assayed in a Packard liquid scintillation spectrometer with a toluene–ethanol scintillation fluid containing 1% Cab-O-Sil. Slices of the acrylamide gels were homogenized in the scintillation fluid for counting. The activity of the enzyme was determined routinely by a spectrophotometric assay based on the decomposition of 5-diazo-4-oxo-L-norvaline (3).

After treatment with DMS,[2] the enzyme was concentrated for electrophoresis by ultrafiltration with No. 100 collodion bags, 25,000 mol wt exclusion (Schleicher & Schuell). Gel electrophoresis was performed on a Canalco model 6 apparatus with 5% gels containing 0.1% sodium dodecyl sulfate as described by Summers *et al.* (6), except that urea was omitted from the preparation. The current was maintained at 8 mamp/column for 3–4 hr with sodium phosphate buffer (0.1 M, pH 7.2) containing 0.1% sodium dodecyl sulfate as the running buffer. Samples of native and modified L-asparaginase were introduced into the apparatus after dilution with an equal volume of a solution containing sodium phosphate buffer (0.2 M, pH 7.2), 0.2% sodium dodecyl sulfate, 20% sucrose, and sufficient bromphenol blue to serve as a marker. The gels were fixed with 12.5% trichloroacetic acid and stained in Coomassie blue dye according to the procedure of Chrambach, Reisfeld, Wyckoff, and Zaccari (7). After destaining, gels were preserved in 5% aqueous glycerol and the band intensity was determined with a Gilford spectrophotometer scanning attachment at 550 nm. The relative activity of the modified enzyme toward β-cyano-L-alanine was determined by substituting this nitrile (1.5 mM) for L-asparagine in the coupled enzyme assay for L-asparaginase described by Cooney *et al.* (8). The rate of inactivation of the catalytic centers in the modified enzyme by DONV was determined by incubation of enzyme (0.4 unit) with DONV (0.8 mM) in 4 ml of Tris-HCl buffer (0.05 M, pH 8.0) at 37°. Residual activity in 0.2-ml portions of the reaction mixture was assayed by the coupled enzyme method (8), in which the L-asparagine concentration was increased to 0.33 mμ. Glutaminase activity was measured by the Nessler reaction according to the method of Meister (9).

[2] The abbreviations used are: DMS, dimethylsuberimidate; DON, 5-diazo-4-oxo-L-norvaline; SDS, sodium dodecyl sulfate.

RESULTS

Evaluation of the optimal parameters for cross-linking L-asparaginase with DMS established the following conditions. To a solution of the enzyme (1 mg/ml) in 0.2 M triethanolamine HCl (pH 8.5) at 4° was added solid DMS (2 mg/ml); the reaction was allowed to proceed for up to 6 hr. Control studies indicated that DMS was stable in the buffer for 6 hr at this temperature.

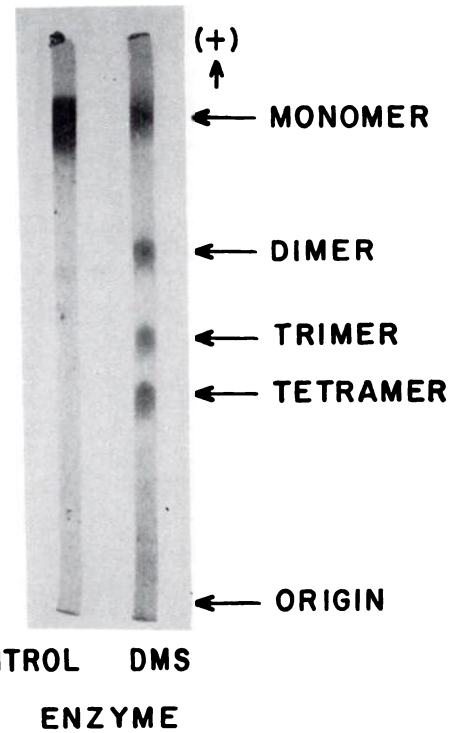

FIG. 1. *SDS–acrylamide gel electrophoresis of native and DMS-cross-linked L-asparaginase*

Left, native *E. coli* asparaginase; right, L-asparaginase after treatment with DMS for 6 hr.

