Biosynthesis of Albumin via a Precursor Protein in Morris Hepatoma 5123tc¹,²

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SUMMARY

The mechanism of albumin biosynthesis was studied in Morris hepatoma 5123tc in vivo and in hepatoma cell suspensions obtained by solubilizing the intercellular matrix with collagenase and hyaluronidase.

In the in vivo experiments, L-[1-¹⁴C]leucine was injected i.v. into rats bearing hepatomas in the muscles of both hind legs. After 14 min, tumors were removed and homogenized. A protein fraction quantitatively precipitable with antialbumin was isolated from the homogenate by acetone fractionation and precipitation with antiserum against serum albumin. This protein fraction was not homogeneous. With the use of 3 consecutive chromatographies on diethylaminoethyl cellulose, a highly radioactive albumin-like protein could be separated from a large amount of only slightly radioactive albumin.

In hepatoma cell suspensions incubated with L-[1-¹⁴C]leucine followed by a chase with excess nonradioactive L-leucine, radioactivity was incorporated first into the albumin-like protein and transferred thereafter into albumin, suggesting that albumin was synthesized via the albumin-like protein as precursor.

In vivo, 1.8% of newly synthesized hepatoma protein was albumin or its precursor, compared with 1.2% in cell suspensions.

INTRODUCTION

Radiochemically pure albumin is obtained easily from the serum of rats given injections of radioactive amino acids, whereas rigorous purification is required to isolate albumin to radiochemical purity from liver or hepatoma homogenates (7, 12, 25). This observation led to the isolation from rat liver microsomes of an albumin-like protein that differed from albumin by an oligopeptide extension at the N-terminus consisting of 5 (22) or 6 (9) amino acids. We now report data demonstrating the presence of a similar albumin-like protein in homogenates and cell suspensions from Morris hepatoma 5123tc. The conversion of the albumin-like protein into albumin could be monitored in pulse-chase experiments with the cell suspensions.

MATERIALS AND METHODS

Chemicals and Isotopes. All chemicals were of analytical or scintillation grade. Tris of the highest purity, 99.0 to 99.5%, was purchased from Sigma Chemical Co., St. Louis, Mo.; DEAE-cellulose, DE 32, microgranular, was from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.; and bovine serum albumin, 100% electrophoretic purity, was from Behring-Werke AG, Marburg/Lahn, Germany. Hyamine hydroxide [p-(diisobutylcresoxyethoxyethyl) dimethylbenzlammonium hydroxide], 1 M in methanol, was obtained from Packard Instrument Co., Inc., Downers Grove, Ill. All other chemicals were bought from E. Merck AG, Darmstadt, Germany, or from Sigma.

Calibrated [¹⁴C]toluene was from either Packard Instrument Co., Inc., New England Nuclear, Boston, Mass., or The Radiochemical Centre, Amersham, U.K.; L-[1-¹⁴C]leucine, 56 Ci/mole, was purchased from The Radiochemical Centre.

Animals and Hepatomas. The animals used were male Buffalo rats, 250 to 350 g body weight, from an inbred colony kept in our laboratory. They were on a 20% protein diet with free access to food and water. Operations were performed between 9 a.m. and 1 p.m. The tumor was hepatoma 5123tc, provided in generation 68 by H. P. Morris, and further retransplanted in our laboratory. Albumin-like protein was isolated from hepatoma in generation 148 and cell suspensions were prepared from hepatoma in generations 152 and 153.

Determination of Albumin and Total Protein. Antiserum was raised in rabbits against purified rat serum albumin (11, 25). Albumin and albumin-like protein were determined by using radial immunodiffusion in agar gel with a modification of the method of Mancini et al. (4). It is not possible to differentiate between the 2 proteins by immunoprecipitation. The obtained values correspond to the sum of albumin plus albumin-like protein. This sum is called anti-albumin-precipitable protein.

Total protein was estimated with a modification of the biuret reaction (1) with bovine serum albumin as a standard.

Determination of Radioactivity. Samples were solubilized with Hyamine hydroxide for the determination of total radioactivity. The scintillation medium consisted of 295.2 g naphthalene, 18.4 g PPO, 0.1839 g α-naphthylphenyloxazole, 1400 ml xylene, 1400 ml dioxane, and 840 ml absolute ethanol. Absolute counting efficiency was calculated from the channel ratio of an external ²²⁰Ra standard or was determined by adding calibrated [¹⁴C]toluene as internal standard.

