Purification and Properties of an Enzyme from Beef Liver Which Catalyzes Sulfhydryl-Disulfide Interchange in Proteins

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SUMMARY

An enzyme which catalyzes sulfhydryl-disulfide interchange in proteins containing "incorrect" disulfide bonds has been isolated from beef liver microsomes. The purified form of this enzyme shows two bands upon electrophoresis in starch gels and polyacrylamide gels. The proteins represented by these bands are considered to be variants of the same protein because both are enzymically active after elution from starch gels and because they appear as a single band after reduction and alkylation. A contaminant of higher molecular weight was identified in the purified enzyme preparation both as a faint third band in starch and polyacrylamide gels and as a faster sedimenting component in the analytical ultracentrifuge. By the latter technique the quantity of the contaminant was estimated to be 8 to 10% that of the microsomal enzyme.

The molecular weight of the enzyme was found to be 42,000 by analytical ultracentrifugation. Amino acid analyses of the enzyme disclosed the presence of 3 half-cystine residues, and 44 arginine plus lysine residues, per molecule. Peptide maps, prepared from tryptic digests of the enzyme, contained 48 ninhydrin-positive spots. The visible and ultraviolet absorption spectra of the enzyme showed no peaks other than one with a maximum at 278 m\(\mu\).

Previous studies in this laboratory have shown that the microsomes of rat and beef liver contain an enzyme that catalyzes rearrangement of "incorrect" pairs of half-cystine residues in bovine pancreatic ribonuclease, egg white lysozyme, and soybean trypsin inhibitor (1–3). The same enzyme has been identified in chicken, pigeon, and pig pancreas tissue (4). After partial purification of the enzyme, it was possible to show that the mechanism of the catalysis involves sulfhydryl-disulfide interchange (5, 6). The enzyme facilitates the rearrangement of unnatural pairs of half-cystine residues in a protein to yield the pairings characteristic of the native form. The enzyme may be assayed by using, as substrate, either a reduced protein (in which case the enzyme facilitates rapid correction of randomly occurring mistakes in the pairing of half-cystine residues during oxidative reactivation) or a fully oxidized protein already containing randomly paired half-cystine residues (in which case the enzyme, in the presence of a small amount of reducing agent, facilitates the rapid rearrangement of half-cystine pairs during nonoxidative reactivation). In either case, the reaction catalyzed by the enzyme appears to be driven by the free energy released when a protein with relatively disorganized conformation (with incorrect disulfide bonds) attains the more stable conformation of the native state (7, 8).

The localization of the enzyme within the microsomal fraction of many tissues suggests that, in vivo, it catalyzes sulfhydryl-disulfide interchange during the process by which newly synthesized polypeptide chains attain specific three-dimensional conformations stabilized by specific disulfide bonds (9).

This report concerns the purification of the enzyme from beef liver, and some physical and chemical properties of the purified enzyme.

MATERIALS AND METHODS

Analytical Methods—The concentrations of protein solutions were determined by the method of Lowry et al. (10), except for solutions of RNase and STI. For these proteins, concentrations were calculated from absorbance at 280 m\(\mu\). Routine measurements of absorbance at 280 m\(\mu\) were made with Zeiss spectrophotometers with quartz cuvettes with 10 mm light paths. The visible and ultraviolet absorption spectra of the sulfhydryl-disulfide interchange enzyme were recorded with a Cary model 15 spectrophotometer. Measurements of pH were

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made with a Radiometer model PHM-25 pH meter. Solutions of urea, recrystallized from 95% ethanol, were prepared immediately prior to use.

Reduction of RNase and STI—RNase (type II-A, Sigma) and STI (crystallized three times, Worthington) were fully reduced with β-mercaptoethanol (Eastman, white label) in 8 M urea, and the reduced proteins were separated from the reagents by gel filtration as described previously (11). Stock solutions of reduced RNase in 0.1 M acetic acid, at a concentration of 0.2 mg per ml, were kept for no longer than 1 day, and at 0°, to prevent spontaneous reoxidation.

