ISOMERIC INCORPORATION OF THE HAEM GROUP INTO TWO MONOMERIC HAEMOGLOBINS OF *CHIRONOMUS THUMMI THUMMI*

A nuclear magnetic resonance study

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1. Introduction

The polymorphism of the haemoglobin components of the larvae of *Chironomus thummi* has been described previously [1]. Even chromatographically pure components like the monomeric component III (CTT III) reveal heterogeneities in the amino acid sequence [2]. The occurrence of heterogeneities may be explained by the fact that the haemoglobins usually are isolated from larvae of different subspecies of *Chironomus thummi* [3]. We would like to show in the following paper that the component CTT III for which an alternative occupancy of the position E6 (Thr or Ile) has been described [2,4,5], may be obtained without heterogeneity by growing larvae of one particular subspecies of *Chironomus thummi*. In addition, we are able to demonstrate by ^{1}H- and ^{13}C-NMR spectroscopy of the two monomeric components CTT III and CTT IV that the haem group is arranged in two ways in the haem pocket which leads to two different conformational isomers [6] of both, CTT III and CTT IV. The two different conformational isomers of either components reveal different functional properties with respect to the Bohr effect [6].

2. Materials and methods

Larvae of *Chironomus thummi* were obtained from Nebelung, Münster, as a mixture of various subspecies. Larvae of one particular subspecies *Chironomus thummi thummi* were grown in our laboratory. The components III and IV were isolated according to the procedure of Sick et al. [7]. A final step of purification by isoelectrofocusing has been added.

NMR investigations are performed with a WH 270 Bruker instrument connected to a BNC 12 Data system. Solutions of CTT III or CTT IV were 1.5 mM in 0.2 M NaCl–D_2O. Extreme care has been applied to always keep the reduced state of the haemoglobins by adding appropriate reducing agents. Chemical shifts of proton resonances are given in ppm relative to trimethylsilyl propionic acid (TSP).

^{13}CO (90% enriched in the isotope ^{13}C) has been purchased from MSD, Munich. ^{13}C chemical shifts are reported in ppm relative to free ^{13}CO dissolved in the haemoglobin–D_2O solution [8].

3. Results and discussion

In the very low field absorption region of ^{1}H-NMR spectra of haem proteins various resonances are observed which can be assigned to protons of the porphyrin ring system [9]. In particular the meso proton resonances are found in a spectral region around 10 ppm which is typical for most diamagnetic myoglobins or haemoglobins [10,11]. In fig.1 this absorption region is shown for two samples of CTT III of different origin.

Remarkable differences are found between the component of a previous preparation and the corresponding preparation of CTT III from larvae of one particular subspecies *Chironomus thummi thummi*. Apparently the alternative occupancy at position E6

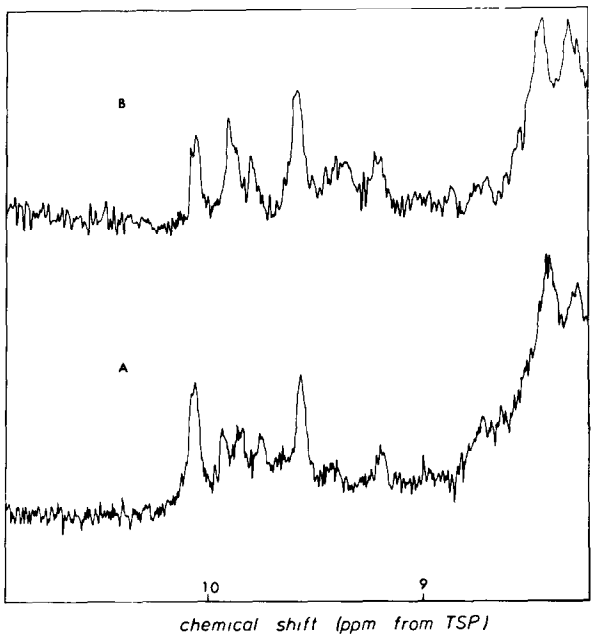

Fig.1. Absorption region of the meso proton resonances at pH = 7.2 of (A) CTT III CO-Hb(II) prepared from larvae of various subspecies of *Chironomus thummi;* (B) CTT III CO-Hb(II) prepared from larvae of one particular subspecies *Chironomus thummi thummi.*

