Molecular Characterization of Limulin, a Sialic Acid Binding Lectin from the Hemolymph of the Horseshoe Crab, *Limulus polyphemus*†

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ABSTRACT: The sialic acid binding lectin, limulin, was isolated by gel filtration and ion-exchange chromatography from the hemolymph of *Limulus polyphemus*. When the purified protein was characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of β-mercaptoethanol, two major protein bands were observed. These two bands, subsequently found to contain carbohydrate as well, corresponded to molecular weights of 25 000 and 27 000. Amino acid sequence analyses were performed on both the intact protein and isolated cyanogen bromide fragments. The following primary structural features were noted in the amino-terminal region of limulin: (1) the absence of histidine and alanine from the NH_2-terminal 50 residues; (2) the presence of five of the total eight prolines of the molecule between positions 13 and 30; and (3) a possible carbohydrate attachment site consisting of only the amino acids proline and serine between residues 13 and 19. The results of cyanogen bromide cleavage studies confirmed the presence of 2 methionine residues per subunit, at positions 25 and 58 respectively. No sequence heterogeneity was observed in this study. While it is quite possible that limulin plays some role in the defense mechanisms of the horseshoe crab, there is no obvious sequence homology between this invertebrate lectin and vertebrate immunoglobulins.

In recent years, the circulatory systems of a wide variety of invertebrates have been shown to contain naturally occurring agglutinins (for a review, see Cohen (1974)). The ability of these proteins to recognize foreign cells and, in some cases, to enhance phagocytosis (Tripp, 1966; McKay et al., 1969; McKay and Jenkin, 1970) has led to the idea that such lectins may play some role in the defense mechanisms of invertebrates. These molecules have also proven useful as specific probes of cell membrane structure and function.

Marchalonis and Edelman have previously isolated and characterized an agglutinin from the hemolymph of the horseshoe crab, *Limulus polyphemus* (Marchalonis and Edelman, 1968). These and subsequent studies by others (Finstad et al., 1974; Roche and Monsigny, 1974; Oppenheim et al., 1974; Nowak and Barondes, 1975) indicated that this lectin, now designated limulin, was a sialic acid binding glycoprotein of approximately 400 000 daltons which contained 18 noncovalently linked subunits of approximately 22 000 daltons. In the present communication we report the amino acid sequence of 76 residues of this molecule, including the amino-terminal 50 amino acids, obtained by sequence analysis of the intact protein and its constituent cyanogen bromide fragments. The sequence data support the conclusion of Marchalonis and Edelman (1968) that the structure of limulin is unrelated to that of vertebrate immunoglobulins.

Materials and Methods

Isolation and Purification of Limulin. Horseshoe crabs of various sizes were obtained from the Marine Biological Laboratory, Woods Hole, Mass. The crabs were bled immediately upon arrival. All subsequent operations were carried out at 4 °C. The hemolymph was allowed to clot for 1–2 h and was then centrifuged for 45 min at 15 000*g* to remove clots and cellular debris. The supernatant was adjusted to pH 8.5 with Tris[1]-HCl buffer. Limulin was then isolated from the clarified hemolymph according to the method of Roche and Monsigny (1974), employing ion exchange chromatography and gel filtration. The purified protein was desalted on a Sephadex G-25 column (0.9 × 90 cm), using 1.0 M propionic acid, and then lyophilized prior to the sequence studies.

Hemagglutination Assay. Hemagglutination assays were performed on microtiter plates (Linbro Scientific Co., Inc.). Each well contained 100 μL of diluent (0.05 M Tris-HCl (pH 8.5)–0.1 M NaCl–0.01 M $CaCl_2$) and 50 μL of a 1% suspension of horse red blood cells in 0.9% NaCl. Serial dilutions were made of 100 μL of each sample to be tested. The plates were incubated at room temperature. The dilution in the last well showing visible hemagglutination was taken as the titer.

Protein Determination. Protein concentration was determined by the Biuret test (Goa, 1954) using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis in Sodium Dodecyl Sulfate. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out on 12.5% slab gels (Laemmli, 1970). The gels were run in Tris–glycine buffer, pH 8.3, and stained with Coomassie brilliant blue. Bovine serum albumin (68 000), ovalbumin (43 000), chymotrypsinogen (23 500), and ribonuclease (13 700) were used as standards.

Double Diffusion in Agarose. Rabbits were immunized with purified limulin by administering four to six biweekly doses of 1.0 mg of protein in Freund's adjuvant. Ouchterlony plates contained 1% agarose in 0.05 M Tris-HCl (pH 8.5)–0.1 M NaCl–0.01 M $CaCl_2$. The antigen, purified limulin at a concentration of 10 mg/mL, was added to the plates in the same Ca^{2+}-containing buffer, except for those samples which were

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Pth, phenylthiohydantoin; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; RBC, red blood cells; DEAE, diethylaminoethyl.

