^{13}C MAGNETIC RESONANCE STUDY OF THE CARBON MONOXIDE DERIVATIVES OF HEMOGLOBIN VALENCY HYBRIDS

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1. Introduction

Oxygen-binding by hemoglobin being highly cooperative, the equilibrium concentrations of partially oxygenated species are small relative to the two limiting forms, deoxyhemoglobin and oxyhemoglobin, which are known to possess different structures [1,2]. Most of the information about the properties of the half-oxygenated hemoglobin tetramer have been obtained using the so called mixed-state hemoglobins, particularly the valency hybrids [3,4]. In these molecules, one kind of subunit, either α or β, is oxidized while the other type is in the normal ferrous state and can combine reversibly with gaseous ligands. Moreover, the oxidized subunit can be made to take alternately a weak or a strong field ligand (H_2O or CN^-, respectively) with different structural consequences to be discussed below.

The study of ^{13}C resonances of the carbonyl ligand bound to various hemoglobins has already been of considerable use, namely for chain identification [5–7] and the investigation of chain heterogeneity [8]. Carbon monoxide bears a close similarity to oxygen as a ligand towards hemoglobin and the ^{13}C-labelled CO, bound directly to the active site is an innocuous reporter of the heme environment. In this work, we have studied the ^{13}C magnetic resonance of the ferrous carbonyl complexes of the hybrids with particular emphasis on chemical shifts. The oxidized chain was alternately bound to H_2O or CN^-. The action of inositol hexakisphosphate (Ins–P_6), considered to be a strong allosteric effector, was also studied.

2. Materials and methods

Phosphate-free (stripped) human oxyhemoglobin was obtained from fresh hemolyzates by extensive dialysis against 0.05 M Tris–HCl buffer, pH 8.1, containing, in addition, 0.1 M NaCl. The α and β chains were prepared following ref. [9] for chain separation and ref. [10] for the regeneration of free sulfhydryl groups. The valency hybrids, $\alpha^{II}\beta^{III}$ and $\alpha^{III}\beta^{II}$ were prepared and purified as described before [3]. The solutions were rendered salt-free by passing through a mixed bed ion-exchange column before being adjusted to pH 7.0 in bis–Tris lactic acid buffer (0.1 M final concentration).

^{13}C-labelled CO (90.5% ^{13}C) was purchased from Merck, Sharp & Dohme, Canada. Complete saturation of the ferrous sites by ^{13}CO was ensured by prolonged exposure of the samples to a slight positive pressure of the gas in a syringe, before being transferred into the NMR sample tube (10 mm o.d.). A 3 mm tube containing ^2H_2O (to serve as a field-frequency lock) and tetramethyl silane, TMS, (in a sealed capillary, to serve as an external reference) was inserted concentrically into the NMR tube which was then flushed with ^{13}CO

and closed by a capsule. ^{13}C NMR spectra were recorded at 25.2 MHz and 34° using a Varian XL-100 spectrometer operated in the Fourier Transform mode with proton noise decoupling. Spectral widths of 5500 Hz (220 ppm) were used for shift measurements (± 0.03 ppm) with respect to the primary external TMS reference; spectral widths of 200 Hz were used for line width measurements. Data accumulation were carried out for 4 – 12 h using 90° r.f. pulses (45 μs) and 0.7 or 1 s acquisition times respectively.

The correction of the shifts for macroscopic susceptibility effects of the hemoglobin solution (valency hybrids) upon the external reference was measured by comparison of the proton resonance of the external TMS with that of internal tetradeuterotrimethylsilyl propionate (TTS). A low field shift of 0.065 ppm for the external reference was observed upon complete oxidation to high spin methemoglobin of a 4 mM diamagnetic HbO_2 solution.

3. Results

Measurements performed on human hemoglobin and its isolated chains in neutral solution essentially confirm earlier reports. In normal hemoglobin, two rather well resolved resonances of equal intensity were observed; their assignment as being due to carbon monoxide bound respectively to the α and β chains is now unequivocal [5–7].

