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THE EFFECT OF LIMITED PROTEOLYSIS ON ENZYMATIC, BINDING AND IMMUNOLOGICAL PROPERTIES OF LIGANDIN

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SUMMARY: The peptide mixture obtained from controlled proteolytic digestion of ligandin with proteinase K or subtilisin retained 40% of glutathione-S-transferase and steroid isomerase activities, immunological reactivity and lower affinity bilirubin binding but binding at the primary site was abolished. When these limited proteolytic digests, which had no intact ligandin as determined by SDS gel electrophoresis, were subjected to Sephadex G-75 column chromatography, 40-50% of the peptide fragments were recoverd in fractions where intact ligandin eluted. The results suggest that intact ligandin is not required for enzymatic activities, binding of bilirubin at the secondary site, or immunological reactivity; steroid isomerase and glutathione-S-transferase activities are modulated in a parallel manner and may be mediated by the same region of the protein, and primary and secondary binding sites for bilirubin are distinct and independent, despite nicks introduced by proteolysis in ligandin's subunits, some of the fragments remain associated under non-denaturing conditions and the susceptibility of the two subunits to the proteases is different.

INTRODUCTION: Ligandin is an abundant protein of rat liver cytosol that binds bilirubin, sulfobromophthalein, azodye carcinogens, steroids and a variety of other organic anions (1), and exhibits glutathione-S-transferase (2) and steroid isomerase activities (3). Liver ligandin consists of two subunits of molecular weights 25K and 22K. Although both subunits have glutathione-S-transferase activity, high affinity binding of sulfobromophthalein and bilirubin occur on the 22K subunit (5). To distinguish between structural domains of the protein that are involved in its multiple functions, we subjected ligandin to limited

Abbreviations: GSH, reduced glutathione; SGP, Streptomyces griseus protease; PK, proteinase K; SL, subtilisin. The counterions are sodium for phosphate and barbital buffers and hydrochloride for Tris.

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proteolytic digestion. These digests were used to determine whether ligandin's binding, enzymatic and immunological properties are interrelated.

MATERIAL AND METHODS: Acrylamide was obtained from Bio Rad and used without further purification. Subtilisin, proteinase K and Streptomyces griseus protease were obtained from Sigma. S. aureus V_8 protease was obtained from Miles Laboratories. The steroid Δ^5- androstene 3,17-dione, used as a substrate for the steroid isomerase assay, was a gift from Dr. Paul T. Talalay.

Preparation of Ligandin: Ligandin was isolated from adult male rat liver and its purity determined by methods described earlier (6,7).

Proteolytic Digestions: Ligandin (1 mg/ml in 0.1 M phosphate buffer, pH 7.4) was incubated at 37°C with S. griseus protease (SGP; final concentration 10 µg/ml), proteinase K (PK; 20 µg/ml) and subtilisin SL; 10 µg/ml). Ligandin was incubated under identical conditions in the absence of protease as a control for all proteolysis experiments.

SDS- polyacrylamide Gel Electrophoresis: Aliquots were withdrawn from proteolytic digests at several intervals, analyzed by SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophroesis was performed in a slab gel apparatus using 15% acrylamide in a discontinuous tris-glycine system described earlier (4). Gels were stained in a solution containing 0.2% Coomassie blue in 20% methanol - 10% acetic acid and destained by diffusion in 20% methanol - 10% acetic acid.

Assay of Glutathione-S-transferase Activity: GSH-S-transferase was assayed by measuring changes in absorbance at 343 nm with GSH and 1-chloro-2,4-dinitrobenzene as substrates (6).

Steroid Isomerase Assay: Isomerization of Δ^5-androstene-3,17-dione was measured at 248 nm in tris-phosphate buffer pH 8.5 containing GSH and dithiothreitol as described by Benson et al. (3).

Determination of Immunoreactivity: It was not possible to perform Ouchterlony double diffusion with the limited proteolytic digests because all proteases interfered with antigen-antibody complex formation. Therefore, prior to incubation with monospecific antiligandin IgG, protease were separated from the peptides by electrophoresis. Immunoelectrophoresis of ligandin and its proteolytic digests was performed for 2 hours in barbital buffer pH 8.5, μ =.01, at 50V (8). Under these conditions, ligandin migrated twoard cathode while the proteases migrated towards anode (data not shown). After electrophoresis, monospecific anti-rat liver IgG was added and incubated at 4°C for 48 hrs. After incubation, plates were washed, stained with 0.2% Amido Black and destained in 7% acetic acid (7).

