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Properties of *Dendrostomum pyroides* Hemerythrin*

Robert E. Ferrell and G. Barrie Kitto

ABSTRACT: The nonheme iron respiratory protein hemerythrin from the sipunculid worm *Dendrostomum pyroides* has been subjected to a detailed physicochemical characterization. The protein is an octamer with a molecular weight of approximately 100,000 and dissociates to a monomer of mol wt 13,000. There are two molecules of nonheme iron bound per subunit and the native molecule combines reversibly with oxygen in a manner which involves no subunit cooperativity as determined from a Hill plot ($n = 1$), and is independent of hemerythrin concentration over a wide range of concentrations.

Immunodiffusion, amino acid analysis, and peptide mapping show that this hemerythrin is closely related to the hemerythrin of the sipunculid *Golfingia gouldii*.

The hemerythrins are a group of nonheme iron-containing, respiratory proteins found in certain members of four phyla: Sipunculoidea, Priapulida, Brachiopoda, and Annelida. A number of recent reviews of the hemerythrin literature are available (Manwell, 1960, 1964; Gihretti, 1962; Boeri, 1963).

Only the hemerythrin of the sipunculid *Golfingia gouldii* has been the subject of detailed physicochemical characterization, and to a lesser degree that of the sipunculid *Sipunculus nudus*. Klotz and his coworkers have investigated the molecular weight and dissociation properties of the hemerythrin from *G. gouldii* in a series of studies (Klotz and Keresztes-Nagy, 1963; Keresztes-Nagy and Klotz, 1963, 1965; Keresztes-Nagy *et al.*, 1965; Klapper and Klotz, 1968; Klapper *et al.*, 1966). The studies have shown the hemerythrin from this species to have a molecular weight of 107,000, and that it is composed of eight similar subunits. The molecule is reversibly dissociable and the octamer $\rightleftharpoons$ monomer equilibrium is dependent on the concentration of hemerythrin and the coordination state of the iron atoms within the molecule. The amino acid sequence of the subunit has been reported (Groskopf *et al.*, 1966a,b; Subramanian *et al.*, 1968; Klippenstein *et al.*, 1968).

The hemerythrin from *S. nudus* has been shown to bind one oxygen molecule per two nonheme iron atoms (Boeri and Ghiretti-Magaldi, 1957) and a detailed study of the oxygen binding properties of the hemerythrin from this species has been reported (Bates *et al.*, 1968). They observed only slight homotropic interaction upon oxygenation and the absence of any Bohr effect.

This paper is concerned with the physicochemical characterization of the coelomic hemerythrin from the sipunculid *Dendrostomum pyroides*, and includes iron analysis, ultracentrifugal analysis, oxygen equilibrium, amino acid composition, and peptide maps of this protein.

Materials and Methods

D. pyroides were obtained from Pacific Bio-Marine Supply Co., Venice, Calif. *G. gouldii* were from Woods Hole Biological Supply, Woods Hole, Mass. Identification of the specimens was confirmed using the key of Fisher (1952). Hemerythrin from the brachiopod *Lingula* was a gift from Dr. Bolling Sullivan, Duke University Medical School, Durham, N. C. Hydrolyzed starch was from Connaught Medical Research Laboratories, Nitroso-R-salt from Wilshire Chemical Co., Inc., and *o*-phenanthroline, *N*-ethylmaleimide were from Sigma. Sephadex G-25, G-100, and G-200 were from Pharmacia, Inc. Tosylamidoethyl chloromethyl ketone treated trypsin was from Worthington Biochemical Corp. All other chemicals were reagent grade.

Preparation of Hemerythrin. Individual specimens were bled from an incision, in the posterior end, into small centrifuge tubes. Hemerythrin-containing cells were collected by centrifugation at 2000*g* for 5 min, and the cells were washed three times by resuspension in cold sea water and centrifugation at 2000*g* for 5 min. The washed cells were suspended in 0.1 M potassium phosphate buffer (pH 7.5) and lysed by freezing and thawing. After thawing, the cell debris was removed by centrifugation at 12,000*g* for 30 min, and the supernatant hemerythrin solution was used immediately or stored at $-70°$. *Dendrostomum pyroides* hemerythrin prepared in this manner sediments as a single symmetrical peak in the ultracentrifuge and stains as a single band after starch gel electrophoresis.

