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THE INTERACTION OF SOME *BIS*-ARYLHYDROXYSULPHONIC ACIDS WITH A SITE OF KNOWN STRUCTURE IN HUMAN HAEMOGLOBIN

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1 Two *bis*-arylhydroxysulphonic acids were previously designed to interact with the known molecular configuration of the 2,3-diphosphoglycerate (DPG) receptor-site of human haemoglobin. These compounds liberate oxygen from the haemoglobin similarly to DPG.

2 Solutions of haemoglobin have now been observed under physiological conditions by nuclear magnetic resonance (n.m.r.) in the presence of DPG and of the compounds.

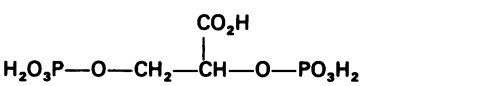
3 Two peaks in the n.m.r. spectrum of haemoglobin are shifted when DPG is added to the solution.

4 The same two peaks in the spectrum are affected by the compounds.

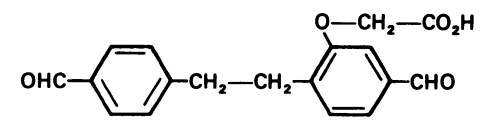
5 The observations are compatible with the predicted interaction between the compounds and the haemoglobin receptor site.

Introduction

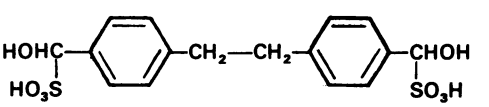
There is a site of known molecular structure in the human haemoglobin molecule, at which 2,3-diphosphoglycerate (DPG) interacts and promotes the liberation of oxygen (Arnone, 1972). This site (Figure 1) has been used as a model pharmacological receptor by Beddell, Goodford, Norrington, Wilkinson & Wootton (1976), who designed and synthesized three novel compounds which should also interact with it:



(DPG)



(I)



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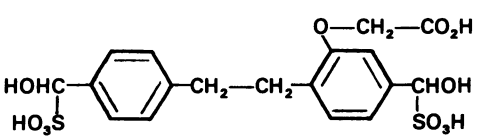

(III)

They predicted that these compounds, like DPG itself, should promote the liberation of oxygen from haemoglobin, and this was found to be the case. To this extent at least their approach was therefore successful but no direct evidence confirmed that the compounds were in fact reacting with the DPG site as intended.

The present paper describes some observations by nuclear magnetic resonance (n.m.r.) on the interaction of two of the compounds (the *bis*-arylhydroxysulphonic acids II and III) with human haemoglobin under physiological conditions similar to those used by the original authors.

Methods

Preparation of solutions

Human haemoglobin was prepared from freshly drawn blood as described by Brown, Halsey & Richards (1976) except that 5 mmol dm^{-3} sodium metabisulphite was added to the final solution as described by Beddell *et al.* (1976). The haemoglobin was deoxygenated in a IL 237 Tonometer which allowed equilibration with oxygen-free nitrogen. pH was measured *in situ*, and was adjusted by admixture with high pH or low pH solutions of haemoglobin. Temperature was controlled by a water jacket thermostatted to 37°C. A permanent over pressure of

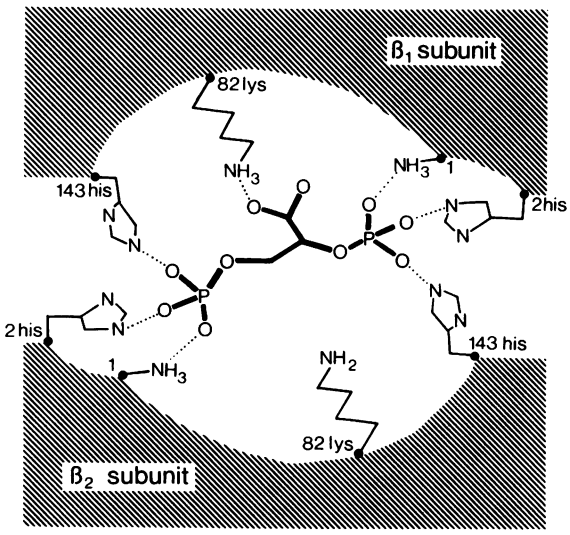

Figure 1 A schematic representation of the 2,3-diphosphoglycerate (DPG) receptor site in the human haemoglobin molecule, with DPG bound as observed by Arnone (1972).

nitrogen minimized contamination of the gas phase from the atmosphere.

