Biochimica et Biophysica Acta, 445 (1976) 437—445

BBA 67899

THE EFFECT OF TRINITROPHENYLATION ON SUBUNIT INTERACTIONS IN L-ASPARAGINASE

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(Received January 19th, 1976)

Summary

L-Asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) from *Escherichia coli* B was modified by treatment with 2,4,6-trinitrobenzene-1-sulfonic acid at pH 7.5. The introduction of 13 trinitrophenyl groups into one mol of the tetrameric enzyme (TNP_{13}-asparaginase) results in a loss of 67% of the catalytic activity while the presence of 20 groups (TNP_{20}-asparaginase) reduces the enzymatic activity by 88%. The modified proteins are homogeneous as judged by disc gel electrophoresis and by the monodisperse boundary in the analytical ultracentrifuge having a sedimentation coefficient of 7.2 S. The rate of dissociation of the TNP_{13}-asparaginase is twice as fast and TNP_{20}-asparaginase three times as fast as that of unmodified asparaginase in 4 M urea. Trinitrophenylated subunits in 8 M urea can reassociate into the tetramer after removal of urea by dialysis or by dilution. Hybridization of unmodified and TNP subunits indicates that trinitrophenyl derivatives qualify as suitable variants for studying subunit interactions in oligomeric proteins.

Introduction

During the past several years we have been concerned with an examination of the nature of the forces through which the subunits of L-asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) are held together in the catalytically active tetramer. In an earlier study [1] we observed that succinylation of 35 out of the 88 lysyl residues of *E. coli* B L-asparaginase did not affect catalytic activity nor did it induce spontaneous dissociation. The present study is concerned with the effect of another reagent which reacts preferentially with lysyl residues; namely, 2,4,6-trinitrobenzenesulfonic acid.

2,4,6-Trinitrobenzenesulfonic acid was shown to be useful for labeling amino groups in amino acids and proteins without modifying the hydroxyl groups of serine, threonine, or tyrosine and without modifying guanidino or imidazole

groups [2,3]. The effect of this reagent on the catalytic and physicochemical properties of L-asparaginase is examined in this report.

Materials and Methods

L-Asparaginase from *Escherichia coli* B (Lot C-D210) was a product of Merck, Sharp and Dohme and was generously supplied to us through the courtesy of the Cancer Chemotherapy Unit of the National Cancer Institute. Stock solutions of the enzyme were generally prepared in 0.05 M phosphate buffer, pH 7.5, unless indicated otherwise. The concentration of the unmodified protein in solution was determined spectrophotometrically using the following extinction coefficients [4]: at 276 nm for the enzyme in 8 M urea, $E^{1\%}_{276\,nm}$ = 6.5, and at 278 nm for the enzyme in buffer, $E^{1\%}_{278\,nm}$ = 7.1. When only a few trinitrophenyl groups are introduced into the protein, the concentration can still be determined spectrophotometrically. However, as the trinitrophenyl content of the protein increases it makes a significant contribution to the absorbance at 278 nm so that the spectrophotometric determination is not accurate. In such cases the protein concentration was determined using the method of Lowry [5] using unmodified enzyme to construct the standard curve.

2,4,6-Trinitrobenzene-1-sulfonic acid ($N_3PhSO_3^-$) was puchased from Aldrich Chemical Co. Urea was ultrapure quality obtained from Schwartz/Mann. All other inorganic reagents were purchased from J.T. Baker Co.

Trinitrophenylation of L-asparaginase was carried out as follows: 15 mg of L-asparaginase contained in 1.0 ml of 0.05 M phosphate buffer, pH 7.5, was treated with 38 mg of $N_3PhSO_3^-$ in a dark container. 30 s after the reagents were mixed, 0.5 ml of the reaction mixture was removed and placed on a Sephadex G-25 column which effected separation of the modified protein from the reagents and its hydrolysis products. The remaining 0.5 ml of the reaction mixture was allowed to incubate an additional 10 min before it was also placed on the Sephadex column.

The enzyme-catalyzed hydrolysis of L-asparagine was followed continuously by monitoring the absorbance of the amide band at 220 nm as a modification of the method described by Howard and Carpenter [6]. The molar extinction coefficient of asparagine at 220 nm is approx. 74 while its hydrolysis product, aspartic acid, has an extinction coefficient of approx. 42.

