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PROALBUMIN IS BOUND TO THE MEMBRANE OF RAT LIVER SMOOTH MICROSOMES

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SUMMARY Rat serum albumin and its immediate biosynthetic precursor, proalbumin, were resolved from rat liver smooth microsomes using two dimensional gel electrophoresis. These polypeptides were identified by considering their charge properties (in first dimensional isoelectric focusing), apparent molecular weights (in second dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis), and in situ radio-iodinated peptide fingerprints in comparisons with pure albumin. Tryptic digestion or alkaline-high salt treatment of microsomes did not affect their content of either polypeptide, while 0.05% deoxycholate completely removed proalbumin and decreased albumin. Lactoperoxidase catalyzed radio-iodination of intact microsomes resulted in significant labelling of proalbumin but virtually no labelling of albumin. These results suggest that a short, internal polypeptide segment of proalbumin is exposed on the outer microsomal surface, whereas the bulk of the protein chain is entrapped by the membrane. It is concluded that some, if not all, of proalbumin in liver smooth endoplasmic reticulum is associated with the membrane which may be important for its subsequent processing.

INTRODUCTION Translation of rat liver albumin mRNA occurs on membrane bound polysomes of rough ER[1] and results in the synthesis of pre-proalbumin (1). The eighteen residue N-terminal "pre-piece" is removed by proteolysis during or immediately after completion of the polypeptide chain resulting in the formation of proalbumin (1). This processed polypeptide is then transported, presumably, via the smooth ER, to the Golgi apparatus, where it is converted to albumin by further proteolysis of the hexapeptide, N-terminal "pro-piece" (2,3). The N-terminal "pre-piece" most likely acts as a signal sequence which directs the nascent polypeptide chain across the membrane of the rough ER (1). On the other hand, the function of the "pro-piece" is not understood at this time.

The state of proalbumin during transport to the Golgi apparatus has not been explicitly investigated. However, it has been shown that serum glycoproteins (or their precursors) are bound to the membrane of smooth microsomes (4) Our investigations on the transverse topology of ER membrane proteins led to the present results which suggest that at least some proalbumin in rat liver smooth microsomes is also bound to the membrane.

1 Abbreviations used: ER, endoplasmic reticulum; RSA, rat serum albumin; Tris, 2-amino-2-hydroxy-1,3-propanediol, TCA, trichloroacetic acid.

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METHODS Total smooth microsomes from livers of phenobarbital induced (0.1% phenobarbital in the drinking water for five days prior to killing) and non-induced male Holtzman rats (150 ± 10 g) were prepared according to the method of DePierre and Dallner (5). RSA was obtained from Sigma; L-(tosylamido 2-phenyl) chloromethyl ketone treated trypsin was from Worthington; carrier free [^{125}I]NaI was a product of ICN; lactoperoxidase and glucose oxidase were from P-L. All other chemicals were reagent grade.

Microsomes were solubilized in a solution containing 3% Triton X-100, 1.5% pH 3.5-10 Ampholytes (LKB), 0.5% pH 6-8 Ampholytes, 5% mercaptoethanol and 10 M urea and two dimensional gel electrophoresis of microsomal polypeptides was performed using the method of O'Farrell (6) with several important modifications (7). In situ radio-iodinated peptide fingerprinting of polypeptide spots excised from two dimensional gels was performed according to the method of Elder et al. (8) as modified by Zweig and Singer (9). Protein concentrations were determined according to Lowry et al. (10).

Tryptic digestion of microsomes was conducted as follows: 10 mg (protein) of microsomes in the presence of 0.1 mg trypsin were incubated at 30° for varying times in one mL of 0.05 M Tris-HCl, 0.1 M KCl buffer, pH 7.5, and the reaction was terminated by removing aliquots and diluting them three-fold with the sample solubilization solution used for first dimensional isoelectric focusing. Alkaline-high salt washing of microsomes was according to Weihing et al. (11). Treatment of microsomes with 0.05% deoxycholate was as described by Nilsson and Dallner (12).

