J. Mol. Biol. (1987) **195**, 453–455

Histidine Proton Resonances of Carbonmonoxyhaemoglobins A and Cowtown in Chloride-free Buffer

A re-examination of the C-2 histidine proton resonances of haemoglobins A and Cowtown (His HC3(146)$\beta \rightarrow$ Leu) in chloride-free Hepes buffer has shown that all the resonances present in haemoglobin A are present in haemoglobin Cowtown, so that the pK_a of His HC3(146)β cannot be determined by nuclear magnetic resonance in this buffer.

By noting the changes in the nuclear magnetic resonance (n.m.r.†) spectrum that accompany the titration of the histidine residues in haemoglobins, one can hope to locate the origins of the Bohr effect, provided that the appropriate n.m.r. resonances can be assigned correctly to the various histidine residues in the haemoglobin molecules. Recently there has been conflicting experimental evidence regarding the assignment of two of the n.m.r. resonances termed H and C in human HbCO. Resonance H was assigned by us (Perutz *et al.*, 1985*a*,*b*) to His HC3(146)β in HbCOA on the basis of its presence in the spectrum of normal HbA, and its apparent absence in the spectrum of HbCO Cowtown (His HC3(146)$\beta \rightarrow$ Leu). However, doubt was cast on the correctness of our assignment by Russu & Ho (1986), who found resonance H to be present equally in both HbCOA and HbCO Cowtown. Russu & Ho (1980) had assigned resonance C to His HC3(146)β on the basis of its absence in HbCO from which that histidine residue had been cleaved enzymically, while Perutz *et al.* (1985*a*) found resonance C to be present in both HbCOA and Cowtown, a finding now confirmed by Russu & Ho.

Since the HbCO Cowtown samples used by the two groups stemmed from the same individual and had both been isolated by one of us (D.T.-b.S.), the conflicting results called for an independent n.m.r. study, which was done as follows. D.T.-b.S. isolated HbCO Cowtown from HbCOA in Portland as described by Shih *et al.* (1984). HbCO Cowtown isolated from the CM-Sephadex column was denatured and digested with trypsin. The abnormal C-terminal peptide of the β-chain was isolated by high-pressure liquid chromatography and its amino acid composition determined. This confirmed the replacement of His 146β by Leu.

D.T.-b.S. then sent the samples of HbCOA and Cowtown to Professor Kurt Wüthrich and Dr Hans Senn in Zürich. Previously, M.F.P. had sent there a sample of HbCOA that he had prepared. In Zürich, their pH values were adjusted by addition of one-third of their volume of 0·6 M-Hepes, bringing the concentration of HbCO to ~0·6 M-haem: 500 MHz ^{1}H n.m.r. spectra were recorded on a Bruker AM 500 spectrometer, the same instrument as had been used by Perutz *et al.* (1985*a*,*b*). Figure 1 shows that all the resonances found in the spectrum of HbCOA were present in the spectrum of HbCO

