Synthesis of catalase in two cell-free protein-synthesizing systems and in rat liver
(organelle biogenesis/peroxisomes)

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ABSTRACT Rat liver polysomal RNA was translated in the rabbit reticulocyte lysate and in the wheat germ cell-free protein-synthesizing systems, using [35S]methionine as label. The catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) that was synthesized was isolated by immunoprecipitation and characterized by electrophoresis in sodium dodecyl sulfate/polyacrylamide gels followed by fluorography. The catalase made in both systems migrated more slowly during electrophoresis than did purified peroxisomal catalase. By comparison with standards of known molecular mass, the cell-free products were estimated to be about 4000 daltons larger than the purified enzyme. We also investigated the biosynthesis of catalase in vivo by injecting [35S]methionine into rats. The precursor of catalase known to be synthesized in liver and found in the high-speed supernatant 8 min later [Lazarow, P. B. & de Duve, C. (1973) J. Cell Biol. 59, 491–506] was isolated immunologically. For comparison, 1-day-old completed catalase was immunoprecipitated from peroxisomes. The migrations in sodium dodecyl sulfate gels of the 8-min-old precursor and the subunit of the day-old enzyme were indistinguishable and approximately the same as the migration of the cell-free products. These results indicate that catalase's apparent size does not change when it enters peroxisomes but rather decreases during the chemical purification procedure.

Little is known about the messenger RNAs for enzymes that are located in the organelles of eukaryotic cells. Equally little is known about the primary translation products of these mRNAs, and of the mechanisms whereby these proteins are integrated into their host organelles. This contrasts with the considerable knowledge that has accumulated about the translation products of secretory protein mRNAs (1–11) and the nature of the secretory pathway itself (12).

Catalase (hydrogen-peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) is a principal and characteristic enzyme of the peroxisomes of rat liver (13). This hemoprotein has a molecular weight of about 240,000 and consists of four identical subunits. Lazarow and de Duve (14) showed that this enzyme is synthesized in the liver as a precursor that (i) is approximately the size of a catalase monomer (by sedimentation analysis), (ii) lacks heme, and (iii) does not accompany catalase through its chemical purification procedure. The exact site of synthesis of this precursor is unknown, but the precursor is first recovered in the high-speed supernatant from carefully prepared homogenates (15). Beginning about 8 min after its synthesis, it is transferred into peroxisomes with a half-time of about 14 min. Inside the peroxisome it acquires heme and then aggregates to form an active tetrameric catalase molecule (15).

In order to learn more about the early steps in catalase biosynthesis, we have translated catalase mRNA in both the reticulocyte lysate and wheat germ protein-synthesizing systems and have characterized the product by electrophoresis in sodium dodecyl sulfate (NaDodSO4)/polyacrylamide gels. We have similarly characterized the in vivo catalase precursor as well as finished peroxisomal catalase.

METHODS

Polysomes were prepared from rat liver according to Taylor and Schimke (16). The polysomes were treated with NaDodSO4 and ethylenediaminetetraacetate (EDTA), precipitated with ethanol, and redissolved as described (16). Polysomal RNA was then precipitated and deproteinized with 4 M urea/2 M LiCl/2 mM EDTA at 0°. The RNA was washed with 3 M Na acetate, pH 6/5 mM EDTA at 0° and reprecipitated three times with ethanol. Rabbit reticulocyte lysate and wheat germ extract were prepared according to Rhoads et al. (17) and Roman et al. (18), respectively.

The polysomal RNA (containing mRNA and rRNA) was translated with the reticulocyte lysate at a final concentration of 0.2 mg/ml as described by Palmiter (19), except that we used [35S]methionine (560 Ci/mmol) at 0.35 mCi/ml and 19 unlabeled amino acids at a concentration of 0.1 mM each and omitted hemin.

The RNA was translated in the wheat germ system in a manner based on that of Roman et al. (18). The most important difference was the addition of the human placental ribonuclease inhibitor described by Blackburn et al. (20) to a final concentration of 4 μg/ml. The inhibitor was dialyzed against 0.15 M NaCl/5 mM dithiothreitol/20 mM 2-amino-2-hydroxy-methyl)-1,3-propanediol (Tris)-HCl buffer, pH 7.5/1 mM EDTA/15% (vol/vol) glycerol. It was stable at −85° for months and was diluted at least 1:40 when added to the translation mixture in order to avoid inhibiting the translation with the solutes that stabilize the inhibitor. Other components of the translation mixture were: 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes)/KOH at pH 7.7, 110 mM K acetate, 1.6 mM Mg acetate, 0.08 mM spermine, 1 mM dithiothreitol, 1 mM ATP, 0.12 mM GTP, 8 mM creatine phosphate, creatine kinase at 0.12 mg/ml, [35S]methionine (560 Ci/mmol) at 0.5 Ci/ml, 19 unlabeled amino acids at 0.02 mM each, liver polysomal RNA at 0.3 mg/ml, and 0.35 ml of wheat germ extract per ml.