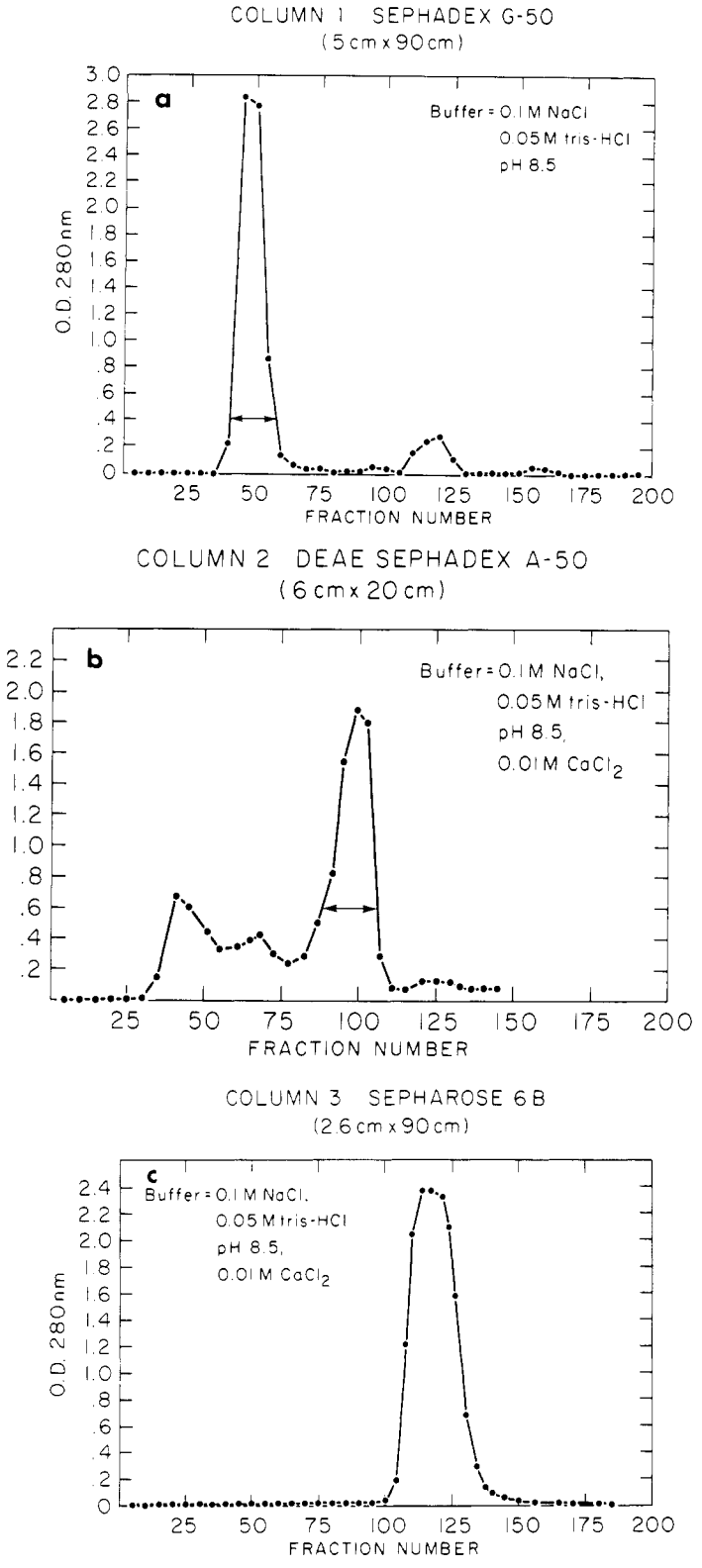

FIGURE 1: Passage of clarified hemolymph over Sephadex G-50 (a), DEAE-Sephadex (b), and Sepharose 6B (c) to obtain pure limulin.

dialyzed against Tris–EDTA.

Reduction and Alkylation. The lyophilized protein was dissolved at a concentration of 10 mg/mL in a buffer containing 8.0 M guanidine hydrochloride and 0.1 M Tris-HCl, pH 8.0. Following the addition of β-mercaptoethanol (to 0.2 M), the mixture was gassed with N_2, capped, and gently stirred for 2 h at room temperature. [^{14}C]Iodoacetamide (10 μCi) was then added (sp. act 73 μCi/mg) and the resultant mixture stirred for 15 min. This was followed by the addition of cold iodoacetamide (one and one-half times the weight of the protein) and an additional 15 min of stirring prior to column application.

TABLE I: Hemagglutination Activity during Limulin Purification.

Fraction	Volume (mL)	Titer	Protein[a] concn (mg/mL)	Spec act. (titer/mg of protein)	% recovery of hemagglutination act.
Whole hemolymph	66	16000	99.6	161	100
Sephadex G-50 fine	390	2560	15.1	170	95
DEAE-Sephadex A-50	270	2048	1.2	1707	52

[a] Biuret test indicated $E_{280nm}^{1\%,1cm} = 14.0$ for limulin. This figure was used for all calculations of specific activity.

