A Comparative Study of the Oxygen Transport Proteins of *Dendrostomum pyroides*

ISOLATION AND CHARACTERIZATION OF HEMERYTHRINS FROM MUSCLE, THE VASCULAR SYSTEM, AND THE COELOM*

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SUMMARY

Hemerythrins have been isolated from muscle, the vascular system, and the coelom of the sipunculid *Dendrostomum pyroides*. Muscle hemerythrin (myohemerythrin), vascular hemerythrin, and coelomic hemerythrin have been purified by ammonium sulfate precipitation and have been partially characterized. The ultraviolet and visible absorption and circular dichroic spectra of myohemerythrin and vascular hemerythrin closely resemble those of the coelomic protein, indicating that these hemerythrins have similar iron sites. Far-ultraviolet circular dichroism measurements reveal that the three hemerythrins also have similar secondary structures. In contrast, amino acid analysis shows that the three proteins differ substantially in primary structure. Vascular hemerythrin and coelomic hemerythrin each occur as oligomeric (probably octameric) molecules while myohemerythrin occurs only as a monomer.

Hemerythrin is the nonheme iron oxygen-carrying protein of sipunculids. Structural studies on hemerythrin have been limited primarily to the protein isolated from the red blood cells of the coelom. However, other hemerythrins have been detected in these animals. A vascular hemerythrin has been isolated from several species of sipunculids (1, 2). This protein is different from the coelomic hemerythrins in oxygen affinity (1), electrophoretic mobility (1, 2), and peptide map patterns (2). Another hemerythrin-like protein has been detected, this in the muscle of a sipunculid. The existence of this muscle hemerythrin ("myohemerythrin") was proposed on the basis of the observation that the retractor muscles of *Dendrostomum zostericola* have a pale violet-pink color which disappears upon removal of oxygen. Extraction of muscle with distilled water gave a pink solution with a faint absorption band at 490 to 500 nm (1).

In studying the structure-function relationships in hemerythrin, we have undertaken to analyze the differences in primary structure of hemerythrins obtained from a variety of sources. It has been found that hemoglobin and myoglobin from a given species have only a limited degree of amino acid sequence homology (3). This fact suggested that coelomic hemerythrin, vascular hemerythrin, and myohemerythrin might also be significantly different from one another in primary structure. Therefore, we have isolated and purified vascular hemerythrin and myohemerythrin as well as coelomic hemerythrin from the sipunculid *Dendrostomum pyroides* and have studied some of the chemical and physical properties of these proteins. The results of this work indicate that the three hemerythrins differ significantly from one another in primary structure. While these proteins have similar iron binding sites and secondary structures, it was found that coelomic and vascular hemerythrin occur as oligomeric molecules, whereas myohemerythrin occurs only as a monomer. This work shows the existence of myohemerythrin and suggests a hemerythrin-myohemerythrin oxygen transport system in some ways analogous to that involving hemoglobin and myoglobin.

EXPERIMENTAL PROCEDURE

Purification of Coelomic, Vascular, and Muscle Hemerythrins—"D. pyroides" were obtained from Pacific Bio-Marine Supply Co., Venice, Calif. All steps in the preparation of the hemerythrin were carried out at 5°. Each worm was cut lengthwise and the coelomic fluid collected. The animal was then pinned out and rinsed thoroughly with 3.5% NaCl solution to remove the last traces of coelomic red blood cells. The main contractile vessel and "Polian vesicles" both of which also contain red blood cells, were then carefully dissected out. The vessels were placed in a beaker of 3.5% NaCl until the vascular hemerythrin could be purified. The worm was once again rinsed with 3.5% NaCl.
solution and finally the two retractor muscles were removed and placed in 3.5% NaCl. The muscles were rinsed several times with the NaCl solution and then frozen.

The coelomic red blood cells were washed, lysed, and the coelomic hemerythrin purified essentially as described by Klotz et al. (4), except that the crystallization step was omitted. Instead, the oxyhemerythrin was dialyzed against 0.1 M NaN₃ to convert it to the metazide form, and then this was precipitated by dialysis against 60% saturated ammonium sulfate solution which had been adjusted to pH 7.0. (All ammonium sulfate solutions used in this work were adjusted to pH 7.0.) The precipitate was collected by centrifugation, washed with fresh 60% saturated ammonium sulfate, and stored frozen.

