Nereis Cuticle Collagen: Isolation and Characterization of Two Distinct Subunits†

Shigeru Kimura‡ and Marvin L. Tanzer*

ABSTRACT: The soluble cuticle collagens of the Polychaetes, *Nereis japonica* and *Nereis virens*, have been isolated from aqueous acetic acid extracts of cuticle fibers which had been previously treated with enzyme inhibitors. Sodium dodecyl sulfate gel electrophoresis and CM-cellulose chromatography of the denatured cuticle collagens showed that two distinct subunits, A chain and B chain, were present in a molar ratio of about 1:2, respectively. Each subunit had a molecular weight of approximately 470 000, as shown by agarose gel filtration. These subunit chains were separated by CM-cellulose chromatography and then analyzed to determine their chemical compositions. B chain was isolated as a single species, whereas A chain was always accompanied by two minor components. Both chains differed significantly from each other in their content of several amino acids, but both were unusual due to their very high content of hydroxyproline. Neither contained hydroxylysine, while both contained galactose as the major carbohydrate moiety. The B chain of *N. virens* cuticle collagen was cleaved by CNBr into two fragments, one of molecular weight approximately 70 000 and the other about 400 000. The amino acid compositions of these polypeptides were similar to each other. The small fragment arose from the COOH terminus of the original B chain. The composite studies indicate that polychaete cuticle collagens are unique with regard to their subunit mass and chemical compositions, compared with other collagenous proteins.

The major cuticle protein of polychaetes is a collagen having several unusual properties, not only in chemical composition, but also in molecular structure (Kimura, 1971, 1973). In many respects, it resembles the cuticle collagen of the oligochaete, earthworms (Josse and Harrington, 1964; Lee and Lang, 1968). The cuticle collagens of both classes are extremely rich in hydroxyproline, but poor in proline and lacking in hydroxylysine. In addition, they mainly contain galactose as the carbohydrate moiety, probably linked to threonine and serine residues (Lee and Lang, 1968; Kimura, 1973). Another feature is that these native cuticle collagens have the highest particle molecular weight, about 1 700 000 to ~1 900 000, when various collagens in the animal kingdom are compared (Josse and Harrington, 1964; Kimura, 1971; Utiyama et al., 1973). Sedimentation velocity analysis of these denatured cuticle collagens suggests that they contain very large subunit chains whose molecular weights are several times higher than the 95 000 daltons of the α chains from most collagens. Structural studies on the subunit chains of cuticle collagens are needed to clarify the complex structure of this unusual collagen in order to provide a suitable model of molecular architecture.

In the present paper, we describe the properties and fractionation of denatured cuticle collagen obtained from polychaetes. Two distinct subunit chains have been isolated by CM-cellulose[1] chromatography and their molecular weights and chemical composition have been determined.

Materials and Methods

Preparation of Nereis Cuticle Collagen. Living worms, *Nereis japonica*[2] and *Nereis virens*, were purchased from local bait dealers at Tokyo and Connecticut, respectively, and preserved in the frozen state at −20 °C until use. After thawing in tap water, the cuticles were isolated by scraping off the adherent tissues with forceps and washed with successive changes of distilled water (Kimura, 1971). The cuticles of both worms were treated with 1 M NaCl–0.05 M Tris (pH 7.5) containing the enzyme inhibitors, 0.025 M ethylenediaminetetraacetic acid, 0.010 M *N*-ethylmaleimide, and 0.001 M phenylmethane-sulfonyl fluoride. After 48 h of constant stirring at 4 °C, the cuticles were washed exhaustively with distilled water and then extracted with 0.1 M acetic acid for 48 h at 4 °C. The acid extracts were centrifuged at 40 000*g* for 1 h and the acid soluble cuticle collagens were precipitated by the addition of 20% NaCl to a final concentration of 2.5%. The precipitates were redissolved in 0.1 M acetic acid, centrifuged, and precipitated with 2.5% NaCl as described above. The procedure of dissolution and precipitation was repeated twice more. The final precipitates were redissolved in 0.1 M acetic acid, dialyzed against the solvent, and lyophilized. In separate experiments, the cuticle of *N. japonica* was partly dissolved in 1 M NaCl–0.05 M Tris (pH 7.5) in the absence of enzyme inhibitors. The resultant solution was centrifuged at 40 000*g* for 1 h and the neutral salt soluble collagen was precipitated by dialyzing the supernatant against successive changes of 0.02 M Na_2HPO_4. The precipitate was redissolved in 0.1 M acetic acid and purified in the same manner as the acid soluble cuticle collagen. All of the isolation procedures were performed in a cold room at approximately 3–5 °C.

Pepsin Digestion. Cuticle collagen was dissolved in 0.1 M acetic acid and incubated with pepsin (enzyme:substrate = 3:100) at 5 °C for 48 h with stirring. The reaction mixture was then dialyzed against successive changes of 0.02 M Na_2HPO_4

† From the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032. *Received December 27, 1976.* This work was supported by a grant from the National Institutes of Health (AM-12683).

‡ Present address: The Laboratory of Biochemistry, Tokyo University of Fisheries, Minato, Tokyo 108.

