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CARBON-13 NMR STUDIES OF $C_2H_5N^{13}C$ BOUND TO HEMOPROTEINS - EVIDENCE FOR A DIFFERENT DISTAL PROTEIC ENVIRONMENT IN β-CHAINS EITHER ISOLATED OR WITHIN HUMAN HEMOGLOBIN

D. Mansuy*, J. Thillet†, B. Cendrier, J.Y. Lallemand and J.C. Chottard

Laboratoire de Chimie de l'Ecole Normale Supérieure associé au CNRS,
24 rue Lhomond,, 75 231 Paris Cedex 05, France

† Unité de Recherche sur les Anémies, U 91 INSERM, Hôpital H. Mondor,
94 Créteil, France

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SUMMARY

The previous assignment of the ^{13}C resonances of $C_2H_5N^{13}C$ bound to the α and β subunits of human adult hemoglobin (HbA) is confirmed by the study of Hb-S^C Louis β 28 (B 10) Leu → Gln. The large chemical shifts differences between the labelled isocyanide in [Fe(II)(protoporphyrin IX)($RN^{13}C$)(L)] complexes (L = DMSO or N-methyl-imidazole) and in the hemoglobins complexes, suggest that the heme proteic environment interacts with the bound ligand. For both the α and β chains, there is a significant difference between the chemical shifts of $C_2H_5N^{13}C$ bound to the subunits in HbA or to the isolated chains, this difference being particularly large for the β-chain. However, for each isolated chain, no difference appears for $C_2H_5N^{13}C$ bound to either the free sulfhydryl form or the p-hydroxymercuribenzoate derivative. This is the first direct evidence, in the completely liganded state, for a difference between the environments of the ligands bound to the β-chain either free or aggregated within the HbA tetramer. From these results and others obtained with the mutants Hb J Calabria β 64 (E8) Gly → Asp, and Hb I Toulouse β 66 (E 10) Lys → Glu, $C_2H_5N^{13}C$ appears as a sensitive probe for the modifications of the distal environment of the heme, because of its steric interactions with the aminoacids of the heme pocket.

We have recently reported (1) that $C_2H_5N^{13}C$ appeared as a more sensitive probe than ^{13}CO (2-7) for hemoprotein NMR studies. Characteristic resonances have been assigned to $C_2H_5N^{13}C$ bound to the heme-iron (II) of the α and β subunits in human adult hemoglobin tetramer (HbA), on the basis of a comparison with the signals observed with human fetal hemoglobin (HbF). The ^{13}C chemical shifts, from TMS, are respectively 173.1, 174.0 and 174.8 ppm, for the

* to whom reprint requests should be addressed.

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ligand bound to the α,β and γ subunits of HbA and HbF (1).

In the same pH range and similar conditions, the difference between the chemical shifts of ^{13}CO bound to the β subunit of HbA and the γ subunit of HbF is not significant (3, 7). In the case of the human mutants M Iwate (α 87 (F 8) His → Tyr), Kansas (β 102 (G 4) Asn → Thr), Hb-S (β 6 (A 3) Glu → Val), and various others that we studied (9), the largest difference observed is 0.41 ppm for the ^{13}CO-β-subunit of Hb M Iwate, compared to that of HbA (4, 8, 7, 9). A study of valency hybrids has given a chemical shift difference of 0.1 ppm for ^{13}CO bound to the α chain in $(\alpha^{II}\beta^{III}H_2O)_2$ compared to that of HbA (10, 11). It seems that there are no reliable differences between the ^{13}CO chemical shifts for the isolated chains, either with free sulfhydryl groups (SH) (6, 10) or as p-hydroxymercuribenzoate derivatives (PMB) (4,7), and those observed for the corresponding subunit within HbA (e.g. β^{SH} : upfield shift of 0.23 ppm (6) or downfield shift of 0.01 ppm (9); α^{PMB} : upfield shifts of 0.2 ppm (7) or 0.06 ppm (4)).

