Characterization of Partially Ligated Hemoglobins: NMR, ENDOR, CD, and Stopped-Flow Studies of Zinc-Containing Hemoglobin Hybrids

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Abstract: A series of hemoglobin hybrids in which Fe is replaced by Zn have been studied. These hybrids have been extensively characterized by measurements of their CO dissociation kinetics and by a variety of spectroscopic techniques including circular dichroism (CD), electron nuclear double resonance (ENDOR), and detailed ^1H and ^{13}C nuclear magnetic resonance (NMR) studies. The ligand dissociation rate ($k = 0.11\ s^{-1}$) is characteristic of a low-affinity structure. Some spectroscopic probes suggest a "deoxy"-like structure for the hybrid, while other probes suggest an "oxy" structure. When combined with other work, the present results suggest that the α-Zn_2-β-$(FeCO)_2$ and α-$(FeCO)_2$-β-Zn_2 models for partially ligated hemoglobins adopt a structure which is distinct from either the limiting oxy or deoxy structures. The results are generally more consistent with a sequential (KNF) model for hemoglobin ligation than with the classic two-state (MWC) model. (Abbreviations used in this paper are as follows: HbCO ≡ α-$(FeCO)_2$-β-$(FeCO)_2$ ≡ α-(FeCO)-β-(FeCO)-α-(FeCO)-β-(FeCO), Fe(II) ≡ Fe(II) deoxy; ZnHb ≡ α-Zn_2-βZn_2 ≡ α-Zn-β-Zn-α-Zn-β-Zn; α-Zn_2-β-$(FeCO)_2$ ≡ α-Zn-β-(FeCO)-α-Zn-β-(FeCO); α-$(FeCO)_2$-β-Zn_2 ≡ α-(FeCO)-β-Zn-α-(FeCO)-β-Zn; HbCN ≡ α-$(Fe^{III}CN)_2$-β-$(Fe^{III}CN)_2$.)

Despite many elegant studies, the basis for cooperative ligand binding in hemoglobin remains incompletely understood.[1] One central question is "how does the structure of hemoglobin change with sequential addition of one, two, three, or four ligands"? To answer this question, stable hemoglobin derivatives are needed to model intermediate ligation states. To this end, a number of studies have appeared over the past decade of "mixed-valence" hemoglobins in which the heme in one type of subunit is oxidized to produce a six-coordinate Fe(III), while the heme in the other type of subunit remains reduced and can be deoxygenated to produce a five-coordinate Fe(II).[2-7] More recently, mixed metal hybrids (e.g., α-Zn_2-β-$(FeCO)_2$) have been investigated as models for partially ligated hemoglobins.[8-10] These systems have an added advantage in that the properties of the added metal can be tailored to provide specific probes of reactivity and structure that are not available for Fe(II) or Fe(III).

Here we report extensive studies of hybrid hemoglobins in which one subunit contains Zn(II) while the other subunit contains Fe(II).[10] Zn(II) provides a particularly valuable probe, since five-coordinate zinc(II) porphyrins are virtually isostructural with five-coordinate, high-spin iron(II) porphyrins (as found in deoxy-Hb).[11,12] Furthermore, Zn(II) does not react with exogenous ligands. The iron subunit may then be oxygenated and deoxygenated without affecting the ligation state of the zinc(II) porphyrin containing subunit. Finally, Zn(II) is diamagnetic, so that amino acids in the immediate vicinity of the zinc(II) porphyrin may be inestigated by high-resolution NMR. Systems studied include α-Zn_2-β-Zn_2, α-Zn_2-β-Fe^{II}_2, α-Zn_2-β-$(FeCO)_2$, α-Zn_2-β-$(Fe^{III}CN)_2$, α-Zn_2-β-$(FeO_2)_2$, α-Fe^{II}_2-β-Zn_2, α-$(FeCO)_2$-β-Zn_2, α-$(FeO_2)_2$-β-Zn_2, α-$(Fe^{III}CN)_2$-β-Zn_2, and HbCO.

A variety of techniques have been used to characterize the structure of these hybrids, including high-resolution (400 MHz) NMR, circular dichroism, and electron nuclear double-resonance (ENDOR) spectroscopy. In addition, kinetic reactivity is studied via stopped-flow techniques. Comparisons are made to literature studies of the analogous mixed-valence hybrids and with the few previous studies of Zn/Fe hybrids. The results are compared with current models of heme–heme interaction in hemoglobins.

Results

CO Dissociation Kinetics. In order to try to relate protein structure and function, we first established the functional behavior of the protein with respect to CO-binding kinetics. Using flash photolysis, Hoffman and co-workers[13,14] have previously measured the CO recombination rate for α-Zn_2-β-$(FeCO)_2$. The on rate so obtained is $k_{on} = 1.3 \times 10^5\ M^{-1}\ s^{-1}$. This rate refers to addition of the second CO:

$$\alpha\text{-}Zn_2\text{-}\beta\text{-(FeCO)-}\beta\text{-}Fe^{II} \xrightarrow[CO]{k_{on}} \alpha\text{-}Zn_2\text{-}\beta\text{-(FeCO)}_2$$

We now report the corresponding CO dissociation rate. This rate was measured by measuring the rate of oxidation of the α-Zn_2-β-$(FeCO)_2$ hybrid by $Fe(CN)_6^{3-}$.

Similar oxidation experiments for oxyhemoglobin were reported by Antonini et al.[15] As Antonini showed, such experiments may be understood by assuming that only the ligand-free hemoglobin is oxidized. The appropriate kinetic scheme is shown below.

$$\alpha\text{-}Zn_2\text{-}\beta\text{-(FeCO)}_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \alpha\text{-}Zn_2\text{-}\beta\text{-(FeCO)-}\beta\text{-}Fe^{II} \xrightarrow{k_2[Fe(CN)_6^{3-}]} \alpha\text{-}Zn_2\text{-}\beta\text{-(FeCO)-}\beta Fe^{III} + CO$$

Thus, the rate of appearance of Fe(III) is given by

$$d[Fe(III)]/dt = \frac{k_2[Fe(CN)_6^{3-}]k_1[\alpha\text{-}Zn_2\text{-}\beta\text{-(FeCO)}_2]}{k_2[Fe(CN)_6^{3-}] + k_{-1}[CO]}$$

The key experimental observation in the present work is that when

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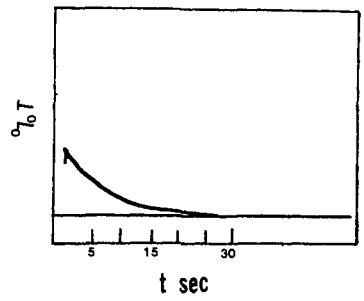

Figure 1. Oxidation of α-Zn_2-β-$(FeCO)_2$ by $[Fe(CN)_6]^{3-}$ followed at 630 nm, pH 6.8 (10 mM BIS-TRIS), T = 297 K, $[Fe(CN)_6]^{3-} = 4 \times 10^3$ M.

