Comparison of Hemerythrins from Four Species of Sipunculids by Optical Absorption, Circular Dichroism, Fluorescence Emission, and Resonance Raman Spectroscopy†

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ABSTRACT: Resonance Raman, optical absorption, circular dichroic, and fluorescence emission spectroscopy of hemerythrins from four species of sipunculids (*Phascolopsis gouldii, Phascolosoma agassizii, Themiste dyscritum,* and *Themiste pyroides*) reveals no major differences in their active site or tertiary structures. This precludes any change in iron ligands or coordination geometry and makes it unlikely that the active-site structures of *P. gouldii* and *T. dyscritum* hemerythrins could be as disparate as indicated by present crystallographic interpretations (Stenkamp, R. E., Sieker, L. C., and Jensen, L. H. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 349; Klotz, I. M., Klippenstein, G. L., and Hendrickson, W. A. (1976), *Science 192,* 335). Resonance Raman enhancement profiles of the stretching modes involving coordinated dioxygen maximize with excitation at ~525 nm, and correspond to the circular dichroic (CD) transition at ~520 nm. For coordinated azide modes in metazidohemerythrins these profiles maximize with excitation at ~505 nm corresponding to the 500-nm CD transition. Hemerythrins also possess another resonance Raman peak at ~510 cm^{-1} which show maximum intensity enhancement at ~530 nm and this vibration is most likely associated with a permanent iron ligand.

The structure of the active site of hemerythrin, the oxygen-carrying protein of sipunculids, priapulids, and some brachipods and annelids, has been the subject of considerable investigation (Klotz, 1971; Okamura and Klotz, 1973). This protein exists in two physiologically active forms, oxy- and deoxyhemerythrin, and in an inactive oxidized form, methemerythrin, which is capable of binding halides and pseudohalides at the active site. The active site is a dimeric iron moiety ligated to the protein through the functional groups of amino acid residues.

Spectroscopic studies of the active site in hemerythrin have predominately been carried out on the protein from the sipunculid *Phascolopsis* (syn. *Golfingia*) *gouldii* (Klotz, 1971). Particular structures and electronic states have been suggested based on comparisons of the spectra of the various forms of hemerythrin and model compounds (Garbett et al., 1969). Although an x-ray diffraction study of metazidohemerythrin from *P. gouldii* is in progress and provides one model of the active-site structure (Ward et al., 1975; Klotz et al., 1976), the crystal structure of methemerythrin from *T. dyscritum* at higher resolution gives a second but substantially different active site (Stenkamp et al., 1976a,b). These two crystal structures provide a basis for the reinterpretation of spectroscopic data obtained from the active-site chromophores. First, however, it must be determined whether the differences in the proposed crystal structures could be due to the fact that they are based on hemerythrins from different species. In order to decide whether the active sites of these homologous proteins are structurally and electronically equivalent, we have undertaken a spectroscopic investigation of hemerythrins from four species of marine worms: *Phascolopsis gouldii, Phascolosoma agassizii, Themiste dyscritum,* and *Themiste pyroides.*

Optical absorption, circular dichroism (CD),[1] and resonance Raman spectroscopy are the methods we have chosen for determining the structural and electronic equivalence of the active sites in hemerythrins from different species. Circular dichroism in the visible and near-UV regions can detect changes in chromophore chirality induced by a protein environment (Coleman, 1968). Resonance Raman spectroscopy has been shown to be a sensitive and selective probe of the chromophoric sites in proteins (Spiro and Loehr, 1975). For example, the resonance Raman spectra of oxyhemerythrin from *P. gouldii* demonstrated that the oxidation state of bound oxygen in this protein is that of the peroxide ion, O_2^{2-} (Dunn et al., 1973). Subsequent Raman studies of oxy- and metazidohemerythrin (Dunn et al., 1975; Dunn, 1974; Kurtz et al., 1976) suggested active-site structures in which only one of the ligand atoms is directly bonded to iron.

The visible absorption spectra of oxyhemerythrin and methemerythrins are composed of several overlapped bands (Keresztes-Nagy and Klotz, 1965; Garbett et al., 1969), but some of the absorption maxima are resolved in CD spectra (Garbett et al., 1969). The nature of the optical transitions giving rise to the intensity enhancement of Raman peaks has not yet been identified. A comparison of CD and visible absorption spectra with resonance Raman enhancement profiles should indicate the energies of the electronic transitions providing intensity enhancement of active-site vibrational modes. Comparable studies with the copper-containing oxygen-transport protein, hemocyanin, have provided considerable insights into the electronic and structural properties of its active site (Freedman et al., 1976).

