FEBS Letters 372 (1995) 126–130

FEBS 16037

Oxygen equilibrium and electron paramagnetic resonance studies on copper(II)–iron(II) hybrid hemoglobins at room temperature

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Received 10 July 1995; revised version received 10 August 1995

Abstract Copper(II)–iron(II) hybrid hemoglobins, in which hemes in either the *α* or *β* subunits are substituted with copper(II) protoporphyrin IX, have been prepared. The affinities of the ferrous-subunits in both hybrids for the first binding oxygen are as low as the affinity of deoxyhemoglobin under various solution conditions, indicating the equality of behavior in copper(II) protoporphyrin IX and deoxyheme. Electron paramagnetic resonance (EPR) examinations on these hybrids at room temperature show that the interaction between copper(II) and the proximal histidine (F8) is specifically weakened in the *α* subunits within a low affinity conformation of hemoglobin. These results suggest that copper(II) protoporphyrin IX is a useful EPR probe at room temperature for investigating the deoxyheme environment in hemoglobin.

Key words: Hemoglobin; EPR; Metal-substitution; Oxygenation; Intermediate species

1. Introduction

Even though the structure and function of human Hb molecule have been extensively studied, the exact role of the heme active site in triggering the allosteric transition of Hb is not fully understood. Perutz [1] first suggested that the transition from the T to the R quaternary structure is triggered by small decrease in the ionic radius of ferrous iron due to its ligand-induced spin-state transition. For further understanding of this mechanism, a detailed knowledge of the relationships between the protein tertiary and quaternary structures and the electronic structure of the heme would be necessary. One approach for studying such relationships is characterization of Hb, in which specific hemes are substituted with another metalloprotoporphyrin.

During recent years, the oxygen equilibrium data on various metal-substituted hybrid Hbs, $\alpha_2(M)\beta_2(Fe)$ and $\alpha_2(Fe)\beta_2(M)$, containing the first transition metal ions (M) that do not react with oxygen or CO, have been accumulated [2–4]. It has been found that the oxygen affinity of the ferrous subunits in M-Fe(II) hybrids increases as the number of d_γ electrons of the metal ions decreases. This finding suggests that the number of electrons occupying d_γ orbitals in the central metal ion is an important determinant in the interaction between metal ion and the globin moiety. This rule is also consistent with the fundamental finding that the iron ions in deoxyHb with low affinity and in oxyHb with high affinity are high-spin (d^6, d_γ^2) and low-spin (d^6, d_γ^0), respectively [1]. However, it has been recently pointed out that this rule cannot be straight-forwardly applied to several other cases, such as (metal-free) protoporphyrin-Fe(II) hybrids [5] and Mg(II)-Fe(II) hybrids [6], both of which exhibit very low affinity for oxygen despite the lack of a d_γ electron.

In this paper, we have prepared Cu(II)-Fe(II) hybrid hemoglobins, $\alpha_2(Cu)\beta_2(Fe)$ and $\alpha_2(Fe)\beta_2(Cu)$, in which heme in either the α or β subunits are substituted with Cu(II)-PPIX, to study the effects of d^9 electrons of the porphyrin metal with d_γ^3 configuration on the structure and function of Hb molecule. Since Cu(II)-PPIX binds neither oxygen nor CO, the oxygen equilibrium properties of the ferrous subunits in both hybrids can be readily determined.

Among metalloporphyrins with the first transition metal ions, Cu(II)-PPIX with one unpaired electron is quite suitable for EPR investigation. In fact, several Cu(II)-substituted heme proteins, such as Cu(II)Mb [7], Cu(II)Hb [8], and Cu(II)-cytochrome *c* [9], have been previously characterized by EPR technique at cryogenic temperature. In the case of Hb, however, EPR experiments at room temperature are preferable to those at cryogenic temperatures, not only because functional characterizations must be carried out under physiological conditions but also because lowering temperature may stabilize the T-state relative to the R-state. Therefore, we have undertaken experiments to assess the utility of Cu(II)-PPIX as a EPR probe at room temperature for the deoxyheme environment in Hb.

