Response of Spinulicidum Hemerythins to Inorganic Ions and CO2

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ABSTRACT
The O2 equilibrium properties of spinulicidum (Hemerythins) are influenced by intracellular effectors. O2 affinity rises and cooperativity decreases when the tentacular O2 carrier is extracted into intercellular ratios of inorganic ions. The effectors include Ca2+ and Cl-, which raise the O2 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 hemerythins O2 affinity decreases and cooperativity increases. Another effector of O2 binding is CO2, which lowers O2 affinity and enhances cooperativity. While the inorganic ion and CO2 effects on coelomic hemerythrin are either absent or very small, the actions of at least the inorganic ions on tentacular Hz clearly have respiratory significance. Physiological variations in intracellular ions are very small, but the low levels of free Ca2+ and Cl- in the cell exaggerate the intracellularly lower O2 affinity of tentacular than coelomic Hz and thus enhance the routing of O2 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 O2 carriers, the hemerythrin Hz are by far the least known. Hz are found in muscle and/or in calcified pink blood cells (PBC) in four phyla (Spinulicida, 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 (80) showed that Hz in the two extracellular fluid compartments of the spinulicidum Thiasmata antarcticum have very different O2 affinities. The O2 affinity in the tentacular compartment, which is ventilated in the water column, is lower than the O2 affinity in the coelomic compartment, which is more stagnant. The two systems are believed to be arranged for O2 transfer, Hz in the mammalian maternal and extra-embryonic hemolymphs. Mangum and Konden (76) and Mangum (80) presented circumstantial evidence that in Phalloepilaxis gouldi coelomic PBCs function as O2 carrier between the body wall epithelium and deep tissues (the tentacular compartment in this species is rudimentary). Like the annelid coelomic Hbs, the coelomic Hz of P. gouldi also function as O2 stores. In this case demonstrated directly (Mangum, 77, Portzer et al. (82) reached a similar conclusion for Spinulicida studies. The ectomeric structure of a typical circula
ting Hz provides one prerequisite for functional plasticity by means of allosteric modulation, a potential that has been exploited only occasionally among the O2 carriers, however (Mangum, 80). The influence of which the Hz are in fact modulated is uncertain. Structural studies predict that anion binding near the active site should influence conformation of the polypeptide chains (Langermann and Klotz, 80; Garbett et al., 71). While several investigations described the effects of various natural ligands on O2 equilibrium or naturally occurring ligands on O2 binding kinetics (DePhillips, 71; de Waal and Wilkins, 76; Portzer et al., 81), the effects of substances known to occur in cells on properties with direction respiratory con-

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sequences have been reported in the for- mal literature. At a physiological level, Manwell (160) and Mangum and Kondon (75) reported differences in the O₂ equilibria of sipunculid PCRs and fibrin extracts. Weber and Fange (160) reached the same conclusion for parasipulid Hr.

There are, however, reasons to regard both the biochemical and the physiological evi- dence with circumspection. Manwell (160) was forced to reduce PCR light scattering in his absorbance measurements by adding bovine serum albumin and Karo syrup, which could have impaired equilibration between the differ- ent phases in the titrator and which, as made clear by the author, eliminated light scattering only in part. The differences re- ported by Manwell and Kondon (75), who used non-optical methods, was small. In her investigation of sipunculid coelomic Hr, Chadwick (79) detected no effect of Co²⁺ or Mg²⁺ on O₂ equilibrium (on the other hand, the active site of her preparation, which was described as old, had clearly undergone alter- nation). Mangum (80) found no difference in O₂ equilibria of Hr extracts and PCRs in the banchipid Lingula. Most importantly, effec- tors responsible for putative differences be- tween PCRs and extracts have not been identified.

To identify possible effectors and to assess their physiological role, we have examined the effect on sipunculid HrO₂ equilibria of the inorganic ions that influence O₂ binding kinetics. We have also investigated the effect of CO₂, CO, was chosen even though both Fleckin (70) and Manwell (60) reported no effect, in part because they examined only the coelomate molecule and in part because Henry (87) has recently shown that sipun- culid PCRs have very high activities of the enzyme carbonic anhydrase.

**MATERIALS AND METHODS**

As indicated above, Phascolopsis gouldii has a tentacular compartment as 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 *Thermastoma intermediterraneum*, whose Hrs are some- what less well known both physiologically and biochemically were purchased as a commercial supplier and held in aerated, re- circulating aquaria (32°C) with about 8 cm sand, into which they readily burrowed.

Coelomic PCRs were obtained by sifting the body wall, draining the coelom, and then washing the inverted coelomic cavity with seawater. Even in this species the volume of the tectal tentacle system is quite small, and pure preparations of tentacular PCRs proved to be difficult to prepare. The coelom was washed until no PCR was observed in the wash with a dissecting microscope. Our first preparation was made by then slitting the tentacular vessels in situ. Because the O₂ binding results suggested possible contami- nation of the preparation with coelomic PCRs, the procedure was subsequently al- tered. 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 PCRs could be obtained from dozens or so animals.

PCRs were either washed, packed by low-speed centrifugation and immediately used in O₂ binding measurements, or their Hr was extracted. For determinations of the response to an inorganic see the PCRs were packed, the supernatant fluid discarded, and the cells extracted with buffered (0.05 M Tris maleate) 10 mM Ca(NO₃)₂ or 50 mM NaCl in a volume equal to the discarded fluid. The extract was thus dialyzed against the extracting solution for 24 hr. The inorganic salt to be tested was added in small aliquots of concentrated solution so that protein dilution (which does not influence O₂ binding in the range examined [Manwell, 86; Petrou et al., 81]) but makes the cell respiration method more difficult. The results of the measurement would be less than 8%.