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[1] Abbreviations used are: CD, circular dichroism; UV, ultraviolet; EPR, electron paramagnetic resonance; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

Experimental Procedures

Hemerythrin from P. gouldii and T. dyscritum. The marine worms *P. gouldii* and *T. dyscritum* were obtained, respectively, from Marine Biological Laboratory, Woods Hole, Mass., and Oregon Institute of Marine Biology, Charleston, Oregon. Oxyhemerythrin was obtained from the coelomic fluid of both marine worms by the method of Klotz et al. (1957). Crystallization of *P. gouldii* hemerythrin was accomplished by dialysis against buffered 20% ethanol, while dialysis of concentrated protein solutions (>20 mg/mL) against 0.05 M KCl, 0.01 M Tris (pH 7.5) was sufficient to crystallize the *T. dyscritum* protein. Laked blood was used for most experiments as it proved to be spectroscopically indistinguishable from protein purified by crystallization.

Since oxyhemerythrin slowly converts to methemerythrin in vitro, samples of oxyhemerythrin were either freshly prepared or regenerated by dithionite reduction and subsequent exposure to air. A particularly rapid extraction procedure was used to prepare oxyhemerythrin samples from *P. gouldii* and *T. dyscritum* for the determination of absorption spectral parameters. In this procedure, collection and centrifugation of blood cells was completed in 15 min. Red blood cells were lysed for 30 min in distilled water and cell ghosts were removed by a 30-min centrifugation at 27 000*g*. The protein was then dialyzed in 6-mm diameter tubing for 2 h against 0.05 M potassium phosphate (pH 7.5) and filtered through a 0.45-μm Millipore membrane. The absorption spectra of these protein samples were obtained within 4 h of collection of coelomic fluid from the worms.

Metazidohemerythrin was prepared from oxyhemerythrin by the usual procedures (Keresztes-Nagy and Klotz, 1965; Garbett et al., 1969). Single crystals of metazidohemerythrin from *T. dyscritum* were obtained by addition of 10^{-4} M sodium azide solution to single crystals of methemerythrin, which had formed during several months storage of sterile laked blood.

Hemerythrin from T. pyroides and P. agassizii. T. pyroides worms were obtained from Pacific Biomarine Supply, Venice, Calif. Specimens of *P. agassizii* were collected on the west coast of Vancouver Island, British Columbia.

To minimize metchlorohemerythrin formation, washings were performed with isotonic Na_2SO_4 solution, instead of chloride medium, and 10 mM EDTA (pH 7) was added to the wash solution to prevent clotting of the coelomic fluid. After cell lysis, these two proteins were purified by gel-permeation chromatography on a (5 × 60 cm) Sepharose 6B column. This procedure removes high-molecular-weight impurities from both proteins as well as a low-molecular-weight green contaminant often found in *T. pyroides* lysates. This contaminant, whose molecular weight appears to be <5000 on a Sephadex G-25 column, has a strong absorption band at 317 nm which interferes with quantitative spectroscopy of *T. pyroides* hemerythrin. The *T. pyroides* protein thus prepared is electrophoretically indistinguishable from the $(NH_4)_2SO_4$ precipitate of Klippenstein et al. (1972). Hemerythrin from *T. pyroides* was stored in the oxy form, whereas that from *P. agassizii* was stored in the deoxy form. Samples of oxyhemerythrin were either freshly extracted or regenerated by reduction with dithionite or formamidinesulfinate and subsequent exposure to air. Excess reductant was removed by rapid passage through a Sephadex G-25 column.

Iron Analyses. The iron content of *T. dyscritum* and *P. gouldii* hemerythrins was determined by atomic absorption spectroscopy using a Perkin-Elmer 305B instrument. For the *T. pyroides* and *P. agassizii* proteins, the iron analyses were performed by photometry of the 1,10-phenanthroline complex of iron(II) extracted from the protein. The procedure of Rill and Klotz (1970) was modified by the substitution of 1 M $NaHSO_3$ for hydroxylamine to improve reproducibility. Ferrous ethylenediammonium sulfate (G. F. Smith Chemical Co., primary standard grade) gave ϵ = 11 200 ± 200 M^{-1} cm^{-1} at 510 nm under these conditions. Protein solutions for iron analysis were made 2 mM in EDTA to chelate any nonspecifically bound iron present. The EDTA species were then separated from the protein by chromatography on Sephadex G-25.

Spectroscopy. Optical spectra were recorded on Cary 14 and 15 spectrophotometers. CD spectra were obtained on a Durrum-Jasco 10 (*T. dyscritum* hemerythrin) or a Jasco-20 spectrometer calibrated with camphor-10-sulfonic acid. EPR measurements were performed on hemerythrin crystals at room temperature, −30 °C, and −150 °C with a Varian E3 spectrometer. Fluorescence emission spectra were obtained on an Aminco-Bowman spectrofluorimeter operated at 2-mm slit width. Wavelength calibration was effected with anthracene, tyrosine, and tryptophan. All optical and CD parameters are based on the iron dimer concentration, since there are two iron atoms in the active site of each protein subunit.

