Evidence for the Association of L-Asparaginase with Cytomembrane Components in the Guinea Pig Liver Soluble Fraction*

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Homogenization of guinea pig liver in isotonic sucrose solution followed by the separation of the subcellular fractions by differential centrifugation releases the liver L-asparaginase (L-asparagine amido-hydrolase, EC 3.5.1.1) activity into the supernatant fraction.

Electron micrographs of the liver L-asparaginase antibody complexes, precipitated from the clear supernatant phase by addition of L-asparaginase-specific antisera, show membrane-like structures and some amorphous material. The attachment of L-asparaginase to the membrane-like structures is indicated by the ferritin-labeled antibody technique.

The immunoprecipitates possess low activities of 5'-nucleotidase, alkaline phosphodiesterase I, NADPH cytochrome c reductase, glucose-6-phosphatase, and acid phosphatase. This observation suggests that L-asparaginase found in the liver supernatant fraction is associated with cytomembrane components.

Analysis of guinea pig serum L-asparaginase antibody complexes by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gives three distinct protein bands. These bands correspond to heavy and light chains of rabbit immunoglobulins and the L-asparaginase subunits. Analysis of the liver L-asparaginase antibody complexes by the above procedure shows similar but more diffuse protein bands.

In the previous communications, evidence was presented concerning the structural differences between the L-asparaginases isolated from the guinea pig serum and the liver (1-3). In the pursuit of the ultimate objective, elucidation of these structural differences, purification of these enzymes was carried out by a variety of protein separation techniques. A 3000-fold purification of the serum L-asparaginase was obtained; this enzyme preparation was found to be homogeneous (2). However, only a 50-fold purification of the guinea pig liver L-asparaginase could be effected; all further attempts to purify the liver enzyme were unsuccessful. Thus, a large fraction of the liver enzyme was always converted into an insoluble form in the course of purification procedure (1).

Rabbits immunized with either guinea pig serum or liver L-asparaginase preparations produced antibodies which precipitated both the serum and liver L-asparaginases (3). Injection of rabbits with the heterologous antigen-antibody complexes gave rise to L-asparaginase-specific antisera. Hence, it was surmised that isolation of the L-asparaginase-antibody complexes could provide a technique for further structural analysis of the serum and liver L-asparaginases. However, all attempts to dissociate the L-asparaginase-antibody complexes remained unsuccessful.

The present study was undertaken to explore the feasibility of the use of sodium dodecyl sulfate in the isolation of liver and serum L-asparaginase subunits.

EXPERIMENTAL PROCEDURE

Subcellular Fractionation—The subcellular fractionation was carried out with a single guinea pig liver (wet weight 18 to 20 g) at a time. The animal was anesthetized with ether, exsanguinated, and the liver rapidly removed and placed into ice-cold 0.25 M sucrose solution. After cooling the liver was blotted dry, weighed, and minced in 30 ml of isotonic sucrose solution. The liquid was decanted and discarded. This procedure for the removal of blood was repeated two more times. The minced liver, suspended in 5 volumes (w/v) of 0.25 M sucrose solution, was homogenized by three consecutive down strokes in a glass-Teflon Potter-Elvehjem type tissue grinder (size C, A. H. Thomas, Philadelphia, Pa.). The smooth tipped pestle was driven mechanically at 1,000 rpm. The homogenate was centrifuged at 600 x g for 10 min to remove the intact cells, nuclei, and cell debris. The supernatant fraction was decanted and the sediment was rehomogenized in 2 volumes of isotonic sucrose solution and centrifuged at 600 x g for 10 min. The supernatant fractions from both centrifugations were combined and, to separate the mitochondrial fraction, centrifuged in a Spinco model L preparative ultracentrifuge (No. 30 rotor) at 6,000 x g for 20 min. The microsomal and soluble cytoplasmic fractions were obtained from the 6,000 x g supernatant fluid by centrifugation at 105,000 x g for 120 min (No. 40 rotor). After centrifugation a small (1 ml) upper lipid layer was removed and discarded. The underlying supernatant phase was withdrawn with a pipette, leaving the bottom 1 ml behind. The

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optically clear supernatant fluid was used immediately for the preparation of the L-asparaginase-antibody precipitates.