2. Material and methods

2.1. *Preparation of Cu(II)-PPIX*

Copper(II) protoporphyrin IX (Cu(II)-PPIX) was synthesized by the following procedures. The disodium salt of protoporphyrin IX (Sigma)

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Abbreviations: Hb, hemoglobin; Hb A, human adult hemoglobin; Mb, myoglobin; EPR, electron paramagnetic resonance; M-Fe hybrid Hb, mixed metal hybrid hemoglobin in which hemes in either the α or β subunits are substituted with metal ion, M; Cu(II)-PPIX, copper(II) protoporphyrin IX; Cu(II)-TPP, copper(II) (meso)-tetraphenylporphyrin; $\alpha_2(Cu)\beta_2(Fe)$, hybrid hemoglobin containing copper(II) protoporphyrin IX in the α subunits and ferrous protoporphyrin IX in the β subunits; $\alpha_2(Fe)\beta_2(Cu)$, hybrid hemoglobin complementary with the preceding one; Cu(II)Hb, hemoglobin in which hemes in both the α and β subunits are substituted with copper(II) protoporphyrin IX; Cu(II)Mb, myoglobin in which heme is substituted with copper(II) protoporphyrin IX; EPR, electron paramagnetic resonance; Ni(II)-PPIX, nickel(II) protoporphyrin IX; Ni(II)Hb, hemoglobin in which hemes in both the α and β subunits are substituted with nickel(II) protoporphyrin IX; Bistris, 2-[bis(2hydroxyethyl)amino]-2-(hydroxymethyl)-1,3-propanediol; Tris, tris(hydroxymethyl)aminomethane; IHP, inositol hexaphosphate.

SSDI 0014-5793(95)00965-5

was dissolved in formic acid, and the solution was neutralized with ammonium hydroxide. The precipitate of ammonium salt of protoporphyrin IX was collected and washed with distilled water several times. A refluxing solution of anhydrous copper(II) chloride in DMF, containing 3% acetic acid, under nitrogen atmosphere was added to another refluxing solution of 0.9-fold stoichiometric amount of ammonium salt of protoporphyrin IX in DMF, containing 3% acetic acid, under nitrogen atmosphere. After heating at 85°C under refluxing for 20–25 s, an equal volume of cold distilled water was added to the solution. The precipitate of Cu(II)-PPIX was collected by centrifugation, and dissolved in ammonium hydroxide. After neutralization with acetic acid, the precipitate was collected and washed with distilled water several times, and then dried under reduced pressure. At this stage, the resulting Cu(II)-PPIX is usually pure as judged by visible spectrophotometry and thin layer chromatography with DMF. If necessary, the purification of Cu(II)-PPIX was performed by a column chromatography on Celite 545 as described previously [2].

2.2. Preparation of Cu(II)-Fe(II) hybrid Hbs

Hb A and its isolated chains were prepared according to the methods of Kilmartin et al. [10]. Cu(II)–Fe(II) hybrid Hbs were prepared by the same procedures for the preparation of Ni(II)–Fe(II) hybrids [2].

2.3. Oxygen equilibrium measurement

Oxygen equilibrium curves of Cu(II)–Fe(II) hybrid Hbs were measured by automatic oxygenation apparatus of Imai et al. [11]. Measurements were carried out at 430 nm at 25°C in 50 mM Bistris or Tris buffer with 100 mM chloride. The concentration of the hybrids was about 15–20 μM on metal basis.

2.4. EPR measurements

EPR spectra were measured at room temperature (21°C) in 50 mM Bistris or Tris buffer with 100 mM chloride by using a Varian E-line EPR spectrophotometer operating at 9.12 GHz (X-band). The concentration of the proteins was 5–10 mM on a metal basis. Deoxygenation for the hybrids were carried out by flushing Ar gas over the samples, followed by addition of a minimum amount of sodium dithionite. Half-liganded samples were prepared by flushing CO gas over the hybrid Hbs. Note here that the conclusions of the present study would not be altered by natural isotope abundance of copper atom, i.e. ^{63}Cu and ^{65}Cu, because the difference between nuclear magnetic moments of ^{63}Cu (69.1%) and ^{65}Cu (30.9%) is less than 10%.

3. Results and discussion

Fig. 1 shows the Hill plots of the oxygen equilibrium curves of Cu(II)–Fe(II) hybrid Hbs and native Hb A in 50 mM Bistris buffer, pH 7.4, in the presence of 0.1 M chloride at 25°C. The Hill plots of both hybrids approximate to the lower asymptote of the sigmoidal plot of Hb A, indicating that the substitution of deoxyheme with Cu(II)-PPIX does not significantly alter the K_1 values, the equilibrium constants of Hb for the first binding oxygen molecule. This was further confirmed quantitatively under various solution conditions (see Table 1). Previously, Manoharan et al. [12] reported that the sulfhydryl reactivity of Cys-93β in Cu(II)Hb toward 4,4′-dithiopyridine (4-PDS) is similar to that in deoxyHb. This observation is consistent with the present finding that Cu(II)-PPIX acts like a deoxyheme in a Hb molecule.

