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Allosteric Interactions in Sipunculid and Brachiopod Hemerythrins†

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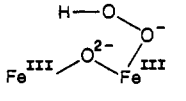
ABSTRACT: Chemical and spectroscopic consequences of allosteric interactions for ligand binding to sipunculid (*Phascolopsis gouldii*) and brachiopod (*Lingula reevii*) hemerythrins (Hrs) have been investigated. Possible allosteric effectors for homotropic effects in sipunculid Hrs have been examined, but only reduction in ligand affinity is observed without cooperativity. In contrast to sipunculid Hr, *L. reevii* Hr binds O_2 cooperatively in the pH range 7–8 and exhibits a Bohr effect. Spectroscopic comparisons of the sipunculid and brachiopod Hrs show no significant differences in the active site structures; therefore, modulation of oxygen affinity is attributable to effects linking the site to quaternary structural changes in the octamer. Oxygen equilibria can be fit with a conformational model incorporating a minimum of three states, tensed (T), relaxed (R), and an R–T hybrid. Resonance Raman spectra of *L. reevii* oxyHr show a shift in the peroxo stretching frequency when the pH is lowered from pH 7.7 (predominantly R oxyHr) to pH 6.3 (a mixture of R, T, and R–T hybrid), but *P. gouldii* Hr does not have a frequency shift under the same conditions. In contrast to hemoglobins, ligand binding to the deoxy and met forms is noncooperative for brachiopod (and sipunculid) Hrs. It is thus suggested that conformational changes in the protein are linked to the oxidation state change that accompanies oxygenation of the coupled binuclear iron site (deoxy [$Fe^{II}Fe^{II}$] → oxy [$Fe^{III}Fe^{III}$]). The total allosteric energy expended in oxygenation is about 1.4 kcal/mol, and such a shift is possible in the relaxed–tense conversion with relatively limited constraints of the iron coordination environment via the protein quaternary structure. The mechanism of cooperativity in the binuclear copper oxygen carrier hemocyanin is discussed in light of these results.

Hemerythrins are non heme iron proteins known to occur in four marine phyla, including *Sipunculida* and *Brachiopoda* (Klippenstein, 1980). As a reversible oxygen carrier, hemerythrin has been implicated in the transport and storage of oxygen in the sipunculids (Mangum & Kondon, 1975), but the precise relationship between its functional characteristics and physiological role has not been defined. This situation derives, in part, from the observation that the oxygen equilibrium curves of cellular hemerythrin are significantly different from those of purified extracts (Mangum & Kondon, 1975; Wells, 1982), with the $p_{1/2}$ values being higher for the intact coelomic cells. In addition, sipunculid cellular hemerythrin has been reported to have cooperativity in oxygen equilibrium that is absent in purified protein (Mangum & Kondon, 1975). These observations suggest the presence of allosteric effects in the ligand binding properties of hemerythrin, a topic that has not been addressed in significant detail. The study reported here involves an investigation of allosteric phenomena in purified octameric hemerythrins isolated from two sipunculids and a brachiopod.

The physical, structural, and spectroscopic properties of coelomic hemerythrins (Hrs) have been reviewed (Kurtz et al., 1977; Klippenstein, 1980; Loehr & Loehr, 1979; Klotz & Kurtz, 1984). A relatively recent development is the agreement by two X-ray crystallographic groups on the ligand environment in the binuclear iron active site. Presently, the oxidized site with bound azide (metazidoHr, [$Fe^{III}Fe^{III}N_3^-$]) is known to have the same general structure in the hemerythrins of two distinct species, *Phascolopsis gouldii* myoHr and *Themiste dyscritum* coelomic Hr (Stenkamp et al., 1984; Hendrickson et al., 1975). From single-crystal polarized spectra (Gay & Solomon, 1978), it was determined that the end-on binding mode found crystallographically for azide is retained in the oxy form, where dioxygen is bound as peroxide:



Extended X-ray absorbance fine structure (EXAFS) (Hendrickson et al., 1982) and low-resolution X-ray crystallographic studies (Stenkamp et al., 1985) have confirmed that the μ-oxo

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bridge is also retained in the oxy state. Resonance Raman results indicate a hydroperoxide description for the protonation state of the peroxide (Shiemke et al., 1984), and other evidence indicates H bonding between the bound peroxide and the oxo bridge (Shiemke et al., 1986).

No X-ray structural data are available for the coelomic hemerythrin from the brachiopods, but our recent spectral comparison (Richardson et al., 1983) has demonstrated a significant conservation of structural features in the active site when *Lingula reevii* and *P. gouldii* oxy- and metazidohemerythrins are compared. Specifically, the μ-oxo bridge and end-on geometries for peroxide and azide are retained in *L. reevii* hemerythrin. In addition, the *L. reevii* protein appears to be octameric, as found for the brachiopod *Lingula unguis* hemerythrin (Joshi & Sullivan, 1973).

The structure of the deoxy active site ([$Fe^{II}Fe^{II}$] deoxyHr) is presently less well characterized. EXAFS studies (Elam et al., 1983; Co, 1983) have shown that the number of ligands to the irons is not reduced markedly, and there is no evidence for the short bond distance associated with a bridging oxide atom as found for the oxidized forms. Recently reported low-resolution X-ray crystallographic results support these conclusions (Stenkamp et al., 1985). Ferrous iron ligand field transitions are found in the 9000–12000- and 4000–5000-cm^{-1} regions of the CD spectrum, thereby demonstrating that the irons have coordination numbers of five and six, respectively (Reem & Solomon, 1984; Reem & Solomon, 1986). The binding of nonoxidizing small molecules to the ferrous site (to yield [($Fe^{II}Fe^{II}$–X^-]) has been studied by use of the resulting shifts in this group of CD bands. Magnetic circular dichroism (MCD) and electron paramagnetic resonance (EPR) studies of the deoxy form indicate a significant antiferromagnetic coupling of the irons, implying the presence of a μ-hydroxo bridge (Reem & Solomon, 1984).

Allosteric effects documented for hemerythrins fall into two groups: (i) *Homotropic interactions* in oxygen equilibria have been reported for hemerythrins from *L. unguis* (Manwell, 1960) and, more recently, *L. reevii* (Richardson et al., 1983). In the former case, unpurified extracts had a maximum Hill coefficient n_{max} of 1.6 for oxygen binding (pH 7.6 phosphate buffer). In the latter, the purified protein has an n_{max} value of 2.0 for pH 7.7, μ = 0.2 phosphate buffer. Purified sipunculid hemerythrins have shown no cooperativity in oxygen binding (Klippenstein, 1980). (ii) *Heterotropic interactions* are found in *Lingula* hemerythrin oxygen binding, where a Bohr effect is observed in the pH range 6–8 (Richardson et al., 1983; Manwell, 1960). This is similar to the "normal" Bohr effect observed for hemoglobin (Wyman, 1964), except n_{max} decreases to $\sim$1.0 at the lower pH values [this pH dependence of n_{max} is the Root effect (Perutz & Brunori, 1982)]. No Bohr effect is found for sipunculid Hr oxygen binding, but perchlorate and other oxyanions are heterotropic effectors for binding of oxygen and other exogenous ligands to *P. gouldii* hemerythrin (DePhillips, 1971; Darnall et al., 1968). Less pronounced effects of perchlorate have been noted for *Themiste zostericola* hemerythrin (Chadwick & Klippenstein, 1983). Variation in ligand binding properties upon covalent modifications of the protein side chains is not included in heterotropic effects considered here.

In this paper, we document some of the chemical and spectroscopic consequences of allosteric interactions in sipunculid and brachiopod hemerythrins. The effect of perchlorate and other possible heterotropic effectors on oxygen equilibria of *P. gouldii* hemerythrin has been reexamined. The cooperativity in oxygen equilibria for *L. reevii* hemerythrin is considered through application of current models for allosteric effects. Results of ligand binding experiments for *L. reevii* and *P. gouldii* deoxyHr are given and considered in relation to possible active site mechanisms for allosteric control of ligand affinity. Finally, these results for hemerythrin are compared to related allosteric effects found for hemoglobin and hemocyanin.

Experimental Procedures

Hemerythrins. *P. gouldii* specimens were obtained live from Marine Biological Laboratories (Woods Hole, MA), and the coelomic oxyhemerythrin was isolated and purified as described in the literature (Klotz et al., 1957). *P. gouldii* metazidohemerythrin was prepared by dialysis of oxyhemerythrin against 0.01 M sodium azide in 0.1 M tris(hydroxymethyl)-aminomethane (Tris)/sulfate buffer at the desired pH. Oxyhemerythrin samples were stored at liquid nitrogen temperature in buffer, and these samples did not autooxidize appreciably over several weeks. Alternatively, storage at 4 °C in the deoxy form was acceptable for a few days prior to oxygen binding experiments.