Both types of valency hybrids with the respective ferri chains in the aquomet form, $(\alpha^{III}H_2O\,\beta^{II}CO)_2$ $(\alpha^{II}CO\,\beta^{III}H_2O)_2$ were examined first in phosphate-free buffer and then again after the addition of 1 equiv. of Ins–P_6 per tetramer. In another series of experiments, the metaquo chains were titrated with KCN in order to replace the water molecule by the cyanide ion. The results presented in table 1 show that for $(\alpha^{III}H_2O\,\beta^{II}CO)_2$ neither the presence of Ins–P_6 nor the tertiary structure changes induced on the partner ferri α chain by replacing the weak field water ligand by the strong field cyanide has any noticeable effect on the β chain resonance. On the other hand, significant effects are produced by both of these reagents in the case of $(\alpha^{II}CO\,\beta^{III}H_2O)_2$ (fig.1 and table 1). Upon the addition of Ins–P_6 to the aquomet derivative of this hybrid, the αCO line broadens (from 8 Hz to 13 Hz) without marked change in position; ligand replacement

Table 1
^{13}C-Chemical shifts of the carbonyl ligand bound to human hemoglobin, its isolated α and β chains, and the valency hybrids

Protein (3.5–4.5 mM ferrous site)	Chemical shifts[a] (α)	(β)
Stripped Hb (bis–Tris lactic acid buffer, pH 7.0)	207.18 (8)	206.68 (8)
Stripped Hb + 1 equiv. Ins–P_6 (bis–Tris lactic acid buffer, pH 7.0)	207.18 (8)	206.70 (8)
α chain (phosphate pH 7.0)	207.14 (5)	–
β chain (phosphate pH 7.0)	–	206.69 (7–5)
Valency hybrids (2 mM ferrous sites) (0.1 M bis–Tris lactic acid buffer, pH 6.5)	–	–
	–	–
$(\beta^{II}\,\alpha^{III}H_2O)_2$ stripped	–	206.65
$(\beta^{II}\,\alpha^{III}H_2O)_2$ + Ins–P_6	–	206.65
$\beta^{II}\,\alpha^{III}CN$	–	206.61
$(\alpha^{II}\,\beta^{III}H_2O)_2$ stripped	207.28 (8)	–
$(\alpha^{II}\,\beta^{III}H_2O)_2$ + Ins–P_6	207.35 (13)	–
$\alpha^{II}\,\beta^{III}CN$	207.12 (8)	–

[a]Displacement downfield with respect to external TMS (± 0.03); corrected for macroscopic paramagnetic susceptibility when required. Figures within brackets represents the line width (in Hz) at half maximum intensity.

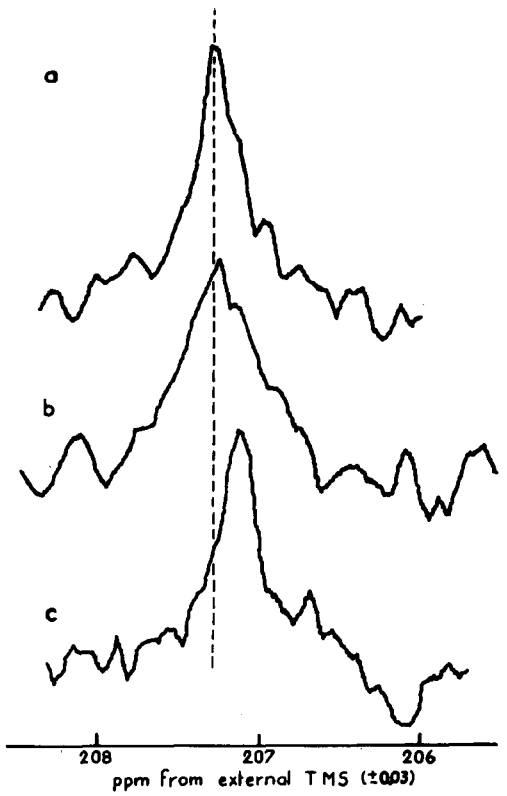

Fig.1. ^{13}C-labelled CO resonance of the valency hybrid $\alpha^{II}CO\ \beta^{III}$: (a) stripped protein in 0.1 M bis–Tris lactic acid buffer pH 7.0; (b) upon the addition of 1 equiv. Ins–P_6/tetramer; (c) upon ligand replacement ($H_2O \rightarrow CN$) on the ferri β chain, same pH.

($H_2O \rightarrow CN^-$) on the β chain results in a significant upfield shift in line position for αCO.