Resonance Raman spectra were recorded on a Jarrell-Ash 25-300 spectrophotometer equipped with an ITT FW-130 (S-20) photomultiplier and photon-counting electronics. A Coherent Radiation Model 52MG mixed-gas ion laser was used for excitation of samples. A 180° back-scattering geometry was employed with spinning sample tubes (7-mm i.d.) to minimize thermal decomposition. Perchlorate ion (ν_1 = 933 cm^{-1}) was used as the internal intensity standard in one series of experiments to determine the excitation profile of metazidohemerythrin. However, oxyhemerythrin decomposed in the presence of perchlorate ion and 0.33 M sulfate ion (ν_1 = 981 cm^{-1}) was used as the internal standard in all subsequent experiments. Intensities were measured as peak heights. Excitation profiles were determined from intensities of resonant protein modes compared to the intensity of the ν_1 vibration of the internal standard. Corrections for absorption were made (Shriver and Dunn, 1974) and intensities were normalized to the value at the wavelength of maximum enhancement for each mode.

Results

Oxyhemerythrin. Optical and CD Spectra. The optical absorption spectra of oxyhemerythrins from all four species show great similarity with essentially constant wavelength maxima and only small variations in extinction coefficients (Table I). Some of this variability is apparently related to the freshness of the hemerythrin sample as evidenced by the higher extinction coefficients at 500 nm for the rapidly extracted samples from *P. gouldii* and *T. dyscritum* than for the older samples from *T. pyroides* and *P. agassizii*.

Aging was found to be particularly deleterious to the coelomic hemerythrin from *T. pyroides*. After several weeks storage at 5 °C, it was found that the protein could no longer be fully reduced by dithionite; that fraction which had been reduced and reoxygenated underwent autoxidation much more rapidly than fresh hemerythrin. Even storage as the metazide derivative did not protect against this aging process, as evidenced by a considerable loss in the intensity of the 370-nm CD band. Thus, all spectral parameters (Tables I and II) were obtained using freshly prepared samples from *T. pyroides*.

The visible and near-ultraviolet CD spectral properties of

TABLE I: Optical Absorption Parameters of Oxyhemerythrin and Metazidohemerythrin from Four Species of Sipunculids.[a]

Species	Oxyhemerythrin[b]				Metazidohemerythrin[c]		
P. gouldii[d]	280 [35 400]	326 [6900]	360sh [5400]	500 [2300]	327 [7200]	370sh [4800]	446 [3800]
T. dyscritum	280 [33 300]	329 [6700]	360sh [5100]	500 [2350]	325 [7750]	375sh [4750]	446 [3600]
T. pyroides	280 [32 800]			500 [2050]	326 [7400]	380sh [4500]	446 [3500]
P. agassizii	280 [29 200]	328 [6100]	360sh [4850]	500 [2000]	327 [6700]	375sh [4450]	446 [3500]

[a] λ_{max} in nm ± 2 nm [ϵ in M^{-1} (iron dimers) cm^{-1} ± 3%]. [b] Oxyhemerythrin from *P. gouldii* and *T. dyscritum* was prepared by a rapid extraction procedure (see Experimental Procedures) and analyzed within 4 h of collection of coelomic fluid from the worms. Oxyhemerythrin from *T. pyroides* was regenerated within 24 h of isolation of the protein; its near-UV data were not quantitated. Oxyhemerythrin from *P. agassizii* was regenerated from several-day-old protein. [c] Metazidohemerythrins were prepared according to the methods of Keresztes-Nagy and Klotz (1965). [d] These results are similar to those reported by Garbett et al. (1969).

TABLE II: Principal Circular Dichroic Bands in Oxyhemerythrin and Metazidohemerythrin from the Four Species of Marine Worms.[a]

	Oxyhemerythrin		Metazidohemerythrin	
Species[b]	λ(nm)	Δε	λ(nm)	Δε
P. gouldii[c]	520	−2.46	500	−4.09
T. dyscritum	520	−2.29	500	−3.98
T. pyroides	520	−2.65	500	−3.55
P. agassizii	520	−2.03	500	−2.68
P. gouldii[c]	336	−3.78	370	−9.02
T. dyscritum	336	−4.01	370	−9.41
T. pyroides	336	−4.12	370	−8.42
P. agassizii	346	−4.06	370	−6.22

[a] λ values ±1 nm; Δε (per dimeric iron unit) ±1%. [b] Both oxy- and metazidohemerythrin from *P. gouldii* were at pH 8.2, while those from *T. dyscritum* were at pH 7.5. *T. pyroides* and *P. agassizii* samples had oxyhemerythrin at pH 8.2 and metazidohemerythrin at pH 7.1. [c] These results are similar to those reported for *P. gouldii*, pH 7.0 by Garbett et al. (1969).

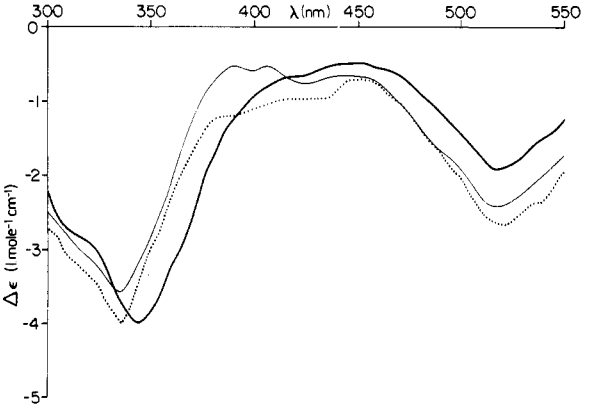

FIGURE 1: Near-UV-visible circular dichroism of oxyhemerythrins from *P. gouldii* (light line), *P. agassizii* (heavy line), and *T. pyroides* (dots).