Oxyhemerythrin was isolated from *T. zostericola* (Venice Marine Supply) as described elsewhere (Klippenstein et al., 1972). It was stored in a freezer as the ammonium sulfate salt until use.

Live specimens of *L. reevii* (length 7–14 cm) were collected in 25 °C water at Kaneohe Bay, Oahu, and were shipped under oxygen. They survived in seawater for at least 3–4 days, and if provided with 3–4 in. of sand, they will feed and live for at least 10 days. Hemerythrin-containing red cells were obtained from the coelomic fluid as follows. The pedicle (stalk) of each animal was amputated at the base of the shells and allowed to drain into isotonic saline. Meanwhile, the body of the animal was bisected through the posterior quarter by inserting a blade between the shells. Without opening the shells and with slight pressure, the coelomic fluid was drained through cheesecloth into ice-cold isotonic saline. The combined cells of 50 animals were washed with saline several times, and the white cells were removed by differential centrifugation (at <100*g*). Some white cells could not be removed in this way, but the contamination was minimized. The packed red cells were lysed with 3–4 volumes of distilled water, followed by centrifugation at 1700*g* for 90 min. The wine red supernatant was dialyzed against phosphate buffer (μ = 0.2, pH 7.7) and then concentrated by ultrafiltration to 10–20 mg/mL. The concentrate was filtered (0.2 μm) and chromatographed on Sephacryl 200 (fine) or Bio-Gel A-0.5M, and fractions with constant A_{330}/A_{280} were concentrated and dialyzed against the appropriate phosphate buffer. Only low molecular weight contaminants were found, and these could also be removed by passing the crude protein through Sephadex G-50 (2.5 × 50 cm). The total yield was $\sim$2 mg of hemerythrin per animal, with some larger animals yielding >5 mg.

The *L. reevii* hemerythrin could be conveniently stored at 4 °C (in the deoxy form) or at liquid nitrogen temperature (in the oxy form). Under these conditions, met formation is very slow, and frozen samples are usable for several weeks. Solutions left at room temperature denature in a few hours, so all operations were done at 4 °C. Stability of the protein in Tris buffer was low, with denaturation occurring after several hours of dialysis at 4 °C. The experiments were therefore done in phosphate buffer.

Molecular Weight Studies. The molecular weight of native *L. reevii* hemerythrin was estimated by sedimentation equilibrium and gel chromatography. In the ultracentrifuge method, 3-mm sample columns were used (Van Holde & Bald-

win, 1958). Samples of *L. reevii* deoxyhemerythrin were loaded anaerobically into ultracentrifuge cells fitted with 12-mm centerpieces. FC-43 layering oil was used (no denaturation was evident at the end of runs). The equilibrium data were obtained on a Spinco Model E equipped with a photoelectric scanner. The scanning wavelength was selected to be appropriate to the concentration of protein (0.1–10 mg/mL). After equilibrium data were obtained at 8000 rpm, the cells were oxygenated, and the resulting oxyhemerythrin solutions were also equilibrated at 8000 rpm. For comparison, samples of *P. gouldii* hemerythrin were also checked. Molecular weights were estimated from the usual ln A vs. r^2 plots with the partial specific volume estimated as 0.73. In all experiments the buffer ionic strength was ≥0.1, and the temperature was 5 °C.

A calibrated 1.5 × 45 cm Sephacryl 200 column was also used to estimate hemerythrin molecular weights. The column was run at 4 °C with phosphate buffer (pH 7.7, μ = 0.1), and a 280-nm absorbance detector was used to monitor elution volumes.

Oxygen Binding Studies. Oxygen equilibrium curves for *P. gouldii* and *L. reevii* hemerythrin were obtained at 22–23 °C by spectrophotometric titration and with a glass tonometer. Oxyhemerythrin in the desired buffer was deoxygenated at ice temperature by repeated evacuation of the tonometer (to not less than 30 Torr) and backfilling with prepurified nitrogen. Oxygen was added by syringe in increments necessary to characterize the binding curves. Equilibration was accomplished by rotation of the tonometer, and oxygenation was usually followed by the absorbance change at 500 nm. Partial denaturation and turbidity were a problem with *P. gouldii* hemerythrin in Tris–sulfate buffer, so a tonometer equipped with an internal fritted glass filter was used. Despite the partial denaturation, the final spectrum was essentially unchanged from that of the original oxygenerythrin sample (the exception being in the 0.1 M perchlorate experiments, where autooxidation of ~3% occurs during the titration). *L. reevii* hemerythrin was sufficiently stable in phosphate buffer, and the small amount of denaturation that did occur in the course of the experiment did not interfere with the spectral absorbance.

DeoxyHr Ligand Binding Studies. DeoxyHr was prepared by anaerobic dialysis of oxy or metHr at ~0.5–1.0 mM against excess sodium dithionite. Aliquots were transferred anaerobically to a 1-cm cuvette that had been purged with nitrogen. The sealed cell was fixed into the sample compartment of the spectropolarimeter. Small volumes (10–50 μL) of concentrated sodium azide in buffer were added anaerobically through a septum on the cell. CD spectra were recorded in the region 700–1000 nm with three replicates. Because of the low intensity of the CD bands and susceptibility to artifacts in the spectra, the protein solution was stirred after each addition with the cell still fixed in the cell holder. Stirring was accomplished with a small magnetic stir bar. In addition, a slow purge of water-saturated nitrogen was maintained through the cell to prevent oxygenation of the protein. Saturation curves were derived from CD intensities at 890 nm, where maximum reproducibility was observed for the replicates. Spectral intensities were corrected for dilution, and saturation curves were calculated from $y = (\theta_{obsd} - \theta_{deoxy})/(\theta_{N_3^- deoxy} - \theta_{deoxy})$.

Hydroxide Binding to MetHr. The method of McCallum et al. (1984) was used to determine hydroxide binding curves for *L. reevii* metHr. MetHr was dialyzed against μ = 0.01 phosphate buffer to fix the initial pH (6.5 or 7.2) for hydroxide binding studies. Concentrated phosphate buffer solutions were added to aliquots of the protein stock solution to give μ = 0.2 solutions in the range pH 6.5–8.0. Each sample was allowed to equilibrate for 1 h at 5.0 °C before difference spectra were obtained. Values of ΔA were obtained from the absorbance differences at 349 and 328 nm, where the maximum absorbance changes occur. Values of K_f and n for the equilibrium

$$\text{metHr} + n\text{OH}^- \overset{K_{OH^-}}{\rightleftharpoons} \text{metHR(OH}^-)_n$$

were obtained by fitting the data to equations given by McCallum et al. (1984). The empirically determined value of n is the Hill coefficient for hydroxide binding.

Spectroscopy. UV–vis spectra were obtained on Cary 14 or Cary 17 spectrophotometers. Circular dichroism spectra were taken on a Jasco J-500C equipped with a UV–vis (S-20) or near-IR (S-1) detector to cover the range 200 to ~1000 nm. X-band EPR spectra were taken at 10 K with a Bruker ER200 instrument and an Air Products helium cyrostat.

Raman spectra were obtained with a spinning cell sample configuration and a Kr^+ or Ar^+ laser source. A Spex Model 1401 monochromator equipped with photon-counting detection was interfaced to a Nicolet 1280 computer used to collect and store data. Multiple scans were taken to improve the signal-to-noise ratio. Once calibrated, the wavelength drive is precise to within ±0.1 cm^{-1} for repeated scans.

RESULTS

Properties of L. reevii Hemerythrin. With the exception of a limited study of *L. unguis* Hr (Joshi & Sullivan, 1973), the physical and spectroscopic properties of brachiopod hemerythrin have not been examined. We have further characterized the chemistry and spectroscopy of *L. reevii* Hr to better compare the active sites of brachiopod and sipunculid Hrs.

Sedimentation equilibrium and sizing column experiments indicate that native *L. reevii* Hr is an octamer with molecular weight of ~105 000. In addition, this octameric structure is retained upon dilution to ~0.1 mg/mL. In contrast, *P.gouldii* Hr is substantially dissociated at this concentration (Langerman & Klotz, 1969). At pH values of 6.3 and 7.7, both the deoxy and oxy forms remain octameric. All molecular weight determinations were at 5 °C due to the room temperature instability of *L. reevii* Hr. One experiment at 20 °C did not indicate any change in molecular weight. Centrifugation under denaturing conditions (5 M urea) demonstrated that subunit molecular weights for *L. reevii* and *P. gouldii* Hr are similar (~13 500).

