Table I. Spectral Comparison of *Lingula reevii* and *Phascolopsis gouldii* Hemerythrins

	UV-vis, nm (M^{-1} cm^{-1})[a]	circular dichroism, nm (M^{-1} cm^{-1})[a]	Raman, cm^{-1} [b]
oxy-Hr			
L.r.	507 (2100), 332 (6000)	795 (−0.4), 518 (−3.5), 340 (−5.3)	505 (Fe-O), 844 (O-O)
P.g.	500 (2300), 326 (6900)	791 (−0.36), 520 (−2.5), 336 (−3.8)	505, 844
metazido-Hr			
L.r	448 (3900), 329 (6900)	500 (−5.2), 367 (−12.0)	376 (Fe-N), 511 (FeOFe), 2052 (N_3^-)
P.g.	446 (3800), 327 (7200)	500 (−4.1), 370 (−9.0)	376, 510, 2054

[a] Principal band maxima. Intensities are based on concentration of iron dimers (2 Fe/subunit) as determined by atomic absorption. Intensities for *P.g.* Hr are quoted from ref 15. [b] Frequencies have been measured relative to sulfate at 984 cm^{-1}.

Results of our tonometric oxygen-binding studies are summarized in Figure 1.[15] The binding is cooperative and shows the Root effect,[16] with the maximum Hill slope[17] highest at pH 7.7 (n_{max} = 2.0) and decreasing at higher and lower pH values (n_{max} = 1.1 at pH 6.3–6.6). Manwell studied the oxygen equilibrium of *Lingula unguis* hemerythrin[18] and reports that n_{max} = 1.7 at pH 7.6 (phosphate) with the value of n_{max} decreasing to ~1 at pH 6.7–6.8.

The oxygen-binding curves have been fit by using a modified relaxed-tense model.[19] It is notable that the oxygen affinity of sipunculid coelomic hemerythrins (~3 mmHg)[3] is close to that estimated for the relaxed form of *L.r.* Hr (K_R = 2 mm). This implies the presence of structural constraints[20] in the tense form (K_T = 20 mm) of *L.r.* Hr. The allosteric energy, given by the difference in free energies of R and T oxygen binding, is ~1.5 kcal/mol.

The absorbance, CD, and resonance Raman data from *L.r.* oxy-Hr and metazido-Hr are correlated to analogous results for *Phascolopsis gouldii* Hr in Table I. All spectral features of *L.r.* Hr are extremely similar to those of *P. gouldii*, and two are particularly characteristic of the peroxide binding mode. Single-crystal polarized spectral studies[10] of the $O_2^{2-} \rightarrow$ Fe(III) charge-transfer band in *P. gouldii* oxy-Hr were initially used to determine that the peroxide is bound end-on (II), and the similar energy (507 nm) and intensity of the band in *L.r.* Hr implies no significant differences in bonding geometry. In the resonance Raman spectrum the Fe–(O_2^{2-}) stretching frequency of oxy-Hr is expected to be sensitive to the peroxide binding mode. A normal coordinate analysis predicts that the terminally bound Fe–O stretch observed at 505 cm^{-1} for *P. gouldii* oxy-Hr would shift to ~350 and 456 cm^{-1} for μ-1,1 and -1,2 bridging geometries, respectively.[21]

The comparison of spectroscopic properties in Table I strongly implies that the terminal exogenous ligand binding mode of sipunculid Hr is maintained in *L.r.* Hr. This demonstrates that the hemerythrin binuclear non-heme iron site is capable of engaging in cooperative interactions and that a relaxed-tense conformational change in the protein quaternary structure can control the affinity of the active site for oxygen. Thus, cooperativity has been found in coupled binuclear metalloprotein oxygen carriers that have either end-on or bridging peroxide geometries. Although the μ-peroxo geometry may be important in homotropic allosteric effects of hemocyanins, the present results indicate that other mechanisms can contribute significantly to cooperativity in this type of active site.

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Registry No. O_2, 7782-44-7; Fe, 7439-89-6.

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Iron–Histidine Stretching Raman Lines of the aa_3-Type Cytochrome Oxidases

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Cytochrome oxidase (EC 1.9.3.1), a terminal oxidase of the respiratory chain, couples cytochrome *c* oxidation with dioxygen reduction and simultaneously translocates protons across an energy-transducing membrane (see ref 2 for review). The aa_3-type cytochrome oxidase is composed of two active centers, namely, cytochrome *a* and a_3. The former consists of a low-spin heme *a* and an EPR-active copper, and the latter contains a high-spin heme *a* and an EPR-silent copper antiferromagnetically coupled with the high-spin iron.[3] The a_3 heme serves as the catalytic site for dioxygen reduction, but for the resting enzyme the occupation of a sulfur ligand in the catalytic site is proposed from the EXAFS study.[4] Thus, elucidation of the iron coordination environments of the a_3 heme is a matter of current spectroscopic concern. We report here the iron–histidine stretching Raman line of the reduced a_3 heme of mammalian, yeast, and bacterial cytochrome oxidases.