The rate of dissociation of TNP-asparaginase was followed by the increase in absorbance of the 287 nm band of the ultraviolet difference spectrum as described previously [7] and the rate of reassociation of the denatured subunits was also followed at this wavelength according to the procedure described earlier [8].

Hybridization studies were carried out by mixing unmodified L-asparaginase with TNP-asparaginase in varying ratios in a dialysis bag. The mixture was dialyzed against 200 vols. of 8 M urea for 2 h. The dialysis bag was then transferred into 0.05 M phosphate buffer, pH 7.5, and the buffer was changed several times over a period of 16 h. The two components of the hybridization mixture were denatured and renatured separately as the controls.

Sedimentation velocity studies were carried out with the Spinco model E analytical ultracentrifuge equipped with ultraviolet absorption scanner optics as

described previously [9]. The observed sedimentation coefficients were corrected to standard conditions ($s_{20,w}$) using the appropriate densities and viscosities of urea solutions [10] and assuming a partial specific volume of 0.73 ml per g for L-asparaginase [4]. The same partial specific volume was assumed for TNP-asparaginase. Centrifugation was carried out at 60 000 rev./min at 20°C with the monochromator set at 346 nm for TNP-asparaginase and at 280 nm for unmodified L-asparaginase. The composition of the protein mixture was determined from the pen deflection for each component in the scanner tracing.

Results and Discussion

Trinitrophenylation of L-asparaginase

Borate versus phosphate buffer. 15 mg of L-asparaginase contained in 1.0 ml of 0.05 M borate buffer, pH 7.5, was added to 26 mg of $N_3PhSO_3^-$ contained in a test tube. The $N_3PhSO_3^-$ dissolved immediately producing a bright yellow solution. After standing at room temperature for 10 min the reaction mixture was placed on a Sephadex G-25 column. The same procedure was carried out with 15 mg of the enzyme contained in 1.0 ml of 0.05 M phosphate buffer, pH

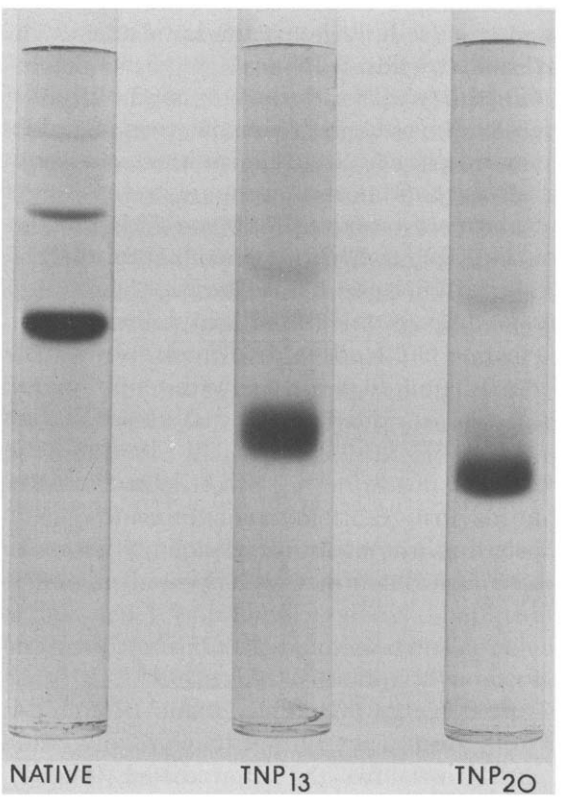

Fig. 1. Polyacrylamide disc gel electrophoresis of native (left gel), TNP_{13}-asparaginase (center gel) and TNP_{20}-asparaginase (right gel) using 7% polyacrylamide. 75 μg of each protein were placed on the gel column.

TABLE I

RELATIVE ENZYMATIC ACTIVITY OF NATIVE AND TRINITROPHENYLATED ASPARAGINASE

The rate of hydrolysis of 0.005 M L-asparaginase was followed spectrophotometrically at 220 nm as described in Materials and Methods.

