CARBON-13 NMR STUDIES OF $C_2H_5N^{13}C$ BINDING TO VARIOUS HEMOPROTEINS

D. Mansuy , J.-Y. Lallemand, J.-C. Chottard and B. Cendrier

Laboratoire de Chimie de l'Ecole Normale Supérieure, associé au CNRS n° 32, 24, rue Lhomond, 75231 Paris Cedex 05, France

and

G. Gacon and H. Wajcman

Institut de Pathologie Moléculaire, Groupe U15 INSERM, 24, Faubourg Saint Jacques, 75014 Paris, France.

Received March 8,1976

SUMMARY : The addition of an excess of $C_2H_5N^{13}C$ to myoglobin and human adult and fetal hemoglobins, gives three characteristic NMR spectra with new ^{13}C resonances respectively at δ = -10,56 ppm, δ = -7,03 and -7,95 ppm and δ = -6,28 and -7,95 ppm (CH_3CO_2Na as external standard). These signals correspond to the $C_2H_5N^{13}C$ bound to the Fe(II) of the different heme units, according to CO exchange experiments. Characteristic resonances can be assigned to $C_2H_5N^{13}C$ bound to α, β and γ subunits. $C_2H_5N^{13}C$ appears as a more sensitive probe than ^{13}CO for hemoprotein NMR studies.

Separated NMR signals can be observed for ^{13}CO bound to the hemes of the α and β hemoglobin chains (1-6). This probe has been used to study the different chemical properties and ligand affinities of the α and β subunits in intact hemoglobin. However the dependence of the bound ^{13}CO chemical shift on variations of the heme environment is rather small. For instance, no difference is observed between normal and fetal human hemoglobins (HbA and HbF) (6), and in our hands the ^{13}CO probe did not give sufficient differences to ensure comparisons between several heme pocket mutant hemoglobins (7). Furthermore the ^{13}CO resonances are pH independent in the range pH 6-8 and do not vary upon addition of an excess of 2,3-diphosphoglycerate (6). This prompted us to look for a ^{13}C labelled ligand which ought to be more sensitive to variations of the heme environment. Isocyanide ligands, R-N=C, seemed promising because of their good affinities for hemoproteins (8) and of the expected interaction between the alkyl group of the bound ligand and the aminoacid residues of the heme pocket. We report here ^{13}C NMR data of $C_2H_5N^{13}C$ complexes with myoglobin (Mb) and human adult and fetal hemoglobins.

MATERIALS AND METHODS

$C_2H_5N^{13}C$ was prepared from C_2H_5I and $K^{13}CN$ using the method reported for the ^{12}C derivative (9).

Pure human adult and fetal hemoglobins were 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 solutions were then at pH 7.4.

Horse myoglobin was purchased from Sigma Chemical Cy. and used without further purification.

In a typical experiment a 10 mm diameter NMR tube, sealed with a rubber septum, containing 1.5 ml of 1 to 2 mM hemoglobin solution was first vacuum degassed and filled with Argon several times before addition of 10 µl of pure $C_2H_5N^{13}C$.

The proton decoupled pulse ^{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 a lock. 90° pulses and 2048 points memory blocks were used to accumulate FID. Sweep width was 1200 Hz giving an acquisition time of 0.83 sec. Chemical shifts are reported in ppm upfield from the carbonyl resonance of the external standard $CH_3{}^{13}CO_2Na$ which was in a sealed capillary tube.

RESULTS

Addition of pure $C_2H_5N^{13}C$ to a solution of pure HbA (pH 7.4) leads to an NMR spectrum with two new signals at δ = -7.03 and -7.95 ppm (Fig. 1b) compared to the spectrum of $C_2H_5N^{12}C$ - HbA recorded under the same conditions (Fig. 1a). These resonances are superimposed with the broad carbonyl resonances of the protein. Visible spectroscopy indicates that all the isocyanide binding sites of HbA are saturated under the conditions of Fig. 1b ; this is confirmed by the intensities of the two NMR bands which are not affected by further addition of $C_2H_5N^{13}C$, even in the presence of sodium dithionite. That the two new signals do not result of irreversible binding of $C_2H_5N^{13}C$ is ascertained by their disappearance after extensive saturation of the solution with ^{12}CO. Vacuum degassing of this solution followed by a new addition of $C_2H_5N^{13}C$ regenerates the original spectrum of Fig. 1b in agreement with the known reversibility of the isocyanide binding.

Similar results are obtained upon an identical treatment of both horse myoglobin (reduced by sodium dithionite in phosphate buffer at pH 7.4) and pure human fetal hemoglobin (pH 7.4). However the former exhibits only one signals at δ = -10.56 ppm and the latter exhibits two signals at δ = -6.28 and -7.95 ppm, one of which has a different chemical shift from the two resonances of the $C_2H_5N^{13}C$ - HbA complex (Table 1).