Lactoperoxidase catalyzed iodination of the outer microsomal surface was accomplished using 5 mg (protein) of microsomes in one mL containing 0.1 M phosphate buffer, pH 7.5, 1 µg/mL butylated hydroxytoluene (13), 0.01 M glucose, 2 mCi carrier free [^{125}I]NaI, 10 µg lactoperoxidase and 0.5 µg glucose oxidase. The reaction was initiated by the addition of glucose oxidase and was incubated at 25° for 10 minutes. The mixture was then diluted to 70 mL with ice-cold 0.1 M phosphate buffer, pH 6.8, in 20% glycerol. After centrifugation at 105K x g for two hours the microsomal pellet was resuspended in 0.2 mL of the same buffer. Samples of labelled microsomes were precipitated with 20% TCA for liquid scintillation counting; other samples (130 µg protein) were submitted to two dimensional gel electrophoretic analysis. Autoradiography of these gels used LKB Ultrafilm ^{3}H. Comparisons of Coomassie blue stained gels with autoradiograms indicated no changes in polypeptide migration as a result of iodination.

RESULTS AND DISCUSSION Microsomal polypeptides designated D6 and D7 in Fig. 1 have been observed in at least fifty two dimensional gels run under a variety of conditions in the first and second dimensions and have consistently migrated as ∿67K dalton species focusing at ∿pH 6.54 and ∿pH 6.38, respectively. Both polypeptides were independent of drug (xenobiotic) induction and it was generally observed that D6 was present to a greater extent than D7 in these smooth microsomes. Furthermore, polypeptide D6 always migrated in the second dimension with a slightly lower mobility than D7 which, in turn, exactly comigrated with pure RSA in two dimensional gels (not shown). These properties suggest that spots D6 and D7 represent proalbumin and albumin, respectively, since purified proalbumin is a slightly longer polypeptide than albumin and focuses at pH 6.23, whereas albumin focuses at pH 6.10 (14)[2]. In addition, proalbumin is present in greater amounts than albumin in microsomes (3). To verify these identifications,

2 These pH values were corrected for measurements in 6 M urea (i.e., the measured values were 0.42 pH units higher (14)). The pH values for

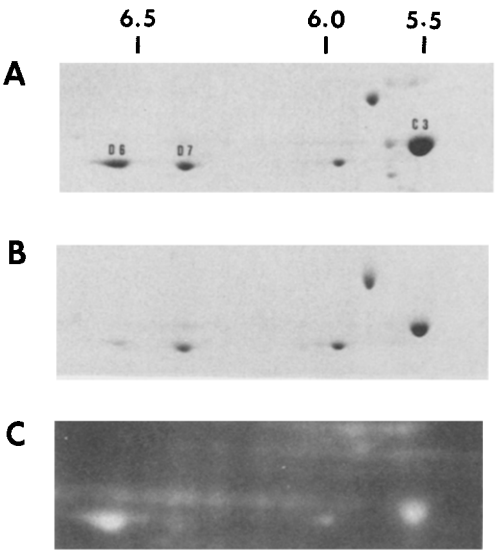

Figure 1. Two dimensional gel electrophoresis of rat liver smooth microsomes resolving albumin and proalbumin. Two dimensional gel electrophoresis, dilute deoxycholate treatment, and lactoperoxidase catalyzed radio-iodination of microsomes were as described under METHODS. 130 µg of microsomal protein was applied. Photographs represent portions of the gels only. A. Untreated microsomes; coomassie blue stained. B. 0.05% deoxycholate treated microsomes; coomassie blue stained. C. Negative print of an autoradiogram for resolution of ^{125}I labelled microsomes (2.5×10^6 cpm applied); film was developed after three day exposure.

in situ radio-iodinated peptide fingerprints were determined for polypeptide spots D6, D7, and pure RSA excised from separate two dimensional gels. Each of these spots in four different gels were analysed in this manner and some typical results are presented in Fig. 2. These peptide maps were distinct from those of ten other microsomal polypeptides[3]. Since no additional iodinatable residues are present at the N-terminus of proalbumin compared with albumin (1), and rat proalbumin can be processed to RSA in vitro with dilute trypsin (16), it was expected that the fingerprints for polypeptides D6, D7 and RSA would be identical and this apparently is the case (minor differences between fingerprints in Fig. 2 were not reproducible). The fact that the radio-iodinated peptide maps for these polypeptides are essentially the same also indicates the homogeneity of spots D6 and D7 in two dimensional gels.