In the present work, the comparison of the chemical shifts of $C_2H_5N^{13}C$ bound to Fe(II)(protoporphyrin IX) model complexes, to HbA and to two β-heme-pocket mutants of HbA, shows that the isocyanide ligand is very sensitive to the proteic environment of the heme. Furthermore the comparison of the chemical shifts of $C_2H_5N^{13}C$ bound to the subunits of HbA and to the corresponding isolated chains (SH or PMB) shows that there is a significant difference, in the liganded state, between the environments of the ligand bound to the β chain either free or aggregated within the HbA tetramer.

MATERIALS AND METHODS

$C_2H_5N^{13}C$ and $n\text{-}C_4H_9N^{13}C$ were prepared from the corresponding iodide and $K^{13}CN$ using the method reported for the ^{12}C derivative (12).

Pure human adult hemoglobin was obtained by DEAE sephadex chromatography followed by removal of the organic phosphates by passage over a mixed-bed ion exchange column (Amberlite IRA 400 and IR 120), the hemoglobin solution was then at pH 7.4.

α^{SH}, β^{SH}, α^{PMB} and β^{PMB} chains were prepared according to Geraci et al. (13) and Yip et al. (14). The purity of each preparation was tested by electrophoresis on cellulose acetate. Equimolar amounts of α^{SH} and β^{SH} were mixed and resulted in a complete reconstitution of native HbA tested by

electrophoresis and functional properties : normal oxygen affinity and cooperativity were restaured. Hb S^t Louis has been prepared according to Cohen-Solal et al. (15). Hb J Calabria was purified as previously described (28). Hb I Toulouse was separated on a column of carboxymethyl cellulose using a 0.01 M phosphate buffer at pH 6.8.

In a typical experiment a 10 mm o.d. NMR tube sealed with a rubber septum, containing 1.0 to 1.5 ml of 1 to 2 mM hemoglobin solution(and a capillary of $CH_3{}^{13}CO_2Na$ in D_2O) was first degassed and flushed with argon several times before addition of 10 µl of pure $C_2H_5N^{13}C$; 100 µl of degassed D_2O were then added. The proton decoupled ^{13}C FT NMR spectra were recorded at 22.63 MHz with a Bruker WH 90 spectrometer, using the deuterium resonance of internal D_2O as lock signal. 90° pulses (19.6 µs), no pulse delay, and 2048 points memory blocks were used to accumulate FID's. Sweep width was 1200 Hz giving an acquisition time of ~ 0.83 s. Chemical shifts were first measured from the carbonyl resonance of the external $CH_3{}^{13}CO_2Na$, with no bulk susceptibility correction, and then converted downfield from TMS, the resonance of which was taken at 181.1 ppm from sodium acetate. Reproducibility of the spectra of one sample was better than 0.05 ppm (digital resolution). In our experimental conditions the exchange rate between the bound and free isocyanides is small (≤ 1 Hz) compared to the corresponding chemical shifts difference (~ 450 Hz), as proved by the presence of the ^{14}N-^{13}C coupling constant of 7 Hz for the free $C_2H_5N^{13}C$. Thus it can be assumed that the free ligand chemical shift may be used as an internal reference. Actually the largest chemical shift variation that we have observed for the free isocyanide, relative to external $CH_3{}^{13}CO_2Na$, was less than ± 0.1 ppm, probably due to the variation of magnetic susceptibility of the hemoglobin solution. Accumulation of 10 000 to 50 000 transients was necessary to achieve sufficient signal to noise ratio. The temperature of the samples was controlled in the 5 to 25°C range.