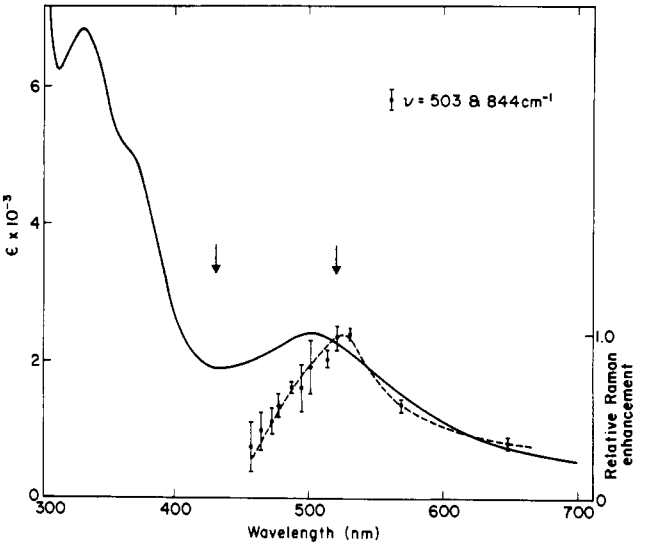

FIGURE 2: Absorption spectrum (—) and resonance Raman enhancement profile (- - -) of vibrational modes associated with dioxygen bound at the active site in oxyhemerythrin from *P. gouldii*. Each error bar on the enhancement profile indicates the standard deviation for two sets of measurements of both the 503- and 844-cm^{-1} resonance Raman peaks. Arrows indicate the wavelengths of CD bands in the visible region.

oxyhemerythrins are shown in Figure 1 and Table II. Apart from the *P. agassizii* case, the results indicate essentially identical band wavelengths and somewhat variable Δε values between species. The *P. agassizii* band is at an unusually high wavelength (346 nm). This is not the result of partial transformation to the oxidized form, as the spectrum is not affected by pH changes in the range 6.5–9.0 where methemerythrin is interconverted between the aquo and hydroxo forms, which have very different CD spectra. Furthermore, autoxidation would have produced a lower Δε value. An additional band at 430 nm has been reported for *P. gouldii* (Garbett et al., 1969); however, we have found that this band varies in intensity and is often missing.

Resonance Raman Spectra. The resonance Raman spectra of oxyhemerythrin from all four species consisted of two major peaks at 503 and 844 cm^{-1}. These have been previously identified in oxyhemerythrin from *P. gouldii* as resulting from Fe–O and O–O stretching modes, respectively, for a bound peroxide ion (Dunn et al., 1973). The enhancement profiles of these modes in oxyhemerythrin from *T. dyscritum* and *P. gouldii* are presented in Table III and Figure 2. Maximum enhancement of the 503- and 844-cm^{-1} peaks in both species occurred at ~525-nm excitation. While enhancement profiles of oxyhemerythrin from *T. pyroides* and *P. agassizii* were not determined as accurately, qualitative inspection of spectra with various excitation frequencies indicated similar results.

Metazidohemerythrin. Visible and CD Spectra. Visible absorption spectra of metazidohemerythrins are very similar for all four species of worms, as shown in Table I for the principal peaks. Similarly, the CD spectra of metazidohemerythrins from these same species exhibit constant band positions but somewhat variable Δε values for the two principal visible CD bands at 370 and 500 nm (Table II). Although the oxy-

TABLE III: Resonance Raman Enhancement Profiles[a]

Resonance Raman Peaks (cm^{-1})	Species	Excitation Wavelength (nm) 488.0	496.5	501.7	514.5	520.8	530.9	568.2	647.1
		Oxyhemerythrin[b]							
503	*P. gouldii*	0.66	0.64	0.67	0.79	0.90	1.00	0.53	0.34
	T. dyscritum	0.68	0.74	0.76	0.83	1.00	0.90	0.89	0.63
844	*P. gouldii*	0.60	0.66	0.89	0.86	1.00	0.95	0.57	0.32
	T. dyscritum	0.49	0.62	0.90	0.88	1.00	0.90	0.90	0.60
		Metazidohemerythrin[c]							
292	*P. gouldii*	0.73	0.86	0.98	1.00	0.75	0.46	0.19	0.00
	T. dyscritum	0.46	0.71	0.89	1.00	0.71	0.42	0.21	0.00
375	*P. gouldii*	0.82	0.82	1.00	0.97	0.69	0.45	0.19	0.00
	T. dyscritum	0.75	0.80	1.00	0.97	0.67	0.48	0.22	0.01
508	*P. gouldii*	0.23	0.30	0.60	0.75	0.96	1.00	0.52	0.26
	T. dyscritum	0.20	0.42	0.66	0.77	0.95	1.00	0.56	0.33
~800	*P. gouldii*[d]	0.61	0.63	1.00	0.41	0.24	0.23	0.11	0.00
2049	*P. gouldii*	0.85	0.92	1.00	0.92	0.59	0.38	0.10	0.00
	T. dyscritum	0.87	0.81	1.00	0.84	0.58	0.40	0.19	0.00

[a] Intensities have been normalized to intensity at wavelength of maximum enhancement. [b] Average of two data sets. [c] Average of four data sets for *P. gouldii* and two data sets for *T. dyscritum*. [d] Average of two data sets although some points represent only one intensity.

