Volume 233, number 2, 383–387 FEB 05972 June 1988

Detection of anion-linked polymerization of the tetrameric hemoglobin from *Scapharca inaequivalvis* by ^{35}Cl NMR spectroscopy

Emilia Chiancone, Torbjörn Drakenberg[+] and Sture Forsén[+]

CNR Center of Molecular Biology, Department of Biochemical Sciences, University 'La Sapienza', 00185 Rome, Italy and [+] *Physical Chemistry 2, Chemical Centre, Lund University, PO Box 124, S-221 00 Lund, Sweden*

Received 22 April 1988

Ion binding to the hemoglobin components of *Scapharca inaequivalvis* has been measured directly in quadrupole relaxation experiments of ^{23}Na and ^{35}Cl. The dimeric and tetrameric hemoglobins interact weakly with sodium ions, but differ in their interaction with chloride ions. The dimeric hemoglobin binds chloride ions with low affinity, whereas the tetrameric protein has high-affinity chloride binding sites. Binding of chloride ions to these high-affinity sites brings about an oxygen-linked polymerization which manifests itself in an unusual dependence of the ^{35}Cl excess linewidth on the concentration of the anion. Polymerization is more pronounced in the deoxygenated than in the oxygenated derivative: in the former, it has been observed previously in sedimentation velocity experiments. The sensitivity of the ^{35}Cl excess linewidth on polymer formation indicates that the residence time of the transiently bound chloride on the tetrameric hemoglobin is not shorter than the correlation time of the molecule (2×10^{-8} s^{-1}).

Hemoglobin; Polymerization; ^{35}Cl-NMR; *(Scapharca)*

1. INTRODUCTION

The dimeric and tetrameric hemoglobins extracted from Arcid molluscs are exceptional in many respects. The three constitutive polypeptide chains possess the typical myoglobin fold (with an additional α-helix at the amino end), but assemble in an inside-out fashion relative to vertebrate hemoglobins. Thus, in the dimeric protein (HbI), the heme-linked E and F helices of two like chains are not exposed to solvent as in vertebrate hemoglobins, but form the intersubunit contact region. In the tetrameric protein (HbII), two heterodimers assembled in the same way give rise to the tetramer by contact between the A helices and the non-helical segments preA-A and GH [1]. In the stabilization of these unique dimer and tetramer contacts, salt bridges and hydrogen bonds play a less important role than in vertebrate hemoglobins as indicated by the characteristic pattern of highly conserved hydrophobic residues in the E and F helices [2] and by the stability of HbI and HbII towards dissociation into subunits in high salt concentrations [3]. However, ionic interactions appear to be at the basis of another interesting property of the tetrameric protein, namely its strong tendency to polymerize upon removal of oxygen [4]. The dependence of polymer formation on several experimental variables (such as pH, temperature, nature and ionic strength of the buffer) and on selective chemical modifications has suggested that one or two reactive lysyl residues may have a specific role in the process by forming an acceptor pocket for anions in the deoxygenated state, but not in the oxygenated one, so that the bound anions can favor the interaction between deoxygenated tetramers [5].

The functional properties of Arcid hemoglobins

Correspondence address: E. Chiancone, CNR Center of Molecular Biology, Department of Biochemical Sciences, University 'La Sapienza', 00185 Rome, Italy

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

are unique like their quaternary structure. At variance with vertebrate hemoglobins, in which cooperativity in oxygen binding is restricted to a tetrameric molecule built by unlike chains, HbI, the homodimer, binds oxygen with significant cooperativity and represents the 'minimum functional unit' of Arcid hemoglobins [4]. However, HbI lacks the sensitivity to allosteric effectors that is characteristic of vertebrate hemoglobins, and in particular its oxygen affinity is not affected by ionic components in the medium in line with the minor role played by electrostatic interactions at the level of the dimer contact. In contrast, the tetrameric protein displays a slight acid Bohr effect below pH 6.5 and its oxygen affinity is lowered upon polymerization of the deoxy derivative [4].