Amino Acid Analysis. Salt-free lyophilized protein was hydrolyzed in 6 N HCl for 24 h at 110 °C in sealed evacuated vials. The samples were then dried in a vacuum desiccator over NaOH pellets. Amino acid analyses were performed on a Beckman Model 121 automatic amino acid analyzer. In order to correct for the destruction or incomplete hydrolysis of certain amino acids during the 24-h hydrolysis, the values obtained for isoleucine, valine, and serine were raised 10%, and those obtained for threonine were increased by 5%.

Cyanogen Bromide Cleavage. The protein was dissolved in 70% formic acid to a final concentration of 15–20 mg/mL. Cyanogen bromide (Aldrich Chem. Co. Inc.) was then added at a weight one and one-half times that of the protein. The reaction vessel was shaken and the mixture allowed to stand 18 h at room temperature. The reaction was stopped by the addition of 10 volumes of distilled water followed by lyophilization.

Automated Sequence Analysis. Automated sequence analysis was performed on an updated Beckman Model 890B sequencer (Edman and Begg, 1967; Kehoe and Capra, 1971; Li et al., 1974). The resultant thiazolinones were converted to phenylthiohydantoins (Pth) by heating for 10 min at 80 °C in 1.0 N HCl.

Identification of Pth-Amino Acids. Pth-amino acids were identified by gas–liquid chromatography (GLC) on a Beckman GC-45 gas chromatograph equipped with columns containing 10% SP-400 (Pisano et al., 1972). Each sample was injected both in the presence (silylated) and absence (unsilylated) of *N,O*-bis(trimethylsilyl)acetamide. In addition, each residue was examined by thin-layer chromatography (TLC) on polyamide sheets (Summers et al., 1973). Pth-arginine was identified by the phenanthrene quinone spot test (Hermodson et al., 1972).

Results

Protein Isolation. Figure 1 shows the results of the purification of limulin according to the method of Roche and Monsigny (1974). In each experiment, the starting material was approximately 70 mL of whole hemolymph, pooled from four separate animals. The final yield in each experiment was near 300 mg.

Hemagglutination Activity. Hemagglutination activity was monitored during the isolation procedure using horse red blood cells (RBC). The results of these experiments are recorded in Table I. Our data indicate that the purification of limulin from

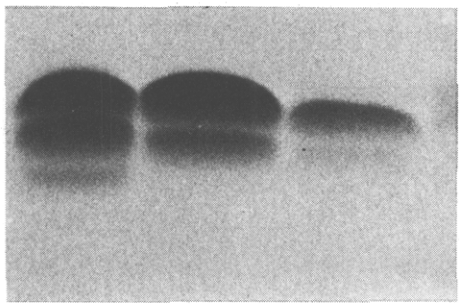

FIGURE 2: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of purified limulin (25, 20, and 5 μg) carried out according to the method described in the text.

whole hemolymph results in an approximate tenfold increase in specific activity, a figure which is lower than other reported values (Oppenheim et al., 1974; Roche and Monsigny, 1974; Nowak and Barondes, 1975). However, when comparisons are made of *initial* specific activities, i.e., those obtained when whole hemolymph is tested with horse RBC, the present results are 4–50 times higher than those obtained by the above investigators. The agglutinating property of limulin may be potentiated by the hemocyanin component of the hemolymph since removal of the hemocyanin by DEAE-Sephadex passage results in a sharp reduction in the total red cell agglutinating activity recovered.

Polyacrylamide Gel Electrophoresis. The results of polyacrylamide gel electrophoresis in sodium dodecyl sulfate and β-mercaptoethanol are shown in Figure 2. All preparations of limulin examined showed two major protein bands corresponding to molecular weights of 25 000 and 27 000. In addition, a third minor band with a molecular weight of 29 000 was sometimes seen. The two major bands were periodic acid–Schiff (PAS) positive (Zacharius and Zell, 1969), indicating the presence of carbohydrate. No information was obtained regarding the carbohydrate content of the third, minor band.

Ouchterlony Analyses. The results of double diffusion in agarose, using purified limulin as the antigen, are shown in Figure 3 (a and b): in both a and b, the antibody in the center well is anti-limulin. In all cases tested, the reaction of limulin with anti-limulin in the presence of calcium ions produced either two or three precipitin lines (Figure 3a). The three lines consisted of a prominent central band, a weak outer line curved toward the antigen well, and a diffuse inner band close to the antibody well. The innermost band was not always present. Our interpretation of these results is that the various precipitin lines reflect the presence of distinct antigenic determinants on the intact protein (18 subunits), as well as on smaller, lower order polymers (number of subunits unknown). In support of this hypothesis is the observation that, when limulin is dialyzed against Tris–EDTA to remove calcium, the outer band is no longer present (Figure 3b). The curvature of this precipitin line and its position close to the antigen well would indicate that it is due to the presence of a high molecular weight antigen. The important role of calcium ions in maintaining both the stability and biological activity of limulin has previously been demonstrated (Marchalonis and Edelman, 1968; Finstad et al., 1974).

