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^{31}P-NMR investigation of trimethylphosphine binding to [αFe(II),βMn(II)] hybrid hemoglobin

A model for partially liganded species

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^{31}P-NMR of trimethylphosphine binding to the ferrous chains of a ([αFe(II),βMn(II)]hemoglobin hybrid is employed to investigate partially liganded species. This study shows that at low pH (6.5), in the presence of inositol hexaphosphate, the resonance at 23.2 ppm (from H_3PO_4) is due to phosphine bonding to α-chains in the T quaternary state. At elevated pH (7.6), phosphine binding to the α-chains produces a resonance at 24.8 ppm which is associated with a T-to-R conversion. These findings are discussed in relation with our previous results on direct observation of intermediate ligation states of hemoglobin.

1. Introduction

The phenomenon of cooperative ligand binding by hemoglobin (Hb) has been the subject of intensive research for several decades. There are two general models which have been used to describe the cooperativity: i.e., the allosteric two-state model of Monod, Wyman and Changeux (MWC) [1] and the sequential induced-fit model (KNF) [2]. In 1970, Perutz [3] proposed a stereochemical mechanism for the cooperative oxygenation of Hb. His mechanism, which describes the concerted conformational transition in Hb, also shows many features of the MWC model. Although different approaches have also been proposed more recently in order to account for variations in the ligand affinity induced by effectors [4,5] or functional heterogeneity between two types of subunits [6], the MWC model has been more widely used. This model states that hemoglobin at any stage of the ligation process exists in equilibrium between two conformations, the T state, with low affinity and the R state, with high affinity, and that the transition between the two conformations occurs via a concerted mechanism. These conformations have been assumed to be T (unliganded) and R (liganded), respectively, on the basis of functional and structural studies.

However, despite considerable effort devoted to the Hb molecule, the detailed molecular mechanism of cooperativity is not fully understood, and a complete description of ligand binding to Hb must include detailed analysis of the intermediate structural states of hemoglobin. Various approaches have been used to carry out studies on Hb intermediate states. These include the use of both synthetic and naturally occurring valency hybrid hemoglobins [7,8], mixed-metal hybrid he-

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moglobins [9–12], the crystallization of partially liganded Hb in the T state [13], and the quenching and isoelectric focusing techniques at subzero temperatures to isolate intermediate compounds between carbonmonoxy- and deoxyhemoglobin [14]. Another effective method for gaining insight into the nature and properties of partially oxygenated species is to investigate the ^{1}H-NMR spectral changes associated with the cooperative oxygenation of Hb [15]. Thus, Ho and co-workers [16] concluded that the ligand-induced structural changes are not concerted and that intermediate structures can exist during the oxygenation process of Hb.

Recently, we have shown that ^{31}P-NMR spectroscopy can also be used as a direct probe of ligand binding to unmodified Hb. The ligand used is a phosphine, trimethylphosphine (PMe_3), which is small enough to complex Hb [17]. By monitoring the resonances due to PMe_3 complexation, it was found that, on comparison of the ^{31}P-NMR spectrum of the fully ligated Hb with spectra at intermediate stages of ligand saturation, two additional resonances are present in the latter spectra [17].

Analysis of the NMR results in the absence and presence of inositol hexaphosphate indicated that the intermediate resonances represent the PMe_3 bond to the α-subunit(s). However, it should be mentioned that these ^{31}P-NMR studies do not provide complete information on the structures of the partially ligated species of Hb. Another approach to the elucidation of the nature of partially ligated species is to study the ^{31}P-NMR spectral changes of metal hybrid hemoglobins. Among previous models, metal hybrid Hbs have a major advantage in that they can be prepared in sufficient amounts for investigation by NMR spectroscopy. Thus, the X-ray structural analysis of partially liganded hemoglobin [α(Fe(II)-CO),βMn(II)]$_2$ carried out by Hoffman and Arnone [9] shows that this mixed-metal hybrid Hb crystallizes in the deoxyhemoglobin quaternary structure (the T state) even though it is half liganded. Moreover, metal substitution in this hybrid is shown to offer a particularly advantageous means to probe both the initial and final stages of CO binding parameters for individual chains within the two major quaternary states of Hb [18]. The purpose of the present paper is to present a preliminary report of the ^{31}P-NMR investigation of the [αFe(II)-PMe_3,βMn(II)] hybrid hemoglobin in combination with our earlier work [17] in order to gain further insight into the structural properties of the intermediate species.