With the aim of getting a deeper insight into this latter process and in view of the proposed role of anions in favoring the interaction between deoxygenated tetramers, the ion binding properties of HbII from *Scapharca inaequivalvis* have been measured and compared with those of HbI. Advantage has been taken of the fact that chloride and sodium ions are amenable for direct NMR spectroscopic studies. In fact, due to the relaxation properties of quadrupolar nuclei (like ^{35}Cl and ^{23}Na) undergoing fast chemical exchange between 'free' and 'bound' ions, their interaction with proteins manifests itself readily as a pronounced broadening of the NMR signal of the free solvated ion [6,7].

2. MATERIALS AND METHODS

2.1. *Hemoglobins and NMR measurements*

The hemoglobins of *S. inaequivalvis* were prepared and purified according to published procedures [4]. The samples for the NMR measurements were obtained by dilution of a hemoglobin stock solution with ^2H_2O and appropriate amounts of a NaCl stock solution. For the measurements on the deoxygenated derivative, the NMR tube was fitted with a gas tight device as described by Norne et al. [8]. For the measurements on the carbonmonoxy derivative the hemoglobin solution was equilibrated with pure CO. The pH was adjusted by additions of 0.05–0.2 M HCl or NaOH. The protein concentration was determined on the oxygenated derivatives from the optical absorption at 578 nm, using as millimolar extinction coefficient on a heme basis 14.3 for HbI and 14.2 for HbII.

The ^{35}Cl and ^{23}Na NMR spectra were recorded at 24.98 MHz and at 67.44 MHz, respectively, on a home made 6 T spectrometer as described previously [9]. The sample temperature was kept at 25°C unless otherwise stated.

2.2. *General principles and data treatment*

The NMR spectroscopic behavior of spin $I = 1/2$ quadrupolar nuclei in the presence of chemical exchange has been detailed in [6,7]. For the present purposes it suffices to recall that when the exchange rate of the ions between the bulk and protein binding sites is rapid compared to the relaxation rate of bound ions, the observed linewidth of the quadrupolar nuclei, $\Delta\nu_{obs}$, is given by:

$$\Delta\nu_{obs} = \Delta \cdot p_0 + \Delta\nu_i \cdot p_i \quad (1)$$

where $\Delta\nu_0$ and $\Delta\nu_i$ are the linewidths of the specific ion in the absence and in the presence of the protein, p_0 and p_i are the fractions of free hydrated ion in solution and of the ion bound to the different protein binding sites, respectively. In general a large excess of ions is used and it is convenient to express the results in terms of an excess linewidth.

$$\Delta\nu_e = \Delta\nu_{obs} - \Delta\nu_0 = \Sigma\, p_i \cdot \Delta\nu_i \quad (2)$$

On the basis of mass law considerations it is possible to relate $\Delta\nu_e$ to the number of i-type protein binding sites, n_i, to their intrinsic binding constant, $K_{i,X}$, and to the total protein and ion concentration, c_p and c_X, by means of the expression [6]

$$\Delta\nu_e = c_p \Sigma \frac{n_i \cdot K_{i,X} \cdot \Delta\nu_i}{1 + K_{i,X} \cdot c_X} \quad (3)$$

As mentioned above, these equations apply when the chemical exchange rate is rapid compared to the relaxation rate of bound ions. This condition can be verified by performing variable temperature studies on the basis of the following considerations. $\Delta\nu_i$ may generally be written as:

$$\Delta\nu_i = 1/\pi(T_{2,i} + \tau_{m,i}) \quad (4)$$

where $T_{2,i}$ is the transverse relaxation time at site i and $\tau_{m,i}$ is the mean lifetime of the ion at site i. If chemical exchange determines the linewidth ($\tau_{m,i} \gg T_{2,i}$), the linewidth will increase with increasing temperature since $\tau_{m,i}$ is bound to decrease. On the other hand, if quadrupole effects dominate ($\tau_{m,i} \ll T_{2,i}$) the linewidth will decrease with increasing temperature since $1/T_2$ is proportional to τ_c, the correlation time for reorientation of the electric field gradient affecting the relaxing nuclei. For ions bound to the protein either irrotationally or in such a way that they are free to rotate rapidly about some axis in the protein, one should expect τ_c to be either the correlation time for rotation of the protein or to be directly proportional to it. Therefore, $1/T_2$ depends on η/T, where η is the viscosity of the medium and T the absolute temperature.