As found for sipunculid Hr, *L. reevii* oxyhemerythrin could be converted to the met form by oxidation with ferricyanide. The rapid autooxidation of oxyHr to metazidoHr in the presence of azide (Bradic et al., 1977) is also observed. The half-life for conversion in 0.05 M azide is ~25 min at pH 7.7. In addition, bleaching of the *L. reevii* oxy spectrum is observed upon addition of azide at higher pH values, as found for *P. gouldii* Hr (Bradic et al., 1977).

Unlike *P. gouldii* metHr (Bradic & Wilkins, 1983; Darnall et al., 1968), however, the aquo–hydroxy equilibrium for *L. reevii* Hr does not shift in the presence of perchlorate. This was checked in Tris/sulfate buffer (pH 7.7), where a mixture of hydroxy and aquo species is observed in the UV–vis spectrum. It is possible that the specific binding sites for perchlorate observed in the sipunculid octamers are not present in *L. reevii* Hr, as also seems to be the case for myohemerythrin from *T. zostericola* Hr (Chadwick & Klippenstein, 1983).

The UV–vis absorption and circular dichroism spectra of oxy and met-N_3^- *L. reevii* Hr are presented in parts A and

Table I: UV–Vis and CD Band Maxima and Intensities for Hemerythrins from Sipunculids and Brachiopods

Hr from		optical λ_{max} (nm) (ϵ [M^{-1} cm^{-1}])	CD λ (nm) ($\Delta\epsilon_{max}$)
L. reevii[a]	oxy	507 (2100)	795 (−0.40)
		332 (6000)	518 (−3.50)
		280 (28500)	430 (−1.26)
			340 (−5.3)
	azidomet	448 (3900)	630 (+0.35)
		329 (6900)	500 (−5.2)
			367 (−12.0)
	deoxy		1070 (+0.4)
	azidodeoxy		955 (0.68)
P. gouldii	oxy[b]	500 (2300)	791 (−0.36)[c]
		326 (6900)	520 (−2.46)
		280 (35400)	336 (−3.78)
	azidomet[b]	446 (3800)	500 (−4.09)
		327 (7200)	370 (−9.02)
	deoxy[c]		1070 (+0.44)
	azidodeoxy[c]		955 (0.68)

[a] All spectral data this work. All intensities are based on the concentration of iron dimers (2 Fe subunits). [b] Dunn et al. (1977). [c] This work.

B of Figure 1 and summarized in Table I. Small shifts in band maxima are observed in comparison to the sipunculid hemerythrins, but the overall characteristics of the spectra are clearly retained. The most noticeable difference in the UV–vis spectrum occurs in the peroxide-to-Fe^{III} charge-transfer band with a maximum found at 500 nm for *P. gouldii* oxyHr (Gay & Solomon, 1978), which is shifted to 507 nm in *L. reevii* oxyHr. Also, the protein absorbance at 280 nm is less intense in *L. reevii* Hr when compared to *P. gouldii* and *T. zostericola* Hr. An amino acid analysis (Joshi & Sullivan, 1973) shows fewer tyrosines in *L. unguis* Hr than in *P. gouldii* Hr and no tryptophan. The low 280-nm intensity in *L. reevii* Hr is consistent with this observation, but the shape of the UV band (shoulder at 292 nm) clearly reflects the presence of tryptophan.

CD spectra of deoxyHr and deoxy-N_3^--Hr for the 700–1100-nm range are given in Figure 1C and included Table I. Counterparts of these bands have been observed in the optical spectrum (Loehr et al., 1980), but they are much better resolved in the CD. The deoxyHr ligand field bands at λ_{max} 1070 nm for *P. gouldii* and *L. reevii* are essentially identical, indicating both contain very similar active sites, with one five- and one six-coordinate high-spin iron(II). The 700–1100-nm band system contains contributions from both five- and six-coordinate irons, so any change in coordination numbers for iron in *L. reevii* Hr would likely appear as a significant change in the spectral features in that range.

The resonance Raman spectrum of *L. reevii* oxyHr is shown in Figure 2. The intra-peroxide and Fe–O modes are observed at 844 and 505 cm^{-1}, respectively, frequencies virtually identical with those of *P. gouldii* Hr (Richardson et al., 1983; Dunn et al., 1977, 1973). The weaker band at ~760 cm^{-1} is also seen in both the *L. reevii* and *P. gouldii* spectra, and has been assigned to an asymmetric Fe–O–Fe stretch (Shiemke et al., 1984).

The 844-cm^{-1} band of oxyHr was examined in some detail under various pH conditions (μ = 0.2 phosphate buffer). In *L. reevii* Hr, this frequency was found to be pH sensitive, shifting about 1.5 cm^{-1} to higher frequency when the pH is lowered from 7.7 to 6.3 (Figure 2b). An attempt was made to produce a correlation of band shape vs. pH, but the poor Raman scattering (at 2-cm^{-1} resolution) and laser sensitivity of the sample made this difficult to do accurately. However, it does appear that both bands are present in roughly equal

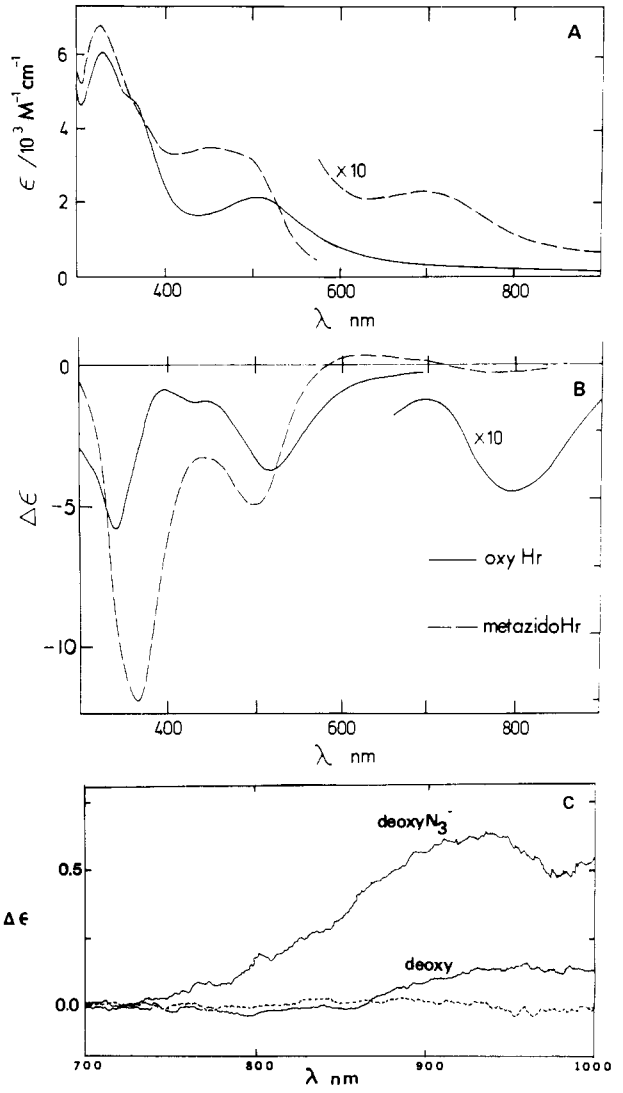


FIGURE 1: UV–vis absorbance (A) and circular dichroism (B) spectra of *L. reevii* oxyHr (solid line) and metazidoHr (dashed line). (C) Near-IR spectra of *L. reevii* deoxyHr and deoxyazidoHr (dashed line is base line). All spectra taken with protein solutions at pH 7.7 (μ = 0.2 phosphate).

amounts around pH 6.8–7.0. In contrast, we found the 844-cm^{-1} band of *P. gouldii* oxyHr to be insensitive (±0.3 cm^{-1}) to pH in this same pH range. These observations are consistent with the pH dependence of O_2 binding found in *L. reevii* Hr but absent in *P. gouldii* Hr (vide infra).