Only hemerythrin-containing cells from the coelomic fluid were used in these preparations to avoid contamination from a vascular hemerythrin reported by Manwell (1963) to be present in the tentacles of certain sipunculid worms. DEAE-cellulose chromatography performed as described by Bates *et al.* (1968) showed no evidence of protein contamination, and

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samples were routinely used after isolation as described above.

Spectral Studies. Spectra were obtained, using a Cary Model 14 recording spectrophotometer, in 0.1 M potassium phosphate buffer (pH 7.5). Methemerythrin was prepared by treating oxyhemerythrin with a 10% molar excess of potassium ferricyanide. Excess ferricyanide was removed by extensive dialysis against appropriate buffer solutions.

Starch gel electrophoresis was carried out in 14% gels using 0.01 M Tris-citrate buffer (pH 8.8). The runs were carried out at 10 V/cm for 20 hr at 4°. Immediately after termination of the run, the gels were cut into slices 2 mm thick and stained specifically for hemerythrin and for protein. The hemerythrin-staining solution was a modification of that reported by Ornstein (1964) for transferrins. The staining solution contained 1.0 g of hydroxylamine·HCl, 2.7 g of sodium acetate·$3H_2O$, 1.5 ml of glacial acetic acid, and 0.5 g of Nitroso-R-salt in 100 ml of deionized water. Gels were incubated in this mixture for 30 min, after which the hemerythrin could be detected as bright green spots. Amido Black was used as a general protein stain. All gels were destained by washing in a H_2O–methanol–acetic acid (5:5:1, v/v) solution.

Protein Determination. Protein concentration was determined by the biuret reaction (Layne, 1957) or the method of Lowry *et al.* (1951).

Iron Analysis. Iron determinations were carried out using the *o*-phenanthroline method (Ballentine and Buford, 1957) after wet ashing. Samples were desalted on a 2.5 × 50 cm column of Sephadex G-25, which had been equilibrated with deionized water, and freeze-dried prior to ashing. Protein concentration was based on amino acid analysis of iron-free hemerythrin prepared according to Groskopf *et al.* (1966a).

Isoelectric focusing was performed in a linear pH gradient between pH 3.0 and 10.0 in a total volume of 110 ml. Carrier ampholytes, the column and the linear density gradient maker were purchased from LKB Produkter, Stockholm, Bromma, Sweden, and all procedures were performed according to the manufacturer's instructions. The temperature was maintained at 4° throughout the run by means of a Fisher Model 45 circulating-water bath.

Isoelectric focusing was achieved at a final potential of 700 V. After 36 hr, 1-ml fractions were collected from the column and monitored spectrophotometrically for hemerythrin at 500 mμ using a Zeiss spectrophotometer PMQ II. The pH measurements were carried out with a pH meter, type PHM 26, from Radiometer, Copenhagen, equipped with a scale expansion and using a combined glass electrode, type GK 2024C, of the same brand.

Oxygen Equilibria. Measurements and calculations were made at 25° by a modification of the spectrophotometric procedure described earlier for hemoglobin (Riggs and Wolbach, 1956). Percentage oxygen saturation was followed at two wavelengths, 510 and 570 mμ, where changes in optical density are proportional to the degree of saturation. Oxyhemerythrin concentration was determined from absorbance at 500 mμ, and methemerythrin estimated from the difference between oxyhemerythrin concentration and the total protein concentration determined from the absorbance at 280 mμ. The amount of methemerythrin did not exceed 10% in these experiments and remained constant during the oxygen equilibrium measurements. The pH was determined immediately after removal of the solution from the tonometer.

