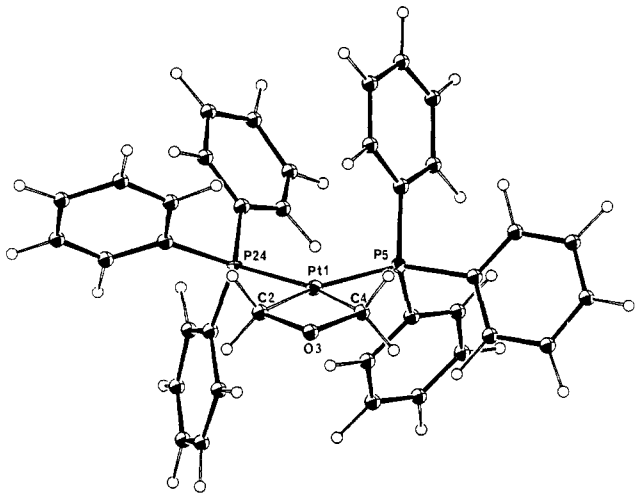

Figure 1. ORTEP drawing of complex **6b**. Selected bond distances (Å): Pt(1)-C(2), 2.061 (22); Pt(1)-C(4), 2.037 (20); O(3)-C(2), 1.434 (26); O(3)-C(4), 1.467 (24); Pt(1)-P(5), 2.302 (5); Pt(1)-P(24), 2.315 (5). Selected bond angles (deg): C(2)-Pt(1)-C(4), 65.1 (8); Pt(1)-C(2)-O-(3), 97.9 (13); Pt(1)-C(4)-O(3), 97.9 (12); C(2)-O(3)-C(4), 99.0 (14); P(5)-Pt(1)-P(24), 102.85 (18); P(5)-Pt(1)-C(4), 94.6 (6); P(24)-Pt-(1)-C(2), 97.5 (6). Final residuals: $R(F) = 0.087$ and $R_w(F) = 0.088$.

lacyclobutane complexes has been accomplished through the controlled manipulation of organometallic precursors possessing multiple reactive sites. The reactivity of these compositionally unique metallacycles toward migratory insertion and oxidative addition is currently being explored.

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Supplementary Material Available: Spectroscopic and analytical data for complexes **6a**, **6c**, and **6d**, details of the X-ray data collection and structure solution (**6b**), atomic positional and thermal parameters, and complete bond distance and angle data (17 pages); a listing of F_o vs F_c (20 pages). Ordering information is given on any current masthead page.

Long-Range {Fe^{2+}(heme) → (M(porphyrin))$^+$} Electron Transfer within [M, Fe] (M = Mg, Zn) Hemoglobin Hybrids

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We report the spectroscopic observation of long-range $Fe^{2+}P \rightarrow (MP)^+$ electron transfer (eq 1) within the charge-transfer

$$(MP)^+ + Fe^{2+}P \xrightarrow{k_b} MP + Fe^{3+}P \quad (1)$$

intermediate of the mixed-metal [β(MP), α(FeP)] (M = Mg, Zn) hemoglobin (Hb) hybrids.[1] We[2,3] and others[4-7] have shown that proteins modified to contain closed-shell metalloporphyrins can be used to study photoinitiated electron transfer from the triplet state (eq 2). In general, however, it is difficult to observe the

$$^3(MP) + Fe^{3+}P \xrightarrow{k_t} (MP)^+ + Fe^{2+}P \quad (2)$$

(1) All experiments here refer to the [β(MP), α(FeP)] hybrid where P = protoporphyrin IX and the symbol in brackets for the hybrid represents an [α_2, β_2] T-state tetramer of hemoglobin (Hb) in which the iron ions in the two β-chains are replaced by M = Mg or Zn; Cc, cytochrome *c*; CcP, cytochrome *c* peroxidase.

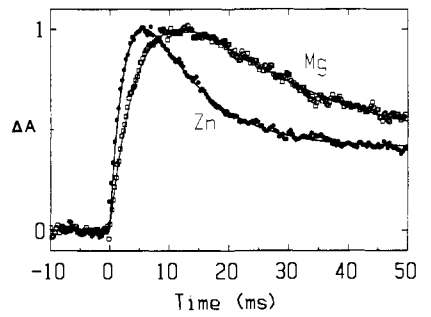
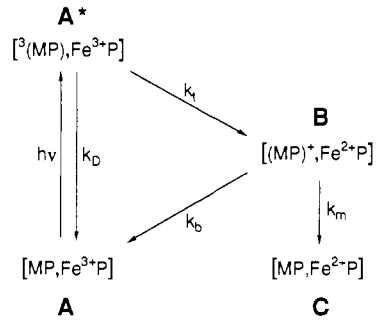