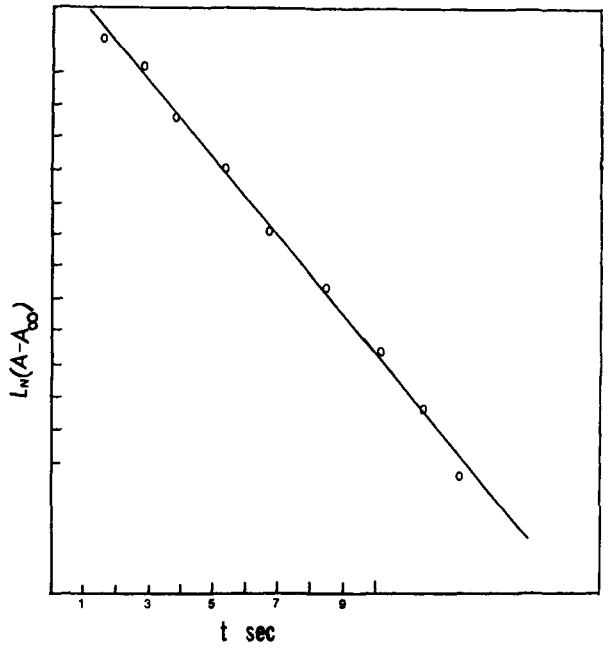

Figure 2. First-order plot of the data in Figure 1; K_{obsd} = 0.11 s^{-1}.

$[Fe(CN)_6^{3-}] > 10^3$ M, the rate of oxidation of the hybrid is independent of ferricyanide concentration (see Figures 1 and 2). This result implies $k_2[Fe(CN)_6^{3-}] \gg k_{-1}[CO]$. Thus, CO dissociation is the rate-limiting step, and $k_{obsd} = k_1 = 0.11$ s^{-1}. This value is close to the dissociation rate constant estimated for a "pure" deoxy quatenary (T) structure ($k_T = 0.11$–0.16 s^{-1}). For comparison, the dissociation constant estimated for a fully ligated (R) hemoglobin quaternary structure is $k_R \approx 0.01$ s^{-1}.[13] Combining the dissociation rate with the association rate gives the equilibrium (Adair) constant for binding the second CO to α-Zn_2-β-(FeCO) β-Fe(II): $K_2 = 1.2 \times 10^6$ M^{-1}.

At least two interpretations of the dissociation kinetics are possible. First, one interpretation is close to the "two-state" model. Dissociation of CO from α-Zn_2-β-$(FeCO)_2$ produces a monoligated α-Zn_2-β-(FeCO)-β-Fe^{II} species. This monoligated species would be expected to have a "T" conformation and "T" reactivity. When the Hammond postulate is adopted,[16] the transition state for CO dissociation might closely resemble the product. Thus a T-state dissociation rate would be observed.

Second, a limiting two state model could be adopted in which the α-Zn_2-β-$(FeCO)_2$ hybrid rapidly interconverts between R and T structures (i.e., k_1, $k_{-1} \gg k_2$).

$$\alpha\text{-}Zn_2\text{-}\beta\text{-}(FeCO)_2\ (R) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \alpha\text{-}Zn_2\text{-}\beta\text{-}(FeCO)_2\ (T)$$

$$\alpha\text{-}Zn_2\text{-}\beta\text{-}(FeCO)_2\ (T) \xrightarrow[k_2]{-CO} \alpha\text{-}Zn_2\text{-}\beta\text{-}(FeCO)\text{-}\beta\text{-}Fe^{II}\ (T)$$

$$\alpha\text{-}Zn_2\text{-}\beta\text{-}(FeCO)_2\ (R) \xrightarrow[k_3]{-CO} \alpha\text{-}Zn_2\text{-}\beta\text{-}(FeCO)\text{-}\beta\text{-}Fe^{II}\ (R)$$

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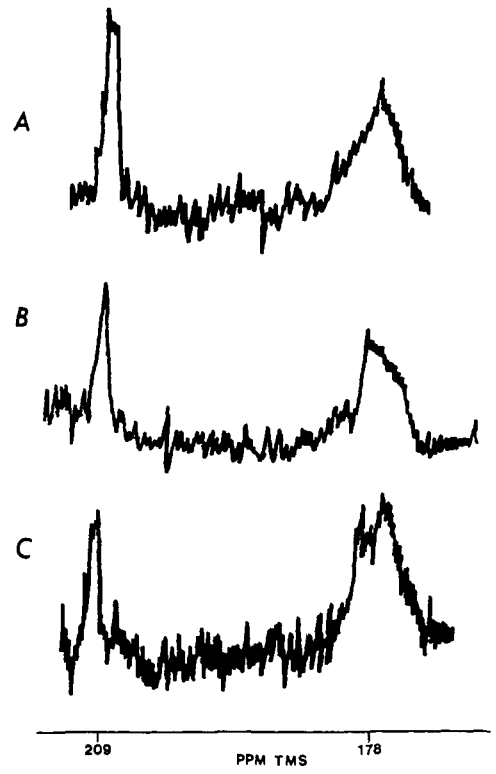

Figure 3. ^{13}C NMR spectra of (A) $Hb^{13}CO$, (B) α-Zn_2-β-$(Fe^{13}CO)_2$, and (C) α-$(Fe^{13}CO)_2$-β-Zn_2.

Taking $k_2 = 0.16$ s^{-1} and $k_3 = 0.01$ s^{-1} required $K = 2$.

This second model is less likely to be correct for two reasons. First, the spectroscopic data detailed in the following sections are generally inconsistent with a 2:1 equilibrium between the two conformations. Second, equilibrium CO-binding studies of the homologous α-Mn^{II}_2-β-$(FeCO)_2$ hybrids show no cooperativity in going from the ligand-free α-Me^{II}_2-β-Fe^{II}_2 to the bis-ligated α-Me^{II}_2-β-$(FeCO)_2$ complex. This result strongly suggests that very little Hb R conformation is present for α-Mn^{II}_2-β-$(FeCO)_2$ at equlibrium.[13]

The kinetic results show that CO-binding rates are different for α-Zn_2-β-$(FeCO)_2$ structure than for fully ligated HbCO. The structural basis for that perturbation is of interest. Therefore, the local structure at the heme iron was investigated in detail by using both ^{13}C NMR, to probe CO binding, and ENDOR spectroscopies to probe the structure of the isostructural paramagnetic species, α-Zn_2-β-$(Fe^{III}CN)_2$ and α-$(Fe^{III}CN)_2$-β-Zn_2.

^{13}CO NMR. The ^{13}C resonance frequency of bound CO is generally assumed to be sensitive to hemoglobin conformation.[17] Thus, initial studies sought to determine the effect of ligation on the resonance frequency of heme-bound ^{13}CO.

Since zinc(II) porphyrins do not react with CO, it was possible to prepare and study ^{13}CO adducts of specific subunits with the α-Zn_2-β-$(Fe^{13}CO)_2$ and α-$(Fe^{13}CO)_2$-β-Zn_2 systems. The data for HbCO, α-Zn_2-β-$(FeCO)_2$, and α-$(FeCO)_2$-β-Zn_2 are summarized in Figure 3. Clearly the resonance frequency for bound CO is essentially constant ($\Delta\nu \leq 0.5$ ppm) for the fully ligated and both half-ligated systems. Thus, within the limit of the conformational sensitivity of ^{13}C NMR, the local environment of the bound CO is equivalent in both the fully ligated and half-ligated systems. However, it is possible that ^{13}C NMR was insufficiently sensitive to conformation to detect a local change. Furthermore, the ^{13}C studies do not provide, a priori, a direct measure of (three dimensional) structure. To obtain such a measure, we studied the (isostructural) paramagnetic α-Zn_2-β-$Fe^{III}CN)_2$ and α-$(Fe^{III}CN)_2$-β-Zn_2 hybrids by using ENDOR spectroscopy.

