J. Mol. Biol. (1985) **186**, 471–473

Comparison of Histidine Proton Magnetic Resonances of Human Carbonmonoxyhaemoglobin in Different Buffers

We have recorded the C-2 proton resonances of the histidines of carbonmonoxyhaemoglobin A and of four abnormal human HbCOs† in different buffers and at different concentrations of haemoglobin. Resonance H assigned by Perutz *et al.* (1985) to His HC3(146)β, is present at both pH 7·30 and pH 6·90, but somewhat broadened when recorded in 5 to 10% HbCO A in 0·1 M-bis-Tris. The broadening disappears on tenfold dilution of the Hb with bis-Tris and the resonance then stands out sharply. Resonance H is absent at both Hb concentrations in HbCO Cowtown (His HC3(146)$\beta \rightarrow$ Leu). HbCO Fort de France (His CD3(45)$\alpha \rightarrow$ Arg) in 0·1 M-bis-Tris of pH 6·90 has a spectrum similar to that of HbCO A. In the same buffer a resonance marked L by Russu *et al.* (1982) is absent from the spectrum of Hb Abbruzzo (His H21(143)$\beta \rightarrow$ Arg), whereas resonance H is present. Hb Barcelona contains an additional histidine in position FG1(94)β; in 0·1 M-bis-Tris buffer of pH 6·90 its resonance is not resolved and resonance H is either shifted or broadened. The resonances of both histidines are resolved in phosphate buffer. At pH 6·90, spectra in 0·1 M-bis-Tris buffer are similar to those previously recorded in 0·2 M-HEPES. Addition of 0·1 M-KCl produces marked changes. Replacement of bis-Tris by 0·2 M-KCl + 0·2 M-phosphate gives rise to a different and much better resolved spectrum.

In an endeavour to resolve a conflict in the interpretation of the alkaline Bohr effect of human haemoglobin, we have recently studied the C-2 protons of its histidines by nuclear magnetic resonance spectroscopy (Perutz *et al.*, 1985). We found that in carbonmonoxyhaemoglobin the resonance with a pK_a of 7·85 assigned by Russu *et al.* (1980) to His HC3(146)β belongs in fact to His FG4(97)β, and that the resonance that does belong to His HC3(146)β titrates with a pK_a of 6·2, consistent with a large contribution by this histidine to the alkaline Bohr effect. Russu *et al.* (1980, 1982) had used bis-Tris and Tris buffer as their solvent, and based their assignment upon comparisons of HbA† with Hb des-His (146β), while we tried to avoid any possible artefacts due to chloride binding by using HEPES buffer, and assigned the resonances by comparison of HbA with three abnormal Hbs: Cowtown (His HC3(146)-$\beta \rightarrow$ Leu), Malmø (His FG4(97)$\beta \rightarrow$ Gln) and Wood (His FG4(97)$\beta \rightarrow$ Leu).

I. M. Russu and C. Ho recently told us that they had been unable to reproduce our results. While we had found resonance H to be present in HbA and absent in Hb Cowtown (Fig. 1 of Perutz *et al.*, 1985), they had found it to be weakly present in both Hbs in bis-Tris buffer of pH 6·90 and pH 7·30. On the other hand, they had found resonance H to be absent in Hb Fort de France (His CD3(45)-$\alpha \rightarrow$ Arg). Furthermore, they had found resonance C, which we had assigned to His FG4(97)β, to be absent in Hb Abbruzzo (His H21(143)$\beta \rightarrow$ Arg) and Barcelona (Asp FG1(94)$\beta \rightarrow$ His). We found these reports so disturbing that we decided to reinvestigate the spectra of these haemoglobins in bis-Tris buffer. Our results confirm our earlier assignments.

Haemoglobins A, Cowtown, Abbruzzo, Barcelona and Fort de France were prepared as described by Perutz (1968), Shih *et al.* (1984), Tentori *et al.* (1972), Wajcman *et al.* (1982) and Braconnier *et al.* (1977). All the haemoglobins were equilibrated against unbuffered salt-free ^2H_2O by repeated pressure dialysis through Amicon filters.