Binding of Bilirubin to Peptide Fragments: A Cary model 60 spectropolarimeter with a 6001 circular dichroism attachment was used to detect bilirubin binding. Measurements were made in a 1 cm light path cell at 25°C. To ligandin (500 µg/ml), or mixtures of proteolytic fragments generated from it, small increments of 0.02 M bilirubin were added to obtain CD spectra in the 550-300 nm range (6).

Sephadex G-75 Column Chromatography of Proteolytic Digests of Ligandin: Ligandin or the mixture of peptides obtained by limited proteolytic digestion with PK or SL were applied to 0.5 x 20 cm Sephadex G-75 columns equilibrated with 0.01 M phosphate buffer, pH 7.4. Elution was performed with the same buffer, and 1 ml fractions were collected. Every fraction was assayed catalytically for glutathione-S-transferase and steroid isomerase activities and for immunological reactivity by Ouchterlony double diffusion method (8).

Protein concentrations were estimated by the method of Lowry et al. (9) using bovine serum albumin as a standard.

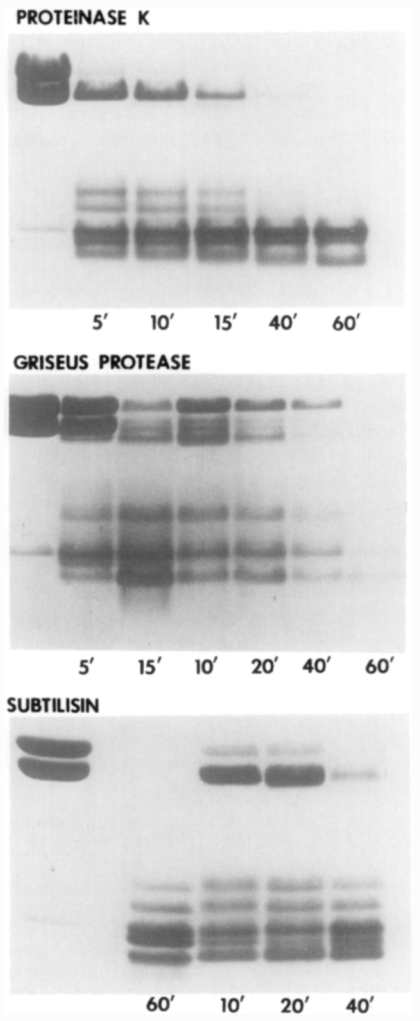

Fig. 1 Kinetic study of digestion of ligandin: Ligandin at a concentration 1 mg/ml was incubated with SGP (10 µg/ml, final concentration) or PK (20 µg/ml) or SL (10 µg/ml) at 37°C. Samples were withdrawn at indicated time intervals (from 5 min-60 min) and analyzed by Tris glycine SDS-polyacrylamide gel electrophoresis. See text for details. The sample in the first lane of each gel contains ligandin which was incubated for 1 hr at 37°C in the absence of protease.

RESULTS:

Analysis of Proteolytic Digests of Ligandin: Fig. 1. shows SDS-polyacrylamide gel electrophoresis patterns of peptides obtained by limited proteolysis of ligandin with SL, PK and SGP. Intact ligandin subunits were not detected after 40 min of digestion with PK or after 60 min digestion with SL or SGP. No further change was observed in the electrophoretic pattern when the digestion was continued for 4 hr at

Table 1: Effect of Limited Proteolysis on GSH-S-transferase and Steroid Isomerase Activities of Ligandin

Protease Treatment	GSH-S-Transferase moles/min/mg/Protein	Steroid Isomerase moles/min/mg/Protein
None	11	1.0
Proteinase K	2.8	.26
S. Griseus Protease	0	0
Subtilisin	4.1	.36

Ligandin was incubated at 37°C with or without protease for 60 min and then assayed for the indicated catalytic activity. GSH-S-transferase was assayed by measuring the change in absorbance at 343 nm with reduced GSH and 1-chloro-2,4-dinitrobenzene as substrates (6). Steroid isomerase was assayed by the change in absorbance at 248 nm at pH 8.5 in the presence of dithiothreitol and GSH with Δ^5 androstene-3,17-dione as a substrate (3).

