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^{31}P NMR Probes of Sipunculan Erythrocytes Containing the O_2-Carrying Protein Hemerythrin†

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ABSTRACT: Reported are the first examinations by ^{31}P NMR of erythrocytes containing the non-heme iron O_2-carrying protein hemerythrin (Hr). Intact coelomic erythrocytes from the sipunculids *Phascolopsis gouldii* and *Themiste zostericola* were shown by ^{31}P NMR to contain *O*-phosphorylethanolamine and 2-aminoethylphosphonate as the major soluble phosphorus metabolites. This combination of major metabolites appears to be unique to sipunculan erythrocytes. Nucleoside triphosphates and mannose 1-phosphate were present in lower concentrations. The concentration of *O*-phosphorylethanolamine within *P. gouldii* erythrocytes was established to be >20 mM. *T. zostericola* erythrocytes contained relatively high levels of 2-aminoethylphosphonate (on the order of 0.1 M) and lower levels of *O*-phosphorylethanolamine compared with those of *P. gouldii*. For *P. gouldii* and *T. zostericola* the intracellular pHs were determined to be 7.2 ± 0.1 and 7.1 ± 0.1, respectively, in air-equilibrated erythrocytes, and 6.5 ± 0.1 in anaerobic *P. gouldii* erythrocytes. *O*-Phosphorylethanolamine was found to bind weakly to *P. gouldii* metHr ($K_f \sim 7\ M^{-1}$). This interaction is best characterized by either negative cooperativity or nonspecific binding. *O*-Phosphorylethanolamine strongly inhibits azide binding to the iron site of *P. gouldii* metHr at pH 7.2. The rate of azide binding decreases by ~85-fold in the presence of 0.33 M *O*-phosphorylethanolamine. However, neither *O*-phosphorylethanolamine nor 2-aminoethylphosphonate at 0.33 M was found to have any significant effect on O_2 affinity of *P. gouldii* deoxyHr. Alternative functions for the two metabolites are suggested.

Hemoglobin has served as a focal point for numerous studies concerning requirements for reversible binding of O_2, as well as the structure, metabolism, and enzymology of erythrocytes. The non-heme iron O_2-carrying protein hemerythrin (Hr),[1] found in a few phyla of marine invertebrates, presents physiological and biochemical contrasts to the more widespread heme oxygen carriers (Klotz & Kurtz, 1984; Mangum, 1985, Terwilliger, 1985). Although a large body of information is available concerning the structure and reactivity of Hr itself (Wilkins & Harrington, 1983; Kurtz, 1986; Wilkins & Wilkins, 1987), relatively little is known about other constituents of Hr-containing erythrocytes.

Hr from erythrocytes of sipunculids and brachiopods usually consists of octamers ($M_r \sim 108\,000$) of essentially identical subunits. Each subunit contains a binuclear iron site that reversibly binds one molecule of O_2:

$$\underset{\text{deoxy}}{[Fe^{II},Fe^{II}]} + O_2 \rightleftharpoons \underset{\text{oxy}}{[Fe^{III},Fe^{III}O_2^{2-}]}$$

OxyHr slowly autoxidizes to $[Fe^{III},Fe^{III}]$metHr, which forms a particularly stable adduct with azide, metHrN_3. In this artificial adduct, azide is coordinated to one iron atom of the binuclear site (Stenkamp et al., 1984; Sieker et al., 1982), as is the peroxide in oxyHr (Stenkamp et al., 1985; Shiemke et al., 1984).

O_2 binding to sipunculan Hrs does not exhibit any significant cooperativity; however, brachiopodan Hrs do exhibit cooperativity in O_2 binding with a maximum Hill coefficient, n_{max} = 1.6-2.0 (Richardson et al., 1987). Intact coelomic cells containing Hr are reported to have a lower O_2 affinity than does isolated and purified deoxyHr (Weber & Fange, 1980; Mangum & Kondon, 1975). However, no physiological effector of deoxyHr has been identified. Ca^{2+}, Mg^{2+}, Cl^-, D- and L-lactate, ATP, and H^+ are all reported to have no appreciable effect on the O_2 affinities of purified sipunculan deoxyHrs (Petrou et al., 1981; Terwilliger et al., 1985; Mangum & Burnett, 1987; Richardson et al., 1987). Perchlorate is known to be an artificial heterotropic allosteric effector of sipunculan Hrs; its binding sites have been located 12-15 Å distant from the iron atoms (Stenkamp et al., 1978, 1983). Perchlorate has been shown to lower the affinities of anions for the iron site of metHr (Garbett et al., 1971a) and has recently been reported to lower the affinity of O_2 for the iron site of deoxyHr (Richardson et al., 1987).

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[1] Abbreviations: Hr, hemerythrin; PEA, *O*-phosphorylethanolamine ($O_3^{2-}POCH_2CH_2NH_3^+$); 2-AEP, 2-aminoethylphosphonate ($O_3^{2-}PCH_2CH_2NH_3^+$); PEP, phosphoenolpyruvate; 2,3-DPG, 2,3-diphosphoglycerate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate; UDPG, uridine diphosphoglucose; NAD/NADH, nicotinamide adenine dinucleotide in its oxidized and reduced forms; PIPES, 1,4-piperazinediethanesulfonate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate.

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With the exception of ATP, phosphorus metabolites represent an unexplored class of potential effectors of Hr in vivo. The presence of 2,3-DPG has been established in human erythrocytes, and this molecule is known to lower the O_2 affinity of human hemoglobin. The analogous properties have been established for inositol polyphosphates or NTP in the hemoglobin-containing erythrocytes of birds, reptiles, or fish (Bartlett, 1978; Gronenborn, 1984; Isaaks et al., 1987).

Detection of phosphorus metabolites in mammalian erythrocytes by ^{31}P NMR spectra was first reported in the classic paper of Moon and Richards (1973), who also first demonstrated the feasibility of using ^{31}P NMR to measure intracellular pH. ^{31}P NMR spectra of sipunculan erythrocytes have not been previously reported, and almost no information is available about phosphorus metabolites or pH in these cells. In this paper we report results of ^{31}P NMR analyses of coelomic erythrocytes isolated from two species of sipunculan worms, *Phascolopsis gouldii* and *Themiste zostericola*. We also report characterizations of interactions with Hr of the major soluble phosphorus metabolites in these erythrocytes.