For *P. gouldii* Hr a paramagnetic mixed-valent [$Fe^{III}Fe^{II}$] form can be obtained that is EPR detectable (Harrington et al., 1978, 1981). As a further spectroscopic comparison, we have studied this half-met (or semi-met) form of *L. reevii* Hr for spectral comparison to the sipunculid half-met EPR signals (Muhoberac et al., 1980). For *P. gouldii*, different half-mets are obtained from oxidation of deoxy ([half-met]$_O$: g = 1.93, 1.70, and 1.67) and reduction of met ([half-met]$_R$: g = 1.95, 1.87, and 1.63). *L. reevii* deoxyHr was prepared by anaerobic reduction of met/oxyHr by dithionite followed by extensive anaerobic dialysis against the desired buffer. If ≤1 equiv of ferricyanide is added followed by rapid freezing, a [half-met]$_O$ + [half-met]$_R$ mixture is obtained rather than the "pure" [half-met]$_O$ result from *P. gouldii* (Bradic et al., 1980; Muhoberac et al., 1980). A typical spectrum is shown in Figure 3A. If the solution is allowed to stand at room temperature, a rapid approach to equilibrium occurs ($t_{1/2}$ ~ 10 min), whereupon most of the half-met signal is of the [half-met]$_R$

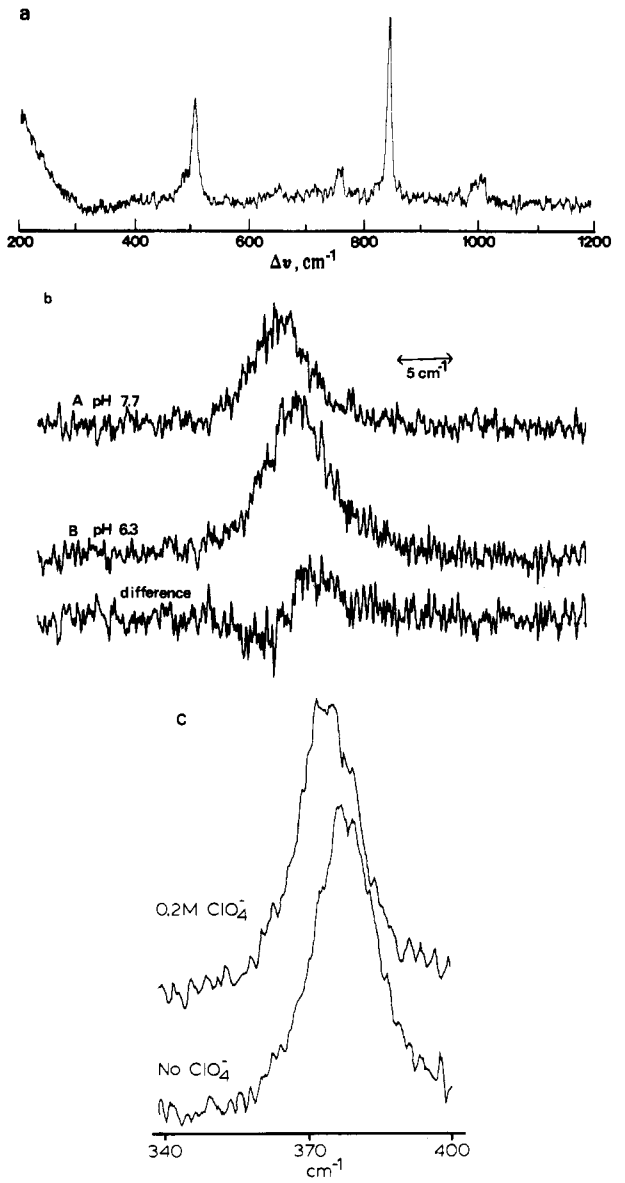


FIGURE 2: (a) Resonance Raman spectra of *L. reevii* oxyHr: spinning cell sampling; pH 7.7, $\mu = 0.2$ phosphate buffer; excitation line 530.9 nm, 60 mW. (b) Effect of pH on peroxide stretching frequency at ~844 cm^{-1} for *L. reevii* oxyHr. Spectra for pH 7.7, pH 6.3, and difference spectrum 0.75b–a are shown. (c) Effect of ClO_4^- on M–N_3^- stretching frequency in *P. gouldii* met-N_3^--Hr. Protein concentration 1.25 mM with no ClO_4^- and 0.25 M ClO_4^- showing a 4-cm^{-1} shift in 376-cm^{-1} band. Excitation line 501.7 nm, 60 mW.

Table II: Effective *g* Values for [Half-Met] Hemerythrins[a]

	$[\text{half-met}]_O$	$[\text{half-met}]_R$	$[\text{half-met}]-N_3^-$
L. reevii[b]	1.95, 1.69, 1.66	1.95, 1.86, 1.67	1.93, 1.85, 1.58
P. gouldii[b]	1.93, 1.70, 1.67	1.95, 1.87, 1.63	1.90, 1.82, 1.50
T. zostericola[c]	1.95, 1.72, 1.68	1.96, 1.88, 1.67	1.94, 1.85, 1.57

[a] *g* values ±0.01. [b] This work. [c] Muhoberac et al. (1980)

type (Figure 3B). Addition of sodium azide results in a rapid color change and an EPR spectrum expected for a [half-met–N_3^-] adduct (Figure 3C) (Muhoberac et al., 1980). The azide adduct of *L. reevii* half-met is quite stable at room temperature, with a significant fraction still intact after 4 days (Figure 3D). The observed *g* values for the two O + R components and the azide adduct can be estimated and are listed in Table II, along with those for half-met derivatives of two sipunculid coelomic Hrs. There is obviously a substantial similarity in the *g* values of the octameric half-met derivatives, but the rapid O ⇄ R equilibrium is reminiscent of that found

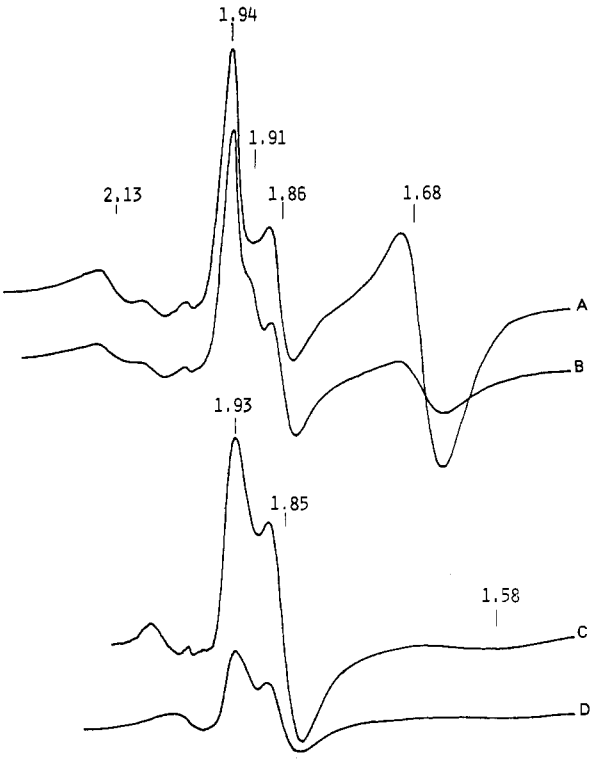


FIGURE 3: X-band EPR spectra of half-metHr at 10 K derived from partial oxidation of *L. reevii* deoxyHr by ferricyanide. Effective *g* values are given in the figure. (A) Spectrum taken 1 min after addition of 1 equiv of ferricyanide; (B) spectrum taken 15 min after addition; (C) spectrum of half-metHr–N_3^- in 0.01 M NaN_3; (D) spectrum of sample C after 4 days at room temperature in a sealed EPR tube.

for myohemerythrin, a monomeric protein (Harrington et al., 1981).

The spectroscopic data presented in this section show that the principal features of the non-heme binuclear iron active site in sipunculid hemerythrins are retained in brachiopod coelomic Hr. Octameric quaternary structure is also retained, and the absence of dissociation at high dilution indicates stronger intersubunit bonding than found in octameric *P. gouldii* Hr.

Oxygen Equilibria of P. gouldii and L. reevii Coelomic Hemerythrins. DePhillips (1971) has reported an unusual effect of perchorate on the oxygen binding curve for *P. gouldii* hemerythrin at pH 7.1. It was reported that when the ClO_4^-:Fe ratio is ~1, a Hill plot of the oxygen binding data showed regions of cooperative ($n = 3.8$) and anticooperative ($n = 0.6$) slopes. The effect also included a decrease of $p_{1/2}$ until ClO_4^-:Fe is >2, whereupon the $p_{1/2}$ becomes higher than in the absence of perchlorate. In addition, the effects described were not found at pH 8.5.

Our initial attempts to reproduce the results of DePhillips for *P. gouldii* Hr by the reported procedure failed due to substantial denaturation of the protein during equilibration with the gas phase at 23 °C. Using the method described under Experimental Procedure, we were able to maintain clear solutions throughout the titrations. When partial denaturation occurred, turbidity was removed with a fritted glass filter built into the tonometer. The small amount of turbidity encountered resulted from relatively insignificant denaturation (~1%).