An exit tube acted as gas outlet and an inlet tube could be connected either to a haemoglobin reservoir for pH adjustment, or to a 3-way tap whose second port held a 1 ml syringe for injection of solutions of the compounds. These were always dissolved in the same phosphate buffer as that used for the haemoglobin preparation. The third port of the tap held a large syringe of nitrogen gas which was used to force the solutions down the tube and into the equilibration chamber. Samples could be withdrawn from the equilibration chamber by pushing the mouth of the exit tube below the surface of the liquid which was then forced through the exit tube by the nitrogen pressure and into an n.m.r. sample tube which had been thoroughly flushed with nitrogen and could then be sealed.

Nuclear magnetic resonance

The samples were studied on a Brucker 270 MHz Fourier Transform n.m.r. Spectrometer to obtain their proton spectra. The data were accumulated by the spin echo method of Campbell, Dobson, Williams & Wright (1975) when a 90°-τ-180°-τ sequence of pulses and delays were applied. This procedure allows for the collection of an echo signal after the second delay, τ. No information appears in the echo from peaks whose relaxation times are short enough for their signal to have decayed within the time 2τ. It was found that if $\tau = 25$ ms, the only peaks remaining in the aromatic region of the spectrum were those from the C2 and C4 protons of surface histidine residues. All the spectra presented here are of this type, and peaks are numbered with Roman numerals according to the nomenclature of Brown *et al.* (1976).

The change in the n.m.r. signal which occurs when an added compound interacts with a resonant atom at a binding site is determined both by the change in chemical shift which occurs on binding, $\Delta\omega$, and by the lifetime t in the bound state. When $t.\Delta\omega \ll 1$, then the compound does not remain bound for long enough for the nuclei at the binding site to distinguish a clearly defined environment from either the bound or the unbound state. Instead, a weighted average is observed dependent on the relative proportions present in each state. This is the fast exchange case and is characterized by a single sharp signal whose position will move between the two extremes. As $t.\Delta\omega$ increases, so the line width increases reaching a maximum at $t.\Delta\omega \simeq 1$. Above this the bound and unbound states begin to become discernible as separate environments and the broad line separates into two lines. In the limit of slow exchange $t.\Delta\omega \gg 1$ the bound and unbound states both behave as completely separate entities.

The value of $\Delta\omega$ can vary greatly with the nature of the interaction. In the case of DPG the perturbation of the histidine ring protonation by the proximity of a negatively charged group (phosphate) is being observed. The effect of the perturbation is to stabilize the positively charged protonated form of the interacting ring in relation to the neutral form. The ring systems in these two forms differ considerably in the magnitude of the field induced in them by the applied magnetic field and hence in the chemical shift of the protons joined to the ring (i.e., the C2 and C4 protons). As a result such protons can be used to monitor the state of the rings whose nitrogen atoms will titrate exhibiting characteristic pKs which one would expect to increase in the presence of an interacting DPG molecule. Such an interpretation assumes that both the DPG and the protonating proton are in fast exchange with the histidine, although this situation could alter at lower pH values when the charges on the histidines and on the DPG change.