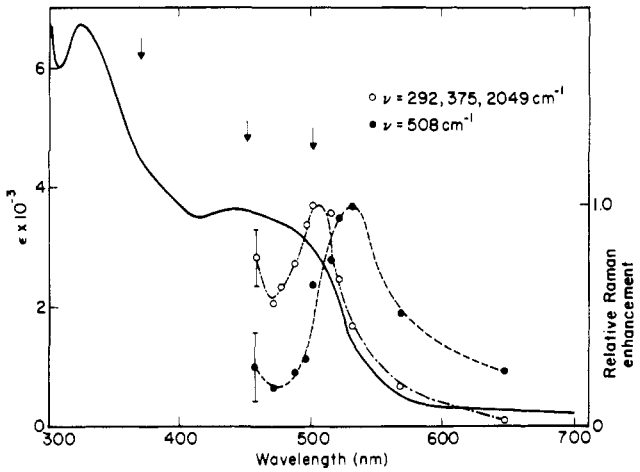

FIGURE 3: Absorption spectrum (—) and resonance Raman enhancement profiles (- • -; - - -) of vibrational modes observed in metazidohemerythrin from *P. gouldii*. Each error bar represents the average standard deviation obtained for the 11 excitation wavelengths from four sets of measurements. Arrows indicate the wavelengths of CD bands.

hemerythrin from *P. agassizii* has an anomalously high-wavelength CD band at 346 nm (compared to 336 nm in the other proteins), there is no evidence for any difference in the CD spectrum of the metazide form. This finding obviates the need to postulate different iron ligands in *P. agassizii* oxyhemerythrin compared with the other species to account for the CD spectral shift.

Resonance Raman Spectra. The resonance Raman spectra of metazidohemerythrin from all four species consist of the following peaks: 295 (weak), 375 (strong), 508 (strong), 750–850 (broad and ill-defined), and 2049 cm^{-1} (moderate). The frequencies of the peaks at 375 and 2049 cm^{-1} are known from previous studies to be dependent on the nitrogen isotope in the bound azide (Dunn et al., 1975). These peaks have, therefore, been assigned to ν_sFe-N_3 (375 cm^{-1}) and ν_{as}N-N-N (2049 cm^{-1}). These two vibrations, involving the bound azide, and the peak at 295 cm^{-1} show maximum enhancement of their Raman intensities at an excitation of ~505 nm (Table III and Figure 3). The broad band at ~800 cm^{-1} in the resonance Raman spectrum may be composed of several weakly enhanced modes and also appears to have maximum enhancement with ~500-nm excitation. The sharp peak at 508 cm^{-1} in the resonance Raman spectra of metazidohemerythrins, which is insensitive to isotopically labeled azide, has not been assigned. However, the resonance Raman enhancement profile of this peak is significantly different from the other modes, showing a maximum at ~530 nm.

The excitation wavelength dependence of the intensities at 375 and 508 cm^{-1} is further demonstrated for *T. dyscritum* metazidohemerythrin in Figure 4. The striking similarity in the resonance Raman enhancement profiles of *T. dyscritum* and *P. gouldii* metazidohemerythrins is shown in Table III. Resonance Raman spectra of metazidohemerythrins from *T. pyroides* and *P. agassizii* indicate a similar dependence of these peak intensities on excitation wavelengths.

Fluorescence Spectra. Fluorescence emission spectra of metazidohemerythrins from *P. gouldii*, *T. dyscritum*, and *T. pyroides* also show close agreement with one another. In each case, excitation at 280 nm leads to an emission peak at 347 ± 2 nm with a half-height bandwidth of 55 ± 2 nm. These spectral parameters are characteristic of tryptophan side chains in a polar medium (Burstein et al., 1973) and indicate that the hemerythrins from the different species must have their tryptophans in very similar environments.

Metchlorohemerythrin. Additional resonance Raman studies were performed on the metchloro derivative of *P. gouldii* hemerythrin in order to differentiate spectral features due to adduct ligands from those due to permanent ligands in the protein. Although metchlorohemerythrin was somewhat unstable to laser irradiation, a peak was observed at 511 cm^{-1} which was maximally enhanced with ~530-nm excitation. This peak, therefore, behaves similarly to that of the 508 cm^{-1} peak observed in the spectra of metazidohemerythrin. It is probable that the vibrational modes and electronic transitions are the same in these two derivatives and represent a chromophore in methemerythrin which is independent of bound ligand.