[1] Abbreviations used: CM-cellulose, carboxymethylcellulose; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

[2] Per the suggestion of Dr. L. C. Oglesby of Pomona College, the scientific name of th6s worm is corrected to *"Nereis japonica"* instead of *"Neanthes diversicolor"*, which was used in previous papers (Kumura, 1971, 1973).

to inactivate the pepsin and to precipitate the pepsin-treated cuticle collagen. The precipitate was collected by low-speed centrifugation and redissolved in 0.1 M acetic acid. After reprecipitation with 0.02 M Na_2HPO_4, the pepsin-treated collagen was redissolved in 0.1 M acetic acid, dialyzed against the solvent, and lyophilized.

Cleavage with CNBr. The samples were dissolved in 70% formic acid (Steers et al., 1965) at a concentration of 5 mg/mL. The sample solution was flushed with nitrogen and crystalline CNBr, equivalent to 1.5 times the weight of the dry material, was added. The mixture was incubated for 6 h at room temperature, diluted tenfold with water and lyophilized, following which it was dissolved in water and relyophilized.

Molecular Sieve Chromatography. Chromatography was performed on a 1.5 × 180 cm column of 6% agarose gel (Bio-Gel A-5m, 200–400 mesh) at room temperature. Samples were dissolved in 1 mL of 1.2 M $CaCl_2$–0.05 M Tris (pH 7.5) and 0.1 mL of tritiated water containing about 2×10^5 dpm was added. The mixture was warmed to 40 °C for 5 min to ensure that denaturation was complete and then applied to the column equilibrated with the same buffer. Elution was achieved by the buffer at a flow rate of 6.5 mL/h. Fractions of 2.7 mL were collected and the absorbance was monitored at 220 nm.

CM-Cellulose Chromatography. Denatured cuticle collagens were fractionated upon a 2.5 × 20 cm column of CM-cellulose (Whatman CM-52) at 40 °C, essentially according to Piez et al. (1963). Samples were dissolved at a concentration of 10 mg/mL in 0.04 M sodium acetate, pH 4.8 (starting buffer), and warmed to 40 °C for 10 min. After centrifuging, the sample solution was applied to the column which had been equilibrated with the starting buffer. Elution was achieved with a linear gradient from 0 to 0.1 M NaCl over a total volume of 1300 mL at a flow rate of 120 mL/h. The effluent was monitored continuously at 230 nm by a Gilson recording UV column monitor and fractions of 8 mL were collected. Appropriate fractions were pooled, desalted by Sephadex G-25, and lyophilized. In a similar way, peptides liberated by CNBr cleavage were fractionated upon a 0.9 × 7.0 cm column of CM-cellulose. Samples were dissolved in 0.02 M sodium acetate, pH 4.8 (starting buffer), and applied to the column. Elution was achieved with a linear gradient from 0 to 0.1 M NaCl over a total volume of 200 mL at a flow rate of 40 mL/h. Fractions of 1.3 mL were collected.

Sodium Dodecyl Sulfate Gel Electrophoresis. Electrophoresis was carried out on 4% precast polyacrylamide gels (Bio-Rad Laboratory), using an electrophoresis buffer of 0.205 M Tris–0.205 M acetic acid–0.1% sodium dodecyl sulfate, pH 6.6. A current of 8 mA per tube was applied for 3 h at room temperature. Following electrophoresis, the gels were stained with Coomassie blue, destained, and then scanned at 600 nm in a Gilson spectrophotometer.

Viscosity. The viscosity of cuticle collagen in the denatured state was measured by a Cannon-Ubbelohde dilution viscometer at 35 °C, since the denaturation temperatures of both *Nereis* cuticle collagens were found to be below 30 °C (unpublished data). Samples were dissolved in 0.15 M potassium acetate, pH 4.8 (Lewis and Piez, 1964), and warmed to 40 °C for 10 min. After centrifugation at 40 000*g* for 30 min, the samples were dialyzed against the same buffer.

Chemical Analyses. Protein concentrations of collagen solutions were measured by a microhydroxyproline determination (Bergman and Loxley, 1963) and the collagen content was calculated on the basis of hydroxyproline contents of 23.9 and 20.8% for the cuticle collagens of *N. japonica* and *N. virens*, respectively. Amino acid analyses were done by a single column system on a Beckman Model 116 amino acid analyzer. In the case of CNBr peptides, homoserine lactone was determined as homoserine by heating the samples in 0.1 M pyridine–acetic acid, pH 6.5, for 1 h at 110 °C (Ambler and Brown, 1967). Neutral sugars were analyzed as the trimethylsilyl derivatives by a Hewlett-Packard gas chromatograph (Laine et al., 1972).

TABLE I: Amino Acid Composition of *Nereis* Cuticle Collagens.[a]

	N. japonica		*N. virens*
	NSCC[b]	ASCC[c]	ASCC
3Hyp	6.5	5.9	3.9
4Hyp	172	170	147
Asp	12	10	10
Thr	43	42	43
Ser	61	59	69
Glu	124	127	133
Pro	30	23	51
Gly	344	352	347
Ala	93	95	88
Cystine	0	0	0
Val	8.4	7.7	7.0
Met	1.1	0.8	0.8
Ile	7.3	8.9	5.0
Leu	19	19	18
Tyr	0.7	0.6	0.4
Phe	2.9	2.3	2.2
Hyl	0	0	0
His	0.2	0.2	0.3
Lys	0.7	0.5	0.6
Arg	75	76	74

[a] Values are given in residues per 1000 total amino acid residues. [b] Neutral salt soluble cuticle collagen. [c] Acid soluble cuticle collagen.