¹ This work was supported by a grant from the Anti-Cancer Council of Victoria.
² Dedicated to Van R. Potter on the occasion of his 65th birthday.

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standard, followed by recounting. Radioactivity in protein was estimated with the filter disk method of Mans and Novelli (5) using purified [14C]albumin as a standard to assess absolute counting efficiency (17). A Packard Model 3330 liquid scintillation spectrometer was used.

Preparation of Cell Suspensions from Hepatoma. Cell suspensions were prepared from hepatoma 5123tc by a slightly modified version of a previously described procedure (6).

Incubation of Cell Suspensions. Cells were incubated in a medium optimized for the incorporation of L-[1-14C]leucine into protein (13).

Preparation of Microsomes as a Carrier. Preparation was as reported previously (22).

Purification of Albumin and Albumin-like Protein from Hepatoma Homogenate and from Cell Suspensions. Frozen hepatomas were homogenized in 3 volumes of distilled water for 3 min at maximum speed in a Sorvall Omnimixer. Ten volumes of acetone, −20°, were slowly added, with continuous stirring. The precipitate was collected by centrifugation and washed twice with acetone and twice with diethyl ether. The resulting material was finely ground in a mortar and dried in a vacuum over paraffin and solid NaOH. All manipulations were carried out at 4°, unless stated otherwise. The acetone-dried powder was extracted several times with 100 mM Tris-HCl buffer, pH 7.7. The combined extracts were incubated for 72 hr with sufficient antiserum to quantitatively precipitate the albumin plus albumin-like protein. The precipitate was washed several times with 100 mM Tris-HCl buffer, pH 7.7, and suspended in acetic acid of pH 1.7. The pH was adjusted to 2.0 with 6 M HCl, and the mixture was incubated overnight. Ethanol was added to a final concentration of at least 80% (v/v), and the pH was readjusted to 2.0. After 6 hr of incubation, insoluble globulins were removed by centrifugation. Soluble protein was precipitated from the supernatant by the addition of 2 volumes of diethyl ether, −20°, collected by centrifugation, dissolved in 100 mM Tris-HCl buffer, pH 7.7, concentrated by ultracentrifugation with either an Amicon UM 20 E or a PM 10 filter (Amicon N.V., Oosterhout, Holland) and diluted to 10 mM Tris-HCl buffer, pH 7.7. Albumin-like protein and albumin were separated by consecutive chromatographies on DEAE-cellulose that had been equilibrated with 10 mM Tris-HCl buffer, pH 7.7. Washing with 75 mM buffer was followed by elution with a linear gradient from 75 to 230 mM Tris-HCl buffer, pH 7.7.

RESULTS

Occurrence of an Albumin-like Protein in Morris Hepatoma 5123tc. Radioactive L-leucine was injected into the caval veins of hepatoma-bearing rats, as described in the legend of Table 1. The tumors were removed 14 min after the injection and homogenized. An acetone-dried powder was prepared from the homogenate and extracted with 100 mM Tris-HCl buffer, pH 7.7. All protein reacting with antialbumin was precipitated by the addition of a sufficient quantity of anti-albumin antiserum to the extract. The values obtained during purification for total protein determined with the biuret reaction (1), for anti-albumin-precipitable protein determined by radial immunodiffusion in agar gel (4), and for radioactive protein in protein are summarized in Table 1. After the immunoprecipitation step, the same value, 800 mg, was obtained for total protein and for protein precipitable with antialbumin. However, the protein precipitable with antialbumin was not yet homogeneous. During chromatography on DEAE-cellulose (Table 1, DEAE-cellulose I) the specific radioactivity of protein in the eluate was not constant. The peaks of radioactivity and protein did not coincide. A highly radioactive protein that was quantitatively precipitable with antialbumin was eluted immediately before albumin. The fractions corresponding to the ascending part and the peak of the radioactivity curve were combined and chromatographed again (Table 1, DEAE-cellulose II). The obtained elution pattern, described in Chart 1, was similar to the