2. Materials and methods

Adult human hemoglobin was prepared according to a standard method [19]. The [αFe(II)-CO,βMn(III)] hybrid was prepared following the procedure of Blough and Hoffman [18] with minor modifications. The α- and β-subunits of human hemoglobin were separated and purified as described by Geraci et al. [20]. Heme-free β-chain globin was obtained by the 2-butanone extraction method of Teale [21]. The apo subunits were dialysed twice against water and then twice against 20 mM Bis-Tris buffer (pH 6.4). This dialysis procedure has already been described by Simolo et al. [12] to avoid precipitation of the apo subunit. The complementary αFe(CO) subunit, also dialysed against Bis-Tris buffer, was slowly added to the apo β-subunit and left for 70 h under a CO atmosphere at 4°C. At this point, the insertion of manganese(III) protoporphyrin IX as well as the purification of the hybrid were performed according to the procedure of Blough and Hoffman [18]. The hybrid was concentrated by using an Amicon ultrafiltration cell containing a PM 30 membrane, up to 10–12%. The decarbonylation was performed by illuminating the concentrated solution with a 200 W lamp under an atmosphere of air. The deoxygenated hybrid was prepared by using a current of humidified argon.

The NMR spectra were recorded with a Bruker AC 300 spectrometer equipped with a 5 mm tetranuclear QNP probe. The ^{31}P-NMR spectra were obtained at 121.49 MHz in pulse Fourier transform mode over 12.5 kHz with 16 K points. A line broadening of 5–15 Hz was applied on the free induction decays. Chemical shifts were referenced to the resonance position of 85% H_3PO_4 as external standard. The deoxy samples (1.5 mM) were introduced in argon-filled NMR tubes. The solu-

tions were prepared in approx. 10% ^2H_2O and 100 mM Tris-HCl at different pH values. The pH values were measured on a Schott-Gerate CG 837 equipped with an Ingold microcombination electrode. Small amounts (20 μl) of a sodium dithionite solution (0.6 M) in the appropriate buffer were then added to reduce the Mn(III) and to deoxygenate the α-subunits completely. Small aliquots of standard phosphine solutions were then added as previously described [17]. Inositol hexaphosphate (IHP) from Sigma was added to the sample up to 10 mM when indicated.

3. Results

The ^{31}P-NMR spectra of partially liganded hemoglobin are shown in fig. 1. In order to achieve approx. 10% saturation, 5 μl of trimethylphosphine (0.26 M) were added both in unmodified Hb and in (Fe,Mn) hybrid. In the latter case, it should be noted that complete saturation is disfavored in the presence of IHP at low pH (6.5). Such behavior has been previously observed in the case of constrained unmodified Hb (with 10 mM IHP) [17]. An increase in phosphine concentration tends to lead to the formation of a signal at approx. 12.5 ppm which is assigned to the ferroprotoporphyrin bis(PMe_3). Such an assignment is based on comparison with our model studies [22]. Here, with a small addition of phosphine standard solution (5 μl) there is no signal at 12.5 ppm, even in the presence of IHP at pH 6.5. Fig. 2 presents the ^{31}P-NMR spectra for the complete saturation by PMe_3 of human hemoglobin A_0 without any phosphate at pH 7.1 [17]. As previously observed, the complete saturation of HbA_0 leads to two resonances at 26 ppm (A) and 24.8 ppm (B) corresponding to the PMe_3 bound in the R state to the β- and α-chains, respectively [23]. The two additional resonances D and C have been attributed to the phosphine bound to the α-chains of hemoglobin in the T quaternary form and to a modified structure which is intermediate between those of R and T.