Experimental Procedures

Isolation and Purification of Erythrocytes. Coelomic erythrocytes from *P. gouldii* were isolated from live worms obtained from Marine Biological Laboratory, Woods Hole, MA. The coelomic fluid from these worms was strained through cheesecloth, and the erythrocytes were collected by centrifugation in a clinical centrifuge for 5 min. The erythrocytes were then washed 3 times by suspension in artificial seawater (Jungle Labs Corp.) followed by centrifugation. Approximately 2.5 mL of packed erythrocytes was then transferred to a 10-mm-diameter NMR tube on ice, and the ^{31}P NMR spectrum was immediately recorded. Coelomic erythrocytes from specimens of *T. zostericola* obtained live from Pacific Biomarine Supply, Venice, CA, were isolated and prepared for NMR in the same manner. The above procedure was used for air-equilibrated erythrocytes. Anaerobic erythrocytes were obtained from two dozen live specimens of *P. gouldii*, which had been placed in a jar of artificial seawater at 15 °C and kept under a N_2 atmosphere for 16 h. After this period, the erythrocytes were isolated, washed, and transferred to the NMR tube as described above, except that all steps were carried out under Ar.

Preparation of Erythrocyte Extract. An ~2-mL packed volume of freshly isolated and washed erythrocytes was placed in an ice bath for 15 min, at which time a 1:1 volume of 6% $HClO_4$ was added. This mixtures was incubated in the ice bath for at least 30 min and then subjected to centrifugation at 16300*g* in a Sorvall centrifuge in order to remove insoluble cellular material. The supernatant was neutralized with either solid K_2CO_3 or 2 M KOH. Solids were removed from the neutralized solution by centrifugation, and the supernatant was subjected to ^{31}P NMR analysis.

^{31}P NMR. Collection of Spectra and Identification of Resonances. Spectra were collected with a Bruker WM300 spectrometer operating at 121.49 MHz for ^{31}P. A 20-μL capillary containing a solution of 0.1 M methylenediphosphonic acid (Alfa Products) in D_2O was used as external standard. This standard possessed a chemical shift of approximately 18.4 ppm relative to an 85% phosphoric acid standard, which was assigned a shift of 0 ppm. The external standard was placed in the center of a 10-mm NMR tube and held in place by a vortex suppressor. Spectra were obtained at either 278 or 283 ± 1 K. Collection parameters included a sweep width of 14 kHz, a pulse time of 13.0 μs (78°), a delay of 0.5 s, and an acquisition time of 0.59 s with 16K block sizes. These parameters resulted in a total accumulation time of 1.09 s per transient. From 25 to approximately 24 000 transients were collected per sample. Usually, a 20-Hz line broadening was applied. Examination of a sample of *P. gouldii* erythrocytes under a microscope after collection of a ^{31}P NMR spectrum showed that the cells were intact.

In order to identify resonances, commercially available phosphorus compounds (from Sigma Chemical Co. or Alfa Products) were added as standards to perchloric acid extracts of the erythrocytes. Spectra of the extracts and of the extracts plus standards were obtained at several pH values from 1 to 13. The pH was adjusted by addition of either 2 M HCl or 2 M KOH.

^{31}P NMR. Measurement of Intracellular pH. Coelomic erythrocytes isolated and washed as described above were placed in an ice bath, and 3-mL aliquots of packed erythrocytes were added to 15-mL centrifuge tubes. PIPES buffer, 0.05 M, pH 6.46, containing 1 mM 2,4-dinitrophenol was adjusted to various pH values by additions of either KOH or HCl. At each pH a 5-mL portion of this buffer was transferred to one of the centrifuge tubes containing the packed erythrocytes. The tubes were permitted to equilibrate for at leat 1 h at 5 °C. Then, after centrifugation, the pH of the solution in each tube was recorded. For these measurements, a Beckman pH meter was first standardized with certified buffer solutions from Fisher Scientific Corp. The tubes were kept in an ice bath until recording of ^{31}P NMR spectra at 5 °C, as described above. Immediately after each spectrum was recorded, the pH was remeasured and found to be in agreement with that measured beforehand. A standard curve was generated from these whole cell titration data by measuring the chemical shift difference between the 2-AEP (δ_1) and mannose 1-phosphate (δ_2) resonances and plotting ($\delta_1 - \delta_2$) as a function of pH. The pH of freshly isolated erythrocytes in artificial seawater without 2,4-dinitrophenol was calculated by measuring the value of ($\delta_1 - \delta_2$) obtained from the ^{31}P NMR spectrum at 5 °C and translating this value to pH by using the standard curve.

Binding and Inhibition Measurements. MetHr. These determinations were made spectrophotometrically on a Perkin-Elmer diode array spectrophotometer (lambda array Model 3840) with 1-cm path length cells. MetHr was prepared from oxyHr, which had been purified by a standard procedure (Klotz et al., 1957) and stored in crystalline form in a liquid N_2 refrigerator. The crystalline oxyHr was dissolved in 8–10 mL of 0.15 M HEPES, pH 7.2, which was the buffer used for all subsequent experiments. A few grains of $K_3Fe(CN)_6$ was added to the solution of oxyHr, and after dissolution by mixing, the solution was allowed to incubate for 3–4 h at room temperature. The excess $K_3Fe(CN)_6$ was removed by dialysis against a large excess of buffer at 4 °C. Concentrations of metHr, expressed as subunits (i.e., binuclear iron sites), were measured by addition of excess NaN_3 and use of ϵ_{446} = 3700 M^{-1} cm^{-1} (Garbett et al., 1969).

Inhibition of N_3^- binding to metHr by allosteric effectors was measured as follows. Stock solutions of 1 M PEA, 1 M 2-AEP, and 1 M $NaClO_4$ were prepared in buffer, and aliquots of these solutions were added to separate 1-mL volumes of 60–110 μM metHr in buffer. The final concentration of effector was 0, 45, or 330 mM in the solutions of metHr. After incubation of these solutions at room temperature for 12 ($NaClO_4$), 15 (PEA), or 3 h (2-AEP), aliquots of 0.1 M NaN_3 were added sufficient to achieve concentrations of 0.9–5 mM. The rate of N_3^- binding at 22 ± 1 °C was monitored by measuring the time course of absorbance change at 447 nm.

