Response of Sipunculid Hemerythrins to Inorganic Ions and CO_2

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ABSTRACT The O_2 equilibrium properties of sipunculid hemerythrins (Hr) are influenced by intracellular effectors. O_2 affinity rises and cooperativity decreases when the tentacular O_2 carrier is extracted into incorrect ratios of inorganic ions. The effectors include Ca^{2+} and Cl^-, which raise the O_2 affinity and lower cooperativity and which are present within the cell in either low levels or an inaccessible form. In the presence of salines approximating the inorganic ion conditions within the cell, tentacular hemerythrin-O_2 affinity decreases and cooperativity increases. Another effector of O_2 bindng is CO_2, which lowers O_2 affinity and enhances cooperativity. While the inorganic ion and CO_2 effects on coelomic hemerythrin are either absent or very small, the actions of at least the inorganic ions on tentacular Hr clearly have respiratory significance. Physiological variations in intracellular ions are very small, but the low levels of free Ca^{2+} and Cl^- in the cell exaggerate the intrinsically lower O_2 affinity of tentacular than coelomic Hr and thus enhance the routing of O_2 from the ambient source to the tentacular compartment and from there to the coelomic compartment and then to metabolizing tissue.

Of the three groups of O_2 carriers, the hemerythrins (Hr) are by far the least known. Hrs are found in muscle and/or nulceated pink blood cells (PBC) in four phyla (Sipunculida, Priapulida, Brachiopoda, and Annelida), which are usually regarded as more or less closely related and at the middle level of animal phylogeny. While considerable progress has been made in elucidating molecular structure of these unique proteins (reviewed by Klippenstein, '80; Klotz and Kurtz, '84), less is known about their physiological function.

Manwell ('60) showed that Hrs in the two extracellular fluid compartments of the sipunculid *Themiste zostericolum* have very different O_2 affinities. The O_2 affinity in the tentacular compartment, which is ventilated in the water column, is lower than the O_2 affinity in the coelomic compartment, which is more distant from the water column. The two systems are believed to be arranged for O_2 transfer, like the mammalian maternal and fetal hemoglobins (Hb). Mangum and Kondon ('75) and Mangum ('78) presented circumstantial evidence that in *Phascolopsis gouldi* coelomic PBCs function as O_2 carriers between the body wall epithelium and deep tissue (the tentacular compartment in this species is rudimentary). Like the annelid coelomic Hbs, the coelomic Hr of *P. gouldi* also functions as an O_2 store, in this case demonstrated directly (Mangum, '77); Portner et al. ('85) reached a similar conclusion for *Sipunculus nudus*.

The octameric structure of a typical circulating Hr provides one prerequisite for functional plasticity by means of allosteric modulation, a potential that has been exploited only occasionally among the O_2 carriers, however (Mangum, '85). The degree to which the Hrs are in fact modulated is uncertain. Structural studies predict that anion binding near the active site should influence conformation of the polypeptide chains (Langerman and Klotz, '69; Garbett et al., '71). While several investigations described the effects of either unnatural ligands on O_2 equilibrium or naturally occurring ligands on O_2 binding kinetics (DePhillips, '71; de Waal and Wilkins, '76; Petrou et al., '81), the effects of substances known to occur in cells on properties with direction respiratory con-

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sequences have not been reported in the formal literature. At a physiological level, Manwell ('60) and Mangum and Kondon ('75) reported differences in the O_2 equilibria of sipunculid PBCs and Hr extracts. Weber and Fange ('80) reached the same conclusion for priapulid Hr.

There are, however, reasons to regard both the biochemical and the physiological evidence with circumspection. Manwell ('60) was forced to reduce PBC light scattering in his absorbance measurements by adding bovine serum albumin and Karo syrup, which could have impaired equilibration between the different phases in the tonometer and which, as made clear by the author, eliminated light scattering only in part. The differences reported by Mangum and Kondon ('75), who used non-optical methods, was small. In her investigation of sipunculid coelomic Hr, Chadwick ('79) detected no effect of Ca^{2+} or Mg^{2+} on O_2 equilibrium (on the other hand, the active site of her preparation, which was described as old, had clearly undergone alternation). Mangum ('85) found no difference in O_2 equilibria of Hr extracts and PBCs in the brachipod *Lingula*. Most importantly, effectors responsible for putative differences between PBCs and extracts have not been identified .