Comparison of the ^{31}P-NMR spectra recorded for unmodified Hb (fig. 1a) and (Fe(II),Mn(II)) hybrid at pH 6.5 with IHP and at pH 7.6 (fig. 1b and c, respectively) allows the identification of ^{31}P resonances to be readily performed. At pH 6.5, in the presence of IHP, the ^{31}P resonance of PMe_3 bound to the α-subunit(s) from the (Fe(II),Mn(II)) hybrid (fig. 1b) shows the same chemical shift (23.2 ppm) as that of resonance D of PMe_3 bound to unmodified Hb (fig. 1a). At pH 7.6, the ^{31}P resonance (fig. 1c) shows the same chemical shift (24.8 ppm) as that of resonance B of PMe_3 bound

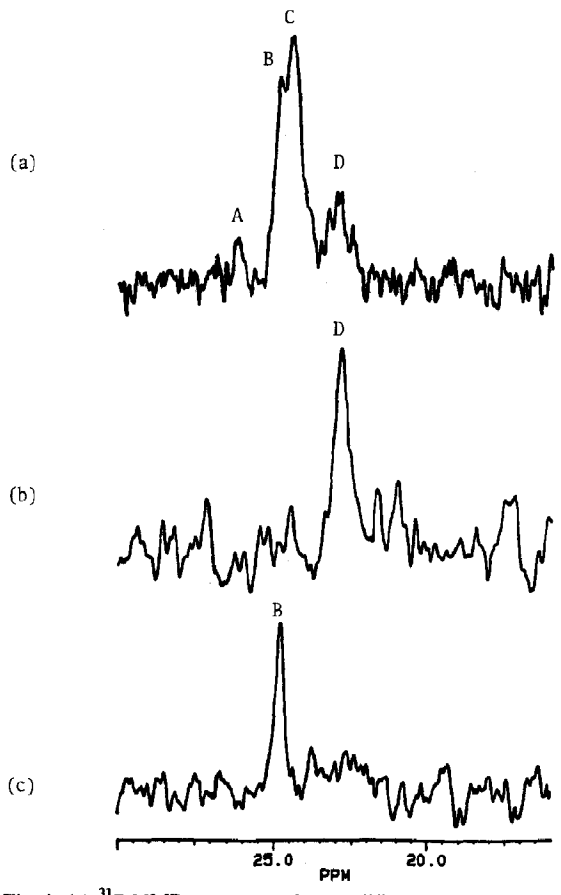

Fig. 1. (a) ^{31}P-NMR spectrum of unmodified Hb (1.5 mM) at low saturation $Y \simeq 8\%$ (in 0.1 M Tris-HCl buffer, pH 7.1). (b) ^{31}P-NMR spectrum of [Fe(II)(PMe_3),Mn(II)] hybrid (1.5 mM) at low saturation (~10%) (10 mM IHP in 0.1 M Tris-HCl buffer, pH 6.5). (c) ^{31}P-NMR spectrum of [Fe(II)-(PMe_3),Mn(II)] hybrid (1.5 mM) at low saturation (~10%) (in 0.1 M Tris-HCl buffer, pH 7.6). Peak A is the absorbance due to the β(PMe_3)-subunit (R state), B to the α(PMe_3)-subunit (R state), C to the partially liganded intermediate state and D to the α(PMe_3)-subunit (T state).

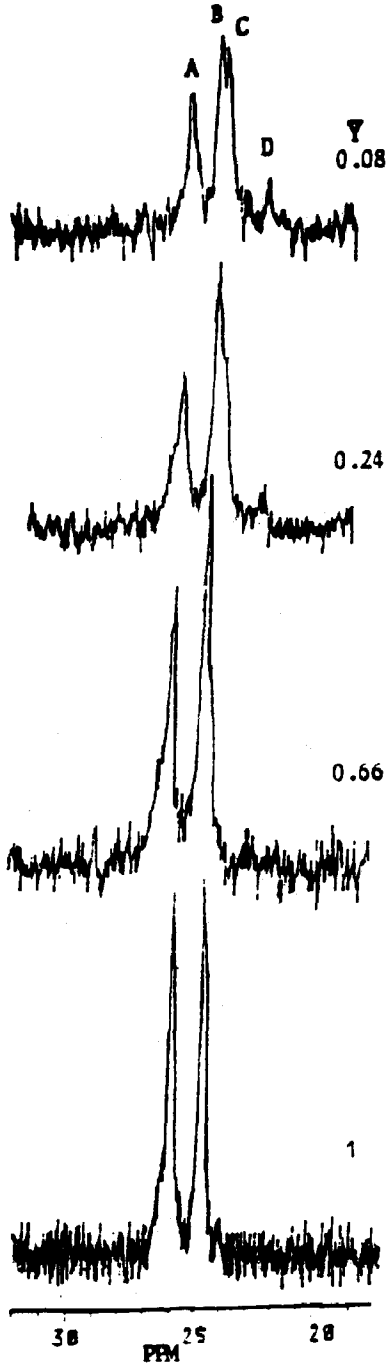

Fig. 2. ^{31}P-NMR spectrum of unmodified Hb (1.5 mM) as a function of increasing fraction bound to PMe_3 (Y) [17].

to unmodified Hb (fig. 1a), presumably due to α-chain(s) in the R state (vide infra). Consequently, two of the observable resonances appearing in the 22–26 ppm region of HbA_0 have now been completely assigned to the α-chains.