Temperature control was maintained by a Brinkman constant temperature bath connected to a thermostated cell holder. The pH was recorded at the conclusion of each kinetic run. The absorbance vs time data were either fit to exponential functions with the nonlinear least-squares program EXPSUM (provided by Dr. J. H. Espenson) or plotted as ln (A/A_0) vs time, in which case least-squares analysis was used. The reported rate constants represent the average of triplicate determinations.

The binding constant of PEA and metHr was measured at 22 ± 1 °C as follows. To 1-mL volumes of 60–100 μM metHr in buffer was added PEA from stock solutions sufficient to give millimolar concentrations of PEA equal to 333, 133, 66, 33, 13.3, 6.6, 3.3, 1.3, 0.66, or 0 after adjustment of the total volume to 1.5 mL with buffer. After incubation of these solutions for at least 3 h at room temperature, absorption spectra were collected against appropriate blanks. A binding constant was determined from the change in absorbance at 378 nm vs [PEA] by use of standard Scatchard and Hill plots. The pH of each solution was measured immediately after its absorbance measurement and found to be between pH 7.1 and 7.2 in all cases. Further details are given under Results and in the supplementary material. The reported value of the binding constant represents the average of three determinations.

Binding and Inhibition Measurements. OxyHr. Solutions of *P. gouldii* oxyHr were prepared as described above in 0.15 M HEPES, pH 7.28 which was the buffer used for all experiments. All dialyses were at 4 °C. DeoxyHr was prepared by anaerobic dialysis of solutions of oxyHr against buffer containing 2 mM $Na_2S_2O_4$ (BDH Laboratories) for 12 h. Excess $Na_2S_2O_4$ was removed by dialysis against several changes of strictly anaerobic buffer. The concentrations of deoxyHr used in these experiments ranged from 0.16 to 0.23 mM (expressed as subunits) and were determined by conversion to oxyHr and use of $\epsilon_{500} = 2200\ M^{-1}\ cm^{-1}$ (Garbett et al., 1969). The solutions of deoxyHr were transferred to 2-dram vials under a blanket of Ar by using gas-tight syringes and a vacuum manifold. The vials were sealed with tight-fitting rubber septa. These solutions were stored in a vacuum desiccator and used within 5 days. PEA or 2-AEP from 1 M stock solutions was anaerobically added via a gas-tight syringe to solutions of deoxyHr to give a final effector concentration of 330 mM. The resulting solutions were incubated for at least 3 h at room temperature prior to measurements of O_2 affinity. Three-milliliter volumes of the equilibrated solutions of deoxyHr were transferred under a blanket of Ar to a tonometer of a design described by Keyes et al. (1967). Oxygen equilibrium curves were obtained at 24 ± 1 °C and pH 7.1 ± 0.1. Equilibration of Hr solutions with various partial pressures of O_2 was achieved by rotation of the tonometer for several minutes. The degree of oxygenation was determined from the absorbance at 500 nm, measured directly in the tonometer. The data, consisting of absorbance at 500 nm vs partial pressure of O_2, were fit to single exponentials by using the nonlinear least-squares program EXPSUM. The O_2 half-saturation pressure, $P_{1/2}$, could then be calculated directly from the value of the exponential. This method of analysis avoids bias in comparing O_2 equilibrium curves. The reported values of $P_{1/2}$ represent the average of triplicate determinations of each O_2 equilibrium curve. In some cases the data were also analyzed by conventional Hill plots. The values of $P_{1/2}$ determined from these plots were, within experimental error, identical with those determined by exponential fits.

RESULTS

Figure 1 shows ^{31}P NMR spectra of air-equilibrated coelomic erythrocytes isolated from *P. gouldii*. An informative spectrum could be obtained after accumulation of as few as 25 transients, and spectra of considerable quality could be achieved after accumulation of 100 transients. No changes in peak intensities or chemical shifts were detected during accumulation of 25, 100, 250, and 2500 transients at 10 °C. A very similar spectrum was obtained at 5 °C. The resonances of Figure 1 are assigned to 2-AEP (2), other phosphonates (3), PEA (4), mannose 1-phosphate (5), phosphoenolpyruvate (6), NTP (7, 8, and 11),[2] NAD/NADH (9), and UDPG (9 and 10). Resonances were assigned by additions of known standards to 6% perchloric acid extracts of the erythrocytes and titrations between pH values of 1 and 13 in the presence or absence of added standards. Identical limiting ^{31}P NMR chemical shifts and pK_a's of standard and unknown were assumed to be sufficient for ensuring proper assignment of a resonance.

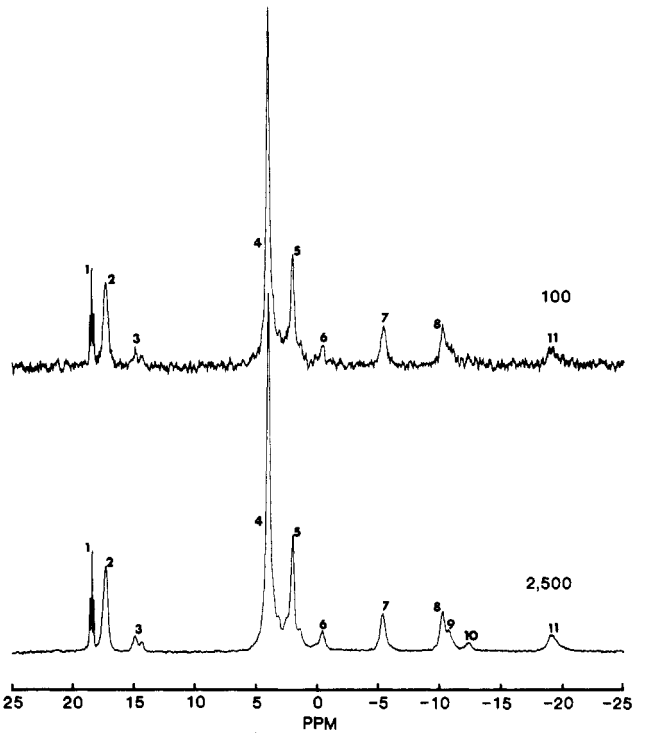

FIGURE 1: ^{31}P NMR spectra of freshly isolated, air-equilibrated *P. gouldii* erythrocytes after collection of 100 (top) and 2500 (bottom) transients at 10 °C. Resonances (in ppm) have been assigned as follows: 1 (18.48), methylenediphosphonic acid standard in a concentric capillary; 2 (17.38), 2-AEP; 3 (14.93 and 14.40), other phosphonates; 4 (4.09), PEA; 5 (2.08), mannose 1-phosphate; 6 (−0.38), phosphoenolpyruvate; 7 (−5.34), γ-P of NTP; 8 (−10.24), α-P of NTP; 9 (−10.77), NAD/NADPH and UDPG; 10 (−12.37), UDPG; 11 (−19.04), β-P of NTP.