Figure 1. Normalized kinetic progress curves at 5 °C for mixed-metal Hb hybrids: [β(Mg), α(Fe)] (□, λ = 432 nm); [β(Zn), α(Fe)] (•, λ = 435 nm). Solid lines are nonlinear least-squares fits to the equations in ref 20 and 21. For [Mg, Fe], k_b = 155 (15) s^{-1}, k_p = 47 (5) s^{-1}, and k_m = 20 (5) s^{-1}; for [Zn, Fe], k_b = 350 (35) s^{-1}, k_p = 112 (10) s^{-1}, and k_m = 40 (8) s^{-1}. Buffer: 0.01 M KP_i, pH 7.0.

Scheme I



charge-separated state and the charge-return reaction, eq 1, the major success to date being with [cytochrome *c*, cytochrome *c* peroxidase] complexes.[3] This report shows that [Zn, Fe] and [Mg, Fe] Hb hybrids are excellent systems for studying the electron transfer reaction of eq 1 as well as that of eq 2. Furthermore, comparison of Mg and Zn hybrids provides a powerful probe of mechanistic, energetic, and electronic aspects of long-range electron transfer in protein complexes.[2-14]

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[Mg, Fe] Hb was prepared by procedures analogous to those for [Zn, Fe] Hb.[2,15] The triplet decay[16] for the reduced hybrid, [Mg, Fe^{2+}], as monitored both by ^{3}(MgP)/MgP absorbance difference spectrum and by ^{3}(MgP)phosphorescence, is exponential (k_D = 20 (2) s^{-1} at 25 °C; Scheme I) with a moderate temperature dependence. When the iron-containing chain of the hybrid is oxidized to the Fe^{3+}P state, the triplet decay curve monitored at λ = 415 nm, the $Fe^{3+/2+}$P isosbestic point, or at 475 nm, where contributions from the charge-separated intermediate are minimal, is still a single exponential, but the decay rate is increased to k_p = 55 (3) s^{-1}. According to Scheme I,

$$k_p = k_D + k_1$$

where k_1 is the photoinitiated electron-transfer rate constant (eq 2); thus, at room temperature, k_1 = 35 (4) s^{-1}. Surprisingly, k_1 for [Mg, Fe] is significantly lower than $k_1 \approx 100$ s^{-1} reported for the corresponding [Zn, Fe] hybrid,[2] although the driving force for eq 2 is greater by about 100 mV in the former.[17-19]

As shown for [ZnC*c*P, C*c*],[3] the time course of B, the charge-separated intermediate formed by the ^{3}(MP) → Fe^{3+}P electron-transfer process, can be measured in a flash photolysis experiment that monitors the (B − A) transient absorbance difference at a ground-state/triplet-state isosbestic point (e.g., λ = 432 nm for M = Mg, and 435 nm for M = Zn). We now have observed this intermediate for both [M, Fe] hybrids; representative kinetic progress curves are shown in Figure 1. In a kinetic scheme that includes eq 2 and 1 as the only electron-transfer processes, when $k_b > k_p$, as is the case here, B appears exponentially with rate constant k_b and disappears completely in an exponential fall, with rate constant k_p.[3] However, the occurrence of a persistent absorbance change (ΔA_∞) for the [M, Fe] hybrids requires an extended kinetic model[20] (Scheme I) in which $(MP)^+$ is reduced not only by Fe^{2+}P (regenerating the [M, Fe^{3+}] state) but also by an as yet unidentified amino acid residue X (eq 3) leading to [M, Fe^{2+}]:

$$[(MP^+), Fe^{2+}] + X \xrightarrow{k_m} [M, Fe^{2+}] + X^+ \qquad (3)$$

In Scheme I, the magnitude of ΔA_∞ is proportional to k_m, and B appears exponentially with rate constant $k_x = k_b + k_m$. Figure 1 shows that the kinetic traces for the Zn- and Mg-substituted hybrids are well-described by nonlinear least-squares fits to the kinetic equations of Scheme I.[21] Identification of the absorbance transients of Figure 1 with the charge-transfer intermediate, B, is confirmed as follows: (i) the magnitude of the transient is proportional to the concentration of Fe^{3+}P; (ii) reduction of [M, Fe^{3+}] to [M, Fe^{2+}] with a stoichiometric addition of $Na_2S_2O_4$ eliminates the transient; (iii) the signs and magnitudes of the absorbance changes at ^{3}(MP)/MP isosbestic points are entirely consistent with formation of Fe^{2+}P in accord with eq 2 (e.g., for Mg, the transient absorbance is positive at 432 nm and is negative at 542 nm, as is the ([Mg, Fe^{2+}] − [Mg, Fe^{3+}]) difference spectrum).[22]

