We report biochemical, immunological, and genetic studies which demonstrate that an accessory protein with the essential features of mouse egasyn is complexed with and stabilizes a portion of $\beta$-glucuronidase in microsomes of rat liver. The accessory protein exists as a complex with $\beta$-glucuronidase since it coprecipitates with $\beta$-glucuronidase after treatment of extracts with a specific $\beta$-glucuronidase antibody. The two proteins are associated by noncovalent bonds since they are easily dissociated at elevated temperatures. Only 20–25% of total liver accessory protein is complexed with microsomal $\beta$-glucuronidase. The remainder exists as a free form. The molecular weight of the accessory protein is 61 to 63 kDa depending upon the rat strain of origin. This protein, like mouse egasyn, has esterase catalytic activity and is concentrated in microsomes.

The accessory protein is genetically polymorphic with at least four alleles. Combined biochemical and genetic evidence indicates it is identical with esterase-3 of the rat. Also, both mouse egasyn and rat esterase-3 react with antisera to egasyn and to rat esterase-3, indicating they are homologous proteins.

Several inbred rat strains lack microsomal $\beta$-glucuronidase. The same strains lack the accessory protein, suggesting that stabilization of $\beta$-glucuronidase in rat microsomes requires egasyn. (Medda and Swank, 1985) and (b) as detailed in this report, microsomal glucuronidase positive and negative inbred rat strains exist, have enabled analysis of the biochemical properties and mechanism of formation and stabilization of $\beta$-glucuronidase in microsomes of the rat.

EXPERIMENTAL PROCEDURES AND RESULTS

The present results demonstrate that the mechanism for localization of rat $\beta$-glucuronidase to microsomes involves an accessory protein similar to mouse egasyn. The evidence for the accessory protein includes a low mobility group of $\beta$-glucuronidase components on nondenaturing gels and the strict association of these low mobility forms with microsomes. Also, an accessory protein of 61–63 kDa was found to be associated with $\beta$-glucuronidase after precipitation of $\beta$-glucuronidase with specific antisera. Perhaps the most convincing evidence that localization of $\beta$-glucuronidase to microsomes requires the binding protein in vivo is the genetic data. Rat strains (90 total) containing the accessory protein contain microsomal $\beta$-glucuronidase while those (13 total) which lack the accessory protein also lack microsomal $\beta$-glucuronidase. Absence of microsomal $\beta$-glucuronidase was also concordant with the absence of esterase-3 in backcross animals.

It is also evident that in rats, as in mice (Medda and Swank, 1985), the accessory protein, egasyn, is an esterase. The protein which copurifies with rat $\beta$-glucuronidase has identical electrophoretic mobility and isoelectric point to rat esterase-3, purified by independent methods. Furthermore, both purified proteins have the same pattern of hydrolytic activities toward a number of typical ester and amide substrates. The strain distribution pattern also indicates that rat egasyn is identical to esterase-3. Recent studies have established that mouse egasyn is identical with esterase-22 (Medda et al., 1986). Thus, structurally and functionally similar esterases are associated with microsomal $\beta$-glucuronidase in the two species.

The biochemical, immunological, and biological similarities (summarized in Table 4) between mouse egasyn and the rat...

1 Portions of this paper (including "Experimental Procedures," "Results," Figs. 1–10, and Tables 1–4) are presented in miniprint at the end of this paper. The abbreviation used is: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-3153, cite the authors, and include a check or money order for $9.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

An Accessory Protein Identical to Mouse Egasyn Is Complexed with Rat Microsomal $\beta$-Glucuronidase and Is Identical to Rat Esterase-3*
accessory protein are so great that there is no doubt the two are homologous proteins. It has been proposed (Womack and Sharp, 1976; Peters, 1982) that the mouse esterase genes of chromosome 8 have developed into two clusters from an ancestral esterase gene by repeated tandem duplication followed by divergent evolution. A similar development of esterase genes appears to have taken place in the rat. It appears that, as in the house mouse, the esterase gene region of the rat (linkage group V) consists of two clusters. The suggested homology between rat Es-3 and mouse Es-22 is now supported by the fact that these genes are located in homologous clusters on linkage group V and chromosome 8, respectively (Hedrich and von Deimling, 1986). It may be expected that duplicated isoforms are free to gradually acquire new functions. The ability of rat esterase-3 and mouse esterase-22 to complex with β-glucuronidase is suggested to be such a new function. This ability has obviously been acquired before evolutionary divergence of the rat and mouse, that means 10 years at least (Britten, 1986).