Vascular red blood cells were obtained by cutting the vessels into fine pieces and filtering through glass wool. The filter was rinsed with 3.5% NaCl. Then the cells in the filtrate were treated as described above for hemerythrin isolation. Vascular oxyhemerythrin was converted to the metazide form by dialysis against 0.1 M NaN₃ and then purified by ammonium sulfate fractionation as follows. The hemerythrin preparation was dialyzed against 50% saturated ammonium sulfate and the precipitate discarded. Dialysis against 60% saturated ammonium sulfate precipitated a small amount of hemerythrin which was collected by centrifugation. Dialysis of the supernatant against 70% saturated ammonium sulfate precipitated the remainder of the hemerythrin which was combined with that obtained at 60% saturation. Finally the vascular hemerythrin was dissolved in 0.1 M NaN₃, dialyzed against this solution, and then stored at 5°.

The retractor muscles from 100 animals were thawed, mixed with 250 ml of 0.1 M Tris-chloride buffer, pH 8.0, which was 0.1 M in NaCl, and homogenized in a Waring Blender. The homogenate was centrifuged at 7500 × g for 90 min. The supernatant was collected and the pellet washed twice with 125-ml portions of the Tris-NaCl buffer. The supernatant fractions were combined and solid NaN₃ was then added to a concentration of 0.1 M. After standing overnight, this fraction was centrifuged at 16,000 × g for 30 min and the pellet discarded. Solid ammonium sulfate was then added to the supernatant to bring it to 80% saturation. After centrifugation at 7500 × g to remove the precipitate, the soluble fraction was dialyzed against 90% saturated ammonium sulfate, and again the precipitate was discarded. Finally the supernatant fraction was dialyzed against 100% saturated ammonium sulfate at which point myohemerythrin precipitated. The final precipitate was washed three times with saturated ammonium sulfate solution, dissolved in 0.1 M NaN₃, and dialyzed exhaustively against this solution. The myohemerythrin solution was then centrifuged at 39,000 × g for 2 hours and the supernatant was stored at 5°.

Disc Electrophoresis—The method of Davis (5) was used for disc electrophoresis of hemerythrin octamer and monomer. Coelomic or vascular hemerythrin monomer was prepared by addition of p-hydroxymercurobenzoate to the protein. Two mols of HMB1 per mol of hemerythrin monomer were added as a 5 × 10⁻⁸ M HMB solution in 0.1 M Tris-chloride buffer, pH 8.6. Myohemerythrin was also modified with HMB, ratios of 3 to 10 mols of HMB per mol of myohemerythrin being used. Samples in a 10 to 20% sucrose solution were layered directly onto the surface of the stacking gel and electrode buffer was then layered onto the sample. While in most cases the standard 7±0.2% gels were used, in one experiment, 5 and 10% polyacrylamide gels were utilized. Electrophoresis was done at 5° at a constant current of 3.5 mA per gel. Before staining, the position of the tracking dye was marked by inserting a piece of fine wire into the gel.

Determination of Molecular Weights—Sedimentation velocity experiments were conducted in a Beckman model E analytical ultracentrifuge, with the AN-D rotor. Solutions of hemerythrin at concentrations of 2 to 10 mg per ml were run in 0.1 M sodium phosphate buffer, pH 7.0, or in 0.2 M Tris-chloride, pH 8.0. 0.1 M in NaCl. Runs were made at 52,640 rpm and 20°. Schlieren optics were used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of vascular hemerythrin was carried out with a slightly modified procedure of Weber and Osborn (6). Standards used were bovine serum albumin, ovalbumin, and glyceraldehyde 3-phosphate dehydrogenase (all from Sigma), trypsin (Worthington), myoglobin and cytochrome c (Calbiochem), ribonuclease and insulin (Mann), and Goliath gouldii coelomic hemerythrin. Gels were approximately 10 cm long and were run at 5°. The reservoir buffer was diluted 1:3 prior to electrophoresis. Myohemerythrin was run under the same conditions but in a 12½% acrylamide gel.

Determination of molecular weights by gel filtration was carried out on an upward flow column of Sephadex G-100 (Pharmacia Fine Chemicals), 2.5 × 75 cm in size. The column was equilibrated with 0.1 M sodium phosphate buffer, pH 7.2, and run at a flow rate of 12 ml per hour. Fractions of 2 ml were collected. The eluant was monitored by measurement of the absorbance of the fractions at 280 nm. The column was calibrated with a sample containing 10 mg each of bovine serum albumin, β-lactoglobulin (Mann), and G. gouldii hemerythrin monomer. This latter standard was prepared as described above for D. pyroideus coelomic hemerythrin monomer. The standards and all samples were also 0.1 M in NaNO₃, the azide ion serving as a low molecular weight marker.