To identify possible effectors and to assess their physiological role, we have examined the effects on sipunculid HrO_2 equilibria of the inorganic ions that influence O_2 binding kinetics. We have also investigated the effect of CO_2. CO_2 was chosen even though both Florkin ('33) and Manwell ('60) reported no effect, in part because they examined only the coelomic molecule and in part because Henry ('87) has recently shown that sipunculid PBCs have very high activities of the enzyme carbonic anhydrase.

MATERIALS AND METHODS

As indicated above, *Phascolopsis gouldi* has a tentacular compartment so small that the amount of material available that could be collected would limit observations to one or two at best. Therefore the West Coast species *Themiste zostericolum*, whose Hrs are somewhat less well known both physiologically and biochemically, was purchased from a commercial supplier and held in aerated, recirculating aquaria (32 o/oo) with about 8 cm sand, into which they readily burrowed.

Coelomic PBCs were obtained by slitting the body wall, draining the coelom, and then washing the inverted coelomic cavity with seawater. Even in this species the volume of the tentacular system is quite small, and pure preparations of tentacular PBCs proved to be difficult to prepare. The coelom was washed until no PBCs were observed in the wash with a dissecting microscope. Our first preparation was made by then slitting the tentacular vessels in situ. Because the O_2 binding results suggested possible contamination of the preparation with coelomic PBCs, the procedure was subsequently altered. The tentacular system was removed intact and in toto and washed, and the highly contractile vessels lanced at numerous places. Finally, the lanced vessels were pressed against the glass container with a spatula. Nonetheless, only small numbers of tentacular PBCs could be obtained from a dozen or so animals.

The PBCs were either washed, packed by low speed centrifugation and immediately used in O_2 binding measurements, or their Hr was extracted. For determinations of the response to an inorganic ion the PBCs were packed, the supernatant fluid discarded, and the cells extracted with buffered (0.05 M Tris maleate) 10 mM $Ca(NO_3)_2$ or 100 mM NaCl in a volume equal to the discarded fluid. The extract was then dialyzed against the extracting solution for 24 hr. The inorganic salt to be tested was added in small aliquots of a concentrated solution so that protein dilution (which does not influence O_2 binding in the range examined [Manwell, '60; Petrou et al., '81] but makes the cell respiration method more difficult) during the measurements would be less than 8%.

O_2 binding was determined by one of two methods: 1) For measurements on PBCs and determination of the responses of extracts to inorganic ions, the cell respiration method (Mangum and Lykkeboe, '79) was used. PBCs were suspended in buffered (0.05 M Tris maleate) saline and the yeast cells added in the same solution. Extracts were diluted by 3.3% with yeast cells in the initial test solution. 2) For measurements on extracts in the presence of CO_2, a tonometric method was used for equilibration, and changes in oxygenation were determined spectrophotometrically (Burnett, '79; Mangum and Burnett, '86).

The physiological range of intracellular ions was estimated using coelomic cells (because of their abundance) incubated in differ-