Methemerythrin Crystals from T. dyscritum. In making spectral and crystallographic comparisons of hemerythrins, it is important to know both the oxidation state of the iron and the kinds of adduct ligands present at the active site. The

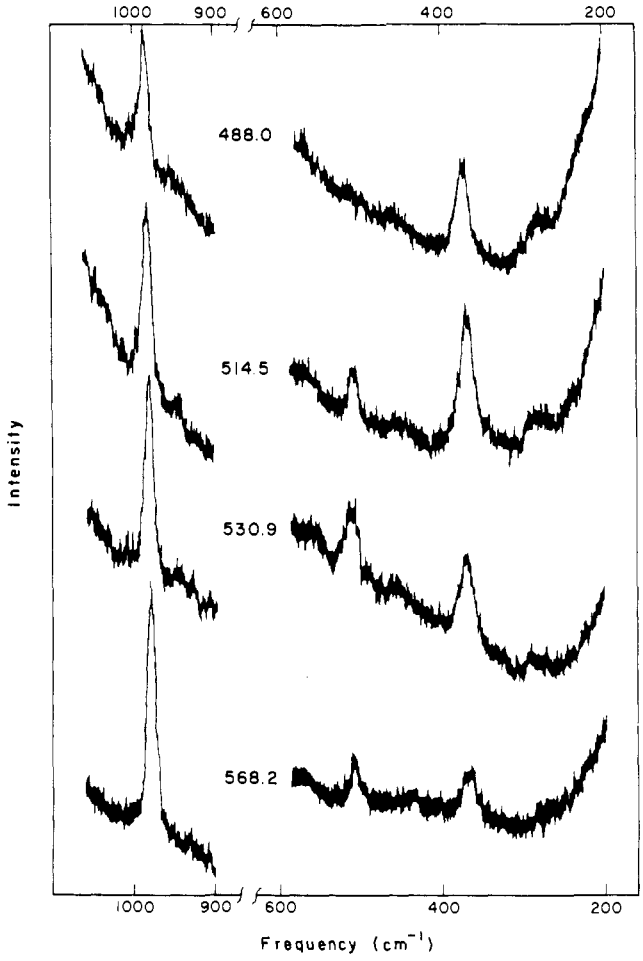

FIGURE 4: Resonance Raman spectra of metazidohemerythrin from *T. dyscritum* demonstrating the excitation frequency dependence of the intensities of the peaks at 375 and 508 cm^{-1}. The peak at 981 cm^{-1} is due to sulfate ion used as internal standard. Concentration of metazidohemerythrin is 4×10^{-3} M. Scan rate 20 cm^{-1}/min; slits 5.5 cm^{-1}; time constant 2 s. Laser power and amplification were varied to maintain approximately equal intensity of the internal standard peak and good S/N for the two hemerythrin peaks.

crystals of *T. dyscritum* hemerythrin used in the crystallographic studies (Loehr et al., 1975; Stenkamp et al., 1976a,b) were originally identified as containing metaquohemerythrin mainly on the basis of color and insolubility (Loehr et al., 1975). The crystals formed after several months storage of sterile laked blood at which time the amber color indicated that a substantial amount of hemerythrin had oxidized.

Additional evidence in favor of the methemerythrin assignment is the external morphology of the *T. dyscritum* crystals used for x-ray diffraction analyses. They were large tetragonal prisms with lengths roughly twice their widths. We have now been able to obtain very similar crystals from both metazidohemerythrin and metchlorohemerythrin, whereas oxyhemerythrin forms only tiny needles and does not crystallize as readily.

Since the hemerythrin had apparently oxidized during storage, we wanted to be certain that no additional decomposition had taken place. We had found previously that aging can reduce the ability of hemerythrins to coordinate with azide, as judged by a decrease in the intensity of the 446-nm peak in the visible spectrum. Crystals of *T. dyscritum* hemerythrin prepared in the same manner as those used for x-ray crystallography reacted readily with azide and gave the normal metazidohemerythrin spectrum (Table I and Garbett et al., 1969) upon dissolution. Thus, no deleterious aging appears to have occurred. Furthermore, EPR spectra of these same crystals showed them to be free of any of the unusual EPR-detectable species observed in aged samples of oxyhemerythrin from *P. gouldii* (Bayer et al., 1972).

The nature of the methemerythrin adduct ligand in the crystallographic samples is still uncertain. A metaquo or methydroxo assignment is favored by the fact that there is no sign of any electron-rich anion bound near the active site in the 2.8-Å resolution electron-density map (Stenkamp et al., 1976a). Supernatants of crystals used for x-ray diffraction studies display spectral peaks at ~320, 360, and 480 nm which are characteristic of methydroxo- but not metaquohemerythrin (Garbett et al., 1969). However, the presence of some bound chloride cannot be ruled out, as the solution in which the crystals formed contained some sodium chloride.