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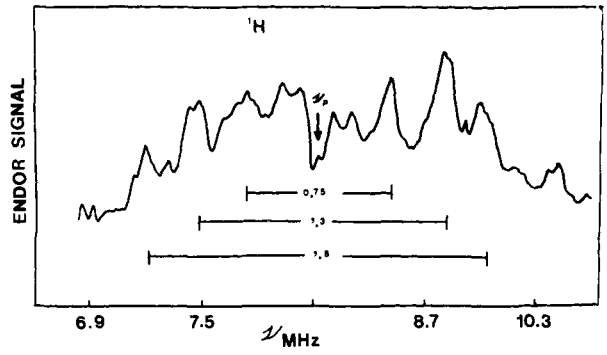

Figure 4. ^{1}H ENDOR spectrum from α-($Fe^{III13}CN)_2$-β-Zn_2: 100-kHz field modulation of 0.3 G peak-to-peak was used and the ENDOR radio frequency field was approximately 0.5 G peak-to-peak. Two-second sweeps (1450) were averaged to produce this spectrum. The ENDOR frequency was swept at a rate of 0.6 MHz 1 s, T = 2.1 K, g_e = 3.45, H = 1.90 kG, Zp = 8.09 MHz.

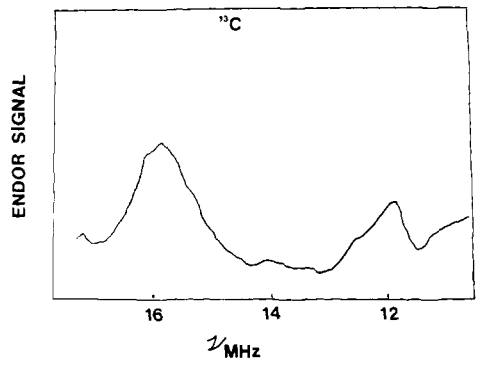

Figure 5. ^{13}C ENDOR spectrum from α-Zn_2-β-($Fe^{III13}CN)_2$: 100-kHz field modulation of 1.5 G peak-to-peak was used and the ENDOR radio frequency field was approximately 0.5 G peak-to-peak. Two-second sweeps (500) were averaged to product this spectrum. The ENDOR frequency was swept at T = 2.1 K, g_e = 3.45, H = 1.9 kG.

ENDOR Experiments. Electron nuclear double-resonance spectroscopy provides a uniquely sensitive probe of structure in the immediate vicinity of paramagnetic centers (e.g., low-spin Fe(III)).[18] This structural sensitivity reflects the strong dependence of the measured (dipolar) coupling constant, A, on the distance between the paramagnetic center and the nucleus of interest. The ENDOR experiment is also sensitive to the angle between the observed nucleus and the principle g tensor(s) of the paramagnet. For dipolar coupling, the coupling constant, A, is given by

$$A = \frac{g_z g_H \beta_e \beta_n}{r^3} (3 \cos^2 \alpha - 1)$$

where r is the distance between the proton and the iron and α is the angle between the direction of the applied magnetic field (in this instance, the heme normal) and the vector from the iron to the proton. In addition, g_z is the electronic g factor along the heme normal, g_H is the proton nuclear g factor, and β_e and β_n are the electronic and nuclear Bohr magnetons, respectively.[18] In principle, the observed coupling constant also may include a contact shift contribution due to "through-bond" electron delocalization. However, previous ENDOR studies of Hb have shown that the observed coupling constants of HbCN can be explained by considering only dipolar interactions. The present data are limited to HbCN derivatives. These were chosen for detailed study since CO and CN^- are isoelectronic. Thus, their heme derivatives are likely to be isostructural. Indeed, crystallographic studies showed that HbCO and HbCN are isostructural.[19,20] Similarly, the small molecule crystal structures of $Fe^{III}CN$ porphyrins and FeCO porphyrins show very similar metal–ligand bond lengths and angles.[12]

The ^{1}H ENDOR spectrum taken at 2 K of α-Zn_2-β-($Fe^{III13}CN)_2$ is shown in Figure 4. The ^{13}C ENDOR spectrum of α-Zn_2-β-($Fe^{III13}CN)_2$ is shown in Figure 5. Preliminary assignments of the proton ENDOR peaks are based both on D_2O substitution results and on comparison with previous literature assignments.[22] Within experimental error, there are no differences among the ^{13}C ENDOR spectra of α-Zn_2-β-($Fe^{III}CN)_2$ and α-($Fe^{III}CN)_2$-β-($FeCO)_2$. Although poorer resolution is obtained for the ^{1}H ENDOR, a similar conclusion can be tentatively drawn. These results suggest that the structure around the metal center of a ligated Hb subunit is independent of whether the other subunits are ligated or unligated. We recently reported EXAFS studies of α-Zn_2-β-($FeCO)_2$ and α-($FeCO)_2$-β-Zn_2 hybrids.[23] These studies showed that the Fe–ligand bond lengths were equivalent in α-Zn_2-β-($FeCO)_2$, α-($FeCO)_2$-β-Zn_2, and HbCO. Furthermore, the Zn–ligand bond lengths were equivalent in α-Zn_2-β-($FeCO)_2$, α-($FeCO)_2$-β-Zn_2, and in the ligand-free ZnHb (which is presumably isostructural with deoxy-Hb).

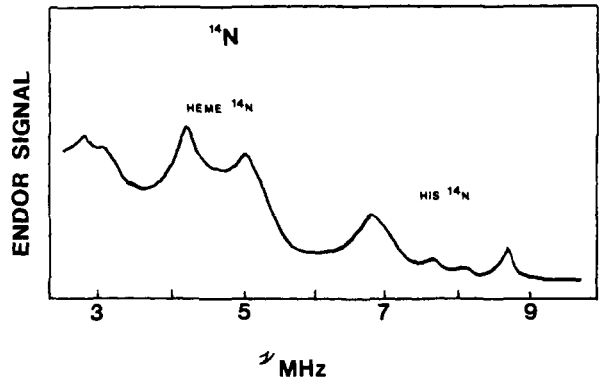

Figure 6. ^{14}N ENDOR spectrum from α-($Fe^{III}H_2O)_2$-β-Zn_2: 100-kHz field modulation of 1.5 G peak-to-peak was used and the ENDOR radio frequency field was approximately 0.5 G peak-to-peak. One-second sweeps (260) were averaged to produce this spectrum. The ENDOR frequency was swept at a rate of 10 MHz/s, T = 1.8 K, g_e = 2.00, H = 3.25 kG.

The present ENDOR results are consistent with the EXAFS work (Figure 6). Since the ENDOR measurement is sensitive to both distance and angle, the combined ENDOR and EXAFS data suggest that any displacements of metal–ligand bond lengths or angles must be quite small in proceeding from HbCO which binds CO strongly, to α-Zn_2-β-($FeCO)_2$ which binds CO weakly.

Since the change in CO-binding affinity between HbCO and α-Zn_2-β-($FeCO)_2$ was not accompanied by noticeable structural changes at the heme, the conformational energy responsible for the change in ligand affinity must be stored elsewhere in the protein. It has been suggested[24] that this structural change may be localized at the $\alpha_1\beta_2$ interface. Therefore, we examined the UV circular dichroism as a probe of the protein structure at the $\alpha_1\beta_2$ interface.