3. RESULTS

3.1. *^{35}Cl NMR*

Both *S. inaequivalvis* hemoglobins at pH values around neutrality give rise to a broadening of the ^{35}Cl NMR signal. Moreover, the temperature dependence of the ^{35}Cl excess linewidth is typical of a fast exchange regime since the linewidth decreases with increasing temperature (not shown). In the case of oxy-HbI, the ^{35}Cl excess linewidth increases with decreasing anion concentration as

expected on the basis of eqn 3 assuming one class of binding sites with $K_{Cl} \leq 10\ M^{-1}$. In the case of HbII, the dependence of the ^{35}Cl excess linewidth on anion concentration was measured on the same samples before and after deoxygenation in view of the proposed role of anions in the polymerization of the deoxy derivative [5]. The line broadening is consistently larger in the presence of deoxy-HbII, but for both derivatives the ^{35}Cl excess linewidth behaves in an unexpected way – it first increases and then decreases as a function of the concentration of chloride ions. Fig.1 shows that this chloride-concentration-dependent increase in linewidth at low Cl^- concentrations is more pronounced in the presence of deoxy-HbII and that the maximum line broadening occurs at about 20 mM NaCl for both derivatives.

The pH dependence of the ^{35}Cl excess linewidth was measured for carbon-monoxy-HbII in 20 mM NaCl. Fig.2 shows that the linewidth decreases monotonically above pH 7.0 without marked inflection points.

3.2. *^{23}Na NMR*

The ^{23}Na excess linewidth was measured in the presence of HbI or HbII at pH values of 6.2–6.3.

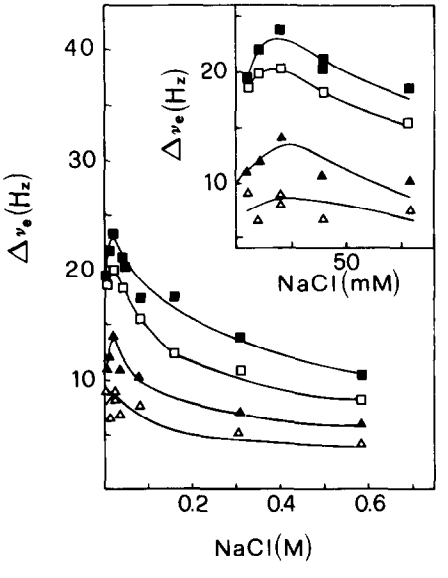

Fig.1. ^{35}Cl excess linewidth as a function of NaCl concentration in the presence of oxy- and deoxy-HbII from *S. inaequivalvis* at pH 6.3. Hemoglobin concentration: (△, ▲) 0.26%; (□, ■) 0.5%. Closed symbols refer to the deoxy-derivative.

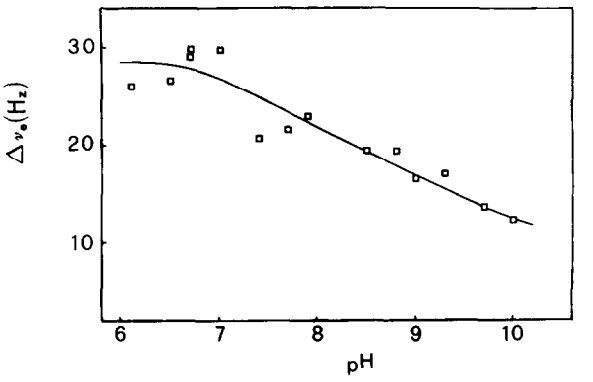

Fig.2. ^{35}Cl excess linewidth as a function of pH in the presence of carbonmonoxy-HbII from *S. inaequivalvis*. Solvent: 20 mM NaCl. Hemoglobin concentration: 0.7%.

In the presence of oxy-HbI, the ^{23}Na excess linewidth depends on sodium ion concentration as expected for weak binding ($K_{Na} \leq 10\ M^{-1}$). In the presence of oxy-HbII, the excess linewidth is very small (a maximum of 10 Hz at a protein concentration of 1.5% and at 20 mM NaCl) indicating a very weak interaction.