Preparation of Randomly Cross-linked RNase and STI—Ribonuclease and STI containing "incorrect" disulfide bonds were prepared as previously described by allowing the reduced proteins to reoxidize spontaneously in 8 M urea. The randomly cross-linked proteins were concentrated by vacuum dialysis, separated from the urea by gel filtration on Sephadex G-25 (Pharmacia), and stored as frozen solutions at -20°.

Reduction and Alkylation—The sulfhydryl-disulfide interchange enzyme was reduced in 8 M urea containing 0.1 M β-mercaptoethanol at 45° for 4 hours. Iodoacetic acid (Eastman) was twice recrystallized from petroleum ether and was added in 5-fold molar excess over the amount of β-mercaptoethanol present. The pH was maintained at 8.4 for 10 min. The reaction was stopped by adding an amount of β-mercaptoethanol equal (mole per mole) to that of the iodoacetic acid, and the entire solution was dialyzed exhaustively against water, and lyophilized.

Electrophoresis in Starch Gel—Electrophoresis of the purified enzyme preparation in starch gels was performed in 0.04 M sodium phosphate buffer, pH 6.5, by the method of Smithies (15). For preparative runs, a representative strip of the gel was first stained with Amido black for protein (15) in order to locate the positions of the bands. The areas of the remaining gels corresponding to those bands were then cut out and eluted. In cutting the gels, care was taken to obtain only the front part of the faster moving band and only the rear portion of the slower moving band in order to avoid cross-contamination. Elution of the enzyme from the gels was accomplished electrophoretically by the method of Hoerman, Balakjian, and Berzinskas (16).

Electrophoresis in Polyacrylamide Gel—Electrophoresis in polyacrylamide gels was employed to monitor the number and characteristics of contaminants throughout the purification procedure. Electrophoreses were performed in 7.5% standard gels with 0.2 M Tris-glycine buffer, pH 8.5, at 4° and at 3.0 ma per tube as described by Ornstein and Davis (17).

Ultracentrifuge Studies—All of the ultracentrifuge studies were carried out on a Spinco model E analytical ultracentrifuge equipped with the standard schlieren and Raleigh interference optical systems. Temperature was controlled at 20° on all runs with the RTC control unit. Sedimentation values were determined, at various protein concentrations (see Fig. 6), for enzyme obtained following DEAE-Sephadex chromatography at pH 6.3 (see below). The buffer system consisted of 0.05 M Tris-HCl, 0.10 M NaCl, pH 7.5. Sedimentation coefficients were calculated as described by Schachman (18) and were corrected to water at 20°.

Molecular weight determinations were carried out by the high speed equilibrium technique of Yphantis (19). Determinations were made on solutions with protein concentrations of 0.2 mg per ml and 0.4 mg per ml at 39,460 rpm, 35,600 rpm, and 33,450 rpm, with Tris-NaCl as solvent. For molecular weight calculations, the partial specific volume of the protein was assumed to have the value 0.725 ml per g.

Amino Acid Analyses—Samples were prepared for amino acid analyses by alkylation after prior reduction. Duplicate samples of the alkylated protein were hydrolyzed in constant boiling HCl in sealed, evacuated tubes at 110° for 21, 45, and 70 hours. Beckman/Spinco amino acid analyzers, equipped with automatic integrators (Infotronics Corporation), were used for the analyses. Amino acid compositions were calculated by extrapolation of the results obtained for the timed hydrolysos to zero time.

Preparation of Peptide Maps—Peptide maps were prepared by the method of Katz, Dreyer, and Anfinsen (20). After reduction and alkylation, the sulfhydryl-disulfide interchange enzyme was dissolved in 0.1 M ammonium bicarbonate buffer, pH 8.2, at a concentration of 10 mg per ml. It was digested at 37° for 4 hours with 1.5% (by weight) trypsin which had been treated with disopropyl phosphorofluoridate to abolish residual chymotryptic activity (21). The reaction mixture was then lyophilized, dissolved in water, and the pH adjusted to 8.0 with ammonium hydroxide. The solution was applied to a sheet of Whatman No. 81MM filter paper, and chromatographed in butanol-acetic acid-water (4:1:5). After drying, the paper was submitted to electrophoresis in pyridinium acetate buffer, pH 3.6, for 80 min, at 2500 volts in a Gilson model D electrophoresis apparatus. After drying, peptides were stained by dipping the paper in a 0.25% solution of ninhydrin in ethanol.