CD Measurements. Previous studies have demonstrated that circular dichroism in the region from 260 to 290 nm provides a sensitive probe of Hb (quaternary) structure at the $\alpha_1\beta_2$ interface.[25-30]

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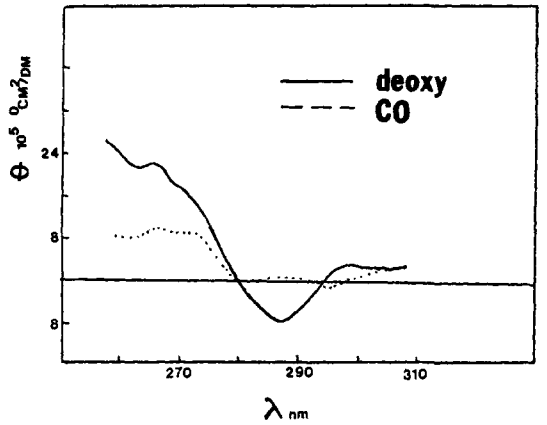

Figure 7. CD spectra of HbCO (···) and deoxy-Hb(—), [Hb] ≃ 5 × 10^{-5}.

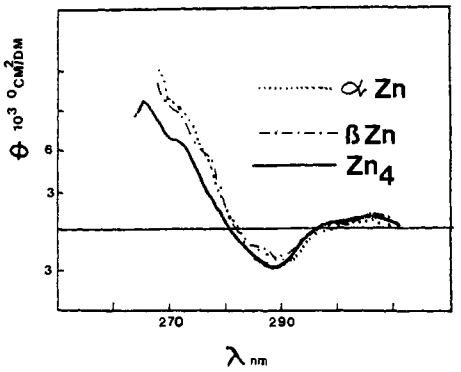

Figure 8. CD spectra of α-Zn_2-β-$(FeCO)_2$ (···), ZnHb (—), and α-$Fe(CO))_2$-β-Zn_2 (-·-·).

For oxyhemoglobin, and other R-state hemoglobins (HbCO, MetHb, Mn^{III}Hb, etc.), a very small positive ellipticity occurs in the region 270–290 nm (Figure 7).[31,32] By contrast, deoxyhemoglobin, and other purported T-state hemoglobins (e.g., ZnHb, MetHb + IHB) show a significant negative ellipticity in the region 270–290 nm.[33] This CD band reflects the local environment of Tyr-α42 and Trp-β37. These amino acids are located at the $\alpha_1\beta_2$ subunits interface, which rearranges significantly between deoxy-Hb and HbCO.[43]

Table I. Assignments of Exchangeable Proton Resonances in Hb

resonance,[a] ppm	hydrogen bond	interface	structure	ref
5.8	Asp-α94–Asn-β102	$\alpha_1\beta_2$	R	65
6.2–6.4[b]	Val-β98–Tyr-β145	$\beta_1\beta_1$	T	36
8.2	Asp-α126–Tyr-β35	$\alpha_1\beta_1$	R, T	6
9.4	Tyr-α42–Asp-β99	$\alpha_1\beta_2$	T	65

[a] In ppm downfield from water signal. [b] This peak found as low as 6.15 ppm in the T state derivative MetHb·IHP.

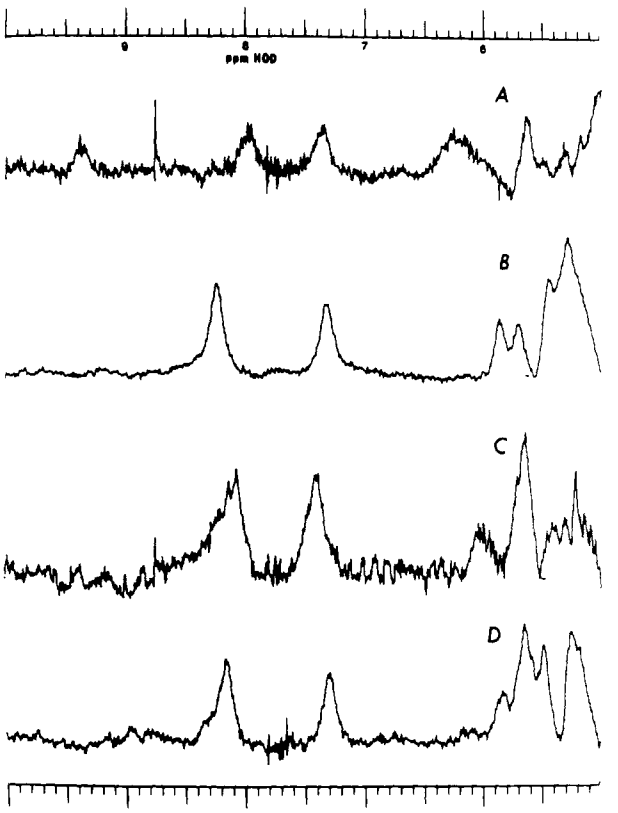

Figure 9. ^{1}H NMR (400 MHz) of the exchangeable protons of (A) ZnHb, (B) HbCO, (C) α-Zn_2-β-$(FeCO)_2$, and (D) α-$(FeCO)_2$-β-Zn_2. Spectra were obtained in H_2O by using a DASWEFT pulse sequence.

As shown in Figure 8, the CD spectra of α-Zn_2-β-$(FeCO)_2$ and α-$(FeCO_2$-β-Zn_2 both show a significant negative ellipticity band centered at 280 nm, analogous to deoxy-Hb (and other T-state Hbs). Therefore, the local structural environment around Trp-β37 reflected in the CD must be similar in both deoxy-Hb and the Zn/Fe hybrid hemoglobins. While this qualitative interpretation is justified, it is not reasonable to *quantitatively* compare the molar ellipticities of the Fe/Zn proteins with those for deoxy-Hb. The precise magnitude of the negative ellipticity depends on several factors including some coupling between the heme-centered CD (τ_{max} ~ 260 nm) and the amino acid-centered CD at ~280 nm.[25,33] The positive ellipticity at ca. 260 is independent of quaternary structure but does differ in magnitude for Fe and Zn, thereby modulating the magnitude (but not the qualitative shape) of the 280-nm band.

In a limiting two-state (T/R) description of hemoglobin structure, the negative ellipticity at 280 nm would suggest that Zn/Fe hemoglobin exists in the T state. However, it is possible that only the structural regions probed by CD resemble the deoxy (T) structure, while other regions may resemble the oxy (R) structure.

In order to obtain a more complete picture of the solution structure of the α-Zn_2-β-$(FeCO)_2$ and α-$(FeCO)_2$-β-Zn_2 hybrids, extensive NMR studies were undertaken, as reported in the following section.

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Magnetic Resonance Experiments. (A) Exchangeable Protons. Pioneering work by Shulman and co-workers[34] demonstrated that conformationally sensitive exchangeable protons in hemoglobin could be observed by NMR. Subsequent elegant work by Ho and co-workers[35-39] identified several of these exchangeable proton resonances by using both specific hemoglobin mutants and specific chemical modification. Some exchangeable protons so identified are listed in Table I. The 6.4 and 9.4 ppm resonances thereby appear to serve as conformational markers for a deoxy-like quaternary structure (involving formation of specific salt bridges) in the immediate vicinity of these specific amino acids.