Immunological Procedures. Rabbit antisera directed against *D. pyroides* and *Lingula* hemerythrin were prepared in the following manner. A normal saline solution (1 ml) containing 10 mg of hemerythrin was mixed with an equal volume of Freund's complete adjuvant and injected into the toepads and thigh muscles. First-course sera were obtained after 3 weeks. Sera were then obtained weekly, with the injection of 5 mg of antigen after each bleeding, for a period of 6 weeks. Double diffusion was performed by the method of Ouchterlony (1948) in 1% ion agar prepared in a saline–borate buffer (pH 8.0). The antibody well contained undiluted antiserum. The antigen wells contained varying amounts of hemerythrin from several species. The antigen wells were 0.5 cm apart and diffusion was allowed to continue, at room temperature, until precipitin lines were clearly visible. At the end of the experiment, the agar plates were washed for 24 hr in saline to remove excess protein and stained with Amino Black.

Ultracentrifugal Analysis and Molecular Weight Determinations. A Spinco Model E ultracentrifuge with an AN-D rotor, electronic speed control, and an RTIC temperature control was used to obtain sedimentation rates. Analyses were carried out in 0.1 M potassium phosphate buffer (pH 7.5) except where specified in the figure legends. The sedimentation coefficients were determined from measurements, obtained with a Gaertner microcomparator, of the maximum ordinate on schlieren photographs. Ultraviolet optics were used at protein concentrations below 0.5 mg/ml and ultraviolet plates analyzed using a Beckman, Model RB, film densitometer. Schlieren and ultraviolet optics were overlapped at a concentration of 0.5 mg/ml.

The molecular weight of native hemerythrin was determined by the sedimentation equilibrium method of Schachman (1957) in a double-sector interference cell equipped with sapphire windows. The equilibrium runs were carried out at 20° for 30 hr. The molecular weights of native hemerythrin and hemerythrin subunits were estimated by gel filtration on Sephadex G-200 and G-100, respectively. Hemerythrin subunits were prepared by the reaction of native hemerythrin with *N*-ethylmaleimide in the presence of N_3 ions as described by Keresztes-Nagy and Klotz (1963). Columns were calibrated for molecular weight determination with proteins of known molecular weight (Andrews, 1964).

Amino Acid Analysis. The amino acid composition of hemerythrin was determined according to the procedure of Moore *et al.* (1958) using a Beckman Spinco automatic amino acid analyzer. Hydrolyses were carried out on iron-free hemerythrin prepared as described by Groskopf *et al.* (1966a,b), for 24 and 48 hr at 110° in 6 N HCl. Cysteine residues were estimated by free sulfhydryl group determination using the method of Ellman (1959) with bovine serum albumin, human hemoglobin A, and trypsin as standards.

Peptide Mapping. Tryptic digests were prepared by dissolving 100 mg of pooled *D. pyroides* or *G. gouldii* hemerythrin in 100 ml of deionized water and heating for 10 min on a boiling-water bath to denature the protein. The solution was allowed to cool to room temperature and made 0.05 M in ammonium bicarbonate. Tosylamidoethyl chloromethyl ketone treated trypsin (5 mg) was added to this solution and digestion was allowed to proceed for 24 hr at 37°. The reaction was terminated by adjusting the pH of the solution to 2.0 by the dropwise addition of 2 N HCl. The resulting solution was reduced to a volume of 20 ml on a rotary evaporator under reduced pressure and stored at 0° until used.

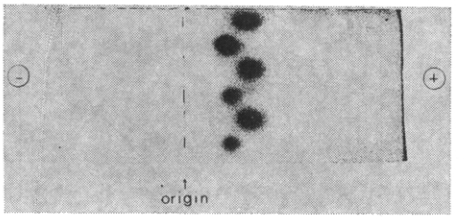

FIGURE 1: Starch gel electrophoresis of hemerythrins. The more anodally moving bands represent *D. pyroides* and the more cathodal bands *G. gouldii*. Electrophoresis was carried out at 10 V/cm for 20 hr at 4° and the gels were stained specifically for hemerythrin.

