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Proton NMR investigation of the reconstitution of equine myoglobin with hemin dicyanide

Evidence for late formation of the proximal His^{93}F8-iron bond

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Reconstitution of apoequine myoglobin (apoEqMb) with hemin dicyanide (FePPIX(CN)$_2$) was monitored by ^{1}H NMR spectroscopy to gain information about the sequence of events leading to metEqMbCN. At least one step in the pathway is slow enough to allow us to follow the time-dependence of formation of the product, a mixture of heme-insertion isomers characterized by others (Jue, T., Krishnamoorthi, R. and La Mar, G.N. (1983) J. Am. Chem. Soc. 105, 5701–5703; Lecomte, J.T.J., Johnson, R.D. and La Mar, G.N. (1985) Biochim. Biophys. Acta 829, 268–274). However, in contrast to all previously reported Mb-FePPIX reconstitutions, we find that the initial ratio of heme-insertion isomers is *not* 1:1. This mixture is, instead, found to be enhanced in the isomer which dominates at equilibrium. This is taken as evidence for a '[FePPIX(CN)$_2$•EqMb]' intermediate which lacks the proximal His^{93}F8-Fe bond and which proceeds quickly toward an equilibrium ratio of heme-insertion isomers. Therefore the heme-insertion isomer ratio is frozen only when the proximal His^{93}F8-Fe bond is formed. The difference in this ratio of heme-insertion isomers between EqMb (4.5:1) and SwMb (2.5:1) likely reflects the amino acid substitution: Lys → Arg^{45}CD3 (EqMb → SwMb).

Heme; Myoglobin; NMR

1. INTRODUCTION

Heme is a classic protein prosthetic, and its contribution to holoprotein stability is a topic of continuing interest. Also of interest is the process by which heme might be inserted into the globin, and efforts over decades have been made to elucidate the steps of heme incorporation into myoglobin (Mb) using optical absorbance [1–5], circular dichroism (CD) [6–8], and NMR [9–12] spectroscopic techniques. Of these, the study of Kawamura-Konishi et al. [4] is of special interest because it reported on the reconstitution of apoEqMb with hemin dicyanide (FePPIX(CN)$_2$) to form metEqMbCN. The use of FePPIX(CN)$_2$ provided for the strong-field ligand CN$^-$, presumed to require a longer time to be displaced from the heme than other ligands such as OH$^-$, thereby slowing the reconstitution pathway. Four independent processes were proposed for the reconstitution process, which was presumed to result in a 1:1 mixture of the two heme-insertion isomers which differ from one another by a 180° rotation about the α,γ-*meso* axis as depicted in the inset to Fig. 1 [9]. At least one of these processes was found to occur over several seconds and so might be observed directly by ^{1}H NMR, because the combination of short longitudinal relaxation times (T_1) and relatively narrow lines allows rapid collection of ^{1}H NMR spectra for the low-spin species derivatives of heme which have cyanide as a ligand [13]. We therefore decided to study the reconstitution reaction between FePPIX(CN)$_2$ and apoEqMb and apoSwMb by ^{1}H NMR, and here provide evidence that formation of the proximal His^{93}F8-Fe bond is late in the reconstitution process. This is done by demonstrating that the metMbCN heme-insertion isomer ratio is different if generated by the reconstitution of apoMb with FePPIX(CN)$_2$ rather than FePPIX.

Abbreviations: EqMb, equine myoglobin; FePPIX, iron (III) protoporphyrin IX, hemin; FePPIX(CN)$_2$; hemin dicyanide; Mb, myoglobin; NMR, nuclear magnetic resonance spectroscopy; PPIX, protoporphyrin IX; SwMb, sperm whale myoglobin

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2. MATERIALS AND METHODS

EqMb and SwMb were from Sigma; the appropriate apoMb was then generated [14]. Reconstitutions were performed in ^2H_2O solvent directly such that the final Mb concentration was ~0.5–1 mM in 0.5 ml. Reconstitutions with heme required dissolving the heme with a minimum amount of NaO^{2}H, which resulted in a pH of at least 11. FePPIX(CN)$_2$ was generated by adding ~5-fold excess of KCN to the heme, then adjusting the pH to 9.0–9.2 (higher pH values result in significant displacement of CN$^-$ by OH$^-$). The apoMb solution pH was adjusted before adding the heme or FePPIX(CN)$_2$. In each case the apoMb was in ~20% excess, as determined by optical titration of an aliquot against the heme solution. After reconstitution the sample

