Conformational disorder of the distal leucine in monomeric *Glycera* hemoglobins and implications for oxygen binding

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Received 21 May 1985

^{1}H NMR studies of the carbon monoxide complexes of the major monomeric hemoglobins from *Glycera dibranchiata* show that distal leucine is conserved at position E7. The observed ring current shifts and nuclear Overhauser enhancements indicate conformational disorder of the leucine E7 side chain. The conformational substates interconvert rapidly on the NMR time scale. The rapid conformational fluctuations of leucine E7 may play a fundamental role in governing diffusion of ligands to the heme.

Glycera dibranchiata *hemoglobin* *Distal leucine* *Ligand binding* *^{1}H NMR* *Heme pocket structure* *Conformational fluctuation*

1. INTRODUCTION

The monomeric hemoglobins from the marine annelid *Glycera dibranchiata* possess an extremely low affinity for oxygen [1–3]. The amino acid sequence [4] and X-ray structure [5] of one of the major *Glycera* hemoglobins show that the distal histidine found in most vertebrate hemoglobins and myoglobins is replaced by leucine. The low oxygen affinity of *Glycera* hemoglobin has been attributed to the distal Leu 58 (E7), which is unable to stabilize the O_2 complex through hydrogen bond formation [5,6]. Leucine E7 also blocks the entrance to the heme pocket [5]. It is probable that in *Glycera* hemoglobin, as in sperm whale myoglobin and human hemoglobin [7,8], motions of E7 and other distal residues are necessary to allow ligands to enter the heme cavity.

The monomer fraction of *Glycera* hemoglobin can be separated into 3 major and 2 minor components [9,10]. Although the oxygen affinity of each of the 3 major monomeric hemoglobins is very low, kinetic studies reveal large differences in the rates of reaction with oxygen [11]. As a first step in understanding these variations, it is essential to identify the amino acid residues surrounding the ligand binding site of each of these hemoglobin components. Here, we describe NMR experiments which show that the distal leucine is conserved at position E7 for the 3 major components. The ring current shifts and nuclear Overhauser enhancements indicate conformational disorder of the leucine E7 side chain, and show that the conformational substates interconvert rapidly on the NMR time scale. These conformational fluctuations may be of fundamental importance in controlling diffusion of ligands to the heme.

2. EXPERIMENTAL

The monomeric *Glycera* hemoglobins A–D were isolated as described [9]. Samples of the carbon monoxide complexes of the hemoglobins were prepared by stirring the protein in sodium phosphate buffer (0.1 M, pH 6.5–7.0) under a stream of carbon monoxide and adding 1–2 mg sodium dithionite. Residual dithionite was removed by passing the solution through a 0.75 ×

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00145793/85/$3.30

8 cm column of Sephadex G-15 equilibrated with sodium phosphate buffer (0.1 M, pH 7) in ^2H_2O. The buffer and column were flushed with carbon monoxide. The protein was collected in an NMR tube under a stream of carbon monoxide. The sample concentrations varied from 2 to 4 mM as measured by the pyridine hemochrome method [12] and the pH ranged from 6.8 to 7.1 (uncorrected meter readings).

^1H NMR spectra were recorded at 40°C using Bruker WM-400 and AM-500 spectrometers. Dioxan was used as an internal reference but all spectra are referred to TSS (trimethylsilylpropanesulfonic acid). One-dimensional spectra were acquired with solvent suppression. Resolution enhancement of one-dimensional spectra was achieved by Lorentzian to Gaussian transformation [13].

Two-dimensional scalar correlated (COSY) and dipolar correlated (NOESY) spectra were obtained using standard pulse sequences [14–17] with gated solvent suppression during the relaxation and incremented (t_1) delays. Experiments were performed with full quadrature detection using N type peak selection. A 45° mixing pulse was used in the COSY experiments. NOESY spectra were recorded with mixing times of 200 and 300 ms. Two-dimensional relayed coherence transfer spectra were recorded as in [18,19]. A mixing time τ = 25 ms was used. For all 2D experiments 256 × 2K data points were Fourier transformed to a 1K × 1K real spectrum. Sine-bell window functions were used in both time domains and absolute value spectra were calculated. The spectra were symmetrized [20] to reduce t_1 noise.