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Protein precipitable with antialbumin (mg)</th>
<th>Total protein (dpm/mg)</th>
<th>Antialbumin-precipitable protein (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>43,200</td>
<td>1,080</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td>14,600</td>
<td>1,130</td>
<td>120</td>
<td>160</td>
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<tr>
<td>Immunoprecipitation</td>
<td>800</td>
<td>800</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td>DEAE-cellulose I</td>
<td></td>
<td>32.9</td>
<td></td>
<td>1,500</td>
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<tr>
<td>DEAE-cellulose II</td>
<td>4.1</td>
<td></td>
<td></td>
<td>4,300</td>
</tr>
<tr>
<td>DEAE-cellulose III</td>
<td>0.8</td>
<td></td>
<td></td>
<td>4,500</td>
</tr>
</tbody>
</table>
pattern for chromatography DEAE-cellulose I. Again, the radioactivity and protein peaks were eluted at different buffer concentrations, and the specific radioactivity of protein was not constant. The fractions containing the ascending portion and peak of the radioactivity curve, i.e., fractions 10 to 23 in chromatography DEAE-cellulose II (Chart 1), were combined and subjected to a third chromatography (Table 1, DEAE-cellulose III). After the third chromatography, the specific radioactivity of protein in the eluate was constant. The peaks of radioactivity and anti-albumin-precipitable protein coincided. The elution pattern was similar to that shown in Chart 3 for a preparation from cell suspensions.

The isolated highly labeled protein was quantitatively precipitable with anti-albumin. It was called albumin-like protein. Its specific radioactivity was 4500 dpm/mg protein, whereas only 30 dpm/mg protein were obtained for albumin upon rechromatography of the fractions containing the descending portion of the protein peak in chromatography DEAE-cellulose I (not shown in a chart).

**Synthesis of Albumin-like Protein in Hepatoma Cell Suspensions and Its Conversion into Albumin.** Cells from Morris hepatoma 5123tc were prepared as indicated in "Materials and Methods" and incubated with L-[1-14C]leucine for 25 min. Sixteen % of the added radioactivity had been incorporated into protein at the end of this period. Then, excess nonlabeled L-leucine was added, and one-third of the incubation mixture was removed and rapidly cooled. The remainder of the cell suspension was incubated further. After another 15 min, one-half of the remainder was removed and cooled. The rest of the cell suspension was incubated for still another 15 min and then cooled. Liver microsomes were added as carrier to all 3 samples. Anti-albumin-precipitable protein was isolated (Table 2) and chromatographed on DEAE-cellulose. The obtained elution patterns are described in Chart 2, A to C. The curves representing the concentration of anti-albumin-precipitable protein in the eluate reflect mainly carrier protein (microsomes) added before purification. They show 2 typical peaks, the 1st corresponding to albumin-like protein (22) and the 2nd corresponding to albumin. They are similar for all 3 samples. In contrast, the radioactivity pattern differed very distinctly between the samples. At the end of the radioactive pulse, labeled protein appeared mainly in the 1st protein peak, corresponding to albumin-like protein. After a 15-min chase, about equal amounts of protein radioactivity were observed in the albumin-like protein and the albumin peaks. After 30 min of incubation with nonlabeled L-leucine, the albumin peak contained more radioactivity than the albumin-like protein peak. The fractions containing the albumin-like protein peak and those containing the albumin peak were combined separately for each time point and rechromatographed several times until constancy of the specific radioactivity throughout the peaks was achieved. The elution pattern of the final chromatography of the albumin portion of the 25-min sample is shown as an example in Chart 3. The protein and radioactivity peaks coincided; the specific radioactivity was constant.

Constancy of the specific radioactivity throughout the peaks in the final 6 chromatographies for the 3 time points (3 chromatographies for albumin and 3 for albumin-like protein) provided very strong evidence for the identity of albumin and albumin-like protein from hepatomas with those from liver, since most of the protein originated from liver, whereas radioactivity originated from tumor only. Albumin-like protein from liver microsomes has been previously characterized and compared with albumin by analysis of the amino acid composition, immunological properties, determination of the molecular weight by gel filtration on Sephadex G 100, electrophoresis in sodium dodecyl sulfate

### Table 2 Isolation of antialbumin-precipitable protein from cell suspensions from hepatoma 5123tc

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Anti-albumin-precipitable protein (mg)</th>
<th>Specific radioactivity of anti-albumin-precipitable protein (dpm/μg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells plus carrier microsomes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-min sample</td>
<td>12,500</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>40-min sample</td>
<td>12,200</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>55-min sample</td>
<td>11,900</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>Acetone precipitation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-min sample</td>
<td>Not determined</td>
<td>93</td>
<td></td>
</tr>
<tr>
<td>40-min sample</td>
<td>3,740</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>55-min sample</td>
<td>3,560</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>Immunoprecipitation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25-min sample</td>
<td>52.7</td>
<td>20.5</td>
<td></td>
</tr>
<tr>
<td>40-min sample</td>
<td>54.6</td>
<td>20.8</td>
<td></td>
</tr>
<tr>
<td>55-min sample</td>
<td>52.6</td>
<td>25.3</td>
<td></td>
</tr>
</tbody>
</table>
K. Edwards et al.