Results

Properties of Cuticle Collagen Preparations. When the cuticle of *N. japonica* was placed in 1 M NaCl–0.05 M Tris (pH7.5) in the absence of enzyme inhibitors, about 40% of the collagen dissolved. This neutral salt soluble collagen was purified by precipitation with 0.02 M Na_2HPO_4. In contrast, when the cuticle was placed in 1 M NaCl–0.05 M Tris, pH 7.5, in the presence of enzyme inhibitors, it was insoluble and no collagen could be detected in the neutral salt solution. When the cuticle was placed directly into 0.1 M acetic acid, it almost completely dissolved, whether or not enzyme inhibitors were present in the solvent. However, all studies were done on acid soluble collagen which had been initially dissolved in the presence of enzyme inhibitors. This acid soluble collagen was purified by precipitation with 2.5% NaCl. As shown in Table I, the amino acid compositions of both salt and acid soluble *N. japonica* cuticle collagens were similar, within experimental error. These data exhibited the characteristic compositional features of polychaete cuticle collagen: the high contents of hydroxyproline, glutamic acid, and arginine, the low contents of aspartic acid and proline, and the absence of hydroxylysine and cystine (Kimura, 1971). The cuticle collagen preparations were then examined with respect to their subunit size. An aliquot of each preparation was denatured and chromatographed on 6% agarose gel column. The acid soluble collagen eluted as a single component (Figure 1B), whereas the neutral salt soluble collagen separated into several relatively low molecular weight components (Figure 1A), each of which had amino acid compositions similar to the unfractionated samples (data not

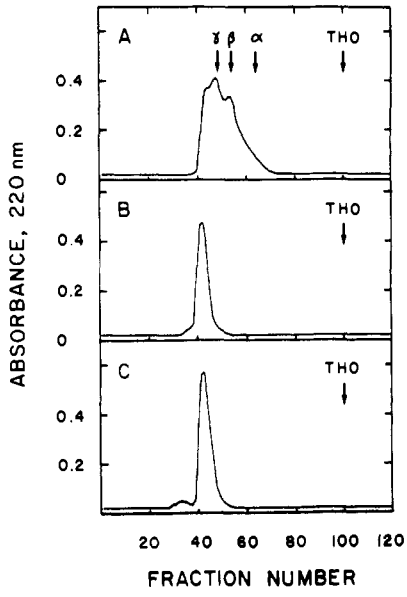

FIGURE 1: Elution patterns of approximately 2 to ~4 mg of denatured cuticle collagens on a column of 6% agarose gel (1.5 × 180 cm). Chromatography was performed as described in the text. Arrows designate the elution positions of tritiated water (THO) and the α, β, and γ components of calf skin collagen. (A) Neutral salt soluble collagen of *N. japonica*. (B) Acid soluble collagen of *N. japonica*. (C) Acid soluble collagen of *N. virens*.

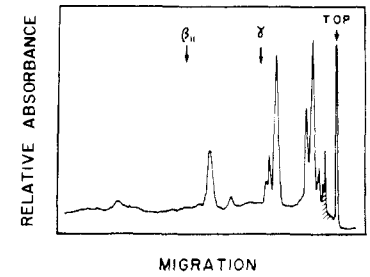

FIGURE 2: Densitometric tracing of sodium dodecyl sulfate-polyacrylamide gel following electrophoresis of the neutral salt soluble cuticle collagen from *N. japonica*. Arrows indicate the migration positions of β_{11} and γ components of calf skin collagen.

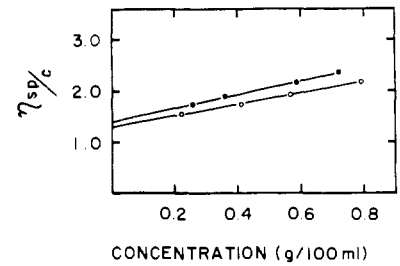

FIGURE 3: Plots of reduced viscosity vs. concentration for the acid soluble cuticle collagens in the denatured state (35 °C). (O—O) *N. japonica*. (●—●) *N. virens*.

shown). This agarose gel column had been previously calibrated with the α, β, and γ components of calf skin collagen. A linear relationship of log M and V_E/V_{THO} was observed, where M is the molecular weight of each component and V_E/V_{THO} is the ratio of the elution volume of the component to that of tritiated water. Thus, the acid soluble collagen in the denatured state was determined to have a molecular weight of approximately 470 000 on the basis of its elution position. On the other hand, 4% sodium dodecyl sulfate gel electrophoresis indicated that the neutral salt soluble collagen was separated into four major and several minor components as shown in Figure 2. As will be shown, this finding is in contrast with the acid soluble collagen, where only two major components were present. The hatched peaks in Figure 2 were found to correspond to these two major components of acid soluble collagen (see below). Of importance is that the acid soluble collagen did not alter in subunit composition on agarose gel chromatography and sodium dodecyl sulfate gel electrophoresis, even after pepsin treatment at 5 °C.

The acid soluble cuticle collagens of *N. japonica* and *N. virens* were compared. The amino acid compositions were very similar except for a slight difference in the degree of proline hydroxylation; the ratio of hydroxyproline to total imino acids was 88% for *N. japonica* and 75% for *N. virens* (Table I). Both proteins had similar chromatographic and electrophoretic behavior on agarose gel filtration and sodium dodecyl sulfate-polyacrylamide gels, respectively. A typical elution pattern of *N. virens* cuticle collagen is shown in Figure 1C, where only a major component having a molecular weight of approximately 470 000 is evident. This elution profile was unchanged following prior exposure of the native cuticle collagen to pepsin.