Excess reagent was removed by dialysis against 1000 volumes of Tris-HCl (0.05 M, pH 8) for 18–20 hr with at least one buffer change. After concentration to 10–20 mg/ml by ultrafiltration, the modified enzyme was analyzed on a 5% acrylamide gel containing 0.1% SDS in the gel and running buffer (Fig. 1). The mobility of the various cross-linked species was directly proportional to the logarithm of multiples of the monomer (mol wt 33,000) (Fig. 2). This demonstration of four major species after cross-linkage affords further confirmation of the tetrameric nature of the enzyme. The relative amounts of cross-linked species larger than the tetramer decreased sharply. Increased amounts of higher forms were observed, however, when higher concentrations of enzyme (3 mg/ml) were present in the reaction mixture. The rate of inactivation of enzyme activity was twice as great with DMS at 2 mg/ml compared to 1 mg/ml under the same conditions, but the final degree of inactivation was the same. Addition of 10 mg of DMS per milliliter caused protein precipitation.

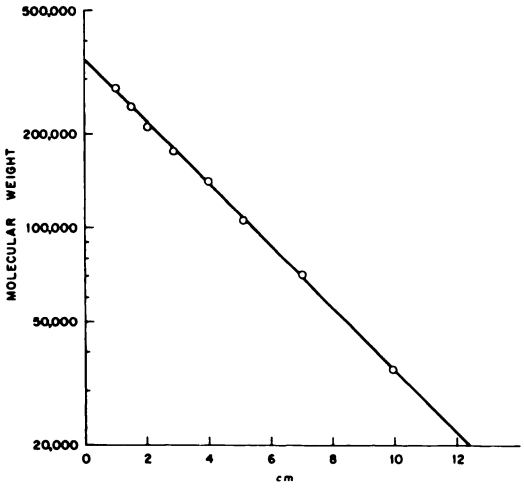

FIG. 2. *Mobility of individual oligomers on SDS–acrylamide gel electrophoresis after reaction of asparaginase with DMS*

The conditions of electrophoresis and determination of mobilities are described in the text. The molecular weight values were based on the mobility of the native monomer (mol wt 33,000).

To facilitate studies of the time course of DMS binding, the reaction was stopped by the addition of acid to protonate lysine ε-amino groups. Portions of the reaction mixture taken at different times were adjusted to pH 4.5 with glacial acetic acid (0.01 ml/ml of reaction mixture before dialysis at pH 6.0) to remove excess reagent. With [1,8-^{14}C] DMS, the rate of covalent attachment to L-asparaginase as well as the degree of cross-linkage and inactivation could be measured.

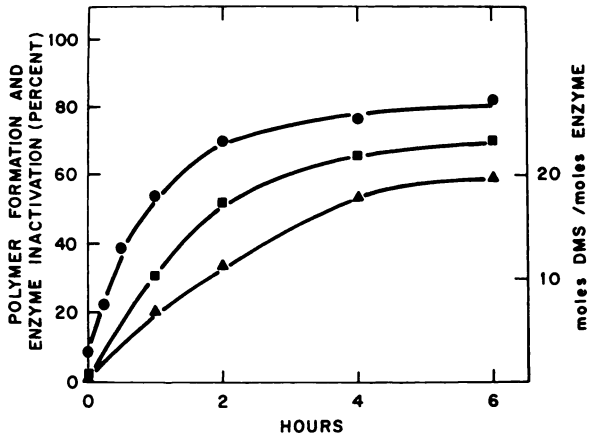

FIG. 3. *Time course of DMS–L-asparaginase interaction*

Conditions for the enzyme assay and radioactivity determination are described in the text. The degrees of cross-linkage are expressed as percentage of dimers or higher forms as determined by gel scanning. The reaction was terminated at each time point as indicated in the text by adjustment to pH 4.5. ●, asparagine hydrolysis; ■, percentage of cross-linkage; ▲, moles of DMS bound per mole of enzyme (mol wt 133,000).