4. DISCUSSION

The present ^{23}Na and ^{35}Cl NMR data bring out interesting differences in the ion binding properties of the dimeric and tetrameric hemoglobins from *S. inaequivalvis*. Thus, HbI and HbII interact with low affinity with sodium ions ($K_{Na} \leq 10\ M^{-1}$), but differ in their interaction with chloride ions. In fact chloride ions bind weakly to HbI ($K_{Cl} \leq 10\ M^{-1}$) and strongly to HbII. Moreover, the location of the specific chloride binding sites on the HbII molecule is such that binding of the anion gives rise to an association-dissociation equilibrium between tetramers and polymers thereof. The existence of a chloride-linked polymerization in both the oxygenated and deoxygenated derivatives of HbII is clearly indicated by the unusual dependence of the ^{35}Cl excess linewidth on the concentration of the anion. Deoxy-HbII was known to polymerize on the basis of sedimentation velocity studies carried out on several species [4,10], but polymerization of the oxygenated form of the protein had never been detected. Actually, there is no reason why polymerization of HbII should be an all or none

process as a function of oxygenation. On the contrary, linkage considerations suggest that the chloride-linked polymerization should be affected by oxygenation to a limited extent and that one should be able to find conditions in which polymerization is observable in the oxygenated derivative.

In qualitative terms the linewidth of the chloride ions bound transiently to HbII is expected to be considerably increased in the larger HbII aggregates as compared to the tetramer if one assumes no drastic change in the Cl^- binding sites during the association process. Since the temperature dependence of the linewidth is indicative of a fast exchange situation, the fact that $\Delta\nu_e$ is sensitive to polymerization in turn means that the residence time of the bound chloride ions is not shorter than the correlation time of the tetramer ($\tau_c = 2 \times 10^{-8}\ s^{-1}$) in agreement with results obtained by Bull et al. [11] on human hemoglobin. In order to account for the maximum in $\Delta\nu_e$ observed as a function of chloride concentration, the anion-dependent association-dissociation equilibrium must be such that: (i) at low chloride concentration the dominant species is the tetramer; (ii) the amount of polymer formed increases with increasing chloride concentration. Such an anion-dependent association phenomenon has been observed in sedimentation velocity experiments carried out with the deoxygenated derivative in phosphate buffer [4,10]. With the oxygenated derivative, however, the sedimentation velocity experiments provide evidence for polymerization only in as much as the diffusion coefficients are indicative of heterogeneity since they are higher than expected on the basis of the tetramer molecular mass (Vecchini, P., personal communication). In accordance with these observations the present data show that association is more pronounced in deoxy- than in oxy-HbII since the ^{35}Cl excess linewidth is consistently larger in the presence of the former derivative.

A quantitative treatment of the ^{35}Cl NMR data concerning HbII is rendered difficult by the fact that the excess linewidth of chloride ions, $\Delta\nu_e$, is a function of the linewidth of the bound ions, $\Delta\nu_i$, and of K_{Cl} which occur as a product in eqn 3. Normally, in a system where no ion dependent association-dissociation takes place, these quantities can be determined separately from the dependence of $\Delta\nu_e$ on the ion concentration as in the case of HbI and of a number of other systems studied previously [12–14]. However, when an ion dependent association takes place, such a separation is no longer possible without independent knowledge of the binding constants involved in the association equilibrium. These difficulties of course are encountered also in the interpretation of the data obtained as a function of pH, since $\Delta\nu_e$ becomes a complex function of polymerization and anion binding to the various states of the protein.

