L-Asparaginase from Erwinia carotovora

PHYSICOCHEMICAL PROPERTIES OF THE NATIVE AND SUCCINYLATED ENZYME

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SIDNEY SHIVHIN, BARBARA G. SOLIS, AND IRIWN M. CHAIKEN

From the Laboratory of Cell Biology, National Cancer Institute, and Laboratory of Chemical Biology, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

The physicochemical properties of the antileukemic L-asparaginase from Erwinia carotovora have been studied and compared with those of the corresponding clinically effective enzyme from Escherichia coli B. Although both enzymes are tetramers with similar molecular weight, the amino acid compositions are distinctly different and the Erwinia enzyme is more basic as judged by polyacrylamide electrophoresis. Of the 48 to 50 tyrosyl residues in native Erwinia asparaginase, only about 10% ionize with a normal pK, while 30% of these residues in the E. coli enzyme titrate normally. The native tetramer (s20,w = 7.2) is considerably more stable than the corresponding tetramer from E. coli. Asparaginase from Erwinia is only partially dissociated in 8 M urea, whereas the E. coli enzyme is completely converted to the 1.7 S monomer under the same conditions. Guanidinium chloride at concentrations of 3.5 M completely dissociates Erwinia asparaginase. Dissociation is accompanied by the appearance of an ultraviolet difference spectrum with a maximum at 288 nm. The rate of dissociation is markedly increased by the addition of alcohols to the denaturant. Dissociation in the presence of alcohols occurs in two distinct steps whose rates increase as the length of the alkyl side chain of the alcohol increases.

Succinylation of the lysyl residues results in marked increases in mobility of the modified enzyme on polyacrylamide gels, but does not affect the state of aggregation of the tetramer or its catalytic activity. In addition succinylation does not affect either the rate of dissociation in guanidinium chloride or the extent of reconstitution to the active enzyme. The presence of five bands on polyacrylamide gel electrophoresis of hybrids prepared from the native and succinylated enzyme indicates that Erwinia carotovora asparaginase is composed of four identical subunits.

In 1961 Broome (1) demonstrated that L-asparaginase was responsible for the antilymphoma activity of whole guinea pig serum (2). A larger supply of the enzyme that would furnish sufficient material for clinical testing was provided by Escherichia coli B (3). Many bacterial asparaginases were subsequently screened to determine their effectiveness as antilymphoma agents (4). The enzyme isolated from Erwinia carotovora is pharmacologically active (5) and is as effective as the enzyme from E. coli B in the treatment of acute lymphocytic leukemia. In an effort to determine the molecular bases of clinical effectiveness, the present study was undertaken to compare the physicochemical properties of the asparaginase from Erwinia carotovora with those of the enzyme from Escherichia coli B (6-9).

MATERIALS AND METHODS

L-Asparaginase from Erwinia carotovora was generously supplied by the Drug Evaluation Branch of the National Cancer Institute. The lyophilized powder was dissolved in 0.05 M phosphate buffer, pH 7.5, to give a stock solution containing 15 mg per ml. This solution was dialyzed against several changes of the same buffer in order to remove salts. The enzyme was homogeneous as determined from the monodisperse boundary obtained in the analytical ultracentrifuge. The enzyme has a sedimentation coefficient (s20,w) of 7.2. The native enzyme showed three distinct bands on polyacrylamide gels which were isolated and all were found to be catalytically active.

Aspartic acid L-β-N-tosylamide was a generous gift from Dr. Osamu Takenaka of the Tokyo Institute of Technology. Urea and guanidinium chloride were recrystallized from aqueous ethanol using decolorizing charcoal to adsorb impurities. The crystalline reagents were stored in the cold in dark containers and solutions were prepared immediately before use. Dodecyl sulfate was a highly pure grade purchased from Mann. All organic solvents were spectroquality grade and were purchased from Matheson, Coleman and Bell. [14C]Succinimide was purchased from International Chemical and Nuclear Corporation and was diluted with unlabeled succinic anhydride (Eastman) to a specific activity of 1.8 X 10⁶ cpm per mg.