At the end of the in vitro translations, we added Triton X-100, sodium deoxycholate, and unlabeled methionine to final concentrations of 1%, 1%, and 10 mM, respectively, and centrifuged for 4 min in the Brinkmann microcentrifuge. Catalase was isolated by direct immunoprecipitation according to Taylor and Schimke (16). We used 5 μg of purified rat liver peroxiso-

Abbreviation: NaDodSO4, sodium dodecyl sulfate.
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mal catalase as carrier and excess goat anticalatalase prepared by Lazarow and de Duve (14) and known to be monospecific for catalase. Control immunoprecipitations were performed with rabbit gamma globulins and sheep anti-rabbit gamma globulins prepared by Robbi et al. (21).

Rat liver peroxisomal catalase was purified as described previously (22). One preparation of catalase was labeled in vivo by the intraperitoneal injection of 5 mCi of [3H]leucine 1 day before killing the rat; it was used in the experiments of Figs. 1, 2, and 4. Another preparation of catalase was labeled with [14C]acetic anhydride (23) and used in other experiments, included in Table 1. Horse skeletal muscle myoglobin, human erythrocyte carbonic anhydrase b, chicken ovalbumin, beef liver catalase, and Escherichia coli β-galactosidase were labeled with [14C]acetic anhydride and used as molecular weight standards.

Immunoprecipitates of catalase and other samples for NaDodSO₄/polyacrylamide gel electrophoresis were dissolved in 50 μl of 2% NaDodSO₄/0.06 M Tris-HCl buffer, pH 6.8/10 mM dithiothreitol/0.001% bromophenol blue/10% sucrose, and heated at 100° for 5 min. The reduced proteins were then alkylated at room temperature with 20 mM iodoacetamide for 30 min. They were electrophoresed for 1800 V-hr (at constant voltage) in 1-mm-thick slab gels containing a linear gradient of 7–15% acrylamide and a 3% acrylamide stacking layer. All other conditions were essentially those used by Scheele (24) for his second-dimension electrophoresis. We obtained highly reproducible absolute migrations of the proteins by keeping the total volt-hours constant. The voltage could be regulated to suit one's convenience; values up to 125 V gave satisfactory results. After electrophoresis, the slab gels were stained and destained, and fluorograms were prepared as described by Bonner and Laskey (25).

Isotopes were purchased from New England Nuclear (Boston, MA), female Sprague–Dawley rats were obtained from Charles River (Wilmington, MA), the electrophoresis materials were from Eastman (Rochester, NY), and most other reagents and the protein standards came from Sigma (St. Louis, MO).

RESULTS

Translation with Reticulocyte Lysate. When rat liver polysomal RNA is translated in the reticulocyte lysate, the pattern of total proteins synthesized (Fig. 1, lane 3) is similar to that of the translation products in the absence of added messenger (lane 2), indicating that most of the mRNAs undergoing translation are endogenous to the reticulocyte lysate. The proteins synthesized are numerous and range in molecular mass from less than 15,000 to several hundred thousand daltons.

Several additional minor bands are apparent when liver polysomal RNA is present during the translation (lane 3, arrows). Serum albumin would be the most prominent of these, but is masked by the heavy endogenous protein band of approximately 71,000 daltons. We find that under these conditions, but using [3H]leucine as label, albumin represents 0.5% of the total proteins synthesized. This is in close agreement with the results of Taylor and Schimke (16) and indicates that both the polysomal RNA and the reticulocyte lysate are highly active.

Catalase is invisible among the total proteins synthesized in the reticulocyte lysate. However, when 5 μg of carrier catalase is added to 130 μl of translation mixture and immunoprecipitated with excess anticalatalase, a single major radioactive protein band is clearly seen (Fig. 1, lane 6). This protein migrates distinctly more slowly during electrophoresis than does purified rat liver catalase (lanes 4 and 7). It is not synthesized by the reticulocyte lysate in the absence of added liver mRNA (Fig. 1, lane 5). The unlabeled carrier catalase in lanes 5 and 6, stained with Coomassie blue (not shown), had the same migration as the labeled catalase in lanes 4 and 7; i.e., it moved farther than the labeled cell-free product during coelectrophoresis.