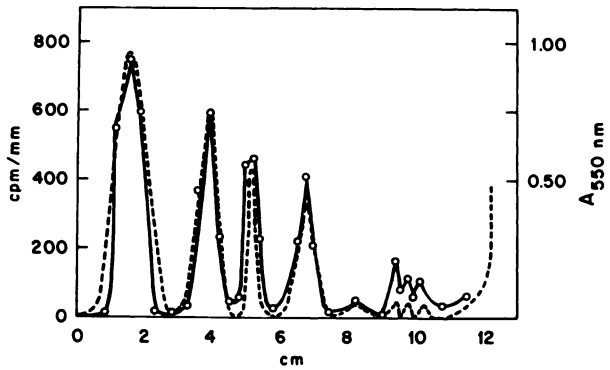

FIG. 4. *Correlation between protein content and radioactivity in* [^{14}C] *DMS-cross-linked* L-*asparaginase after SDS–gel electrophoresis*

Enzyme was treated with [^{14}C] DMS under the same conditions as described in Fig. 1 and was analyzed as described in the text. - - -, densitometer reading (protein); O, radioactivity.

Figure 3 indicates that the rate of polymer formation decreased throughout the time course and that inactivation of the enzyme paralleled polymer formation. The rate of binding of radioactive DMS to the enzyme, however, proceeded at a more constant rate during the first 2 hr but essentially stopped after 6 hr. Further additions of DMS after 6 hr did not change the cross-linkage or enzymatic activity.

Each subunit of asparaginase contains 21 lysine residues (10). A minimum of 5 and a maximum of 10 of these residues react with DMS under the experimental conditions, presumably because the other lysines are inaccessible within the tertiary structure. As indicated in Fig. 4, the total number of molecules of [^{14}C]DMS bound to the various oligomeric forms was the same as for the monomer. This might be predicted, since only a small percentage of the DMS residues in any one subunit may be involved in intermolecular linkages.

Since the residual enzymatic activity could be attributed to monomers that had not been cross-linked, the activity of the various species of oligomers was determined. Individual segments of the SDS–acrylamide gels were eluted by macerating the gel in buffer containing 6 M urea. The SDS was removed by passage through a column of Biorad AG1-X2, 100–200 mesh (formate form), as described by Weber and Kuter (11), and the effluent was dialyzed against 0.05 M Tris, pH 8, to remove urea. After concentration by ultrafiltration, the specific enzymatic activity of the eluted enzyme was assayed and the purity of the oligomeric species was determined by SDS–acrylamide gel electrophoresis. Table 1 indicates that all cross-linked species are active but that the specific activity of the monomeric bands is somewhat greater. The lower specific activity in all bands compared to the activity before SDS–acrylamide electrophoresis reflects inactivation during the separation procedures, as noted by Weber and Kuter (11). Since renaturation of the higher oligomeric species after exposure to SDS and urea may have been less complete than for the monomer, the activity recovered in these species must be considered the minimal value for their activity before electrophoresis.

TABLE 1

Enzymatic activity of cross-linked oligomeric species of L-*asparaginase*

Gel fraction	Composition	Specific activity
		units/mg
1	100% monomer	32
2	100% dimer	23
3	41% dimer, 59% trimer	15
4	18% trimer, 82% tetramer	13
5	69% tetramer, 31% higher oligomers	8

Table 2 indicates the relative activity with

TABLE 2

Relative enzymatic activity of native and cross-linked L-asparaginase

The cross-linked enzyme had been treated with DMS for 6 hr as shown in Fig. 3. Enzymatic activity was determined as described under MATERIALS AND METHODS.

Substrate	Substrate concentration	Control enzyme	DMS–cross-linked enzyme	Percentage of control
	mM	*μmoles/min/mg*	*μmoles/min/mg*	
L-Asparagine	20	300	52	17.2
L-Glutamine	25	11.4	2.4	21.2
β-Cyano-L-alanine	40	7.2	1.8	24.8
5-Diazo-4-oxo-L-norvaline	3	7.2	1.7	23.2

alternative substrates at concentrations approaching their maximal velocities. The reaction velocity with all substrates was reduced to the same degree. However, the rate of inactivation of the catalytic site by DONV was essentially unaffected (control, 0.40 unit inactivated per hours; cross-linked enzyme, 0.42 unit inactivated per hour).

