Catabolism of Desialylated Ceruloplasmin in the Liver*

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SUMMARY

When desialylated ceruloplasmin (ASCPN), labeled with 64Cu and with 3H in its galactose residues, is injected intravenously into a rat, more than half of the radioactivity can be recovered within minutes in ASCPN precipitated immunologically from liver homogenates. Both radioautographic studies and measurements of hepatic uptake of ASCPN following blockade of the reticuloendothelial system indicate that ASCPN enters only hepatocytes. Serial measurements on the immunoprecipitated ASCPN indicate that cleavage of 64Cu is 80% complete within 30 min of injection, whereas galactose is split from the protein even faster. Sucrose gradient fractionation of liver homogenates from rats treated with Triton WR 1339 or dextran reveals the principal site of the catabolic cleavage of copper and galactose to be lysosomes.

We were thereby enabled to show that cleavage of copper and galactose from ASCPN occurred within minutes, principally in hepatocytic lysosomes. Since native serum albumin had previously been shown by Freeman, Gordon, and Humphrey (3) to enter hepatocytes, rather than Kupffer cells, it seemed probable that our findings had physiological significance.

EXPERIMENTAL PROCEDURE

Materials—The sources and chemical forms of 64Cu and 3H, the preparation of human ASCPN, its labeling with 64Cu and 3H (the latter in the galactose moieties), and the assay of both forms of radioactivity have been described previously (1, 4).

To prepare rat ASCPN labeled with 64Cu, three to four rats were injected intravenously with 10 µg each of 64Cu, as copper acetate, and killed 24 hours later. Their sera were pooled, diluted with 2 volumes of 0.05 m sodium phosphate buffer, pH 7.0, and passed through a DEAE-cellulose column equilibrated with the same buffer containing 0.3% NaCl. The column was washed with this buffer until the A280 of the eluate was less than 0.150, whereupon ceruloplasmin was eluted with 0.05 m sodium phosphate buffer, pH 7.0, containing 1.3% NaCl, and precipitated with 2 volumes of ethanol. The precipitate was dissolved in 0.1 m sodium acetate buffer, pH 5.6, containing 1% NaCl, and centrifuged in a Spinco model L ultracentrifuge at 100,000 × g for 5 min. The supernatant solution of ceruloplasmin was passed through a column of Chelex 100 (Calbiochem) equilibrated with the sodium acetate buffer. The ratio of A280/403 nm of the eluted ceruloplasmin was 0.046, which is the same as that obtained for purified human ceruloplasmin (4). Desialylation was carried out in 0.1 m sodium acetate buffer, pH 5.6, by incubation, at 37° for 60 min, with 0.001 unit of Clostridium perfringens neuraminidase (Worthington) per mg of ceruloplasmin. Heat-denatured human albumin was prepared as described by Benacerraf et al. (5).

p-Nitrophenyl phosphate, cytochrome c, DL-β-glycerophosphate (disodium salt, grade I), and dextran (type 100 C, average mol wt 135,000) were purchased from Sigma; p-nitrophenyl-β-D-galactopyranoside from General Biochemicals; Triton WR 1339 from Ruge Chemical Company, Irvington-on-Hudson, New York; and Triton X-100 from Hartman-Leddon, Philadelphia, Pennsylvania. Antihuman ceruloplasmin goat serum was kindly supplied by Dr. John B. Robbins, National Institute of Child Health and Development, Bethesda, Maryland.

Methods—In the principal experiments of this study, 300 µg of human ASCPN, in 1 ml of 0.1 m sodium acetate buffer, pH 5.6, containing 0.9% NaCl, were injected rapidly into the tail veins of adult male albino rats (Wistar) weighing about 200 g. At intervals after the injection, the rats were killed by decapitation;
the livers were quickly removed, washed with an ice-cold solution of 0.9% NaCl, blotted with filter paper, and weighed. Aliquots of 2 to 6 g were minced with scissors, suspended in 4 ml of 0.3 m sucrose per g of liver, and homogenized for 2 min at low speed using a Potter-Elvehjem homogenizer and Teflon pestle (6). Centrifugation of the homogenate in the cold at 600 × g for 10 min precipitated the nuclear fraction. The crude mitochondrial-lysosomal fraction was obtained by centrifuging the supernatant at 14,000 × g for 30 min. The second supernatant was centrifuged at 100,000 × g in a Spinco model L ultracentrifuge to yield a microsomal fraction and a third supernatant. Each of the three particulate fractions was washed twice with 0.3 m sucrose, suspended in either 10 ml of the sucrose solution or distilled water, and subjected to appropriate analytical procedures.