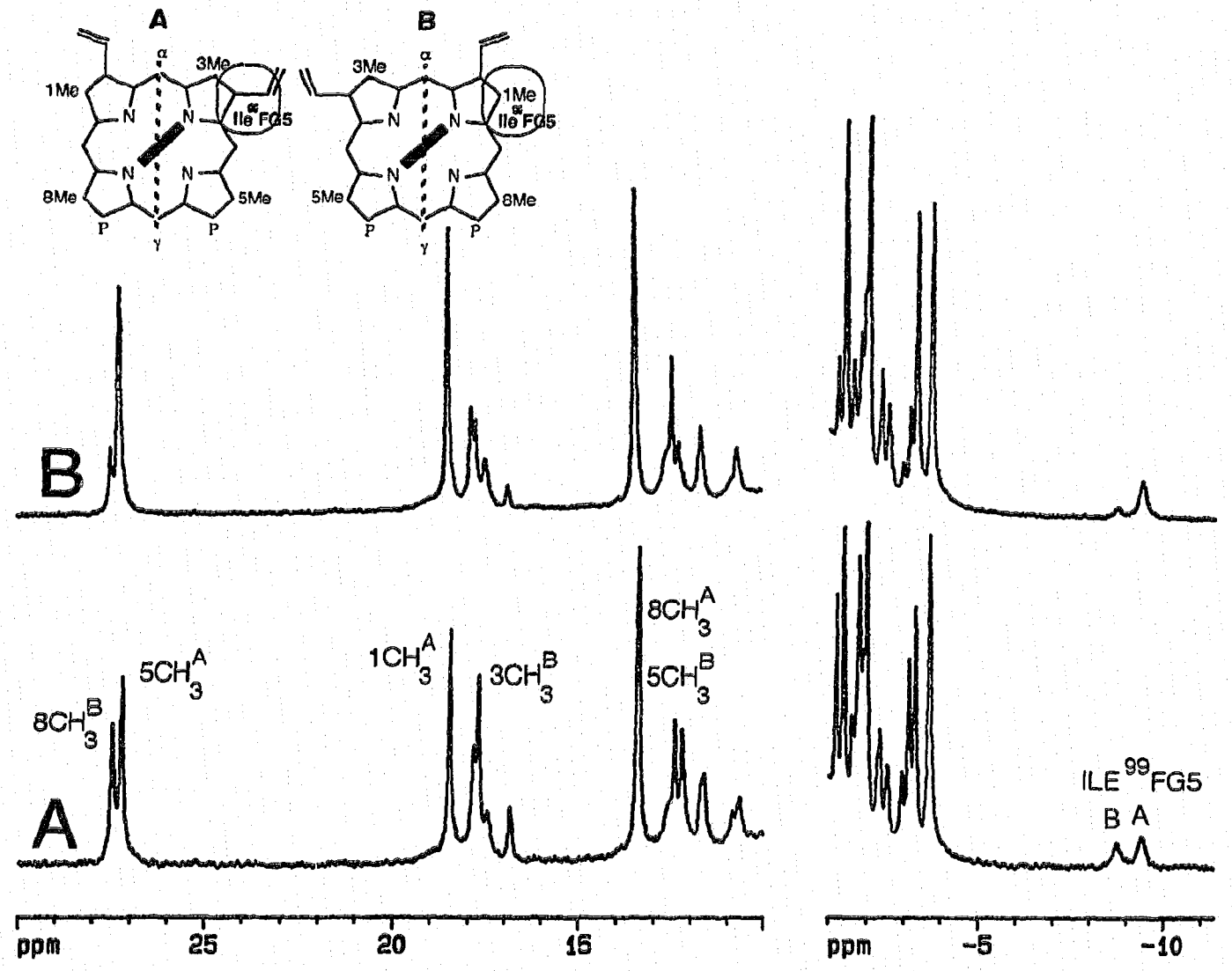

Fig. 1. Products of reconstitution reactions. A. Product of FePPIX + apoEqMb, then cyanide added, at pH 9.2 and 25°C in ^2H_2O. B. Product of $FePPIX(CN)_2$ + apoEqMb at pH 9.2 and 25°C in ^2H_2O. Note that the product ratio (major:minor) for trace B is significantly larger than the ~1:1 ratio shown in trace A. The *inset* at the top illustrates the two heme-insertion isomers which differ from each other by a 180° rotation about the α,γ-*meso* axis. Two proximal residue positions are shown: $Ile^{99}FG5$ and the $His^{93}F8$ ring (end on as the bar in the middle).