Spectra—Ultraviolet and visible spectra were determined with a Hitachi-Perkin Elmer 124 spectrophotometer. Circular dichroic spectra in the far-ultraviolet and near-ultraviolet-visible regions were measured in a Cary model 60 spectropolarimeter. The metazide form of hemerythrin was used in all spectral studies. Concentrations of hemerythrin used in calculating extinction coefficients were determined by the Lowry method as discussed by Oyama and Eagle (7) with G. gouldii coelomic hemerythrin as a standard, or by amino acid analysis.

Amino Acid Analysis—Amino acid compositions were determined on a Beckman 120 C amino acid analyzer (8). Proteins were hydrolyzed for 24 hours at 110° in 2 ml of triply distilled 5.7 M HCl to which had been added 0.1 ml of 0.5 M hydrazine (9) to prevent the destruction of tyrosine. In the case of coelomic hemerythrin, the quantities of threonine and serine were extrapolated to zero time to correct for hydrolytic losses.

RESULTS

Isolation and Purification of Hemerythrins—Coelomic and vascular hemerythrins occur in morphologically distinguishable red blood cells. Our observations confirm Manwell's (1) assertion that the coelomic protein is contained within nucleated cells whereas vascular hemerythrin is found in smaller, non-nucleated cells. Hemerythrin comprises nearly all of the soluble...
protein in these cells. Hence, purification of these hemerythrins was readily accomplished by washing the cells, lysing them, centrifuging the lysate, and crystallization or precipitation.

Myohemerythrin, in contrast, comprises a small fraction of the soluble protein in the muscle homogenates. However, the fact that myohemerythrin is soluble in 90% saturated ammonium sulfate solution and is precipitated only at 100% saturation makes the purification of this protein surprisingly simple. Approximately 70 to 80 mg of purified myohemerythrin was obtained from the muscles of 100 worms.

Disc electrophoresis patterns of coelomic, vascular, and myohemerythrin both in the presence and absence of the mercurial HMB are shown in Fig. 1. Addition of a mercurial to either G. gouldii or D. pyroides coelomic hemerythrin is known to dissociate the octameric molecule into subunits (10,11). The increase in electrophoretic mobility of D. pyroides coelomic hemerythrin upon HMB treatment (Gel 2) is thus undoubtedly caused both by the addition of one charged group (HMB) and by the 8-fold reduction in molecular weight. Vascular hemerythrin gives a similar HMB-induced mobility change probably due as well to an octamer to HMB-monomer conversion. Myohemerythrin, on the other hand, gives a pattern upon addition of HMB which is not as easily explained by an oligomer-monomer transformation. When a preparation similar to that shown in Gel 6 was run on gels of different acrylamide concentrations (5, 7½, and 10%) and the log of the relative mobilities plotted against acrylamide concentration (12), parallel lines were obtained for the three bands, a good indication that all three species are of a similar molecular weight. Molecular weight studies (see below) show that this is indeed the case.

Coelomic hemerythrin octamer and monomer each give more than one electrophoretic band, a reflection of the occurrence of molecular variants of this hemerythrin (13). While vascular hemerythrin generally gives a diffuse band in the unmodified form, it seems unlikely that this is due to molecular variants since vascular HMB-monomer moves as a sharp single band.

The gel electrophoresis patterns indicate that myohemerythrin, vascular hemerythrin, and coelomic hemerythrin are pure. In addition, the mobilities of the unmodified proteins (as seen in Gels 1, 3, and 5) and the change in mobilities in response to addition of HMB clearly show that each is a distinct hemerythrin.

Spectroscopic Properties—The ultraviolet-visible absorption spectra of coelomic hemerythrin, vascular hemerythrin, and myohemerythrin, all in the metazide form are shown in Fig. 2. Although there are small variations in the extinction coefficients from protein to protein, each has a spectrum characteristic of metazidehemerythrin (14, 15). The ultraviolet-visible circular dichroic spectra provide additional evidence that these three proteins are indeed hemerythrins (Fig. 3). Again, despite small variations in the molar extinction coefficients, the spectra are nearly identical with one another and compare favorably to the ultraviolet-visible circular dichroism spectrum reported for G. gouldii.
gouldii coelomic hemerythrin (15). Since these spectral properties are a reflection of the structure of the iron binding site in hemerythrin, these results suggest that the iron binding sites in these three hemerythrins are similar.