Heat-denatured human albumin and 64Cu-labeled human ASCPN were injected into rats in an experiment designed to confirm or contradict our preliminary finding (1) that hepatocytes, and not cells of the reticuloendothelial system, take up injected ASCPN.

Subfractions of the mitochondrial-lysosomal fraction (7) were obtained by density gradient centrifugation. Two solutions, one containing 59.7 and the other 117.0 g of sucrose per 100 g of water, were delivered by a density gradient mixer (Buchler Instruments, Inc., Fort Lee, New Jersey) into plastic Spinco centrifugation tubes (6-ml capacity). One milliliter of the mitochondrial-lysosomal fraction in 0.3 m sucrose, corresponding to 0.6 g of liver, was layered on top of 5 ml of the sucrose gradient. The tubes were centrifuged at 39,000 rpm for 150 min in the swinging bucket rotor of the Spinco model L ultracentrifuge, and 0.5-ml fractions were carefully pipetted off. In these experiments, an intraperitoneal injection of 1 ml of 0.0% NaCl alone, or containing 200 mg of dextran, or 200 mg of Triton WR 1339, was given to experimental rats 3 days after the 64Cu-ASCPN.

Immunoprecipitation of ASCPN was carried out by mixing 2 to 4 ml of liver homogenate, or 2 to 4 ml of a subcellular fraction, with an equal volume of 0.4% sodium dodecyl sulfate and stirring for 30 min in the cold. After centrifugation at 100,000 × g for 1 hour, 0.02 ml of a 2% solution of human ceruloplasmin and 2 ml of antihuman ceruloplasmin goat serum were added to the supernatant, which always contained more than 95% of the sample's total radioactivity. The mixture was incubated at 37° for 1 hour and centrifuged in the cold at 3,000 rpm for 10 min. After washing with cold 0.9% NaCl, the precipitate was dissolved in 8% acetic acid and assayed for radioactivity (1).

Enzyme Assays—Prior to determining enzymatic activity, Triton X-100 was added, to a final concentration of 0.1%, to homogenates, subcellular fractions, or fractions obtained from density gradient centrifugation. p-Nitrophenyl phosphate or

TABLE I

<table>
<thead>
<tr>
<th>Material infused</th>
<th>64Cu in whole liver %</th>
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<tr>
<td>64Cu-ASCPN (0.26 mg)</td>
<td>95.0</td>
</tr>
<tr>
<td>64Cu-ASCPN (0.26 mg) and heat-denatured albumin (20.0 mg)</td>
<td>93.5</td>
</tr>
</tbody>
</table>

Fig. 1. Recovery of 64Cu from entire rat liver (—) following injection of 300 μg of human 64Cu-ASCPN. Each point is the average of the recoveries in two or three animals (—). 64Cu in immunoprecipitable ASCPN.

Fig. 2. Intracellular distribution of 64Cu in fractions of rat liver as a function of time following injection of 300 μg of human 64Cu-ASCPN. The height of each bar represents 64Cu in the fraction; the shaded portion of the bar represents 64Cu recovered in immunoprecipitable ASCPN. Values are the means of two to five rats. At a given time, the sum of 64Cu recovered in each fraction was 80 to 95% of the radioactivity. N, nuclear fraction; ML, crude mitochondrial and lysosomal fraction; P, microsomal fraction; S, cytosol. Of the acid phosphatase activity present in the homogenate, the mitochondrial-lysosomal fraction contained 45.1% ± 4.3 with p-nitrophenyl phosphate as substrate and 35.6% ± 3.1 with l-glycerophosphate as substrate.
dl-β-glycerophosphate were used as substrates for acid phosphatase activity (8, 9) and p-nitrophenyl-β-D-galactopyranoside for β-galactosidase activity (10). The method of Cooperstein and Laarow (11) was used for the determination of cytochrome c oxidase activity and that of Lowry et al. (12) for total protein in the mitochondrial-lysosomal fractions.