Fig. 2A shows the X-band EPR spectra of deoxygenated Cu(II)–Fe(II) hybrids, $\alpha_2(\mathrm{Cu})\beta_2(\mathrm{Fe})$ and $\alpha_2(\mathrm{Fe})\beta_2(\mathrm{Cu})$, at room temperature. Both EPR spectra are well-resolved even at room temperature and differ in their (low-field) parallel and (high-field) perpendicular signals from each other.

The parallel Cu(II) hyperfine splitting, $A_{\parallel}^{\mathrm{Cu}}$, of $\alpha_2(\mathrm{Fe})\beta_2(\mathrm{Cu})$ is smaller than that of $\alpha_2(\mathrm{Cu})\beta_2(\mathrm{Fe})$ (Table 2), indicating that significant part of the electron spin density at the Cu(II) center of the β subunit is removed by coordination of the fifth-ligand, most likely the proximal histidine (F8). On the basis of the comparisons of the parallel parameters of Cu(II)–Fe(II) hybrids with those of Cu(II)-TPP model compounds [7], we can assign that Cu(II)-PPIX in the α subunit is four-coordinated with no axial ligand while Cu(II)-PPIX in the β subunit is five-coordinated with the proximal histidine (F8) (Table 2). This assignment has been further confirmed by our spectrophotometric data on deoxy-Cu(II)–Fe(II) hybrid Hbs, although their visible absorption spectra are complicated by the spectral overlap of deoxyheme and Cu(II)-PPIX (data not shown). We should point out here that the perpendicular EPR pattern of $\alpha_2(\mathrm{Fe})\beta_2(\mathrm{Cu})$ is very similar to that of Cu(II)Mb, in which Cu(II) protoporphyrin IX was previously assigned to be five-coordinated with the proximal histidine (F8) [8]. This spectral similarity between $\alpha_2(\mathrm{Fe})\beta_2(\mathrm{Cu})$ and Cu(II)Mb also supports our EPR assignment.

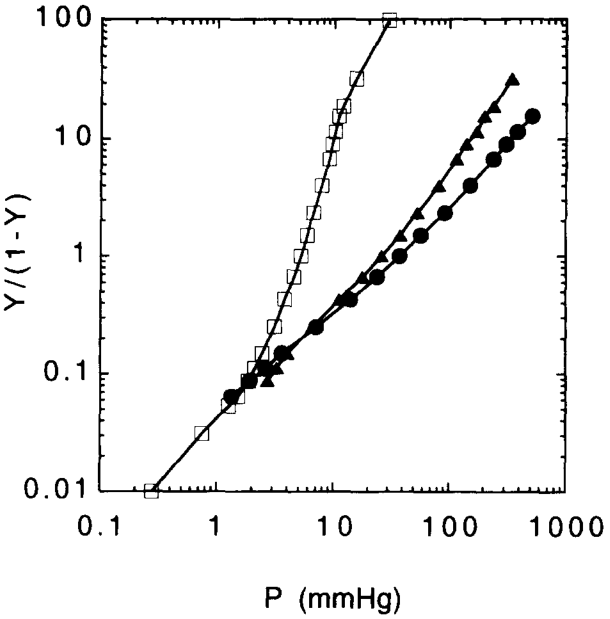

Fig. 1. Hill plots of oxygen equilibrium curves of $\alpha_2(\mathrm{Cu})\beta_2(\mathrm{Fe})$ (●), $\alpha_2(\mathrm{Fe})\beta_2(\mathrm{Cu})$ (▲), and native Fe(II)Hb (□) in 50 mM Tris buffer, pH 7.4, in the presence of 100 mM chloride at 25°C. P is partial pressure of oxygen in mmHg (1 mmHg = 133.3 Pa), and Y is fractional oxygen saturation of ferrous subunits. Measurements were carried out at 430 nm by automatic oxygenation apparatus of Imai et al. [11]. The concentration of the hybrids was about 15–20 μM on metal basis.