Enzyme	Relative activity (%)
Native L-asparaginase	100
$TNP_{6,8}$-asparaginase	40
TNP_{13}-asparaginase	33
TNP_{20}-asparaginase	12

7.5. Upon addition of this enzyme solution to 26 mg of $N_3PhSO_3^-$, the latter did not completely dissolve and the heterogeneous reaction mixture turned orange. After a 10-min incubation of this mixture at room temperature, it was placed on the Sephadex G-25 column. The labeled proteins gave absorption spectra that are characterized by a maximum at 346 nm and a broad, less intense band with a maximum near 420 mm. These spectra are characteristic of TNP-amine derivatives [2,3]. The extent of labeling was determined from the absorbance in neutral solution at 346 nm using a molar extinction coefficient [11] of 14 500 or at 367 nm using a molar extinction coefficient [11] of 10 500. If we assume that ϵ-TNP-α-acetyllysine in peptide linkage has the same extinction coefficient as the labeled free amino acid, 6.8 trinitrophenyl groups are introduced into 133 000 daltons of L-asparaginase in borate buffer, while 20 TNP groups are introduced when phosphate buffer is used. This slower rate of trinitrophenylation in the presence of borate buffer is consistent with the observations reported by Burton and Josse [12] in their studies on inorganic pyrophosphatase. The remainder of the studies to be described in this report were carried out in phosphate buffer since the presence of the borate causes inactivation of L-asparaginase.

Thirteen trinitrophenyl groups are introduced into one mol of L-asparaginase tetramer within 30 s after mixing 26 mg of $N_3PhSO_3^-$ with 15 mg of the enzyme in phosphate buffer and this derivative will be designated TNP_{13}-asparaginase. The other derivative whose properties will be described is the one obtained after a 10-min incubation as reported above and will be designated TNP_{20}-asparaginase.

Sedimentation velocity studies of the three modified proteins were carried out at concentrations of 1 mg/ml or less and each showed a single monodisperse boundary having a sedimentation coefficient of 7.2 S which is characteristic of the tetrameric enzyme [4].

Polyacrylamide gel electrophoresis. If trinitrophenylation takes place on the ϵ-amino group of lysyl residues, the migration of L-asparaginase in an electric field should be altered since the positively charged amino group of lysine is replaced by the neutral trinitrophenyl group. The protein patterns that were obtained on polyacrylamide gels are shown for the unmodified (native), TNP_{13}-, and TNP_{20}-asparaginase in Fig. 1. The gel on the left shows the native enzyme containing 95% of the 7-S tetramer and 5% of the higher molecular weight octamer [13,14]. The gel in the center of the figure represents the pat-

tern of TNP_{13}-asparaginase in which both components travel farther toward the anode than did the unmodified enzyme. These bands are also slightly more diffuse than those of the native protein. The gel on the right of the figure represents TNP_{20}-asparaginase which again travels even farther toward the anode and appears to be slightly contaminated with the TNP_{13}-asparaginase.

Catalytic activity of TNP-asparaginase. The catalytic activity of several trinitrophenylated derivatives of L-asparaginase in 0.05 M phosphate buffer, pH 7.5, relative to that of the native enzyme is summarized in Table I. The results show that the introduction of 6.8 TNP groups per tetramer produces a 60% loss of catalytic function. TNP_{13}-Asparaginase has only 33% of the original enzymatic activity and the introduction of 20 TNP groups per mol of enzyme produces a loss of 88% of the initial activity. The ability to hydrolyze asparagine was not recovered after treating the derivatives with either 1 M KCN or 1 M hydroxylamine hydrochloride which would have removed the trinitrophenyl moiety from an imidazole group. (Cohen, L.A., personal communication).

On the basis of the electrophoretic and spectrophotometric behavior we have assumed that the modification takes place on the amino group of a lysyl residue. However, earlier studies [1] indicated that 40% (35) of the lysyl residues could be succinylated without altering the catalytic properties of the enzyme. Therefore the loss in activity that results from trinitrophenylation may not be a direct result of blocking lysyl residues, but the introduction of the hydrophobic trinitrophenyl group might produce conformational changes in the structure of the protein which secondarily affect the catalytic activity.