To determine the L-asparaginase activity in both the mitochondrial and microsomal fractions, the pellets were suspended in 0.25 M sucrose solution by homogenization and centrifuged at 105,000 x g for 90 min. Aliquots of the washed mitochondrial and microsomal fractions, the 600 x g sediment, and the 105,000 x g total supernatant solution were used for the L-asparaginase assay.

**Enzyme Assays**—The activity of L-asparaginase was determined as described previously (1).

The following assays were carried out on guinea pig liver L-asparaginase-antibody precipitates. Glucose-6-phosphatase, 5'-nucleotidase, and alkaline phosphodiesterase I activities were assayed according to the procedures described by Emmelot et al. (4). Acid phosphatase activity was measured by the method of Reid (5), using p-nitrophenyl phosphate as the substrate, however, omitting the freeze-thawing steps. NADPH cytochrome c reductase activity was determined by the procedure of Gigno and coworkers (6). Immunoprecipitates derived from the soluble fraction equivalent to 5 g of liver were used in each sample. Fractionation of the liver and preparation of the immunoprecipitates and the enzyme assays were carried out on the same day.

**Immunochromatographic Techniques**—The preparation of antisera to L-asparaginase has been reported previously (3). In the present studies rabbits were immunized either with 3000-fold purified guinea pig serum L-asparaginase (2) or with liver L-asparaginase-antibody precipitates.

The immunoprecipitates were prepared by the modification of a previously reported procedure (3). Guinea pig serum or freshly prepared 105,000 x g supernatant fraction of the liver homogenate was mixed by gentle stirring with predetermined optimal, volume of the antiasparaginase serum. The mixtures were kept, with occasional stirring, at room temperature for 30 min and the immunoprecipitates were collected by centrifugation at 0 to 4° (500 x g, 5 min). The precipitates were washed three times by suspending in ice-cold 0.25 M sucrose solution and centrifuged as described above. Depending on the further use, the washed immunoprecipitates were suspended in different buffer solutions.

Double immunodiffusion analysis was carried out on Hyland immunoplates.

Rabbit serum y-globulin fraction was isolated by column chromatography on DEAE-cellulose (7).

**Electrophoresis**—The polycrylamide gel electrophoresis system used in these studies was a modification of the discontinuous slab polycrylamide gel electrophoresis (2). Gel slabs of 6-mm thickness were used in a vertical electrophoresis cell with the previously described buffer systems, with the modification that sodium dodecyl sulfate was added to all buffers to give a final sodium dodecyl sulfate concentration of 0.1% (w/v), Electrophoresis in 8.0% Cyanogum-41 gels was run at room temperature with tap-water cooling until the bromphenol blue band had traveled 12 cm from the origin. The applied current was 10 mA/cm. The gels were stained with 0.2% Coomassie blue. Samples of the immunoprecipitates (700 to 900 µg of protein/slot), prepared for the gel electrophoresis, were suspended in 0.25 ml of 0.02 M Tris-HCl buffer, pH 6.8, containing 5% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 8% urea. The samples were incubated for 20 min at 60°, cooled to room temperature, and applied to the gel slab.

After the electrophoretic run, in some experiments, the individual protein bands were extracted from sliced (2-mm wide sections) gels with a small volume of Tris-glycine buffer, pH 8.3, containing sodium dodecyl sulfate (0.02%). After 24 hours at 4° the protein-sodium dodecyl sulfate extracts were withdrawn and tested for the positive precipitin reaction with L-asparaginase-specific antiserum by the agar gel double diffusion technique. Another section of the gels was developed with Coomassie blue and used as a marker.