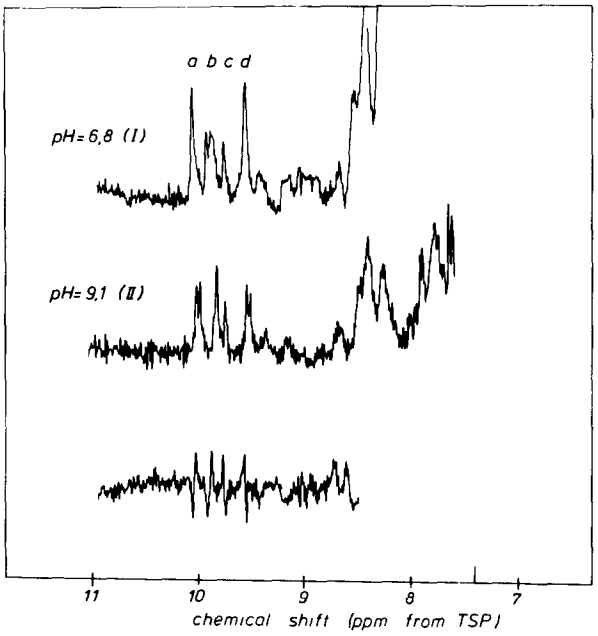

Fig.2. Absorption region of the meso proton resonances of CTT II CO-Hb(II) at two different pH values. (I), pH = 6.8; (II), pH = 9.1 and the difference spectrum II–I. The CTT III material has been prepared as described in fig.1B.

(Thr or Ile) has been replaced by a homogeneous occupancy which is Thr according to a subsequent peptide analysis. The position E6 is located on the distal site near the haem group according to the X-ray analysis of Huber et al. [5,12]. Depending on whether this position is kept by a threonine or isoleucine the meso proton resonances behave differently. In position E7 a histidine residue is located. The C2 proton resonance of this histidine is found to be split by about 12 Hz in the ^{1}H-NMR spectra of CTT III prepared in the previous manner from larvae of a mixture of subspecies of *Chironomus thummi.* There are many additional evidences from the comparison of both preparations that the position E6 influences the structure of the distal site of CTT III and thereby also the functional properties of this haemoglobin. Hence the NMR investigations which will be described in the following were performed exclusively with CTT III and CTT IV material prepared from larvae of one particular subspecies *Chironomus thummi thummi.*

A detailed analysis of the pH dependence of the meso proton resonances has been possible with this preparation of CTT III. Seven resonances are observed four of which shift position with pH (fig.2). Compared to other proton resonances the intensity of each of these signals corresponds to 0.5 proton equivalents.

Since the material is supposed to be homogeneous this behaviour can only be explained assuming an isomeric incorporation of the haem into the haem pocket. The titration curves which can be derived from the pH dependence of four meso proton resonances reveal a pK value of 7.7 (fig.3). This pK value is also found from the pH dependence of the C2 proton resonance of one histidine. Since this pK value is changing with the nature of the ligand this histidine is considered to represent the Bohr proton bearing group [13]. The assignment of its C2 proton resonance to His G2 of the sequence will be described elsewhere. However, also the His G2 C2 proton resonance is split into two signals, again indicating that CTT III consists of two conformational isomers one

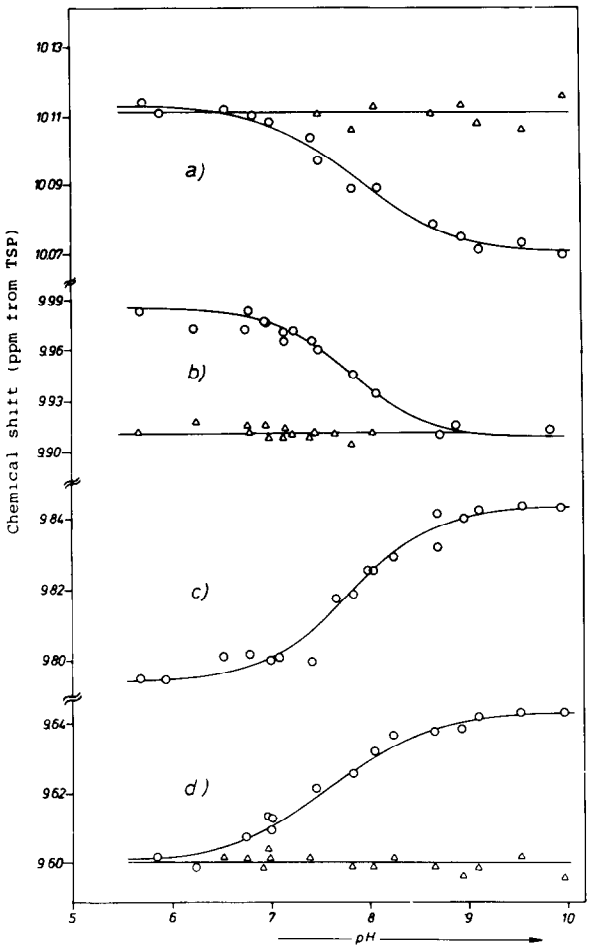

Fig.3. Chemical shifts of the meso proton resonances of CTT III CO-Hb(II) in dependence of pH (CTT III material as in fig.2).