Previously, Manoharan et al. [8] reported the X-band and Q-band EPR spectra of Cu(II)Hb and Cu(II)Mb at 12K, and suggested the presence of two distinct Cu(II) coordination structures in Cu(II)Hb; namely, a five-coordinated complex with a strong proximal histidine bond as in the case of Cu(II)Mb, and a four-coordinated complex with a much weaker axial bond. Their previous results could not give any basis for the evaluation of subunit heterogeneity of Cu(II)Hb, but the present findings enable the unambiguous assignment that the five-coordinated and four-coordinated complexes in Cu(II)Hb are localized in the β and α subunits, respectively.

The perpendicular EPR signals of both hybrids are so complex that they could not be simulated by assuming an isotropic environment of the paramagnetic center which produces four equivalent sets of nine superhyperfine lines with the intensity

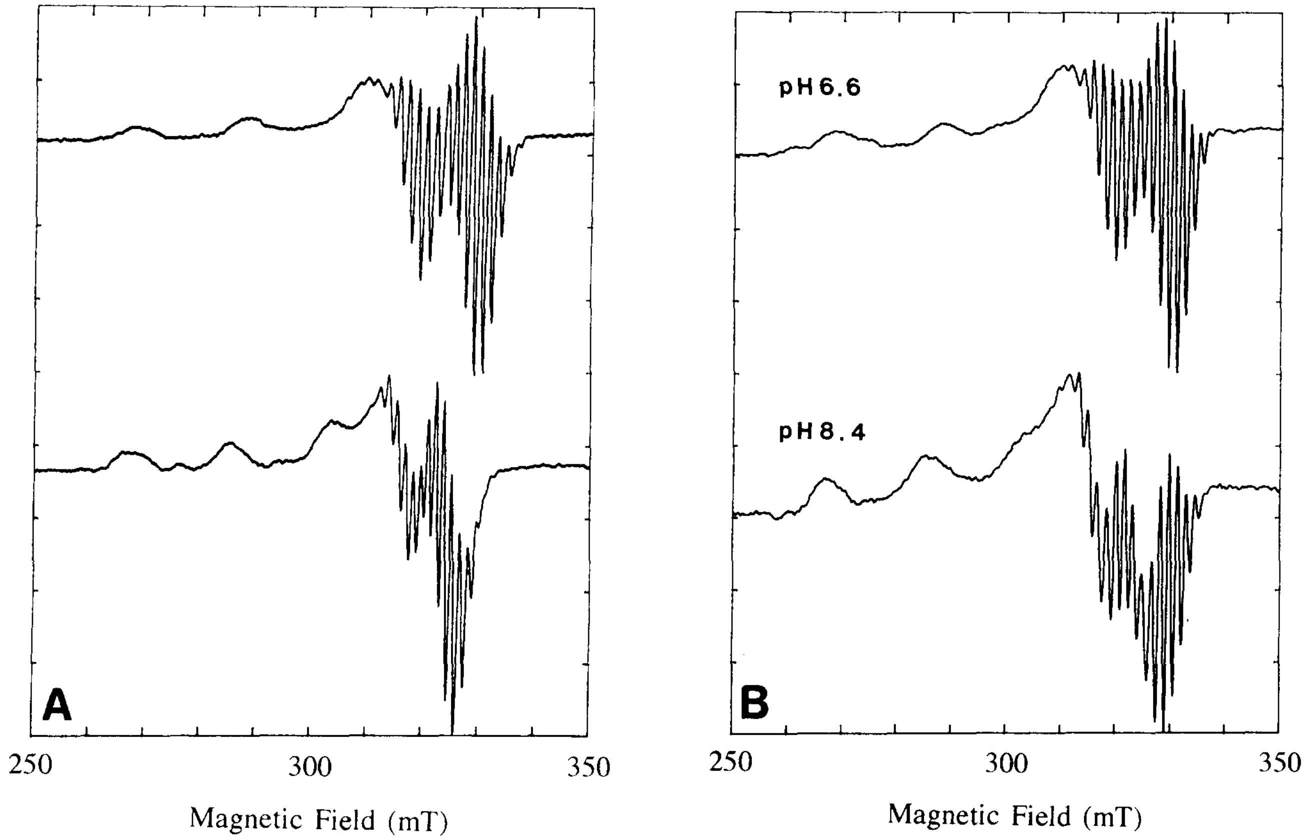

Fig. 2. (A) X-band (9.12 GHz) EPR spectra of $\alpha_2(Cu)\beta_2(Fe)$ (upper) and $\alpha_2(Fe)\beta_2(Cu)$ (lower) in 50 mM Tris buffer, pH 7.4, in the presence of 100 mM chloride at 21°C. (B) X-band EPR spectra of $\alpha_2(Cu)\beta_2(Fe\text{-}CO)$ at 21°C, at pH 6.6 (upper) and at pH 8.4 (lower), in 50 mM Bistris or Tris buffer with 100 mM chloride. The concentration of the proteins was 5–10 mM on metal basis. Deoxygenation for the hybrids were carried out by flushing Ar gas over the samples, followed by addition of a minimum amount of sodium dithionite. Half-liganded samples were prepared by flushing CO gas over the hybrid Hbs.

ratio of 1:4:10:16:19:16:10:4:1. One of the most plausible origin of this complexity is the in-plane anisotropy of Cu(II)-PPIX. To clarify the extent of the in-plane anisotropy, EPR characterizations of single crystals of ^{63}Cu(II)–Fe(II) hybrids should be required.