^{1}H nuclear magnetic resonance spectra were recorded at 30°C and 500 MHz on a Bruker AM500 spectrometer. A total of 200 to 2000 transients were averaged for each spectrum using a 70° observation pulse, an acquisition time of 0·5 s and a relaxation delay of 1·5 s, and using quadrature detection. Before Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line-broadening of 2 Hz.

Figure 1 shows the histidine resonances of four HbCOs at pH 6·90 in 0·1 M-bis-Tris with [Hb] = 5 to 10%. In 0·2 M-HEPES at pH 6·90 resonance H was prominent in HbCO A and absent in HbCO Cowtown (Perutz *et al.*, 1985). In 0·1 M-bis-Tris resonance H is less sharp, and thus more difficult to detect, but distinct in both HbCO A and HbCO Abbruzzo, and its absence in HbCO Cowtown is marked by a trough. We wondered whether its broadening might be due to association of Hb molecules and therefore diluted the Hb solution tenfold with 0·1 M-bis-Tris. We then found resonance H to be strong in HbCO A and distinct though slightly shifted in HbCO Fort de France, and absent in HbCO Cowtown (Fig. 2). At pH 7·30 resonance H was distinct in HbCO A and absent in HbCO Cowtown, even at high Hb concentrations (Fig. 3). The histidine C-2 proton resonance region

† Abbreviations used: Hb, deoxyhaemoglobin; HbCO, carbonmonoxyhaemoglobin.

0022-2836/85/220471-03 $03.00/0

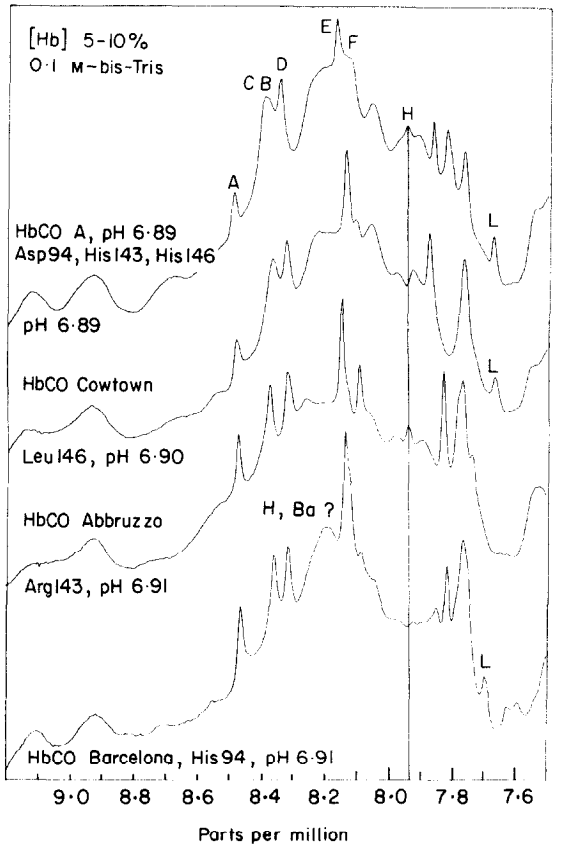

Figure 1. 500 MHz ^{1}H nuclear magnetic resonance (n.m.r.) spectra of the histidine C-2 proton resonances of HbCO A, HbCO Cowtown (His HC3(146)$\beta \rightarrow$ Leu), HbCO Abbruzzo (His H21(143)$\beta \rightarrow$ Arg), and Hb Barcelona (Asp FG1(94)$\beta \rightarrow$ His). The labelling of the C-2 proton resonances is the same as that of Russu *et al.* (1980). Resonance H is absent in Hb Cowtown and shifted, possibly to the position marked H,Ba? in Hb Barcelona. Resonance L is absent in Hb Abbruzzo. Experimental conditions: 5 to 10% HbCO in ^2H_2O containing 0·1 M-bis-Tris of pH 6·90±0·01 at 30°C.