The Fe–His stretching Raman line of the five-coordinate ferrous high-spin hemoproteins[5,6] is strongly intensity enhanced upon excitation around 442 nm, and its frequency is noticeably sensitive to a state of the coordinated histidine.[7] Presence of appreciable strain in the Fe–His bond of the low-affinity deoxy-Hb was first revealed by the observation of the Fe–His stretching Raman line,[8] and now its frequency difference between the α and β subunits is discussed.[9,10] The structural implication of the frequency difference between the Fe–His stretching modes of peroxidases and oxygen carriers was also interpreted satisfactorily.[11] A large

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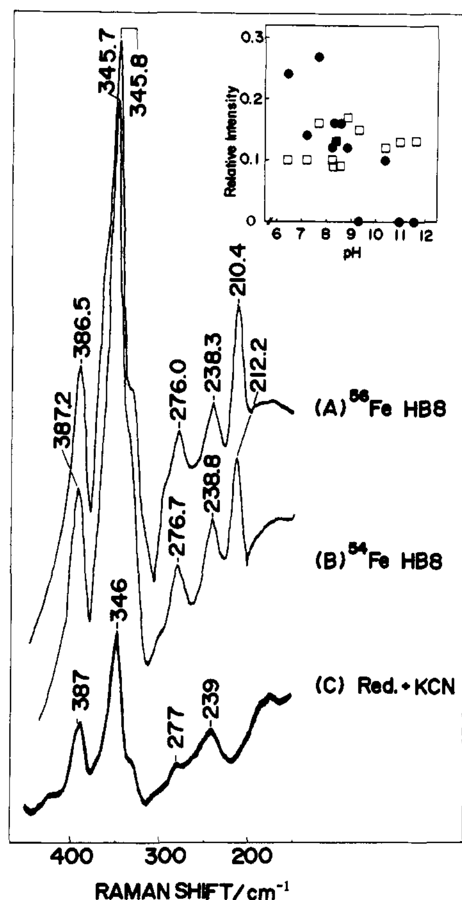

Figure 1. Resonance Raman spectra of ^{56}Fe- (A) and ^{54}Fe-substituted (B) reduced cytochrome oxidase of *T. thermophilus HB8* and its reduced and cyanide-bound form (C) excited at 441.6 nm. Enzyme was dissolved in a 20 mM Tris/HCl buffer (pH 7.7) containing 0.5% Triton X-100 and 0.1 mM EDTA. Heme *a* concentration was ca. 20 μM. Reduction of the enzyme was performed by addition of a minimal amount of solid dithionite after mixing ascorbate (final concentration 50 mM) and *N*,-*N*,*N'*,*N'*-tetramethylphenylenediamine (final concentration, 2.5 mM) with the enzyme solution. Immediately after the reduction, the cell was degased under 10^{-2} mmHg at room temperature. Cyanide-bound form was obtained by addition of cyanide solution to the reduced enzyme (final concentration, 40 mM) followed by pH readjustment to 7.1 with HCl. Instrumental conditions: laser 80 mW; slit width 5 cm^{-1}, sensitivity 1000 counts/s for spectra A and B and 2500 counts/s for spectrum C, scan speed 10 cm^{-1}/min, time constant 8 s. The inset plots the relative intensities of Raman lines against pH: I_{210}/I_{346} (●) and I_{276}/I_{346} (□). Upon the pH variation, a small amount of HCl or NaOH was added to the resting enzyme, and then it was reduced in the same way as above. The pH values were determined after the Raman measurements with the use of a Beckman pH meter (Model $\phi 71$).

number of Raman studies are reported about cytochrome oxidases and model compounds (ref 12 and references therein), and the coordination of histidine to the axial position of the a_3 heme of cytochrome oxidase is demonstrated.[13] Nonetheless, there has been no conclusive evidence for the assignments of the Fe–His stretching Raman line presumably due to serious difficulty in reconstitution of the enzyme with an isotope-substituted heme *a*. We have overcome this problem through growing a bacterium in an isotope-replaced medium.

A thermophilic bacterium (*Thermus thermophilus HB8*) was cultivated in a ^{56}Fe- and ^{54}Fe-enriched (ORNL, 97.08%) synthetic medium,[14] and cytochrome oxidase was isolated with reported method.[15] Preparation of beef heart cytochrome oxidase is described elsewhere.[16] Raman scattering was excited at 441.6 nm with a He/Cd laser (Kinmon Electrics, CDR80MGH) and recorded with a JEOL-400D Raman spectrometer. Calibration of the spectrometer was performed with CCl_4 as a standard.