Peptide maps were prepared by two-dimensional electrophoresis on paper, essentially as described by Brown and Hartley (1966). The tryptic digest was spotted on Whatman No. 3MM paper and electrophoresis carried out at pH 6.0 for 20 min at 3000 V. A marker strip was cut from this sheet and stained with cadmium–ninhydrin to locate the positions of the peptides. And unstained strip was then cut an sewn to a second sheet of Whatman No. 3MM paper and electrophoresis carried out at pH 3.5 for 40 min at 2500 V in a direction perpendicular to the direction of the original run. The complete maps were then stained with cadmium–ninhydrin to locate the final positions of the peptides.

Results

Starch gel electrophoresis of the hemerythrin from approximately 100 individual members of the species *D. pyroides* gave no detectable electrophoretic variants among the animals used in these experiments. Starch gel electrophoresis of the hemerythrin from a similar group of the species *G. gouldii* showed a low level of electrophoretic variants as observed by others (Manwell, 1963; Groskopf *et al.*, 1966a,b). Figure 1 shows the typical electrophoretic pattern for hemerythrin obtained from these two species and stained specifically for hemerythrin.

The hemerythrin from *Dendrostomum* is shown to migrate slightly more toward the anode than does that of *Golfingia*. The spectrum of hemerythrin from *D. pyroides* is shown in Figure 2. The visible spectrum of oxyhemerythrin shows a characteristic peak at 500 mμ which is lost upon deoxygenation under vacuum or on treatment with dithionite. It has been shown that the visible spectrum of hemerythrin is affected by the environment around the iron atoms within the molecule. The visible spectrum of hemerythrin from *Dendrostomum* does not change at protein concentrations as low as 0.01% although the results of ultracentrifugal analysis indicate that the molecule is completely dissociated at this concentration (see below). This is in agreement with results obtained from the hemerythrin of *G. gouldii*, indicating that there are no gross changes in the conformation of the subunits upon dissociation (Keresztes-Nagy and Klotz, 1965; Darnall *et al.*, 1969). The ultraviolet spectrum of *D. pyroides* hemerythrin shows a peak at 315 mμ in the oxy and met states. This probably corresponds to the 330-mμ peak observed in *G. gouldii*. Whether this difference is due to structural differences between the two hemerythrins or to differences in experimental conditions is not certain.

The extinction coefficients for hemerythrin at 280 and 500 mμ were determined by measuring the absorbance at these

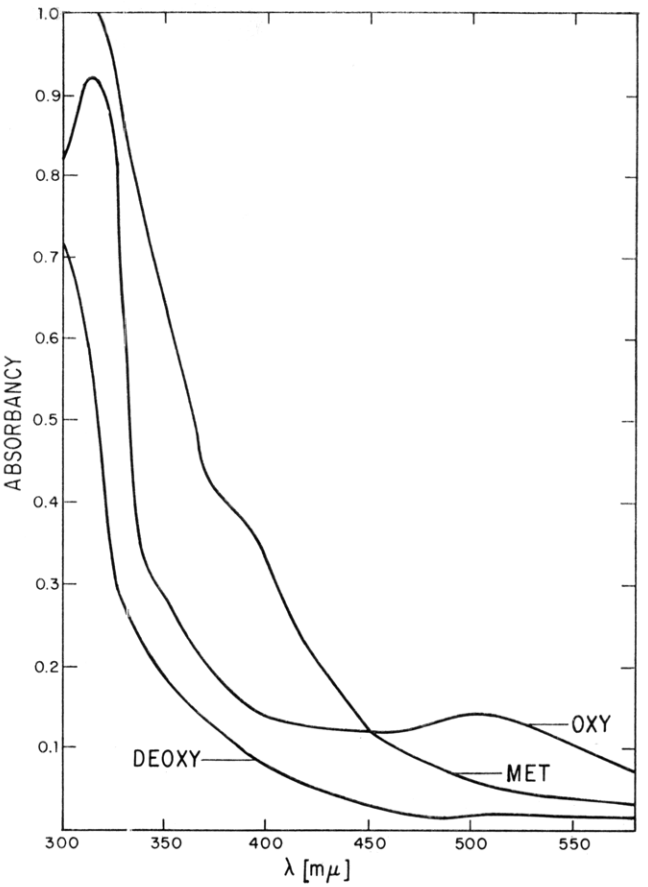

FIGURE 2: Spectra of oxy-, deoxy-, and methemerythrin from *D. pyroides* in 0.1 M potassium phosphate buffer (pH 7.5).