**Preparation of Samples for Electron Microscopy**—The liver L-asparaginase-antibody precipitates were prepared immediately after the fractionation of the liver as described above. The precipitates were suspended in ice-cold 0.1 M phosphate buffer, pH 7.3, divided into two portions, and transferred into 12-ml centrifuge tubes. After centrifugation for 5 min at 500 x g one of the duplicate pellets was covered with 3 ml of ice-cold 3% glutaraldehyde, prepared with 0.1 M sodium phosphate buffer, pH 7.3. After 15 min at 4° the pellet was divided into 2 parts and loosened from the wall of the centrifuge tube. The fixation was continued for an additional 120 min. The fixed pellets were washed four times with 0.1 M sodium phosphate buffer, pH 7.3, and postfixed for 60 to 90 min at 0 to 4° in phosphate-buffered 2% osmium tetroxide (8). After rinsing with the phosphate buffer, the pellets were dehydrated and embedded in Epon 812 (9). Thin sections of the pellets were double-stained with uranyl acetate and lead citrate (10). The second portion of the initial liver L-asparaginase-antibody precipitate was suspended in 1.0 ml of 0.1 M sodium phosphate buffer and mixed with 0.2 ml of ferritin-conjugated goat antirabbit IgG (Cappel Laboratories, Downingtown, Pa.). The mixture was kept at room temperature, with occasional stirring, for 30 min, then stored overnight at 4°. The precipitate was sedimented and washed by suspending it in 5 ml of 0.1 M sodium phosphate buffer, pH 7.3, and centrifuged at 500 x g. This procedure was repeated three times and then the pellet was fixed and processed as described above.

**RESULTS AND DISCUSSION**

**Electrophoretic Analysis of Serum and Liver Immunoprecipitates**—The guinea pig serum and liver immunoprecipitates were prepared with the antiasparaginase serum raised in rabbits against 3000-fold purified guinea pig serum L-asparaginase. The specificity of the antiserum was tested by the agar gel double diffusion technique. On reaction with the antiserum, both the guinea pig serum and liver-soluble fractions gave single precipitin lines.

The immunoprecipitates were solubilized in the sodium dodecyl sulfate solution containing a reducing agent and were analyzed by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. The serum L-asparaginase-antibody complex showed four major protein bands (Fig. 1). The predominant protein (molecular weight 51,000) band and the fastest moving protein (molecular weight 25,000) band correspond to the heavy and light chains of the rabbit immunoglobulins. The above apparent molecular weights compare closely

![FIG. 1. Electrophoretic analysis of sodium dodecyl sulfate-solubilized and reduced protein samples on sodium dodecyl sulfate-polyacrylamide gels. a, 3000-fold purified guinea pig serum L-asparaginase, 35 µg; b, rabbit y-globulin, 500 µg; c and e, mixtures of ovalbumin, aldolase, chymotrypsinogen A, and ribonuclease A, 20 µg each; d, guinea pig serum L-asparaginase-antibody complex, 800 µg; f, guinea pig liver L-asparaginase-antibody complex, 800 µg. High concentrations of immunoprecipitates were applied to the gels to visualize the protein band corresponding to L-asparaginase. For details see "Experimental Procedure."](http://www.jbc.org/)
with those reported by Small and co-workers (11). The third,
minor protein band, migrating just ahead of the major band,
had the \( R_g \) value identical with that of the purified serum
L-asparaginase run under the identical conditions. An appar-
ent molecular weight of 43,000 ± 1,000 for the L-asparaginase
subunit was indicated by the comparison of the electropho-
sic mobility with those of the reduced ovalbumin and
aldolase subunits. Molecular weight for the serum L-asparagi-
nase has been reported to be 158,000 (12). After elution of the
protein bands with buffer, only these extracts containing the
protein with the molecular weight of 43,000 gave positive
precipitin reactions with the antisera to L-asparaginase. The
fourth, remaining protein band, with the lowest mobility,
could be an aggregate of two heavy chains or an undissociated
immunoglobulin. In comparison with the serum immunopre-
cipitate the liver L-asparaginase-antibody precipitate has a
differently different protein distribution (Fig. 1). It is seen that
some of the Coomassie brilliant blue-stained protein had not
penetrated the gel. The bands corresponding to the heavy and
light chains of the immunoglobulin are present, but the
relative concentrations are apparently different from those in
the serum L-asparaginase immunoprecipitate. It may be sur-
sumed that the band zone corresponding to the heavy chain
contains also other polypeptides. Furthermore, a low molecular
weight (< 13,700) polypeptide band, migrating together with
the bromphenol blue marker, is present. The protein band
containing the liver L-asparaginase subunit is diffuse and
weak. Nevertheless, an extract from this gel zone gives the
precipitin line with the antisera to L-asparaginase. The
weakness of the band could be due to the fact that the guinea pig liver
immunoprecipitates contain less enzyme per milligram of
protein than those of the guinea pig serum. It has been reported
that at an optimal antibody-enzyme ratio, twice the amount of
the antisera to L-asparaginase is required for the precipitation of
an equivalent number of liver L-asparaginase activity units,
compared to the serum L-asparaginase (3).