Ho[40] subsequently demonstrated that on reacting deoxy-Hb with O_2, the intensities of the 6.4 and 9.4 ppm resonances do not decrease concertedly. This suggests that intermediate ligation states may adopt intermediate conformations, in which some salt bridges are made while others are broken. In the Zn/Fe hybrids, the "bis" ligation is precisely dictated by Zn substitution. Figure 9 shows the exchangeable proton region of Zn-substituted Hbs, from 5 ppm to 10 ppm vs. HOD, obtained by using a DASWEFT pulse sequence.[71] ZnHb (Figure 9A), like deoxy-Hb, shows strong resonances at 6.2, 7.3, 8.2, and 9.4 ppm. In the α-Zn_2-β-$(FeCO)_2$ hybrid system (Figure 9C), the 6.2 ppm resonance (i.e., the β98 $\rightarrow$ β145 salt bridge) is still present although diminished, but the 9.4 ppm resonance (β99-α42) is not observed at all. Changing the temperature does not reveal any new resonances between 9 and 10 ppm, precluding exchange broadening. It appears that, for hemoglobins with an intermediate degree of ligation, like the Fe/Zn hybrid hemoglobins, some hydrogen bonds characteristic of the deoxy structure have been broken while others remain intact. The loss of the Tyr-α42-Asp-β99 salt bridge (9.4 ppm) before loss of the Asp-β98-Tyr-β145 salt bridge (6.2 ppm) is consistent with Ho's[40] results in that the resonances do not decrease concertedly. The α-$(FeCO)_2$-β-Zn_2 hybrid (Fiure 6D) does *not* show any peaks at 6.1-6.4 ppm, nor at 9.4 ppm, but instead exhibits a new resonance at 5.8 ppm which is assigned[56] to an Aspα94-Asnβ102 H bond in the high affinity conformation.

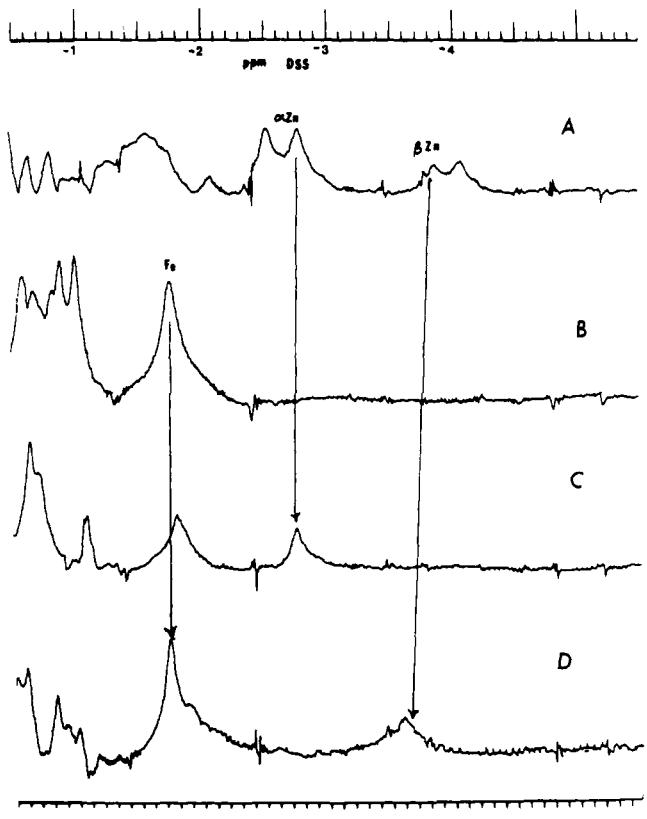

Figure 10. Upfield NMR spectra (in the Val-E11 region) of (A) ZnHb, (B) HbCO, (C) α-Zn_2-β-$(FeCO)_2$ and (D) α-$(FeCO)_2$-β-Zn_2.

(B) Upfield (Ring Current) Shifts—Val E11. Previous studies[42] have identified an upfield resonance in diamagnetic hemoglobin derivatives (e.g., HbCO ν = ⁻1.8 ppm vs. DSS, HbO_2 ν = −2.3 ppm vs. DSS). This resonance has been assigned to the γ_2 methyl of valine E11, for which an important stereochemical role in controlling ligand binding has been suggested.[43,44] The observed ring current shift is very sensitive to the geometry (distance and angle) between the Val γ_2 methyl and the heme. It would be useful, therefore, to compare the ring current shifts and thus the valine E11 geometries, in deoxy-Hb and partially ligated hemoglobins. However both deoxy-Hb and the previously examined mixed-valence hybrid hemoglobins are paramagnetic. The paramagnetic iron produces a large induced paramagnetic (pseudocontact) shift of the nearby γ_2 methyl of a Val-E11 to an unknown resonance frequency. Although zinc(II) porphyrins are structural homologues of high-spin Fe(II),[12] Zn(II) is diamagnetic. Therefore, the ring current shift may be monitored directly in Zn(II)-substituted hemoglobins.

Figure 10 compares the spectra of ZnHb, HbCO, α-Zn_2-β-$(FeCO)_2$, and α-$(FeCO)_2$-β-Zn_2 in the upfield region. The methyl resonances at −1.8 ppm for HbCO and at −1.2 ppm for HbO_2 are in good agreement with previous studies. The small shift for HbCO reflects the movement of Val-E11 away from the heme normal due to a structural contact with the linear CO. Such a contact does not occur in HbO_2.[45] The most interesting results are found for the hybrid hemoglobins. In these half-ligated models, the resonance positions of both the Fe Val-E11 methyls (associated with the ligated subunit) and the Zn Val-E11 methyls (associated with the unligated Zn(II) subunits) can be observed. Figure 10 shows that the resonance frequencies of the Fe γ_2 methyls of Val-αE11 in the hemoglobin hybrids are identical with the corresponding resonance in fully ligated HbCO. A similar result is obtained for HbO_2. By inference, the stereochemistry of the valine–heme contact is identical in both the half-ligated and fully ligated systems. This identity is somewhat surprising. Previous workers[43,45] suggested that Val-E11 may help modulate hemoglobin ligand affinity by "pushing" against the ligand in the deoxy (T) quaternary structure. The present NMR study does not

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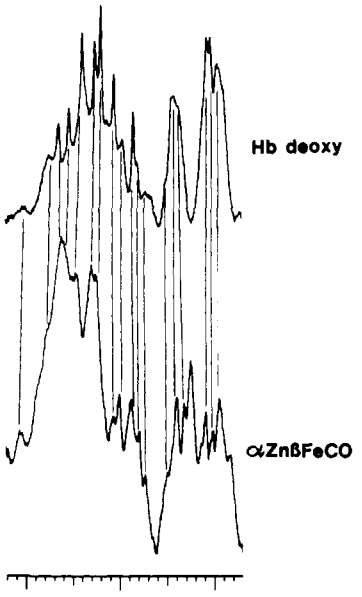

Figure 11. ^{1}H NMR spectra in the histidine proton region of deoxy-Hb and α-Zn_2-β-$(FeCO)_2$.

support this idea. Zn/Fe hybrid Hbs have a low CO affinity, and several conformational indicators discussed in this paper suggest that the Zn/Fe hybrid hemoglobins adopt (at least in part) a deoxy-like structure. However, the NMR studies show *no* evidence of any dislocation of Val-E11 within the ligated FeCO subunit in the half-ligated Fe/Zn hemoglobin hybrids.