There are, on the other hand, several differences in the rat and mouse β-glucuronidase-egasyn complexes. One is that the rat complex is much more readily dissociated into the uncomplexed X β-glucuronidase form by heat treatment. The significant levels of X form, even in nonheated extracts, may reflect this lability of the rat complex. The high levels of free rat esterase-3 (egasyn) in liver homogenates indicate that egasyn levels are not limiting in formation of the complex. Secondly, the rat microsomal β-glucuronidase-egasyn complex is more heterogeneous upon nondenaturing electrophoresis, not forming the discreet M₁-M₃ components present in mice (Swank and Paigen, 1973). This may reflect intrinsic electrophoretic heterogeneity of rat β-glucuronidase, evident in lysosomal β-glucuronidase on the same gels.

Strawser and Touster (1979) have reported a protein(s) of rat liver homogenate binds to β-glucuronidase-Sepharose 2B affinity columns. This interesting protein has some similarities to the rat egasyn of this report but for several reasons is likely to be a separate protein. First, it is an intrinsic membrane protein requiring 2% Triton X-100 for extraction from microsomal membranes while egasyn and the β-glucuronidase-egasyn complex are extracted at less than 0.06% Triton X-100. Also, unlike rat egasyn, it self-aggregates and forms very high molecular weight complexes with β-glucuronidase that do not enter typical polyacrylamide gels. Finally, unlike rat egasyn, it does not cross-react with antibody to mouse egasyn.

The reasons why the rat microsomal β-glucuronidase-egasyn complex eluded detection in previous studies (Owens and Stahl, 1976) of rat microsomal β-glucuronidase may be due to the above described lability of the rat complex and/or to the use of rats which genetically lacked egasyn. As discussed by Festing (1979), commercially available Wistar rats have considerable genetic variation.

The finding of a microsomal β-glucuronidase-egasyn complex in another species raises the possibility the complex may have general rather than limited distribution among mammals and that it may have physiological importance. Recent studies (Belinsky et al., 1984) suggest microsomal β-glucuronidase may function to modulate levels of circulating glucurononidated drugs and endogenous compounds.

The functional role of the esterase activity of egasyn is under investigation. We previously speculated (Medda and Swank, 1985) that the esterase may serve in proteolysis of the β-glucuronidase precursor to mature form. However, mixing purified egasyn with purified β-glucuronidase precursor have thus far not revealed processing of the precursor under a variety of in vitro conditions. Also, the fact that processing of most pro-lysosomal forms including β-glucuronidase (Brown et al., 1981) occurs at later times, when the precursor form would be expected to have left the endoplasmic reticulum (the subcellular location of the egasyn-β-glucuronidase complex), argues against a direct processing role for egasyn. A second hypothetical function of the esterase activity, namely that it might serve to bind to and/or hydrolyze the naturally occurring phosphodiester linkage on the oligosaccharide unit of newly synthesized β-glucuronidase, is also unlikely. We have not detected either binding to or modification of naturally occurring oligosaccharides with phosphodiesterases by egasyn. Recent studies do suggest, however, that the esterase active site is important in binding egasyn to β-glucuronidase since inhibitors of the esterase active site cause rapid dissociation of the complex. Also, in regard to possible physiologic functions of egasyn, it has recently been discovered (Mentlein et al., 1985b) that esterase-3 (egasyn) has potent and specific activity in the hydrolysis of certain long chain fatty acid esters such as palmityl carnitine (Mentlein et al., 1985b), monoglycerides, and lysophospholipids (Mentlein et al., 1984b).

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REFERENCES


Hayashi, M., Nakajima, Y., and Fishman, W. H. (1964) J. Histochem. Cytochem. 12, 283-297


Koima, D. J. (1963) J. Histochem. Cytochem. 11, 619-623


3 S. Medda, unpublished results.

**Egasyne (Esterase-3) Is Complexed with Rat β-Glucuronidase**


**SUGGESTED MATERIALS**

As an accessory protein identified to house Egacyne in complex with Rat Microsomal β-Glucuronidase.

**DETERMINATION OF ACTIVITY**

1. **Electrophoresis and molecular analysis**

   1. Use a polyacrylamide gel electrophoresis method to determine the molecular weight of the complex.
   2. Use a gel filtration method to determine the molecular weight of the complex.

2. **Western blot analysis**

   1. Use a western blot analysis method to determine the presence of the complex.
   2. Use a gel filtration method to determine the presence of the complex.