Unsuccessful attempts were made to fractionate the sodium
dodecyl sulfate-solubilized and reduced polypeptides from the
liver L-asparaginase-antibody precipitates by gel filtration on
Sephadex G-200, using buffers containing either sodium do-
decyl sulfate or sodium dodecyl sulfate and urea. Some protein
was eluted as a high molecular weight aggregate; other protein
fractions were mixtures of several polypeptides as shown by the
sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Immunological Studies with Liver L-Asparaginase-Anti-
body Complexes—Antisera raised in rabbits against the liver
L-asparaginase-antibody precipitates showed two precipitin
lines when tested with either the guinea pig serum or liver solu-
tion. Also, antisera from different rabbits showed variations in their antibody composition patterns. Some rabbits
produced antibodies mainly against L-asparaginase, others
elicited sera with high concentrations of antibodies against the
contaminating protein. These observations are not in com-
plete agreement with our previous findings where the use of
asparaginase-antibody precipitates had made possible prepa-
rations of the L-asparaginase-specific antisera (3). The dispara-
rate results could be due to the different experimental condi-
tions, e.g., in the present studies only freshly prepared guinea
pig liver-soluble fractions were used in the preparation of
immunoprecipitates, while in the previous studies soluble
fractions employed had been stored at −20° and thawed
extracts had been recently refuged (105,000 × g, 90 min) prior to
the preparation of immunoprecipitates. Furthermore, the
washing procedures for the immunoprecipitates were also
modified. In the previous experiments the precipitates had
been washed with 1 M sodium chloride solution, then with 0.1 M
sodium borate buffer, pH 10.5. The above treatment solubi-
lized some protein as shown by the increase of the specific
activity of L-asparaginase-antibody precipitates. To preserve
the basic structures of the native forms of the enzymes milder
conditions were used in the present study, thus the immuno-
precipitates were washed only with the 0.25 M sucrose solution.

Electron Microscopic and Enzymatic Studies on Liver
L-Asparaginase-Antibody Precipitates—Electron micrographs
prepared of the liver L-asparaginase-antibody precipitates show
membrane-like long filaments and some amorphous
material (Fig. 2, A and B). To detect the distribution of the
L-asparaginase-antibody complexes the immunoprecipitates
were reacted with ferritin-labeled goat antirabbit IgG. The
ferritin-conjugated antibody method for the localization of
macromolecular antigens was developed by Singer (13) and
both the direct and indirect methods have been used (14).
Indirect evidence for the attachment of L-asparaginase to the
membrane-like filaments was provided in the present study by
the location of the ferritin label alongside the filaments after
the reaction of L-asparaginase-attached rabbit IgG with the
ferritin-labeled goat antirabbit IgG (Fig. 2, C and D). Ferritin-
labeled antibodies were present also in the amorphous mat-
erial. Since the liver had not been perfused prior to the
homogenization, the amorphous material may contain either
several or liver L-asparaginase antibody complex.

It is known that membranes can be dispersed either by
chemical agents and/or mechanical forces and that the mem-
brane fragments may reaggregate to form morphologically
membrane-like by guest on September 10, 2017 http://www.jbc.org/ Downloaded from
Guinea Pig Liver L-Asparaginase

FIG. 2. A and B, electron micrographs of a section through a pellet of liver L-asparaginase-antibody complex. Some amorphous material (arrow) is seen among the long filaments. The globular substructure observed may reflect an artifact of the staining. C and D, electron micrographs of the same immunoprecipitate preparation after reaction centrifuged at 105,000 x g for 120 min and, to avoid possible contamination of the supernatant fraction with the microsomal fraction, the supernatant immediately above the residual pellet was retained in the tube and was not used in the preparation of the immunoprecipitates.