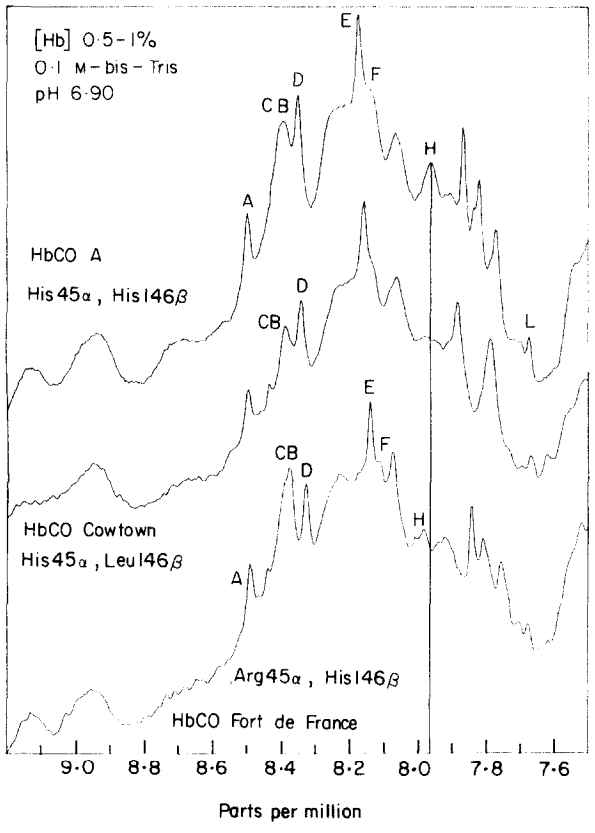

Figure 2. 500 MHz ^{1}H n.m.r. spectra of HbCO A, HbCO Cowtown and HbCO Fort de France (His CD3(45)$\alpha \rightarrow$ Arg). Conditions as for Fig. 1 except that the HbCOs were diluted 10 times to 0·5 to 1%.

of the spectrum of HbCO Fort de France is very similar to that of HbCO A. Our spectra, contrary to those of Russu *et al.* (1980, 1982) show resonance C to be present in all four Hbs, consistent with our assignment of this resonance to HisFG4(97)β. In agreement with them, we find resonance L to be absent in Hb Abbruzzo. Hb Barcelona contains all the same histidines as HbA plus an additional one, yet in bis-Tris buffer at pH 6·90 resonance H is missing and no resonance clearly due to the additional histidine or the shifted resonance H is visible. They may contribute either to peak Ba, which lies in the same position as peak H plus an additional peak visible in the spectrum of Hb Barcelona in 0·2 M-KCl+0·2 M-phosphate of pH 6·90 (not shown), or be buried as broad resonances in the region between 8·1 parts per million and 8·3 parts per million. These observations suggest that the conformation of the C terminus is altered in Hb Barcelona.

Our results show that in bis-Tris at pH 6·90 and [Hb] = 5 to 10%, resonance H, assigned by Perutz *et al.* (1985) to His HC3(146)β, is broad and small, but becomes prominent on tenfold dilution of the haemoglobin. In Hb Cowtown, where that histidine is replaced by leucine, it is clearly absent at both HbCO concentrations. At pH 7·30 it is also present