Dissociation of TNP-asparaginase

In order to determine the effect of trinitrophenylation on subunit-subunit interactions we examined the rates of dissociation of TNP_{13}- and TNP_{20}-asparaginase compared with the rate of dissociation of native L-asparaginase. We previously reported [1,7,8] that dissociation of tetrameric L-asparaginase into its monomers could be monitored by following the appearance of the 287 nm maximum of the ultraviolet absorbance difference spectrum. The spectral shifts that result when TNP_{20}-asparaginase is transfered from 0.05 M phosphate buffer, pH 7.5, to 8 M urea are shown in Fig. 2. The spectra in the upper portion of the figure demonstrate that the 346 nm maximum obtained in buffer (solid curve) is shifted to 348 nm in the presence of the denaturant (broken curve). This shift to longer wavelengths is also observed with the 420 nm band. These shifts in position of the maxima together with changes in the intensity of the bands are most easily visualized using ultraviolet difference spectroscopy as shown in the lower portion of Fig. 2. The arrangement of the cuvettes is such that the spectral shifts due to the trinitrophenylamine group appear as minima and the spectral shift assigned to the aromatic amino acid residues of the protein appear as maxima at 287 and 280 nm. The apparent minima are at 365 nm and 440 nm.

When TNP-aspraginase was added to 4 M urea, the minima of the difference spectrum at 365 nm and 440 nm appear immediately while the absorbance of the 287 nm band increases at a rate that corresponds to the rate of dissociation of the tetramer. This suggests that the environment of the trinitrophenyl moiety does not change during the course of dissociation while the Tyr & Trp residues

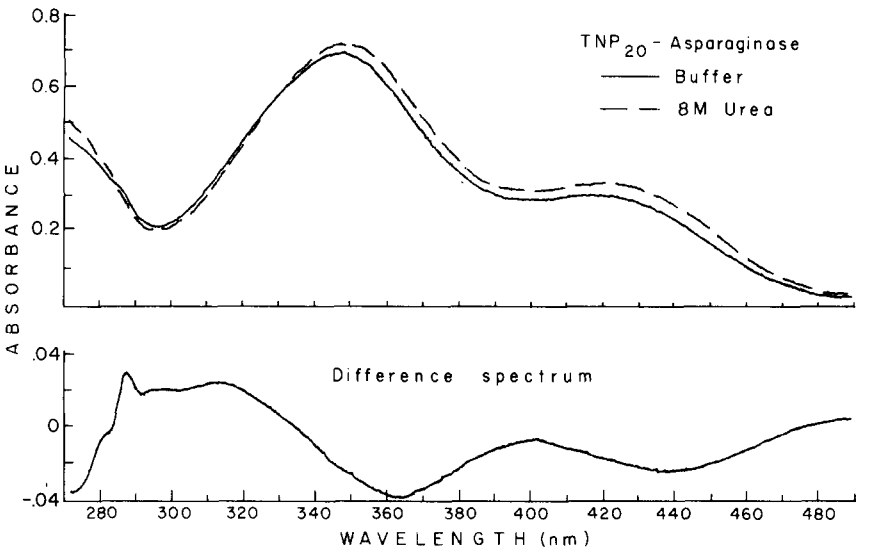

Fig. 2. Ultraviolet absorption spectrum of TNP_{20}-asparaginase. Upper portion: solid curve, $2.5 \cdot 10^{-6}$ M enzyme in 0.05 M phosphate buffer, pH 7.5; broken curve, $2.5 \cdot 10^{-6}$ M enzyme in 8 M urea. Lower portion: difference spectrum using tandem cuvettes in which the enzyme in buffer is placed in the sample beam and the enzyme in urea is used as the blank.

are transferred from their hydrophobic milieu in the native protein into a hydrophilic one in the dissociated monomer. The rates of dissociation of native, unmodified L-asparaginase, TNP_{13}- and TNP_{20}-asparaginase were followed at 287 nm as shown by the curves in Fig. 3. The results show that TNP_{13}-asparaginase dissociates more rapidly than the native enzyme and that TNP_{20}-asparaginase dissociates most rapidly. The relative rates of dissociation were taken from these curves at the four-minute interval and are summarized in Table II.

Reassembly of TNP-asparaginase subunits

Previous reports [8] indicated that unmodified L-asparaginase subunits in 8 M urea will reassemble into active tetramers after removal of the urea either by dialysis or by dilution into buffer or into dilute urea solution. The presence of the trinitrophenyl group interferes with the quantitative reassembly into active tetramers. When TNP_{13}-asparaginase subunits contained in 8 M urea were diluted into 0.8 M urea, there was no detectable amount of tetramer formed as examined by sedimentation velocity studies and shown in the upper portion of Fig.4.