The molecular weight of denatured acid soluble cuticle collagen was also estimated by its intrinsic viscosity. The relation between molecular weight M and intrinsic viscosity $[\eta]$ is expressed in the equation of Houwink (1940), $[\eta] = KM^{\alpha}$, where K and α are calculated to be 7.93×10^{-5} and 0.744, respectively, from the data of α_1 and β_{12} components of rat skin collagen (Lewis and Piez, 1964). As shown in Figure 3, the intrinsic viscosities of acid soluble cuticle collagens from *N. japonica* and *N. virens* were found to be 1.30 and 1.40 dL/g, respectively. Using these values, we obtained the molecular weights of 462 000 for *N. japonica* and of 513 000 for *N. virens*, which are consistent with the values obtained by agarose gel chromatography.

These results indicated that the acid soluble collagen of *Nereis* cuticle was composed of subunit chains whose molecular weights were approximately 4.7 to $\sim 5 \times 10^5$. In addition, the neutral salt soluble fraction of *N. japonica* cuticle was thought to arise from enzymatic cleavages of the cuticle collagen since its appearance was prevented by enzyme inhibitors and its subunits were smaller in size than the acid soluble collagen.

Isolation and Characterization of Two Distinct Subunits. The acid soluble cuticle collagen of *N. japonica* was found to consist of two major components, designated A and B, together with two minor components, A′ and A″, when examined by sodium dodecyl sulfate gel electrophoresis (Figure 4). The quantitative ratio of A and B chains was calculated to be about 1:2 by measuring the areas under the peaks of the electrophoretic pattern. In order to isolate the A and B chains, the cuticle collagen was chromatographed on a column of CM-cellulose at 40 °C. As shown in Figure 5, it seemed to be poorly separated into two fractions, which were pooled as indicated by each bar. Following rechromatography both fractions were examined by sodium dodecyl sulfate gel electrophoresis and the results are presented in Figures 4II and 4III. On the basis of mobility, fraction 1 was found to largely contain the A chain with small amounts of A′ and A″, while fraction 2 was exclusively the B chain. This was confirmed by the fact that a 1:2 mixture of fractions 1 and 2 (Figure 4IV) gave the same electrophoretic pattern as the original sample. As might be

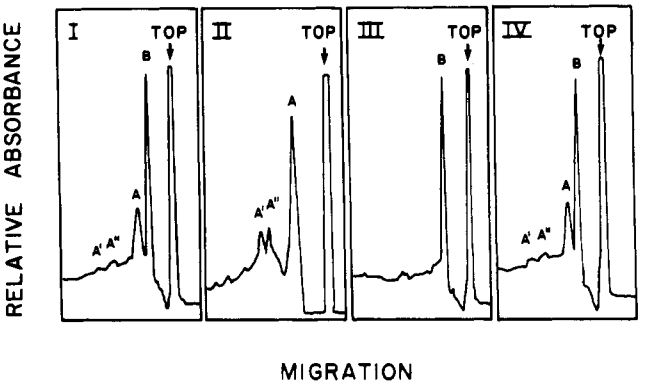

FIGURE 4: Densitometric tracings of sodium dodecyl sulfate-polyacrylamide gels following electrophoresis of the acid soluble cuticle collagen from *N. japonica* and the fractions indicated in Figure 5. (I) Acid soluble cuticle collagen; (II) fraction 1; (III) fraction 2; (IV) 1:2 mixture of fractions 1 and 2.

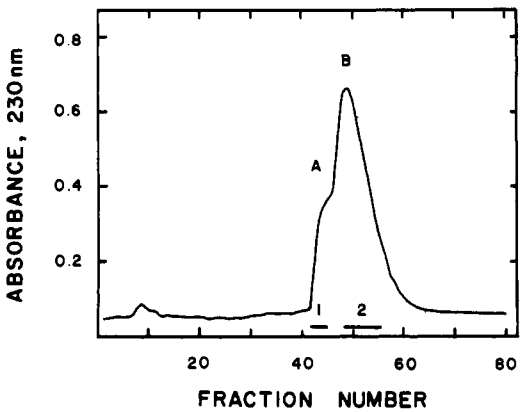

FIGURE 5: Elution pattern of approximately 35 mg of the acid soluble cuticle collagen from *N. japonica* on a column of CM-cellulose (2.5 × 20 cm) at 40 °C. Chromatography was performed as described in the text. Fractions indicated under each bar were pooled and subjected individually to sodium dodecyl sulfate gel electrophoresis and agarose gel chromatography.

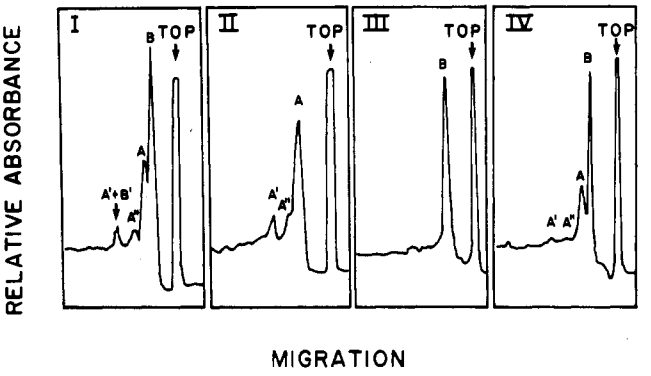

FIGURE 6: Densitometric tracings of sodium dodecyl sulfate-polyacrylamide gels following electrophoresis of the acid soluble cuticle collagen from *N. virens* and the fractions indicated in Figure 7. (I) Acid soluble cuticle collagen; (II) fraction 1; (III) fraction 3; (IV) 1:2 mixture of fractions 1 and 3.