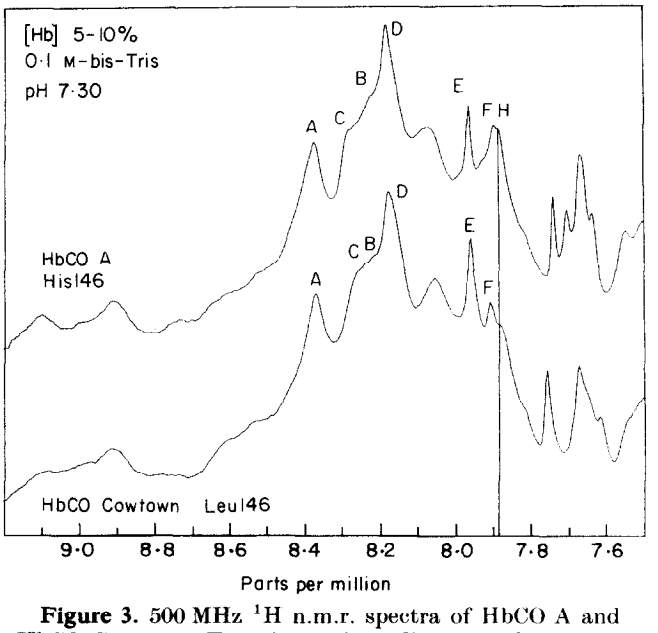

Figure 3. 500 MHz ^{1}H n.m.r. spectra of HbCO A and HbCO Cowtown. Experimental conditions as for Fig. 1. except that the pH was raised to 7·30.

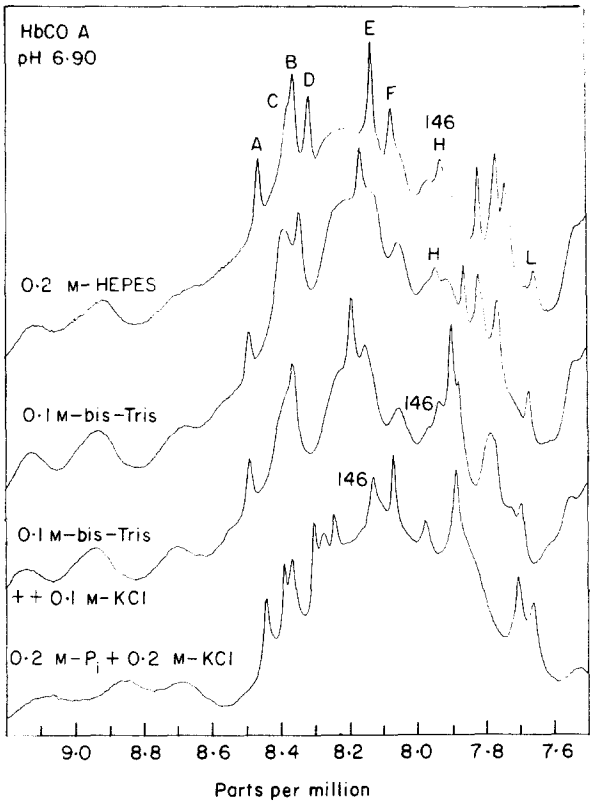

Figure 4. Comparison of 500 MHz ^{1}H n.m.r. spectra of HbCO A at pH 6·90 in 4 different buffers. [Hb] = 5 to 10%.

in HbCO A and absent in HbCO Cowtown. These results confirm our earlier assignment of resonance H to His HC3(146)β. Judged by its position, resonance L, absent in Hb Abbruzzo, should titrate with a low pK_a, consistent with the location of His H21(143)β between Lys EF6(82)β and Lys HC1(144)β, and with its contribution to the acid Bohr effect (Perutz *et al.*, 1980). Titration by deuterium exchange in 0·1 M-bis-Tris + 0·1 M-KCl showed it to have pK_a values of 6·0 in HbCO and 5·2 in deoxy Hb (Matsukawa *et al.*, 1984). The spectrum of HbCO Fort de France was very similar to that of HbCO A under all conditions. His CD3(45)α, which is replaced by Arg in Hb Fort de France, donates a hydrogen bond to one of the haem propionates and may be so rigidly clamped that its resonance is broadened beyond detection. Comparison of the histidine spectra in different buffers shows them to be strongly affected by chloride and phosphate (Fig. 4). The pK_a of His HC3(146)β shifts from 6·2 in chloride-free HEPES to 7·1 in 0·2 M-NaCl + 0·2 M-phosphate (Perutz *et al.*, 1985; Kilmartin *et al.*, 1974). The large spectral changes suggest that the pK_a values and chemical shifts of other histidines may also undergo substantial changes. Note the improved resolution of the histidine spectra in 0·2 M-NaCl + 0·2 M-phosphate. These changes may be due in part to electrostatic screening at high ionic strengths and in part to specific binding of chloride and phosphate ions.