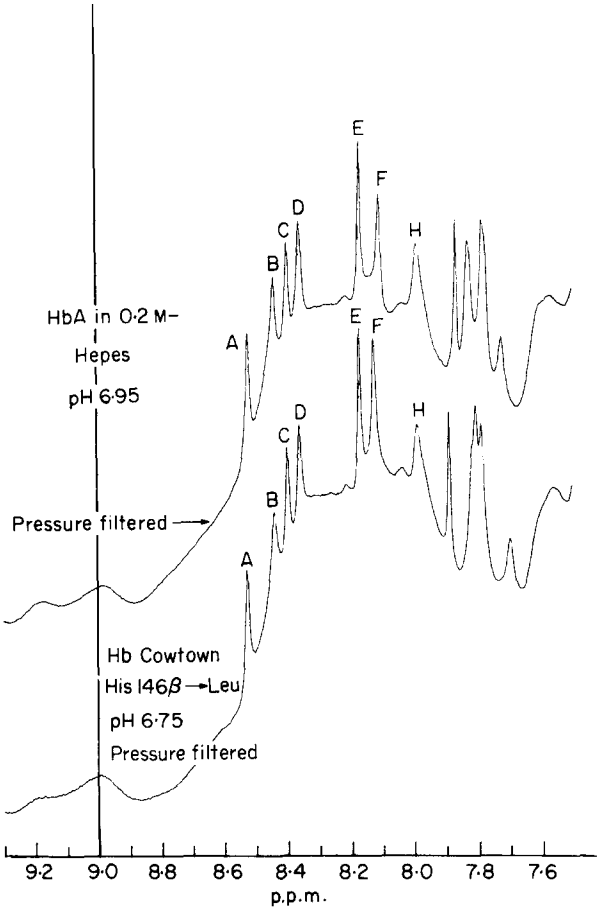

Figure 1. 500 MHz ^{1}H n.m.r. spectra of the histidine C-2 proton resonance region at 30 °C in ^2H_2O containing 0·2 M-Hepes of HbCOA and Cowtown at pH 6·75. The differences beyond resonance H are due to slightly different overlap of 4 closely spaced resonances. p.p.m., parts per million.

† Abbreviations used: n.m.r., nuclear magnetic resonance; Hb, haemoglobin; HbCO, carbonmonoxyhaemoglobin.

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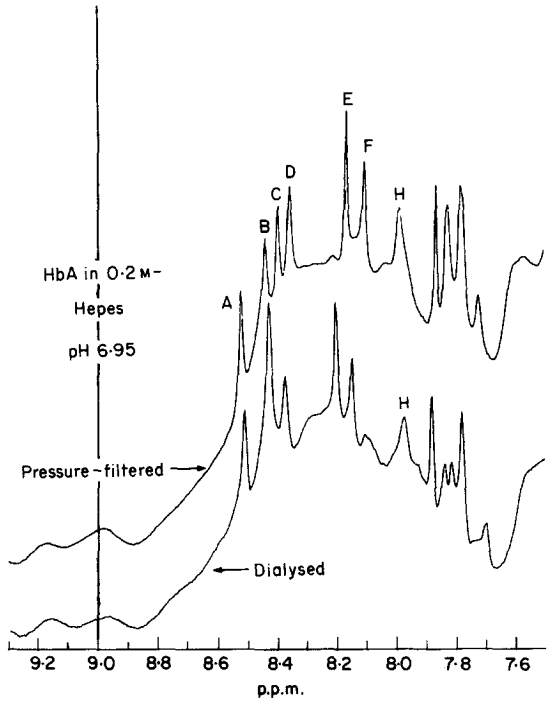

Figure 2. Comparison of 500 MHz ^1H n.m.r. spectra of the histidine C-2 proton resonance region at 30°C in ^2H_2O containing 0·2 M-Hepes of HbCOA equilibrated against ^2H_2O by pressure filtration and by dialysis in Visking tubing (pH 6·95).

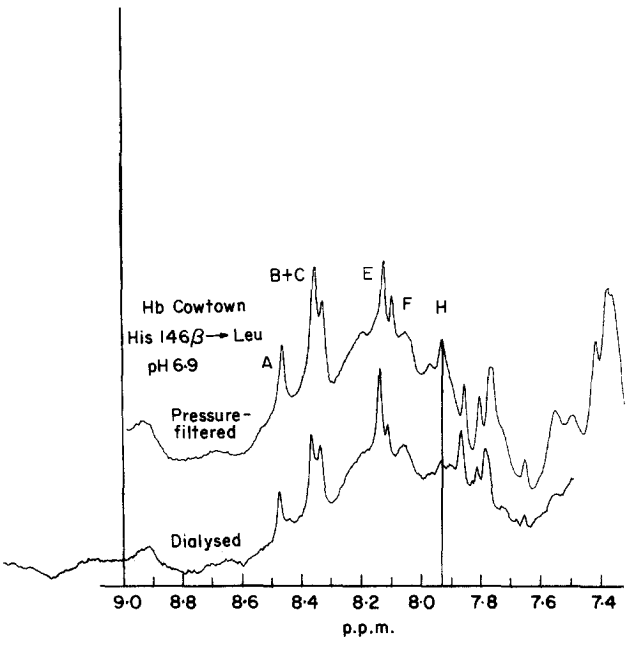

Figure 3. Same comparison as Fig. 2 for HbCO Cowtown. These spectra are not as sharp as those shown in Fig. 1, probably because the sample had by then been stored for some weeks.

