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)β→Leu). HbCO Fort de France (His CD3(45)β→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)β→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 pKₐ 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 pKₐ 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 HbA1 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)β→Leu), Malmo (His FG4(97)β→Gln) and Wood (His FG4(97)β→Arg).

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 II 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.
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.
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 pKₐ, 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 pKₐ 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 3(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 pKₐ 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 pKₐ 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.

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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

Reference


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Note added in proof. One of us recently compared the C-2 histidine resonances of deoxy Hbs A and Malmo (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 pKₐ of 8.07, close to its pKₐ 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.