pH was checked, resulting in the uncorrected value of 9.2. ^1H NMR spectra were then recorded on a Bruker AMX-400 NMR spectrometer equipped with a dedicated 5 mm ^1H probe at 25°C, operating at 400.14 MHz. The data in Fig. 1 were collected as 1024 transients with a repetition time of 1 s^{-1} employing quadrature; the acquisition was initiated within 0.5 h after mixing the heme and apoEqMb. The data for Fig. 2 were obtained from accumulations of 16 transients for each time value; the pH was measured after the NMR spectra were recorded. Chemical shifts are referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonate) through the residual water signal at 4.76 ppm.

3. RESULTS

Fig. 1 presents a comparison of products from the two reconstitution protocols for generating metEqMbCN. Fig. 1A shows the hyperfine-shifted downfield and upfield regions for FePPIX added to apoEqMb, with CN^- added immediately thereafter. As expected, this trace shows a ~1:1 ratio of heme-insertion isomers. Possible reasons for observing somewhat >1:1 ratio of heme-insertion isomers include progress toward the native ratio of 9:1 before quenching with CN^-, and incomplete incorporation of FePPIX which would then be converted to $FePPIX(CN)_2$ before insertion. Fig. 1B shows the hyperfine-shifted downfield and upfield regions for $FePPIX(CN)_2$ added to apoEqMb. In marked contrast to Fig. 1A there is a strong predominance of the heme-insertion isomer which dominates at equilibrium, with a heme-insertion isomer ratio ~4.5:1. The same trend between the two reconstitution protocols also exists at pH 7 (not shown). Reconstitution of apoSwMb with $FePPIX(CN)_2$ at pH 9.2 was found to produce the heme-insertion ratio ~2.5:1 (not shown).

A series of spectra was collected at intermediate times after reconstitution of apoEqMb with $FePPIX(CN)_2$. The hyperfine-shifted resonances for metcyano EqMb were seen to grow; Fig. 2 shows the rate-dependence for the increase in intensity of the $5CH_3{}^{\wedge}$ peak. The heme-insertion isomer ratio ~4.5:1 did not change during the course of the reconstitution, and no other resonances were observed in the hyperfine-shifted regions of the spectrum.

4. DISCUSSION

We propose the following mechanism to account for the data presented in Figs 1 and 2. $FePPIX(CN)_2$ is assumed to insert into the heme pocket, giving a 1:1 ratio of heme-insertion isomers of an intermediate, '[$FePPIX(CN)_2$•EqMb]'. Equilibration toward the native heme-insertion isomer ratio would then occur before proximal cyanide is displaced by $His^{93}F8$. Rapid porphyrin-insertion isomer equilibration in PPIX•SwMb and PPIX•EqMb has been suggested [10,15]. An alternate possibility that apoMb directly interacts with the ratio of heme-insertion isomers shown in Fig. 1 is difficult to disprove, but is unlikely on the basis of the ~1:1 ratio in Fig. 1A. Facile formation of [$FePPIX(CN)_2$•EqMb] may be a consequence of F-helix deformation in apoMb relative to holoMb [16].

Others [17] have used reconstitution of apoSwMb with $FePPIX(CN)_2$ to obtain a final heme-insertion isomer ratio of ~1:1. This is in apparent disagreement with our data, but may result from different relative amounts of OH^- and CN^- ligands complexed to the iron in the heme stock solutions; this equilibrium is known to be pH-sensitive [18]. If the $FePPIX(CN)_2$ solution pH were very high the reconstitution might more closely resemble addition of FePPIX to apoMb, then quenching with CN^-, as in Fig. 1A. There is also a fundamental difference between the SwMb and EqMb heme pockets: SwMb has $Arg^{45}CD3$ while EqMb has $Lys^{45}CD3$ [19,20]. $Lys(Arg)^{45}CD3$ is a surface residue which may have a direct role in the mechanism of O_2 diffusion into and out of its distal site in heme pocket [20]. The CD3 side chain is salt-bridged to the heme 7-propionate. This salt bridge involving $Arg^{45}CD3$ in SwMb is supposed to be more stable than the salt bridge involving $Lys^{45}CD3$ in EqMb [11,12,21], by ~0.6 kcal/mol. For this energetic difference to account for the 1:1 ratio of heme-insertion isomers reported for SwMb, but not EqMb, two conditions would have to be satisfied: (i) the salt bridge to $Arg(Lys)^{45}CD3$ would have to be formed before reorientation between isomers happens, and (ii) the reorientation rate would have to be sufficiently retarded in SwMb to allow formation of the Fe-$His^{93}F8$ bond before equilibration happens. Reconstitution of apoSwMb with $FePPIX(CN)_2$ indicates that this may play a role because the heme-insertion isomer ratio for this reconstitution is reduced to ~2.5:1.