wavelengths in 0.1 M potassium phosphate (pH 7.5) of a carefully dialyzed and clarified solution of the protein and then assaying for protein by the biuret (Layne, 1957) or Lowry *et al.* (1951) procedures. The reaction was standardized using known concentrations of bovine serum albumin. The extinction coefficients at 280 mμ of a 0.1% solution of *D. pyroides* hemerythrin were 3.03 (biuret) and 3.10 (Lowry *et al.*, 1951). These values are similar to those reported for other hemerythrins (Ghiretti, 1962).

Iron analysis by the *o*-phenanthroline method gave a value of 0.87% iron which corresponds to 6500 g of protein/mole of iron. Using a subunit molecular weight of 12,800 ± 1300 obtained from gel filtration on Sephadex G-100, this represents 2 moles of iron/mole of subunit. The oxidation state of the iron in native hemerythrin was not determined.

The isolectric point of oxyhemerythrin was determined, by the method of isoelectric focusing, to be 6.71. Pooled samples of hemerythrin were used in these experiments and no evidence for multiple forms of hemerythrin was observed although the method of isoelectric focusing offers a particularly sensitive means of detecting heterogeneity (Vesterberg and Svensson, 1966).

Sedimentation Behavior. The concentration-dependent dissociation of hemerythrin is shown graphically in Figure 3. At hemerythrin concentrations of 1 mg/ml or greater, hemerythrin moves as a single distinct boundary with a sedimentation coefficient between 6.5 and 6.8 S. At a concentration of 0.1

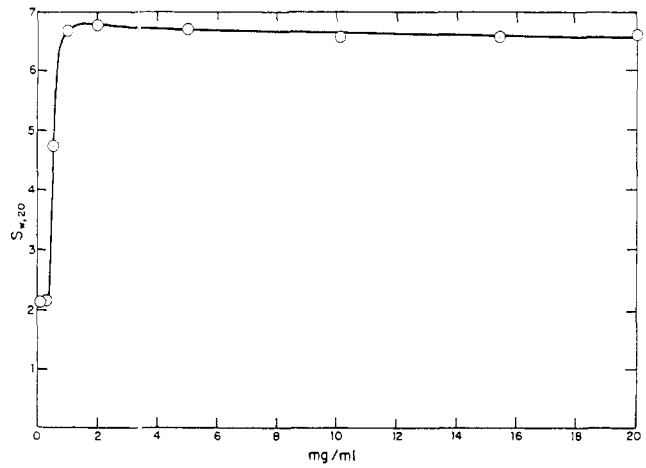

FIGURE 3: Concentration dependence of the sedimentation velocity of oxyhemerythrin of *D. pyroides* in 0.1 M potassium phosphate buffer (pH 7.5) at 20°.

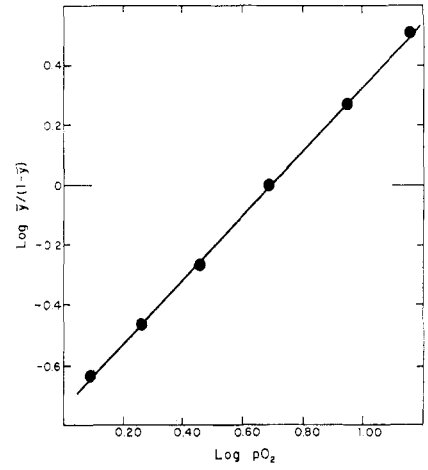

FIGURE 5: Hill plot of the oxygen equilibrium of *D. pyroides* hemerythrin in 0.1 M potassium phosphate buffer (pH 7.5) at 25°. The straight line drawn through the points corresponds to a Hill parameter value of $n = 1.01$.