**RESULTS**

- The complex is stable under a variety of conditions, including high and low pH, and is resistant to heat and cold.
- The complex is susceptible to proteolytic degradation, indicating a potential role in the control of enzymatic activity.
- The complex is resistant to chemical inactivation, suggesting a stable interaction between the components.

**DISCUSSION**

- The complex may play a role in the regulation of enzymatic activity, possibly by controlling the availability of the enzyme for substrate binding.
- The complex may have implications for the understanding of the mechanisms of disease, as it may be involved in the development of certain conditions.
- The complex may have potential applications in the development of therapeutic strategies, as it may be targeted for selective inhibition or activation.

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**REFERENCES**


**MATERIALS**

- Electrophoresis and molecular analysis: Rat microsomal β-glucuronidase complex fraction isolated from rat liver, prepared by detergent (10 mg/ml) against 4% w/v sodium dodecyl sulfate as described by Luisi et al. (1978). Antibodies to purified β-glucuronidase were obtained from Dr. J. W. Owens.

- Western blot analysis: Rat liver old and young adult β-glucuronidase from young rats (1 mg/ml) and young adult β-glucuronidase from adult rats (1 mg/ml).

- Electrophoresis: Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970).
Egasy (Esterase-3) Is Complexed with Rat β-Glucuronidase

The accessory protein has esterase activity

The accessory protein has esterase activity. It is recently demonstrated (Hendler and Bank) that, in the presence of the accessory protein, the esterase activity is increased. This result suggests that the accessory protein is involved in the mechanism of esterase activity.

In a typical experiment, the ratio of esterase activity to total activity was determined. The activity of the esterase was measured using the method of Bank and Hendler (1985). The results are shown in Figure 1. Figure 1 shows the relationship between the ratio of esterase activity to total activity and the concentration of the accessory protein.

Figure 1. Esterase activity in the presence of the accessory protein.

- Figure 1: Graph showing the relationship between the ratio of esterase activity to total activity and the concentration of the accessory protein.

- Table 1: Summary of the experiment results.

- Table 2: Additional information on the experiment.

- Table 3: Comparison of the results with previous studies.

Identity of esterase activity with ES-3

It is apparent that ES-3 is an esterase activity associated with the accessory protein. The esterase activity was measured using the method of Bank and Hendler (1985). The results are shown in Figure 2. Figure 2 shows the relationship between the esterase activity and the concentration of the accessory protein.

Figure 2. Esterase activity in the presence of the accessory protein.

- Figure 2: Graph showing the relationship between the esterase activity and the concentration of the accessory protein.

- Table 4: Summary of the esterase activity results.

- Table 5: Additional information on the esterase activity.

Stability of esterase activity with ES-3

The esterase activity was measured at various pH values in the presence of the accessory protein. The results are shown in Figure 3. Figure 3 shows the relationship between the esterase activity and the pH value.

Figure 3. Esterase activity at various pH values.

- Figure 3: Graph showing the relationship between the esterase activity and the pH value.

- Table 6: Summary of the esterase activity results at various pH values.

- Table 7: Additional information on the esterase activity at various pH values.

In conclusion, the accessory protein plays an important role in the mechanism of esterase activity. Further studies are needed to understand the mechanism in more detail.

References:


Egasyn (Esterase-3) is Complexed with Rat β-Glucuronidase

Figure 1. Analysis of an accessory protein with β-glucuronidase in microsomal β-glucuronidase-positive strain and its absence in a negative strain.

Accessory proteins from various rat strains were purified from total liver homogenate by co-purification with β-glucuronidase using specific anti-β-glucuronidase serum. GSA-3 was purified as described by Newman and Pimentel (1981). Samples were electrophoresed on SDS gels and stained with silver staining. The position of the accessory protein is shown by the arrow. L and R represent the heavy and light chain, respectively. The presence of only two subunits in the SGS-3 binding complex was confirmed by gel filtration on a Sephadex column (Fr. 40, 400 ml), ovalbumin (Fr. 48, 400 ml), and soybean trypsin inhibitor (Fr. 31, 400 ml), and soybean trypsin inhibitor (Fr. 21, 500 ml).

Figure 2. Autoradiography of the accessory components of liver lysosomal, microsomal and soluble fractions of livers of H1G192M rats were prepared as described. Each lane of each gel was stained with silver and stained for β-glucuronidase activity. Lane 1, 2, and 3: left, middle, and right with anti-β-glucuronidase antibody and Lane 4, 5, and 6: right with anti-β-glucuronidase antibody and stained for total activity. Lane 7, 8, and 9: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity. Lanes 2, 4, 6, 8, and 10: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity. Lanes 3, 5, 7, 9, and 11: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity. Lanes 1, 2, and 3: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity. Lanes 4, 5, and 6: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity. Lanes 7, 8, and 9: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity. Lanes 10 and 11: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity. Lanes 12 and 13: left, middle, and right with anti-β-glucuronidase antibody and stained for total activity.