Amino Acid Sequence Analyses. Direct sequence analysis was performed on the intact purified protein and the first 30 residues definitively identified (Table II). Figure 4 shows the results of gas chromatographic analyses (with silylation) of residues 1 and 2 of intact limulin, and attests to the purity of the protein preparation. Quantitative estimates of these two

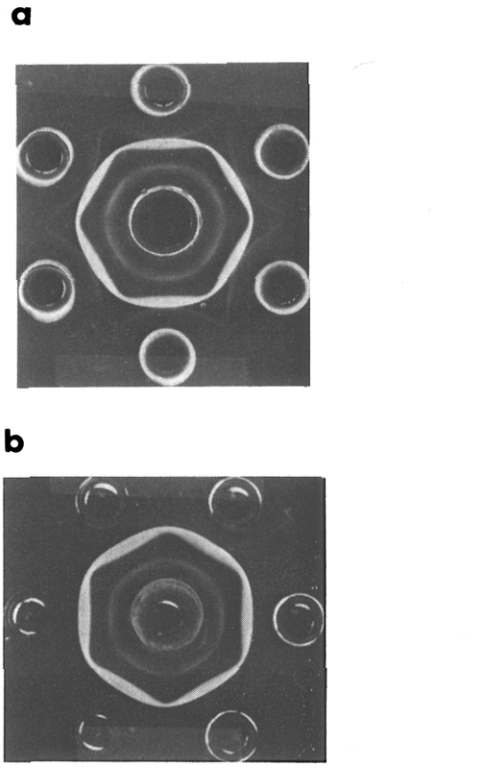

FIGURE 3: Ouchterlony analysis of purified limulin using anti-limulin antiserum in center cell. (a) Calciums ions present in buffer as described in the text. (b) Limulin sample dialyzed against Tris–EDTA to bind calcium.

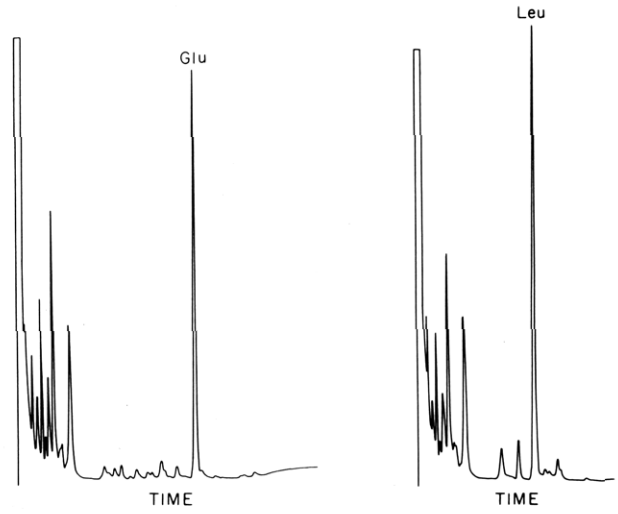

FIGURE 4: Gas chromatography analysis of the Pth derivatives of positions one (Leu) and two (Glu) of intact limulin.

residues showed that these two amino acids occurred in a yield of approximately 75% based upon the amount of starting material and comparative measurements of the gas chromatography peaks. This finding was important in view of the presence of two major bands on polyacrylamide gel electrophoresis of purified, intact limulin. The yields obtained for the other residues identified on the direct degradation of intact limulin are shown in Figure 5.

Cyanogen bromide (CNBr) cleavage of limulin was chosen as the next step in the sequence analysis for two reasons: (1) the amino acid composition of limulin indicated the presence of only 2 methionine residues per subunit and (2) sequence analysis performed on the intact protein permitted the as-

TABLE II: Assignment of Amino Acids for Intact Limulin and Cyanogen Bromide Fragments CBII and CBIII.[a]

Native Limulin					Native Limulin				
	GC			Deduced		GC			Deduced
Step	−s	+s	TLC	residue	Step	−s	+s	TLC	residue
1	LI	L	LI	Leu	26	V	V	V	Val
2		E	E	Glu	27	G	G	G	Gly
3		E	E	Glu	28	PT	T	T	Thr
4	G	G	G	Gly	29	LI	L	LI	Leu
5		E	E	Glu	30	PT	P	P	Pro
6	LI	I	LI	Ile	31				
7	PT	T	T	Thr	32	LI	L	L	Leu
8	S	S		Ser	33				
9			K	Lys	34				
10	V	V	V	Val	35	LI	LI	LI	Leu/Ile
11			K	Lys	36				
12	F	F	F	Phe	37	LI	LI	LI	Leu/Ile
13	PT	P	P	Pro	38				
14	PT	P	P	Pro	39		Y	Y	Tyr
15	S	S		Ser	40				
16	S	S		Ser	41		F	F	Phe
17	S	S		Ser	42				
18	PT	P	P	Pro	43	V	V	V	Val
19	S	S		Ser	44				
20	F	F	F	Phe	45				
21	PT	P	P	Pro	46	LI	LI	LI	Leu/Ile
22			R	Arg	47				
23	LI	L	LI	Leu	48	G	G	G	Gly
24	V	V	V	Val	49				
25	M	M	M	Met	50	LI	LI	LI	Leu/Ile