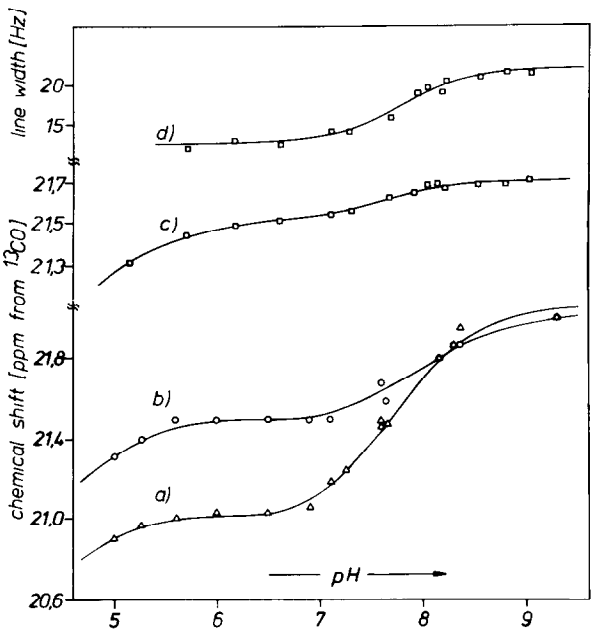

Fig.4. Chemical shifts of the ^{13}CO carbon resonances of CTT IV ^{13}CO-Hb(II) (a and b) and CTT III ^{13}CO-Hb(II) (c) and line width of the ^{13}CO carbon resonance of CTT III ^{13}CO-Hb(II) (d) in dependence of pH. Both CTT III and CTT IV have been prepared from larvae of one particular subspecies *Chironomus thummi thummi.*

of which reveals the Bohr effect, the other one does not.

Similar results are also obtained from an analysis of the ^{1}H-NMR spectra of CTT IV. From the pH dependence of the meso proton resonances and the C2 proton resonance of the Bohr proton bearing histidine it can be concluded that both conformational isomers of CTT IV reveal the Bohr effect although the extent of proton release upon ligand binding is different in both isomers.

The isomerism which is brought about by two different arrangements of the haem in CTT III or CTT IV is also evident from the pH dependence of the ^{13}CO carbon resonance of the two haemoglobins in the corresponding complexes (fig.4). For CTT IV ^{13}CO-Hb(II) the carbon resonance is split into two signals. Two titration curves with slightly different pK values have been obtained indicating that in both isomers of CTT IV the ligand at the sixth coordination site of the haem is influenced by the ionization of the Bohr proton bearing histidine thus mediating the Bohr effect. The ^{13}CO carbon resonance of CTT III ^{13}CO-Hb(II) is not split. However, the signal position and the line width are changing with pH (fig.4). From these changes a pK value of 7.7 is derived which again agrees with the pK value of His G2. It is assumed that the ^{13}CO carbon resonances of the two isomers of CTT III ^{13}CO-Hb(II) are overlapping; one of the signals is changing slightly position with pH which results in a line width broadening of the total unresolvable resonance.

The change in the carbon resonance position of all

investigated ^{13}CO-Hb(II) species at lower pH values are assumed to be produced by a reversible acid denaturation process at low pH values [14]. Analogous changes are observed for ^{13}CO myoglobin complexes [8]. However, the ^{13}CO carbon resonance of ^{13}CO-Mb(II) complexes are not split [8] nor are the meso proton resonances split into a complex signal pattern [15]. Apparently in myoglobins the discussed isomerism does not occur.

4. Conclusions

As a result of an isomeric incorporation of the haem into the haem pocket two conformational states of each of the momomeric haemoglobins CTT III and CTT IV are derived. The conformational states differ with respect to the Bohr effect. The two different arrangements of the haem in the protein may be thought of as being performed by a 180° rotation around the $\alpha-\gamma$ methine axis which leads to an exchange of the vinyl and methyl groups in the positions 1,2,3 and 4 [10]. It should be mentioned that X-ray analysis of CTT III has not been able to provide the coordinates of the vinyl groups; at least one of the two vinyl groups is not fixed in the crystal. According to the tertiary structure a vinyl group in the position 4 of the haem is located adjacent to the side chains of Val FG5 and Met H22. The C-terminal carboxyl group of Met H22 is forming an H bond with the imidazole ring of His G2, which is assumed to be the Bohr proton bearing group [6,13]. The presumably different position of the vinyl groups in the two isomeric structures may thus lead to the two different functional properties of the haemoglobins.

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