ent salinities. The cells were first washed several times with seawater (35 o/oo) and then packed by centrifugation at 3,900 RPM for 10 min. Packed cells (0.59–0.63 ml) were brought to 5 ml with water (22–100 o/oo) buffered to pH 7.77 (the coelomic fluid pH at the acclimation temperature of 18.5°C) with 0.05 M Tris maleate and incubated at 4°C for 90 min. The low end of the salinity range was chosen from data on osmotic fragility of *P. gouldi* cells collected by Demanche ('80) and D. Simpson (unpublished), indicating that 22 o/oo is the salinity of incipient lysis. The cells were then brought to room temperature, gently agitated for 15–30 min, and their packed volume measured again. The supernatant fluid was removed, and the cells were first washed with isotonic $NaNO_3$ and then extracted into 0.05 M Tris maleate buffer (pH 7.60) in a volume equal to the final volume of the packed cells. The activities of Ca^{2+}, Mg^{2+}, and Cl^- were determined with ion-selective electrodes (Mangum and Lykkeboe, '79), using stirred samples diluted with 0.05 M Tris maleate buffer to control pH and total ionic strenth. To improve the accuracy of the determination of Mg^{2+} with a total divalent cation electrode, 0.05 M ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid was added in a volume equal to that of the sample, after first determining Ca^{2+} activity. Equilibration of the electrodes was ascertained with a recorder. The electrodes were calibrated with a series of six standards prepared from IAPSO seawater (19.3760 o/oo chlorinity) treated identically to the samples. The extracellular space in a preparation of packed mammalian red cells is 3.5% of total volume (Kim and Luthra, '76). The average size of *T. zostericolum* PBCs is not known, but it appears to be greater than that of a mammalian red cell. The data were corrected for extracellular space by a factor of 7%. While the correction factor is approximate, it in fact alters the raw data by a factor so small (< 0.5 mM) that the effect on O_2 binding is trivial. The data were also corrected for dry matter using the measurements of water content made by Oglesby ('82).

Unless otherwise specified the data were analyzed according to Student's t-test.

RESULTS

O_2 binding of PBCs and extracts

Our first preparation of tentacular PBCs, which was made by slitting the tentacular system in situ, yielded enough material to make eight measurements spanning the pH range 7.10–8.22. The slopes of regression lines describing n_{50} or log P_{50} as a function of pH in the range 7.10–8.22 did not differ from zero ($P > .05$). Mean values (± SE) were P_{50} = 19.9 (± 0.8) mmHg and n_{50} = 2.04 (± 0.07). When we succeeded in isolating the tentacular system prior to slitting it, however, we obtained a lower O_2 affinity (Table 1). While we have no concrete reason to discard the data reported above we suggest that the first preparation might have been contaminated with coelomic cells. The data, however, confirm Manwell's ('60) observation that tentacular Hr is not pH sensitive, even though the present results show that it is distinctively cooperative (Table 1; Fig. 1). Regardless it is clear from both sets of data that tentacular PBCs have a much lower O_2 affinity than coelomic PBCs (Table 1; $P < .001$) (Manwell, '60).

We decided to use the remainder of the tentacular material available for additional experiments on extracts rather than further replication of previously reported results. Note, however, that the control point (P_{50} =

TABLE 1. Oxygen equilibrium properties of Themiste zostericolum *PBCs and extracts*[a]

	Saline	pH	P_{50} (mmHg)	n_{50}
Cells				
Coelomic	Physiological[b] + 0.05 M Tris maleate	7.07–8.22	4.26 ± 0.16 (8)	1.08 ± 0.03 (8)
Tentacular		7.77	23.6 ± 1.1 (3)	2.16 ± 0.10 (3)
Extracts				
Coelomic	10 mM $Ca(NO_3)_2$ + 0.05 M Tris maleate	7.00–7.64	3.77 ± 0.15 (5)	1.07 ± 0.09 (5)
Tentacular		7.69	14.8 (1)	1.54 (1)
Tentacular	"Intracellular"			
	Freel et al., '73	7.59	25.5 ± 0.3 (6)	2.42 ± 0.09 (6)
	Present data	7.55	27.0 ± 0.3 (3)	2.05 ± 0.17 (3)

[a]Mean ± S.E. (N). 20°C. Data collected by the cell respiration method.
[b]300 mM NaCl, 11 mM KCl, 10 mM $CaCl_2$, 35 mM $MgCl_2$, 25 mM Na_2SO_4, and 2 mM $NaHCO_3$ (prepared from data reported by Oglesby, '69, '82).