mg/ml this boundary is replaced by a boundary with a sedimentation coefficient of 2.1 S. At protein concentrations between 0.1 and 1.0 mg per ml there appear to be boundaries representing both the fast- and slow-sedimenting components, however, resolution using the ultraviolet optical system was not sufficient to allow accurate estimation of the relative amounts of the two species. The reaction boundary theory (Nichol *et al.*, 1964) suggests that a slow peak should be present at both high and low concentrations, however, the Schlieren optics used at high concentrations would not reveal the presence of a minor slow peak. At no concentration was there evidence for the appearance of a stable component of intermediate sedimentation velocity, and the dissociation appears to be an all or none process. This is in agreement with the dissociation data reported for the hemerythrin of *Golfingia* by Klapper *et al.* (1966). At hemerythrin concentrations ranging from 1 to 10 mg per ml no difference was observable between the sedimentation velocities of oxyhemerythrin and deoxyhemerythrin.

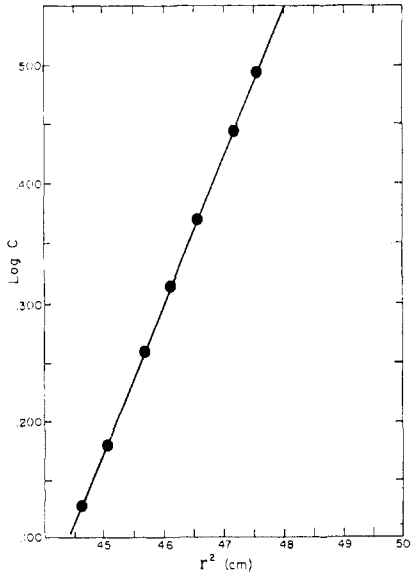

FIGURE 4: Sedimentation equilibrium analysis of *D. pyroides* hemerythrin. Protein concentration as 1 mg/ml in 0.1 M potassium phosphate buffer (pH 7.5). Operational speed was 7000 rpm. Liquid column length was 3 mm.

Molecular Weight. The results obtained from sedimentation equilibrium of oxyhemerythrin are shown in the log c *vs.* r^2 plot in Figure 4. Sedimentation equilibrium was carried out at a concentration of 10 mg/ml of hemerythrin to avoid dissociation which is observed at lower concentrations. Using a value for the partial specific volume of 0.730 as reported by Klotz and Keresztes-Nagy (1963) for *G. gouldii* hemerythrin, a value for the molecular weight of *D. pyroides* hemerythrin of 101,000 was calculated. This value is in agreement with a value of 100,000 ± 10,000 calculated from gel filtration on Sephadex G-200, and is in agreement with the molecular weights previously reported for several hemerythrins (Klotz and Keresztes-Nagy, 1963; Bates *et al.*, 1968). A subunit molecular weight of 12,800 ± 1300 was obtained by gel filtration, on a calibrated Sephadex G-100 column, of *N*-ethylmaleimide-treated *Dendrostomum* hemerythrin.

Oxygen Equilibrium. Experimental data from a typical oxygen equilibrium determination are shown in Figure 5 in the form of a Hill plot. The values for the Hill parameter, n, determined from a series of oxygen equilibrium experiments ranged from 0.99 to 1.10, indicating that there is little, if any, cooperative interaction between oxygen binding sites. The P_{50} for the coelomic hemerythrin from *D. pyroides* is 4–5 mm which is in agreement with values obtained for other *Dendrostomum* species by Manwell (1960). Attempts to determine the effects of *N*-ethylmaleimide on the oxygenation reaction of hemerythrin were not successful because of the progressive denaturation of hemerythrin upon oxygenation in the presence of even low levels (10^{-5} M) of this reagent.