The ^{31}P NMR spectrum of a 6% perchloric acid extract of *P. gouldii* erythrocytes after neutralization of the extract to pH 7.6 is shown in Figure 2. The pH titration profiles of resonances corresponding to 2-AEP, PEA, mannose 1-phosphate, and phosphoenolpyruvate in a perchloric acid extract of *P. gouldii* erythrocytes are shown in the inset to Figure 2. High-pH spectra of these extracts indicate that inorganic phosphate may contribute some minor intensity to the resonance assigned to mannose 1-phosphate in Figure 1. The major resonances of Figure 2 have nearly the same intensities and line widths as do their counterparts in Figure 1. These

[2] Resonances corresponding to NTP/NDP are principally composed of ATP/ADP with minor contributions from UTP/UDP, GTP/GDP, and CTP/CDP. These resonances normally reflect interaction with cellular Mg^{2+} (Gupta et al., 1984).

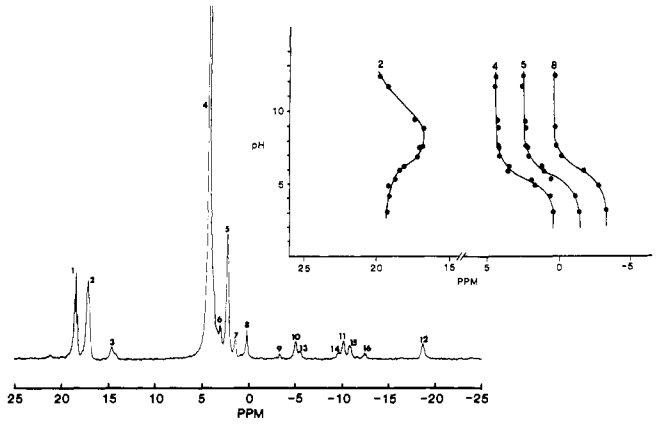

FIGURE 2: ^{31}P NMR spectra (24 000 transients) of a perchloric acid extract of *P. gouldii* erythrocytes neutralized to pH 7.6. Resonances (in ppm) are assigned as follows: 1 (18.45), methylenediphosphonic acid in a concentric capillary; 2 (17.16), 2-AEP; 3 (14.64), other phosphonates; 4 (4.25), PEA; 5 (2.31), mannose 1-phosphate; 6 and 7 (3.09 and 1.43), unidentified; 8 (0.21), phosphoenolpyruvate; 9 (−3.30), a phosphagen; 10 (−5.05), γ-P of NTP; 11 (−10.13), α-P of NTP; 12 (−18.66), β-P of NTP; 13 (−5.55), β-P of NDP; 14 (−9.66), α-P of NDP; 15 (−10.77), NAD/NADH and UDPG; 16 (−12.40), UDPG. (Inset) pH titration profile of ^{31}P NMR resonances of a perchloric acid extract of *P. gouldii* erythrocytes. Numbers nearest each curve correspond to numbered resonances in the main figure.

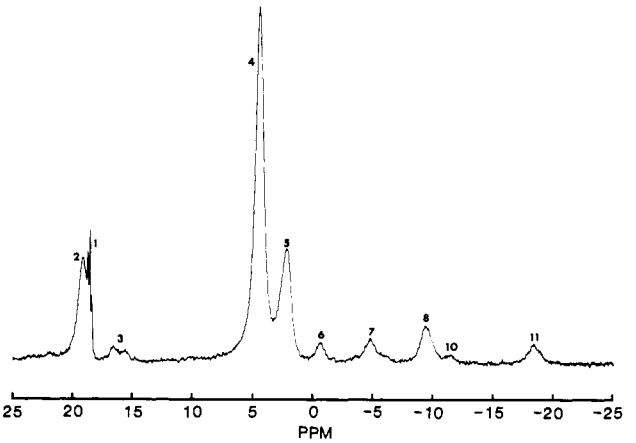

FIGURE 3: ^{31}P NMR spectra (5300 transients) of anaerobic *P. gouldii* erythrocytes at 5 °C. Resonances (in ppm) are assigned as follows: 1 (18.42), methylenediphosphonic acid in a concentric capillary; 2 (19.04), 2-AEP; 3 (16.45 and 16.22), other phosphonate; 4 (4.39), PEA; 5 (2.12), mannose 1-phosphate; 6 (−0.70), phosphoenolpyruvate; 7 (−4.97), γ-P of NTP; 8 (−9.91), α-P of NTP; 10 (−11.56), UDPG; 11 (−18.42), β-P of NTP.

similarities show that the major ^{31}P NMR resonances in spectra or intact *P. gouldii* erythrocytes are derived from nonproteinaceous low molecular weight components. The presence of PEA and 2-AEP as the major soluble phosphorus metabolites in these erythrocytes is well established by these methods. By addition of a known concentration of PEA to a perchloric acid extract, the concentration of PEA in *P. gouldii* erythrocytes was established to be >20 mM. Other resonances in the range expected for phosphonates, namely, resonances 3 of Figures 1 and 2, have not been conclusively identified. We have established that resonances 3 do not correspond to the structural isomer 1-aminoethylphosphonate. From their chemical shifts (14.93 and 14.40 ppm at pH 7.6), it is possible that one of these resonances corresponds to 1-hydroxy-2-aminoethylphosphonate (Deslauriers et al., 1980). Although this compound occurs naturally (Korn et al., 1973), it was not commercially available, and therefore, we did not test this possible assignment. The perchloric acid extracts also revealed a resonance (resonance 9 of Figure 2) in the region expected for phosphagens. However, the position of this resonance does not match those of either phosphoarginine or phosphocreatine, and this resonance was not conclusively identified. Resonances 6 and 7 of the perchloric acid extract also remain unassigned. Hr, by far the most abundant soluble protein in these erythrocytes, is not known to be phosphorylated, and we found that purified *P. gouldii* oxyHr does not give rise to any ^{31}P NMR spectrum.