Figure 3. Recognition of "M" components by anti-β-glucuronidase antibody. Liver homogenate (10 μl) of the H1G192M rat was left without anti-β-glucuronidase antibody and stained for total activity. Liver homogenate (10 μl) of the H1G192M rat was left without anti-β-glucuronidase antibody and stained for total activity. Liver homogenate (10 μl) of the H1G192M rat was left without anti-β-glucuronidase antibody and stained for total activity.

Figure 4. Immunoprecipitation of β-glucuronidase and esterase "M" components.

10 μl of liver homogenate (100 μg/ml) was applied to each lane. After electrophoresis under non-denaturing conditions, the gel was divided. One part was stained for β-glucuronidase and the other for esterase activity.

1 - Micromolar β-glucuronidase positive (H1G192M) strain.
2 - Micromolar β-glucuronidase negative (H2G192M) strain.
3 - Estrogenic liver homogenate (10 μg/ml) was applied to each lane. After electrophoresis under non-denaturing conditions, the gel was divided. One part was stained for β-glucuronidase and the other for esterase activity.
4 - Micromolar β-glucuronidase positive (H1G192M) strain.
5 - Micromolar β-glucuronidase negative (H2G192M) strain.
6 - Micromolar β-glucuronidase positive (H1G192M) strain.
7 - Micromolar β-glucuronidase negative (H2G192M) strain.
8 - Micromolar β-glucuronidase positive (H1G192M) strain.
9 - Micromolar β-glucuronidase negative (H2G192M) strain.
10 - Micromolar β-glucuronidase positive (H1G192M) strain.
11 - Micromolar β-glucuronidase negative (H2G192M) strain.
12 - Micromolar β-glucuronidase positive (H1G192M) strain.
13 - Micromolar β-glucuronidase negative (H2G192M) strain.

Figure 5. Estrogen is free of purified accessory proteins of various rat strains.

Accessory proteins were purified from rat strains by cation-exchange chromatography on a Sephadex column (Fr. 40, 400 ml), ovalbumin (Fr. 48, 400 ml), and soybean trypsin inhibitor (Fr. 31, 400 ml), and soybean trypsin inhibitor (Fr. 21, 500 ml).

1 - Liver homogenate; 2 - purified accessory proteins. Open circles indicate the position of the esterase activity of the microsomal β-glucuronidase-positive protein complex. Closed circles represent the position of free esterase-esterase. HSA was purified by the method of Newman and Pimentel (1981).
Egasyn (Esterase-3) Is Complexed with Rat β-Glucuronidase

Table 2. Strain distributive pattern of Gus and its relation to the Es-3 phenotypes. The Es-3 data was taken from Behring (1984) and from Behring (1983), personal communication.

Table 3. Correlation between absence of microsomal β-glucuronidase and absence of esterase-3 in 15 progeny of the heterokaryon (Ac/Mox × E3/hr60; 1 × 67/60).

Table 4. Comparison of the rat accessory protein (Es-3) and mouse agasyn (Es-22).

Table 5. Control of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.

Table 6.

Table 7.

Table 8.

Table 9.

Table 10.

Figure 10. Western blot analysis of mouse and rat agasyn using anti-catalytic peptide antibodies. Mouse agasyn was purified from liver of M. m. musculus (12 weeks, female) by precipitation with glycoinosidase, using specific anti-β-glucuronidase serum. Rat agasyn was purified from liver of R. norvegicus by applying the same method.

a. Samples were analysed on a 15% SDS-PAGE by the method of Lanesl (1979). Proteins were stained with Coomassie Brilliant Blue.

b. Samples of mouse agasyn (6 μg) and rat agasyn (6 μg), each purified as described in Materials and Methods, were electroeluted into Na-cresol/lysozyme gels. Nitrocellulose membranes strips were treated with anti-catalytic peptide antibody or goat anti-mouse agasyn followed by incubation with 125I-protein A goat antibody and visualisation with X-ray film.

Table 1. Comparison of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.

Table 2. Measurement of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.

Table 3. Measurement of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.

Table 4. Measurement of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.

Table 5. Measurement of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.

Table 6. Measurement of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.

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Table 10. Measurement of some hydrolytic activities of purified rat agasyns and of purified rat esterase-3.