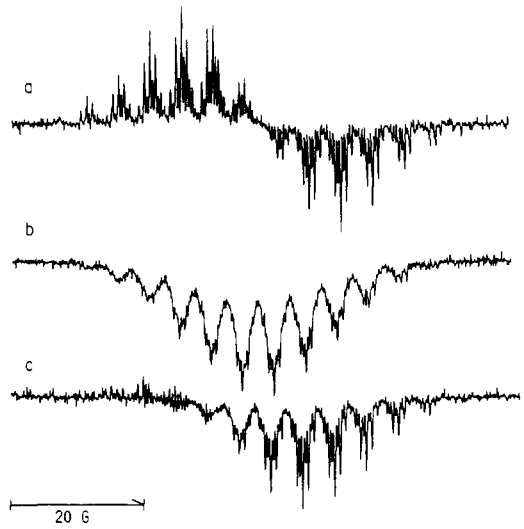

Figure 1. Transient ESR spectra of the 1,4-dimethylpyridinyl radical observed 1 μs after the laser pulse photolysis of the dimer at −45 °C in toluene: (a) without sensitizer, (b) in the presence of 2-acetonaphthone (0.1 M), and (c) in the presence of benzophenone (0.2 M).

The transient spectrum shown in Figure 1a was obtained by the laser photolysis of the pyridinyl dimer at −45 °C. In our previous paper,[4] a singlet radical pair precursor was proposed for the photolytic generation of the radical on the basis of the observation of A/E polarization. This proposal is supported by the subsequent results: (a) the CIDEP signal was clearly weakened by the addition of the singlet quencher, biacetyl (E_s = 272 kJ/mol),[5] and (b) oxygen showed no influence on the spin polarization. The dimer of the present radical has the lowest excited singlet state at about 330 kJ/mol above the ground state, as estimated roughly from the absorption spectrum, though no fluorescence spectrum has been observed.

Addition of 2-acetonaphthone, a triplet sensitizer, to the dimer solution caused a significant change of the CIDEP spectrum, as shown in Figure 1b. This sensitizer has a relatively long lifetime of the $\pi\pi^*$-phosphorescence (τ_p = 0.95 s)[6] and a large quantum yield of the intersystem crossing (ISC) (Φ_{ISC} = 0.84).[7] The complete emission spectrum of Figure 1b is essentially due to the 1,4-dimethylpyridinyl radical, though its hyperfine structure shows broadening. The half-life of the E polarization ($\tau_{1/2} \simeq 4$ μs) is longer than that of A/E polarization (1 μs) of Figure 1a. The observation of E spectrum suggests the participation of triplet mechanism in the process of the radical generation and it would be considered that the pyridinyl was generated by homolytic cleavage of excited state of the dimer formed through an energy transfer from the polarized triplet state of 2-acetonaphthone. In spite of the lower absorption coefficient of 2-acetonaphthone at 337 nm ($\epsilon_{337} \simeq 1600$) than that of the dimer ($\epsilon_{337} \simeq 4700$), triplet photosensitization occurred efficiently in the CIDEP. This is probably due to the higher possibility of photolytic cleavage of the dimer from the triplet state, as compared with that from the singlet state, and also to the large quantum yield of ISC of the excited 2-acetonaphthone. The E polarization also indicates that a highly spin-specific ISC to the triplet sublevel occurred in 2-acetonaphthone and the energy transfer was faster than the spin–lattice relaxation of the triplet state in this fluid system.

The effect of sensitizer was also examined by using benzophenone which has a $n\pi^*$ triplet of the first excited state. The quantum yield of ISC of the excited benzophenone is 1.0 and the ISC proceeds in highly spin-specific manner, while the half-life of the triplet state is very short (τ_p < 80 ms at 77 K).[8] Figure 1c shows the time-resolved ESR spectrum of the radical generated from the dimer in the presence of 0.2 M benzophenone observed 1 μs after the laser pulse irradiation. In this spectrum with an A/E* polarization, the absorption signal is compensated at the low field by the emission due to the sensitization by benzophenone. The complete E polarization was observed 3 μs after the photolysis in this system, because the E polarization due to triplet mechanism decayed slowly ($\tau_{1/2} \simeq 4$ μs). This result means that the triplet excitation transfer takes place in this system regardless of the short lifetime of the triplet state and the low absorption coefficient at 337 nm ($\epsilon_{337} \simeq 200$) of benzophenone. The mutual relation of the levels of excited states in the pyridinyl dimer and benzophenone makes double energy transfer possible, as demonstrated in the coumarine–benzophenone system,[9] that is, energy transfers from S_1 of the dimer to S_1 (320 kJ/mol) of benzophenone and then from T_1 (290 kJ/mol) of the latter to the dimer after the spin-specific ISC. During the ISC, the highest sublevel, P_+, of benzophenone is occupied with highest population at high magnetic field, because the zero-field parameter, D, is negative for the $n\pi^*$ state and the ISC rate into T_z is significantly larger than those into T_x and T_y (in which the z axis was chosen parallel to the C=O direction).