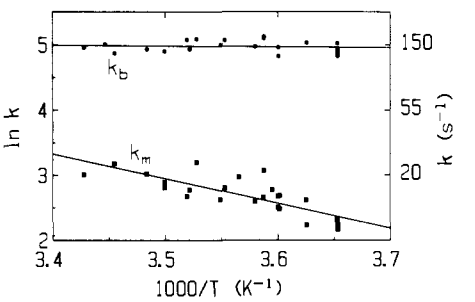

Figure 2. Arrhenius plots of k_b (●) and k_m (■) for the [β(Mg), α(Fe)] hybrid (left ordinate, ln *k*; right ordinate, *k* (s^{-1})). Solid lines are least-squares fits to the data: $E_a(k_b^{Mg}) \approx 0$ eV; $E_a(k_m^{Mg})$ = 0.30 (6) eV.

As seen in Figure 1, the time course of the intermediate [$(MP)^+$, Fe^{2+}] (B) strongly depends on M. At 5 °C for M = Mg, k_b = 155 (15) s^{-1}, k_p = 47 (5) s^{-1}, and k_m = 20 (5) s^{-1}; for M = Zn, k_b = 350 (35) s^{-1}, k_p = 112 (10) s^{-1}, and k_m = 40 (8) s^{-1}. That k_b^{Mg} is not equal to k_b^{Zn} rules out a conformational "gate"[23] for the Fe^{2+}P to $(MP)^+$ thermal electron transfer (eq 1) and further indicates that this is a direct (single-step) process.[24]

Figure 2 shows Arrhenius plots of temperature dependence for k_b and k_m in the [Mg, Fe] hybrid. Over the temperature range of 0 °C to 20 °C, k_b does not vary significantly, suggesting either that tunnelling is important near ambient temperatures or that the reorganization energy for the Fe^{2+}P → $(MgP)^+$ process is $\lambda_b \approx -\Delta G^\circ \approx 1$ eV. In contrast, over the same temperature range, k_m is highly temperature-dependent, with E_a = 0.30 (6) eV. This value is large compared to activation energies reported for long-range ET in modified proteins[2-14] and suggests the reaction in eq 3 is associated with a large reorganization energy, λ_m.[25]

Observation of B not only directly gives the thermal electron-transfer rate constants, k_b and k_m, but also quantitatively confirms our interpretation that the rate constant, k_1, represents electron-transfer quenching of the [^{3}(MP), Fe^{3+}P] (A*) excited state.[2]

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(16) E_{00} (at ambient) for [β(Mg), α(Fe)] was 1.74 (2) eV, as measured on a degassed sample in a phosphorimeter.

(17) Due to the long-term instability of $(MP)^+$ in Hb, direct measurements of $E^{\circ\prime}$ for $(MP)^+ + e^- \rightarrow$ MP have not been made. However, in several porphyrins[18] as well as in Mg- and Zn-reconstituted horseradish peroxidase[19] the reduction potention for $(MgP)^+$ is approximately 100 mV less than for $(ZnP)^+$. By using published values for $E^{\circ\prime}$ $(ZnP^+) + e^- \rightarrow$ ZnP ≈ 0.70 V vs NHE, we estimate the driving forces as follows:

$$^3(ZnP) + Fe^{3+}P \rightarrow (ZnP)^+ + Fe^{2+}P \qquad E^{\circ\prime} \approx 0.9 \text{ V}$$

$$^3(MgP) + Fe^{3+}P \rightarrow (MgP)^+ + Fe^{2+}P \qquad E^{\circ\prime} \approx 1.0 \text{ V}$$

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(20) Kinetic equations:

$$d[B]/dt = k_t[A^*] - k_b[B] - k_m[B]; \; d[C]/dt = k_m[B]; \text{ and } [A^*(t)] = A_o^* e^{-k_p t}$$

$$[B(t)] = k_t A_o^* [e^{-k_p t} - e^{-k_x t}]/(k_x - k_p)$$

$$[C(t)] = k_m k_t A_o^* (k_p(e^{-k_x t} - 1) + k_x(1 - e^{-k_p t}))/k_x k_p (k_x - k_p)$$

where $k_x = k_b + k_m$. As $t \rightarrow \infty$, [B] → 0, [C] → $k_m k_t A_o^*/k_x k_p$.