2 cont.
isoelectric focusing gels reported in Fig. 1 were determined by equilibrating gel slices (15). It is noteworthy that the difference between pH values for focusing polypeptides D6 and D7 was essentially the same as that between proalbumin and albumin.

3 Manuscript in preparation.

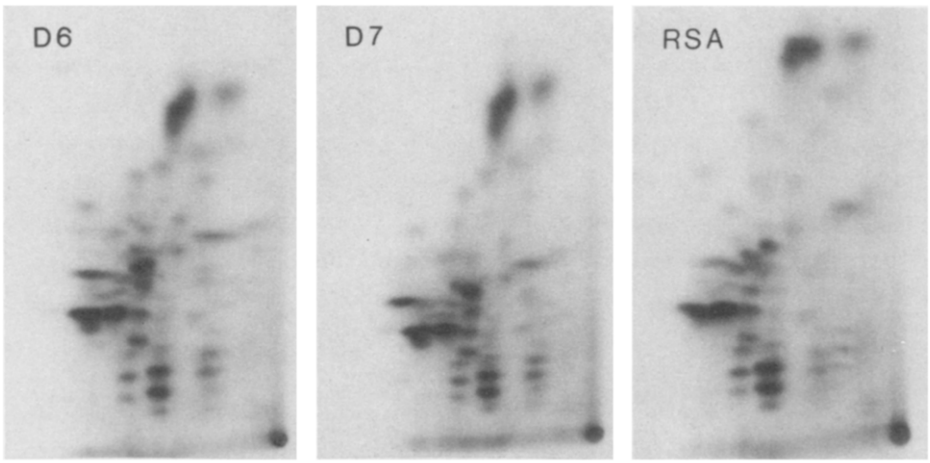

Figure 2. Radio-iodinated peptide fingerprints of polypeptide spots resolved in two dimensional gels. Polypeptide designations are those in Fig. 1; RSA represents rat serum albumin run in a separate two dimensional gel. Origins are in the lower right corners. Electrophoresis was from right (anode) to left (cathode) and chromatography was from bottom to top. Exposure of Kodak SB5 film was for two days.

Proalbumin and albumin in smooth microsomes strongly resist proteolysis by trypsin since electrophoretic analysis of samples after two hour digestion results in the same display of spots D6 and D7 as shown in Fig. 1,A. However, major microsomal polypeptide spots representing cytochrome b_5 and putative phenobarbital induced cytochrome P-450s disappear under these conditions[3]. These results suggest that trypsin sensitive bonds of albumin and proalbumin (i.e., the bulk of their polypeptide chains) are occluded by the microsomal membrane. Alkaline-high salt treatment of smooth microsomes (10) was also without effect on the microsomal contents of proalbumin and albumin which indicates that these are not peripheral proteins or scavenged on the microsome surface during isolation. The effects of these treatments were anticipated since it had been shown that secreted proteins are synthesized on rough endoplasmic reticulum membranes and vectorially displaced onto the cisternal side (17)

Considering the presumption that most secreted proteins are discharged as solutes into the cisternal space of rough endoplasmic reticulum (17,18) it was surprising that proalbumin was significantly labelled when smooth microsomes were submitted to lactoperoxidase catalyzed iodination as shown in Fig. 1,C. The microsomal membrane is impermeable to macromolecules such as lactoperoxidase (12) and no labelling of proalbumin occurred in the absence of this enzyme (not shown); therefore, lactoperoxidase catalyzed labelling of proalbumin in intact microsomes indicates that it reveals an iodinatable residue(s) on the outer microsomal surface. That lactoperoxidase only has access to the outer surface

is demonstrated by the fact that albumin in smooth microsomes evidences little, if any, labelling even though it is present in reasonable amounts and is ∿99% homologous with proalbumin (see Fig. 1,C). Identical labelling results were obtained in four separate experiments using different microsome preparations[4]. Even though spots D6 and D7 appear to be homogeneous (see above), a remote possibility is that a minor microsomal polypeptide exists which is extremely susceptible to lactoperoxidase catalyzed iodination and shares the charge and molecular weight characteristics of proalbumin. This possibility was tested by treating outside radio-iodinated microsomes with trypsin or alkaline-high salt washing. These treatments were without effect on the radioactivity in spot D6 detected by autoradiography; furthermore, the shape of spot D6 in autoradiograms was congruent with that in coomassie blue stained gels (see Fig. 1). Therefore, it is safe to conclude that the radioactivity in spot D6 represents labelled proalbumin. The present results cannot discriminate whether all or a portion of the total smooth microsomal proalbumin is exposed on the outside surface. Nevertheless, proalbumin was one of the most extensively labelled polypeptides in two dimensional gels following lactoperoxidase catalyzed radio-iodination of microsomes[3]. In any event, the presence of a significant amount of membrane bound proalbumin in this heterogeneous smooth microsome preparation is an interesting observation which might be important for understanding subsequent processing events.