The far-ultraviolet circular dichroism spectra of these proteins are essentially the same, indicating a high content of \( \alpha \) helix in each case (Fig. 4). The \( \alpha \) helical content, based on the ellipticity at 222 nm (16, 17), is 69% for coelomic, 66% for vascular, and 69% for myohemerythrin. These values compare with 72% \( \alpha \) helix computed for G. gouldii coelomic hemerythrin (18).

**Molecular Weights**—Coelomic hemerythrin from \( D. \) pyroides has been reported to have a molecular weight near 100,000 and to be dissociated to a 12,800 molecular weight monomer by dilution or by treatment with sulfhydryl reagents (11). The disc electrophoresis studies described above suggest a similar pattern of molecular weights for vascular hemerythrin. Sedimentation velocity studies on vascular hemerythrin in sodium phosphate buffer gave a \( s_{20,w} \) of 6.7 S. In Tris-chloride-sodium azide buffer, a \( s_{20,w} \) value, uncorrected for the buffer, of 6.7 S was also obtained. This value is in good agreement with the 6.6 to 6.9 S sedimentation coefficients reported for \( G. \) gouldii hemerythrin (19) and the 6.5 to 6.8 S values reported for the coelomic \( D. \) pyroides hemerythrin (11). It is clear, therefore, that both coelomic and vascular hemerythrin from this species occur with molecular weights near 100,000.

The molecular weight of the monomeric form of vascular hemerythrin was obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Vascular hemerythrin monomer gave a molecular weight value of 14,900 ± 1,500 (standard deviation).

The molecular weight of myohemerythrin was determined by ultracentrifugation, gel filtration, and sodium dodecyl sulfate-polyacrylamide electrophoresis. Sedimentation velocity experiments yielded a \( s_{20,w} \) value (in Tris-chloride-sodium azide buffer) of 1.6 S. This value compares favorably with the \( s_{20,w} \) of 1.95 S obtained for the 13,500 molecular weight monomer of \( G. \) gouldii coelomic hemerythrin (19). Furthermore, myohemerythrin did not aggregate at concentrations as high as 10 mg per ml. Gel filtration of HMB-treated myohemerythrin on a Sephadex G-100 column gave a molecular weight of 14,500. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis, myohemerythrin consistently ran with a mobility identical with that of monomeric \( G. \) gouldii hemerythrin, indicating a minimum molecular weight for myohemerythrin near 13,500. Thus myohemerythrin, whether native, HMB-treated, or denatured, gave similar molecular weights, all near 13,500.

**Amino Acid Analyses**—The amino acid compositions of coelomic hemerythrin, vascular hemerythrin, and myohemerythrin from \( D. \) pyroides are given in Table I. Also included is the amino acid composition of \( G. \) gouldii coelomic hemerythrin and that reported for \( D. \) pyroides coelomic hemerythrin by Ferrell and Kitto (11). Significant differences between the amino acid compositions of the three \( D. \) pyroides proteins are readily apparent. Vascular hemerythrin is obviously different from the other two in its content of methionine (5 residues) and histidine (3 residues). Myohemerythrin is also distinctive in comparison to the coelomic and vascular proteins, containing, for example, relatively smaller quantities of histidine, arginine, aspartic acid, and tyrosine and a larger quantity of lysine. Overall, there are differences in amino acid composition of approximately 26 residues between coelomic and vascular hemerythrin, 34 residues between coelomic hemerythrin and myohemerythrin.

![Fig. 4. Far ultraviolet circular dichroism spectra of D. pyroides coelomic hemerythrin (---), vascular hemerythrin (--), and G. gouldii coelomic hemerythrin (—) (18). Molecular ellipticities [θ] in degrees cm\(^2\) decimole\(^{-1}\) are calculated in terms of moles of amino acid residue per liter.](http://www.jbc.org/)

<p>| Amino acid composition of coelomic hemerythrin, vascular hemerythrin, and myohemerythrin |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Coelomic (^a)</th>
<th>Vascular (^b)</th>
<th>Myohemerythrin (^c)</th>
<th>Coelomic (^d) of ( G. ) gouldii</th>
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<tr>
<td>Lysine (^e)</td>
<td>9.0</td>
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<td>H histidine (^f)</td>
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<td>6.0</td>
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<td>17.0</td>
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<td>6.1</td>
<td>3.7(^j)</td>
<td>4.8(^k)</td>
</tr>
<tr>
<td>Serine (^l)</td>
<td>3.4</td>
<td>1.5(^m)</td>
<td>3.6(^n)</td>
</tr>
<tr>
<td>Glutamic acid (^o)</td>
<td>10.6</td>
<td>13.1</td>
<td>13.0</td>
</tr>
<tr>
<td>Proline (^p)</td>
<td>3.6</td>
<td>5.7</td>
<td>5.6</td>
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<tr>
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<td>2.8(^t)</td>
<td>1.9(^u)</td>
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<td>3.0(^\gamma)</td>
<td>2.9(^\delta)</td>
<td>2.3(^\epsilon)</td>
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</table>