(21) Data were fit to the equation $\Delta A(t) = (\epsilon_C - \epsilon_A)[C(t)] + (\epsilon_B - \epsilon_A)\cdot[B(t)]$ with fit parameters k_x, k_p, $\Delta A(\infty)$, and δ, where $\Delta A_\infty = (\epsilon_C - \epsilon_A)C(\infty)$, $\delta = (\epsilon_B - \epsilon_A)k_t A_o^*$, and ϵ_i is the extinction coefficient of species i as identified in Scheme I. Using these parameters, $k_m = k_x k_p \Delta A_\infty R/\delta$, where $R = (\epsilon_B - \epsilon_A)/(\epsilon_C - \epsilon_A)$; $k_b = k_x - k_m$. For M = Mg, $R \approx 0.93$ (6); for M = Zn, $R \approx 0.89$ (6).[22]

(22) Manuscript in preparation.

(23) (a) If the "electron-transfer rate" actually were a measurement of the rate of a conformational change, as has been observed in protein-protein complexes,[6a] a change from Zn to Mg would not change the observed rates, as shown in ref 23b. (b) Hoffman, B. M.; Ratner, M. R. *J. Am. Chem. Soc.* **1987**, *109*, 6237-6243.

(24) If the B → A charge recombination process were indirect, it would imply the existence of an amino acid, y, which mediates electron flow to form one of the discrete species, $[(MP)^+, y^-, Fe^{3+}]$ or [MP, y^+, Fe^{2+}]. However, the Fe^{2+}P of Hb cannot reduce an amino acid, which eliminates the former. The latter implies rapid oxidation of y by $(MP)^+$, with k_b representing slow oxidation of Fe^{2+}P by y^+. If this were the case, k_b should be the same for M = Mg and Zn, contrary to observation.

(25) (a) For the [Zn, Fe] hybrid at room temperature the quantum yield for the B → C irreversible step is $\phi_m = k_m/(k_b + k_m) \approx 0.1$. This is lower than our earlier estimate of $\phi_m \approx 0.37$ because of the removal of impurities that cause the B → C reaction, giving the appearance of a larger k_m. This may have contributed to the anomalous k_m reported recently.[25b] (b) Magner, E.; McLendon, G. *Biochem. Biophys. Res. Commun.* **1989**, *159*, 472-476.

Kinetic Scheme I implies a quantitative relationship among k_t, the absorbance changes associated with B, and the concentration of A* formed by the actinic flash;[26] analysis based on this relationship demonstrates that within experimental error, triplet-state quenching is associated only with the electron-transfer process, eq 2, and that other mechanisms leading to deactivation of the excited state in A* (e.g., energy transfer) are relatively insignificant (<20%).[22]

With these results in hand, the effects of heme ligation, porphyrin substituents, and the protein matrix on the $Fe^{2+}P \rightarrow (MP)^+$ long-range electron-transfer process now can be investigated.

Acknowledgment. We thank Professor J. Torkelson for the use of his phosphorimeter. This research has been supported by a NIH NRSA postdoctoral fellowship (HL07531) to M.J.N. and by NIH Grants HL 13531 and HL 40453 and NSF Grant DMG 8606575 to BMH.

(26) When monitoring triplet decay at 475 nm, $\Delta A(0) = A_o^*(\epsilon_A^* - \epsilon_A)$. Thus, $k_t = (\epsilon_A^* - \epsilon_A)\delta/\Delta A(0)(\epsilon_B - \epsilon_A)$ where terms are defined in footnote 21.

Synthesis of Seryl Threonyl Phosphate. A Model Compound Designed To Study the Spectroscopic and Chemical Features of a Phosphodiester Linkage Similar to the One Proposed To Exist in *Azotobacter* Flavodoxin

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A well-established post-translational event in proteins is the formation of intermolecular and (or) intramolecular disulfide linkages which, inter alia, confer[1] on these molecules their unique structure and physiological activity. On the other hand, phosphorylation is another post-translational modification of increasing importance[2] in proteins. For example, the regulatory action of phosphorylation was nicely illustrated[3] in the conversion of glycogen phosphorylase *b* to phosphorylase *a* by phosphorylation of serine-14.

