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Proton nuclear magnetic resonance study of the ferrous derivatives of the dimeric and tetrameric hemoglobin from the mollusc *Scapharca inaequivalvis*

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Proton NMR spectra have been measured for the two hemoglobins from the mollusc *Scapharca inaequivalvis:* HbI, a homodimer, and HbII, a heterotetramer. These hemoglobins are endowed with a unique subunit assembly, since the heme carrying E and F helices are involved in the major intersubunit contact. In the far-downfield region of hyperfine-shifted resonances the spectra of HbI and HbII in the deoxy state show respectively one (66.7 ppm) and two (67.8 and 63.6 ppm) exchangeable signals of the proximal histidine $N_\delta H$ groups, the resonance position being indicative of a significant strain in the iron-imidazole interaction. In the hydrogen-bonded proton region, inter- and intrasubunit hydrogen-bonded proton signals have been detected for both hemoglobins. Deoxy-HbI shows two unique downfield resonances at 11.83 and 11.51 ppm which disappear in the oxygenated state, suggesting that the corresponding hydrogen bonds are involved in the stabilization of the tertiary and/or quaternary structure of the deoxy form. HbII shows even smaller changes in this region upon changes in ligation state. These results therefore provide further proof that, at variance with the vertebrate hemoglobin tetramer, the unique subunit assembly of these proteins is stabilized mainly by hydrophobic interactions.

^{1}H-NMR; Hemoglobin; (*Scapharca inaequivalvis*)

1. INTRODUCTION

The interest in the dimeric (HbI) and tetrameric (HbII) hemoglobins of Arcid molluscs (such as *Scapharca inaequivalvis, Anadara broughtonii* and *A. trapezia*) arises from the recognition that they are endowed with a unique subunit assembly which in turn must be reflected in a distinctive molecular machinery underlying cooperativity in ligand binding. HbI and HbII are constructed from three different kinds of polypeptide chain [1] having the typical myoglobin fold with a proximal histidine as the axial heme ligand. The assembly of these globin chains into the dimeric protein is unique in that the heme carrying E and F helices are not exposed to solvent as in vertebrate hemoglobins, but form the intersubunit contact. The tetrameric HbII in turn is assembled from two such heterodimers that interact via the A helices and the non-helical AB and GH corners [2]. Due to their involvement in the major intersubunit contact the E and F helices display several specific structural features: they are highly conserved in all known Arcid hemoglobin sequences (78 and 95%, respectively), while the overall homology is low [3–5]. Moreover, they have a characteristic pattern of additional hydrophobic residues with respect to vertebrate hemoglobin and myoglobin chains [3]. The importance of these 'sticky patches' in the stabilization of the basic dimeric unit is brought out by the lack of dissociation into subunits in the presence of NaCl at high concentrations and in the pH range 5–9 [6].

The functional properties of Arcid hemoglobins

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are unusual like their assembly [1], since the homodimeric HbI displays a high cooperativity in ligand binding ($n = 1.5$). This behavior is in marked contrast to that of mammalian hemoglobins where the presence of unlike chains and a tetrameric molecule are required for cooperativity [7]. The cooperative Arcid dimer however lacks the sensitivity to allosteric effectors that is typical of the vertebrate hemoglobin tetramer [1].

The present ^{1}H-NMR study on the ferrous derivatives of HbI and HbII from *S. inaequivalvis* was undertaken in order to obtain information on the control of heme reactivity in these unique hemoglobins and to search for structural features related to the structural transition from the oxy to the deoxy form. To this end, the hyperfine-shifted region (where the proximal histidyl imidazole exchangeable $N_\delta H$ resonance occurs) and the hydrogen-bonded proton region (where inter- and intrasubunit hydrogen bonds in the human hemoglobin molecule are observed) have been investigated.

2. MATERIALS AND METHODS

The hemoglobins were isolated and purified according to established methods [1]. For NMR measurements the samples were prepared in 0.1 M potassium phosphate buffer (pH 7.0) at 2–3 mM in terms of hemoglobin molecule. The deoxy derivatives were obtained by addition of a few grains of solid sodium dithionite to the oxygenated samples under an N_2 atmosphere; the carbon monoxide derivatives were obtained by flashing CO gas over the deoxygenated samples.