In contrast to the above results, fluorenone (E_T = 226 kJ/mol, $\pi\pi^*$) showed no effect on the CIDEP pattern of the radical, though the intensity decreased and the polarized signal diminished at high concentration (0.5 M) of fluorenone. The result is ascribed to that either the spin polarization conservation occurs less effectively or no energy transfer is caused because of the lower triplet energy of fluorenone compared with that of the pyridinyl dimer.

The present results clearly prove that the spin polarization is conserved during the triplet energy transfer in the system involving both $\pi\pi^*$ and $n\pi^*$ states, even in fluid solution. Therefore, the excitation transfer rate is faster than the triplet spin–lattice relaxation $1/T_1$. No T_1 value of benzophenone in fluid solution is available, while T_1 = 19 μs at 77 K.[10] Since the energy transfer proceeds with diffusion controlled rate, T_1 values of benzophenone and acetonaphthone would be larger than the order of nanosecond.

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MCD–EPR Studies of Deoxy[Fe^{II},Fe^{II}]hemerythrin: Probes of Endogenous Bridging Ligands and Exogenous Ligand Binding

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The geometric structure of the binuclear nonheme iron active site in met[Fe^{III},Fe^{III}]hemerythrin[1] (Hr) is known to high resolution from crystallography[2] and is best described as two ferric ions bridged by a μ-oxo anion and the carboxylate side chains of glutamate and aspartate amino acid residues. In addition, one iron binds the imidazole groups from three histidines, while the other binds two histidines and the exogenous ligand.[3] The ex-

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ogenous ligand in the oxygenated complex is peroxide,[4] which can be replaced by anions in the met X^- forms. Since ferrous iron is typically EPR silent, and has no easily detected optical features, deoxyhemerythrin is not well characterized. Weak ligand field absorbance bands have been reported[5] near 1000 nm, and Mössbauer spectroscopy[6] shows high-spin Fe(II) ($S = 2$) is present, with low-temperature magnetic results[6,7] interpreted as indicating large zero field splitting (ZFS).

The binuclear high-spin ferrous ($S_1 = 2$, $S_2 = 2$) active site can be considered from two limiting types of magnetic ground states. In one the irons behave as noninteracting, isolated ferrous ions, each having magnetic properties governed by zero field splitting. In the limit of axial geometry, this generates an $M_S = 0$ singlet, and $M_S = \pm 1$ and $M_S = \pm 2$ doublets, split by D and $3D$, respectively, for each iron. Alternatively, the ions may interact to produce an exchange-coupled binuclear iron system, giving five levels described by their total spin $S_T = 0$, 1, 2, 3, and 4, split by energies of $2J$, $4J$, $6J$, and $8J$, respectively. In the intermediate case, ZFS and exchange coupling are of comparable magnitude, resulting in complicated splitting of the binuclear ferrous ground-state energy levels.

We have observed large CD features[8] in the IR region due to spin-allowed ligand field transitions in the ferrous forms of hemerythrin. The unusual temperature dependence of these bands in MCD spectroscopy can be used to directly probe the lowest sublevels of the ground state and the splitting to higher levels,[9] allowing us to distinguish among these types of ground states and to estimate the values of D and J.

Oxyhemerythrin was obtained from *Phascolopsis gouldii* by a procedure similar to that of Klotz et al.[10] and was reduced with $Na_2S_2O_4$. Excess reductant was removed by anaerobic dialysis against Tris–sulfate buffer (0.1 M, pH 7.7). The resultant deoxy protein was added to 1.5 vol of degassed glycerol, and NaCl was added (0.2 M final concentration) to enhance the optical quality of the glass.[11] This sample was injected through a rubber spacer into a quartz disk sandwich and placed into an Oxford SM4-6T magnet system. Spectra were taken at 1100 nm on a Jasco J500C spectropolarimeter with a specially constructed sample compartment.[12]

The results for deoxy-Hr are shown in Figure 1A. At 4.5 K the 5-T spectrum is coincident with the zero field spectrum, therefore showing no observable MCD signal. The broad band at 1000 nm is simply the CD spectrum of deoxy-Hr, with both irons contributing ligand field transitions in this region.[8] As the temperature is increased while keeping the applied field constant at 5 T, a very weak MCD signal appears, reaching its greatest intensity at about 65 K and then losing intensity slowly with increasing temperature. The lack of signal at lowest temperatures requires a singlet ground state for the binuclear site, while the temperature dependence can be fit in the two limiting cases with $D = 35 \pm 10$ cm^{-1} or alternatively with $-J = 20 \pm 7$ cm^{-1}.