Based on the distribution of L-asparaginase activity in the four subcellular fractions from differential centrifugation, L-asparaginase appears to be a soluble enzyme (Table I). The enzyme activity is distributed mainly between the 600 x g sediment and the 105,000 x g supernatant fraction. The relative amounts of L-asparaginase found in these two fractions depend on the intensity of homogenization. In a typical experiment, where the liver was homogenized by three consecutive strokes in the Potter-Elvehjem type grinder, 57% of the total L-asparaginase activity was recovered in the supernatant phase; 75 and 81% of the activity was found in the respective supernatant fractions when the 600 x g sediment was rehomogenized either once or twice. The activity remaining in the 600 x g sediment appears to derive from the incompletely ruptured cells. To avoid extensive damage to the particulate

with ferritin-labeled goat antirabbit IgG. The electron-dense clusters of ferric hydroxide micelles are seen alongside the filaments and amorphous material. All sections were stained with uranyl acetate and lead citrate. For details see "Experimental Procedure." Magnification: a and C, × 33,250; B and D, × 98,000.
parts of the cell and cross-contamination of the individual subcellular fractions, the low speed sediment was rehomogenized only once.

Both the homogenization of guinea pig liver and fractionation of the homogenate by differential centrifugation were carried out according to the established techniques (15, 18, 19). A commonly accepted procedure for homogenization of liver tissue is to disrupt cells in 0.25 M sucrose solution in the Potter-Elvehjem type tissue grinder. Although this technique is thought to result in minimal damage to the cellular components, de Duve (18) considers this a multilating procedure. Various types of cytomembranes, especially the plasma membranes and endoplasmic reticulum, may suffer extensive fragmentation during the cell fractionation. Particles of different sizes may be produced, contributing to the pellet heterogeneity in differential centrifugation. Both the optical clarity and failure to sediment are often taken to indicate solubility. In fact, the above two criteria may reflect only a sharp reduction in the particle size. The difficulty in trying to define the physical meaning of solubility is emphasized by Rosenberg and McIntosh (20) who fragmented human erythrocyte membrane by sonication and obtained particles that failed to sediment at high centrifugal fields but were otherwise identical with the starting membranes.

Based on our studies, no determination could be made whether the liver L-asparaginase is membrane-bound in the cell or associates with the unsedimentable, dispersed membrane during the fractionation process. To account for the relative ease of release of the cytomembrane-bound L-asparaginase into the supernatant phase it could be hypothesized that, compared to other regions of the cytomembrane, the L-asparaginase-associated regions of the membrane are fragmented more readily and produce unsedimentable particles. Addition of the antiasparaginase serum may initiate aggregation of the dispersed membrane components to form filaments that sediment at low force of centrifugation.

There are several conflicting literature reports on the subcellular localization of guinea pig liver L-asparaginase. Krebs (21) reported that L-asparaginase was associated with the cellular particles and remained in the sediment on centrifugation. Unfortunately, no experimental details of the fractionation were given. On the other hand, after dialysis of L-asparaginase preparations against water, Matthews and Brown (22) isolated from the liver supernatant phase two fractions with the L-asparaginase activity.

In retrospect, earlier observations made in the course of the attempted purification of the liver enzyme could indicate its association with the membranous material. These observations also include a pronounced tendency of the enzyme to aggregate and conversion of the liver enzyme into the serum type enzyme by digestion with papain (2).

Formation of a variety of particles with different structures in a clear solution of rat liver-soluble phase, obtained by centrifugation at 105,000 × g for 60 min, has been reported by Anderson (23). Following storage of the soluble fraction at 2° either in the undialyzed state or dialyzed against a variety of solutions, small granules, round vesicles, translucent sheets, and fibrous material were observed. The electron micrographs of these structures were given but no enzymatic studies were reported. Except for the above study, no other information has been found on the presence of particles in the 105,000 × g supernatant fraction of liver homogenate.

A detailed analytical study of subcellular membranes from rat liver has been reported by Amar-Costescu and co-workers (24). A distribution pattern of 25 enzymes was studied and low activities of membrane marker enzymes were found in the 105,000 × g supernatant fraction. This raises the question whether the observed enzyme activities arise from the nonsedimentable particles in the supernatant phase or from the truly soluble enzymes.

In summary, the experimental data do not provide sufficient information to determine the localization of L-asparaginase in the guinea pig liver cell. The significant finding of the present study is the association of the liver enzyme with the membrane fragments present in the supernatant fraction. The previous attempts to purify the liver L-asparaginase failed apparently because the purification procedures employed were based on the assumption that L-asparaginase was a soluble enzyme. Different techniques, applicable for purification of membrane-bound enzymes, should be employed for the isolation of this particular enzyme.

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