From a difference of 13.5 ppm in the chemical shifts of 2-AEP and mannose 1-phosphate at 5 °C, and the standard curve (included as supplementary material) determined as described under Experimental Procedures, an intracellular pH of 7.2 ± 0.1 was calculated for air-equilibrated *P. gouldii* erythrocytes in artificial seawater.

The ^{31}P NMR spectrum of anaerobic *P. gouldii* erythrocytes shown in Figure 3 reveals significant and uniform broadening of all resonances compared to those of Figure 1. We attribute this broadening to increased paramagnetic susceptibility gradients across the erythrocyte membrane due to increased levels of deoxyHr. Because of a large decrease in extent of antiferromagnetic coupling between iron atoms, deoxyHr exhibits much greater paramagnetism than does either oxy- or metHr (Okamura et al., 1969; Maroney et al., 1986; Reem & Solomon, 1987). The analogous explanation has been given for uniform broadening of ^{31}P NMR resonances of human erythrocytes upon deoxygenation (Labotka, 1984). Resonances of Figure 3 are the same as those identified in Figure 1. With the standard curve, an intracellular pH of 6.5 ± 0.1 was calculated for anaerobic *P. gouldii* erythrocytes. This latter determination will be accurate assuming that, upon deoxygenation, any paramagnetic shift effects on 2-AEP and mannose 1-phosphate are identical and no changes occur in interactions of these two metabolites with other cellular constituents, particularly the paramagnetic deoxyHr. The 2-AEP resonance undergoes the largest shift upon deoxygenation, and 2-AEP does not show any detectable interactions with deoxy-, oxy-, or metHr (vide infra).

The ^{31}P NMR spectrum of air-equilibrated erythrocytes isolated from a second sipunculid, *T. zostericola*, is shown in Figure 4A. A much more intense 2-AEP resonance (2) and much less intense PEA resonance (5) are seen in comparison to those of *P. gouldii* in Figure 1. From the intensity of its ^{31}P NMR resonance, we estimate the concentration of 2-AEP to be on the order of 100 mM in *T. zostericola* erythrocytes. Freshly isolated erythrocytes from specimens of *T. zostericola* that had been collected several months apart showed the same pattern of intensities. A resonance (4) corresponding to an unidentified phosphomonoester is also observed in Figure 4. The intracellular pH of air-equilibrated *T. zostericola* erythrocytes in artificial seawater was calculated to be 7.1 ± 0.1, assuming that the standard curve generated from *P. gouldii* erythrocytes is applicable to *T. zostericola* erythrocytes. This assumption is reasonable, since one expects the intracellular environment of coelomic erythrocytes from these two sipunculids to be quite similar to each other. Resonances, including the unusual metabolite mannose 1-phosphate, were identified by titration of perchloric acid extracts of *T. zostericola* erythrocytes. A ^{31}P NMR spectrum of such an extract is shown in Figure 4B. This spectrum once again contains an unidentified resonance (11) in the region expected for phosphagens. Surprisingly, no resonance corresponding to inorganic phosphate, which should appear at ~5.8 ppm under the conditions of Figure 4B, is apparent. Resonances 7–9 of Figure 4A correspond to NTP/NDP. These resonances, particularly resonance 9, which is assigned to the β-phosphate of NTP, are relatively broad compared with the corresponding *P. gouldii*

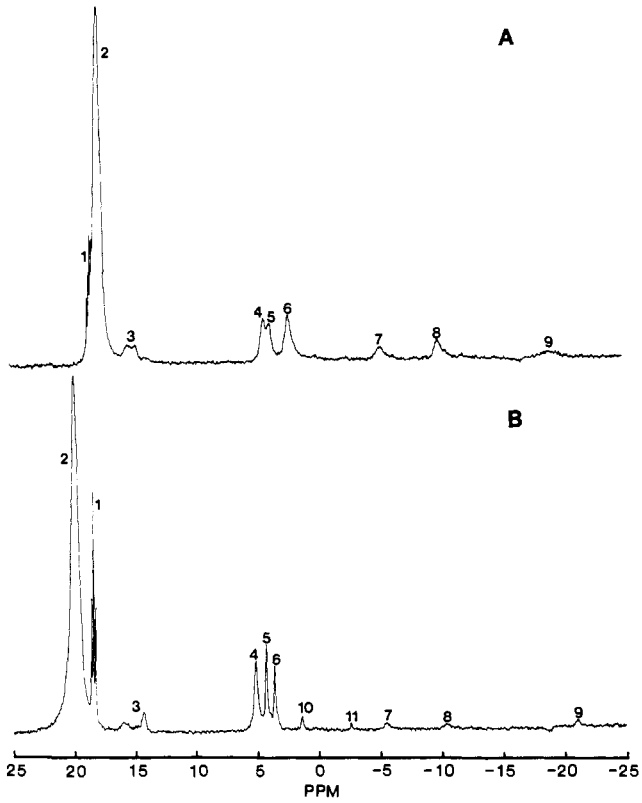

FIGURE 4: (A) ^{31}P NMR spectrum (500 transients) of freshly isolated *T. zostericola* erythrocytes at 5 °C. Resonances (in ppm) are assigned as follows: 1 (18.34), methylenediphosphonic acid in a concentric capillary; 2 (17.69), 2-AEP; 3 (15.32 and 14.61), other phosphonates; 4 (4.18), a phosphomonester; 5 (3.65), PEA; 6 (2.12), mannose 1-phosphate; 7 (−5.21), γ-P of NTP; 8 (−10.07), α-P of NTP; 9 (−19.64), β-P of NTP. (B) ^{31}P NMR spectrum (4700 transients) of a perchloric acid extract of *T. zostericola* erythrocytes. The extract was adjusted to pH 12.7 with 2 M KOH. Resonances (in ppm) are assigned as follows: 1 (18.39), methylenediphosphonic acid in a concentric capillary; 2 (19.88), 2-AEP; 3 (15.98 and 14.33), other phosphonates; 4 (5.12), a phosphomonoester; 5 (4.27), PEA; 6 (3.59), mannose 1-phosphate; 7 (−5.51), γ-P of NTP; 8 (−10.35), α-P of NTP; 9 (−21.05), β-P of NTP, 10 (1.36), a phosphodiester; 11 (−2.62), a phosphagen.