RESULTS

Hepatic Uptake and Intracellular Distribution of ASCPN—

Three minutes after intravenous administration of human 64Cu-ASCPN, 50% of the radioactivity was present in the rat's liver. Uptake was maximal, with over 90% of the label recoverable from the liver 9 to 15 min after injection (Fig. 1). With homologous rat 64Cu-ASCPN, 89% of the label was recovered in the liver 9 min after administration and 97.2% 15 min after administration. Blockade of the Kupffer cells by the simultaneous injection of 20 mg of heat-denatured albumin (2) (Table I) did not alter the rapidity of uptake or the intracellular localization of ASCPN.

Following injection of 64Cu-ASCPN, the radioactivity found in various subcellular fractions and in immune precipitates prepared from them is shown in Fig. 2. Up to 15 min after injection, most of the radioactivity is found in the microsomal and mitochondrial-lysosomal fractions. Total radioactivity in the former decreases with time, whereas that in the latter increases up to 15 min. In the latter fraction, the ratio of 64Cu-ASCPN to total 64Cu falls sharply with time.

To investigate more precisely the site of ASCPN uptake in the mitochondrial-lysosomal fraction, rats were previously treated with either Triton WR 1339 or dextran, which are known to decrease (13) or increase (14), respectively, the specific gravity of lysosomal, but not mitochondrial, particles. In control rats previously treated with NaCl solution, 27% of the immunoprecipitable 64Cu-ASCPN appeared in the region of light Fractions 2 and 3 of the gradient, and 48% was found in the heavier Fractions 5 to 8 (Fig. 3a), a bimodal distribution strikingly similar to that of the two lysosomal enzymes, acid phosphatase and β-galactosidase (Fig. 3b). In rats previously treated with Triton WR 1339, 78% of 64Cu-ASCPN, 72% of acid phosphatase, and 65% of β-galactosidase were recovered in the light Fractions 2 and 3 (Fig. 3, d and e), and little, if any, of the three was in Fractions 5 to 8. In the rats previously treated with dextran, 39% of 64Cu-ASCPN and 34 to 36% of the lysosomal enzymes were recovered in the heavy Fractions 8 to 10 (Fig. 3, g and h), although 28% of ASCPN and 13 to 14% of
residues, the ratio of $^3$H:$^{64}$Cu in the specific precipitate of ASCPN was the same as that in the injected material (Fig. 5). By 30 min after injection, this ratio had decreased to 0.46, indicating that galactose had been removed from the protein before the molecule had lost either an equivalent amount of copper or its immunochemical specificity. In the mitochondrial-lysosomal fraction, cleavage of both tritiated galactose and $^{64}$Cu occurred in the light and in the heavy subfractions (Table II). Catabolism also occurred in the microsomal fraction, where the ratio of $^3$H:$^{64}$Cu, unchanged after 9 min, had decreased by 30% at 30 min.

**DISCUSSION**

Although much is known about the synthesis of plasma proteins, relatively little is known of the mechanism by which they are catabolized. The fundamental reason for this discrepancy is technical. It is relatively easy to follow a labeled precursor to a synthesized, identifiable protein product. Its individual catabolites, on the other hand, cannot be readily isolated and identified because of two consequences of the rapid intracellular degradation of the protein. First, the intracellular concentration of protein which still possesses measurable characteristics of the circulating intact protein is always very low in comparison with the latter. Thus, the protein which is intracellular cannot be distinguished from that unavoidably present in the plasma which contaminates homogenates. Second, the continual entry of the protein's catabolic products, whether labeled or not, into various pathways and pools makes their identification extraordinarily difficult. Only if nearly instantaneous transfer of all of the labeled injected protein from the circulation into the target cells can be achieved can the blood be effectively freed of the protein, and at least some of the protein's catabolites identified and assayed.