Chart 2. DEAE-cellulose chromatography of protein isolated with antialbumin from hepatoma 5123tc cells incubated with L-[1-14C]leucine followed by a chase with excess nonlabeled leucine. The protein obtained as described in Table 2 was applied to a 10- x 390-mm DEAE-cellulose column, washed with 300 ml of 10 mM Tris-HCl buffer, pH 7.7, and eluted with 280 ml of a linear gradient from 75 to 230 mM Tris-HCl buffer, pH 7.7, at a flow rate of 20 ml/hr. Collection of fractions was started immediately after application of the gradient. Determination of radioactivity and precipitation of protein with antialbumin in a small sample of each fraction are described in "Materials and Methods." A, 25-mm sample; B, 40-mm sample; C, 55-mm sample.

Chart 3. Purification of anti-albumin-precipitable protein to constant specific radioactivity. The elution pattern of the final DEAE-cellulose column chromatography of albumin of the 25-mm sample is shown as an example. Conditions for chromatography were the same as those described in the legend of Chart 2. Determination of radioactivity and precipitation of protein by antialbumin were as described in the legend for Chart 2.

polyacrylamide gel followed by autoradiography of the gels, and sequencing of the N-terminus (22).

The albumin peak in the chromatography shown in Chart 3 was eluted somewhat later than the albumin peak in Chart 2A, due to removal of the albumin-like protein during the course of purification. This albumin-like protein contaminated the left flank of the albumin peak more heavily than the right flank, thus producing an apparent shift of the position of the peak.

The specific radioactivities of purified albumin and albumin-like protein are plotted against incubation time in Chart 4. The specific radioactivity of total protein is also given for comparison.

**DISCUSSION**

**Isolation of an Albumin-like Protein from Hepatoma Homogenate.** It is very difficult to isolate albumin to radiochemical purity from livers or Morris hepatomas 5123tc or 9121 of rats given injections of radioactive amino acids 12 to 14 min before removal of the tissues (7, 12, 25). However, radiochemically pure albumin can be prepared easily from serum, testis, or kidney (25). The explanation for this, in the case of the liver system, was found to be the presence of a highly labeled, albumin-like protein reported to differ from albumin by an oligopeptide extension at the N-terminus (9, 22). A large amount of radioactivity is found in the albumin-like protein if it is isolated shortly (up to 15 min) after i.v. injection of radioactive leucine, whereas albumin is only slightly radioactive. The situation is reversed at later times. For example, 150 min after intraportal injection of L-[1-14C]leucine, the radioactivity in the albumin-like protein has decreased to a very small value, whereas a major amount of radioactivity can be measured in albumin, suggesting that the albumin-like protein is a precursor protein in the biosynthesis of albumin in the liver (24). A more detailed analysis of the in vivo time course of labeling of both proteins has been published recently (3, 21). A lack of secretion of serum protein into the bloodstream of eviscerated rats (10), a reduced synthesis rate of albumin (7, 12), and an extravascular accumulation of antialbumin-precipitable protein (23) for Morris hepatomas 5123tc and 9121, have been described. An explanation relating these observations to each other could be that the lack of secretion leads to an intracellular accumulation of albumin and to a decrease in its rate of synthesis by a regulatory mechanism as yet unknown. The discovery of an albumin precursor protein probably requires some modifications of current ideas on the mechanism of albumin synthesis and secretion. The special situation in hepatomas also has to be reexamined. For example, it cannot be decided from earlier work (23) what proportion of extravascular anti-albumin-precipitable protein accumulated in hepatoma consists of albumin precursor. The data presented in Table 1 and Chart 1 suggest that Morris hepatoma 5123tc is able to synthesize albumin precursor protein in vivo. Its conversion into albumin cannot be studied in the in vivo tumor system.