The general order of the Val-E11 ring current shifts are β-Zn > α-Zn > FeCO. This ordering is just that predicted by the structures of deoxy-Hb and -HbCO. A more quantitative treatment of these data is possible by using the empirical porphyrin ring current shift parameters obtained by Wüthrich et al.[70]

$$\Delta\nu \text{ v } 305 \left[\frac{(3(R\cos\theta + 6)^2/(R+6)^2 - 1}{R^3 + 4^3} \right]$$

In using this equation, it is assumed that the structure of the Zn subunit is identical with that in deoxy-Hb. The ring current shifts are determined by the distance, R (and the angle), between the porphyrin center and the γ-Val-E11 methyl group. For deoxy-Hb and -HbCO, the distances and calculated ring current shifts (assuming α-FeCO = −1.8 ppm vs. DSS) are as follows:

$$\alpha_{deoxy} \quad R = 4.35 \text{ Å}, \quad \delta = -2.6 \text{ ppm}$$

$$\beta_{deoxy} \quad R = 3.81 \text{ Å}, \quad \delta = -4.6 \text{ ppm}$$

$$\alpha_{CO} \quad R = 4.78 \text{ Å}, \quad \delta = -1.8 \text{ ppm}$$

$$\beta_{CO} \quad R = 4.63 \text{ Å}, \quad \delta = -1.8 \text{ ppm}$$

By comparison with the FeCO/Zn hybrids, the upfield spectrum of ZnHb is surprisingly complex. The resonances at −3.6 ppm for the β subunit of α-$(FeCO)_2$-β-Zn_2 and at −2.75 ppm for the α subunit of α-Zn_2-β-$(FeCO)_2$ are found at identical frequencies in ZnHb. This suggests that the Val-E11 in the unligated (Zn) subunits has not moved in going from the ligand-free ZnHb to the half-ligated FeCO Zn hybrids. Note, however, that the peaks at −3.6 and −2.7 are split in ZnHb. We are currently investigating the reasons for this splitting.

(C) Histidine Resonances. Histidine β146 is the carboxy terminal residue of the Hb β chain. Perutz and Kilmartin[53-56] have suggested that this histidine is involved in an important hydrogen bond network which stabilizes the structure of deoxy-Hb and contributes to the Bohr effect. The importance of His-β146 for the Bohr effect[57] has been questioned by Russo and Ho,[57] on the basis of NMR data. Studies in our lab have focused on comparisons of the C_2H and C_4H protons of HbCO, deoxy-Hb, ZnHb, α-Zn_2-β-$(FeCO)_2$, and α-$(FeCO)_2$-β-Zn_2. Spectra of deoxy-Hb and α-Zn-β-FeCO are compared in Figure 11. The specific C_2H and C_4H resonances of His β146 were assigned by use of carboxypeptidase-treated Hb. The β146 resonances so obtained for several derivatives are summarized in Table II.

Table II. Assignments of β146 Histidine Protons of Several Hemoglobin Derivatives

	C-2 (ppm) vs. OSS	C-4, ppm	soln cond	struct
Hb^{CO}	(8.38)	7.71	*a*	R
	8.11		*b*	R
Hb^{II}	8.56	7.51	*a*	T
α-Zn-βFeCO	8.48		*c*	T

[a] In pH 7.0, 0.1 M BIS-TRIS buffer. [b] In pH 7.0, 0.2 M NaCl and 0.2 M phosphate buffer. [c] In pH 7.1, 0.01 M BIS-TRIS buffer. On CBPA treatment of HbCO, resonances were abolished at both 8.38 *and* 8.11 ppm.

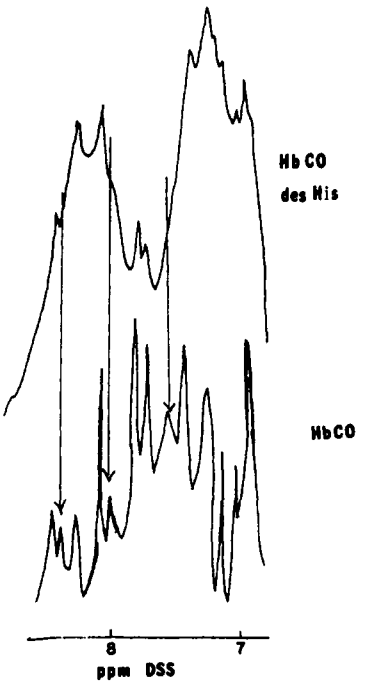

Figure 12. Assignment of His-β146 protons by comparison of HbCO with des β146 HbCO.

When previously established conditions[58,59] are used, carboxypeptidase removes only His-β146 and no other histidines. Thus, the resonances due to β146 can be assigned unambiguously by noting which resonances disappear on carboxypeptidase treatment. (For examples see Figure 12 and ref 58 and 59.) A detailed analysis of the histidine C_2H resonances is beyond the scope of this paper, and results for a series of ligated hemoglobins will be presented elsewhere. Assignment of the C_4H is straightforward, since only one peak disappears on CBP treatment. The C_4H assignments are in complete agreement with Russo and Ho's work.[57]

The resonance frequencies (in parts per million vs. DSS) so obtained for deoxy-Hb (ν_{C_2H} = 8.6, ν_{C_4H} = 7.5) and HbCO (ν_{C_4H} = 7.7) are in good agreement with Ho's recent reports.[57] The key finding of the present work on metal-substituted systems is that under our conditions, the resonance frequencies of α-Zn_2-β-$(FeCO)_2$ are essentially identical (±0.05 ppm) with those of deoxy-Hb for the conformationally sensitive histidines: β146, β143, and β2 (assigned by comparison to the work of Ho et al.).[57] Indeed, all observed histidines resonances appear to allign for deoxy-Hb and the ligated α-Zn_2-β-$(FeCO)_2$ systems except for a resonance at 7.2 in $Zn_2(FeCO)_2$. The more limited resolution of ZnHb precludes a detailed analysis of its His resonances. If the histidine residues form critical links in the quaternary structure of Hb, as argued by Perutz,[53] then α-Zn_2-β-$(FeCO)_2$ must be assumed to have a deoxy quaternary structure as well.

Table III. Summary of Apparent Quaternary Structure of Zn-Substituted Hemoglobins Using Different Techniques

Technique	α-Zn_2-β-$(FeCO)_2$	α-$(FeCO)_2$-β-Zn_2
CO off rate	T	T
CD_{280}	T	T
Val-E_{11} NMR resonance	R	R
Asp-β99–Tyr-α42 exchangeable proton	R	R
Val-β98–Tyr-β145 exchangeable proton	T	R
Asp-α94–Asp-β102 exchangeable proton	T	R
His-β146 (and other His resonances)	T	T
ENDOR (of Fe^{III} C^--substituted species)	R	R

Discussion

The two-state (NMR) model of cooperativity has been generally successful in interpreting the thermodynamics and kinetics of ligand binding to hemoglobin.[13,14,48] However, the structural features which influence the equilibrium between ligated and unligated conformations of Hb remain elusive. Indeed detailed NMR studies by Ho and others on partially oxygenated systems[21,40] raised doubts about the structural validity of a two-state model. The present work has probed the conformation of "model" bis-ligated systems in which a deoxy-Fe(II) heme is replaced by an essentially isostructural Zn(II) heme.

A wide variety of independent conformational probes have been used in this work. The key results are summarized below.