Fig. 2B shows the pH-dependent EPR spectra of $\alpha_2(Cu)\beta_2(Fe\text{-}CO)$ at room temperature. At pH 6.6, the EPR spectrum of $\alpha_2(Cu)\beta_2(Fe\text{-}CO)$ retains the features of the four-coordinated Cu(II)-PPIX. However, at pH 8.4, the EPR spectrum of $\alpha_2(Cu)\beta_2(Fe\text{-}CO)$ becomes rather similar to that of

Table 1
Oxygen equilibrium parameters of Cu(II)–Fe(II) hybrid Hbs

Sample	pH	Anion	K_1 (mmHg)	P_{50} (mmHg)	n_{50}
$\alpha_2(Cu)\beta_2(Fe)$	6.5	0.1 M Cl$^-$	69	69	1.0
	7.4	0.1 M Cl$^-$	38	38	1.0
	8.5	0.1 M Cl$^-$	6.5	12	0.7
	6.5	0.1 M Cl$^-$, 2 mM IHP	164	164	1.0
	7.4	0.1 M Cl$^-$, 2 mM IHP	113	113	1.0
$\alpha_2(Fe)\beta_2(Cu)$	6.5	0.1 M Cl$^-$	68	68	1.0
	7.4	0.1 M Cl$^-$	26	32	1.1
	8.5	0.1 M Cl$^-$	8.7	4.7	1.3
	6.5	0.1 M Cl$^-$, 2 mM IHP	152	152	1.0
	7.4	0.1 M Cl$^-$, 2 mM IHP	150	150	1.0
$\alpha_2(Fe)\beta_2(Fe)$*	6.5	0.1 M Cl$^-$	55		
	7.45	0.1 M Cl$^-$	21		
	8.5	0.1 M Cl$^-$	8.1		
	6.5	0.1 M Cl$^-$, 2 mM IHP	170		
	7.45	0.1 M Cl$^-$, 2 mM IHP	110		

*Data on Hb A under similar experimental conditions are taken from Imai et al. [24] and Imaizumi and Imai (unpublished results). K_1 = the dissociation equilibrium constant for the first binding oxygen in mmHg (1 mmHg = 133.3 Pa); P_{50} = oxygen pressure at half-saturation in mmHg; n_{50} = the slope of the Hill plot at half-saturation.

Table 2
EPR parameters of Cu(II)–Fe(II) hybrid Hbs at 21°C

Sample	pH	$g_{\parallel}$	$A_{\parallel}^{Cu}$ (mT)	$g_{\perp}$	Reference	Notes
$\alpha_2(Cu)\beta_2(Fe)$	pH 7.4	2.197	19.5	2.02	this work	pH-independent
$\alpha_2(Fe)\beta_2(Cu)$	pH 7.4	2.216	17.6	2.026	this work	pH-independent
$\alpha_2(Cu)\beta_2(Fe\text{-}CO)$	pH 6.6	2.197	19.5	2.02	this work	
	pH 8.4	2.213	17.9	2.016	this work	
$\alpha_2(Fe\text{-}CO)\beta_2(Cu)$	pH 7.4	2.216	17.6	2.026	this work	pH-independent
Cu(II)Mb		2.214	17.3	2.045	[8]	at 12K, Q-band
Cu(II)-TPP in chloroform		2.1871	19.9	2.0354	[7]	without axial ligand
Cu(II)-TPP in 1-methyl-imidazole		2.2253	17.9	2.0546	[7]	with axial ligand

$A_{\parallel}^{Cu}$ = parallel Cu(II) hyperfine splitting in mT.