The effect of 0.05% deoxycholate in removing proteins from microsomes was previously suggested to result solely from its ability to render the microsomal membrane permeable to macromolecules (12,19). This effect is probably responsible for the decreased content of RSA (spot D7) in microsomes after incubation with this detergent (see Fig. 1,B) since RSA most likely exists as a solute in the inner microsome compartment (20). However, proalbumin and several other microsomal proteins[3], which are apparently bound to the membrane, are almost completely removed by this treatment (see spot D6 in Fig. 1,B). On the other hand, most integral membrane polypeptides of smooth microsomes such as cytochrome b_5, cytochrome P-450s, and their reductases are completely stable to dilute deoxycholate (7,12). These results indicate that the stabilizing forces maintaining proalbumin in the membrane bound state are considerably different from those involved with most other integral membrane polypeptides of smooth microsomes. It is possible that the membrane bound state of proalbumin is metastable as a consequence of its vectorial synthesis on the membrane surface.

Histidine residues occur near the N-terminus of RSA at positions 3, 9, and 18; whereas, tyrosine occurs first at position 30 (21); however, it is not

4 Labelling patterns for frozen-thawed microsome preparations were similar to that in Fig. 1 except that albumin (spot D7) was more noticeably labelled.

probable that these are iodinated in proalbumin by lactoperoxidase treatment of smooth microsomes since exposure of the N-terminus of proalbumin would most likely subject it to trypsin action which was not observed (see above). On the other hand, the C-terminus of RSA is devoid of iodinatable residues (i.e., at least from residue 549 to the C-terminal residue at position 584 (21)) and apparently is the same as that of proalbumin since C-terminal alanine is retained after processing (22). Therefore, it is likely that a limited, internal polypeptide segment of proalbumin is present on the outer microsomal surface exposing histidine and/or tyrosine side chains which are susceptible to lactoperoxidase catalyzed iodination. The conclusion that the same internal sequence of albumin is not subject to iodination in smooth microsomes suggests that in vivo conversion of rat proalbumin to RSA results in the displacement of the protein from the membrane into the cisternal space.

The preparation of smooth microsomes used in this study is mainly derived from smooth ER but also contains Golgi fractions (5). However, the location of proalbumin and RSA in these microsomes appears to be homogeneous since they completely resist high salt washing and trypsin proteolysis. It can be calculated that the membrane of rat hepatocyte smooth endoplasmic reticulum (including Golgi elements) accounts for a large portion of the total organelle volume[5]. Therefore, these membranes represent a quantitatively significant phase that could be utilized for the transport of secreted proteins. The presence of membrane bound proalbumin in smooth ER could serve to counter the apparent concentration gradient against which albumin is transported (2,17). It is possible that membrane bound proalbumin is necessary for further segregation (e.g., by aggregation in the lateral plane of the membrane) prior to vesicularization at the Golgi apparatus. It is also conceivable that the limited exposure of proalbumin on the outer (cytoplasmic) side of the membrane is important for recognition and fusion with Golgi vesicles that is apparently required for ultimate processing to albumin (25). We are currently using more refined fractionation procedures to isolate microsomes representing smooth ER, rough ER and Golgi elements for extending this study of proalbumin interactions with microsomal membranes. In addition, we are attempting to corroborate the present results using photo-chemical labelling probes for exposed proteins on the microsomal surface and are investigating the disposition of other plasma protein precursors in microsomes.

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5 Rat liver smooth endoplasmic reticulum occupies a volume of 289 μ^3 per hepatocyte and has a surface area of 25,100 μ^2 per hepatocyte (23). If the thickness of the smooth endoplasmic reticulum membrane is 0.005 μ (24) then the membrane volume of this organelle is $\sim$43% of the total.

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