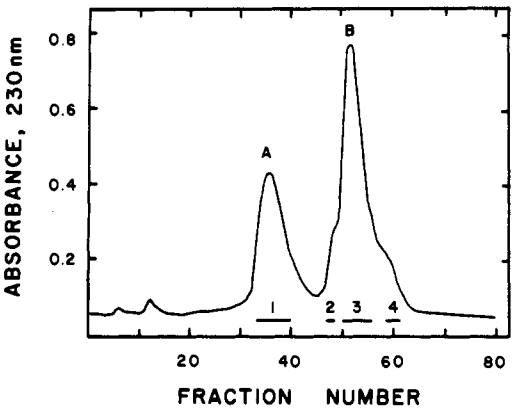

FIGURE 7: Elution pattern of approximately 50 mg of the acid soluble cuticle collagen from *N. virens* on a column of CM-cellulose (2.5 × 20 cm) at 40 °C. Chromatography was performed as described in the text.

expected, agarose gel chromatography of each fraction indicated the presence of a single peak at a region corresponding to a molecular weight of about 470 000. Thus, the molar ratio of B to A was found to be about 2.

The presence of A and B chains was also shown in the acid soluble collagen of *N. virens* cuticle (Figure 6I). In addition to the minor components A′ and A″, another minor component, designated B′, Was present in this collagen. The complete separation of A and B chains was successfully achieved by CM-cellulose chromatography as shown in Figure 7. The fractions indicated under each bar were examined by sodium dodecyl sulfate gel electrophoresis. Fraction 1 largely contained the A chain with small amounts of A′ and A″, while fraction 3 was exclusively the B chain (Figures 6II and 6III). The shoulder preceding the B chain peak, fraction 2, was found to be mostly the minor component B′ and the tail on the B chain peak, fraction 4, consisted ofsome degradation products (data not shown). However, there were no marked differences between fractions 2, 3, and 4 in their amino acid compositions, so that fractions 2 and 4 were presumably derived from some degradation of B chain. The quantitative ratio of A and B chains was about 1:2 by measuring the peak areas of both the electrophoretic pattern and the chromatogram of the original sample (Figures 6I and 7). Moreover, fractions 1 and 3 were eluted from the agarose gel column as a single peak in a region coincident with the elution position of the original sample (Figure 1C), indicating that the A and B chains were each about 470 000 molecular weight and were initially present in a molar ratio of 1:2.

The chemical compositions of the A and B chains which had been fractionated by CM-cellulose are given in Table II. The two minor compones A′ and A″ were not separated from A chain by rechromatography on CM-cellulose and agarose gel. Thus, fraction 1, which contains A′ and A″, is identified in the analyses as "A chain". For each *Nereis* cuticle collagen, the A and B chains exhibited significantly different, but obviously related, amino acid compositions. Generally, these subunit chains contained large amounts of 4-hydroxyproline and small but significant amounts of 3-hydroxyproline. Hydroxylysine and cystine were completely absent, while tyrosine, lysine, and histidine were present in very small amounts. When compared with the B chain, the A chain was rich in aspartic and glutamic acids, valine, methionine, isoleucine, leucine, and phenylalanine, but was poor in 4-hydroxyproline, threonine, and serine. These data also indicated that a calculated 1:2 mixture of A and B chains had an amino acid composition (Table II) almost identical with that of the original sample (Table I), within experimental error. This is in good agreement with the fact that A and B chains are present in a molar ratio of 1:2. The results of carbohydrate analyses are also shown in Table II. In both collagens, the A chain was rich in carbohydrate compared with the B chain. Of the total carbohydrate, about 80% was found

TABLE II: Amino Acid and Carbohydrate Composition of A and B Chains from *Nereis* Cuticle Collagens.[a]

	N. japonica			*N. virens*		
	A	B	A·$(B)_2$[b]	A	B	A·$(B)_2$
3Hyp	5.9	5.5	5.6	4.9	2.9	3.6
4Hyp	162	179	173	128	151	143
Asp	10	8.9	9.3	12	7.2	8.8
Thr	33	44	40	35	45	42
Ser	57	61	60	53	74	67
Glu	131	125	127	146	129	135
Pro	19	24	22	51	50	50
Gly	360	351	354	361	351	354
Ala	93	97	96	85	94	91
Cystine	0	0	0	0	0	0
Val	9.7	5.1	6.6	9.3	4.1	5.9
Met	1.3	0.3	0.6	0.9	0.2	0.5
Ile	12	6.5	8.3	9.0	4.1	5.7
Leu	27	15	19	27	13	18
Tyr	0.1	0.1	0.1	0.2	0.1	0.1
Phe	4.2	0.7	1.9	4.7	0.5	1.9
Hyl	0	0	0	0	0	0
His	0.1	0.1	0.1	0.1	0.1	0.1
Lys	0.1	0.1	0.1	0.1	0.1	0.1
Arg	75	77	76	73	74	74
Galactose[c]	15	10	12	11	6.2	7.8
Glucose	1.6	1.4	1.5	1.1	0.6	0.8
Mannose	3.9	1.0	2.0	1.8	0.4	0.9

[a] Values are given in residues per 1000 total amino acid residues. [b] Calculated from A and B columns. [c] Content of each monosaccharide is calculated on the basis of a mean amino acid residue weight of 92.