This work was supported by the Medical Research Council, by INSERM, by National Institutes of Health grant no. HL31461-02 and National Science Foundation grant no. PCM-8312414 to M.F.P. and by the Max-Planck Gesellschaft (G.M.C. and A.M.G.). We thank Janice H. Fogg for equilibrating the Hbs against ^2H_2O and Professor K. Wüthrich for helpful discussion.

Max F. Perutz
Medical Research Council Laboratory of Molecular Biology
Cambridge CB2 2QH, England

Angela M. Gronenborn
G. Marius Clore
Max-Planck-Institut für Biochemie
D-8033 Martinsried, West Germany

Daniel T.-b. Shih
Department of Biochemistry, School of Medicine
Oregon Health Sciences University
Portland, OR 97201, U.S.A.

Constantin T. Craescu
INSERM U.91, Hopital Henri Mondor
94010 Creteil, France

Received 15 July 1985

References

Braconnier, F., Gacon, G., Thillet, J., Wajcman, H., Soria, J., Maigret, P., Labie, D. & Rosa, J. (1977). *Biochem. Biophys. Acta*, **493**, 228–233.

Kilmartin, J. V., Breen, J. J., Roberts, J. C. K. & Ho, C. (1974). *Proc. Nat. Acad. Sci., U.S.A.* **70**, 1246–1249.

Matsukawa, S., Itatani, Y., Mawatari, K., Shimokawa, Y. & Yoneyama, Y. (1984). *J. Biol. Chem.* **259**, 11479–11486.

Perutz, M. F. (1968). *J. Cryst. Growth*, **183**, 54–56.

Perutz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J. G. & Rollema, H. S. (1980). *J. Mol. Biol.* **138**, 649–670.

Perutz, M. F., Gronenborn, A. M., Clore, G. M., Fogg, J. H. & Shih, Daniel T.-b. (1985). *J. Mol. Biol.* **183**, 491–498.

Russu, I. M., Ho, N. T. & Ho, C. (1980). *Biochemistry*, **19**, 1043–1052.

Russu, I. M., Ho, N. T. & Ho, C. (1982). *Biochemistry*, **21**, 5031–5043.

Shih, T.-b., Jones, R. T., Bonaventura, J., Bonaventura, C. & Schneider, R. G. (1984). *J. Biol. Chem.* **259**, 967–974.

Tentori, L., Carti, A., Sorcini, M. & Bucella, C. (1972). *Clin. Chim. Acta*, **38**, 258–262.

Wajcman, H., Aguilar, J. L., Bascompte, I., Labie, D., Poyart, C. & Bohn, B. (1982). *J. Mol. Biol.* **156**, 186–202.

Edited by G. A. Gilbert

Note added in proof. One of us recently compared the C-2 histidine resonances of deoxy Hbs A and Malmø (His FG4(97)β → Gln) and found resonance 1 in Figure 1 of Russu *et al.* (1982) to belong to His FG4(97)β. According to their titration curve, this histidine has a pK_a of 8·07, close to its pK_a of 7·85 found by us in HbCO, which was to be expected since it caps the C terminus of helix H in deoxy Hb exactly as it does in HbCO (C. T. Craescu, J. Mispelter, C. Schaeffer & Y. Beuzard, unpublished results). This result corroborates our assignment of resonance C in HbCO to His FG4(97)β.