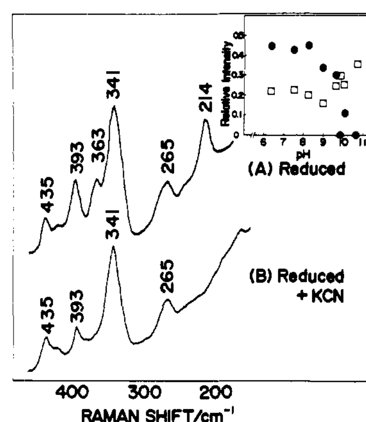

Figure 2. Resonance Raman spectra of reduced (A) and reduced and cyanide-bound (B) forms of beef heart cytochrome oxidase. Enzyme was dissolved in a 20 mM sodium phosphate buffer (pH 7.4) containing 0.5% Brij 35. Heme *a* concentration was ca. 20 μM. Reduction of the enzyme and formation of the cyanide-bound form were accomplished in the same way as for the *T. thermophilus* enzyme except for pH 8.0 used for the cyanide-bound form. Sensitivity of the spectrometer was 2500 counts/s. Other instrumental conditions were the same as those in Figure 1. The inset plots the relative intensities of Raman lines against pH: I_{214}/I_{341} (●) and I_{265}/I_{341} (□).

Figure 1 shows the resonance Raman (RR) spectra of the ^{56}Fe- (A) and ^{54}Fe-incorporated (B) *T. thermophilus* cytochrome oxidase in the reduced state. The Raman line at 210 cm^{-1} exhibited an upward frequency shift by 1.8 cm^{-1} upon the ^{54}Fe substitution while other lines showed little frequency shift.[17] For an isolated Fe–His stretching vibration, the expected frequency shift upon ^{54}Fe substitution is 2.1 cm^{-1} in the two-body harmonic approximation, and actually the Fe–His stretching Raman lines of deoxy-Mb[6] and ferro-HRP[11] exhibited a 2-cm^{-1} shift upon the ^{54}Fe substitution of the heme iron. Accordingly, the 210-cm^{-1} line is considered to arise primarily from the Fe–His stretching vibration of the five-coordinate ferrous high-spin a_3 heme.

This Raman line disappeared upon addition of cyanide as shown at the bottom trace of Figure 1. This feature is also consistent with the present assignment, since the Fe–His stretching Raman line of ferro-HRP disappeared upon binding of cyanide.[18] The inset of Figure 1 plots the relative intensities of Raman lines, I_{210}/I_{346} and I_{276}/I_{346}, against pH. It is apparent that the 210-cm^{-1} line lost intensity around pH 9.5 while the 276-cm^{-1} line and other porphyrin modes as well remained unaltered until pH 11.6. Such pH-dependent intensity change was previously noticed only for the Fe–His stretching Raman lines of several plant peroxidases[19] and is again consistent with the present assignment.

Figure 2 displays the RR spectra of reduced (A) and reduced and cyanide-bound (B) forms of beef heart cytochrome oxidase. The Raman spectral pattern somewhat differs between the bacterial and beef enzymes due to dissimilar heme environments. For the beef enzyme the 214-cm^{-1} line disappeared upon binding of cyanide similar to the 210-cm^{-1} line of the bacterial enzyme. The

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inset of Figure 2 plots the pH dependence of the relative intensity of the 214- and 265-cm^{-1} peaks of beef enzyme. In the same way as in Figure 1, the 214-cm^{-1} line diminished around pH 10, while the 265-cm^{-1} and other lines exhibited no change until pH 11. Accordingly, the 214-cm^{-1} line of beef heart cytochrome oxidase is also assignable to the Fe–His stretching mode.

With regard to the spectral changes upon cyanide binding and pH variation, the Raman line of yeast cytochrome oxidase at 220 cm^{-1} and that of thermophilic bacterium PS3 enzyme at 213 cm^{-1} displayed very similar behavior to that of the 210-cm^{-1} line of the *T. thermophilus* cytochrome oxidase.[20] Consequently, these lines are also considered to involve mainly the Fe–His stretching vibration. In the parallel study of Raman spectra and the enzymatic activities of PS3 cytochrome oxidase, it was found that the incubation-temperature dependence of the intensity of the 213-cm^{-1} line was close to that of the proton-pump activity measured after incorporating the enzyme used for Raman experiments into phospholipid liposome, in contrast with the fact that the incubation-temperature dependence of the intensity of the 1667-cm^{-1} line of the a_3 formyl stretching mode was almost coincident with that of cytochrome *c* oxidase activity.[20] The present assignment will provide a new experimental base for more advanced interpretation of those Raman data.