Erwinia asparaginase was succinylated with [14C]Succinimide at pH 8.0 according to the method described previously (7). The extent of reaction with lysyl residues was determined from titration of free amino groups with ninhydrin, according to the method of Moore and Stein (10), and with trinitrobenzene sulfonic acid (11).

Enzyme activity of asparaginase was determined with Nessler's
1 E. Henderson, personal communication.
reagent by measuring the ammonia released from asparagine (12). Catalytic activity was also determined from the increase in absorbance of aspartic acid \(\beta\)-p-nitroanilide at 410 nm after hydrolysis (13). The synthetic substrate was used at concentrations of 2 \(\text{mg/mL}\) or less and was prepared in pH 8.0 buffer. There was no detectable hydrolysis of the substrate in the absence of enzyme.

Disc electrophoresis was carried out using 7% polyacrylamide gels as described previously (6). Difference spectra were obtained with the Cary model 15 spectrophotometer using tandem double cuvettes such as those described by Herskovits and Laskowski (14). In order to record spectra as far as possible into the ultraviolet region while maintaining a reasonable degree of resolution, it was necessary to increase the voltage on the photomultiplier to its maximum level.

Circular dichroic spectra were recorded with the Aminco-Bowman spectrophotofluorometer which was modified according to the specifications described by Chen (15).

Sedimentation velocity studies were carried out using the Spinco model E analytical ultracentrifuge equipped with ultraviolet absorption scanner optics. When urea and GdnCl solutions were used, the observed sedimentation coefficients were corrected to the density and viscosity of water at 20\(^\circ\)C (82,94), assuming a partial specific volume of 0.73 \(\text{ml/gm}\) obtained with the enzyme from \(E.\ coli\) B (16). In the case of velocity studies carried out in dodecyl sulfate, the sedimentation coefficients that are reported are observed values since dodecyl sulfate has a tendency to aggregate, and reliable values for its density and viscosity are not available. Similarly partial specific volumes of the succinylated derivatives were not determined and sedimentation coefficients are reported as observed values.

The enzyme was dried to constant weight in a vacuum oven at 55\(^\circ\). The extinction coefficient was determined to be \(\varepsilon_{280}^{\text{nm}} = 6.4\). This value was used to determine protein concentrations throughout these studies.

Amino acid analyses were carried out with the Spinco model 120C automatic amino acid analyzer. Asparaginase was hydrolyzed in \(\text{NCl}\) with constant boiling HCl at 110\(^\circ\) in the presence of 10 \(\mu\text{l}\) of 5% phenol and 1 \(\mu\text{l}\) of mercaptoethanol for 24, 48, and 72 hours. Performic acid oxidation was carried out by a modification of the procedure described by Hirs (17).

The rate of dissolution of \(E.\ coli\) asparaginase was followed at 288 nm. A solution of the enzyme (from 1 to 5 \(\text{mg/mL}\) per ml) in 0.05 \(\text{M}\) phosphate buffer, pH 7.5, was placed in the sample compartment of the spectrophotometer while the same concentration of enzyme in the denaturing medium was placed in the solvent compartment. The increase in 288 nm absorbance was recorded within 15 s after mixing enzyme and denaturant and the change was monitored continuously for 10 min. In separate experiments the spectral change from 200 to 350 nm was scanned at 1-min intervals.

Reconstitution of the tetramer was effected by dialyzing the denatured subunits in GdnCl against gradually decreasing concentrations of the denaturant. For example, the enzyme in 6 \(\text{M}\) GdnCl was first dialyzed against 3 \(\text{M}\) GdnCl followed by dialysis against 1.5 \(\text{M}\) GdnCl, etc., until all of the denaturant was removed. Enzyme concentrations from 1 \(\text{mg/mL}\) to 10 \(\text{mg/mL}\) per ml were used.

Circular dichroic spectra were obtained with a Cary model 60 recording spectropolarimeter fitted with a model 6001 circular dichroism attachment and set for a half band width of 15 \(\text{A}\). Measurements were made at 27\(^\circ\). The concentration of enzyme was 0.01% and a path length of 1.0 mm was used.