Discussion

Species Comparison. The crystallographic models of hemerythrin from *P. gouldii* and *T. dyscritum* have very similar peptide backbone structures and conformations but substantially different active-site structures (Ward et al., 1975; Klotz et al., 1976; Stenkamp et al., 1976a,b). In the model for methemerythrin from *T. dyscritum,* the active site is viewed as a pair of iron octahedra sharing a face (Stenkamp et al., 1976a). One iron is coordinated to three His, a bridging Glu, and a bridging Asp. The other iron is coordinated to two His, one Tyr, and the same two bridging ligands, Glu and Asp. These ligand assignments are based on the identification of His-25, His-54, and Glu-58 in the amino acid sequence of *T. dyscritum* hemerythrin (J. S. Loehr and M. A. Hermodson, personal communication) and for the rest of the ligand positions on the analogous amino acids in *P. gouldii* hemerythrin (Klippenstein et al., 1968). The third bridging position of the two octahedra is lacking in electron density from a polypeptide side chain and is possibly the ligand binding site. The iron–iron distance appears to be less than 3.0 Å (L. H. Jensen, personal communication).

The active-site model for metazidohemerythrin from *P. gouldii* is quite different (Klotz et al., 1976). Each iron is coordinated by two His, one Tyr, an oxo bridge, and a bridging peroxide in oxyhemerythrin or other bridges in methemerythrins. The iron–iron distance of 3.45 Å in this model is based on data obtained with crystals of *T. pyroides* myohemerythrin (Hendrickson et al., 1975). The structure and choice of ligands differs from the *T. dyscritum* model by substitution of a Tyr for a His ligand and the involvement of an oxo bridge instead of bridging Glu and Asp. The *T. dyscritum* model rests mainly on a 2.8-Å resolution electron-density map, while the *P. gouldii* model is derived from a combination of 5.5-Å resolution x-ray data and spectroscopic and chemical data.

The two different active-site models could derive from actual differences between species, from the use of methemerythrins containing different adduct ligands, or from a misinterpretation of the crystallographic information. Although hemerythrins do show species-specific differences in physical properties, our probe of the chromophoric active site with various spectroscopic techniques demonstrates a rather conserved structure about the iron atoms for both oxyhemerythrin and metazidohemerythrin. A major modification, such as ligand substitution or a significant change in iron–iron distance, should result in differences in transition energies and probabilities and in resonance Raman frequencies and enhancement profiles, similar to the effects observed when different adduct

ligands are substituted into methemerythrin. For example, replacement of peroxide by azide results in a ~15-nm increase in the 360-nm absorption band and a ~20-nm decrease in the 525-nm metal-adduct ligand charge-transfer band identified by resonance Raman excitation profiles (see below). However, the excellent agreement in the absorption maxima, CD maxima, Raman frequencies, and enhancement profiles for the hemerythrins from all four species indicates that a basic active-site structure has been maintained.

Additional evidence for the basic structural similarity of all hemerythrin protomers is that myohemerythrin, a muscle protein, from *T. pyroides* has essentially the same visible absorption and CD spectra as coelomic hemerythrin from *P. gouldii* (Klippenstein et al., 1972). This is particularly impressive since myohemerythrin differs considerably from coelomic hemerythrin. It has only 45% homology in its amino acid sequence and exists as a monomer rather than an octamer (Klippenstein et al., 1976). X-ray data obtained with myohemerythrin from *T. pyroides* were used in determining the structure of *P. gouldii* hemerythrin at 5.5-Å resolution (Ward et al., 1975).

The small species-specific variations noted in visible extinction coefficients and ellipticity values have been observed to be somewhat dependent on the age of the hemerythrin samples. However, it is quite likely that these variations are also due to minor differences in ligand orientation or environment in the active-site complexes. Thus, for example, CD spectra of transition-metal complexes are quite sensitive to the conformational properties of coordinated ligands (Butler and Snow, 1971). Furthermore, small species-specific changes in protein conformation are quite likely, since the hemerythrins do exhibit the following differences in physical properties: (1) hemerythrin from *T. dyscritum* readily crystallizes from aqueous solutions while the others do not; (2) we have observed different rates for the autoconversion of oxyhemerythrin to methemerythrin between the species; (3) the oligomeric structure of hemerythrin from *P. agassizii* is a trimer (Liberatore et al., 1974) while the others are octamers; (4) the known amino acid sequences show mutations between and among species (Ferrell and Kitto, 1971; Klippenstein, 1972; Liberatore, 1974); and (5) one of the CD bands in oxyhemerythrin from *P. agassizii* is red shifted by 10-nm relative to the 336-nm band of the other species. Such species-dependent changes in protein conformation could result in perturbations of ligand orientation or environment at the active site. However, it is unlikely that these small changes at the active site would be detectable at the resolution levels in the present crystallographic studies.