**Synthesis of Albumin-like Protein in Hepatoma Cell Suspensions and Its Conversion into Albumin.** Cell suspensions prepared from liver by perfusion with hyaluronidase and collagenase (2) are a very appropriate system for the study of protein synthesis (for review see 13). Synthesis (26) and secretion (27) of albumin have been demonstrated in such suspensions. By solubilizing the intercellular matrix with collagenase and hyaluronidase, cell suspensions can also be prepared from Morris hepatoma 5123tc (6, 14, 15).
Synthesis of Albumin Precursor in Rat Hepatoma

\[ y = a \frac{A - X}{Y - X} \]

Thus, values of 3 and 8% were obtained for the fractional precursor pool size in hepatoma 5123tc (this paper) and liver (21), respectively.

Amino Acid Sequence of the Oligopeptide Extension in Albumin Precursor Protein. There is as yet no unanimity about the precise amino acid sequence of the oligopeptide extension in the albumin precursor protein in liver. The same (or a similar) N-terminus, namely, glutamate (or glutamine), was reported for albumin precursor and albumin in 1973 by Russell and Geller (8). In 1974, we found an oligopeptide extension with the amino acid sequence Gly-Val-Phe-Ser-Arg- in the albumin-like protein (22). Serine could be identified in those experiments only by thin-layer chromatography of the phenylthiohydantoin derivatives of the amino acids obtained in the Edman degradation. In 1975, Russell and Geller (9) published an oligopeptide extension for the albumin-like protein consisting of Arg-Gly-Val-Phe-Arg-.

It is also not clear whether the oligopeptide extension is removed as such during the conversion of albumin precursor protein into albumin or whether the amino acids of the extension are cleaved off sequentially during the conversion. The latter reaction mechanism would produce intermediate products in the conversion. A small oligopeptide split off from the precursor could have specific functions in secretion of albumin or regulation of its synthesis rate (16).

ACKNOWLEDGMENTS

We are very much obliged to S. Bourke for her skillful technical assistance.

REFERENCES


In such suspensions, radioactive protein appeared in the medium about 10 min after starting incubation with [1-14C]leucine (14, 15). The addition of cycloheximide (13) or puromycin (15) interrupted protein synthesis but did not influence the appearance of radioactive protein in the medium. Thus, hepatoma cells in suspension seem to secrete protein into the medium, in contrast to the lack of secretion of serum protein into the bloodstream observed earlier (10) in eviscerated rats. The results of the experiments described in Table 2 and Charts 2 to 4 indicate clearly that hepatoma 5123tc cells in suspension are able to synthesize albumin precursor protein and to convert it into albumin.

Pool Size of Albumin Precursor. The amounts of albumin precursor in livers from hepatoma 5123tc cells incubated with [1-14C]leucine followed by a chase with nonlabeled leucine. For experimental details, see legend to Table 2.

Previously, the ratio of albumin to total protein synthesis was calculated from the specific radioactivity of albumin purified from homogenate. The concentration of albumin in the homogenate, and the incorporation of radioactive leucine into total protein. Values of 3.3 to 3.7. 1.4, 0.36, and 0.8% were obtained for normal liver (7, 12, 17), regenerating liver (17), and Morris hepatomas 9121 (7), and 5123tc (12), respectively. These calculations were based on the assumption that radioactive leucine is incorporated into both albumin and total protein linearly with respect to time. and that both are synthesized directly from the same precursor pool of amino acyl-tRNA's. The discovery of a precursor protein for albumin invalidates this assumption. Replacing in these calculations the radioactive activity contained in albumin by that contained in albumin + albumin precursor gives values of 13, 1.8, and 1.2% for the ratio of albumin to total protein synthesis in liver in vivo (21), hepatoma 5123tc in vivo (this paper), and hepatoma 5123tc cell suspensions (this paper), respectively.

Pool Size of Albumin Precursor. The amounts of albumin, \( x \), albumin precursor, \( y \), and antialbumin-precipitable protein, \( a \), in the immunoprecipitates obtained from hepatoma or liver homogenates, and their specific radioactivities, \( X \), \( Y \), and \( A \), are related through the following 2 equations:

\[ x + y = a \]
\[ xX + yY = aA \]

From these equations, the pool size of albumin precursor can be calculated according to the equation:

\[ y = a \frac{A - X}{Y - X} \]
K. Edwards et al.

Biosynthesis of Albumin via a Precursor Protein in Morris Hepatoma 5123tc

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