$\alpha_2(Fe)\beta_2(Cu)$. This observation suggests that the CO-ligation to the β subunits of $\alpha_2(Cu)\beta_2(Fe)$ at alkaline pH changes the α heme environment, involving the formation of the Cu(II)-histidine (F8) bond. In contrast, the EPR spectra of Cu(II)-PPIX in the β subunits are little affected by CO ligation of the α subunits or pH values (pH 6.6–8.4) (data not shown). Thus, we can conclude that Cu(II)-PPIX in $\alpha_2(Fe)\beta_2(Cu)$ is throughout five-coordinated with the proximal histidine.

It has been suggested that Cu(II)Hb and Ni(II)Hb have common features with respect to the quaternary structures and the coordination geometry of the metal ions [8,13]. Moreover, our recent observation that the bond between Ni(II) and the proximal histidine is specifically cleaved in the α subunits within a low affinity conformation of Hb [2,14] is fully correlated with the EPR results on Cu(II)–Fe(II) hybrids. Such strong parallelism between Ni(II)-PPIX and Cu(II)-PPIX as a prosthetic group of Hb may be due to the facts that: (i) both Ni(II)-PPIX and Cu(II)-PPIX prefer four-coordination geometry even in the presence of strong basic ligands [15,16]; and (ii) both metalloporphyrins resemble deoxyheme in the 3D electronic configurations. Also, striking subunit inequivalency observed in Ni(II)–Fe(II) and Cu(II)–Fe(II) hybrids is qualitatively consistent with the previous findings deduced from a variety of measurements on Fe(II)Hb, i.e. the Fe–histidine interaction in the α subunit is much more susceptible to the Hb quaternary structures than that in the β subunit [17–19].

Combining the oxygen equilibrium data on Cu(II)–Fe(II) hybrids with available data on other hybrids, the oxygen affinities of M-Fe(II) hybrids, containing the first transition metal ions, are in the following order: Zn(II)–Fe(II) (d^{10}; d_{γ}^{4}) [4] < Cu(II)–Fe(II) (d^{9}; d_{γ}^{3}) ≈ Ni(II)–Fe(II) (d^{8}; d_{γ}^{2}) [2] < Fe(III)-F^{-}–Fe(II) (d^{5}; d_{γ}^{2}) [20] ≈ Mn(III)$H_2$0–Fe(II) (d^{4}; d_{γ}^{1}) [4,21] ≈ V = O(II)–Fe(II) (d^{1}; d_{γ}^{0}) [4] < Co(III)CN^{-}-Fe(II) (d^{7}; d_{γ}^{0}) [4] ≈ Fe(III)CN^{-}-Fe(II) (d^{5}; d_{γ}^{0}) [22] ≈ Cr(III)$H_2$0-Fe(II) (d^{3}; d_{γ}^{0}) [3]. Thus, our present results add to the evidence that the number of d_{γ} electrons is important for determining the structure of globin moiety. In addition, it is likely that the presence or absence of the distal ligand of metalloporphyrin is another important factor to control the oxygen affinity of Hb.

Recently, Perutz [23] proposed that the distance from the proximal histidine to the heme plane is the only determinant of the allosteric equilibrium visible in the α subunits while in the β subunits the location of the distal valine relative to the heme is a key factor to control the oxygen affinity. This view is basically consistent with the observations on various metal–Fe(II) hybrids that contain the first transition metal ions. This proposal also enables to explain why porphyrin–Fe(II) hybrids exhibit very low affinity for oxygen [5], because less constraints on the proximal histidine in the metal-free subunits may result in a long distance between the proximal histidine and the porphyrin plane.

Finally, these findings reported here have indicated not only the equality of behavior in Cu(II)-PPIX and deoxyheme but also demonstrated the novel utility of Cu(II)-PPIX as a EPR probe at room temperature. Therefore, Cu(II)–Fe(II) hybrids can be used as models for the oxygenation intermediates of Hb, and these systems have an additional advantage over the other metal-substituted hybrids in that Cu(II)-PPIX can be used as a EPR reporter group at room temperature for investigating the deoxyheme environment of Hb in solution.

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