Fragment CBII						Fragment CBIII				
	GC				Deduced		GC			Deduced
Step	−s	+s	TLC	cpm	residue	Step	−s	+s	TLC	residue
1	V	V	V	390	Val	1	F	F	F	Phe
2	G	G	G	406	Gly	2	S	S		Ser
3	PT	T	T	453	Thr	3	Y	Y	Y	Tyr
4	LI	L	LI	443	Leu	4	A	A	A	Ala
5	PT	P	P	401	Pro	5	PT	T	T	Thr
6		D	D	425	Asp	6	A	A	A	Ala
7	LI	L	LI	416	Leu	7		K	K	Lys
8		Q	Q	410	Gln	8		K	K	Lys
9		E	E	397	Glu	9		D	D	Asp
10	LI	I	LI	429	Ile	10		N	N	Asn
11	PT	T	T	423	Thr	11		E	E	Glu
12	LI	L	LI	555	Leu	12	LI	L	LI	Leu
13				5206	Cys	13	LI	L	LI	Leu
14		Y	Y	1794	Tyr	14	PT	T	T	Thr
15		W	W	748	Trp	15	F	F	F	Phe
16	F	F	F	637	Phe	16	LI	L	LI	Leu
17			K	599	Lys	17		D	D	Asp
18	V	V	V	543	Val	18		E	E	Glu
19		N	N	435	Asn	19		Q	Q	Gln
20		Q	Q	400	Gln	20	G	G	G	Gly
21	LI	L	LI	528	Leu	21		D	D	Asp
22			K	572	Lys	22	F	F	F	Phe
23	G	G	G	448	Gly	23	LI	L	LI	Leu
24	PT	T	T	490	Thr	24	F	F	F	Phe
25	LI	L	LI	430	Leu	25		N	N	Asn
						26	V	V	V	Val

[a] GC, gas chromatography; +s, with silylation [addition of *N*,*O*-bis(trimethylsilyl)acetamide]; −s, no silylation; TLC, thin-layer chromatography; the source of the radioactivity is [^{14}C]iodoacetamide used to specifically identify the half-cystine residues. Each identification is based on two or more sequencer runs.

signment of a methionine residue at position 25. The resultant CNBr fragments were separated on a Sephadex G-75 column, in 30% formic acid (Figure 6a). Sequence analysis of an aliquot of the major protein peak (peak 2) gave a double sequence, one with an amino-terminal valine, the other with an amino-terminal phenylalanine. It seemed likely that the two CNBr fragments were joined together by a disulfide bridge. Therefore, peak 2 was pooled, lyophilized, reduced, and alkylated ([^{14}C]iodoacetamide), and then rerun on Sephadex G-75 (Figure 6b). Upon examination of the five protein peaks by sequencing, it was determined that peak 5 was a pure CNBr fragment with an amino-terminal valine while peak 2 contained the amino-terminal phenylalanine CNBr fragment. Peaks 1, 3, and 4 were impure mixtures.

The results of sequence analyses performed on the intact protein and CNBr fragments are summarized in Table II.

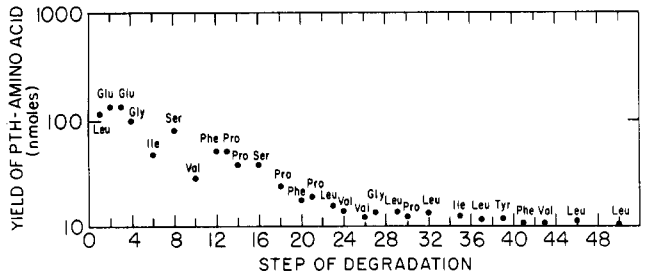

FIGURE 5: Semilogarithmic plot of yields obtained during automated Edman degradation of intact limulin. The value shown for each position was determined either by comparing the sample gas chromatographic patterns with that of an appropriate phenylthiohydantoin standard, or by amino acid analysis of the hydrolyzed phenylthiohydantoins.

TABLE III: Amino Acid Compositions (Residues per Mole) of the Intact Protein and Three CNBr Fragments.