However, when dilution was made into 0.05 M phosphate buffer, pH 7.5, 67% of the 346 nm-absorbing material sediments as the 7.2-S tetramer while the remaining amount of protein sediments as the 1.5-S monomer. There was no indication of any intermediate oligomeric species as had been reported with succinylasparaginase [1]. When TNP_{20}-asparaginase in 8 M urea was diluted into phosphate buffer, less than 20% of the protein appeared as the tetramer.

Hybridization studies

Meighen and Schachman [15] have summarized the criteria that should be

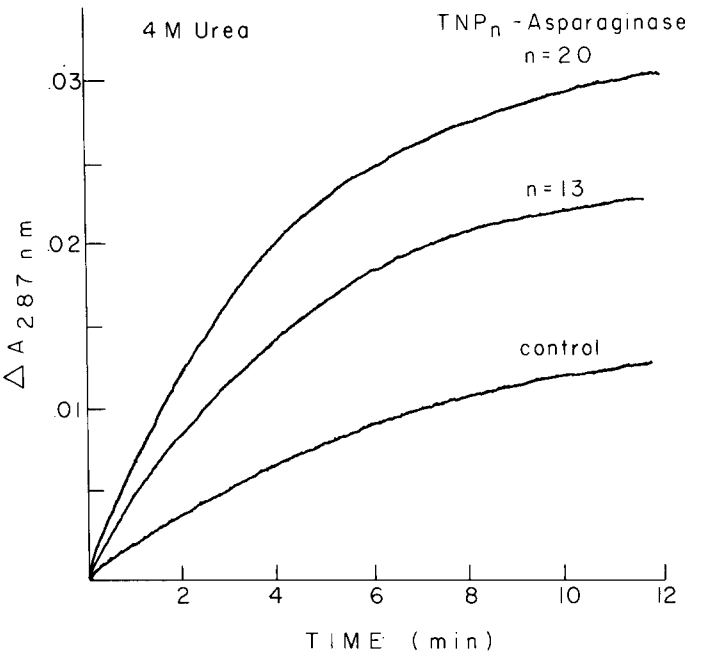

Fig. 3. Rates of dissociation of native L-asparaginase (control) TNP_{13}-asparaginase and TNP_{20}-asparaginase as measured by the increase in absorbance of the 287 nm band of the ultraviolet difference spectrum. The concentration of enzyme was $5.0 \cdot 10^{-6}$ M in each sample.

met if a variant is to be considered suitable for hybdridization studies. The four criteria are:

(1) "The modified protein should possess an electrophoretic mobility which is substantially different from the native enzyme." The polyacrylamide patterns shown in Fig. 1 indicate that both derivatives satisfy this requirement.

(2) "The chemical treatment must be relatively specific so as to cause uniform modification of all the enzyme molecules in the preparation and thereby produce a reasonably homogeneous derivative." The homogeneity of the trinitrophenyl derivatives as determined by polyacrylamide gel electrophoresis and by analytical ultracentrifugation indicates that TNP_{13}-asparaginase satisfies this second criterion.

(3) "The modified enzyme must not be much less (or much more) stable than the native enzyme." We have shown that TNP_{13}-asparaginase dissociates

TABLE II

RELATIVE RATES OF DISSOCIATION OF UNMODIFIED AND TNP-ASPARAGINASE IN 4 M UREA

(Data taken from Fig. 3)

Enzyme	Relative rate of dissociation
Native L-asparaginase	1.0
TNP_{13}-asparaginase	2.1
TNP_{20}-asparaginase	3.0