Results

Figure 2a shows the aromatic region of the nuclear magnetic resonance spectrum from a 2 mmol dm^{-3} aqueous solution of human deoxyhaemoglobin at pH 7.7. The spectrum is well resolved under our experimental conditions, and a number of sharp resonances from the protein can be identified and numbered. At this particular pH, however, peaks II and IV overlap, and so do peaks VIII and IX, and it

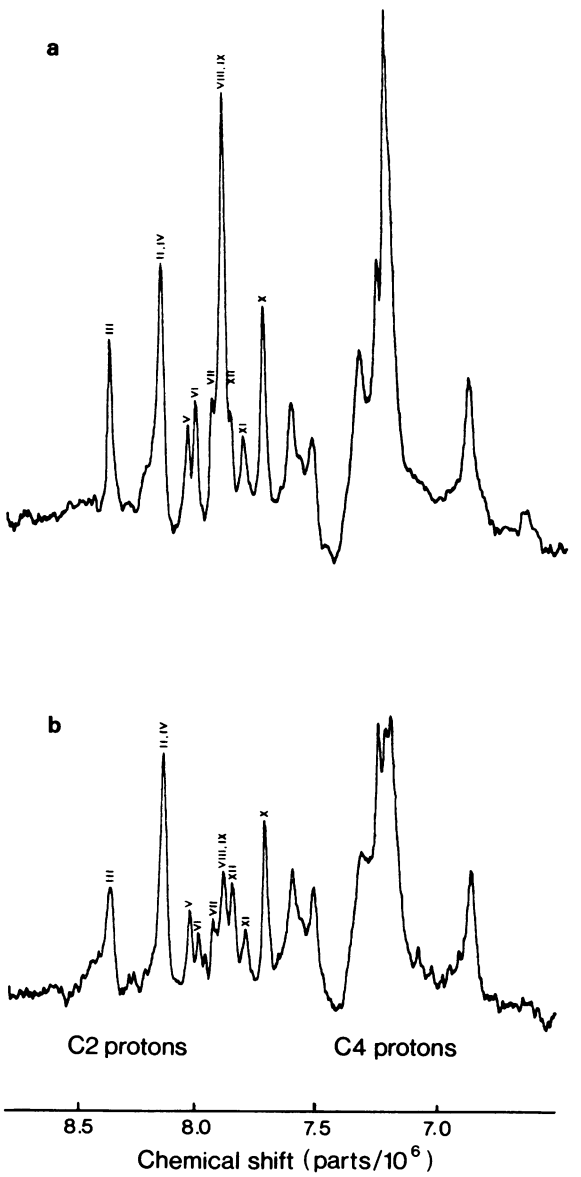

Figure 2 (a) The aromatic region of the n.m.r. spectrum from 2 mmol dm^{-3} human deoxyhaemoglobin at pH 7.7. All chemical shifts are measured relative to sodium 2,2-dimethyl-2-silapentane-5-sulphonate. (b) The same deoxyhaemoglobin sample in the presence of 1 mmol dm^{-3} compound III. This compound has aromatic peaks in the region 7–7.6 parts/10^6 and would not interfere with the C2 spectrum. Note the collapse of peaks VIII and IX in the presence of the compound.