The proteins used in the two crystallography studies were both methemerythrins. However, they were different derivatives in that the hemerythrin from *P. gouldii* was in the azide form while that from *T. dyscritum* was either in the hydroxide or chloride form. Although there is some uncertainty about the adduct ligand for *T. dyscritum*, there is no evidence for any abnormalities or decomposition of the protein in the crystals. Furthermore, it is very unlikely that the difference between azide and chloride or hydroxide binding could alter the protein ligands and iron–iron distances to the extent suggested by the two active-site models, since the azide, chloride, and hydroxide forms of *P. gouldii* methemerythrin have such similar near-UV absorptions (Garbett et al., 1969). In addition, the azide and chloride forms of *P. gouldii* methemerythrin have very similar magnetic properties (Dawson et al., 1972; Moss et al., 1971) and Mössbauer spectra (Okamura et al., 1969; Garbett et al., 1971; York and Bearden, 1970); these data are not available, however, for methydroxohemerythrin. Thus, it appears that neither species differences nor adduct ligand differences can explain the disparity of the two active-site models. The differences in the two models most likely arise from difficulties in the interpretation of the crystal structure data.

Resonance Raman Spectra. The resonance Raman spectra of oxyhemerythrin were previously reported to consist of two peaks associated with the vibrations of a bound peroxide ion (Dunn et al., 1973). The 844-cm^{-1} peak was assigned to O–O stretching and the 503-cm^{-1} peak was assigned to an iron–peroxide stretch. We have now determined that these peaks are both maximally enhanced at ~525 nm. A transition at 520 nm is indicated in the CD spectra of oxyhemerythrin, and is likely to arise from charge transfer between O_2^{2-} and Fe(III). An additional peak was revealed (Dunn et al., 1975) at ~500 cm^{-1} after the dioxygen ligand mode was shifted from 503 to 478 cm^{-1} by isotopic substitution of $^{18}O_2$, and it was suggested to be an iron–tyrosine vibrational mode.

The resonance Raman spectra of metazidohemerythrin consist of several peaks which give rise to two different enhancement profiles. The two Raman peaks associated with vibrations of bound azide (375 and 2049 cm^{-1}) (Dunn et al., 1975) are maximally enhanced at ~505-nm excitation. Since this is nearly coincident with an electronic transition indicated at ~500 nm by CD spectra, it is apparent that this transition is responsible for resonance enhancement of the azide modes. The absorption at 500 nm is, therefore, most likely the azide to iron charge transfer transition expected for the azide–hemerythrin complex. The previous assignment at 446 nm was based on a broad absorption maximum (Garbett et al., 1969); through the resonance enhancement profile this complex absorption band is partially resolved to reveal a true charge-transfer transition at ~500 nm.

A weaker Raman peak at 295 cm^{-1} is also in resonance with the 500-nm absorption. Isotopic studies did not give any evidence for azide involvement at this frequency (Dunn et al., 1975), but it does appear to be in resonance with the iron–azide chromophore. Both of the models of the hemerythrin active site suggest the presence of a bridging adduct ligand and at least one other intrinsic bridging ligand (Stenkamp et al., 1976a; Klotz et al., 1976). This ring system would couple the vibrations of the bridged atoms and, thereby, induce vibronic interaction. It is likely that the 295 cm^{-1} Raman peak arises from the vibration of a second bridge bond or possibly another iron–ligand bond. Its low frequency is consistent with its assignment as an iron–carboxylate or iron–imidazole vibration, both of which have been observed in the 200–300 cm^{-1} range in metal complexes (Herlinger et al., 1970; Cornilsen and Nakamoto, 1974).

The peak at 508 cm^{-1} in the resonance Raman spectra of metazidohemerythrin, however, has a unique enhancement profile. This frequency is independent of nitrogen mass (Dunn et al., 1975) and its intensity is maximally enhanced at ~530-nm excitation. Although no CD bands are present in this region, there is evidence for an electronic transition from a minor inflection in this broad absorption band. Based on the ligand assignments from the crystallographic studies (Klotz et al., 1976; Stenkamp et al., 1976a), the ~510-cm^{-1} peak is possibly due to an iron–tyrosine mode in resonance with a tyrosine → iron charge-transfer transition. It remains unclear, however, why tyrosine-ring modes are not enhanced in these Raman spectra, as they are in the spectra of Fe(III)–transferrin (Gaber et al., 1974).

Although metchlorohemerythrin is rather unstable to laser excitation, a single weak peak at 511 cm^{-1} has been observed

which is maximally enhanced between 520- and 530-nm excitation. The correlation of vibrational frequency and excitation maximum suggests that this is the same mode observed in this region of the resonance Raman spectra of metazidohemerythrin. Furthermore, resonance-enhanced Raman peaks at ~295 and ~510 cm^{-1} have also been observed for methiocyanato- and metcyanamidohemerythrins (D. M. Kurtz, D. F. Shriver, and, I. M. Klotz, personal communication) and at ~500 cm^{-1} in oxyhemerythrin (Dunn et al., 1975). The presence of these modes in a number of hemerythrin derivatives suggests a common chromophoric structure in all hemerythrins.

Noted Added in Proof

A recent comparison of metazidohemerythrins from *P. gouldii* and *T. dyscritum* by extended x-ray absorption fine structure (EXAFS) spectroscopy has shown that (1) the two proteins have identical spectra and (2) the spectra suggest octahedral iron coordination (J. R. Herriott, W. W. Parson, and E. A. Stern, personal communication).

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