Hybrids of the native and succinylated enzyme were prepared by first dialysing the proteins in 6 \(\text{M}\) GdnCl. Aliquots of each were withdrawn and were reconstituted. The remainder of the solutions in GdnCl were mixed in the following ratios of succinylated to native: 9:1, 7:3, 6:4, 5:5, and 2:8. These mixtures were dialyzed against buffer and the products were analyzed using polyacrylamide gel electrophoresis.

**RESULTS AND DISCUSSION**

**Chemical and Physical Properties**

**Amino Acid Composition**—The complete amino acid composition of \(E.\ coli\) asparaginase is summarized in Table I. The moles of each amino acid in a monomer were calculated on the basis of a molecular weight of 33,500 for the subunit (18). Two amino acids that were present in the \(E.\ coli\) enzyme in small amounts and that are completely absent in \(E.\ coli\) asparaginase

| Amino acid composition of \(E.\ coli\) asparaginase |
|-----------------------------------------------|
| Amino acid | Mole per 33,500 g<sup>a</sup> |
|------------|-----------------------------|
| Lysine     | 15.7                        |
| Histidine  | 6.1                         |
| Arginine   | 15.9                        |
| Aspartic acid<sup>b</sup> | 34.6                   |
| Threonine  | 23.9                        |
| Serine     | 19.1                        |
| Glutamic acid<sup>b</sup> | 21.2                   |
| Proline    | 11.1                        |
| Glycerine  | 31.3                        |
| Alanine    | 30.3                        |
| Half-cystine<sup>c</sup> | 0                     |
| Valine     | 27.1                        |
| Methionine<sup>d</sup> | 6.6                     |
| Isoleucine | 16.3                        |
| Leucine    | 20.2                        |
| Tyrosine   | 12.0                        |
| Phenylalanine | 6.7              |
| Tryptophan | 0.0                         |

<sup>a</sup>Calculated from analysis of acid hydrolysates of 0.2-mg aliquots of protein (hydrolysed for the times indicated) and corrected to 33,500 g, the molecular weight determined for the \(E.\ coli\) asparaginase subunits (18). The calculated composition represents a yield of 103.7% of the hydrophilic weight of the protein sample subjected to hydrolysis.

<sup>b</sup>Except where otherwise noted, all calculated values are averages of those obtained by analysis at the three different times of acid hydrolysis.

<sup>c</sup>The sum of aspartic acid plus asparagine.

<sup>d</sup>The value obtained by extrapolation of the time-dependent yields to zero time, in order to correct for destruction during acid hydrolysis.

<sup>e</sup>The sum of glutamic acid plus glutamine.

<sup>f</sup>Determined as cysteic acid after performic acid oxidation.

<sup>g</sup>The average of values obtained at 48 and 72 hours, in order to correct for the time-dependent release during acid hydrolysis.

<sup>h</sup>Determined as methionine sulfone after performic acid oxidation.

<sup>i</sup>Determined spectrophotometrically.
FIG. 1 (left). Disc gel electrophoresis of L-asparaginase from *Erwinia carotovora* (left) compared with the enzyme from *Escherichia coli* B (right). Electrophoresis was carried out on 7% polyacrylamide gels using 0.1 mg of protein. The gels were stained with Amido Schwarz.

**FIG. 2** (right). Ultraviolet difference spectra of L-asparaginase are tryptophan and cystine (19). There are also many other marked differences in amino acid composition of the two enzymes. These differences are also reflected by the patterns obtained on polyacrylamide gels which are shown in Fig. 1. L-Asparaginase from *Erwinia carotovora* (gel on left) shows three distinct bands very near the top of the gel column. All three bands have the ability to catalyze the hydrolysis of aspartic acid \(\beta\)-p-nitroanilide. The gel on the right indicates the greater mobility of asparaginase from *Escherichia coli* B. The major band which has traveled more than three-fourths the length of the polyacrylamide column represents 95% of the total protein and is the 7.2 S tetramer, while the remaining 5% of the protein is assigned to the higher molecular weight octamer (20, 21).