1. Different spectroscopic techniques provide different assignments of the conformation of Zn/Fe hybrid hemoglobins (Table III). Two interpretations of this result are possible. The techniques used may probe only local tertiary structure, and not quaternary structure. However, the exchangeable proton resonances, histidine resonances (including His-β146), and UV CD all probe the conformation of amino acids at the $\alpha_1\beta_2$ interface. The conformation of this interface is believed to be critical to cooperative ligand binding. It has therefore been argued[24,54] that changes in secondary and tertiary structure at the $\alpha_1\beta_2$ interface *necessarily* change ligand binding, assuming MWC linkage.[60] Indeed, the network of H bonds at this interface might serve as one structural definition of quaternary structure.[24] A second interpretation is simpler. In a two-state equilibrium, all probes of the equilibrium must give the same estimate of the position of equilibrium. This condition is not met by our studies. Some probes suggest a T structure by comparison with deoxy-Hb while other probes suggest an R structure (Table III). Thus with the reservation noted above, we concur with Ho[40] that a two-state description of hemoglobin conformation appears inadequate to explain the available spectroscopically based structural results. Quite recently, Miura and Ho reported elegant studies[72] of a unique cross-linked hybrid hemoglobin, to which one, two, or three ligands were bound to $Fe^{III}CN$ subunits. On the basis of detailed magnetic resonance studies, they conclude[72] that "the spectral changes of these cross-linked mixed valency hybrids as a function of ligation are not concerted, and cannot be explained by two structure concerned models".

The present studies of noncross-linked metal hybrid Hbs confirm and extend these conclusions.

Finally, in a recent paper, Ackers presented an elegant and detailed model for a hemoglobin cooperativity which directly predicts the quaternary structure of hemoglobin in various ligated states. One prediction of this model is that the α-$(deoxy)_2$-β-L_2 hybrid will exist primarily in a low-affinity conformation ([R] > 10), while the α-L_2-β-$(deoxy)_2$ hybrid will adopt primarily a high affinity conformation on [R] < 0.1.

With the caveat that we have studied L = CO, while Ackers examined L = O_2, our results provide tests of this model.

In contrast to Ackers prediction, there are no major differences observed in either the conformation *or* reactivity of α-Zn_2-β-$(FeCO)_2$ and α-$(FeCO)_2$-β-Zn_2. We are presently extending our studies to O_2 adducts to determine whether the difference between CO and O_2 is responsible for this discrepancy.

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2. A continuing controversy has surrounding the role of specific protein–heme interactions in modulating the binding of ligands. Previous resonance Raman[49] and EXAFS studies[23,50] suggested that the structure of the heme site remains constant when comparing normal Hb with both high-affinity and low-affinity Hbs. The present studies confirm and extend this conclusion to partially ligated hemoglobins. The Zn/Fe hybrid hemoglobins are functionally different from native hemoglobin with respect to both ligand dissociation and association rate constants. This functional difference is *not* mirrored in any observable structural difference at or near the site, as measured by ^{13}C NMR spectroscopy, ENDOR spectroscopy, EXAFS measurements, and NMR ring current shift measurements (on Val-E11). Thus, in comparing the heme site structure of HbCO, α-Zn_2-β-$(FeCO)_2$, and α-$(FeCO)_2$-β-Zn_2, any distance dislocations > 0.01 Å and angular dislocations > 10° can be ruled out. This result need not be surprising. If the heme site is much less flexible than other segments of the protein (e.g., the $\alpha_1\beta_2$ interface), then any "strain" at the heme will be relaxed and distributed among other, more flexible sites. This idea is quite similar in spirit, but differs in detail, to Hopfield's distributed energy model.[51]

We believe that the range of experiments reported here represents one of the most complete structural and dynamic studies of a hemoglobin derivative. When *all* the results are considered, the data are most consistent with a sequential model[61] of ligand binding to hemoglobin. When a ligand binds to hemoglobin, it appears that the Fe–ligand bonds and the amino acids immediately surrounding the heme adopt a tertiary structure which is indistinguishable from that of fully ligated hemoglobin. However, in adopting this local ligated structure, some (atom) displacements occur. Any strain associated with these displacements is distributed within the protein and may be largely localized at the $\alpha_1\beta_2$ interface. The present results, and others,[40] suggest that some interactions at the $\alpha_1\beta_2$ interface are stronger than others. Thus, stepwise ligand addition may produce associated stepwise changes in the $\alpha_1\beta_2$ interface. In keeping with this idea, not all indicators of Hb quaternary ($\alpha_1\beta_2$) conformation change concertedly in the bis-ligated model Zn/Fe hemoglobins. In principle, this model may best be checked by preparing and studying stable monoligated and tris-ligated hemoglobins. We[52] recently reported a unique method for preparing such systems. The required spectroscopic and dynamic studies will be reported in future publications.

Materials

Unless otherwise stated, all preparative procedures were carried out at 5 °C. Any protein solutions containing Fe–CO were routinely flushed with CO. Isoelectric focusing[69] was performed after every major step in the preparative procedure. The protein was considered pure only if one narrow band was present. If necessary, Chromatofocusing[70] (Pharmacia) was run to purify the protein.

Hb was prepared from fresh whole blood by the method of Fanelli et al.[62] The Hb was then flushed with CO until all the Hb was in the CO form. Subunits were separated by reaction of Hb with PMB,[66] followed by separation of the mercuriated subunits on a CM column.[67] The sulfhydryl groups of the separated subunits were regenerated by 2-mercaptoethanol chromatography.[68] The yield of pure subunits is ca. 25%. The desired aposubunits were prepared by the butanone extraction method of Teale[64] after the pH of the subunits had been adjusted to pH 3.0. The aposubunits were then dialyzed twice against distilled water for 0.5 h each followed by two dialyses, for 1 h each, vs. pH 6.4, 20 mM BIS-TRIS buffer. It was found that the above dialysis procedure prevented the precipitation of the aposubunits that occurs when phosphate buffer is used. All subsequent steps were carried out in the dark.

A 10% excess of zinc(II) protoporphyrin-IX[10] was dissolved in a minimum amount of 200 mM KOH and then immediately diluted 10-fold by the addition of distilled water. This solution was slowly added by using a syringe pump with stirring to the aposubunits over a period of 90 min. During the addition, the pH was monitored and kept in the range of pH 6.2–6.4 by the slow addition of 10 mM HCl. The resulting mixture was then allowed to incubate overnight. After centrifugation, excess porphyrin was removed by desalting on a Biorad P-2 column equilibrated with 20 mM phosphate, pH 6.4. The complimentary Fe-containing subunit was dialyzed against the above buffer and added slowly with stirring to the Zn PPIX containing subunits. An excess of α subunits was always used to facilitate purification. The resulting mixture was allowed to equilibrate overnight. The excess α subunits were

removed by running a Sephadex G-50 (Pharmicia) exclusion column. The purified protein was stored in liquid N_2 after dropwise addition to liquid N_2. The yield of isoelectrically pure hybrid from subunits was always greater than 80%. An equivalent procedure yields the Mn hybrid in excellent yield.

The ^{13}CO hybrids were prepared by oxidizing the CO hybrid to the met hybrid by ferricyanide oxidation (4 equiv $K_3Fe(CN)_6$ per heme, stirring at room temperature until oxidation was complete). The met hybrid was then desalted on a Biorad P-2 column (equilibrated with 20 mM phosphate, pH 6.6) to remove excess ferricyanide and ferrocyanide. ^{13}CO and dithionite were added quickly until the met bands disappeared and only CO bands were present in the visible spectra. The ^{13}CO hybrid was then quickly desalted by using a P-2 column as above. The samples were then exchanged with 15% D_2O, 20 mM phosphate, and pH 6.6 buffer and concentrated by using an Amicon Minicon macrosolute concentrator.