4. Discussion

The results are to be considered in relation to plausible structural effects associated with the ligand replacement reaction. Since none of these hybrids have been crystallized, no X-ray information about their structures are yet available. They can at best be characterized by the spin state of the ferric iron of the oxidized chains. In the low spin cyanomet hybrid, the iron is expected to have its equilibrium position in the plane of the porphyrin ring, whereas in the high spin aquamet hybrid the iron should lie about 0.3 Å out of the porphyrin plane [11], compared to 0.7–0.8 Å for the high spin ferrous protein [2]. The tertiary structure of the oxidized high spin subunits may be intermediate between that of deoxygenated and oxygenated chains [12], or otherwise may be rather close to that of the deoxy chains as one would judge from the identity of ESR spectra of $(\alpha^{II}NO\ \beta^{II})_2$ and $(\alpha^{II}NO\ \beta^{III}H_2O)_2$ [13]. Whatever may be the case, ligand replacement ($H_2O \rightarrow CN^-$) undoubtedly causes a tertiary structural change of the oxidized subunit. If one keeps in mind only such tertiary structure effects and considers conformational interactions within a given quaternary structure, our results would imply that the heme environment of the α subunit is sensitive to changes of the tertiary structure of the β subunit while the converse is not true. It is of interest to recall that similar results were obtained for the nitrosyl hybrids studied earlier [13]. Recent optical measurements [14,15] also show that spectral changes, when observed on suitable derivatives of hemoglobin by the action of external agents, arise principally due to the contribution of the α chains, though the reaction may occur preferentially on the β chains.

However, one should also consider the possibility that the high and low spin hybrids have different quaternary structures. It is reasonable to assign a quaternary 'oxy' conformation to both types of low spin hybrids: $(\alpha^{III}CN\ \beta^{II}CO)_2$ and $(\alpha^{II}CO\ \beta^{III}CN)_2$ [16,17]. For the respective aquamet derivatives, the 'oxy' conformation should be destabilized and their allosteric equilibria [18] may be shifted towards the 'deoxy' form, though not necessarily to the same extent for the two hybrids. If one follows the assumptions of Perutz et al. [17], the allosteric equilibria for $(\alpha^{II}CO\ \beta^{III}H_2O)_2$ and $(\alpha^{III}H_2O\ \beta^{II}CO)_2$ would be different, the former being more to the 'deoxy' side than the latter which is more 'oxy'. If such were the case, the observed effects would correspond to the original allosteric model [18] as well as to views expressed by Perutz et al. [17] that the properties of the heme site could change only as a result of the change of quaternary structure of the oligomeric protein. It may be noted, however, that the ^{13}C resonance of $(\alpha^{III}H_2O\ \beta^{II}CO)_2$ was unchanged on the addition of 1 equiv. of Ins–P_6. This effector has been proposed previously [16] to shift, towards the 'deoxy' form, the allosteric equilibrium of a hybrid of comparable composition, namely $(\alpha^{II}\ \beta^{III}CN)_2$. The

effector probably acts similarly on the two hybrids; the unchanged ^{13}C resonance for the former may again imply the insensitivity of the β heme to conformational stress even when resulting from a change of quaternary structure.

Finally, we should point out that the upfield displacement of the ^{13}C chemical shift for the $(\alpha^{II}CO\ \beta^{III}H_2O)_2$ hybrid on ligand replacement, though significative, is small (0.16 ppm). ^{13}C chemical shifts are known to be large even for small variations of charge densities [19]. An 0.16 ppm difference represents only a very small change which may be due either to a difference in the magnetic susceptibility of the environment or to a small change of the electron charge at the carbon atom due to metal–ligand charge transfer or ligand polarization. If the ^{13}C chemical shifts are in some way related to the stability of the carbonyl complexes, the difference of binding energies as deduced from the observed change of chemical shift will also be small. Indeed, from experiments designed to 'calibrate' the ^{13}C chemical shifts in terms of stability constants of different hemoprotein carbonyl complexes [20], we estimate the observed 0.16 ppm upfield displacement as corresponding to an increase of binding energy of about 200 cal/mol-site. Previous binding studies [4] for a similar ligand replacement reaction $(\alpha^{II}CO\ \beta^{III}H_2O)_2 \xrightarrow{N_3^-} (\alpha^{II}CO\ \beta^{III}N_3)_2$ have also indicated that the cooperativity, if any, is very small; also a theoretical analysis of the data for the complete ligand replacement reaction $(\alpha^{III}H_2O\ \beta^{III}H_2O)_2 \xrightarrow{CN^-} (\alpha^{III}CN\ \beta^{III}CN)_2$ shows that the free energy of cooperativity is only about 0.5 kcal/mol-site as compared to the value of 3 kcal/mol-site for the oxygenation reaction ([12] and M. Karplus, private communication). The estimated value of 200 cal/mol-site is thus of the order of cooperative interaction expected for the case studied here.

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