Specific cation-linked associations of macromolecules have been observed in a number of systems (e.g. [15–17]), whereas to our knowledge *S. inaequivalvis* HbII is the only anion-linked associating system. The anion-linked association of *S. inaequivalvis* HbII must be considered as rather specific since the maximum effect is observed at fairly low concentrations, around 20 mM NaCl, in both oxy- and deoxy-HbII. The maximum effect of phosphate buffer on the polymerization of deoxy-HbII, as measured in sedimentation velocity experiments, also occurs at roughly the same concentrations ([4]; and Vecchini, P., personal communication). Thus, it is reasonable that positively charged amino acid sidechains near or at the surface of the molecule are involved in anion binding. In this connection it is relevant that modification of only one or two lysine residues drastically decreases polymerization of the deoxy-derivative [5]. However, in addition to the involvement of such reactive lysine residues, one has to invoke the contribution of other stabilizing interactions, such as salt bridges, in order to account for the decrease in polymerization at higher ionic strengths. The present data therefore confirm the picture of the polymerization-linked anion binding sites proposed previously for deoxy-HbII and extend it to the oxygenated derivative. Hence it becomes tempting to speculate on the possible location of these binding sites. The lysine residues which are in a cluster of positively charged residues at the end of the pre-A helix in the contact region between two dimers in the tetramer (Lys-15 and Arg-19,20 in the so-called α-chain; Lys-17 and Arg-21 in the so-called β-chain) appear as appealing candidates. Due to their location such lysine residues are likely to feel conformational differences between the

oxy- and deoxy-derivatives and in addition may favor polymer formation since the pre-A and H helices form an extensive contact in the HbII crystals [1].

In conclusion, this study of the ion binding properties of the hemoglobins from *S. inaequivalvis* has brought new insight into the oxygen- and anion-linked polymerization of the tetrameric component. Moreover, it has shown that, under appropriate conditions, ^{35}Cl NMR can be used as a sensitive means to detect polymerization, a hitherto unexploited application of the relaxation properties of quadrupolar nuclei.

REFERENCES

[1] Royer, W.E., jr, Love, W.E. and Fenderson, F.F. (1985) Nature 316, 277–280.
[2] Petruzzelli, R., Goffredo, B.M., Barra, D., Bossa, F., Boffi, A., Verzili, D., Ascoli, F. and Chiancone, E. (1985) FEBS Lett. 184, 328–332.
[3] Gattoni, M., Verzili, D., Chiancone, E. and Antonini, E. (1983) Biochim. Biophys. Acta 743, 180–185.
[4] Chiancone, E., Vecchini, P., Verzili, D., Ascoli, F. and Antonini, E. (1981) J. Mol. Biol. 152, 577–592.
[5] Boffi, A., Gattoni, M., Santucci, R., Vecchini, P., Ascoli, F. and Chiancone, E. (1987) Biochem. J. 241, 499–504.
[6] Forsén, S. and Lindman, B. (1981) Methods Biochem. Analysis 27, 289–486.
[7] Forsén, S., Drakenberg, T. and Wennerström, H. (1987) Q. Rev. Biophys. 19, 83–114.
[8] Norne, J.-E., Chiancone, E., Forsén, S., Antonini, E. and Wyman, J. (1978) FEBS Lett. 94, 410–412.
[9] Drakenberg, T., Forsén, S. and Lilja, H. (1983) J. Magn. Resonance 53, 412–422.
[10] Furuta, H., Ohe, M. and Kajita, A. (1981) Biochim. Biophys. Acta 668, 448–455.
[11] Bull, T.E., Andrasko, J., Chiancone, E. and Forsén, S. (1973) J. Mol. Biol. 73, 251–259.
[12] Chiancone, E., Norne, J.-E., Forsén, S., Antonini, E. and Wyman, J. (1972) J. Mol. Biol. 70, 675–688.
[13] Chiancone, E., Norne, J.-E., Forsén, S., Brunori, M. and Antonini, E. (1975) Biophys. Chem. 3, 56–65.
[14] Norne, J.-E., Gustavsson, H., Forsén, S., Chiancone, E., Kuiper, H.A. and Antonini, E. (1979) Eur. J. Biochem. 98, 591–595.
[15] Klarman, A., Shaklay, N. and Daniel, E. (1972) Biochim. Biophys. Acta 257, 150–157.
[16] Blair, D. and Van Holde, K.E. (1976) Biophys. Chem. 5, 165–170.
[17] Chiancone, E., Brenowitz, M., Ascoli, F., Bonaventura, C. and Bonaventura, J. (1980) Biochim. Biophys. Acta 623, 146–162.