**Nature of Tyrosyl Residues—*Erwinia* and *E. coli* asparaginase** have a similar number of tyrosyl residues (44 in *E. coli* and 48 in *Erwinia*), but there is a marked difference in the number of residues that titrate with a normal pK. The number of normal tyrosines can be most readily determined from a comparison of the spectrum at pH 6.5 (where all of the tyrosyl residues are protonated) with the spectrum at pH 10.2, where only the normal tyrosines will be ionized. Ionization is accompanied by a shift in the absorption maximum from 274 nm (nonionized) to 294 nm (ionized) along with a doubling of the extinction coefficient. The dashed curve in Fig. 2A was obtained when the spectrum of *Erwinia* asparaginase at the lower pH was subtracted from the spectrum of the enzyme at pH 10.2 and should represent the number of “normal” tyrosines. The presence of two maxima (290 nm and 288 nm) is not the behavior expected of these groups.

The usual difference spectrum is shown by the dashed curve in Fig. 2B which was obtained with *E. coli* asparaginase. The higher concentration of *Erwinia* asparaginase was used in these samples so that the difference spectrum at pH 10.2 could be more easily seen.

All of the tyrosyl residues (normal and abnormal) are ionized at pH 12.8 as shown by the solid curves in these figures. These results show that only 10% of the tyrosyl residues of *Erwinia* asparaginase ionize normally, while 30% of these residues in the *E. coli* enzyme have a normal pK.

**Fluorescence Studies—**When a solution of *Erwinia* asparaginase in 0.05 M phosphate buffer, pH 7.5, was excited at 280 nm, a fluorescent band appeared with maximum intensity at 303 nm. This is the behavior exhibited by free tyrosine as well as by proteins that contain tyrosyl residues and no tryptophan (22). In contrast, the *E. coli* enzyme, which contains a single tryptophan residue along with 11 tyrosyl groups per subunit, exhibits an intense fluorescence at 317 nm. This emission band is almost exclusively derived from the tryptophan (6).

In order to determine whether or not the fluorescence of tyrosyl residues in *Erwinia* asparaginase was quenched as it is in a large number of proteins (22), we compared the fluorescence intensity of the 303 nm band obtained from a solution of the enzyme with an absorbance of 0.200 at 275 nm with that from a solution of tyrosine with the same absorbance. The intensity of the 303 nm band was identical in both cases, indicating that fluorescence quenching of tyrosyl residues does not occur in *Erwinia* asparaginase.
Circular Dichroic Spectra—There have been many attempts to correlate the shape of circular dichroic spectra and the magnitude of ellipticities at particular wavelengths with the folding of the polypeptide chain (23, 24). Fig. 3 shows the circular dichroic spectrum of asparaginase from *Erwinia carotovora* (dashed curve) compared with the spectrum of the *E. coli* enzyme (solid curve). Although the shape of the two curves is identical, there is a very significant difference in the magnitude of the ellipticities. If we use the equation suggested by Greenfield and Fasman (23) to give a rough approximation of the helical content of proteins, 13% of the polypeptide chain of *Erwinia* asparaginase is in the α-helix compared with 30% for the *E. coli* enzyme.

**Dissociation and Reconstitution**

The extent of dissociation of the tetrameric aggregate was measured by sedimentation velocity ultracentrifugation in urea and in guanidinium chloride. The greater stability of the oligomeric structure of *Erwinia* asparaginase compared with the *E. coli* enzyme is indicated by its behavior in urea. Exposure of *Erwinia* asparaginase to 8 M urea for 5 hours results in 50% dissociation of the 7.2 S tetramer to its 1.7 S monomer (Fig. 4a). By contrast the *E. coli* enzyme is completely dissociated in 4 M urea during the same 5-hour time period. Complete dissociation of *Erwinia* asparaginase can be effected in 3.5 M GdnCl or by the addition of 10% n-propyl alcohol to the solution of 8 M urea (Fig. 4b).