Translation with Wheat Germ. Rat liver polysomal RNA was also translated in the wheat germ in vitro protein-synthesizing system, in the presence of placental ribonuclease inhibitor (20). As illustrated in Fig. 2, lane 2, many proteins are synthesized by the wheat germ system under the direction of the ex-
Fig. 2. Fluorogram of NaDdSO₄ gel showing products of translation of rat liver polysomal RNA in the wheat germ protein-synthesizing system. Lanes 1 and 2: total proteins synthesized in 0.2 μl of translation mixture in the absence and presence of liver RNA, respectively. Lane 3: purified rat liver catalase labeled in vivo with [³⁵S]methionine for 1 day. Lanes 4–7: immunoprecipitates from [³⁵S]L-leucine of the translation of liver RNA without (lanes 4 and 5) and with (lanes 6 and 7) ribonuclease inhibitor. Catalase immunoprecipitates are shown in lanes 4 and 6. Control immunoprecipitates of rabbit gamma globulins by sheep anti-rabbit gamma globulins are shown in lanes 5 and 7. Exposure was for 3 days except for lane 3, which was for 1 month. The arrow by lane 2 indicates the band tentatively identified as catalase. The positions and molecular weights (in thousands) of the standards in this gel are indicated in the left margin.

Orogenous liver mRNAs. The most prominent one has been identified as serum albumin by immunoprecipitation (not shown). None of these proteins are synthesized in the absence of liver mRNA (lane 1). The wheat germ does make small amounts of its own proteins, but they are not visible at this exposure.

Catalase was specifically immunoprecipitated from the wheat germ translations. As illustrated in Fig. 2, lane 6, a single strongly radioactive band is visible; it has migrated more slowly than the purified catalase shown in lane 3. As a test of the specificity of the immunoprecipitation, a sheep anti-rabbit gamma globulin immunoprecipitate was prepared, using an identical aliquot of the translation mixture. Three faint bands are visible (lane 7). They correspond to three of the faint bands in the catalase immunoprecipitate (lane 6), and to three of the stronger bands in the total translation (lane 2). These clearly are nonspecific contaminants. The catalase immunoprecipitate also contains some additional faint bands of unknown identity. They might be incomplete catalase chains or be due to very small amounts of unrelated antibodies in our anticalatalase serum.

Lanes 4 and 5 of Fig. 2 illustrate the results of a duplicate experiment in which the placental ribonuclease inhibitor was omitted during the translation. No catalase was synthesized. In addition, no catalase is synthesized by the wheat germ in the absence of added liver polysomal RNA, whether or not inhibitor is present (not illustrated). The arrow in lane 2 indicates a faint band among the total proteins that is likely to be the catalase, on the basis of its position and intensity.

The catalase synthesized in the wheat germ was immunoprecipitated, digested with trypsin, and fractionated by ion exchange chromatography. Preliminary results indicate that the pattern of [³⁵S]methionine-labeled tryptic peptides from the in vitro product is similar to the pattern obtained from purified catalase labeled in vivo.

**Biosynthesis In Vivo.** It had previously been demonstrated...
that rat liver catalase is synthesized in vivo as an apomonomer (14). We labeled this precursor by injecting 500 μCi of [35S]-methionine intraperitoneally into a rat and excising the liver 5 min later. A high-speed supernatant was prepared from the homogenate of the liver and catalase was isolated by immunoprecipitation.

For comparison, we also immunoprecipitated some catalase from hepatic peroxisomes that were isolated from a rat 1 day after it received 1 mCi of [35S]methionine by intraperitoneal injection.

As illustrated in Fig. 3, the 8-min-old supernatant precursor of catalase and the 1-day-old peroxisomal catalase have indistinguishable mobilities; both migrate more slowly than does peroxisomal catalase that has been subjected to chemical purification. As shown in Fig. 4, the in vitro precursor of catalase has approximately the same mobility as the wheat germ cell-free product.

**Apparent Molecular Masses.** The apparent size of the catalase synthesized in the two cell-free protein-synthesizing systems was estimated by comparison with standards run in the same gels. In seven experiments it was 65,800 ± 700 daltons. Purified catalase (four batches) in these seven experiments was about 4000 daltons smaller (Table 1). However, both species of catalase immunoprecipitated after in vivo labeling (the apomonomer precursor and the completed enzyme from peroxisomes) were about the same size as the cell-free products (Table 1). Therefore, the major change in size that we observe occurs not when catalase enters peroxisomes, but rather when the peroxisomal catalase is chemically purified.

**DISCUSSION**

These results demonstrate the translation of the mRNA of the major peroxisomal enzyme, catalase, in two cell-free protein-synthesizing systems. The identity of the product is based on its immunoprecipitation by an antibody known to be specific for catalase (14), by its migration as a single band in NaDodSO4 gels with a mobility similar to that of catalase, and by preliminary tryptic peptide analysis. The lack of appreciable nonspecific contaminants in the immunoprecipitates of Figs. 1 and 2 illustrates the remarkable selectivity that may be obtained by immunoprecipitation: catalase represents approximately 0.1% and 0.005% of the total proteins that were synthesized in the wheat germ and reticulocyte lysate systems, respectively, based on [3H]leucine incorporation. The translation of catalase mRNA has been reported previously by several workers (26, 27), including the second author of this paper (28), but the product was not characterized in those studies.