For the model studies, solutions of 5 mM hemin chloride (from Calbiochem) in DMSO d6 are deoxygenated with argon; the iron reduction is obtained by stirring with iron powder. Pure $C_2H_5N^{13}C$ or $n\text{-}C_4H_9N^{13}C$ are added up to a 50 mM concentration. In these conditions an equilibrium between [Fe(II) (protoporphyrin IX) $(RN^{13}C)_2$] and [Fe(II) (PP IX) $(RN^{13}C)$ (DMSO)] is obtained. Addition of N-methyl-imidazole gives an equilibrium between [Fe(II) (PP IX) $(RN^{13}C)_2$] and [Fe(II) (PP IX) $(RN^{13}C)$ (N-MeIm)].

RESULTS AND DISCUSSION

In order to confirm our previous assignment of the two carbon-13 resonances of $C_2H_5N^{13}C$ bound to the α and β subunits of HbA (1), we have first examined the ^{13}C NMR spectrum of the isocyanide bound to hemoglobin S^t Louis β 28 (B 10) Leu →Gln, whose β heme is permanently in a ferric state (15, 16). We observed only one signal at 173.1 ppm which is the resonance previously attributed to the $C_2H_5N^{13}C$-α chain of HbA (1). Moreover this result is in agreement with the X-ray structure of fully reduced Hb S^t Louis, which led to the conclusion that : "the amino acid replacement seems to have no effect on the α subunit" (17).

The apparent sensitivity of the $C_2H_5N^{13}C$ ligand, compared to ^{13}CO (1),

prompted us to look at its ^{13}C chemical shifts in model complexes. It has been pointed out that the ^{13}CO resonance of the CO adduct of the N-methyl-imidazole complex of protoheme dimethyl ester differs in chemical shift by only 1 ppm from the ^{13}CO resonances of human hemoglobin (5). Other model studies have confirmed this similarity of chemical shift between ^{13}CO bound to HbA and to protoheme with N-methyl-imidazole as the sixth ligand (18). In all these cases, the bound ^{13}CO resonances are shifted downfield (about 20 ppm) from that of free ^{13}CO, which is a general trend for carbonyl complexes of transition metals (19). However upfield shifts (10 to 20 ppm) are observed for various $RN^{13}C$ bound to non porphyrinic Fe(II) and Cu(I) complexes (19, 20). The various complexes [Fe(II)(protoporphyrin IX)($RN^{13}C$)L], with L = $RN^{13}C$, DMSO and N-methyl-imidazole, and R = C_2H_5 or n-C_4H_9, in DMSO d6, all give ^{13}C signals between 147 and 152 ppm versus respectively 152.2 and 155.9 ppm for the free isocyanides (to be published). Therefore in these porphyrinic complexes the ^{13}C resonances of the bound isocyanides are also shifted upfield from those of the free ligands, whereas we have shown that they are shifted downfield (20 to 25 ppm) in the case of the $C_2H_5N^{13}C$-hemoglobin and myoglobin complexes (1). Such a difference must be mainly due to an interaction between the bound isocyanide and its close proteic environment which should interfere with its coordination to Fe(II). These results prompted us to look for any difference between the $C_2H_5N^{13}C$ liganded- α and β subunits of HbA and α and β isolated chains.

The ^{13}C NMR signals at 172.4 and 171.7 ppm observed for $C_2H_5N^{13}C$ bound respectively to the isolated α^{SH} and β^{SH} chains of human hemoglobin (Fig. 1), in tris-buffer at pH 7.4, are significantly different from those of the isocyanide bound to the α and β subunits of HbA. Upon mixing stoechiometric amounts of $C_2H_5N^{13}C - \alpha^{SH}$ and $C_2H_5N^{13}C - \beta^{SH}$, the reconstituted $(C_2H_5N^{13}C)_4$ HbA gave two signals at 173.1 and 174.3 ppm, in good agreement respectively with those of the α and β subunits of HbA (Fig. 1). As previously observed with HbA (1), the simultaneous presence in the ^{13}C NMR spectra of the ligan-