Some years ago Edmondson and James[4] proposed on the basis of chemical degradation and ^{31}P NMR studies the presence of a phosphodiester linkage between the hydroxyl groups of serine and threonine in *Azotobacter* flavodoxin. Evidence supporting the occurrence of this unusual linkage was recently corroborated by Live and Edmondson[5] by using ^1H-^{31}P two-dimensional NMR. Although this appears to be the only example sofar[6] to support the existence of a phosphodiester linkage between the hydroxyamino acids serine and threonine in a protein, it is not excluded that phosphodiester bonds may also serve a similar purpose in proteins as disulfide linkages.

As part of a program directed toward the preparation of phosphopeptides[7] and nucleopeptides,[8] we report the synthesis of model compound **6** having a phosphodiester bond between an isolated serine and threonine and evaluate the chemical properties and spectroscopic (NMR) features of **6** with those published on the protein-bound seryl threonyl phosphodiester bond. The synthetic route[9] to **6** is depicted in Scheme I and consists of the following steps.

Phosphitylation of the L-serine derivative[10] **1** with benzyloxybis(*N*,*N*-diisopropylamino)phosphine[11] (**2**), in the presence of 1*H*-tetrazole, resulted, after column chromatography, in the isolation of phosphite derivative **3** (80% yield, δ_p 149.05 and 149.35 ppm). 1*H*-Tetrazole-mediated coupling of **3** with the L-threonine derivative[10] **4** gave an intermediate phosphite triester, which was oxidized in situ with *tert*-butyl hydroperoxide.[12] Purification of the crude phosphotriester product afforded homogeneous **5** (δ_p −1.56 and −1.68 ppm) in a yield of 80%. Hydrogenolysis of **5** (0.19 mmol) in the solvent mixture tBuOH/H_2O/HOAc (4/1/1, v/v/v), followed by workup and conversion into the sodium salt (via SP-Sephadex C25, Na^+ form) yielded **6** (Na^+ salt, 56 mg, δ_p −1.28 ppm, D_2O, pD 7.1, external standard: 85% H_3PO_4). The identity of **6** was unambiguously determined by ^1H NMR[13] (see Figure 1 and Table I), ^{13}C NMR[14] and ^{31}P NMR spectroscopy.

The presence of a seryl threonyl phosphodiester in *Azotobacter* flavodoxin was supported[5] from the proton-detected ^1H-^{31}P multiquantum 2D NMR spectral data. Thus the proton projection of the 2D spectrum reveals an AB type pattern with major peaks at 3.4 and 3.7 ppm and a doublet at 4.0 ppm, which were assigned to the two geminal β-protons of serine and the α-proton of threonine, respectively. The supposed absence of a cross peak for the α-proton of serine with phosphorus was attributed to the smaller Hα-P coupling constant in serine than in threonine. The latter assumption[16] is to some extent supported by the observed difference in magnitude between these coupling constants in model compound **6** (see Table I). On the other hand, the assumed nonappearance in the spectrum of the phosphothreonyl β-proton, which has a higher Hβ-P coupling constant than the phosphoseryl β-proton in compound **6**, cannot be explained solely by a reduction in peak height arising from the multiple couplings of this proton.

Apart from the NMR characteristics of compound **6**, we also observed that the phosphodiester bond in **6** was rather resistent toward alkaline treatment. This finding is in contrast with the result of Edmondson and James[4] who found that base treatment of the protein resulted in the relative fast elimination of phosphate and concomitant formation of the dehydro forms of the appropriate hydroxyamino acids. Our finding may be explained by the presence of two free carboxylic functions in **6** which will decrease

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(9) The synthetic route to **6** proved also to be suitable to couple serine or threonine with tyrosine: Dreef-Tromp, C. M. et al., to be published.
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(13) The simulated ^1H NMR spectrum (PANIC program from Bruker) of **6** was in excellent agreement with the experimental one.
(14) ^{13}C NMR data (δ values, D_2O, pD 7.1) 19.42 (Cγ, Thr), 55.74 (d, Cα, Ser, $J_{C\alpha,P}$ = 8.8 Hz), 60.12 (d, Cα, Thr, $J_{C\alpha,P}$ = 8.8 Hz), 64.84 (d, Cβ, Ser, $J_{C\beta,P}$ = 4.4 Hz), 72.49 (d, Cβ, Thr, $J_{C\beta,P}$ = 4.4 Hz), 172.13 and 172.84 (2 × C=O, Ser and Thr).
(15) Marginal changes in chemical shifts (0.01-0.21 ppm) and coupling constants (0.2-0.6 Hz) were observed for model compound **6** at pD 2.
(16) It is, however, not excluded that the motion of the phosphodiester bond in the protein may be restricted and would not necessarily give identical coupling constants as the model compound.

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