RESULTS AND DISCUSSION

Purification of Sulphydryl-Disulfide Interchange Enzyme

Purification of the sulfhydryl-disulfide interchange enzyme from beef liver was accomplished in seven steps.

Steps 1 Through 5—The isolation of microsomes, preparation and extraction of microsomal acetone powders, ammonium sulfate fractionation of the extract, and chromatography on CM-Sephadex were carried out as described previously (5)
except that these procedures were modified so as to allow the processing of 24 kg of beef liver at one time.\textsuperscript{3}

\textbf{Step 5}—The yellow fraction, containing the sulphydryl-disulfide interchange enzyme, obtained by chromatography on CM-Sephadex (Pharmacia), was dialyzed against 0.1 m Tris buffer, pH 7.8. After dialysis, the volume was 575 ml, and the protein concentration was approximately 92.8 mg per ml. A column (11.2 \( \times \) 60 cm) of Sephadex G-100 (Pharmacia), equilibrated with 0.1 m Tris buffer, pH 7.8, was used for gel filtration of this material, 115 ml at a time, in each of five runs. The pattern obtained in a typical experiment is shown in Fig. 1.

\textbf{Step 6}—The active fractions from all five runs on Sephadex G-100 were pooled. After concentration by vacuum dialysis the total volume was 1000 ml, and the protein concentration was 26.6 mg per ml. It was separated into four parts, and each part separately adsorbed onto a column (4 \( \times \) 40 cm) of DEAE-Sephadex A-50 (Pharmacia) equilibrated with 0.1 Tris buffer, pH 7.8. Elution was accomplished with a linear gradient of NaCl (0 to 0.7 m) in 0.1 m Tris buffer, pH 7.8, with 800 ml in each chamber. The pattern obtained in a typical experiment is shown in Fig. 2.

\textbf{Step 7}—The active fractions eluted from the four runs on DEAE-Sephadex, at pH 7.8, and at approximately 0.22 m NaCl, were pooled and dialyzed against 0.1 m sodium phosphate buffer, pH 6.3. This fraction, containing approximately 3.4 g of protein, was then concentrated by vacuum dialysis and adsorbed onto a column (2.5 \( \times \) 96 cm) of DEAE-Sephadex A-50 equilibrated with the same buffer. Elution was accomplished with a linear gradient of NaCl (0.1 to 0.7 m) in 0.1 m sodium phosphate buffer, pH 6.3, with 900 ml in each chamber. The pattern obtained in a typical experiment is shown in Fig. 3. The fractions obtained with constant specific activity (shaded area in Fig. 3) were pooled and stored as a frozen solution at \(-20^\circ\) for use in all of the studies reported here except where indicated.

\textit{Enzymic Activity}

Reduced RNase and randomly cross-linked RNase were used as substrates for the microsomal enzyme for routine screening of large numbers of fractions during the purification procedure. For comparing the specific activities of the active fractions from all steps in the procedure, however, randomly cross-linked STI was used as substrate. The recovery of protein and the specific activity achieved at each step are shown in Table I. No values for the specific activity of the enzyme in whole liver homogenates or in microsomal suspensions are given since they were so low as to be virtually unmeasurable by the STI assay. The 956-fold increase in specific activity of the purified enzyme over microsomal acetone powder extracts was obviously not due entirely to the removal of nonspecific extraneous protein. It is apparent that, as judged by the total enzyme units recovered at each step (Column 5 in Table I), inhibitory material was removed at several stages in the purification procedure. It has been known for some time that crude and partially purified preparations of the enzyme contained such material, since inhibition was produced in assays when more than optimal amounts of the enzyme were used. In addition, it may be that accurate determinations

\textsuperscript{3} The isolation of microsomes from beef liver was performed in the laboratory of Dr. David E. Green, Institute for Enzyme Research, Madison, Wisconsin. The microsomes, frozen in Madison, were transported to Bethesda packed in solid carbon dioxide.
of specific activity were not possible because of the nature of the assays employed, in which the substrate is, by necessity, rate-limiting, and in which catalytic rates may be influenced by the quantity of sulfhydryl groups present (see Footnote 1 of Reference 2).