Rapid clearance from the circulation and prompt uptake by reticuloendothelial cells occurs when denatured plasma proteins are injected into animals (2). But information about the catabolism of denatured proteins is unlikely to be applicable to native proteins (10). Further, there is evidence, already cited, that native albumin is catabolized in hepatocytes rather than in Kupffer cells, since blockade of reticuloendothelial cells with carbon particles does not affect the rate of hepatic uptake of screened $^{131}$I-labeled albumin (3).

ASCPN is, of course, a modified form of ceruloplasmin which is not known to be present under physiological conditions. Yet it is very little different from native ceruloplasmin, possessing almost all of the physical, chemical, and immunochemical characteristics of the latter (1), to which it can even be converted by resialylation (16). Furthermore, our earlier radioautographic finding that ASCPN is taken up only by hepatocytes in the rabbit (1), has been confirmed in the rat both by radioautography* and by blockade of the reticuloendothelial system (Table I). Because of such minimal and probably insignificant modification, and because of its rapid transfer from blood into hepatocytes,

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*The decrease in the ratio of $^3$H:$^{64}$Cu was shown not to be due to contamination of the specific precipitate by $^{64}$Cu released from the protein. Rat $^{64}$Cu-ASCPN (0.14 mg and 4000 cpm) and 0.16 mg of human unlabeled ASCPN were injected into a rat, and the rat liver was removed 30 min later. No radioactivity was found in the specific precipitate obtained with antiserum to human ceruloplasmin.

1. Sternlieb and M. Ma, unpublished observations.
ASC PN appears a more nearly ideal material with which to study protein catabolism than such foreign proteins as egg albumin (17) or horseradish peroxidase (18). For such a study, it is probably even preferable to hemoglobin, which, although a native protein, only enters hepatocytes when it is administered in unphysiological amounts that far exceed the capacity of the reticuloendothelial system, to which it usually goes (19, 20).

Our results indicate that 75% of the injected dose of ASC PN is taken up by the liver in 6 min and that catabolism of copper from the molecule is about 80% complete within 30 min of injection (Fig. 1). Yet the protein's galactose residues are split, by a process which is presumably enzymatic, about twice as rapidly as the copper since the ratio of $^{64}$Cu in ASC PN falls to 0.46 in 30 min.

The principal subcellular site of the catabolism of ASC PN appears to be lysosomal. Light and heavy particles, both possessing acid phosphatase and β-galactosidase activity, and both, therefore, lysosomal, are distinguishable by sucrose gradient centrifugation of the mitochondrial-lysosomal fraction (Fig. 3b). Prior treatment of rats with Triton WR 1339 decreases the specific gravity of the heavy lysosomes, and both the immunoprecipitable ASC PN and the two lysosomal enzyme markers are shifted to the left, as a comparison of Fig. 3d with 3a and Fig. 3e with 3b shows. Prior treatment of rats with dextran increases the specific gravity of the heavy lysosomes, and both the immunoprecipitable ASC PN and the two lysosomal enzyme markers move to the right, as a comparison of Fig. 3g with 3a and 3h with 3e shows. Those portions of the ASC PN, a and b) are not appreciably affected by Triton WR 1339 or dextran treatment.

The lysosomal cleavage of the copper and galactose of ASC PN in vivo is consistent with the finding of Mahadevan, Dillard, and Tappel (21) and of Coffey and de Duve (22) that plasma proteins can be degraded by lysosomal extracts in vitro. Some catabolism of ASC PN in vivo occurs in microsomes as well (Fig. 2), but this may merely be apparent, representing contamination of the microsomal fraction by lysosomes remaining in the second supernatant.

The rapid transfer of ASC PN to hepatocytes from blood permits pulse labeling of these cells. This has made it possible to investigate the rates and sites of the catabolic cleavage of the copper and galactose moieties of this protein. Similar studies with other desialylated glycoproteins may prove feasible and may yield information about both their catabolism and the functioning of lysosomes.

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