Oxygen binding curves for *P. gouldii* Hr are shown in Figure 4 and summarized in Table III for pH 7.1 Tris/sulfate buffer and varying amounts of perchlorate. Under all conditions of Fe:ClO_4^-, the $p_{1/2}$ value is found to be *higher* than found with no ClO_4^- present, and no significant deviation from a slope of 1.0 is found in the region $0.10 < y < 0.9$. Addition of small

Table III: Effect of Perchlorate on Oxygen Equilibria of *P. gouldii* Hemerythrin[a]

$[ClO_4]$:[Fe][b]	$p_{1/2}$ (mmHg), pH 7.13	$p_{1/2}$ (mmHg), pH 8.75
0	3.5	4.0
1:1	5.6	
22:1	6.6	
0.1 M ClO_4^-	7.6 ± 0.2	8.5

[a] At 23 °C. Hill coefficients are all 1.1 ± 0.1. The buffer is 0.1 M Tris/SO_4^{2-}. [b] The Hr octamer concentration was ~50 μM in all experiments, or ~1 mM in Fe.

aliquots of perchlorate to a partially oxygenated Hr solution only *reduced* the degree of oxygenation. Similar results were found at pH 8.75, and all data are summarized in Table III. A recent study of *T. zostericola* Hr by Chadwick and Klippenstein (1983) reports that ClO_4^- causes effects similar to those we observed for *P. gouldii* Hr. A kinetic study (Dewaal & Wilkins, 1976) also found a perchlorate effect and lack of cooperativity in O_2 binding.

Addition of the heterotropic effector ClO_4^- also has an effect on the metHr derivatives. Resonance Raman spectra of met-N_3^--Hr (Figure 2c) show that upon binding of perchlorate the metal–azide stretch moves 4 cm^{-1} to lower energy (to 372 cm^{-1}), while the iron–oxo stretch moves 1.5 cm^{-1} to higher energy (to 511 cm^{-1}). The intra-azide stretch is not affected. This indicates a direct structural effect at the binuclear ferric active site. The optical spectrum of azidometHr is also affected by added perchlorates (Garbett et al., 1971).

The effect of Ca^{2+} and NaCl on the oxygen equilibria of hemocyanin, the copper oxygen carrier, is well-known; these components of seawater can cause significant shifts in $p_{1/2}$ values and Hill coefficients (Brouwer et al., 1978). We have examined the effect of these ions on O_2 binding to octameric *T. zostericola* Hr. Our studies were motivated by a kinetic investigation of O_2 binding to *T. zostericola* Hr (Petrou et al., 1981). It was reported that the on rate for O_2 was somewhat influenced by Ca^{2+}, Mg^{2+}, and NaCl, while the off rate was not changed. This was not regarded as significant, however, and our O_2 binding curves do not show any significant shifts in $p_{1/2}$ or n in the presence of these ions (Table IV).

Two cases of apparent homotropic allosteric effects in the binding of O_2 to sipunculid Hr in the coelomic fluid "red cells" have been reported (Wells, 1982; Mangum & Kondon, 1975). The blood pigment of *P. gouldii* is reported to bind O_2 in two distinct phases, one with $n \simeq 3.2$ [$p(O_2) < 6$ mm] at 23 °C.

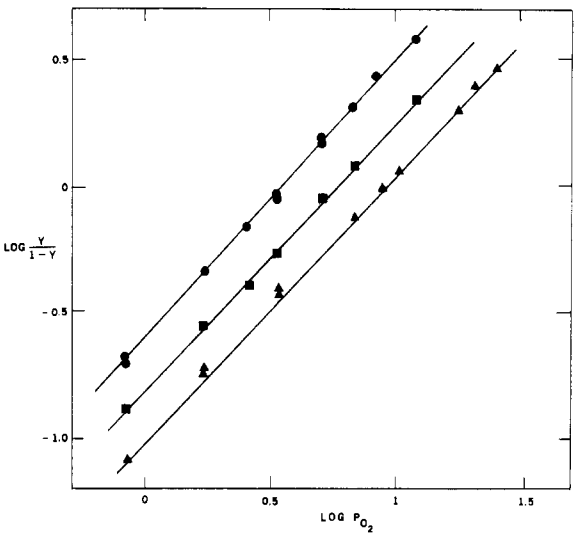


FIGURE 4: Effect of perchlorate on oxygen binding by *P. gouldii* Hr, in 0.2 M Tris/sulfate, pH 7.1, at 23 °C: (●) no ClO_4^-, (■) ClO_4^-:Fe = 1, and (▲) 0.1 M ClO_4^-. Hr concentration 0.5 mM in subunits.

Wells (1982) reports that *Xerosyitron mundanus* coelomic fluid binds O_2 with $n_{max} = 1.8$. No Hill plots were presented in the latter report, but it was stated that the Bohr effect was "normal" in the range 6.8–7.7 and reversed at pH <6.5. Although these results would appear to imply the presence of an effector for homotropic interactions in cellular Hr, no conditions have thus far been found to induce cooperativitiy in purified coelomic hemerythrin from sipunuclids. It is noteworthy that the effect of ClO_4^- is to lower O_2 affinity of purified sipunculid Hr for O_2 (Figure 4). The same trend is observed for Cl^-, 2,3-DPG, and other heterotropic effectors with hemoglobin. Thus, ClO_4^- introduces a "constraint" on O_2 binding, a fundamental feature of cooperative effects (Monod et al., 1965). It is possible that an unidentified effector can induce the structural changes and subunit interactions necessary for cooperativity in *P. gouldii* Hr. The O_2 affinity of cellular Hr is about half that of purified "stripped" protein, again suggesting structural constraint.

The report of cooperativity in oxygen binding by *L. unguis* Hr (Manwell, 1960) led us to examine the oxygen equilibria of *L. reevii* Hr. The data points are presented in Figure 5 and are summarized in Table IV. The O_2 binding is indeed cooperative, and the maximum Hill coefficient ($n_{max} = 2.0$ at pH 7.7) is higher than that deduced from replotting the pH

Table IV: Oxygen Equilibria of Coelomic Hemerythrins[a]

Hr	pH	$p_{1/2}$ (mmHg)	n_{max}	buffer conditions	ref
L. reevii	6.3	14.1	1.1	μ = 0.2 phosphate	this work
	6.8	15.0	1.1		
	7.5	10.2	1.8		
	7.7	7.6	2.0		
	8.0	5.1	1.8		
L. unguis	7.6	8.0	1.6	phosphate	*b*
	6.7–6.8	16.0	1.0–1.1		
P. gouldii	7.1	3.5	1.0 ± 0.1	0.1 M Tris/SO_4^{2-}	this work
	8.8	4.0	1.1 ± 0.1	0.1 M Tris/SO_4^{2-}	this work
	7.0–8.2	3.1	0.9 ± 0.1	μ = 0.2 phosphate	*c*
	7.7	3.3	1.1	μ = 0.2 phosphate	this work
T. zostericola	5.8–7.6	3.5	1.2–1.3	phosphate	this work
	8.2	4.8	1.2 ± 0.1	0.03 M Tris/SO_4^{2-} (μ = 0.1 Na_2SO_4)	this work
	8.2	4.8	1.1 ± 0.1	0.03 M Tris/SO_4^{2-} (0.5 M NaCl, 0.01 M Ca^{2+}, μ = 0.55)	this work

[a] At 22 °C unless otherwise noted. [b] Manwell, 1960. [c] Magnum & Condon, 1975.

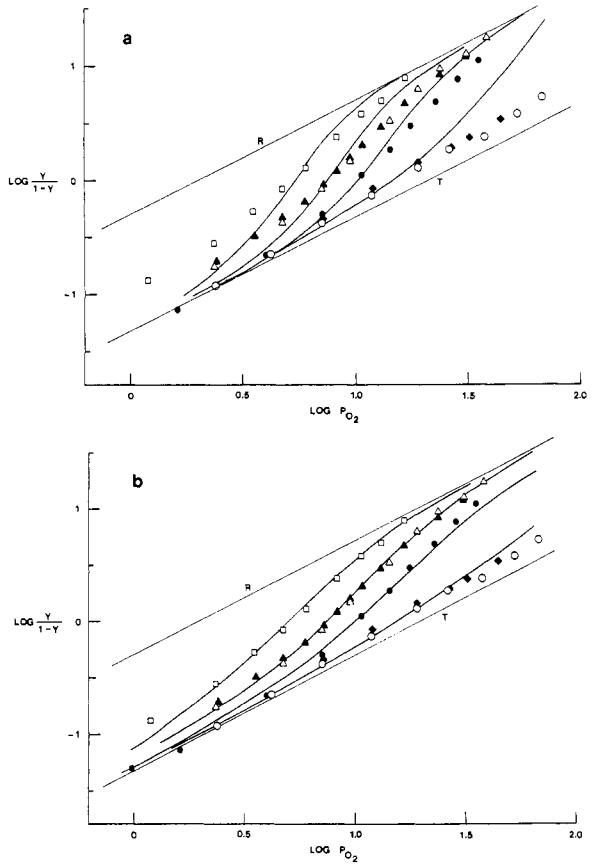


FIGURE 5: (a) Oxygen equilibria for *L. reevii* oxygen binding. Data shown for pH 6.3–6.8 (○, ◆), pH 7.5 (●), pH 7.7 (△, ▲), and pH 8.0 (□), μ = 0.2 phosphate buffer and temperature 22 °C. An attempted fit of the oxygen binding curves with the two-state allosteric model is shown with the lines. Parameters: c = 0.1, log L' = 6.4 (pH 6.3–6.8), 5.1 (pH 7.5), 4.4 (pH 7.7), and 3.5 (pH 8.0). Limiting binding curves for relaxed (R) and tense (T) forms are indicated. (b) Fit of *L. reevii* oxygen binding curves using the hybrid three-state allosteric model (eq 7). Parameters: c = 0.1, q = 2.1, log L' = 7.0 (pH 6.3–6.8), 5.4 (pH 7.5), 4.44 (pH 7.7), and 3.3 (pH 8.0).