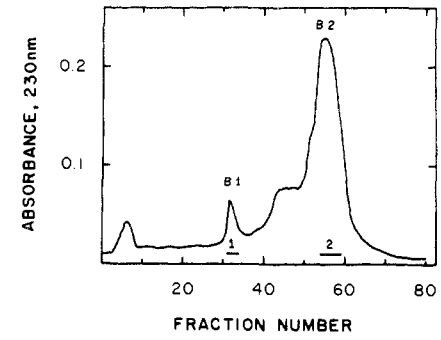

FIGURE 8: Elution pattern of approximately 8 mg of the CNBr-cleavage products from B chain of *N. virens* cuticle collagen on a column of CM-cellulose (0.9 × 7.0 cm) at 40 °C. Chromatography was performed as described in the text.

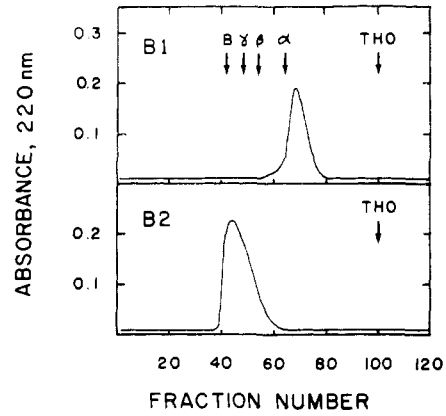

FIGURE 9: Elution patterns of approximately 1 ~1.5 mg of the CNBr peptides on a column of 6% agarose gel (1.5 × 180 cm). Chromatography was performed as described in the text.

to be galactose and the remainder was approximately equivalent amounts of glucose and mannose.

Cleavage of B Chain with CNBr. The B chain was the major subunit which had been isolated by CM-cellulose chromatography. Therefore, it was further characterized with respect to its CNBr peptides. The B chain of *N. virens* cuticle collagen was treated with CNBr in 70% formic acid for 6 h at room temperature. Figure 8 depicts the elution pattern of the cleavage products from a CM-cellulose column, in which the presence of two distinct peaks is apparent. Each fraction indicated by the bars was pooled and rechromatographed on agarose gel (Figure 9). Fraction 1 eluted as a single component with a molecular weight of approximately 70 000 (designated (B1). Fraction 2 eluted as a single, but somewhat asymmetric and broad, peak whose top was located in a region corresponding to a molecular weight of 400 000 (designated B2). When examined by sodium dodecyl sulfate gel electrophoresis, fraction 1 migrated as a single band. Fraction 2, however, migrated as a single major band together with a series of fuzzy bands corresponding to relatively low molecular weights, compared with the major band. In this regard, the shoulder preceding the B2 peak in Figure 8 was found to contain the fuzzy band components. These components increased with the reaction time in CNBr, following the initial decrease of uncleaved B chain, suggesting that they are derived from nonspecific cleavages of the B chain. It is noteworthy that the B chain was very susceptible to nonspecific cleavages under the usual reaction conditions. For instance, the occurrence of such cleavages was not observed in the case of bovine skin collagen α chains recently reported by Scott and Veis (1976). A precise determination of the stoichiometry of B1 and B2 fragments was difficult, because of the considerable amounts of nonspecific cleavage products. However, the weight ratio of approximately 1:7.5 for B1 and B2 fragments was obtained by measuring the area under the peak of each fraction in Figure 8. Assuming the molecular weights of 70 000 and 400 000 for B1 and B2, respectively, the molar ratio was calculated to be about 1:1.3.

Both fragments were analyzed for amino acid composition. The compositions, expressed as residues per mole (Table III), indicate that the sum of B1 and B2 fragments is close to the intact B chain except for a relatively large decrease in leucine. This result, together with the stoichiometry of the fragments, supports the interpretation that the B chain comprises 1 mol of each fragment. The small fragment, B1, contained no homoserine and must represent the carboxyl terminus peptide. Moreover, the compositions, expressed as residues per 1000 residues, were similar to each other except that B1 was rich in 3-hydroxyproline and leucine compared with B2.

Discussion

It is evident from the present work that the acid soluble collagen of *Nereis* cuticles contains two distinct subunits, A and B, in a molar ratio of about 1:2, and each polypeptide has a molecular weight of approximately 470 000. Studies using the acid soluble cuticle collagen preparation from a single individual of *N. virens* confirmed this result and indicated it was not a consequence of mixing a population of cuticles. These subunit chains seemed to be single polypeptide chains rather than cross-linked ones, at least as defined by their behavior under denaturing conditions; addition of dithiothreitol did not affect the results shown. Recently we obtained some evidence supporting the characteristic triple helical structure of collagen,

TABLE III: Amino Acid Composition of CNBr Peptides (B1 and B2) of B Chain from *N. virens* Cuticle Collagen.[a]