3. RESULTS AND DISCUSSION

The sharp single resonances of the heme meso protons can be readily identified in NMR spectra of the carbon monoxide complexes of the *Glycera* hemoglobins and have been assigned specifically from NOESY spectra [21]. In such experiments nuclear Overhauser effects (NOE) are observed between resonances of heme protons and those of neighboring amino acid side chains. Previous experiments on the carbon monoxide and oxygen complexes of leghemoglobin and myoglobin have revealed strong NOEs between the γ meso proton resonance and resonances of the histidine at position E7 [22,23]. The NOEs observed from the γ meso proton resonance may be used to identify the important E7 residue in the monomeric *Glycera* hemoglobin components.

The cross-section through the γ meso proton resonance in the NOESY spectrum of the CO complex of HbC is shown in fig.1C. NOEs are observed onto 2 resonances of approx. 3 proton intensity at −0.52 and −0.66 ppm. The COSY spectrum of HbC (fig.2) shows these two resonances to be coupled to a resonance at 0.07 ppm. Weaker connectivities are observed between 0.07 and 0.89 ppm, and between 0.89 and 3.43 ppm. The relayed coherence transfer spectrum (not shown) reveals additional cross-peaks between the resonances at −0.52 and −0.66 ppm and that at 0.89 ppm, and between the resonance at 0.07 ppm and that at 3.43 ppm. This identifies the residue at position E7 as leucine. The resonances at −0.66, −0.52, 0.07, 0.89 and 3.43 ppm are assigned to the δCH_3, δCH_3, γCH, one or both of the βCH_2 protons and the αCH, respectively. A strong NOE is observed between the αCH resonance and both δCH_3 resonances. Assignment to the residue at position E7 is further supported by the observation of NOEs between the Leu δCH_3 resonances and resonances of valine E11 and phenylalanine CD1

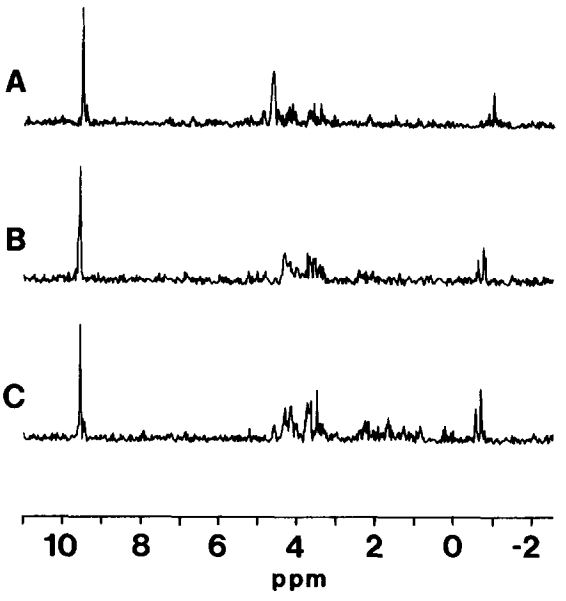

Fig.1. (A–C) Cross-sections through the γ meso proton resonance in the NOESY spectra of HbA, HbB and HbC, respectively.

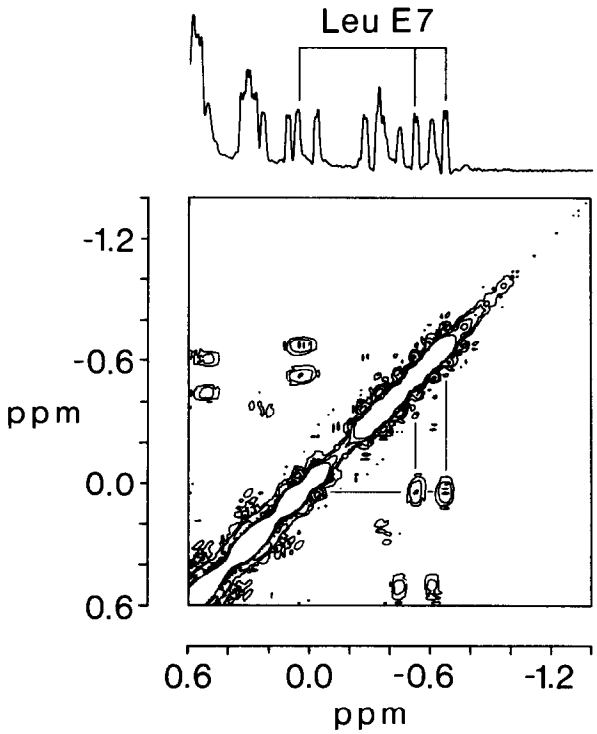

Fig.2. A section of the COSY spectrum of HbC. The two $\delta CH_3/\gamma CH$ connectivities of the Leu E7 spin system are indicated.