7.6 data of Manwell (n_{max} = 1.6). At pH values less than 6.2, the protein is unstable, so data were obtained with phosphate buffer in the pH range 6.3–8.0. Changing to borate buffer modifies the curves at a given pH (for example, the pH 8.5 borate curve is about the same as that for pH 7.7 phosphate buffer). Evidently, the nature of the buffer affects the equilibrium to a significant degree, but a systematic examination of heterotropic effectors was not undertaken. The effect of protein concentration was examined at pH 7.7 over a limited range (2–5 mg/mL), and no change was observed in $p_{1/2}$ or n_{max}.

Ligand Binding to L. reevii and P. gouldii Deoxyhemerythrin. The ligand binding properties of deoxyHr have not previously been studied in detail. We found that the near-infrared ligand field transitions of deoxyHr can be observed easily for *P. gouldii* in the 700–1100-nm region of the CD spectrum (Reem & Solomon, 1984). Upon addition of certain anions (N_3^-, OCN^-), large changes are seen in these Fe(II) d–d bands, and azide and cyanate are each found to bind with binding constants of $\sim 10^2$ M^{-1} at pH 7.7. This is consistent with an earlier study by Wilkins and co-workers (Bradic et al., 1977). Table V contains these kinetically derived binding constants as well as our equilibrium binding constants. It is clear that these values are in agreement, thereby supporting the mechanism of anation suggested by Bradic et al. (1977) for the process oxyHr + X^- → metHrX^-, which involves a binuclear ferrous intermediate.

Table V: Azide Binding to DeoxyHr from *L. reevii* and *P. gouldii*

source	pH	$K_{N_3^-}$	n	ref
L. reevii	7.7	2.9×10^2	1.0 ± 0.3	this work
P. gouldii	6.5	1.0×10^3	1.1 ± 0.1	this work
	7.7	1.3×10^2	1.1 ± 0.1	this work
	6.3	1.6×10^3		*a*
	5.3	1.9×10^4		*a*

[a] Bradic et al., 1977. These values of $K_{N_3^-}$ were derived from the kinetics of converting oxyhemerythrin to metazidohemerythrin in the presence of azide.

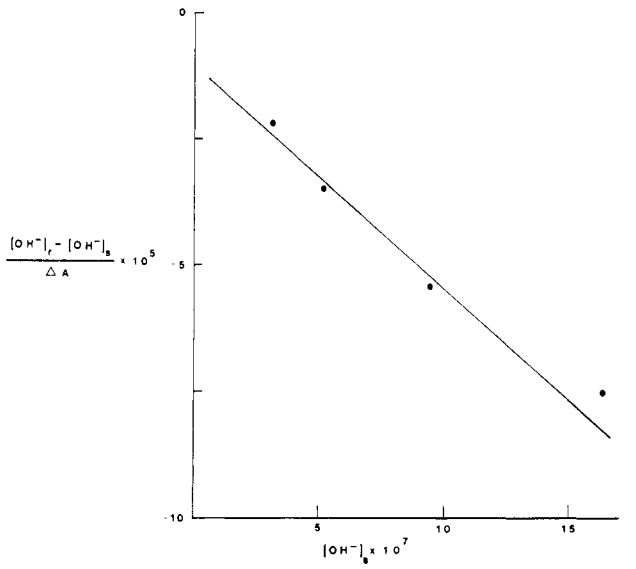


FIGURE 6: Plot for OH^- binding to *L. reevii* metHr in μ = 0.2 phosphate buffer using the absorbance difference method of McCallum et al. (1984). The subscripts s and r refer to sample and reference cells, respectively. The line was produced for n = 1.0.

L. reevii deoxyHr also has CD bands (Figure 1C) with shapes, energies, and intensities similar to those in *P. gouldii*. These are used here to probe for possibly cooperative effects in exogenous ligand binding to deoxyHr. The saturation curves for N_3^- binding to deoxyHrs from *P. gouldii* and *L.reevii* have been examined in some detail. Hill plots for N_3^- binding to *L. reevii* and *P. gouldii* deoxyHrs show only minor, insignificant deviations from n = 1.0, and the binding constants at 23 °C are summarized in Table V.

Hydroxide Binding to L. reevii MetHr. On the basis of X-ray crystallographic results, the active site for metHr is believed to have a five-coordinate iron(III) center where ligand binding occurs producing a six-coordinate center (Stenkamp et al., 1985). The binding of OH^- to metHr from *P. gouldii* was investigated with a difference method by McCallum et al. (1984). From the observed binding constant for OH^-, K_{OH^-}, they were able to calculate the effective pK_a for the active site.

We have studied OH^- binding to metHr from *L. reevii*, which on the basis of the electronic spectra appears to have an active site similar to other metHrs (Richardson et al., 1983). Difference spectra relative to the pH 6.5 or 7.2 spectrum were taken, and with the published equations (McCallum et al., 1984), the values of K_{OH^-} and n, the Hill coefficient for OH^- binding, could be deduced. The small amount of protein available limited the number of experiments and protein concentrations, but reasonably good data were obtained (Figure 6). Least-squares analysis of the data did not yield a unique value for n, but constraints on the possible value of K_{OH^-} led to constraints on n. A plot of ΔA vs. pH required that the effective pK_a of metHr be in the range ~7.0–7.5 (the region of maximum slope). Fits using n values of $0.8 < n < 1.2$ led to pK_a values acceptably close to this range, and when

n was set at 1.0, the effective pK_a is found to be 7.2 ± 0.3. Therefore, no substantial cooperativity is observed in OH^- binding to *L. reevii* metHr.

Application of the Conformational Models to Oxygen Binding. The cooperativity in oxygen binding to *L. reevii* Hr led us to apply the conformational model for allosteric effects (Monod et al., 1965) in order to identify the nature of the quaternary structure under various pH conditions. A fit of this type can predict conditions under which different allosteric states may be spectroscopically studied and provides an estimate for the amount of allosteric energy expended in oxygen binding. An attempt was first made to fit the oxygen binding curves for *L. reevii* Hr within the framework of the pure two-state conformational model. Examination of the binding data (Figure 5) shows that two limiting lines of unity slope at $p_{1/2} \sim 2$ and 20 mm can be drawn to encompass the data at high and low pH. These then define the affinities of the relaxed (R) and tense (T) forms, respectively. The parameter c [$=p_{1/2}(R)/p_{1/2}(T)$] is therefore ~0.1. The Hill plots are calculated with eq 1, where $\bar{Y}$ is the fractional oxygen saturation, α equals $p_{O_2}/p_{1/2}(R)$, and L' is the apparent allosteric equilibrium constant, given by

$$\bar{Y} = \frac{\alpha(1+\alpha)^7 + L'\alpha c(1+\alpha c)^7}{(1+\alpha)^8 + L'(1+\alpha c)^8} \quad (1)$$

$$L' = \frac{[T]_t}{[R]_t} = \frac{\alpha_{1/2} - 1}{1 - \alpha_{1/2}c}\left(\frac{1+\alpha_{1/2}}{1+c\alpha_{1/2}}\right)^7 \quad (2)$$

where $\alpha_{1/2}$ is the oxygen concentration required for half-saturation [$p_{1/2}/p_{1/2}(R)$] (Rubin & Changeux, 1966) and the subscript t indicates the total of all T and R conformations. A fit to the experimental data is shown in Figure 5a. The two-state approximation is obviously inadequate to fit the binding curve, and a more flexible model must be used.