Cowtown, including C and H, in agreement with the findings of Russu & Ho (1986), but contrary to those of Perutz *et al.* (1985*a*,*b*).

At this stage we noticed that the spectrum of HbCOA sent to Zürich from Cambridge was identical to that published by Perutz *et al.* (1985*a*,*b*), but different from that of the HbCOA sent to Zürich from Portland (Fig. 2). This suggested that the spectra of both HbCOA and Cowtown might have been affected by the method of preparation. Previous samples had been prepared in Portland and had then been equilibrated against ^2H_2O in Cambridge by dialysis in Visking tubing at 5°C and saturated with CO. Their absorption spectra showed peaks of equal intensity at 538 and 568 nm, characteristic for HbCO, and no detectable peak at 630 nm that would have indicated the presence of methaemoglobin. The samples sent direct from Portland to Zürich had been equilibrated against ^2H_2O by pressure filtration; dialysis was not used.

Since dialysis appeared to be the only difference between the two methods of preparation, we took portions of the samples of HbCOA and Cowtown that had been sent from Portland to Zürich and dialysed them in Visking tubing against ^2H_2O for four days at 5°C. We then concentrated them by pressure filtration in Centricon tubes, saturated them with CO and recorded their ^1H n.m.r. spectra, together with the spectra of the samples sent directly from Portland to Zürich. Figure 3 shows that dialysis made resonance H disappear. Dialysis in Visking tubing, boiled twice in 10 mM-EDTA and washed in distilled water, is a mild method widely used in protein chemistry. We do not understand why it should affect the n.m.r. spectra of haemoglobin.

Our results confirm that resonance H cannot be assigned to His HC3(146)β, but neither can resonance C, which had been assigned to that histidine by Russu *et al.* (1980). The assignment of resonance C to His FG4(97)β by Perutz *et al.* (1985*a*,*b*) was based on its absence in two abnormal HbCOs in which this histidine is replaced by glutamine and leucine, respectively, on the absence of a resonance with a similar pK_a in one of the corresponding abnormal deoxyhaemoglobins and on the position of His FG4(97)β capping the carboxyl terminus of helix F in both HbCO and deoxyHbA. None of this is invalidated by the present results. In HbCO from which His 146β has been cleaved enzymically, Perutz *et al.* (1985*a*) found a peak in the same position as resonance H, which they assigned to His (FG4(97)β on the basis of transfer of saturation experiments and which they called C_2. Our present results suggest that this is in fact resonance H.

Since no resonance can at present be assigned to His 146β, its pK_a in chloride-free solution cannot be measured by n.m.r. On the other hand, its contribution to the alkaline Bohr effect in such solutions has been determined by chemical methods, which showed that it is independent of chloride concentration (Van Beek *et al.*, 1979; Kilmartin *et al.*, 1980). To that evidence, Shih & Perutz (1987) have added an experiment which demonstrates that in the absence of chloride Hb

Cowtown exhibits no Bohr effect, even though its reaction with oxygen remains co-operative ($n_{50} = 2{\cdot}2$). This shows that all the chloride-independent part of the alkaline Bohr effect of HbA, about 40% in the absence of 2,3-diphosphoglycerate, must be due to His 146β (Shih & Perutz, 1987).

We wish to thank Professor Wüthrich and Dr Senn for recording the n.m.r. spectra of our samples. D.T.-b.S.'s work was supported by United States Public Health Service research grant HL20142. M.F.P.'s work was supported by the Medical Research Council, by National Institutes of Health grant no. HL31461-03 and National Science Foundation grant no. PCM-8312414. A.M.G. and G.M.C.'s work was supported by the Max-Planck Gesellschaft.

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Received 9 October 1986, and in revised form
15 December 1986

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Edited by G. A. Gilbert