	B1	B2	B1 + B2	B chain
3Hyp	8	6	14	15
4Hyp	118	638	756	776
Asp	8	30	38	37
Thr	26	203	229	231
Ser	54	311	365	379
Hse	0	Trace	Trace	0
Glu	91	595	686	663
Pro	41	238	279	259
Gly	273	1506	1779	1799
Ala	58	397	455	481
Cystine	0	0	0	0
Val	4	16	20	21
Met	0	0	0	1
Ile	6	15	21	21
Leu	21	34	55	65
Tyr	1	0	1	1
Phe	1	1	2	2
Hyl	0	0	0	0
His	0	1	1	1
Lys	0	1	1	1
Arg	55	351	406	382
Total	765	4343	5108	5135

[a] Values are given as residues per mole using molecular weights of 70 000, 400 000, and 470 000 for B1, B2, and B chain, respectively.

using wide angle x-ray diffraction of intact cuticle and optical rotatory dispersion studies of solutions of *N. virens* cuticle collagen (unpublished data). These results suggest that the relation of B to A chain is analogous to that of the $\alpha 1$ and $\alpha 2$ chains in type I collagen of vertebrates (Piez et al., 1963). Thus, it is reasonable to speculate that 2 mol of B chains and 1 mol of A chain make up a three-stranded particle containing a polyproline II configuration. The resultant basic molecule may be represented as $A{\cdot}(B)_2$, with a molecular weight of about 1 400 000 and molecular dimensions of the order of approximately 14 000–15 000 Å in length by 13 Å in diameter. Its calculated intrinsic viscosity would be about 240 dL/g, from the equation of Simha (1940). However, this is in marked contrast to the measured intrinsic viscosities of native cuticle collagens, 70 dL/g for *N. japonica* (Kimura, 1971) and 62 dL/g for *L. terrestris* (Josse and Harrington, 1964). The intrinsic viscosity of such an extremely asymmetric particle must be measured at very low velocity gradients. In this regard, a strong dependence of the viscosity of *N. japonica* cuticle collagen on velocity gradients was observed in the previous studies (Kimura, 1971). The reported values for these cuticle collagens should be considered minimal since they were obtained by measurement at relatively high velocity gradients. In fact, Utiyama et al. (1973) reported a higher value of 109 dL/g for the cuticle collagen of earthworm, *Pheretma communissima*, using a rotatory viscometer designed for the measurement of intrinsic viscosity and extrapolating to zero velocity gradient. On the other hand, the acid soluble cuticle collagen of *N. japonica* in the native state was estimated to have a particle molecular weight of 1 700 000, a length of 9500 Å, and a diameter of 16.2 Å from the values of the sedimentation constant and intrinsic viscosity (Kimura, 1971). Similar results had been obtained for the neutral salt soluble cuticle collagen of *L. terrestris* (Josse and Harrington, 1964). Judging from the intrinsic viscosity values described above, these molecular weights and molecular dimensions should be considered provisional. The molecular structure of annelid cuticle collagen in solution should be reexamined in order to elucidate how the A and B chains are organized into a native molecule.

As for the subunits of annelid cuticle collagen, Josse and Harrington (1964) reported the presence of only one component, having a molecular weight of approximately 600 000, from the viscosity and sedimentation velocity analyses of *L. terrestris* cuticle collagen in the denatured state. However, one of the authors (Kimura, 1971) found two subunit components, one of molecular weight roughly 600 000, and the other of 300 000, from the sedimentation velocity analysis of *N. japonica* cuticle. This is apparently inconsistent with the present data. As will be discussed later, the neutral salt extracts of *N. japonica* cuticle fiber have a collagenolytic-like activity. Thus, the 300 000 molecular weight component may have arisen from some enzymatic degradation products since the cuticle fibers used in the previous studies (Kimura, 1971) were not pretreated with enzyme inhibitors. However, this discrepancy still remains to be elucidated.

In addition to the subunit A and B chains, a few minor components were detected by sodium dodecyl sulfate gel electrophoresis. The B chain could be isolated by CM-cellulose chromatography, whereas the A chain was always accompanied by two minor components, A′ and A″, which were present in various collagen preparations of both *Nereis* cuticles. Another minor component, B′, was present only in *N. virens* cuticle collagen and it eluted just before the B chain on the CM-cellulose column. Moreover, B′ had an amino acid composition similar to the B chain. Possibly, A′ and A″ are enzymatic products of the A chain in vivo (or during extraction), while B′ arose from the B chain, although there is no direct evidence to support these interpretations.

The purity of B chain was further confirmed by the analysis of its CNBr peptides obtained from *N. virens* cuticle collagen. The B chain was found to be composed of two primary fragments, one of molecular weight approximately 70 000 and the other of 400 000. This result is consistent with the fact that the B chain contains only one methionine residue per mole (Table III). The amino acid analyses are noteworthy in that both fragments have a glycine content of 35–36 residue % and an imino acid content of more than 20 residue %, indicating that they are capable of forming a collagen triple helix along the length of the chain.

The neutral salt soluble fraction of *N. japonica* cuticle contains relatively low molecular weight components as seen in Figure 1A, while its electrophoretic pattern appears to indicate the occurrence of specific cleavages of cuticle collagen (Figure 2). These cleavages are presumably collagenolytic since this fraction was obtained only in the absence of enzyme inhibitors and since the native cuticle collagen, i.e., acid soluble collagen, was resistant to pepsin digestion. These results suggest the necessity of using enzyme inhibitors in the preparation of soluble cuticle collagen.

Acknowledgment

We thank Mr. Timothy Housley for performing the amino acid analyses and Mrs. Diane Douglas for the carbohydrate analyses.