Hyperfine-shifted proton resonances were recorded by an NIH-270 spectrometer consisting of a Nicolet NIC-270 spectrometer equipped with a Nicolet NIC-1180 computer system operating at 270.03 MHz. Paramagnetically shifted resonances of hemoglobins in non-deuterated buffer were taken with a super WEFT method, which is essentially identical with the water eliminate FT (WEFT) sequence except for the application of the pulse sequence significantly faster than the T_1 of un-

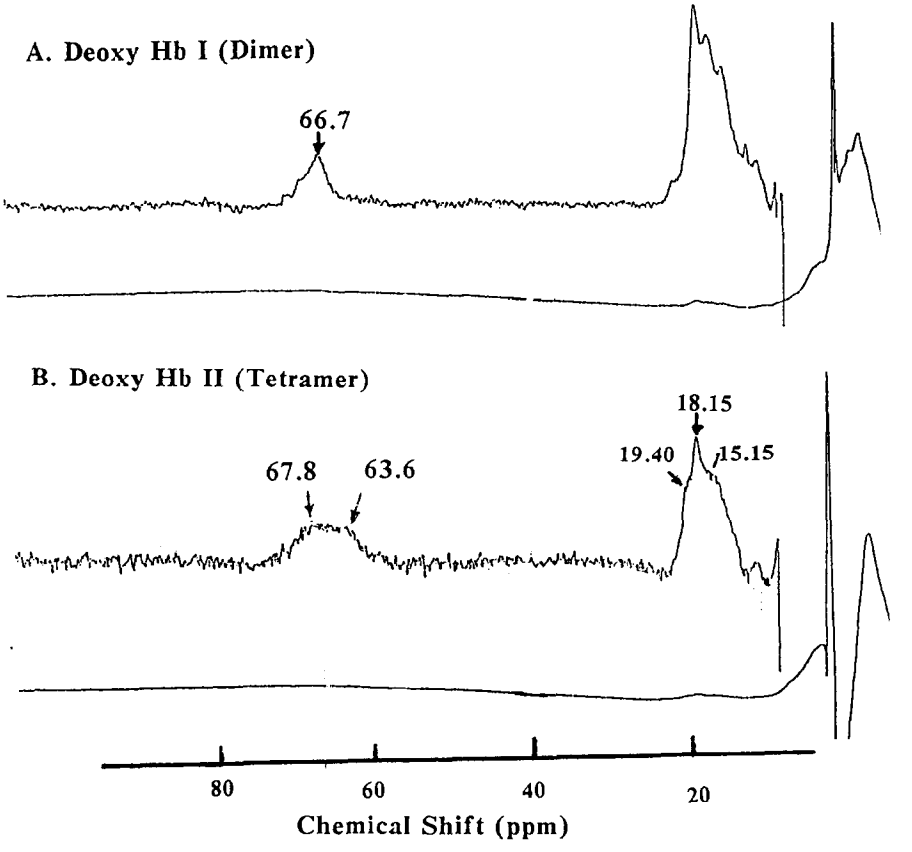

Fig.1. Hyperfine-shifted proton NMR (270 MHz) signals of deoxy *S. inaequivalvis* HbI (A) and HbII (B) obtained by super WEFT. Signals observed in the extremely downfield region are from the proximal histidine $N_\delta H$ and those in the region between 10 and 20 ppm are from heme peripherals.

wanted resonances, such as H_2O, protein and buffer signals [8]. Typically 0.1 s of repetition rate with 10 μs, 90° pulse width was applied by 0.2 s spacing between 180 and 90° pulses. To achieve a steady state of the suppressed magnetization of unwanted signals in their recovery process, 4–8 dummy scans were applied prior to the acquisition. Typically 1000–4000 transients were accumulated to obtain a reasonable signal-to-noise ratio. Inter- and intrasubunit hydrogen bonded signals were detected at 10°C by a Nicolet NT-500 spectrometer equipped with an NIC-1280 system. To eliminate the solvent water peak a modified Redfield 2-1-4-1-2 pulse sequence was used [9], where the middle 4/10 of the long tailored pulse was optimized to minimize the residual water peak in the samples. Four to eight blocks of Fourier-transformed spectrum after 256 scans were accumulated and stored by using floating point calculation.

3. RESULTS

3.1. *Hyperfine-shifted NMR signals*

In the far-downfield region deoxy-HbI at 21°C exhibits only one hyperfine-shifted resonance at 66.7 ppm which originates from the proximal histidine $N_\delta H$ (fig.1a). This assignment is based on the magnitude and direction of the paramagnetic shift, the largest downfield one observed in this hemoglobin, and on the broadness of the peak (approx. 500 Hz) that is typical of the proximal histidine $N_\delta H$ in the $S = 2$ state according to