The purest fractions of the microsomal enzyme catalyzed the reactivation of randomly cross-linked ST1 at an initial rate of 0.885 pmole per min per mg. This value represents an 1800-fold increase over that previously reported (2), although all of the absorbance at 280 nm of the eluate; O---O, the salt gradient; • • • • • •, the enzymic activity (in arbitrary units). The fractions with constant specific activity (shaded area) were pooled, divided into aliquots, and stored as a frozen solution at -20°C.

**Table I**

Purification of microsomal enzyme

<table>
<thead>
<tr>
<th>Material</th>
<th>Step in purification (see text)</th>
<th>Protein</th>
<th>Specific activity*</th>
<th>Enzyme units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef liver</td>
<td>1</td>
<td>654</td>
<td>0.61</td>
<td>168</td>
</tr>
<tr>
<td>Microsomes</td>
<td>2</td>
<td>275</td>
<td>6.4</td>
<td>428</td>
</tr>
<tr>
<td>Acetone powder extract</td>
<td>3</td>
<td>67.2</td>
<td>9.2</td>
<td>488</td>
</tr>
<tr>
<td>Anion exchange resin</td>
<td>4</td>
<td>53.3</td>
<td>30.7</td>
<td>816</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>5</td>
<td>26.6</td>
<td>30.7</td>
<td>816</td>
</tr>
<tr>
<td>DEAE-Sephadex (pH 7.8)</td>
<td>6</td>
<td>3.42</td>
<td>361.0</td>
<td>1233</td>
</tr>
<tr>
<td>DEAE-Sephadex (pH 6.3)</td>
<td>7</td>
<td>1.14</td>
<td>885.0</td>
<td>970</td>
</tr>
</tbody>
</table>

* Assayed by reactivation of randomly cross-linked ST1 (see text).

Tissue weight was 24 kg.
the measurements of activity are done under conditions of limiting substrate (2). This rate is in the range that would be expected for an enzyme which catalyzes the proper folding of a newly synthesized polypeptide chain as judged by estimates for the rate of protein synthesis in mammalian cells (22, 23).

Stability of Sulphydryl-Disulfide Interchange Enzyme

The enzyme was quite stable when stored as a frozen solution. The small amount of inactivation that occurred over several months could be reversed, at least partly, by treatment with 0.4 M β-mercaptoethanol for 1 hour at 25°C (followed by removal of the β-mercaptoethanol by gel filtration). This phenomenon is presently under investigation.

Electrophoresis in Polyacrylamide Gels

Electrophoresis of the various fractions obtained during the preparation of the sulphydryl-disulfide interchange enzyme proved to be a sensitive and reproducible guide in monitoring the stepwise removal of contaminants. The pattern obtained from the final enzyme preparation is shown in Fig. 4. Two bands of approximately equal intensity were present. After reduction and alkylation, however, only one band was found. It thus appears that both bands originally present represent variants of the same protein, differing only in charge. In addition, a faint third band was noted. This band, which is difficult to discern in the photograph shown in Fig. 4, represented a contaminant of higher molecular weight. It was also identified on starch gels and by ultracentrifuge studies.

**TABLE II**

Amino acid composition of microsomal enzyme

Amino acid analyses were performed in duplicate on timed hydrolysates of the reduced, carboxymethylated enzyme, as described in the text, and the results were averaged and extrapolated to zero time. The values shown in Column 2 were calculated on the basis of micromoles recovered from the columns. These figures were converted to residues per molecule, assuming a molecular weight for the enzyme of 42,000 (exclusive of tryptophan). The latter numbers, rounded off to the nearest whole integer, are shown in Column 3.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Concentration</th>
<th>Residues per molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>11.46</td>
<td>41</td>
</tr>
<tr>
<td>Threonine</td>
<td>4.23</td>
<td>15</td>
</tr>
<tr>
<td>Serine</td>
<td>4.47</td>
<td>16</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>15.84</td>
<td>57</td>
</tr>
<tr>
<td>Proline</td>
<td>3.85</td>
<td>14</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.46</td>
<td>23</td>
</tr>
<tr>
<td>Alanine</td>
<td>9.40</td>
<td>34</td>
</tr>
<tr>
<td>Valine</td>
<td>5.16</td>
<td>19</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.83</td>
<td>3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>4.47</td>
<td>16</td>
</tr>
<tr>
<td>Leucine</td>
<td>9.05</td>
<td>33</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.25</td>
<td>8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>7.00</td>
<td>25</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>99.92</strong></td>
<td><strong>359</strong></td>
</tr>
</tbody>
</table>
Electrophoresis in Starch Gels