The flattened nature of the Hill plots when compared to predicted curves of the two-state model is indicative of a larger number of conformational (allosteric) states available in the octamer. This would be consistent with one or more states of intermediate "tension", for example. We have used one possible model for an intermediate state (or hybrid) based on the supposition that a similar quaternary structure is found for brachiopod octameric Hr as for *T. zostericola* Hr (Stenkamp et al., 1985). In this structure, symmetry defines two types of subunit interactions, one between four subunits of a tetramer and the other between the "upper and lower" tetramers. Since these two contacts involve different interactions, it is reasonable to expect some degree of independence between the two halves of the octamer. The hybrid state proposed is therefore R_4T_4, which is related to the fully tense (T_8) and fully relaxed (R_8) quaternary structure by the equilibrium

$$R_8 + T_8 \overset{K}{\rightleftharpoons} 2R_4T_4 \quad (3)$$

If there were no interaction between the tetrameric halves, the population of the hybrid state would be statistical, and $K = 4$.

We describe the equilibria between T_8, R_8, and R_4T_4 further using

$$T_8 \rightleftarrows R_4T_4 \qquad 2/(qH') = [R_4T_4]/[T_8] \quad (4)$$

$$R_8 \rightleftarrows R_4T_4 \qquad 2H' = [R_4T_4]/[R_8] \quad (5)$$

$$R_8 \rightleftarrows T_8 \qquad L' = [T_8]/[R_8] \quad (6)$$

If $K = 4$, then the parameter $q = 1$, and a value of $q \neq 1$ indicates a coupling between the allosteric equilibria of the two tetramers. The hybrid model can be incorporated into the conformational model to produce a "three-state" model. Similar approaches have been used to fit oxygen equilibria of hemocyanins and hemoglobins (Edelstein, 1975; Brouwer et al., 1978). Following the derivation give by Brouwer et al. (1978), the saturation and state functions are

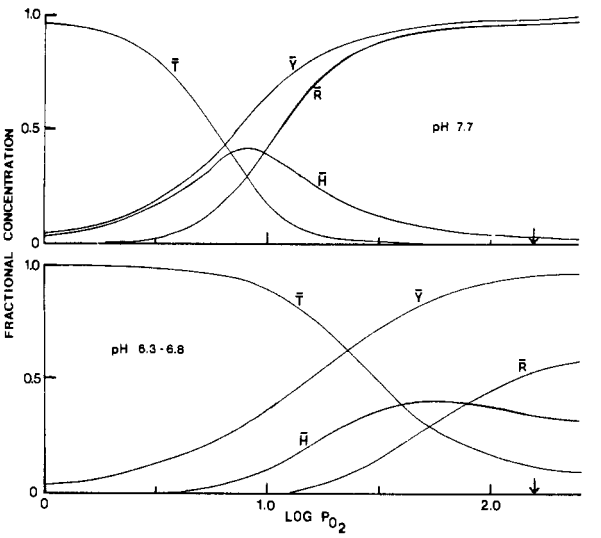


FIGURE 7: The state functions $\bar{R}$, $\bar{T}$, and $\bar{H}$ (from eq 8–10) for pH 7.7 (top) and pH 6.3–6.8 (bottom). Model parameters are given in Figure 5b caption. Small arrows indicate the partial pressure of O_2 in air. The state functions give the fractional population of the relaxed, tense, and hybrid forms at various values of O_2 partial pressure. The fractional saturation of the oxygen-binding sites is given by the function $\bar{Y}$.

$$\bar{Y} = (\alpha/W)\{(1+\alpha)^7 + L'c(1+\alpha c)^7 + (L'/q)^{1/2}[(1+\alpha)^3(1+\alpha c)^4 + c(1+\alpha c)^3(1+\alpha)^4]\} \quad (7)$$

$$W = (1+\alpha)^8 + L'(1+\alpha c)^8 + 2(L'/q)^{1/2}[(1+\alpha)^4(1+\alpha c)^4]$$

$$\bar{R} = (1+\alpha)^8/W \quad (8)$$

$$\bar{T} = L'(1+c\alpha)^8/W \quad (9)$$

$$\bar{H} = 2(L'/q)^{1/2}(1+\alpha)^4(1+c\alpha)^4/W \quad (10)$$

Equations 8–10 are the state functions that give the fractional population of relaxed $\bar{R}$, tense $\bar{T}$, and hybrid $\bar{H}$ forms at a given value of α. A value of $q = 2.1$ was derived by the method of Buc et al. (1973). The fit of the three-state model with this q value and L' values required to fit the observed $p_{1/2}$ values is shown in Figure 5b. A clear improvement is seen over the two-state model, but deviations from the experimental points are still observed. The model analysis indicates that the octameric *L. reevii* Hr oxygen binding can be approximately represented by a two-tetramer model with a $-RT \ln q$ (0.45 kcal/mol) interaction between tetrameric halves. The total allosteric energy (given by the model values for $\Delta G_{O_2}{}^R - \Delta G_{O_2}{}^T = RT \ln c$) is about 1.4 kcal mol^{-1}.

State functions (eq 8–10) for the concentrations of [T_8], [R_8], and [R_4T_4] quaternary structures derived from the three-state model are shown in Figure 7 along with the saturation function $\bar{Y}$. At pH values of maximum cooperativity (7.7 ± 0.2), the unliganded form is predominantly tense (T_8) while the oxygenated form is relaxed (R_8). At lower pH values (6.3–6.8), where the O_2 binding is virtually noncooperative, the state functions show a substantial contribution of the tense forms (R_4T_4 and T_8) to the oxygenated quaternary structure. The observed pH dependence of the O–O stretching frequency in the resonance Raman spectrum of *L. reevii* oxyHr can then

be correlated with increased population of the T state at lower pH values.

Discussion

Comparison of Sipunculid and Brachiopod Hemerythrins. The spectroscopic comparison of hemerythrins from sipunculids and brachiopods indicates that the coupled binuclear iron site is essentially the same in both proteins (Richardson et al., 1983). In addition, the octamer molecular weights and Stokes radii are very close as determined by sedimentation equilibrium and gel chromatography. We can therefore use available structural data for sipunuclid hemerythrins to discuss the general active site features of the cooperative Hr from *L. reevii*, since it is probable that the ligands at the active site are retained. We ascribe the differences in O_2 binding behavior of the purified octamers from sipunculids and brachiopods to structural differences in the intersubunit contacts. In the cooperative case, the affinity of O_2 binding is linked to conformational changes in the octameric quaternary structure.

Mechanism of Allosteric Effects. The results for *L. reevii* Hr reported here demonstrate the allosteric control can operate in a binuclear metallic site where O_2 binding is accompanied by a large change in valency. To examine the mechanism of cooperativity more closely, we can divide the oxygenation reaction for Hr into two thermodynamic steps (at pH 6–9):

$$[Fe^{II}Fe^{II}\text{–}H^+] + O_2 \overset{K_{11}}{\rightleftharpoons} [Fe^{III}Fe^{II}] + HO_2^- \quad (11)$$

$$[Fe^{III}Fe^{III}] + HO_2^- \overset{K_{12}}{\rightleftharpoons} [Fe^{III}Fe^{III}\text{–}O_2H^-] \quad (12)$$

In this scheme, the bound peroxide is shown as protonated (Shiemke et al., 1986). The proton in eq 11 apparently is derived from an intraprotein acidic site in *P. gouldii* Hr, likely the OH^- bridge in deoxyHr, as no pH dependence is found for oxygen binding. In *L. reevii* Hr, the Bohr effect requires exchange of protons with solvent upon oxygenation. These will most likely be from secondary sites not directly involved in the oxygenation. The oxygen affinity is given by $K_{O_2} = K_{11}K_{12}$. Note that eq 11 and 12 represent a convenient thermodynamic separation of the oxygenation process and do not imply a molecular mechanism. We then examine features of (11) and (12) that are subject to allosteric control.