The extent of dissociation as determined by sedimentation methods is correlated with the absorbance of the 288 nm peak in the ultraviolet difference spectrum of *Erwinia* asparaginase. The completely dissociated *Erwinia* enzyme in 6 M GdnCl gives the ultraviolet difference spectrum shown in Fig. 5. Yanari and Bovey (25) suggested that ultraviolet difference spectra of proteins arise when the chromophore of the amino acid residue is transferred from a hydrophobic environment in the native enzyme to a hydrophilic one in the denatured state. The two bands in the difference spectrum of *Erwinia* asparaginase (281 nm and 288 nm) are both assigned to tyrosyl residues. Previous studies with *E. coli* asparaginase (6) and with ribonuclease (26) suggested that each tyrosyl residue that gets transferred from its microenvironment of low dielectric constant in the native enzyme to the aqueous environment has an extinction coefficient Δε of 1000 in the 287 nm band of the difference spectrum. The extinction coefficient of the 288 nm band shown in Fig. 5 is 42,000 which suggests that 42 out of the total of 48 tyrosyl residues are "buried" in native *Erwinia* asparaginase.

The rate of dissociation of the *Erwinia* tetramer could be monitored by following the rate of appearance of the 288 nm band in the ultraviolet difference spectrum. The most convenient concentration of GdnCl for these studies was found to be 3.5 M. The rate of dissociation of *Erwinia* asparaginase is shown in the bottom curve of Fig. 6. The addition of alcohols with increasing chain length at concentrations of 5% by volume is also shown in this figure. In the presence of the alcohols it is apparent that dissociation takes place in two distinct steps, both of which increase as the length of the alkyl chain of the alcohol increases. The results suggest that the alcohols aid in disruption of the hydrophobic forces that participate in subunit interactions. These conclusions are qualitatively in agreement with those found by Hercovite et al. (27) and by Tan and Lovrien (28).

The effect of increasing concentrations of dodecyl sulfate on the dissociation of *Erwinia* asparaginase was also studied by sedimentation velocity and ultraviolet difference spectroscopy. The sedimentation coefficient of the enzyme decreased from 7.2……
Fig. 6. Rate of appearance of the 288 nm band as a function of time in 3.5 M GdnCl, pH 7.5. The enzyme at a concentration of 1 mg per ml was placed in 0.05 M phosphate buffer, pH 7.5, in the sample compartment. At zero time the enzyme was mixed with the appropriate denaturing solvent and the sample was placed in the solvent compartment. The first reading was taken within 15 s after mixing and changes in absorbance were monitored for 20 min.

S in 0.05 M phosphate buffer, pH 7.5, to 5.9 S in 0.5% SDS and to 5.0 S in 5% SDS. This behavior is in sharp contrast to the results obtained with E. coli asparaginase, which had a sedimentation coefficient of 2.4 S in 0.1% SDS. The ultraviolet absorption spectrum of the Erwinia enzyme was the same in 0.5% to 5% SDS as it was in buffer, which also contrasts with the results obtained with the coli protein (6). All of these results support the observation that the tetrameric structure of Erwinia asparaginase is considerably more stable than the enzyme isolated from Escherichia coli B.

Reconstituted Erwinia asparaginase was prepared by removal of GdnCl from solutions of the denatured enzyme by dialysis against 0.05 M phosphate buffer, pH 7.5. Concentrations of protein greater than 1 mg per ml were avoided in this procedure due to formation of a precipitate. The reconstituted product had 90% of the original catalytic activity and showed only one band on disc gels. Since the native enzyme from Erwinia showed three bands on disc gels (Fig. 1), it appears that dissociation followed by reconstitution results in random aggregation of the slightly different subunits.

Intergeneric Hybrids—Although the strength of intersubunit forces is quite different between the Erwinia and E. coli monomers, an attempt was made to prepare a hybrid tetramer composed of subunits from the two different bacterial genera. Different ratios of the two enzymes in 6 M GdnCl were mixed and then dialyzed against buffer. Electrophoresis on polyacrylamide gels showed that the E. coli subunits aggregated with each other as was true with the Erwinia enzyme and there was no evidence of the formation of intergeneric hybrids.