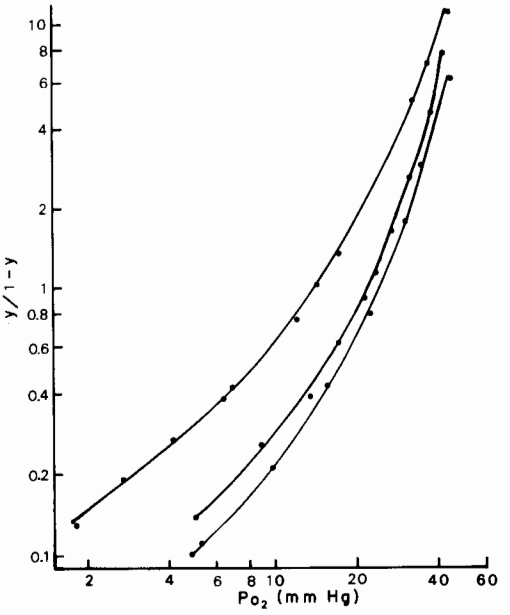

Fig. 1. Examples of O_2 equilibria of (from left to right) tentacular Hr extracts in 0.05 M Tris maleate buffer + 10 mM Ca $(NO_3)_2$ (pH 7.71), tentacular PBCs in 0.05 M Tris maleate buffered physiological saline (pH 7.69) and tentacular Hr extracts in 0.05 M Tris maleate buffered "intracellular" saline (pH 7.59). Y = Percentage oxygenation. Cell respiration method, 20°C.

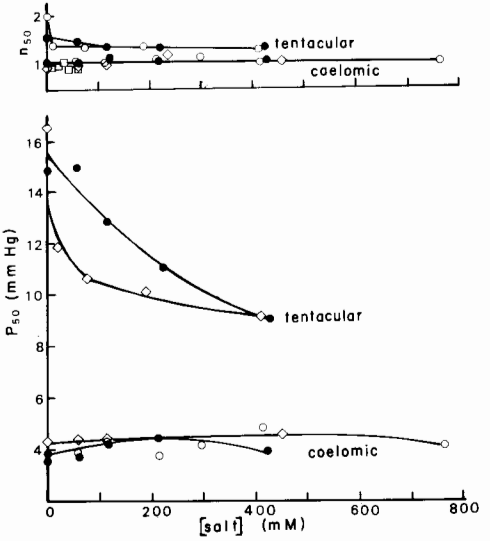

Fig. 2. Effects of (●) NaCl, (○) choline Cl, and (◇) Ca $(NO_3)_2$ on O_2 equilibria of Hr extracts. For effects of Ca^{2+}, extracts were in 0.05 M Tris maleate buffer + 100 mM NaCl (pH 7.69). Otherwise extracts were in 0.05 M Tris maleate + 10 mM $Ca(NO_3)_2$ (pH 7.45–7.59 depending on test salt). Cell respiration method, 20°C.

14.8 mmHg) for the inorganic ion experiments in 10 mM $Ca(NO_3)_2$ is almost identical to the control point (15.0 mmHg, in the same saline) for the CO_2 experiments despite the different methods used (Figs. 2, 4). More importantly the two points for both P_{50} and n_{50} of tentacular Hr extracts fall well outside of the 95% confidence interval (C.I.) for the intact tentacular PBCs. Some of the cooperativity of tentacular PBCs is also lost when the Hr is extracted from the cells into an unphysiological saline (Table 1; Fig. 1).

Coelomic PBCs, as well, have a lower O_2 affinity than extracts (P = .02) and the percentage difference is about the same as that for tentacular Hr. The absolute difference, however, is very small. This Hr is not very cooperative (if at all), regardless of whether or not it is in the PBC (Table 1).

Effects of inorganic ions on O_2 binding of extracts

Like Chadwick ('79), we were unable to demonstrate a clear effect of Ca^{2+} on oxygen affinity of coelomic Hr (Fig. 2). Logarithmic regression lines describing the data do not differ from zero at P = 0.05. Cooperativity