The first step is a redox change, and K_{11} is subject to change via a shift in the $E°$ of the iron dimer:

$$[Fe^{III}Fe^{III}] + 2e^- \rightleftarrows [Fe^{II}Fe^{II}]\ (E°_{Hr}) \quad (13)$$

The reduction potential $E°_{Hr}$ has been determined to be 0.23 V (vs. NHE) for *T. zostericola* Hr (Armstrong et al., 1983), which has a $p_{1/2}$ for O_2 binding (Table IV) close to that of the relaxed form of *L. reevii* Hr. The 1.4 kcal/mol allosteric energy found for oxygen binding to *L. reevii* Hr would be equivalent to a change of $E° = 30$ mV for the $2e^-$ reduction potential (eq 13) in the relaxed–tense conversion. Variations in reduction potentials of this magnitude would certainly be possible within the active site. The most direct way to shift reduction potentials of metal centers is to change the coordination environment by ligand substitution. However, no such drastic change in the ligand environment occurs in the Hr active site upon oxygenation (Stenkamp et al., 1985). Smaller shifts in $E°$ can be achieved with subtle changes in the inner coordination sphere. Obvious examples occur in the macrocyclic complexes. For comparative purposes, a metal redox couple should be selected where the reduction involves addition of an electron into the πd orbital, as occurs in high-spin Fe^{II}/Fe^{III}. Appropriate data can be found for macrocyclic Ru^{II}/Ru^{III} complexes (Walker & Taube, 1981). Variation of a tetraza macrocycle in size from 14 to 16 members changes the redox potentials 55–70 mV, depending on the nature of the axial ligands. Solvation effects will contribute to these shifts in $E°$, but it is clear that the requisite allosteric shift in $E°$ of 30 mV is attainable with relatively subtle changes in the ligand environment at a high-spin iron active site.

The second step (12) involves the binding of peroxide to the met site. Adduct formation at met sites (reaction 14) might

$$[Fe^{III}Fe^{III}] + X^- \rightarrow [Fe^{III}Fe^{III}\text{–}X^-] \quad (14)$$

be considered models for reaction 12. As a model for ligand binding to *L. reevii* metHr, we have studied the equilibrium

$$\text{metHr} + OH^- \rightleftarrows \text{metHr}(OH^-)$$

and find no evidence for cooperativity in this reaction, as also found for *T. dyscritum* metHr (McCallum et al., 1984) and *P. gouldii* metHr (Gorman & Darnall, 1981). If reactions of type 14 are generally noncooperative for *L. reevii* Hr, then it is strongly implied that the O_2 affinity along the binding curve is coupled to variation of the redox potentials of the binuclear iron sites (reaction 11) rather than structural alterations that occur upon ligand binding to the met states. In other words, the relaxed (R) and tense (T) forms would have reduction potentials differing by about 30 mV. A useful analogy for this point is found in Co(II) complexes, which oxidatively bind O_2 to form stable peroxo complexes (McLendon & Martell, 1976; Neiderhoffer et al., 1984). In these reversible cobalt oxygen carriers, the value of K_{O_2} depends systematically on the value of $E°$ for the cobalt complex. We also note that the Raman data show that the Fe–O frequency shifts only slightly (<1 cm^{-1}) in the R–T interchange induced in *L. reevii* Hr by pH changes. It therefore appears that the Fe–O bond strength is not important in the modulation of the Fe–peroxide affinity.

The above discussion should be qualified by the recognition that typical X^- ligands in eq 14 (such as OH^-, N_3^-, etc.) do not necessarily mimic the binding of HO_2^- in the active site. X-ray structural studies (Stenkamp et al., 1985) indicate the peroxy species binds in the same position as N_3^-, but an additional feature of H bonding to the oxo bridge by HO_2^- is suggested by other workers (Shiemke et al., 1986). Constraint on O_2 binding in the tense state could be related to disruption of the H binding in the thermodynamic step 12, but a specific mechanism for this process is not readily identifiable.

Although equilibria 11 and 12 represent the total deoxy–oxy conversion, a third possible source of allosteric control must be considered. In this alternate thermodynamic scheme, ligation to deoxyHr is considered as the first step:

$$[Fe^{II}Fe^{II}\text{–}H^+] + O_2 \rightleftarrows [Fe^{II}Fe^{II}\text{–}H^+\text{–}O_2] \quad (15)$$

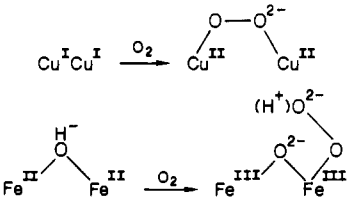
$$[Fe^{II}Fe^{II}\text{–}H^+\text{–}O_2] \rightleftarrows [Fe^{III}Fe^{III}\text{–}O_2H^-] \quad (16)$$

Factors influencing the affinity of the ferrous site for ligands (in the absence of redox change) are then considered as possibly subject to allosteric control. In eq 16, a redox change again is involved, but as an inner sphere rather than outer sphere process (eq 11). Equilibria involving the binding of ligands to deoxy have been studied, where one X^- binds per binuclear site (reaction 17). In view of spectroscopic information, it is assumed that the binding is to the ferrous center involved in O_2 binding (Reem & Solomon, 1984). Our results show no cooperativity linked to this process when X^- is azide. It is therefore unlikely that control of oxygen affinity for the deoxy site involves steric constraints in the $[Fe^{II}Fe^{II}]$ state.

$$[Fe^{II}Fe^{II}] + X^- \rightleftarrows [Fe^{II}Fe^{II}\text{–}X^-] \quad (17)$$

However, it should be noted that binding azide to the deoxy active site leads to loss of antiferromagnetic exchange coupling of the iron centers, apparently by protonating or displacing the hydroxo bridge (Reem & Solomon, 1984). In contrast, the binding of dioxygen leads to oxidation of the irons and formation of an oxo bridge, which provides for strong antiferromagnetic coupling of the iron centers. Therefore, adduct formation with deoxyHr may not be a totally appropriate model for equilibrium 15.

Comparison to Hemocyanin and Hemoglobin. Although details of active site and polypeptide structures in hemocyanin (Hc) are becoming clear (Gaykema et al., 1984), the level of structural knowledge does not approach that for hemoglobin (Hb) and Hr. However, the Hr site has much in common with that of Hc, especially in the binding of oxygen as peroxide by a two-electron oxidative-addition process:



The observation of significant cooperativity in a Hr provides for informative comparisons to Hb and Hc.

Hemocyanin usually binds O_2 cooperatively, but binding of CO is simply hyperbolic (Bonaventura et al., 1981). It has been suggested that this difference is a result of steric changes in the site that accompany the formation of a 1,2-μ-peroxo bridge (Co & Hodgson, 1981; Solomon, 1981; Brunori et al., 1983). The allosteric control would then involve a tension in the Cu–Cu distance, analogous to the tension of the axial base suggested as a method for control of ligand affinity at the heme iron site of Hb (Perutz, 1979). Thus, if the Cu–Cu distance is constrained to be larger than the distance in the oxy state, reduced affinity for O_2 would presumably result. This possibility was based only on the observation that CO does not bind cooperatively to Hc. Our observation of cooperativity in O_2 binding to hemerythrin weakens this argument since no bridging by O_2 is involved. Our results suggest the possible linkage of cooperativity in O_2 binding to the valency change at the coupled binuclear metal site. Therefore, in Hr and Hc, the binding of innocent ligands that do not induce oxidation state changes (such as N_3^- and CO, respectively) is noncooperative. The control of ligand affinity at the active site may then be a result of constraint in the tense form on the rearrangement of the metal environments upon change of oxidation state.

The total allosteric energy ($RT \ln c$) for oxygen binding in hemocyanins can be as high as 5 kcal mol^{-1} (Brouwer et al., 1978), which is larger than maximum values for *L. reevii* Hr ($\sim$1.4 kcal mol^{-1}) and Hb ($\sim$3 kcal mol^{-1}). An oxygen binding free energy difference of 5 kcal mol^{-1} in the relaxed–tense conversion would require a shift of $\sim$110 mV in the $E°$ value for the binuclear copper site $2e^-$ reduction potential. In view of the large differences in preferred geometries for copper(I) and copper(II), it does not seem unreasonble that a protein conformational change could induce the $E°$ shifts required for cooperativity in hemocyanins. The copper(I/II) reduction potential is in fact rather sensitive to the geometry of the ligand field (Addison, 1983; Solomon et al., 1980), thereby making copper an ideal metal center for allosteric control of oxygen affinity.

In the case of hemoglobin, CO binds with cooperativity little different from that of O_2 binding, albeit with a much lower $p_{1/2}$. It is apparent, therefore, that constraints of CO binding in Hb can be achieved without a concomitant change in iron oxidation state. This constraint is usually attributed to the out-of-plane motion of the iron in the porphyrin ring (Perutz, 1979). In addition, ligand exchange in the metHb state can be cooperative if spin-state changes occur in the iron(III) center (Szabo & Karplus, 1972, 1975); once again, an oxidation-state change is not required for modification of ligand affinity via structural constraint. In the case of metHr from *L. reevii*, neither the met (with OH^-) nor the deoxy (with N_3^-) species binds innocent ligands cooperatively, implying the absence of allosteric linkage in those processes. Available evidence for *L. reevii* met binding is therefore consistent with the hypothesis that homotropic allosteric control is coupled to the oxidation-state changes in the Hr active site.

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