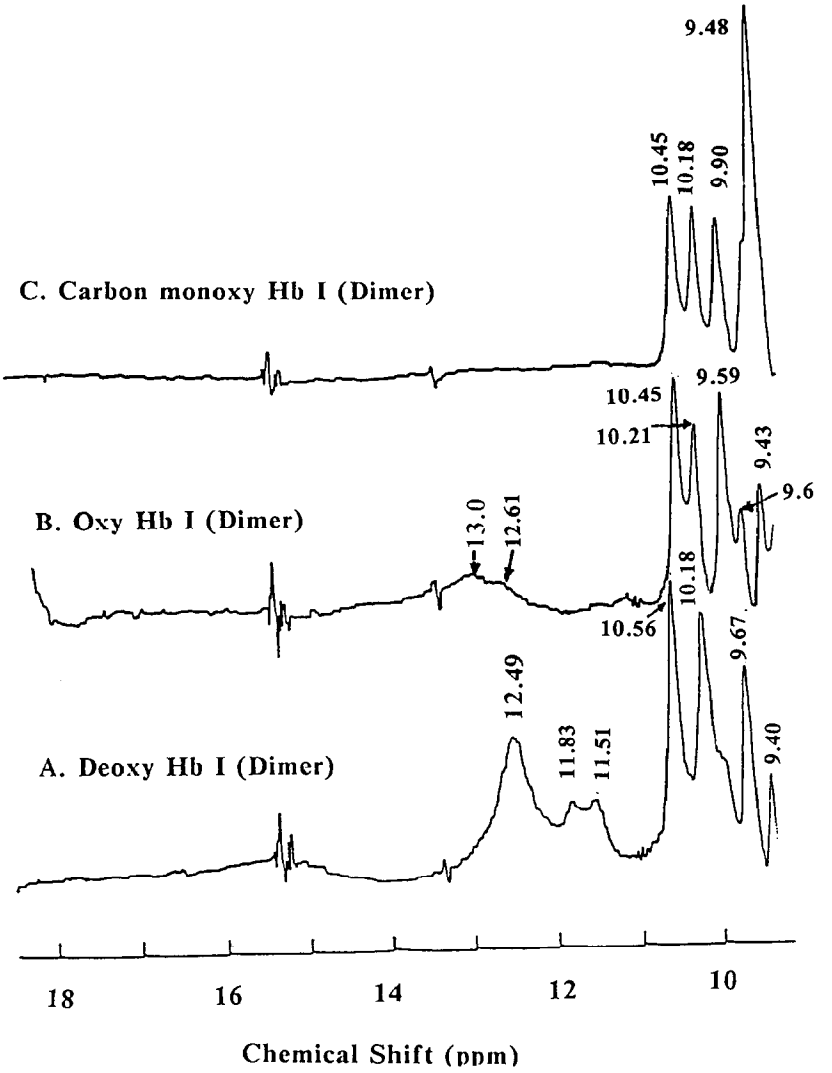

Fig.2. Proton NMR (500 MHz) spectra of the hydrogen-bonded region of dimeric *S. inaequivalvis* HbI in the deoxy (A), oxy (B) and CO (C) forms. Experimental conditions are described in the text.

literature data on deoxygenated myoglobin and human hemoglobin [10,11].

In the same spectral region deoxy-HbII shows two distinct hyperfine-shifted proximal histidyl resonances at 67.8 and 63.6 ppm (fig.1B) in line with the presence of two different polypeptide chains. The resonance position in the lower field is close to that observed for the dimeric HbI. Further, the chemical shift difference between the two signals is approx. 4.2 ppm, as compared to the 13 ppm difference displayed by human deoxyhemoglobin at 20°C [10,11].

3.2. *Inter- and intrasubunit hydrogen-bonded proton signals*

In hemoglobin samples in non-deuterated buffer some hydrogen-bonded proton resonances associated with inter- and intrasubunit interactions can be detected in the downfield edge of the protein signals. Typical spectra of this region for both hemoglobins in various liganded states are shown in figs 2 and 3.

Deoxy-HbI exhibits two exchangeable proton signals of hydrogen-bonded protons at 11.83 and 11.51 ppm. The intensity of these signals is significantly smaller than that of the non-exchangeable resonances, being about 30% of that of the single proton peak at 10.56 ppm. Other non-exchangeable resonances were found at 12.49, 9.96 and 9.40 ppm. Especially the broad peak at 12.49 ppm is in a downfield hyperfine-shifted region; hence it is likely to originate from heme peripherals or amino acid residues in the proximity of the paramagnetic heme moiety. Two additional exchangeable proton signals were also observed in the upfield region at 10.18 and 9.67 ppm. In the oxy form the two exchangeable proton peaks observed at 11.83 and 11.51 ppm in the deoxy state disappear and a characteristically broad exchangeable proton signal appears in the downfield region around 13.0 ppm (fig.1B). Two more exchangeable protons are observed at 10.21 and 9.89 ppm near the protein aromatic region. Peaks at 10.45, 9.67 and 9.43 ppm are non-exchangeable resonances. HbI complexed with CO does not show exchangeable proton signals below 11 ppm. However, similarly to the oxy form it exhibits two exchangeable proton resonances at 10.18 and 9.90 ppm. Again the signals at 10.45, 9.59 and 9.48 ppm are non-exchangeable resonances (fig.1C).