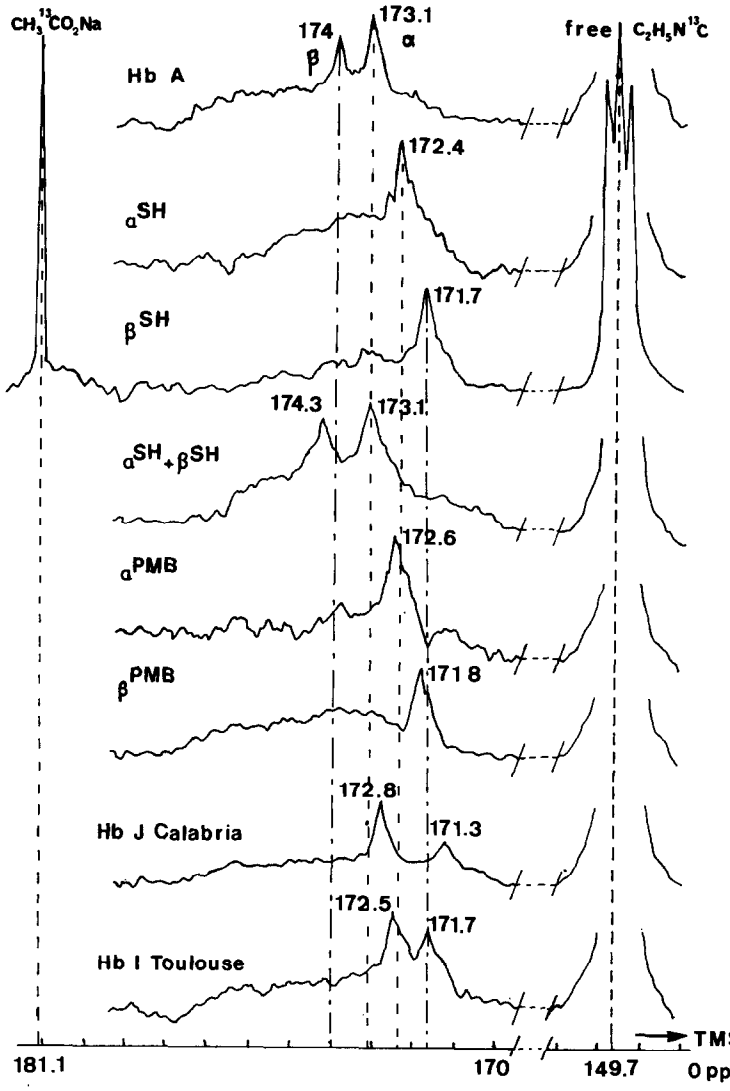

Figure 1 : ^{13}C NMR spectra of $C_2H_5N^{13}C$-liganded hemoglobins and isolated chains (pH 7.4). The chemical shifts are in ppm from TMS (converted from external $CH_3{}^{13}CO_2Na$ standard). The precision is ± 0.1 ppm (see Materials and Methods).

ded isolated chains, of the signals of free and bound $C_2H_5N^{13}C$ and of the N-^{13}C coupling ($J_{N-^{13}C}$ = 7 Hz) for the free ligand, precludes an exchange faster than 1 s^{-1} between bound and free isocyanide in our experimental conditions. The chemical shifts of $C_2H_5N^{13}C$ bound to the α^{PMB} and β^{PMB} chains are respectively 172.6 and 171.8 ppm (Fig. 1). The upfield shifts of the