The oxy hybrids were prepared as above except that air and sodium ascorbate were used instead of ^{13}CO and dithonite.

Methods

The stopped-flow data were obtained on a Durrum stopped-flow spectrophotometer Model D-110 by mixing equal volumes of 0.4 mM heme α-Zn_2-β-$(FeCO)_2$ and CO-saturated $K_3Fe(CN)_6^{3-}$ (5-50 mM). Both species were in pH 6.6, 20 mM BIS-TRIS buffer. The rate of oxidation was determined by monitoring the increase in absorbance of the met band at 630 nm. Spectra were checked on a Cary 118 spectrophotometer to ensure that met formation was complete.

The ENDOR spectra were obtained at 2 K by using the instrument previously described.[18]

Circular dichroism spectra were recorded on a JASCO J-40 automatic recording spectropolarimeter, interfaced with a Digital PDP 11/23 computer which enabled both instrumental control and signal averaging. The instrument was calibrated for each run by using camphor-d_{10}-sulfonic acid as a standard. Molar ellipticities were calculated based on absorbance measurements of the hemoglobin samples. Spectra were drawn on a Tektronix 4662 interactive digital plotter after the base line was subtracted on a Tektronix 4051 graphics system. Samples were typically 15 μM tetramer solutions, pH 7.1, 100 mM BIS-TRIS. Generally, three separate spectra were computer-averaged.

Nuclear magnetic resonance spectra were obtained with a WH-400 MHz Bruker FT-NMR spectrometer equipped with an Aspect 200 computer system. Homonuclear decoupling of the residual HDO peak was accomplished with a WEFT pulse sequence. Typical spectra widths were 6-8 KHz; 16K data points were used. Either 2,2-dimethyl 2-silapentane-5-sulfonate (DSS) or HDO were used as references for proton chemical shifts; downfield chemical shifts were assigned positive values.

Studies involving ring-current shifted resonances were undertaken by exchanging the hemoglobin hybrids with D_2O, pD 7.1, 10 mM BIS-TRIS buffer. The samples were greater than 95% D_2O-enriched. For such studies, relaxation delays of zero were satisfactory and flip angle values of 21° optimized spectral resolution. Typically, 2000 scans were collected.

Studies involving exchangeable proton resonances were undertaken by using a DASWEFT pulse sequence.[71] The samples were run in pD 7.1, 10 mM BIS-TRIS buffer, enriched to 10% D_2O. All NMR spectra were collected at 24 °C, unless otherwise noted. For the CO hybrids, the 5 mM NMR tubes were capped by rubber septa and were purged with CO for 25-30 min prior to spectrum collection. Typically, 3000 scans were collected.

The ^{13}CO samples were prepared as noted above. The samples were run in pH 6.6, 20 mM phosphate, enriched to 15% D_2O. Thus, no decoupling of solvent was required. Typically, 600 scans were collected.

The Stability of Alkyl Radicals

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Abstract: All the data on the decomposition of simple alkyl radicals have been reviewed. Together with results on the reverse addition reactions, alkyl radical combination rates, and the entropies of the alkyl radicals, the data lead to $\Delta H_f(n\text{-}C_3H_7\cdot) = 100.5 \pm 2.1$ kJ/mol, $\Delta H_f(i\text{-}C_3H_7\cdot) = 93.3 \pm 2.5$ kJ/mol, $\Delta H_f(sec\text{-}C_4H_9\cdot) = 71.0 \pm 1.6$ kJ/mol, $\Delta H_f(t\text{-}C_4H_9\cdot) = 51.7 \pm 2.2$ kJ/mol (zero barriers for CH_3 rotors) and 46.2 ± 2.2 (10 kJ barrier for CH_3 rotors), and $\Delta H_f(t\text{-}C_5H_{11}\cdot) = 32.6 \pm 4$ kJ/mol. These values are fully consistent with determinations based on the decomposition of aliphatic compounds and combination of radicals and lead to $D(n\text{-}C_3H_7\text{-H}) = 422.5$ kJ, $D(i\text{-}C_3H_7\text{-H}) = 415.3$ kJ, $D(sec\text{-}C_4H_9\text{-H}) = 414.2$ kJ, $D(t\text{-}C_4H_9\text{-H}) = 404.6$ (zero barrier), and $D(t\text{-}C_5H_{11}\text{-H}) = 402.5$ kJ. They are all significantly higher than those generally used values recommended in a recent review but are in accord with values we suggested several years ago. It appears that previous rejection of measured alkyl radical decomposition rates is due to the general acceptance of the earlier bond energies. The rate expressions for alkyl radical decomposition which satisfy the new thermochemistry and detailed balance over the temperature range 300-800 K are the following: $k(n\text{-}C_3H_7\cdot \rightarrow C_2H_4 + CH_3\cdot) = 10^{13.1}\exp(-15300/T)$/s, $k(i\text{-}C_3H_7\cdot \rightarrow C_3H_6 + H\cdot) = 10^{13.34}\exp(-18700/T)$/s, $k(sec\text{-}C_4H_9\cdot \rightarrow C_3H_6 + CH_3\cdot) = 10^{12.97}\exp(-14700/T)$/s, $k(sec\text{-}C_4H_9\cdot \rightarrow C_4H_8\text{-}1 + H\cdot) = 10^{13.11}\exp(-18300/T)$/s, $k(sec\text{-}C_4H_9\cdot \rightarrow c\text{-}C_4H_8\text{-}2 + H\cdot) = 10^{12.62}\exp(-17500/T)$/s, $k(sec\text{-}C_4H_9\cdot \rightarrow t\text{-}C_4H_8\text{-}2 + H\cdot) = 10^{12.66}\exp(-17100/T)$/s, $k(t\text{-}C_4H_9\cdot \rightarrow i\text{-}C_4H_8 + H\cdot) = 10^{13.92}\exp(-18900/T)$/s, $k(t\text{-}C_5H_{11} \rightarrow i\text{-}C_4H_8 + CH_3) = 10^{13.03}\exp(-15000/T)$/s. Some consequences of the new bond energies with respect to our understanding of hydrocarbon free radical chemistry and the diradical mechanism for the decomposition of small ring compounds are discussed.

This paper is concerned with the thermodynamic and kinetic stability of a number of simple alkyl radicals. It is centered about the heats of formation of these radicals and represents a continuation of investigations begun several years ago.[1] In that work we demonstrated that the experimental data on the kinetics of the decomposition of the simple alkanes (butane, 2,3-dimethylbutane, and 2,2,3,3-tetramethylbutane) and the reverse radical combination processes are incompatible with the generally used[2] heats of formation of ethyl, isopropyl, and *tert*-butyl radicals. Instead, values for ΔH_f of the order of 10-20 kJ/mol higher were required. This has aroused a certain degree of controversy. In the case of ethyl, Hase[3] has demonstrated that a value for $\Delta H_f(C_2H_5\cdot)$ of 118 kJ/mol (298 K) is derivable from the kinetics of the process $C_2H_5 \rightleftarrows C_2H_4 + H$. There has been a considerable amount of experimental work on *tert*-butyl radicals.[4,6] This has

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