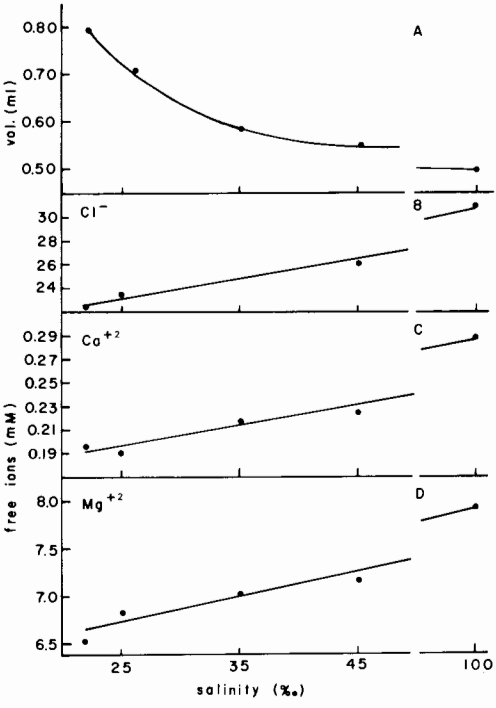

Fig. 3. Effects of salinity on coelomic PBC volume (A) and ionic activity (B–D).

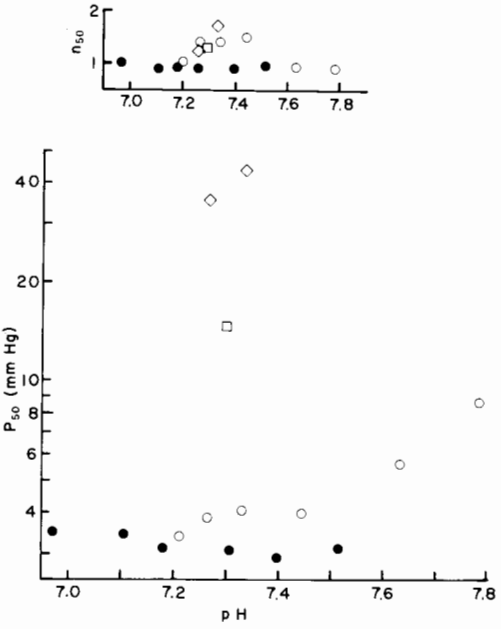

Fig. 4. Effects of CO_2 on HrO_2 binding. (●) Coelomic, PCO_2 = 0 mmHg; (○) coelomic, PCO_2 = 14.8 mmHg; (□) tentacular, PCO_2 = 0 mmHg; (◇) tentacular, PCO_2 = 14.8 mmHg. 10 mm Ca $(NO_3)_2$ + 0.05 M Tris maleate buffer. Tonometric method, 20°C.

also remains unchanged. In the presence of 10 mM $Ca(NO_3)_2$, NaCl as well has little or no effect.

In contrast, $Ca(NO_3)_2$ and NaCl clearly raise the O_2 affinity of tentacular Hr (Fig. 2). Logarithmic regression lines describing the data in Figure 2 have high coefficients of determination (r^2 = 0.993 [P < .001] for $Ca(NO_3)_2$ and 0.884 [P < .01] for NaCl) and slopes significantly different from zero (b = −0.030 ± 0.012 95% C.I. for $Ca(NO_3)_2$ and −0.014 ± 0.007 for NaCl). $Ca(NO_3)_2$ and NaCl also clearly lower cooperativity (P < .05).

Estimation of intracellular ions and their physiological range

The estimated changes in the intracellular Ca^{2+}, Mg^{2+}, and Cl^- in the salinity range 22–35 o/oo, probably the ecological limits, are quite small (Fig. 3). Even when the range is extended to extremely hypersaline conditions, it is clear that physiological variation would not appreciably influence O_2 binding (Figs. 2, 3).