Amino acid	Intact protein	CBI[e]	CBII	CBIII
Lys	11.0 (11)	2.6 (2)	2.0 (2)	8.0 (8)
His	8.2 (8)	0.6 (0)	1.1 (1)	6.9 (7)
Arg	3.3 (3)	1.9 (1)	1.3 (1)	2.0 (2)
CM-Cys			0.8 (1)	2.1 (2)
Asp	16.2 (16)	1.5 (0)	2.6 (3)	12.1 (12)
Thr	9.8 (10)	2.2 (1)	2.6 (3)	5.8 (6)
Ser	11.4 (12)	5.0 (5)	0.9 (1)	6.2 (6-7)
Glu	21.9 (22)	5.5 (3)	2.9 (3)	14.0 (14)
Pro	7.8 (8)	4.4 (4)	1.0 (1)	2.9 (3)
Gly	15.3 (15)	2.1 (1)	2.5 (3)	10.1 (10)
Ala	8.4 (8)	0.4 (0)	0.2 (0)	5.5 (6)
½-Cystine	3.7 (3-4)			
Val	11.7 (12)	3.7 (2)	2.8 (3)	6.3 (7)
Met	2.1 (2)			
Ile	8.2 (9)	1.4 (1)	1.1 (1)	4.1 (5)
Leu	15.3 (15)	3.5 (2)	5.6 (6)	8.6 (9)
Tyr	2.6 (3)	0.4 (0)	1.2 (1)	1.3 (1-2)
Phe	6.9 (7)	2.7 (2)	1.3 (1)	3.3 (3-4)
Hse + Hsl[a]		(1)	(1)	
Trp	(3-4)[b]		(1)[c]	(2-3)[d]
Sum	167-169	25	33	103-107

[a] Homoserine and homoserine lactone. [b] From Roche and Monsigny (1974). [c] Identified by sequence analysis. [d] Deduced from composition of intact protein. [e] Raw composition values based on incompletely purified fragment. Integer values deduced from sequence analysis.

Table III contains the amino acid compositions obtained for the intact protein and CNBr fragments. It was determined that the CNBr fragment with amino-terminal valine (termed fragment CBII) is 33 amino acids long. The sequence of this fragment corresponded to that of the native protein starting at position 26 of intact limulin (Table II). The sequence of the intact protein thus provided an overlap of five residues to permit the proper ordering of residues 1-25 (cyanogen bromide fragment CBI) and fragment CBII. Another fragment, with an amino-terminal phenylalanine, is approximately 105 amino acids long and appears to be the carboxy-terminal peptide (termed CBIII), because of the absence of homoserine and/or homoserine lactone in its composition. This fragment was very insoluble in a variety of solvents and it is conceivable that this region of the molecule could be the site of important intersubunit interactions in the native protein. The CNBr fragment with amino-terminal leucine (the first 25 amino acids of the intact protein, referred to as CBI) was recovered during subsequent studies in which reduction and alkylation of limulin

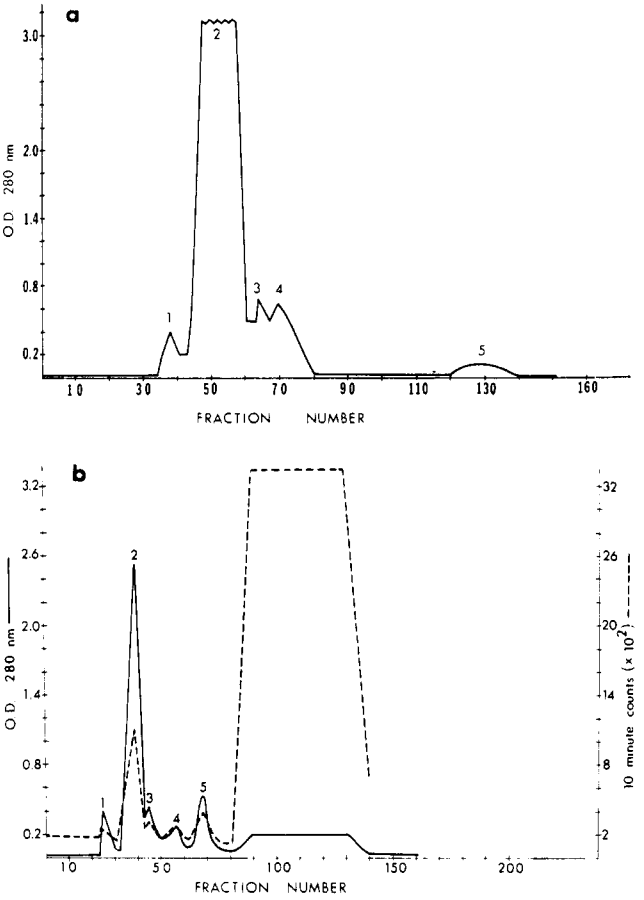

FIGURE 6: Cyanogen bromide cleavage studies of limulin. (a) Cleavage of intact limulin and passage on G-75 in 30% formic acid. (b) Passage of fraction two from above on G-75 after reduction and alkylation with [^{14}C]iodoacetamide.

were performed prior to CNBr cleavage. The composition of this peptide, which was difficult to purify away from the CBII and CBIII fragments, is also included in Table III. Thus the accumulated data allowed the ordering of the cyanogen bromide fragments as CBI-CBII-CBIII, as shown in Figure 7.

Discussion

A main goal of the present study was to search for a possible relationship between this hemagglutinating protein produced by *Limulus polyphemus* and members of the immunoglobulin family of higher vertebrates. Neither the quaternary structure of the intact molecule nor the comparison of selected stretches of amino acid sequence with immunoglobulin sequences (Gally, 1974) suggested that such a relationship exists. A comparison of the amino acid sequence information determined for limulin (Figure 7) with the very limited information that is currently available for the hemagglutinin of influenza virus (Bucher et al., 1976) also failed to show any obvious relationship between these two proteins that possess the same sialic acid binding specificity.