resonances (7, 8, and 11 of Figure 1). This relative broadening may reflect qualitative and/or quantitative differences in interactions with Mg^{2+} (Vasavada et al., 1984).[2]

Interactions of PEA and 2-AEP with purified *P. gouldii* metHr were investigated by UV–visible absorption spectroscopy. The effect of 50 mM PEA on the absorption spectrum of metHr is shown in Figure 5. Approximately 3 h at room temperature is required to reach the final spectrum, which has λ_{max} at 330 and 378 nm. Spectral changes of the type shown in Figure 5 were used to calculate a formation constant after incubation of solutions of metHr with various concentrations of PEA. The pH of each solution used for these measurements was checked after recording the final spectrum, and all solutions were found to be at pH 7.1 ± 0.1. A Scatchard plot of these data (included in the supplementary material) is concave, and a Hill plot of the data (also included in the supplementary material) has a slope of ~0.8. From the Hill plot, a value of the formation constant, K_f, for binding of PEA to metHr was calculated to be 7.0 ± 0.4 M^{-1} at pH 7.1 and 22 °C, assuming $n = 1$. In contrast to the behavior shown in Figure 5, no change in the absorption spectrum of metHr was observed upon addition of 2-AEP, up to a concentration of 450 mM, even after several hours of incubation at neutral pH.

We found that PEA strongly inhibits binding of azide to the iron site of *P. gouldii* metHr. The second-order rate

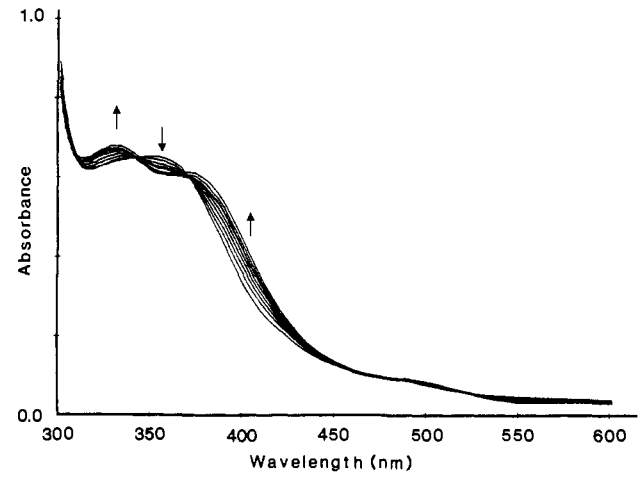

FIGURE 5: UV–visible absorption spectra of 0.10 mM metHr at 15, 30, 45, 60, 105, 120, and 180 min after addition of PEA to a concentration of 50 mM. The buffer was 0.15 M HEPES, pH 7.14. Arrows indicate directions of absorbance changes with time.

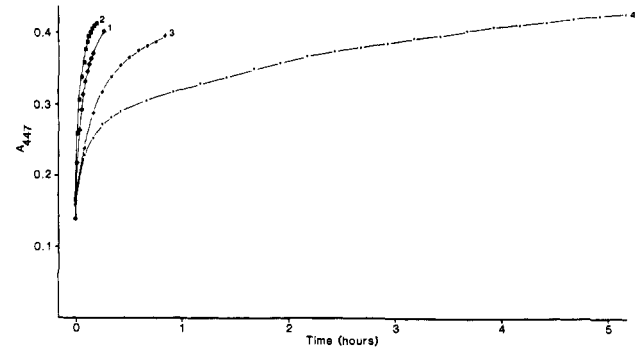

FIGURE 6: Time course of absorbance change at 447 nm after addition of 0.91 mM NaN_3 to 0.11 mM metHr in the presence of (1) no effector, (2) 2-AEP, (3) $NaClO_4$, and (4) PEA. Effector concentration was 45 mM, buffer was 0.15 M HEPES, pH 7.1, and temperature was 22 °C.

constant at 22 °C and pH 7.1 for azide binding to metHr in the presence of 330 mM PEA was determined to be 0.048 ± 0.01 M^{-1} s^{-1}. Rate constants determined at the same temperature and pH with other effectors or no effector were 1.13 ± 0.15 M^{-1} s^{-1} (45 mM $NaClO_4$), 3.68 ± 0.15 M^{-1} s^{-1} (45 mM 2-AEP), and 4.10 ± 0.15 M^{-1} s^{-1} (control, no effector). Our value for the second-order rate constant for azide binding to metHr in the absence of effectors at pH 7.1 agrees well those previously published at pH 6.3 and 8.2 (Wilkins & Harrington, 1983). We obtained satisfactory fits of the absorbance vs time data to single exponentials under all of the preceding conditions. At 45 mM PEA, the absorbance vs time data required the use of two exponentials to achieve a satisfactory fit. These two exponentials were found to generate second-order rate constants of 3.64 ± 0.15 M^{-1} s^{-1} and 0.034 ± 0.01 M^{-1} s^{-1}. These values are close to those obtained for the control and for the solution containing 330 mM PEA, respectively. An example of this two-exponential behavior is shown in Figure 6. The results of exponential fits as well as of conventional ln (A/A_0) vs time plots (included as supplementary material) show that the rate constant for azide binding to metHr is decreased by ~85-fold in the presence of excess PEA at pH 7.1. This decrease may be compared with our observation of an ~4-fold inhibition by 45 mM $NaClO_4$. Bradić and Wilkins (1983) report that 150 mM $NaClO_4$ induces an ~2-fold decrease in the rate of N_3^- binding to metHr at pH 8.5, with no biphasic behavior. Thus, perchlorate appears to exert its maximal effect at a lower concentration than does PEA. This latter observation is consistent with the much larger formation

constant reported for ClO_4^- binding to metHr (Garbett et al., 1971a).