The hydrogen-bonded proton signal region for the oxy and deoxy forms of the tetrameric HbII is shown in fig.3. For the deoxy form an ex-

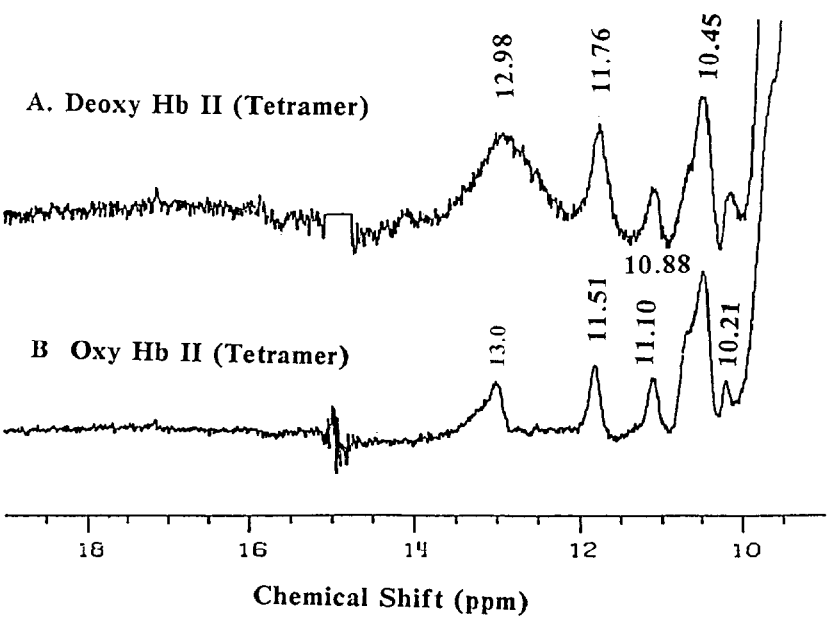

Fig.3. Proton NMR (500 MHz) spectra of the hydrogen-bonded region of tetrameric *S. inaequivalvis* HbII in the deoxy (A) and oxy (B) forms. Experimental conditions are described in the text.

changeable broad signal is observed at 12.98 ppm and three sharp exchangeable peaks between 12 and 10 ppm. Comparison of the peak intensities gives a relative ratio of 3:1 for the peak at 12.98 ppm with respect to that of the single proton resonance at 11.76 ppm. As in HbI some of the non-exchangeable resonances are due to hyperfine-shifted signals of heme peripherals. In the oxy form the broad hydrogen-bonded signal in the deoxy state is replaced by a sharp signal at 13.01 ppm, with relative intensity 2:1 vs the single proton peak at 11.51 ppm. Again, three hydrogen-bonded protons were detected in the 12–10 ppm region. The resonance position of all these peaks, including the non-exchangeable proton signals at 11.10 and 10.88 ppm, is very similar in the deoxy and oxy forms.

4. DISCUSSION

4.1. *Iron-imidazole linkage*

The bond between the proximal histidine imidazole and the iron is central to the control of heme reactivity. An indicator of the iron-histidine interaction is the hyperfine-shifted proximal histidyl imidazole exchangeable $N_\delta H$ resonance [12].

In the hyperfine-shifted region the spectra of deoxygenated HbI and HbII show respectively 1 and 2 exchangeable signals due to the $N_\delta H$ proximal histidine. The similarity in position of these resonances (66.7 ppm for HbI and 63.6 and 67.8 ppm for HbII) indicates that the microenvironment of the proximal histidine is similar for the three molluscan polypeptide chains in accordance with the high homology of the heme-carrying E and F helices [3–5]. The $N_\delta H$ chemical shifts are comparable to that observed in human hemoglobin for the α-chains (64 ppm), but smaller than that of the β-chains (76 ppm) [10,11]. The relatively small shift for the $N_\delta H$ resonances in the two *S. inaequivalvis* hemoglobins can be interpreted along the same lines as that of the human hemoglobin α-chains. It can be taken as indicative of the presence of strain in the histidine-iron linkage. This strain can be envisaged as due to simple bond stretching or tilting of the imidazole [13]; in either case it will lead to reduced Fe-N_ϵ covalency and hence to smaller $N_\delta H$ contact shifts [14]. It should be pointed out that the existence of a distorted coordination of the hindered proximal histidine in the deoxy derivatives has been suggested by the unusually high ellipticity of the Q_0 band [1] and by the anisotropy of the EPR spectrum in Co-substituted HbI [15].