The placental ribonuclease inhibitor described by Blackburn et al. (20) was found to be extremely useful for wheat germ translations, in agreement with previous observations by Scheele and Blackburn (ref. 29 and personal communications). In the experiment of Fig. 2, no catalase was synthesized when the inhibitor was omitted. However, not all batches of wheat germ are as dependent on inhibitor as was this one.

Our observation that the in vitro translation product of catalase mRNA appears to be about 4000 daltons larger than purified catalase recalls the findings that many secretory proteins [with the exception of ovalbumin (30)] are initially synthesized with an extra NH2-terminal peptide believed to play a role in directing the nascent polypeptide into the endoplasmic reticulum (signal hypothesis) (1-11). In the present case, however, the in vitro product is not appreciably smaller than that synthesized in vitro, and no difference is found between the in vitro precursor of catalase and the subunit of the mature enzyme. The only molecular species that is significantly different in size is the peroxisomal catalase that has gone through the chemical purification procedure. This may be an artifact of purification: other cases are known in which a contaminating protease cleaves an enzyme during its purification (31).

That catalase should differ from secretory proteins in terms of its biosynthetic history is not particularly surprising, because it follows a different intracellular pathway and ends up in an organelle. After all, catalase newly synthesized in liver is found in the high-speed supernatant (15) while secretory proteins are found in microsomes. Furthermore, catalase is not taken up into peroxisomes until between 8 min and 1 hr after its biosynthesis (15), whereas the vectorial discharge of secretory proteins into the endoplasmic reticulum occurs during translation (12, 32).

**Table 1. Molecular masses of catalase species estimated by NaDodSO4/polyacrylamide gel electrophoresis***

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Apparent mass, daltons</th>
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<tbody>
<tr>
<td>Cell-free products*</td>
<td>65,800 ± 700</td>
</tr>
<tr>
<td>In vivo precursor</td>
<td>65,000–66,000</td>
</tr>
<tr>
<td>Peroxisomal catalase</td>
<td>65,000–66,000</td>
</tr>
<tr>
<td>Immunoprecipitated</td>
<td>65,000–66,000</td>
</tr>
<tr>
<td>Purified</td>
<td>61,800 ± 800</td>
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</tbody>
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* Mean and standard deviation of five experiments with wheat germ and two with reticulocyte lysate. [3H]Leucine, [14C]leucine, or 14C-labeled algal hydrolysate was used in place of [35S]methionine in some of the experiments.
Some recent observations on the biosynthesis of plant organelar enzymes may be relevant to our results. The mRNA for the small subunit of ribulosebisphosphate carboxylase (EC 4.1.1.39), a chloroplast enzyme, has been translated in the wheat germ system. The product appears to be larger than the authentic enzyme subunit according to NaDodSO₄ gel electrophoresis: the difference in molecular mass is reported to be 3500 daltons in the case of *Chlamydomonas reinhardtii* (33) and 6000 daltons for the pea enzyme (34). Very recently, the translation product of watermelon cotyledon glyoxysomal malate dehydrogenase mRNA was reported to be 5000 daltons larger than the purified enzyme, again according to NaDodSO₄ gel electrophoresis (35). These mass differences are considerably larger than the sizes of the known “signal peptides” of secretory proteins. Curiously, the 4000 dalton mass difference between the catalase cell-free product and the purified enzyme falls into the same size range.

The chloroplast differs from the peroxisomes by having two membranes rather than one, as well as by being a plant organelle. On the other hand, the glyoxysome is a close evolutionary relative of the peroxisome (36): both organelles metabolize H₂O₂ (13, 37), catalyze the β-oxidation of fatty acids (37, 38), and have single membranes. One might expect them to have similar mechanisms of biogenesis. The size of the glyoxysomal enzyme synthesized in *vitro* has not been determined without preliminary purification, as has been done for the animal catalase, so further comparison is not possible.

In summary, these results indicate that the size of the catalase subunit does not change appreciably as the enzyme precursor moves from its site of synthesis to the peroxisomes in rat liver and is processed to form finished catalase. Moreover, the results demonstrate the need for caution in deducing biogenetic relationships by comparing cell-free translation products with purified enzymes.

Further experiments are required on the uptake of catalase into peroxisomes. It may be kept in mind that the catalase precursor could contain structural information directing it to the peroxisome, without necessarily undergoing proteolytic processing.

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