We observed no significant effect of either 330 mM PEA or 330 mM 2-AEP on O_2 affinity of *P. gouldii* deoxyHr. Values of the O_2 half-saturation pressure, $P_{1/2}$, in millimeters of Hg determined in this work at 24 °C and pH 7.1 are 3.7 ± 0.2 (330 mM PEA), 3.7 ± 0.5 (330 mM 2-AEP), and 3.6 ± 0.5 (control, no effector). Within experimental error, these values are identical with each other and with the value of 3.5 mmHg determined by Richardson et al. (1987) at the same temperature and pH without effector. Hill plots were linear with no evidence for induction of cooperativity by PEA.

Discussion

Our results show that ^{31}P NMR is quite useful for identification of the major soluble phosphorus metabolites in sipunculan erythrocytes. Coelomic erythrocytes from the two species of sipunculids examined in this study display a novel ^{31}P NMR spectral pattern compared to those of vertebrate erythrocytes. The novelty is due primarily to the appearance of intense resonances assigned to PEA and 2-AEP and to the absence of resonances expected for 2,3-DPG or inositol polyphosphates. According to our ^{31}P NMR results, PEA and 2-AEP are the major soluble phosphorus metabolites of coelomic erythrocytes from *P. gouldii* and *T. zostericola*. Other soluble phosphorus metabolites common to erythrocytes of the two sipunculids are, in order of decreasing concentration, NTP/NDP, mannose 1-phosphate, and a phosphagen. This phosphagen was not conclusively identified but is almost certainly not phosphoarginine or phosphocreatine. Other tissues of sipunculids have been reported to contain the phosphagen *N'*-phosphoryl-2-guanidoethanesulfinate (Thoai & Robin, 1969). The sipunculan erythrocytes examined in our study reveal little or no NMR-detectable concentrations of inorganic phosphate. *P. gouldii* erythrocytes also revealed the presence of NAD/NADH, phosphoenolpyruvate, and UDPG. *T. zostericola* erythrocytes showed a resonance characteristic of a phosphomonoester, possibly a hexose 6-phosphate. The major difference between ^{31}P NMR spectra of erythrocytes from the two sipunculids is the relative abundance of PEA and 2-AEP. Relatively high levels of PEA (>20 mM) and low levels of 2-AEP were found in erythrocytes from *P. gouldii*, whereas the reverse was true for erythrocytes isolated from *T. zostericola*. We estimate the concentration of 2-AEP to be on the order of 100 mM in these latter erythrocytes.

We measured intracellular pH values of 7.2 ± 0.1 and 7.1 ± 0.1 for air-equilibrated erythrocytes from *P. gouldii* and *T. zostericola*, respectively. The pH in *P. gouldii* erythrocytes decreases to 6.5 ± 0.1 upon deoxygenation. This decrease is unlikely to be due directly to deoxygenation of Hr, because no H^+ uptake or release is observed upon oxygenation of Hr (Okamura & Klotz, 1973). A decrease in pH upon anaerobiosis has previously been observed in the coelomic fluid of the sipunculid *Sipunculus nudus* (Porter et al., 1984). However, our results represent the first measurements of pH within sipunculan erythrocytes. Our method for measuring intracellular pH relies on the chemical shift difference between intracellular phosphonate and phosphate resonances. This method is essentially the same as that described by Labotka (1984) for human erythrocytes, although a nonphysiological phosphonate was used in the latter case. Because the chemical shifts of the phosphonate and phosphate resonances have opposite dependences on pH [as can be seen from Figure 2 (inset)], this method is especially sensitive in the physiological pH range.

Since we found PEA and 2-AEP to be present in the highest concentration within sipunculan erythrocytes, we investigated their interactions with Hr. Absorption spectral changes of the iron site show that PEA binds weakly to *P. gouldii* metHr (K_f ~7 M^{-1}) in a manner suggestive of either negative cooperativity or nonspecific binding. No evidence for binding of 2-AEP to *P. gouldii* metHr was found.

Several studies have shown that coordination to iron in Hr is limited to exogenous ligands consisting of three or fewer non-hydrogen atoms (Garbett et al., 1969; Kurtz et al., 1977; Wilkins & Wilkins, 1987). We, therefore, assume that PEA is much too large to bind directly to the iron site and conclude that PEA is a heterotropic allosteric effector of metHr. We have not tested the effect of Cl^- on binding of PEA. Chloride is known to interact at noncoordinating sites on Hr (Rao & Keresztes-Nagy, 1973), and the absorption spectral changes induced by PEA (λ_{max} 330 and 378 nm) are very similar to those induced by Cl^- (Garbett et al., 1969).[3] While we cannot rule out PEA-induced coordination of OH^-, the PEA-induced spectral changes at neutral pH are distinct from those induced by OH^- (Garbett et al., 1971b; Bradič & Wilkins, 1983).

The artificial heterotropic allosteric effector perchlorate is known from X-ray difference maps (Stenkamp et al., 1978, 1983) to bind to octameric sipunculan metHrs at two different subunit interfaces and to induce changes in electron density near the exogenous ligand coordination site. However, the effect of perchlorate on the absorption spectrum of metHr (Garbett et al., 1971a) is quite different from that of PEA, suggesting different binding sites for the two effectors. This suggestion is supported by the results of our studies of N_3^- binding to metHr. PEA was found to lower the rate of N_3^- binding ~85-fold compared with an ~4-fold lowering by ClO_4^-. Furthermore, ClO_4^- has been reported to lower the O_2 affinity of *P. gouldii* deoxyHr (Richardson et al., 1987), whereas using concentrations of PEA known to inhibit N_3^- binding to metHr, we observed no significant effect of PEA on the O_2 affinity of *P. gouldii* deoxyHr. This result implies some differences in conformation between sipunculan met- vs deoxy- and oxyHr.