protons, which are situated above the β and δ meso protons, respectively. The assignments for Val E11 and Phe CD1 have been reported [21]. Cross-sections through the γ meso proton resonance in NOESY spectra of the CO complexes of HbA and HbB are shown in fig.1A and B. NOEs are observed onto methyl group resonances at -0.97 and -0.82 ppm for HbA and -0.74 and -0.58 ppm for HbB. In each case, COSY and relayed coherence transfer spectra identify these resonances as part of leucine spin systems. The chemical shifts of the assigned resonances of the leucine E7 side chain in hemoglobins A, B and C are summarized in table 1. Preliminary one-dimensional truncated driven nuclear Overhauser effect experiments on the CO complex of the minor hemoglobin component HbD indicate an NOE between the γ meso proton resonance and a resonance at -0.90 ppm. It thus appears that a leucine may also be conserved at position E7 in this hemoglobin.

Table 1

Chemical shifts of the assigned resonances of Leu E7

Resonance	Chemical shift (ppm)		
	HbC	HbB	HbA
δCH_3	-0.66	-0.74	-0.97
δCH_3	-0.52	-0.58	-0.82
γCH	0.07	-0.05	-0.46
βCH_2	0.89	0.86	0.72
αCH	3.43	3.48	3.30

The NMR spectra show clearly that each of the major *Glycera* hemoglobin components contains a leucine residue at position E7 in place of the distal histidine found in most vertebrate hemoglobins. Thus, the greatly reduced oxygen dissociation rate observed for HbA (HbIII in the notation of Parkhurst et al. [11]) relative to the other *Glycera* hemoglobins [11] cannot be explained by hydrogen bonding interactions with an appropriate distal group. Previous investigations of other hemoglobins and myoglobins [24–26] have established that hydrogen bonding to distal histidine can stabilize the oxygen complex and decrease oxygen dissociation rates.

Examination of the NOEs observed between the resonances of Leu E7 and those of the surrounding amino acid side chains allows its orientation to be determined. If Leu E7 were to exist in a single conformational state, the similarity of the ring current shifts experienced by the δCH_3 resonances and the observation of NOEs between both these resonances and the γ meso proton resonance would dictate that the δCH_3 groups straddle the γ meso proton and are approximately equidistant from the iron. In this situation one δCH_3 resonance would show NOEs onto Phe CD1 resonances whilst the other would show NOEs onto Val E11 resonances.

However, as shown in fig.3 for HbC, NOEs are observed from each Leu E7 δCH_3 resonance onto both the Val E11 γCH_3 resonance at -1.98 ppm and the Phe CD1 2,6H and 3,5H proton resonances at 6.83 and 5.59 ppm, respectively. Comparison of NOESY spectra recorded with different mixing times shows that this does not result from spin diffusion. Thus, significant rotation of Leu E7 about the C_β–C_γ axis is indicated, to bring each of its methyl groups close to the neighboring

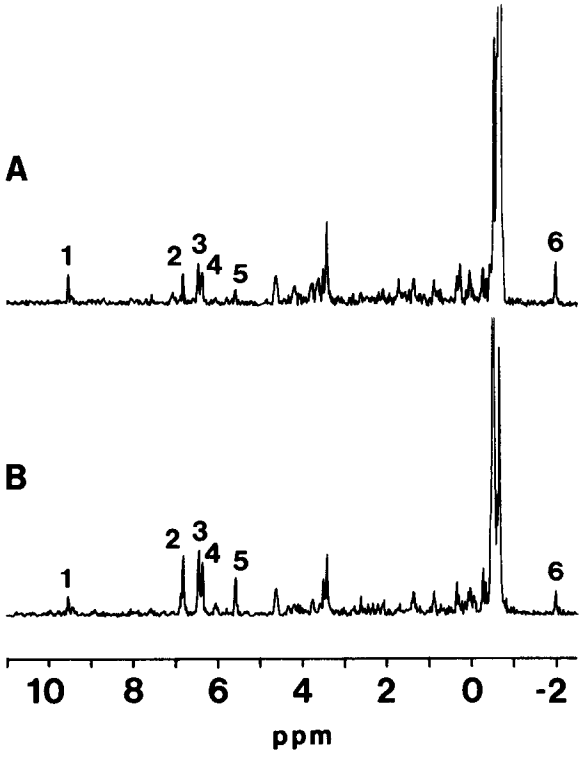

Fig.3. Cross-sections through the two Leu E7 δCH_3 resonances in the NOESY spectrum of HbC at (A) -0.66 ppm, and (B) -0.52 ppm. The assignments of the numbered resonances are: 1, γ meso proton; 2, Phe CD1 2,6H; 3, Phe CD3 3,5H; 4, Phe CD3 4H; 5, Phe CD1 3,5H; 6, Val E11 γCH_3.