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Registry No. Fe, 7439-89-6; His, 71-00-1; cytochrome oxidase, 9001-16-5.

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Facile Syntheses of Bridge-Oxygen-Labeled Pyrophosphates: The Preparation of Adenosine 5′-[β,γ-^{18}O]Triphosphate

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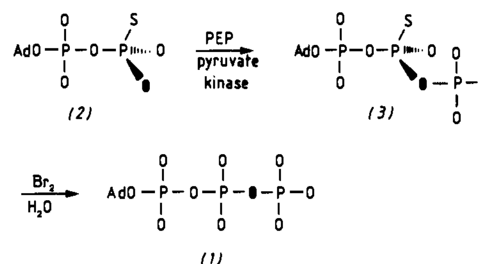
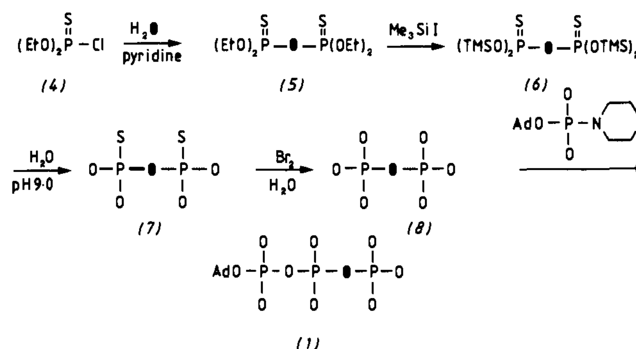
We report here the first syntheses of specifically bridge-oxygen-labeled pyrophosphates illustrated by two synthetic approaches to adenosine 5′-[β,γ-^{18}O]triphosphate (1) that exploit the facile displacement of sulfur from phosphorothioates using bromine in water.[1]

The synthesis of nucleotides stereospecifically enriched with the stable isotopes of oxygen has allowed detailed probing of the mechanisms of many important enzyme-catalyzed reactions at phosphorus; these studies include the positional isotope exchange analysis first reported by Midelfort and Rose[2] and the many stereochemical analyses that have recently been reported.[3] Hitherto there has been *no* method for specifically labeling the bridge position in pyrophosphates, despite the fact that such species would indeed be powerful mechanistic probes, simplifying the positional isotope exchange analysis and facilitating the measurement of heavy-atom kinetic isotope effects. The synthetic difficulties presumed to be inherent in this objective have meant that previous studies have been done with nucleoside triphosphates that are labeled in the *nonbridging* positions.[2,4] The syntheses

Scheme I



Scheme II



described here overcome these difficulties.

Our first synthesis exploits the stereospecific enzyme-catalyzed phosphorylation of ADPβS (2).[5] Pyruvate kinase, for example catalyzes the phosphorylation of the *pro-S* position of P_β whereas acetate kinase catalyzes phosphorylation of the *pro-R* position.[6] The synthesis of (R_P)- and (S_P)-[β-^{18}O]ADPβS has been reported,[7] and by choosing the appropriate enzyme it was possible to phosphorylate exclusively the ^{18}O site. We synthesised (S_P)-[β,γ-^{18}O]ATPβS (3) in this way and converted it to [β,γ-^{18}O]ATP (1) by treatment with bromine in water[1] as shown in Scheme I. The yield of **1** based on [β-^{18}O]ADPβS (2) was 32%, and the material was shown to be identical with authentic ATP by enzyme assay. The location of ^{18}O in the $\beta\gamma$-bridge follows from the synthetic route and was confirmed by ^{31}P NMR (data not shown).

There are a number of objections to the route shown in Scheme I. The major drawback is that the synthesis and separation of the diastereoisomers of [β-^{18}O]ADPβS (**2**) is far from trivial, and it would seem excessive to generate isotopic chirality that is ultimately lost in the product. Other objections are that the method gives poor yield with respect to isotope, the level of enrichment in **1** is only ~80%, and the strategy lacks generality. These considerations led us to develop a second route that addresses these difficulties.

The synthesis is outlined in Scheme II. Diethoxythiophosphoryl chloride (**4**) when treated with 0.5 equiv. of water in the presence of base reacts to give tetraethyl dithiopyrophosphate in almost quantitative yield.[8] The reaction was carried out with $H_2{}^{18}O$ (98.3 atom %) to give **5**. The ethyl groups were removed by treatment with trimethylsilyl iodide[9] to give the tetrakis(trimethylsilyl) dithiopyrophosphate (**6**). The trimethylsilyl groups are extremely hydrolytically labile and were removed by stirring in sodium bicarbonate buffer, pH 9.5. The symmetrical dithiopyrophosphate (**7**)[10] was isolated by ion-exchange chromatography on DEAE sephadex in a yield of 54% based on $H_2{}^{18}O$.

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