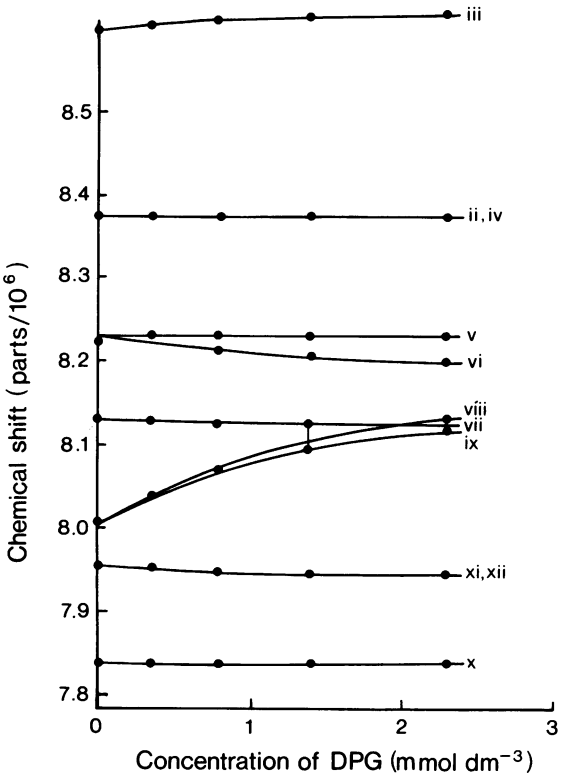

Figure 3 The chemical shifts of the C2 protons of histidine residues in 2 mmol dm^{-3} human deoxyhaemoglobin at pH 7.3 plotted as a function of added 2,3-diphosphoglycerate (DPG) concentration. Correction has been made, from pH titration curves, for the small changes in chemical shift due to small fluctuations in pH in different samples. Peak numbering corresponds with the Roman numerals in Figure 2 (see text). Note the shift of peaks VIII and IX in the presence of DPG.

would be necessary to make observations at different pHs in order to resolve these resonances.

Control observations on 2,3-diphosphoglycerate

When increasing concentrations of DPG are added to the solution the twin peaks VIII and IX are progressively shifted to a much greater extent than any other resonances. This is shown in Figure 3 where the position of each peak in the n.m.r. spectrum is plotted as a function of DPG concentration. Peaks VIII and IX move downfield corresponding to an increase in pK which is in line with the above expectations.

These DPG observations were made at a physiological pH of 7.3, but below pH 7.0 the

situation changes and the peaks broaden in acid solutions. The effects have already been studied in some detail (Brown & Campbell, personal communication), and pH titration curves have been determined. The relevant point in the present context is that DPG is always observed to affect peaks VIII and IX most strongly (Figure 3). On the basis of the X-ray crystallographic evidence (Arnone, 1972) for the DPG-haemoglobin interaction, these two peaks are therefore tentatively assigned to the C2 protons of histidines $\beta 2$ and $\beta 143$. A similar effect is observed for the C4 protons (not shown here).

Observations on the compounds

Examination of the effects of compounds II and III revealed a collapse of the same two resonances at all pH values observed. As a result the peaks apparently vanish from the n.m.r. spectrum. Figure 2b illustrates this effect for compound III which is the more effective of the two. It is an unfortunate coincidence that peaks VIII and IX cannot be distinguished because they super-impose in this physiological range of pH (Figure 2).

These results strongly suggest that both DPG and compounds II and III interact at the same binding site, even though the assignment to histidines $\beta 2$ and $\beta 143$ may not be established unequivocally. For the interaction of DPG it appears that the exchange is fast ($t.\Delta\omega \ll 1$), and the shift is of the order of 40 Hz corresponding to a pK increase of a little over 0.5 for peaks VIII and IX in the bound state. In the case of compounds II and III the exchange is slower ($t.\Delta\omega \gg 1$) and the collapse of peaks VIII and IX in the unbound haemoglobin is not matched by the emergence of a signal from the bound state, so that an estimate for $\Delta\omega_{cpd}$ cannot be made.

Apart from the main effects observed for peaks VIII and IX there are a number of minor effects. It will be observed in Figure 3 that DPG causes a slight downfield shift of peak III which is known to be histidine $\beta 146$ (Kilmartin, Breen, Roberts & Ho, 1973), and a slight upfield shift of peak VI. It is quite possible that peak VI could be histidine $\beta 97$ since histidines $\beta 2$, $\beta 97$, $\beta 143$ and $\beta 146$ all lie quite near each other. Compounds II and III are also observed (Figure 2b) to affect these resonances. Peak III is broadened and peak VI collapses, and although the effect is very much less marked than for peaks VIII and IX, this observation again supports the prediction of Beddell *et al.* (1976) that compounds II and III should interact at the DPG site. However, there is one difference observed between the compounds and DPG and that is an upfield shift of peak IV. This secondary difference from DPG may be due to more subtle conformational changes induced, perhaps, by covalent binding of the compounds.