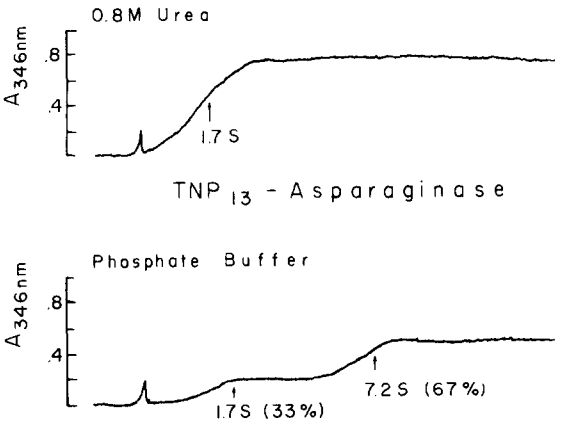

Fig. 4. Sedimentation velocity ultracentrifugation of TNP_{13}-asparaginase subunits contained in 8 M urea diluted into 0.8 M urea (top curve) and into 0.05 M phosphate buffer, pH 7.5 (bottom curve). The monochromator was set at 346 nm, the rotor speed was 60 000 rev./min at 20° C and the final concentration of protein was $5.0 \cdot 10^{-6}$ M. The tracings shown here were taken 56 min after the rotor had reached operating speed.

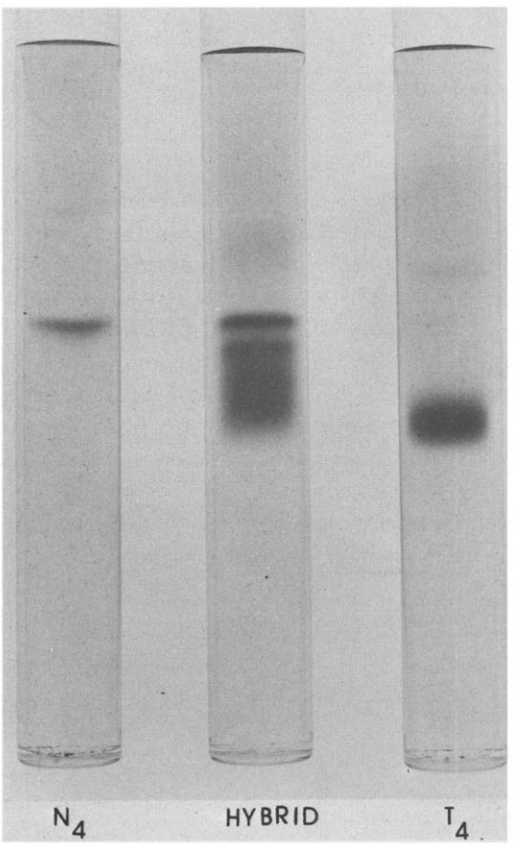

Fig. 5. Polyacrylamide gel electrophoresis of renatured, unmodified L-asparaginase (N_4), renatured TNP_{13}-asparaginase (T_4) and hybrids using a ratio of 1 : 2 (native: TNP_{13}-asparaginase). 75 µg of protein were placed on each gel.

only slightly more rapidly than the unmodified enzyme and therefore fulfills the third requirement.

(4) "The chemically altered enzyme, like the native enzyme, must be reconstitutable after dissociation into subunits." We have indicated that the subunits derived from TNP_{13}-asparaginase will reassemble into tetramers although only to 67% of quantitative recovery. TNP_{20}-asparaginase does not fulfill this requirement.

Native and TNP_{13}-asparaginase were mixed in ratios of 1 : 3, 1 : 2 and 1 : 1, respectively, and exposed to 8 M urea for 2 h followed by dialysis against 0.05 M phosphate buffer, pH 7.5, for 16 h. The products were examined by polyacrylamide gel electrophoresis and the pattern obtained using the 1 : 2 ratio of proteins are shown in the center of Fig. 5. The gel on the left represents the renatured, native (N) enzyme and the gel on the right shows the renatured trinitrophenylated (T) asparaginase. The gel in the center of the figure shows the series of protein bands representing hybrid structures consisting of unmodified and trinitrophenylated subunits. The distance between each band is uniform and the intensity of the band decreases as the content of the trinitrophenylated subunit increases. The uppermost band represents the unmodified tetramer (NNNN) followed by the hybrids (NNNT, NNTT, NTTT) and finally the weakly stained renatured derivative (TTTT).

Conclusions

We have demonstrated that 23% of the lysyl residues of L-asparaginase from *Escherichia coli* B can be modified with trinitrophenyl groups without causing dissociation and without inducing higher degrees of aggregation. The presence of the trinitrophenyl group enhances the rate of dissociation of the tetramer and interferes with the quantitative reassembly of the subunits into active tetramers. TNP_{13}-asparaginase satisfies all of the criteria outlined by Meighen and Schachman [15] for a derivative to be considered suitable as a variant in hybridization studies.

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