Although the sequence analyses of the intact protein and its CNBr fragments gave no evidence of primary structure heterogeneity in limulin, it is not yet possible to totally rule out the existence of some structural heterogeneity, especially in the carboxy-terminal segment of the protein. This, and/or differences in the structure of certain of the carbohydrate components, could explain the concomitant finding of a unique amino-terminal protein sequence and the demonstration of multiple bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

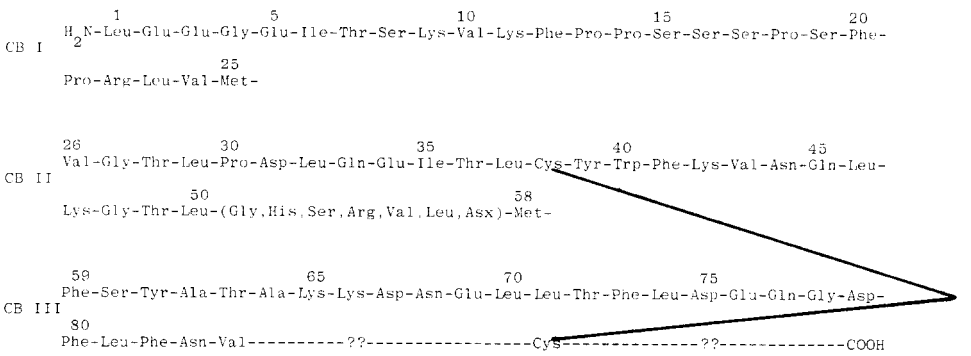

FIGURE 7: Summary of primary sequence data and organization of constituent cyanogen bromide fragments of limulin.

The finding of multiple bands following sodium dodecyl sulfate–polyacrylamide gel electrophoresis is in agreement with the starch gel electrophoresis results of Marchalonis and Edelman (1968) and the isoelectric focusing data of Finstad et al. (1974) and Roche and Monsigny (1974). The presence of multiple bands after gel electrophoresis of a pure glycoprotein can be due, in principle, to any one of a number of factors. If the gel separates on the basis of charge, multiple bands could be due to deamidation of glutamine and/or asparagine residues or to differences in the amount of charged carbohydrate present. In the case of sodium dodecyl sulfate–polyacrylamide gels, however, multiple bands are generally due to differences in carbohydrate content and/or length of the polypeptide chain. In a recent publication, Furthmayr and Marchesi (1976) have pointed out that numerous methodological problems may be encountered when sodium dodecyl sulfate–polyacrylamide gel electrophoresis is used to determine the purity or molecular weight of a glycoprotein. They demonstrated that, in the case of glycophorin A, a human erythrocyte membrane glycoprotein, the electrophoretic mobility was dependent upon the protein concentration, sodium dodecyl sulfate concentration, type of buffer system used, temperature during incubation and length of incubation of samples prior to electrophoresis. Similar variables could be responsible for the observations made in the present study.

The sequence studies indicated a number of additional interesting features of the limulin molecule. The first fifty residues of the protein contain no histidine or alanine, even though there are numerous representatives of these amino acids present in the whole molecule. In contrast, five of an available eight proline residues of the entire protein are present between positions 13 and 30. A shorter sequence stretch between positions 13 and 19 is composed entirely of proline and serine. This region is a logical candidate for a carbohydrate attachment site since Spiro (1973) has recently pointed out that, in many glycoproteins, proline residues are found near serines to which carbohydrate moieties are attached.

The two methionine residues of the limulin subunit were localized to positions 25 and 58. The total number of amino acid residues contained in the three constituent CNBr fragments was 163, very close to the number expected per subunit on the basis of amino acid compositional analysis.

The composition of the intact subunit indicated that either three or four half cystine residues would be found per subunit. The current sequence study showed that there was only one half-cystine between residues 1 and 58 of the intact protein and that this residue was located within cyanogen bromide fragment CBII at position 38. The CNBr fragmentation studies demonstrated that this half-cystine is connected via a disulfide bridge, to another half-cystine located at a presently undetermined position in the CBIII fragment. The amino acid composition of the CBIII fragment showed that two half-cystine residues were present therein. The combined data thus suggest that the intact protein actually contains two half-cystines and one cysteine, rather than four half-cystines, a finding consistent with compositional analyses reported by others (Roche and Monsigny, 1974; Nowak and Barondes, 1975).

Although no sequence homologies with vertebrate immunoglobulin were observed, there is too little sequence information currently available for other invertebrate lectins to discern any relationships among this group of molecules. However, Acton et al. showed that a hemagglutinin from the oyster, *Crassostrea virginica,* shared a number of structural features with limulin including a subunit molecular weight of approximately 20 000, noncovalent interactions between the subunits of the native protein, a requirement for calcium ions to maintain both structural stability and biological activity, and some degree of similarity in amino acid composition. Clearly, much more data on the primary structure of other invertebrate lectins are required to reveal any common structural features they may possess. Limulin itself would seem an ideal probe for additional studies of cell membrane structure and further analyses of the response of various cell types to such proteins. Increased knowledge of the molecular structure of this lectin will make the interpretation of the consequences of such protein–cell interactions much more meaningful.