This large a value of D is inconsistent with the zero field splitting observed for a large number of mononuclear ferrous complexes of coordination numbers 4, 5, and 6.[13] For high-spin systems,

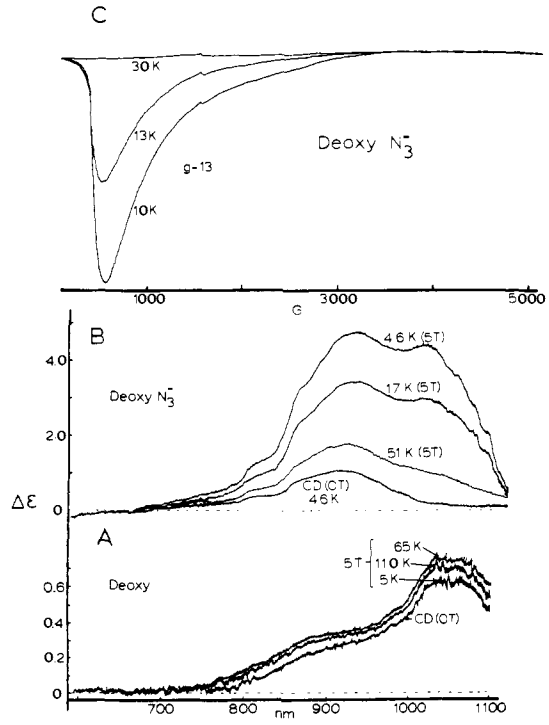

Figure 1. MCD and EPR spectra of reduced hemerythrin species. (A) Deoxyhemerythrin in 0.1 M pH 7.7 Tris–SO_4 buffer, in 60% glycerol, 0.2 M NaCl glass. MCD spectra at 65, 110, and 5 K, all at 5-T field, and CD (0 T) base line at 5 K. Note 5- and 0-T spectra at 5 K coincide, and signal is most intense at 65 K. (B) Deoxy N_3^- hemerythrin (0.2 M NaN_3 replaces NaCl). MCD spectra at 4.6, 17, and 51 K, all at 5 T, and zero field MCD base line. (Note scale change between A and B.) (C) N_3^-–deoxy-Hr EPR. Gain 8×10^3, time constant 50 ms, frequency 9.388 gHz, 100–5100-G scan. Protein was 3 mM, in 0.1 M Tris–SO_4^{2-} buffer pH 7.7, 0.2 M NaN_3^-. The EPR spectra shown are for 30, 13, and 10 K and a base line.

the largest D values[14] are 10–12 cm^{-1}, with the majority[14b] lying between 2 and 8 cm^{-1}. If we then assume the largest reasonable value[15] for D of 12 cm^{-1} and fit the deoxy Hr MCD temperature dependence with the general model which allows for exchange coupling together with ZFS, we can estimate a lower limit on the value for the exchange coupling of $-J = 13 \pm 5$ cm^{-1}. On the basis of existing model complexes,[16] carboxylate bridges, which might be present in deoxy based on the met structure, are expected to mediate exchange coupling of at most 4 cm^{-1} and cannot adequately account for the strong diamagnetic nature of deoxy-Hr shown by the MCD results.

Thus, an additional endogenous bridging ligand is required to account for this coupling. On the basis of the met structure, the

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(15) Ligand field theory relates the ground-state ZFS in low-symmetry ferrous complexes to spin–orbit interactions ($\zeta L \cdot S$) with excited states, with the dominant contribution arising from the split orbital components (^5E and ^5B) of the $^5T_2$ octahedral ground state. A rough estimate of the D value is thus obtained from the expression[13b]

$$D = \frac{\zeta^2}{16} \frac{1}{[E(^5E) - E(^5B)]}$$

Here the ZFS is predicted to be inversely proportional to the $^5T_2$ splitting. This behavior is qualitatively observed in the ferrous complexes listed above.[14b] The Mössbauer spectrum[6] of deoxy Hr indicates an orbital singlet ground state, with a large $^5T_2$ splitting of greater than 500 cm^{-1}. For $\zeta = 280$ cm^{-1}, this equation gives $D < +9.8$ cm^{-1}.