37°C. Since most of the binding, catalytic and immunological determinations were done within this time period, the degree of proteolysis is identical in preparations used for these analysis and those used for SDS gel electrophoresis. The 25K subunit of ligandin was digested more rapidly by Sl and PK than was the 22K subunit. With SGP, however, the 22K subunit was more susceptible. In all cases, 5-8 discrete fragments ranging in molecular weight from 5-12K were observed. Lower molecular weight peptides may have escaped detection by this method.

GSH- S-transferase and Steroid Isomerase Activites of Proteolytic Digests: Approximately 25% and 40% of both catalytic activities were retained by the mixture of peptides generated by PK and SL (Table I), respectively. Both catalytic activities of ligandin were abolished by SGP (Table I), but were not altered by incubation at 37°C for 60 min in the absence of protease.

Immunological Reactivity of Proteolytic Digests of Ligandin: When protease was removed from the proteolytic digests by electrophoresis prior to incubation with antibody, the peptide mixture obtained from PK or SL digestion retained immunological reactivity, while that from SGP was not immunoreactive (Fig. 2). It is not possible to ascertain from

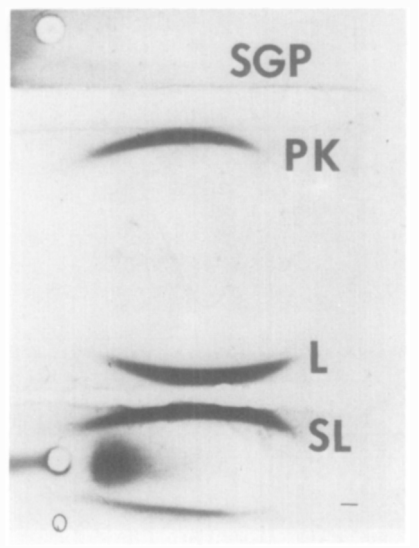

Fig. 2 Immunoelectrophoresis of ligandin and its proteolytic digests: Immunoelectrophoresis of ligandin (L) and its sixty minute proteolytic digests was performed in barbital buffer, pH 8.6, μ = 0.01 at 80V. After incubation with anti-rat liver ligandin IgG for 48 hr at 4°C plates were washed, stained with 0.2% Amido Black and destained in 7% acetic acid. O and - indicate the origin and cathode respectively.

these data if one or more peptides contribute to immunoreactivity, but the immunoprecipitin arc was more acidic with the peptide mixture than with the native ligandin molecule.

Binding of Bilirubin to Peptide Fragments: Proteolysis with SGP abolished bilirubin binding at primary and secondary binding sites. With PK and SL, the binding at the primary site was undetectable, while substantial amounts of lower affinity binding was retained (Fig. 3). These results suggest that the primary binding site for bilirubin is located in a domain that is disrupted by proteolytic action and is probably distinct from that of the low affinity binding.

Separation of Peptides by Column Chromatography and Determination of Catalytic Activities and Immunological Reactivities: As can be seen from Fig. 4, 40-50% peptides generated by limited proteolytic digestion of ligandin with PK or SL can associate themselves into a protein that elutes at the position of ligandin on column chromatography. This peak also contains steroid isomerase and GSH-S-transferase activities. Fractions 8-14 were immunoreactive with antibody to ligandin, but

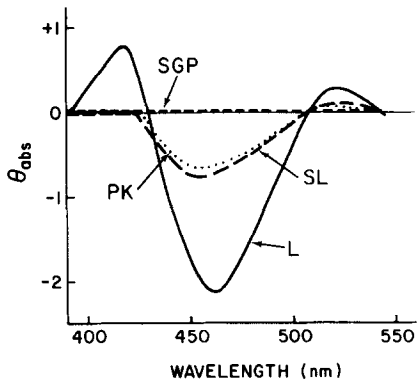

Fig. 3 Circular dichroism spectra of bilirubin complexes with ligandin and its proteolytic digests: To ligandin (1 mg/ml) and its proteolytic digests in 0.01 M phosphate buffer, pH 7.0, increments of 10^{-2} M bilirubin in 20 mM NaOH were added until saturating ellipticity values were attained. A 1 cm path length cell was used. Observed ellipticity values are expressed as degrees per 10^{-5} M protein. For the proteolytic fragments, calculations were based on total protein concentration.