4. Discussion

The finding that PMe_3 complexation to Hb leads to three different signals due to the ligation of α-chains [17] implies the existence of three distinct structural forms and raises the intriguing question as to what these structures might be. In a previous work, Hoffman et al. [18] observed pH dependence of the α-chain affinity constants for CO binding to the ferrous atom in a $(Fe,Mn)_2$ hybrid. The authors concluded that the quaternary structural transition from the T-like to R-like state occurs on raising the pH. Furthermore, at low pH (6.5), the hybrid remains predominantly in the T state for the first two CO ligation steps when binding to the α-chains [24]. Hence, this mixed-metal hybrid hemoglobin (αFe(II),βMn(II)) can serve as a useful model for partially liganded species which occur during the ligation of Hb. It is also important to compare the results obtained with the CO system and those obtained for trimethylphosphine binding.

Our results on the resonance due to $^{31}PMe_3$ bound to the α-subunits of the (Fe(II),Mn(II)) hybrid are consistent with those observed from $^{31}PMe_3$ bound to the chains of unmodified Hb. Namely, ^{31}P resonances from the ligated phosphine are observed as changing upon pH variation: (i) a direct piece of evidence that the 23.2 ppm peak is characteristic of the T state is provided by a comparison of the two spectra obtained for Hb (fig. 1a) and (αFe(II)(PMe_3),βMn(II)) at pH 6.5 with IHP (fig. 1b); (ii) at elevated pH (7.6), the 23.2 ppm resonance is replaced by the 24.8 ppm resonance (fig. 1c) previously assigned to α-PMe_3 in the R state during ligation of unmodified Hb. Our results on the (Fe,Mn) hybrid suggest that the two extreme quaternary structures (T and R) are similar to those found in native hemoglobin. Accordingly, the identity of the chemical shifts observed in fig. 1 is of major interest in

relation to the high sensitivity of the ^{31}P-NMR signal for the structural environment of the phosphorus atom [17]. Furthermore, the close correspondence between our NMR results and flash photolysis studies of CO binding to the ferrous chains of the same hybrid supports the conclusion of Hoffman and Blough that the (αFe(II), βMn(II)) hybrid remains in the T quaternary state at pH 6.5 for CO ligation to the α-chains [18,24].

The implications of the present NMR results for the model of cooperative oxygenation of HbA can now be considered. In this regard, we shall discuss these preliminary results in terms of the molecular mechanism with comparison between results obtained with unmodified Hb and with the hybrid. We have found that peak C at 24.5 ppm due to α-chains in an intermediate state was missing. This means that the presence of Mn atoms in the β-chains in the Hb tetramer is sufficient to suppress the intermediate state, even in the presence of organic phosphate. At this stage of the discussion, a recent theoretical paper dealing with a three-state molecular switch for control of ligand affinity is relevant to our studies [25,26]. These authors concluded that the finding that Hb assumes three cooperative energy states during ligand binding suggests the existence of at least three significant structural forms. It should be also noted that in a different study, Miura and Ho [16] indicated the necessity of considering more than two structural forms of hemoglobin during the course of ligand binding. Furthermore, Gill and Di Cera [27] proposed a model where ligation occurs first at the α-chains in the T state on the basis of CO-binding curves to hemoglobin. However, it should be emphasized that our present NMR studies on this hybrid do not provide information about the degree of ligation of the α-subunit (singly or doubly ligated species) and the complete characterization of these two resonances (B and D) awaits further study. Certainly, studies in the near future on trimethylphosphine binding properties of the [αMn(II),βFe(II)] hybrid will also help to characterize the 'structural' properties of the intermediate state. Phosphine binding appears to be an excellent tool for the investigation of the conformation of the molecule, since the real structure of various modified hemoglobins is difficult to test in solution [28].

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