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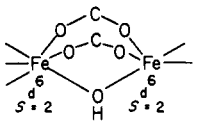
most reasonable choice is a hydroxo (or oxo) bridge, although a large protein rearrangement could lead to additional possibilities. No hydroxo or oxo bridged binuclear ferrous complexes are presently known, but it can be estimated that exchange in these systems should be somewhat larger than that of analogous Fe(III) dimers.[17] Values of $-J = 17$ and $\cong 100$ cm^{-1} have been reported for similar ferric model complexes[18] with hydroxide and oxo bridges, respectively. Thus while the actual magnitude of the exchange coupling is dependent on a number of factors, such as bridging angle, these values do indicate that a hydroxo or oxo bridging ligand is capable of mediating a $-J \geq 13$ cm^{-1} in a binuclear Fe(II) site. Thus the variable-temperature MCD is consistent with the presence of a hydroxo (or possibly oxo) bridge in deoxyhemerythrin. This, combined with an analysis of the near-IR–CD spectrum[8] which indicates one six- and one five-coordinate Fe(II), leads to a preliminary model for the deoxy active site.



Pertubations of the d–d bands in the CD spectrum (compare Figure 1, parts A amd B, zero field spectra) show that N_3^- and OCN^- ($K_B \cong 70$ M^{-1} at pH 7.7) and also F^- ($K_B \cong 7$ M^{-1}) bind to deoxy-Hr, making both irons six coordinate.[8] No other ligands (including Cl^-, Br^-, SCN^-, NCN^{2-}, or CN^-) bind with $K_B \geq 0.5$ M^{-1}. The MCD of these ligand-bound forms is dramatically different from that of deoxy-Hr, as shown for deoxy N_3^- in Figure 1b. The intensity now increases upon going to low temperatures requiring a paramagnetic ground state. In addition, the signal saturates very easily at low temperatures and high fields, generating a saturation–magnetization curve[19] consistent with $g_{eff} > 8$. This behavior indicates the ground state of N_3^-–deoxy-Hr is dominated by a large negative ZFS. Although some contribution from exchange coupling may also be present, its magnitude cannot be accurately determined from the present data. This ground state can give rise to EPR signals if limited rhombic splitting exists and relaxation is slow.[20] We do in fact observe an EPR signal (Figure 1C) that behaves as expected for an $M_S = \pm 2$ doublet, having $g_{eff} \cong 13$ and broadening rapidly with increasing temperature, finally disappearing above ~40 K.

It should be noted that these results provide a reasonable explanation for exchange of ^{18}O into the oxo bridge in the met derivatives, which is observed with only certain exogenous ligands present.[21] The ligands that lead to rapid ^{18}O exchange when the met species are formed (a deoxy intermediate has been implicated[22] in this process) are those that we find to bind strongly to deoxy-Hr and drastically alter the ground-state properties. This is consistent with exogenous ligand binding labilizing an endogenous hydroxo bridge.

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(17) This estimate results from the relation of the many-electron J to the one-electron J_{ij}, $J = (1/n^2) \sum_{ij} J_{ij}$, where i and j are the electrons on each iron center and n is the number of unpaired electrons on each Fe. The J for Fe(II) can be estimated to be on the order of 25/16 of J for Fe(III).

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Neither the Retinal Ring nor the Ring Double Bond Is Required for Proton Pumping in Bacteriorhodopsin: Acyclic Retinal Bacterioopsin Analogues

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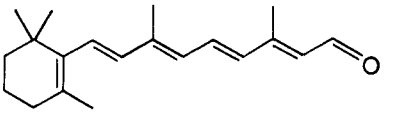
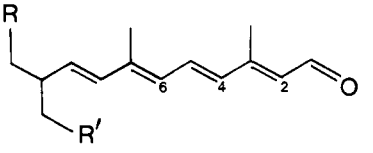
The light-transducing pigment bacteriorhodopsin is the protein in the purple membrane of the halophilic bacteria *Halobacterium halobium* responsible for proton pumping. Like the visual pigment rhodopsin, its chromophore, retinal **1**, binds with the apomembrane



(1)



(2) R=R′=CH_3

(3) R=H; R′=CH_3

(4) R=R′=H

by a protonated Schiff base linkage with the ϵ-amino group of a lysine residue. However, the mechanism of the recombination and interaction of retinal with the respective apoproteins, bacterioopsin and opsin, may be quite different. In the case of the rhodopsin, a specific binding site for one or more of the ring methyl groups has been suggested based on the data that β-ionone[1] and methylated cyclohexanes[2] inhibit the rate of rhodopsin regeneration and that the acyclic retinals lacking "ring methyl groups" do not form pigments with opsin.[3] However, β-ionone and cyclocitral have no inhibitory effect on the regeneration rate of bacteriorhodopsin.[4] Moreover, several retinal analogues such as the spin-labeled retinal[5] and phenyl retinal,[6] which have drastic modifications of the ring, do form pigments with bacteriorhodopsin although the λ_{max} are significantly blue shifted. These shifts may be due to a disruption of the secondary interaction of the chromophore with the charged group in the ring region, which was proposed to account for the red shift of bacteriorhodopsin.[7] All

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