Succcinylation

Erwinia asparaginase was succinylated using increasing molar concentrations of [14C]succinic anhydride at pH 8.0. The number of lysyl residues modified by succinylation are summarized in Table II together with the catalytic activity, sedimentation coefficients, and the distance the modified enzyme migrates on polyacrylamide gels. The bands obtained with the 1:2, 1:1, and 2:1 succinylated samples were rather diffuse in contrast to the very sharp bands obtained with the 1:4, 4:1, and 8:1 derivatives.

| Sample | Moles lysine succinylated per 133,000 g | Enzyme activity | Sedimentation coefficient | Migration on gels |
|--------|----------------------------------------|-----------------|---------------------------|-------------------|
| 1:4    | 5-8                                    | 100             | 7.25                      | 0.7               |
| 1:2    | 12-16                                  | 100             | 7.2                       | 1.2 ± 0.2         |
| 1:1    | 28-31                                  | 100             | 7.2                       | 2.5 ± 0.5         |
| 2:1    | 47-49                                  | 100             | 7.2                       | 5.2 ± 0.5         |
| 4:1    | 54-56                                  | 100             | 7.2                       | 7.0               |
| 8:1    | 50-59                                  | 100             | 7.2                       | 7.0               |

* Moles of succinic anhydride added per lysine residue.

It is apparent that extensive succinylation does not destroy catalytic activity nor does it cause the tetramer to dissociate. The retention of the tetrameric structure is in sharp contrast to the behavior of the E. coli enzyme (7) and with the behavior of a large number of oligomeric proteins (29-35). Not only is the Erwinia tetramer retained after succinylation, but the rates of dissociation of the modified enzyme in 3.5 M GdnCl remain unaffected.

The succinylated tetramer could be fully reconstituted from 6 M GdnCl solutions without the formation of a precipitate in solutions where the protein concentration was as high as 2 mg per ml. The presence of even a few succinyl groups in E. coli asparaginase had been found to interfere with reconstitution (7).

Hybridization

All of the succinylated samples obtained with Erwinia asparaginase fulfilled the criteria described by Meighen and Schachman (29) for a proper chemical derivative that could form hybrids with the native subunit. The 4:1 and 8:1 succinylated samples formed the sharpest bands on disc gels and migrated farthest from the native sample, making them most suitable for hybridization studies.

The native and succinylated samples were dissociated separately in 6 M GdnCl. Aliquots from each were withdrawn and dialyzed against pH 7.5 buffer to serve as the controls (Fig. 7). Varying ratios of the dissociated enzymes were mixed and then dialyzed. The reconstituted product obtained from a 70:30 mixture of succinylated-native enzyme gave the disc gel pattern shown by the middle gel in Fig. 7. Five bands can be detected. The slowest migrating band represents the unmodified enzyme and the most rapidly migrating band corresponds to the 4:1 succinylated derivative. The three intermediate bands represent hybrid tetramers. The formation of five bands in the hybridization experiment constitutes evidence that L-asparaginase from Erwinia carotovora is composed of four identical subunits.

Conclusions

Although the molecular weight of the 1 asparaginases from Escherichia coli (19) and Erwinia carotovora (18) are identical and both are composed of four identical subunits (7 and the present study), there are some very marked differences in their amino acid composition and the stability of the tetrameric aggregate. Tryptophan and cystine are both present in E. coli but are completely absent in the Erwinia enzyme. The over-all difference in the composition of proteins is also reflected in their migration in an electric field. The E. coli enzyme travels three-quarters of the distance down a column of polyacrylamide gel, while the Erwinia asparaginase barely penetrates the gel matrix.

The stability of the Erwinia tetramer is reflected in its resist-
weakens intersubunit forces, and does not interfere with reconstitution. These results might be explained by the greater basicity of the *Erwinia* enzyme, which neutralizes the negatively charged succinyl residue. Since asparaginase from *Erwinia carotovora* is as effective as the *E. coli* enzyme in the treatment of leukemia, it appears that clinical effectiveness of the enzyme does not depend on the ease with which the tetramer can dissociate nor does it appear to depend on the net charge on the enzyme. It would be of interest to study physicochemical properties similar to those reported in this paper for L-asparaginase samples that are pharmacologically inactive.

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