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Nonenzymatic Reduction of Prostaglandin H by Lipoic Acid†

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ABSTRACT: Lipoic acid has recently been found to stimulate prostaglandin biosynthesis by sheep vesicular gland microsomes (Marnett, L. J., and Wilcox, C. L. (1977), *Biochim. Biophys. Acta 487,* 222). The increase in oxygenated products is predominantly in the formation of prostaglandin F and its structure has been verified by gas chromatography–mass spectrometry. Endoperoxide trapping experiments employing reduced glutathione show that the conversion of prostaglandin H to prostaglandin F is slow in lipoate containing incubation mixtures. Therefore, the net effect of the addition of lipoic acid to vesicular gland microsomes is the stimulation of prostaglandin endoperoxide biosynthesis. Further experiments reveal that the reduction of prostaglandin H to prostaglandin F by lipoate is nonenzymatic and occurs *after* the termination of biosynthesis in the work-up mixture. The reduction takes place preferentially in the organic phase of a Folch extract (chloroform–methanol–2% formic acid 8:4:3). Authentic prostaglandin H_2 is reduced by lipoic acid to prostaglandin $F_{2\alpha}$ in high yield under these conditions.

The conversion of PGH[1] to PGF can occur by either of two pathways. In the first, the cyclic peroxide PGH is isomerized spontaneously or enzymatically to the hydroxy ketone PGE. PGE is then reduced by a pyridine nucleotide to PGF (Leslie and Levine, 1973; Hensby, 1974; Lee and Levine, 1974). Both enzymes of this pathway—prostaglandin endoperoxide E isomerase and prostaglandin E 9-ketoreductase—have been purified and characterized (Ogino et al., 1977; Lee and Levine, 1975). In the second pathway, PGH is directly reduced to PGF. No evidence for the existence of a prostaglandin endoperoxide reductase has been found and it has been suggested that agents such as Cu^{2+}–dithiol complexes which selectively stimulate PGF biosynthesis do so by the nonenzymatic reduction of PGH (Chan et al., 1975).

We have recently observed high yields of PGF in incubations of sheep vesicular gland microsomes with eicosatrienoic and arachidonic acids in the presence of lipoic acid (Marnett and Wilcox, 1977). Lipoate stimulates overall prostaglandin biosynthesis two- to fourfold and selectively enhances the biosynthesis of PGF at the expense of other prostaglandins. In view of the uncertainty surrounding the biosynthesis of PGF, we have investigated in detail its formation in the presence of lipoate. Our study conclusively demonstrates that lipoic acid nonenzymatically reduces PGH to PGF and it illustrates in a novel fashion the importance of nonenzymatic transformations of prostaglandin endoperoxides in vitro.

Experimental Section

Materials

Arachidonic acid was kindly furnished by Dr. John Paulsrud, Hoffmann-La Roche. Eicosatrienoic acid and authentic prostaglandins were generously provided by Dr. John Pike, the Upjohn Co. Diclofenac sodium (GP-45840) was a gift of Dr. William Cash, Ciba-Geigy. [1-^{14}C]Eicosatrienoic acid was purchased from New England Nuclear and [1-^{14}C]arachidonic acid from Applied Science Laboratories. α-Lipoic acid (Sigma) was recrystallized twice with hexane/chloroform. The sulfhydryl content of the recrystallized material was below the limit of detection (~0.1%) using 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman, 1959).

The microsomal fraction of sheep vesicular glands was

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[1] Abbreviations used are: PGD_1, 9α,15α-dihydroxy-11-oxo-13-*trans*-prostenoic acid; PGD_2, 9α,15α-dihydroxy-11-oxo-5-*cis*,13-*trans*-prostadienoic acid; PGE_1, 11α,15α-dihydroxy-9-oxo-13-*trans*-prostenoic acid; PGE_2, 11α,15α-dihydroxy-9-oxo-5-*cis*,13-*trans*-prostadienoic acid; $PGF_{1\alpha}$, 9α,11α,15α-trihydroxy-13-*trans*-prostenoic acid; $PGF_{2\alpha}$, 9α,11α,15α-trihydroxy-5-*cis*,13-*trans*-prostadienoic acid; PGG_1, 15α-hydroperoxy-9α,11α-peroxido-13-*trans*-prostenoic acid; PGG_2, 15α-hydroperoxy-9α,11α-peroxido-5-*cis*,13-*trans*-prostadienoic acid; PGH_1, 15α-hydroxy-9α,11α-peroxido-13-*trans*-prostenoic acid; PGH_2, 15α-hydroxy-9α,11α-peroxido-5-*cis*,13-*trans*-prostadienoic acid; GSH, reduced glutathione; PG, prostaglandin; TMS, trimethylsilyl.