PEA appears to be the first example of a molecule that can distinguish deoxy- from metHr without either binding directly to or engaging in redox chemistry with the iron site.[3] This apparently selective effect on the met form may signify a means for intracellular recognition of molecules of Hr that have lost their ability to bind O_2. The formation constant for binding of PEA together with the lower limit of 20 mM intracellular PEA determined in this work means that at least 16% (calculated on a subunit basis) of the intracellular metHr would exist as a Hr–PEA complex, assuming no competition from other intracellular constituents. This percentage represents an average of greater than one PEA molecule per metHr octamer. Previous evidence from our laboratory (Utecht & Kurtz, 1985) is consistent with a small, steady-state intracellular concentration of *P. gouldii* metHr. We have recently discovered and characterized an enzymatic system in *P. gouldii* erythrocytes that is capable of catalyzing reduction of met- to deoxyHr by NADH (Utecht & Kurtz, 1987). The effects of PEA and of the other phosphorus metabolites on this metHr reduction system remain to be investigated.

In the case of 2-AEP, the lack of significant effects on either the absorption spectrum of or the rate of N_3^- binding to metHr

[3] Detection of a heterotropic allosteric effect of Cl^- on metHr is complicated by the possibility of coordination to Fe(III).

or on the O_2 affinity of deoxyHr indicates little or no interaction. This contrast with PEA may mean that the phosphate end of PEA is involved in interactions with metHr. However, phosphate itself binds even more weakly to metHr than does PEA (Garbett et al., 1971a).

PEA has previously been reported to account for 20% of the acid-soluble phosphates in hemoglobin-containing erythrocytes isolated from the Southern Fiddler Ray *Trygonorhina fasciata guanerius* (Coats et al., 1979). The function of PEA in these erythrocytes has apparently not been established. In other cell types, the metabolic role of phosphomonesters such as PEA as lipid precursors is considered to be well established.

2-AEP was the first compound containing a carbon–phosphorus bond ever reported in biological materials (Horiguchi & Kandatsu, 1959). 2-AEP has since been found in a wide variety of organisms and tissues although apparently not heretofore in erythrocytes (Hilderbrand et al., 1983). Some organisms appear to use 2-AEP as a source of the more oxidized phosphate phosphorus (Meneses et al., 1987). Such a function in sipunculan erythrocytes may be reflected in the detection of little or no inorganic phosphate by ^{31}P NMR. Metal ion transport and osmoregulatory roles have been suggested for 2-AEP in marine organisms (Mohan & Abbott, 1978; Kiss et al., 1987; Hilderbrand et al., 1983). We have qualitatively observed that 2-AEP increases the solubility of *P. gouldii* deoxyHr. This presumed ionic strength effect may help maintain the high concentration of Hr (≥10 mM on a subunit basis (Utecht, 1986) within sipunculan erythrocytes.

Whatever their functions may be, the combination of high intracellular concentrations of PEA and 2-AEP appears to be unique to sipunculan erythrocytes. The phosphorus metabolites and pH values identified and quantitated in this study represent previously unknown parameters that must be taken into account in any descriptions of the metabolism and enzymology of sipunculan erythrocytes. With our results as a basis, rapid and convenient ^{31}P NMR methods for measuring such parameters are now available.

Supplementary Material Available

Figures depicting Scatchard and Hill plots of PEA binding to metHr, ln (A/A_0) vs time plots comparing inhibition of N_3^- binding to metHr by PEA, 2-AEP, and $NaClO_4$, and the standard curve used to determine pH within erythrocytes (4 pages). Ordering information is given on any current masthead page.

Registry No. PEA, 1071-23-4; 2-AEP, 2041-14-7; NAD, 53-84-9; NADH, 58-68-4; UDPG, 133-89-1; PEP, 73-89-2; mannose 1-phosphate, 19993-19-2; azide, 14343-69-2.

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Crystallographic Analysis of the Binding of NADPH, NADPH Fragments, and NADPH Analogues to Glutathione Reductase

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ABSTRACT: The binding of the substrate NADPH as well as a number of fragments and derivatives of NADPH to glutathione reductase from human erythrocytes has been investigated by using X-ray crystallography. Crystals of the enzyme were soaked with the compounds of interest, and then the diffraction intensities were collected out to a resolution of 3 Å. By use of phase information from the refined structure of the native enzyme in its oxidized state, electron density maps could be calculated. Difference Fourier electron density maps with coefficients $F_{soak} - F_{native}$ showed that the ligands tested bound either at the functional NADPH binding site or not at all. Electron density maps with coefficients $2F_{soak} - F_{native}$ were used to estimate occupancies for various parts of the bound ligands. This revealed that all ligands except NADPH and NADH, which were fully bound, showed differential binding between the adenine end and the nicotinamide end of the molecule: The adenine end always bound with a higher occupancy than the nicotinamide end. Models were built for the protein–ligand complexes and subjected to restrained refinement at 3-Å resolution. The mode of binding of NADPH, including the conformational changes of the protein, is described. NADH binding is clearly shown to involve a trapped inorganic phosphate at the position normally occupied by the 2′-phosphate of NADPH. A comparison of the binding of NADPH with the binding of the fragments and analogues provides a structural explanation for their relative binding affinities. In this respect, proper charge and hydrogen-bonding characteristics of buried parts of the ligand seem to be particularly important.

Glutathione reductase (EC 1.6.4.2) is a ubiquitous enzyme responsible for the maintenance of a high ratio of GSH/GSSG by the reaction GSSG + NADPH + $H^+ \rightleftharpoons$ 2GSH + $NADP^+$. In the course of the reaction the reduction equivalents are transferred from the nicotinamide to an FAD prosthetic group and then, via a redox-active disulfide bridge, to GSSG (Williams, 1976; Pai & Schulz, 1983). Glutathione reductase belongs to a family of structurally and mechanistically related enzymes that includes lipoamide dehydrogenase (Williams, 1976), asparagusate dehydrogenase (Yanagawa, 1979), trypanothione reductase (Shames et al., 1986; Krauth-Siegel et al., 1987), and mercuric reductase (Fox & Walsh, 1983). The kinetics of catalysis and the binding of nucleotides to the NAD(P)H binding site have been well studied for a number of these enzymes.

The structure of the oxidized, substrate-free form of glutathione reductase from human erythrocytes has recently been refined at 1.54-Å resolution (Karplus & Schulz, 1987), providing a context in which medium-resolution substrate binding studies can be interpreted with a reasonable level of accuracy. This paper describes the NADPH binding site as derived from 3-Å resolution studies of the enzyme complexed with either NADPH or other related ligands. The mode of binding to glutathione reductase is discussed in light of the results of

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