\(^a\) Average of 10 analyses.
\(^b\) Average of seven analyses.
\(^c\) Average of eight analyses.
\(^d\) From Ferrell and Kitto (11).
\(^e\) From Groskopf et al. (20).
\(^f\) Not corrected for destruction during hydrolysis.
\(^g\) As cysteic acid.
\(^h\) Determined spectrophotometrically (21).
and 32 residues between vascular hemerythrin and myohemerythrin. In comparison, there are approximately 16 residues which are different in the compositions of *G. gouldii* and *D. pyroides* coelomic hemerythrins.

**DISCUSSION**

Three distinct hemerythrins have been found in the sipunculid *D. pyroides*. In addition to the coelomic protein, vascular hemerythrin, and myohemerythrin have been isolated, purified, and characterized. All three proteins show the characteristic optical absorption and circular dichroism properties of hemerythrin. These methods also indicate that the secondary structures of the three hemerythrins are quite similar. However, the primary structures of these hemerythrins are significantly different, the proteins being clearly distinguished by their electrophoretic properties, amino acid compositions, and in the case of myohemerythrin, its substantially lower molecular weight.

Coelomic and vascular hemerythrins occur with molecular weights near 100,000. By analogy with *G. gouldii* coelomic hemerythrin, these are probably octameric molecules. In contrast to this, myohemerythrin occurs as a monomer with a molecular weight near 13,500. The octameric coelomic and vascular proteins can be dissociated by reaction with HMB, their subunits having molecular weights near 13,500. Myohemerythrin undergoes a change in electrophoretic mobility upon reaction with HMB but dissociation appears not to occur. The presence of 2 residues of half-cystine in myohemerythrin provides an explanation for the reaction of this protein to reaction with HMB. The two new bands appearing in gel electrophoresis correspond to proteins with either one or two cysteines blocked with the mercurial, the change in mobility being caused by the carboxylate group in HMB. When a large excess of HIV is added, both cysteines are completely blocked, and a single fast moving band is then observed.

The amino acid composition of myohemerythrin provides information about the active center of the hemerythrin molecule. Four residues of histidine (22) and two (23) or three (24) of tyrosine have been postulated to be coordinated to iron in hemerythrin. The presence of approximately 4 residues of histidine in myohemerythrin is consistent with the idea that no more than four histidines can be coordinated to iron. The localization of these histidines in the primary structure of myohemerythrin will provide important information as to the identity of iron-linked histidines in hemerythrin. This work is now in progress in this laboratory.

The amino acid compositions of coelomic hemerythrins from *G. gouldii* and *D. pyroides* and of vascular hemerythrin and myohemerythrin from the latter species reveal a good deal of variability in primary structure within a species but considerably less among the coelomic hemerythrins in two different species. A minimum estimate of the variability between proteins, based on the amino acid compositions, indicates that there is nearly twice as much variability between the hemerythrins in *D. pyroides* as between the coelomic proteins of the two species.

Ferrell and Kitto (25) have determined the amino acid sequence of the first 34 residues of *D. pyroides* coelomic hemerythrin and have constructed the remainder of the sequence by comparison of tryptic peptides with the known sequence of *G. gouldii* coelomic hemerythrin (26). They have found only four amino acid sequence differences between the two proteins. The sequence which they have postulated with this procedure raises some questions. Notably their proposed sequence differs significantly both from their determined amino acid composition (11) and from the one reported in this paper (Table I) and further their analysis does not take into account the occurrence of variant hemerythrins (13). Nevertheless, the close structural similarity between the coelomic hemerythrins of these two species is clear.

Some analogies on the structural level between the hemerythrin-myohemerythrin and hemoglobin-myoglobin systems are apparent. Hemoglobin and myoglobin occur as oligomer and monomer, respectively, have similar secondary structures, and are significantly different in primary structure. These generalizations hold for hemerythrin and myohemerythrin as well. However, construction of a similar analogy between the two systems on the functional level must await measurement of the relative oxygen affinities of the three hemerythrins.

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**REFERENCES**

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