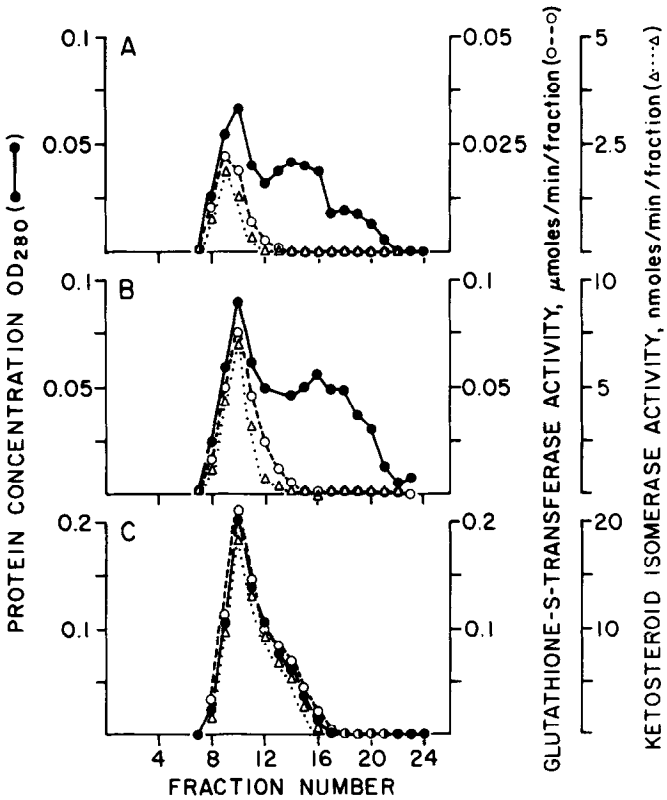

Fig. 4 Sephadex G-75 column chromatography of ligandin and its limited proteolytic digests: 1 mg ligandin digested with either PK (A) or SL (B) or undigested (C) was applied on 1 x 20 cm Sephadex G-75 column. For details of limited proteolysis, see the text. Elution was performed with 0.1 M phosphate buffer, pH 7.4. One ml fractions were collected and aliquots assayed for protein, GSH-S-transferase activity and steroid isomerase activities by methods described in the text.

fractions 15 to 22 were not. Thus, the smaller peptides are neither catalytically nor immunologically reactive.

DISCUSSION:

These studies indicate that fragments obtained by digestion with SGP, PK and SL are incapable of bilirubin binding at the primary site, but retain GSH-S-transferase and ketosteroid isomerase activities, immunological reactivity and bilirubin binding at the secondary site.

Although little is known about the specificity of the proteases used, discrete peptides are generated under the conditions described. Further degradation into smaller peptides occurs after prolonged incubation times (> 6 hr at 37°C) suggesting that some susceptible peptide regions are rapidly cleaved, whereas other sequences are relatively resistant to the action of the same protease.

The observation that the high affinity bilirubin binding site is abolished by SL and PK and that 30-40% binding at the low affinity site and catalytic activities are retained suggests that high and low affinity sites are located on different domains of the molecule and are independent of each other. These results provide further support for our suggestion that bilirubin binding at the high affinity site and GSH-S-transferase activity of ligandin are independent (4). The observation that GSH-S-transferase and steroid isomerase activities are altered to the same extent by limited proteolysis is compatible with the thesis that they may be located at the same or overlapping regions of the protein.

The results also show that, despite proteolytic nicks in ligandin, the peptide fragments tend to reassemble under non-denaturing conditions and mimic activities of native ligandin. This nicked derivative exhibits catalytic activities and immunological reactivity but does not bind bilirubin at the high affinity site. The loss of "high affinity" binding could also be due to conformational changes due to proteolytic nicks at this site.

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