The oxygen equilibrium of hemerythrin is independent of hemerythrin concentration over a range from 1 to 10 mg per ml. These experiments were not performed at lower hemerythrin concentration where the hemerythrin is dissociated into subunits.

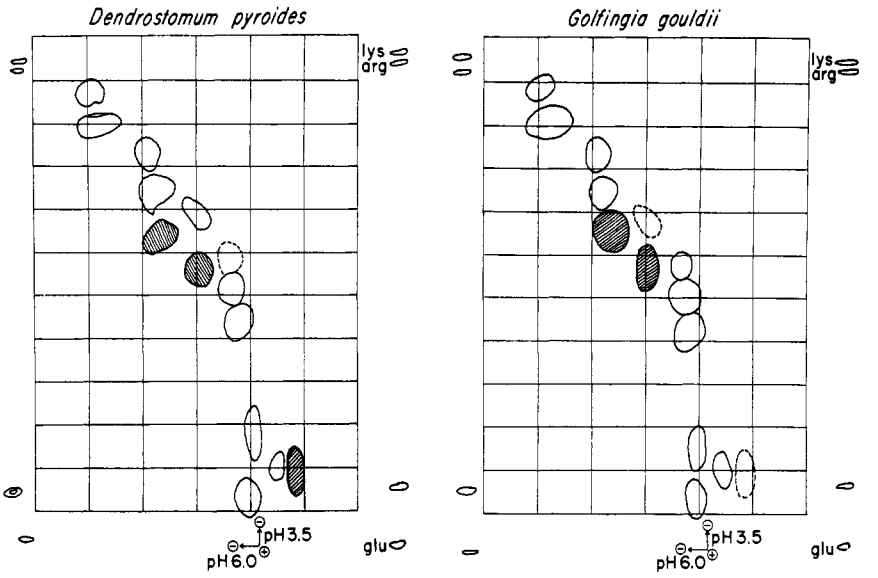

FIGURE 6: Peptide maps of *D. pyroides* and *G. gouldii* hemerythrins. Broken lines represent difference between the two species. Cross-hatched spots represent fluorescence prior to ninhydrin staining.

Immunology. When tested by double diffusion in agar, the antisera to *D. pyroides* hemerythrin gave a single, sharp precipitin band with the homologous antigen. This provides a further indication that a homogeneous protein was obtained when prepared as described in Materials and Methods. When anti-*Dendrostomum* hemerythrin was tested against the hemerythrin of *G. gouldii*, a line of partial identity was observed. No precipitin line could be observed when the antisera to *Dendrostomum* hemerythrin were tested against the hemerythrin of *Lingula*. When the antisera prepared against *Lingula* hemerythrin was tested against either the *Dendrostomum* or *Golfingia* hemerythrin there was no cross-reactivity, although a strong single, sharp line was observed with the original antigen. Further comparative immunological studies are in progress.

Amino Acid Analysis. The results presented in Table I were obtained with duplicate analyses of several hemerythrin preparations. There was a slight increase in the yield of valine and isoleucine between 24- and 48-hr hydrolysates. The higher values were chosen to represent complete liberation of these residues. Other residues were extrapolated to zero time of hydrolysis. Methionine was determined as methionine sulfone after performic acid oxidation of the protein. The value of one methionine per subunit was also supported by experiments, to be reported in detail elsewhere, in which cyanogen bromide cleavage of the protein was carried out, yielding only two polypeptide fragments. Determination of cysteine by the method of Ellman (1959) yields a value of one cysteine per subunit for the native molecule. Amino acid analysis after performic acid oxidation gives a value of slightly less than one residue of cysteic acid per subunit. These results indicate that there are no inter- or intrachain disulfide bonds in the molecule. The amino acid composition of the hemerythrin of *G. gouldii*, obtained from the reported sequence (Klippenstein *et al.*, 1968), is shown for comparison. A close relationship in the structure of the two hemerythrins is reflected in the similarity of amino acid compositions. The *Dendrostomum* hemerythrin does differ considerably from that of *Golfingia* in content of basic amino acids and in the amount of phenylalanine present. The lower number of basic amino acids in the *Dendrostomum* hemerythrin is reflected in its increased anodal mobility on starch gel electrophoresis.