DISCUSSION

The formation of covalent linkages between the subunits of L-asparaginase from *E. coli* and SDS–acrylamide gel electrophoresis has provided further confirmation of the tetrameric structure. During the course of this cross-linking reaction, considerable enzymatic activity is lost, but finite activity remains despite exhaustive treatment with DMS. The data in Fig. 4 and Table 2 for covalent modification by DMS suggest that lysine amino groups accessible to DMS are not essential to enzymatic activity but that the process of cross-linkage somehow alters the catalytic site, either through steric interference created by the DMS residues or by distortion of the subunit near the active site. The covalent reaction of DONV with the enzyme in the region of the active site, however, proceeded at the same rate. Thus, the amino acid residue with which DONV reacts to form a covalent linkage may not be directly involved in catalytic activity. Another modification of L-asparaginase in this laboratory by treatment with tetranitromethane also causes cross-linking in addition to the expected formation of nitrotyrosine residues (12). Although the catalytic activity of the nitro-enzyme is reduced, the relative activity toward the various substrates is greatly modified, presumably because of the participation of tyrosine residues in the catalytic center.

The quantitative data presented in this report emphasize that many DMS molecules do not react to cross-link subunits. Some of the lysine amino groups can react with DMS through only one end of the reagent, and the subsequent reaction of the other imido ester group with water to form an ester precludes its participation in cross-linking. Other molecules of DMS may react by forming intrasubunit bonds through 2 lysines residues in the same subunit. Since a minimum of 5 and a maximum of 10 of the 21 lysine residues in each subunit react with DMS, it would seem from Fig. 4 that conditions are optimal for cross-linking between subunits in the early stages of the reaction. Subsequent reaction of DMS with less accessible amino groups of lysine apparently does not create a spatial relationship favorable to intersubunit cross-linkage.

The covalently cross-linked enzyme prepared in this study provides a useful reference material for current studies directed at the preparation of monomeric forms of the enzyme. Comparative biological properties will be the subject of a future report.

ACKNOWLEDGMENTS

The authors are indebted to Dr. P. K. Chang for her assistance in the preparation of dimethylsuberimidate. The collaboration of Dr. Yung Pin Liu and Mr. J. Wagge in portions of this work is gratefully acknowledged. We are indebted to

Dr. L. Stryer for bringing to our attention the potential use of dimethylsuberimidate in this problem and providing the initial supply of this compound.

REFERENCES

1. E. Grundmann and H. F. Oettgen (eds.), "Experimental and Clinical Effects of L-Asparaginase." Springer Berlin 1970.
2. D. A. Cooney and R. E. Handschumacher, *Annu. Rev. Pharmacol.* **10**, 421 (1970).
3. R. C. Jackson and R. E. Handschumacher, *Biochemistry* **9**, 3585 (1970).
4. B. H. Frank, A. H. Pekar, A. J. Veros and P. P. K. Ho, *J. Biol. Chem.* **245**, 3716 (1970).
5. G. E. Davies and G. R. Stark, *Proc. Nat. Acad. Sci. U. S. A.* **66**, 651 (1970).
6. D. F. Summers, J. V. Maizel, Jr., and J. E. Darnell, Jr., *Proc. Nat. Acad. Sci. U. S. A.* **54**, 505 (1965).
7. A. Chrambach, R. A. Reisfeld, M. Wyckoff and J. Zaccari, *Anal. Biochem.* **20**, 150 (1967).
8. D. A. Cooney, R. L. Capizzi and R. E. Handschumacher, *Cancer Res.* **30**, 929 (1970).
9. A. Meister, *Methods Enzymol.* **2**, 380 (1955).
10. P. P. K. Ho, E. B. Milikin, J. L. Bobbitt, E. L. Grinnan, P. J. Burck, B. H. Frank, L. D. Boeck and R. W. Squires, *J. Biol. Chem.* **245**, 3708 (1970).
11. K. Weber and D. J. Kuter, *J. Biol. Chem.* **246**, 4504 (1971).
12. Y. P. Liu and R. E. Handschumacher, *J. Biol. Chem.* **247**, Jan. (1972).