4.2. *Inter- and intrasubunit hydrogen-bonded proton signals*

In human hemoglobin exchangeable proton NMR signals of inter- and intrasubunit hydrogen bonds have been useful tools for the definition of the tertiary and quaternary structures [16]. In such studies a large body of data obtained on normal and abnormal hemoglobins having known quaternary structures has been compared. However, no proton NMR data have been reported for hemoglobins from invertebrates. In *S. inaequivalvis* HbI and HbII, despite the importance of hydrophobic interactions in the stabilization of the quaternary structure, specific hydrogen bonds participating in intra- and/or intersubunit interactions exist as indicated clearly by the present data. However, the assignment of such hydrogen-bonded proton signals cannot be carried out in the absence of a high-resolution three-dimensional structure for both the liganded and the unliganded derivative.

Deoxy-HbI in the far-downfield region shows two characteristic exchangeable signals at 11.83 and 11.51 ppm which disappear in the oxy form (fig.2a,b). Therefore, the two corresponding hydrogen bonds are unique to deoxy-HbI and are related with its tertiary and/or quaternary structure. It should be noted that the intensity of these signals is weaker with respect to other single proton peaks in this region. A straightforward interpretation is that these hydrogen bonds are dissociated in part on the NMR time scale, indicating that the hydrogen-bonding interactions are weak. This situation is very unusual, since in human hemoglobin the intensities of the signals that are associated with hydrogen bonds specific to the T and R structures (e.g. those between Tyr β_2 145–Val β_2 98 and between Asp β_2 99–Asn α_1 97) always correspond to an integer number of protons [17,18]. The resonance pattern of non-exchangeable resonances of deoxy-HbI is similar to that of its oxy form. The oxy form shows a broad exchangeable resonance at 13.0 ppm. The significant broadening of this peak indicates that it

corresponds to a substantially weaker interaction as compared with other hydrogen bonds observed in the region between 9.5 and 11 ppm. This weak hydrogen-bonding interaction does not exist in the CO form, suggesting that there is a small difference in inter- or intrasubunit hydrogen bonding between the oxy and CO derivatives. In any case, the similarity of the resonance positions of exchangeable and non-exchangeable peaks in oxy- and CO-HbI suggests that the structures of the two forms are very similar, and may differ only in one weak hydrogen bond.

In the tetrameric HbII there are smaller changes in the pattern of resonances upon changes in ligation state with respect to HbI. Thus, the number of signals and the position of exchangeable and non-exchangeable resonances in the hydrogen-bonded proton region, especially in the range between 10 and 12 ppm, are essentially identical for both the deoxy and oxy forms. Such lack of sensitivity to the ligation status suggests that the inter- and intrasubunit hydrogen bonds are maintained almost unaltered during the transition from the deoxy to the oxy structure. The only differences observed in the hydrogen-bonded region of the HbII spectra are the signal intensities and line shapes of the peak at 12.98 ppm. Thus, the deoxy form contains one more hydrogen bond associated with the tertiary and/or quaternary structure than the oxy form. The extremely broad line shape (approx. 450 Hz) of the resonance at 12.98 ppm in the deoxy state deserves a comment. It is anomalous among the hydrogen bonds observed in hemoglobins, also when compared with the 125 Hz line width observed for the oxy state (fig.3). The broadness of the line is probably caused by a rapid exchange phenomenon in a weak hydrogen bond.

In conclusion, the shape and intensity of the exchangeable proton NMR signals detected in *S. inaequivalvis* hemoglobins suggest that hydrogen-bonded interactions are weak in comparison with those involved in the stabilization of the structure of vertebrate hemoglobins, such as human hemoglobin. In the latter proteins distinctive exchangeable resonances, which relate to specific hydrophilic interactions and hydrogen bonds, are always seen and are diagnostic of a given tertiary and quaternary structure [16–18]. The unusual character in the hydrogen bonds of *S. inaequivalvis* hemoglobins must be associated with their unique subunit assembly which involves mainly hydrophobic interactions [3]; accordingly, the change in the pattern of the hydrogen-bonded interactions is less significant than observed in human hemoglobin and actually may not represent the most sensitive probe of the structural transition from the oxy to the deoxy state.

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