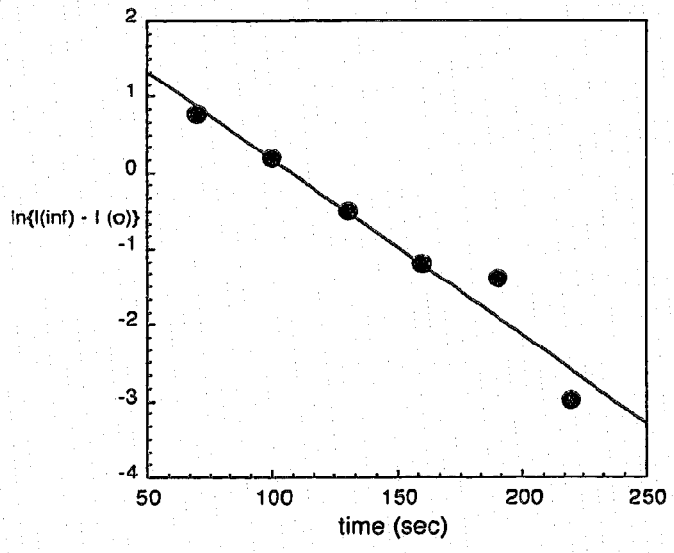

Fig. 2. Kinetics for the formation of metEqMbCN from $FePPIX(CN)_2$ and apoEqMb at pH 9.2 and 25°C. This plot represents the increase in the intensity of the $5CH_3{}^{\wedge}$ peak from metEqMbCN. Each data point reports the time a spectrum was initiated; each spectrum required ~0.25 min to accumulate.

The rate constant k_{obs} ~3×10^{-2} s^{-1} can be extracted from the data shown in Fig. 2. This rate corresponds most closely to the longest process reported by Kawamura-Konishi et al. [4], k ~5×10^{-3} s^{-1};* all other processes seen by optical spectroscopy are much too fast to observe by our NMR measurements. Because $FePPIX(CN)_2$ is supposed to insert into the heme pocket within 1 s, it appears that there is at least one intermediate species which is not observed in the hyperfine-shifted regions of the spectra. Why were we not able to observe such species? It may be that more than one initial set of products is formed (Kawamura-Konishi et al. reported four steps [4]), and that there is exchange between at least two of these. If exchange were to be in the 'correct' rate domain we would not be able to distinguish the broadened peaks from the baseline [22]. This problem can be very important for ^1H NMR of paramagnetic systems due to the large chemical shift separation between exchanging species. Alternatively it may be that peaks for an intermediate are very broad and/or heterogeneous, so as not to be observed. Should the isomer ratio shown in Fig. 1B reflect the equilibrium ratio for [$FePPIX(CN)_2$•EqMb], it would not be surprising for such an intermediate to have a different isomer distribution than the native Mb because at least the proximal side of the heme pocket would be considerably deformed in [$FePPIX(CN)_2$•EqMb].

*There are differences in the conditions of the experiments which could easily lead to this factor of ~10 difference. For example Kawamura-Konishi et al. performed their reconstitution reactions at 15°C, while we maintained our reconstitution reactions at 25°C.

The only process which we can observe directly by NMR using the methods reported here (data acquisition within 1 min of mixing) is formation of the heme-insertion isomer mixture product. Nevertheless the product ratio of heme-insertion isomers permits us to probe the mechanism of reconstitution. Based on these results we must advise caution when studying reconstitutions which have heme ligands which might be slow to dissociate, although reconstitution with stock solutions of 'hemin dicyanide' at very high pH might also be used, but only if insertion of the heme and displacement of OH^- by $His^{93}F8$ is fast relative to displacement of OH^- by CN^-. We have found this to be the case for EqMb, as it appears to be for SwMb.

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