DISCUSSION

Like ^{13}CO, $C_2H_5N^{13}C$ binds to HbA giving two NMR signals and to Mb giving only one. As shown by the ^{12}CO exchange experiments, these

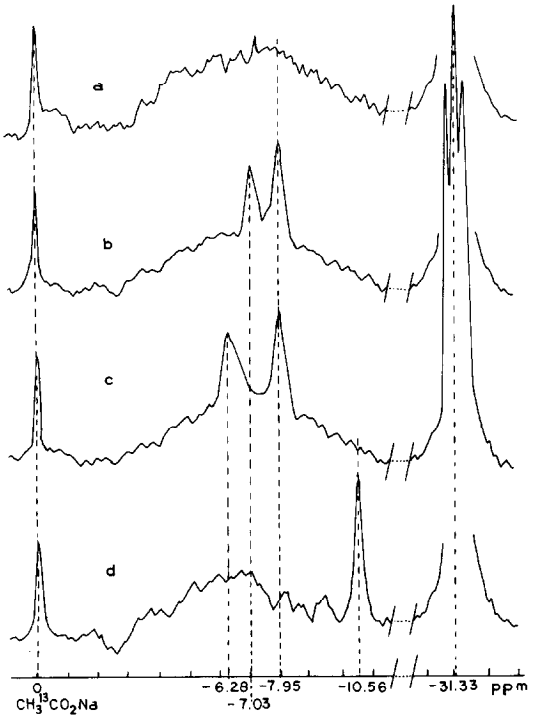

Fig.1. ^{13}C NMR spectra of $C_2H_5N^{13}C$ bound hemoproteins. Chemical shifts upfield from $CH_3{}^{13}CO_2Na$ as external standard. (a) $C_2H_5N^{12}C$ - HbA (pH 7.4), 13000 pulses ; (b) $C_2H_5N^{13}C$ - HbA (pH 7.4), 13000 pulses ; (c) $C_2H_5N^{13}C$ -HbF (pH 7.4), 15000 pulses ; (d) $C_2H_5N^{13}C$ - Mb (pH 7.4 in phosphate buffer, in the presence of 2 mg of sodium dithionite), 25000 pulses.

Table 1. Chemical shifts of h e-bound $C_2H_5N^{13}C$ (a)

	δ_1	δ_2	$\Delta\delta$	$\Delta\delta_{^{13}CO}$ (b)
HbA (human)	-7.03	-7.95	0.92	0.56
HbF (human)	-6.28	-7.95	1.67	0.52
Mb(horse)	-10.56			
free $C_2H_5N^{13}C$	-31.33 ($J_{N-^{13}C}$=7Hz)			

(a) ppm from $CH_3{}^{13}CO_2Na$ ± 0.06 ; (b) from (6), each δ ± 0.04 ppm.

signals should correspond to the isocyanide bound to the Fe(II) of the Hbα, Hbβ and Mb heme units. The simultaneous presence in the two spectra of the signals of free and bound $C_2H_5N^{13}C$ implies a slow

exchange, on the NMR time scale, between the two species in the recording conditions. It is noteworthy that the N-^{13}C coupling ($J_{N-^{13}C}$ = 7 Hz) observed for free $C_2H_5N^{13}C$ (Table 1) does not appear for the Fe(II) bound isocyanide, this is probably due to the change of hybridization of ^{13}C upon coordination.

The signals corresponding to $C_2H_5N^{13}C$ bound to the α and β subunits in HbA are separated by 0.92 ppm instead of 0.56 ppm for bound ^{13}CO (6) (Table 1). Futhermore two different characteristic spectra are obtained for HbA and HbF, leading to the assignment of the δ = -7.95, -7.03 and -6.28 ppm signals respectively to $C_2H_5N^{13}C$ bound to the α, β and γ subunits. Thus $C_2H_5N^{13}C$ appears as an NMR probe more sensitive to the heme environment than ^{13}CO. This suggests that the presence of the ethyl group of the bound isocyanide makes the ligand more sensitive to the aminoacid substitutions differentiating the γ and β subunits (10, 6).

The sensitivity of the isocyanide ligand to variations of the heme environment, resulting from conformational changes, is further supported by preliminary results showing a dependence of the chemical shifts of the bound $C_2H_5N^{13}C$ on the pH of the solution and the presence of phosphate effectors.

The labelled isocyanide appears as a promising probe for NMR investigation of the variations of the heme pocket of various hemoproteins, particularly because of its versatility based on the choice of the alkyl group.

ACKNOWLEDGEMENTS

We thank Dr Labie for her interest in this work and fruitful discussions. H. Wajcman and G. Gacon are indebted to INSERM for financial support.

REFERENCES

1 - Conti, F. and Paci, M. (1971), FEBS Letters, 17, 149-152.
2 - Moon, R.B. and Richards, J.H. (1972), J. Amer. Chem. Soc., 94, 5093-95.
3 - Vegamini, P.J., Matwiyoff, N.A., Wohl, R.C. and Bradley, T., (1973), Biochem. Biophys. Res. Comm., 55, 453-461.
4 - Matwiyoff, N.A., Vergamini, P.J., Needham, T.E., Gregg, C.T., Volpe, J.A., and Caughey, W.S. (1973), J. Amer. Chem. Soc., 95, 4429-31.
5 - Antonini, E., Brunori, M., Conti, F. and Geraci, G. (1973), FEBS Letters, 34(1), 69-70.
6 - Moon, R.B., and Richards, J.H. (1974), Biochemistry, 13, 3437-43.
7 - to be published

8 - Antonini, E. and Brunori, M. (1971), Hemoglobin and Myoglobin in their reactions with ligands, p. 33, North-Holland Publish. Comp., Amsterdam - London.
9 - Jackson, H.L. and McKusick, B.C. (1963), Org. Syntheses, Coll. vol. 4, 438-440.
10- Perutz, M.F. (1969), Proc. Roy. Soc., Serie B, 173, 113.