bound $C_2H_5N^{13}C$, from α-HbA to α^{SH} (- 0.7 ppm), α-HbA to α^{PMB} (- 0.5 ppm) and from β-HbA to β^{SH} (- 2.3 ppm) and β-HbA to β^{PMB} (- 2.2 ppm) are all significant and large compared to those reported for the ^{13}CO probe (4, 6, 7, 10) and are particularly important for the β-chains. However there is no significant chemical shift difference, for each isolated chain, between the liganded free SH form and the PMB derivative. In the case of the β chain, this means : first that the $C_2H_5N^{13}C$ probe does not reflect the fact that the β^{SH} chains exist largely as tetramer whereas the β^{PMB} chains exist as a monomer (14), second that the $C_2H_5N^{13}C$ probe does not reflect the perturbation produced by the presence of PMB at the proximal β 93 position whereas this perturbation leads to a lower affinity of the β^{PMB} chains for isocyanides (21) due to a structural change (22, 23). Therefore the $C_2H_5N^{13}C$ probe seems to be mainly sensitive to variations of the distal environment when bound either to the β chain in the HbA tetramer or to the isolated β^{SH} and β^{PMB} chains. This can be related to previous findings, which attributed the heme spectral change associated with the aggregation of isolated deoxygenated subunits primarily to the α chains, and which then suggested that the corresponding variation of reactivity of the β subunits should rather involve an alteration in the protein structure near the sixth coordination position (24, 25). This suggests that upon aggregation into HbA the interactions between subunits lead to a structural change which affects mainly the heme distal environment in the β chains. Our results show that such a change can be observed between the liganded-β isolated chains and these chains in the completely liganded state of the HbA tetramer. The sensitivity of the $C_2H_5N^{13}C$ ligand to this structural change can be interpreted as a consequence of an interaction of the ethyl group of the bound isocyanide with the proteic environment of the heme pocket. One could tentatively correlate this interaction within the β-heme pocket to its size, which is known to be smaller than in the α chain (23) ; to the greater rate of dissociation of ethyl iso-

cyanide from the β chain than from the α chain (the two association rates being equal) (26) ; and to the closer proximity between the bound ligand and the C_γ of valine E 11 within the β chain compared to the α chain (27, 23).

In order to further investigate the sensitivity of the $C_2H_5N^{13}C$ ligand to the β-heme distal environment, we looked at two human mutant hemoglobins : HbJ Calabria β 64 (E 8) Gly → Asp (28) and HbI Toulouse β 66 (E 10) Lys → Glu (29). Hb J Calabria exhibits a high affinity for oxygen which has been explained by a destabilization of the E helix leading to an easier access to the heme pocket (28). In contrast Hb I Toulouse presents normal functional properties but its autoxidation rate is increased and this has been related to a more acidic environment of the iron (29). With $C_2H_5N^{13}C$, Hb J Calabria and Hb I Toulouse exhibit two signals each, respectively at 172.8, 171.3 and 172.5 , 171.7 ppm (Fig. 1). In both cases the second signal is smaller than expected when comparing the relative intensities of the two observed signals with those of liganded HbA. In the case of Hb J Calabria, the unstability of the mutant could be responsible of the smaller signal at 171.3 ppm ; therefore we assign this signal to the abnormal β chain. Furthermore we have observed a significant upfield shift of 0.19 ppm for this ^{13}CO-abnormal β chain compared to that of HbA (30). In the case of Hb I Toulouse, more than the unstability of the mutant, the larger autoxidation rate of the abnormal β chain compared to the β chain of HbA (31), supports the assignment of the signal at 171.7 ppm to that chain. On the basis of these assignments, the upfield shifts of $C_2H_5N^{13}C$ bound to the α chains, from HbA to Hb J Calabria (- 0.3 ppm) and HbA to Hb I Toulouse (- 0.6 ppm) are relatively small, whereas the corresponding upfield shifts for the ligand bound to the abnormal β chains (respectively - 2.7 and - 2.3 ppm) are large. This result shows the great sensitivity of the $C_2H_5N^{13}C$ ligand to the two studied β chain E 8 and E 10 mutations, which are close to the binding site of the distal ligand and which probably induce a significant structural perturbation of the heme pocket.

Our results show that $C_2H_5N^{13}C$ is a sensitive probe for the NMR study of the distal environment of the heme within hemoglobins, and could be used for the study of the heme pocket of various hemoproteins.

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