The final preparation of enzyme was subjected to electrophoresis in starch gel after dialysis against 0.04 M sodium phosphate buffer, pH 6.5, and concentration by vacuum dialysis. The pattern obtained in a typical experiment is shown in Fig. 5. Two major components of the same intensity were close together. A third, minor component ran more slowly. The proteins of the two major bands were eluted from the gel electrophoretically and assayed for enzymic activity, care being taken to avoid cross-contamination (see "Materials and Methods"). Both fractions were found to be enzymically active, the faster moving band having approximately twice the specific activity of the slower moving band. The fact that both of the bands were enzymically active is a further indication that they represent variants of the same protein.

Visible and Ultraviolet Absorption Spectrum

The absorption spectra of the final enzyme preparation showed no peaks between 650 m\(\mu\) and 250 m\(\mu\) other than a single peak with a maximum at 278 m\(\mu\).

Ultracentrifuge Studies

Fig. 6 represents a plot of the sedimentation values for the microsomal enzyme at various protein concentrations. The \(s_{20,w}\), extrapolated to zero protein concentration, was calculated to be 3.26. Fig. 7 represents a schlieren pattern for the enzyme preparation at a concentration of 8 mg per ml. The heterogeneity evident on the leading edge of the sedimenting boundary was due to contamination with faster sedimenting material which represented approximately 8 to 10\% (by weight) of the sample. A weight average molecular weight, obtained by the high speed equilibrium technique, was calculated to be 42,000. There was no significant variation of the calculated molecular weight as a function of either concentration or ultracentrifugal speed within the ranges examined (0.1 to 0.4 mg per ml and 33,450 to 39,000 rpm). The heavier contaminant seen in the schlieren patterns obtained during the velocity centrifugations (Fig. 7) was also apparent in the high speed equilibrium experiments, as evidenced by a calculated Z average molecular weight for the entire sample of 49,000. The smallest species in the sample, representing greater than 90\% of the material, was estimated to have a molecular weight of 42,000.

Amino Acid Composition

The amino acid composition of the final preparation of the microsomal enzyme is shown in Table II. The notable points from these data are that no amino acid was missing, that there were 3 S-carboxymethylcysteine residues per molecule and that the total number of lysine plus arginine residues per molecule was 44.

The presence of 3 half-cystine residues per molecule in the sulfhydryl-disulfide interchange enzyme is of special interest because of the unique catalytic activity of this enzyme. Studies to determine the essentiality of one or more of these residues and to define the interactions in which they may be involved during the catalysis are now in progress.

Peptide Maps

Peptide maps prepared from tryptic digests of the enzyme reproducibly showed 48 ninhydrin-positive spots. This figure agrees quite closely with the theoretical figure of 45 expected, on the basis of the arginine plus lysine content, if the protein is assumed to be pure, to have a molecular weight of 42,000, and not to contain identical subunits.

Acknowledgments—We wish to thank Dr. David E. Green, Institute for Enzyme Research, Madison, Wisconsin, without whose generosity and help in the large scale preparation of beef liver microsomes the study reported here would not have been possible. We are also grateful to Mr. Clifford Lee for running the automatic amino acid analyzers, Dr. K. Hoerman, Naval Medical Research Institute, Bethesda, Maryland, for carrying out the electrophoreses in starch gels, and Dr. Pál Venetianer, Institute of Medical Chemistry, University of Medicine, Budapest, for his critical reading of the manuscript.

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