O_2 binding of extracts in physiological saline

To test the adequacy of an hypothesis that the effects of extraction and dialysis on O_2 binding can be explained by inorganic ions we first attempted to make an "intracellular" saline from data on total inorganic ion levels in annelid body wall muscle (Freel et al., '73), the closest information we could find. To make up the anion deficit we used PO_4. The saline consisted of 10 mM $CaPO_4$, 20 mM $MgCl_2$, 6 mM Na2HPO4, 55 mM KH_2PO_4, and 3 mM $MgCO_3$. After dialysis against a buffered preparation, both P_{50} and cooperativity of tentacular Hr not only returned towards but actually exceeded the original values (P < .001; Table 1). We next attempted to make an intracellular saline based on our own measurements of ionic activity at 35 o/oo (Fig. 3); the attempt was not entirely successful. In our experience electrometric measurements are best converted to molar units (e.g., Fig. 3) graphically, a process that requires considerable time. And yet it was necessary to use for O_2 binding the tentacular Hr of the animals whose coelomic cells were used for ionic activity determinations. To circumvent the problem of alteration of the active site with aging, we designed the saline from ionic activity data obtained by visual inspection of the data in mv. This intracellular saline, which contained 6.1 mM $MgCl_2$, 0.18 mM $CaCl_2$ and 9 mM KCl, proved to be slightly deficient in divalent cations and Cl^- (see data for 35 o/oo in Fig. 3, which were obtained by graphic analysis). Once again P_{50} and n_{50} exceeded the original values (P < .05; Table 1), although the excess diminished to a very small value due, we suggest, to the greater accuracy of the saline. We made no further attempts to improve the saline, in part because of the unavailability of material and in part because of the inevitable inaccuracy of an intracellular saline lacking proteins as the chief anions. Substitution for it with other inorganic anions would be difficult since the Hrs bind so many of them (Klotz and Kurtz, '84).

Effects of CO_2 on O_2 binding

CO_2 lowers the O_2 affinity, raises cooperativity, and, somewhat surprisingly, brings about a reversed Bohr shift of coelomic Hr (Fig. 4). Semilogarithmic regression lines describing the data for the absence and presence of CO_2 differ significantly (P < .05) in the common pH range examined (7.1–7.7), as do mean values for cooperativity (P = .012). While the data for tentacular hr are too few for a meaningful probablity statement, an

effect of CO_2 and O_2 affinity also seems clear (Fig. 4).

DISCUSSION

Although our values for tentacular Hr extracts in the presence of 10 mM $Ca(NO_3)_2$ do not differ from the value (15 mmHg, also at 20°C) reported by Manwell ('60) for extracts in the presence of only PO_4 buffer, our values for both coelomic and tentacular PBCs are different, suggesting that his early method of reducing light scattering may have introduced other problems. Specifically, the difference between PBCs and extracts in our data is smaller (Fig. 1; Table 1).

Our findings show that the intracellular milieu and the effects of Ca^{2+} and Cl^- on sipunculid coelomic Hr are either absent or so small that they are unlikely to play a physiological role of any importance. Sipunculids have a limited range of salinity tolerance (Oglesby, '82), and ionic variations within the PBC would have not appreciable respiratory effect (Figs. 2, 4).

In contrast, the effects of inorganic ions on tentacular Hr are great enough that they are of considerable physiological significance. Salinity-induced variations within the cell are not (Figs. 2, 3). But the impoverishment of free divalent cations and Cl^- with the PBC, by further (almost two times) lowering the already lower O_2 affinity of tentacular than coelomic Hr, would appear to be an important component of an O_2 transfer-transport system. The designation "modulator" for these effectors of tentacular HrO_2 binding would seem to be warranted.

CO_2 has more dramatic effects than inorganic ions on coelomic Hr, but the levels used in our experiments were chosen to yield unequivocal results; it is not clear that they are physiological (see Portner et al., '86; for CO_2 levels in the extracellular fluid of another species) even though CO_2 could be enriched in the aerobically metabolizing PBC. Earlier investigators (Florkin, '33; Manwell, '60) may have failed to detect a CO_2 effect because they used lower levels, although such details are not entirely clear in the original accounts. Lack of information on the CO_2 level at which an appreciable effect first occurs also renders physiological relevance difficult to assess. From a molecular view, however, CO_2 sensitivity may not be surprising given the broad spectrum sensitivity of O_2 binding kinetics to monovalent anions, especially if the effective species proves to be HCO_3^-. The response of tentacular Hr to CO_2 is even more difficult to evaluate in terms of physiological significance. In this case, the levels of extracellular CO_2 levels in the tentacular compartment are not known in any species.

In conclusion, our findings show that, like the other two classes of O_2 carriers, the Hrs are capable of being modulated, but that within the class, not all members possess the capability.

ACKNOWLEDGMENTS

This work was supported by NSF DCB 84-14856 (Regulatory Biology).

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