Val E11 and Phe CD1 protons. Further, large NOEs are observed between the two δCH_3 resonances of Leu E7 and the 3,5H and 4H proton resonances of Phe CD3 at 6.47 and 6.37 ppm, respectively. Similar results are observed for Hbs A and B. Both the X-ray structure of *Glycera* hemoglobin [5] and our NMR studies show that Phe CD1 is between Phe CD3 and the heme, making the distance between Phe CD3 and the heme >6 Å. Thus, for NOEs to occur between the resonances of Leu E7 and Phe CD3 the conformational fluctuations of the former must allow the methyl groups to reach a considerable distance above the heme plane.

The conformational substates of Leu E7 consistent with these observations are shown in fig.4. Note that one of these (fig.4B) is consistent with the orientation shown in the X-ray structure [5]. Although rapid interconversion between just two

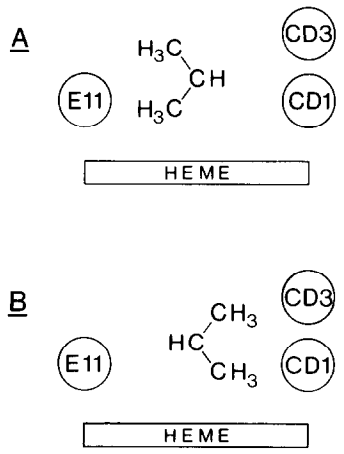

Fig.4. Two orientations of leucine E7 consistent with the NMR results. The δCH_3 and γCH groups are shown and the view is along the C_β–C_γ bond towards C_β. The approximate positions of Val E11, Phe CD1, Phe CD3 and the heme are indicated. Conformations A and B are related by a 180° rotation about the C_β–C_γ axis.

conformational substates is sufficient to explain our present NMR data at a qualitative level, we cannot exclude averaging over even more conformations. Interconversion between the two conformations shown in fig.4 occurs by rapid flipping about the leucine C_β–C_γ bond. The NMR spectra show that the ring current shifts experienced by the δ^1CH_3 and δ^2CH_3 proton resonances are averaged over the conformations of fig.4. Since only a single resonance is observed for each methyl group we can thus place a lower limit on the flip rate of $\sim 10^4$ s^{-1}. Since the ring current shifts experienced by the δ^1CH_3 and δ^2CH_3 resonances are comparable it is likely that the populations of the two conformational substates are approximately equal. The conformational freedom of Leu E7 is not enjoyed by the side chain of Val E11. The NOEs observed from the valine γ^1CH_3 and γ^2CH_3 resonances respectively are quite distinct. Flips about the valine C_α–C_β bond may occur, but occupation of any alternative conformational states is only transient. The NMR spectra do provide evidence for rapid (greater than 10^4 s^{-1}) flips about the C_β–C_γ bond of Phe CD1, since its 2,6 protons are magnetically equivalent, as is also the case for the 3,5 protons [21]. It is thus evident that at least a part of the distal side of the heme pocket of *Glycera* hemoglobin is a dynamic region of the

protein structure, with conformational fluctuations occurring on a time scale shorter than 10^{-4} s.

4. CONCLUSIONS

The present NMR studies of *G. dibranchiata* monomeric hemoglobins provide several important results. Firstly, they establish that leucine is conserved at the critical E7 position in each of the major monomeric hemoglobin components. Thus, hydrogen bonding interactions with distal residues cannot be the explanation for the variations in oxygen dissociation rate. Secondly, the leucine E7 side chain is shown to occupy at least two conformational substates which interconvert rapidly on the NMR time scale. In the case of myoglobin and hemoglobin, diffusion of ligands into and out of the heme pockets appears to be governed by conformational fluctuations between open and closed pathways [27]. The side chain of histidine E7 is believed to function as a gate which must be opened to allow access of ligands to the heme via the classical pathway through the E helix [7,8]. The X-ray structure of *Glycera* hemoglobin shows that leucine E7 blocks the entrance to the heme pocket [5]. The rapid conformational fluctuations detected for this residue may thus play a fundamental role in governing diffusion of ligands to the heme.

ACKNOWLEDGEMENTS

This work was supported in part by the National Institute of Health (grant no.AM:34909) and by the Australian Research Grants Scheme (grant no.D2 8215284). R.M.C. is the recipient of a Commonwealth Postgraduate Research Award.

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