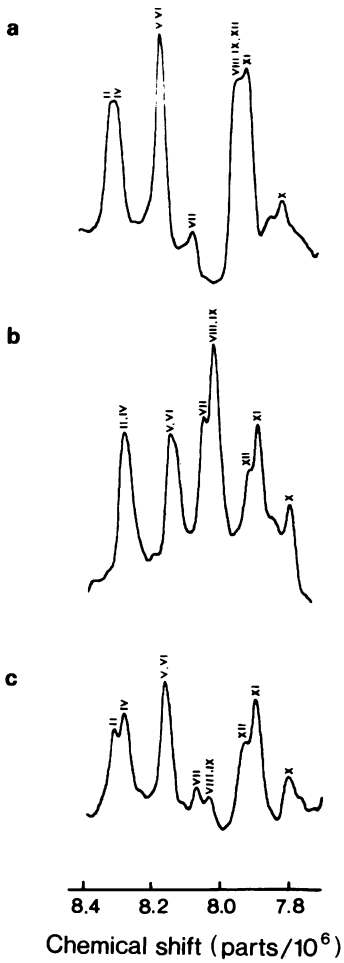

Figure 4 (a) The histidine C2 proton resonances from 2 mmol dm^{-3} deoxyhaemoglobin at pH 7.4. (b) The same sample in the presence of 1 mmol dm^{-3} 2,3-diphosphoglycerate (DPG) which shifts peaks VIII and IX away from peaks XII and XI, and onto the side of peak VII. (c) The same sample to which a further 1.5 mmol dm^{-3} compound III has been added. The shifted peaks VIII and IX collapse as would be expected if both DPG and compound III had a common binding site.

Observations on 2,3-diphosphoglycerate and the compounds together

Although the previous observations are strongly indicative of a common binding site for DPG and the bibenzyl compounds, they are still open to the objection that different but coincidentally superimposed resonances might have been affected by the different agents. To deal with this criticism DPG

was first used to shift the double peak (VIII + IX) into another position before it was collapsed by one of the compounds. Figure 4a is the control spectrum and Figure 4b shows a downfield shift of the double peak (VIII + IX) in the presence of DPG, exposing peak XII and covering peak VII. On the further addition of compound III shown in Figure 4c, the collapse of this double peak in its new position is observed. Because of the large difference in rates of exchange for the two compounds, one does not observe any change in the shift caused by DPG. Instead one still observes the fast exchange of DPG with the few remaining binding sites which are not blocked by compound III. Thus the shifted resonances simply collapse, which would be expected under these conditions if both DPG and compound III have a common binding site. Furthermore the residual peaks XI and XII are unaffected by the addition of compound III to the mixture of haemoglobin and DPG, showing that the effect on the region of the spectrum between 7.8 and 8.1 parts/10^6 is confined exclusively to the resonances which are influenced by DPG itself. Similar observations were also made with compound II.

Discussion

The results illustrated here appear to be entirely consistent with the mode of binding already postulated by Beddell *et al.* (1976) for compounds II and III. The primary effect of these compounds on the n.m.r. spectrum is to collapse the same two resonances which are shifted by DPG. Their long lifetimes in the bound state relative to DPG are exactly what would be anticipated from compounds such as these which would be expected to bind in a covalent manner at the predicted site. The effect of compound III is more pronounced than compound II in terms of its ability to collapse peaks VIII and IX, which suggests weaker binding by compound II and is in line with the relative abilities of the compounds to liberate oxygen from haemoglobin as described in the original paper.

The differences observed between compounds II and III and DPG are at present a matter of pure speculation. It would be surprising if no small differences between the two classes of compounds were observed since DPG is basically hydrogen-bonded to the site, whereas the compounds II and III may form covalent links to the terminal amino groups of the β-chains. However, the prime objective of this study was to identify the site of binding, and the present observations are compatible with the predictions of Beddell *et al.* (1976) for compounds II and III, although they do not preclude the presence of other binding sites elsewhere on the protein and throw no light on the binding of compound I. Subject to these limitations, however, they support the suggestion that it may be possible to design drugs to interact with receptor sites of known molecular structure and produce predefined biological effects.

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