O₂ binding was determined by one of two methods. 1) For measurements on PCRs and determination of the responses of extracts to inorganic ions, a spectrophotometric method (Mangum and Lykkeboe, 79) was used. PCRs were suspended in buffered (0.05 M Tris ma- leate) saline and the yeast cells added in the same solution. Extracts were diluted by 3.5% with yeast cells in the initial test solution. 2) For measurements on extracts in the pres- ence of CO₂ the isometric method was used for equilibration, and changes in oxygena- tion were determined spectrophotometrically (Burnett, 76; Mangum and Burnett, 86).

The physiological range of intracellular ions was estimated using coelomic cells be- cause of their abundance incubated in differ-
were corrected for extracellular space by the factor of 76. 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 O₂ binding is trivial. The data were also corrected for dry matter using the measurement of water content made by Oglesby ('82).

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

RESULTS

O₂ binding of PBCs and extracts

Our first preparation of tentacular PBCs, which was made by4 sitting the tentacular system in situ, yielded enough material to make eight measurements spanning the pH range 7.0–7.22. The degree of regression lines describing n_0 or log P_0 as a function of pH in the range 7.0–7.22 did not differ from zero (P > 0.55). Mean values (± SE) were P_0 = 19.9 ± 0.8 mmHg and n_0 = 2.04 ± 0.07. When we succeeded in isolating the tentacular system prior to sitting it, however, we obtained a lower O₂ 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 osmotic cells. The data, however, confirm Mannwell and Gowing's (60) observation that tentacular Hr is not pH sensitive, even though the present results show that it is distinctly cooperative (Table 1, Fig. 1). Regardless, it is clear from both sets of data that tentacular PBCs have a much lower O₂ affinity than coelomic PBCs (Table 1, P < 0.001)(Mannwell, '69).

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 = 0.586 for n_0 and 0.036 for P_0) was similar from set to set.

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<table>
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<th>Salts</th>
<th>pH</th>
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*Sus is osmotic cells. Data collected in the cell respiration method. Mean±SE of values from 11 isolates. Coelomic fluid made from 20 mm Ca(OH)₂, 10 mm MOPS, 10 mm NaCl, and 1 mm NaSO₄, and 3 mm NaF (prepared from data reported by Oglesby, '69).

TABLE 1. Oxygen equilibrium properties of Thermatia tectocerica PBCs and extracts
Effects of inorganic ions on O₂ binding of extracts

Like Chadwick (79), we were unable to demonstrate a clear effect of Ca⁺⁺ on oxygen affinity of coelomic Hr (Fig. 2). Logarithmic regression lines describing the data do not differ from zero at P = 0.05. Cooperativity
also remains unchanged. In the presence of 10 mM Ca(NO3)2, NaCl as well has little or no effect.

In contrast, Ca(NO3)2 and NaCl clearly raise the 02 affinity of tentacular HR (Fig. 2). Logarithmic regression lines describing the data in Figure 2 have high coefficients of determination (r^2 = 0.998 [P < .001] for Ca(NO3)2 and 0.884 [P < .01] for NaCl) and head significantly different from zero (b = -0.030 ± 0.012 85% C.I. for Ca(NO3)2 and -0.014 ± 0.007 for NaCl). Ca(NO3)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+, Cl^-, and O2 in the salinity range 22-35 \( \sigma_{sw} \), probably the ecological limits, are quite small (Fig. 3). Even when the range is extended to extremely hyperhaline conditions, it is clear that physiological variation would not appreciably influence O2 binding (Figs. 2, 3).

O2 binding of extracts in physiological saline

To test the adequacy of an hypothesis that the effects of extraction and dialysis on O2 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 PO4.

The saline consisted of 10 mM Ca(NO3)2, 20 mM MgCl2, 6 mM Na2HPO4, 55 mM KH2PO4 and 3 mM MgO2. After dialysis against a buffered preparation, both PO4 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 ion activity at 35 \( \sigma_{sw} \) (Fig. 2); the attempt was not entirely successful. In our experience, electro-metric measurements were best converted to molar units (e.g., Fig. 3), particularly a process that requires considerable time. And yet it was necessary to use for O2 binding the tentacular HR of the animals whose electro-metric 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 mmv. This intracellular saline, which contained 6.1 mM MgCl2, 0.18 mM CaCl2 and 9 mM KCl, proved to be slightly deficient in divalent cations and Cl- (see data for 35 \( \sigma_{sw} \) in Fig. 3, which were obtained by graphic analysis). Once again PO4 and PO4 exceeded the original values (P < .05; Table 1), although the error 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 \( \sigma_{sw} \) bind so many of them (Klotz and Kurtz, 84).

Effects of CO2 on O2 binding

CO2 lowers the O2 affinity, misfits cooperativity, and, somewhat surprisingly, brings about a reversed Bohr shift of chemiosmotic HR (Fig. 4). Semilogarithmic regression lines describing the data for the absence and presence of CO2 differ significantly (P < .05) from the common pH range examined (7.1-7.7), so do mean values for cooperativity (P < .01). While the data for tentacular HR are too few for a meaningful probability statement, an
effect of CO$_3$ and O$_2$ affinity also seems clear (Fig. 4).

**DISCUSSION**

Although our values for tentacular Hr extr.

tractions in the presence of 10 mM Ca(NO$_3$)$_2$ do not differ from the values (15 mmHg, also at 20°C) reported by Maxwell (90) for extractions in the presence of only PO$_4$ buffer, our values for both caecoline and tentacular PCBs are different suggesting that his early method of reducing light scattering may have intro-

duced other problems. Specifically, the diff""