References

Ambler, R. P., and Brown, L. H. (1967), *Biochem. J. 104*, 784.

Bergman, I., and Loxley, R. (1963), *Anal. Chem. 35*, 1961.

Houwink, R. (1940), *J. Prakt. Chem. 157,* 15.
Josse, J., and Harrington, W. F. (1964), *J. Mol. Biol. 9,* 269.
Kimura, S. (1971), *Bull. Jpn. Soc. Sci. Fish. 37,* 419.
Kimura, S. (1973), *Bull. Japan. Soc. Sci, Fish. 39,* 311.
Laine, R. A., Esselman, W. T., and Sweeley, C. C. (1972), *Methods Enzymol. 28,* 159.
Lee, Y. C., and Lang, D. (1968), *J. Biol. Chem. 243,* 677.
Lewis, M. S., and Piez, K. A. (1964), *Biochemistry 3,* 1126.
Piez, K. A., Eigner, A., and Lewis, M. S. (1963), *Biochemistry 2,* 58.
Scott, P. G., and Veis, A. (1976), *Connect. Tiss. Res. 4,* 107.
Simha, R. (1940), *J. Phys. Chem. 44,* 25.
Steers, Jr., E., Craven, G. R., Anfinsen, C. B., and Bethune, J. L. (1965), *J. Biol. Chem. 240,* 2478.
Utiyama, H., Sakato, K., Ikehara, K., Setsuiye, T., and Kurata, M. (1973), *Collagen Symposium (Jpn.) 10,* 29.

Myoglobin as an Oxygen Indicator for Measuring the Oxygen Binding Characteristics of a Modified Myoglobin Derivative Containing Covalently Bound Mesoheme†

Philip D. Ross and Paul K. Warme*

ABSTRACT: By measuring the visible spectrum of a mixture of myoglobin and a modified derivative containing mesoheme in place of the normal protoheme, it is possible to evaluate the relative amounts of the oxidized, reduced, and oxygenated forms of each type of myoglobin. If the oxygen affinity of one myoglobin derivative is known, the oxygen affinity of the other can be determined from measurements at various oxygen partial pressures. In the absence of excess reducing agent, the rate of autoxidation can also be evaluated during the same experiment. The method described is suitable at very low oxygen partial pressures, where most previous methods are inaccurate, and it is very convenient to use, since no time-consuming calibration procedures are required. Using protoheme myoglobin as an oxygen indicator, the oxygen pressure at half saturation ($P_{1/2}$) of mesoheme myoglobin was shown to be 11% higher than the $P_{1/2}$ of a modified myoglobin derivative containing covalently bound mesoheme. The autoxidation rate of the covalent derivative is faster than that of the noncovalent derivative, but it is less dependent on oxygen pressure.

Mesoheme monosulfuric anhydride reacts specifically with lysine residue 45 in horse heart apomyoglobin to produce a modified myoglobin in which one of the heme propionic acid side chains is connected by an amide linkage to the lysine ϵ-amino group (Warme and Hager, 1970b). The visible spectrum of this covalent mesoheme myoglobin is almost identical with that of myoglobin containing noncovalently bound mesoheme, in both the oxidized and the reduced (oxy and deoxy) forms. In this paper, a novel equilibrium oxygen binding assay is utilized to demonstrate that the covalent mesoheme myoglobin derivative retains the ability to bind oxygen reversibly with an affinity very similar to that of noncovalent mesoheme myoglobin. The autoxidation rate of the covalent derivative is somewhat greater than that of the noncovalent derivative.

The extremely high oxygen affinity of myoglobin makes it quite difficult to measure its oxygen affinity accurately using current methods. This difficulty is reflected by the wide variation among reported values for the oxygen affinity of horse myoglobin, as measured by gasometric methods (Theorell, 1934; George and Stratmann, 1952), the tonometer method (Brunori et al., 1966), and the oxygen electrode method (Tamura et al., 1973a). Oshino et al. (1972) and Tamura et al. (1973a) have also used luminescent bacteria as an oxygen indicator for measuring the oxygen affinity of myoglobin derivatives.

As first shown by Hill (1939) and later by Davenport (1949), hemoglobin can be used as an indicator of oxygen concentrations in aqueous solutions. The fact that the visible absorption bands of mesoheme myoglobin are shifted about 10 nm to the red with respect to the absorption bands of normal (protoheme) myoglobin permits utilization of either of these proteins as an indicator of oxygen concentrations while measuring the oxygen affinity of the other. Thus, if the oxygen affinity of one derivative is known, the relative oxygen affinity of the other can be evaluated. Since each myoglobin derivative can exist in the oxidized state (met), the reduced state (deoxy), or the oxygenated state (oxy) and each of these forms has a distinct and characteristic absorption spectrum, the concentrations of each component of the equilibrium mixture can be determined by absorbance measurements at six different wavelengths.

Experimental Section

Materials

Horse heart myoglobin (type III), protoheme (type III), ferredoxin (type III), ferredoxin reductase, glucose 6-phosphate, glucose-6-phosphate dehydrogenase (type XV), NADP, NADH, and catalase (type C-100) were all purchased from

† From the Department of Biochemistry and Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802. *Received January 10, 1977.* This work was supported in part by grants from the National Science Foundation (BMS74-15221, PCM76-13451), from the National Institute of General Medical Sciences of the National Institutes of Health, United States Public Health Service (GM20742), and from The Pennsylvania State University.