It is of interest that *Dendrostomum* hemerythrin, like that of *Golfingia*, contains only a single cysteine residue per subunit, thus ruling out the possibility that each iron

TABLE I: Amino Acid Composition of Hemerythrins.

Amino Acid	Residues/Subunit (13,600 g)	
	D. pyroides[a]	*G. gouldii*[b]
Lysine	9.2 ± 1.1[c]	11
Histidine	7.1 ± 1.3	7
Arginine	3.2 ± 0.5	3
Aspartic acid + asparagine	17.7 ± 1.5	17
Threonine	5.3 ± 0.2	4
Serine	3.8 ± 0.3	4
Glutamic acid + glutamine	10.5 ± 0.3	10
Glycine	6.1 ± 0.2	7
Alanine	5.3 ± 0.2	5
Valine	3.3 ± 0.2	5
Methionine	1.0	1
Isoleucine	6.5 ± 0.3	9
Leucine	9.1 ± 0.2	8
Tyrosine	5.2 ± 0.2	5
Phenylalanine	6.4 ± 0.3	9
Cysteine	1[d]	1
Tryptophan[e]	3.8	4

[a] All analyses based on one methionine per subunit (13,600 molecular weight). [b] Data from Klippenstein *et al.* (1968). [c] Standard deviation. [d] See Materials and Methods for details of the method of analysis. [e] Determined spectrophotometrically (Beaven and Holiday, 1952).

is bound through a sulfhydryl residue, as had been suggested earlier (Klotz and Klotz, 1955).

Peptide Mapping. Figure 6 shows peptide maps prepared from tryptic digests of hemerythrin from *D. pyroides* and *G. gouldii*. Spots shown in broken lines represent differences between the two hemerythrins. Although the lysine plus arginine content of the hemerythrin of *Dendrostomum* is lower than that of *Golfingia*, as seen from amino acid analyses, the number of tryptic peptides obtained from *Dendrostomum* is consistently greater. The reason for this result is not clear, although it may represent the absence of Lys-Lys or Arg-Arg sequences, present in the hemerythrin from *Golfingia*. Clarification of this point awaits further characterization of tryptic peptides.

Discussion

The results of the present work show that the coelomic hemerythrin of *Dendrostomum* pyroides has the same general characteristics as other sipunculid hemerythrins. The protein has a molecular weight of approximately 100,000. Molecular weight studies of the dissociated protein and a comparison of the number of tryptic peptides with the lysine plus arginine content of the hemerythrin indicate that it is composed of eight similar, if not identical, subunits. Each subunit contains two atoms of iron, and combines reversibly with oxygen in a ratio of one oxygen molecule per two atoms of iron. No cooperative subunit interactions were observed on the binding of ligands to the hemerythrin.

The preliminary immunological comparison of the hemerythrins from *Dendrostomum*, *Golfingia*, and *Lingula* is consistent with the phylogenetic relationships of these organisms. *Dendrostomum* and *Golfingia* are both sipunculid worms though placed in different genera and they show partial immunological identity. The lack of cross-reaction between *Dendrostomum* and *Lingula* hemerythrins is consistent with the classification of these species in different phyla. The structural similarity of the *Dendrostomum* and *Golfingia* hemerythrins is seen also in their amino acid compositions and tryptic peptide maps. The *Dendrostomum* map shows two ninhydrin-positive spots not present in the *Golfingia* digest and the absence of one prominent *Golfingia* spot. All other ninhydrin-positive spots occupy essentially equivalent positions. Work in progress on the composition and sequence of the tryptic peptides should reveal the specific differences in sequence between the *Dendrostomum* and *Golfingia* hemerythrins. This information, together with that on the sequence of *Lingula* hemerythrin (B. Sullivan, personal communication, 1969), may serve as a basis for establishing common structural features essential to the function of hemerythrin as a respiratory pigment.

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