CROSS CORRELATION OF TITRATING HISTIDINES IN OXY- AND DEOXYHAEMOGLOBIN; AN NMR STUDY

F. F. BROWN and I. D. CAMPBELL
Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, England

Received 15 April 1976

1. Introduction

Haemoglobin is the best known example of a protein exhibiting co-operative binding properties and continues to excite active interest [1–3]. One feature of the co-operative behaviour is the release of protons on binding oxygen, a phenomenon known as the Bohr effect. Perutz in his classic X-ray work [4] identified three groups which might be involved in the Bohr effect and Kilmartin et al. [5] in an n.m.r. study of CO and deoxy forms of haemoglobin confirmed that one of these groups was His β146. Val α1 has also been identified, by chemical modification studies [6], as another group involved but there is still uncertainty about the role of other groups, although these are expected to be histidines. N.m.r. seems to be the technique most likely to provide the detailed information about ionization states necessary to resolve this problem, but at present it is limited by difficulties in resolving and assigning the titrating histidine resonances.

The key factor in an n.m.r. study of the involvement of histidines in the Bohr effect lies in the correlation of peaks in oxy- and deoxyhaemoglobin. In this communication we illustrate a powerful method for correlating peaks whose movements in an oxy–deoxy titration are discontinuous and therefore impossible to follow by stepwise titration procedures. The method, which has been used previously to correlate resonances of reduced and oxidised cytochrome *c* [7] requires that the rate of exchange between liganded and unliganded forms be faster than the nuclear spin lattice relaxation rate ($1/T_1$). This requirement is shown to be met for at least one histidine resonance in haemoglobin when oxygen is the ligand.

2. Experimental

Samples of human haemoglobin were prepared in D_2O with 0.1 M NaCl, 0.021 M phosphate and sulphate as an ionic strength compensator. All samples of oxy, deoxy, and mixtures of the two were prepared using an IL 237 tonometer as described previously [8]. The n.m.r. measurements were made on a Bruker HX270. The pH measurements, which were made with a Corning EEL blood gas analyser, are uncorrected meter readings and are denoted pH*. All measurements were made at 37°C.

All spectra were obtained using the simplifying $90°$-τ-$180°$-τ spin echo pulse sequence described previously [9]. The solvent peak was saturated with a second frequency and a third frequency was used in the transfer of saturation experiments [10]. The second and third frequencies were applied at all times except during data acquisition. The total accumulation time was about 9 min for each spectrum; a typical example is shown in fig.1.

The relaxation times were measured using an additional $180°$ pulse before the simplifying $90°$-τ-$180°$-τ sequence.

3. Results and discussion

A simple spin echo pulse sequence is an effective way of separating the titratable proton resonances of histidine from other resonances in the same region

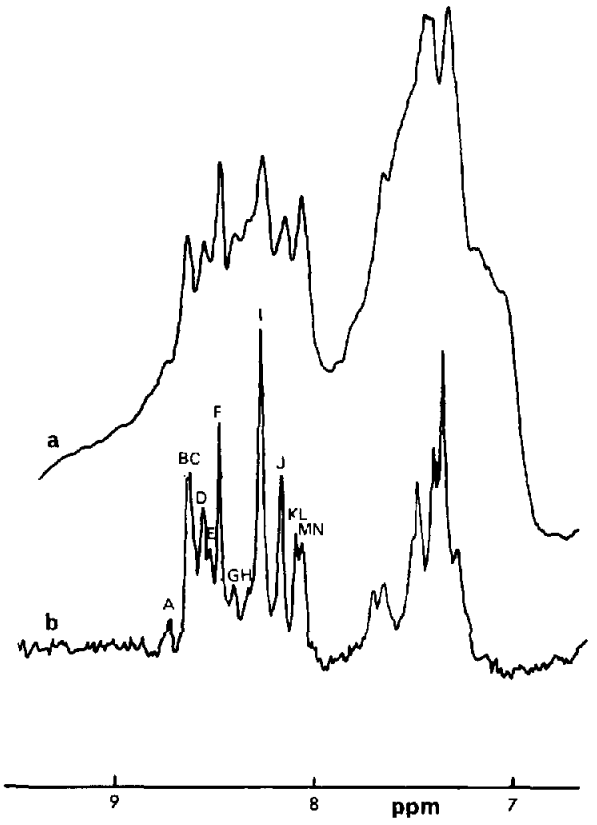

Fig.1. The 270 MHz Fourier transform n.m.r. spectra of the aromatic region of oxy human haemoglobin at 37°C, pH 6.4. (a) Normal spectrum, 512 scans with one 70° pulse per scan. (b) Spin echo spectrum [9], 512 scans with a 90°-τ-180°-τ. pulse sequence every scan, τ = 30 msec. The labelling scheme corresponds to that shown in the titration curves of fig.2.

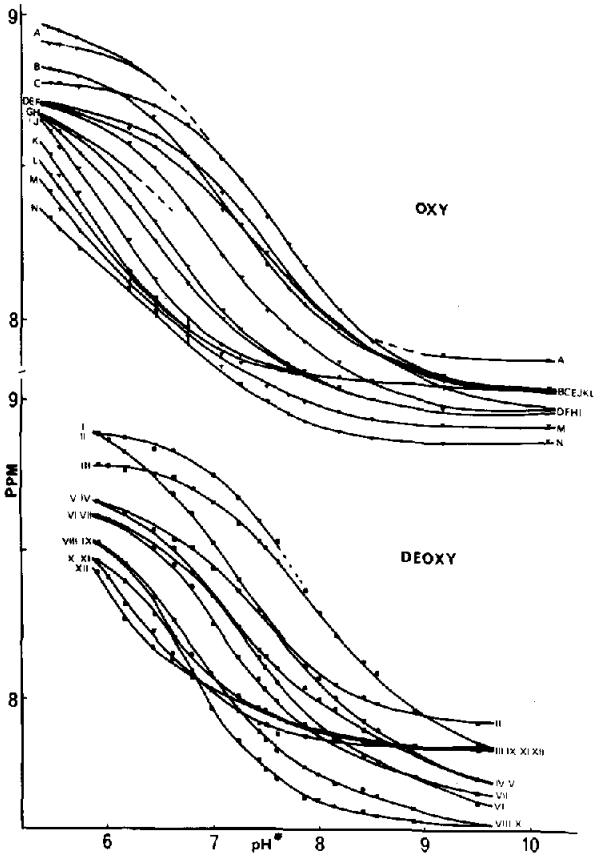

Fig.2. Titration curves of the C(2) proton resonances of some of the histidine of haemoglobin on (a) the oxy form and (b) the deoxy form. The chemical shifts are indicated as downfield from 2,2 dimethyl-2-silapentane-5-sulphonate.

of the spectrum since the histidine resonances have relatively long T_2 values [10] (see fig.1). From such spectra, pH titration curves for the C(2) histidine resonances, have been constructed (fig.2). These diagrams contain a greater number of titration curves than has been previously observed in studies of haemoglobin (compare [8 and 11]).

The overall contribution of histidine residues to the Bohr effect will be related to the shift in the average pK_a of all the titration curves in figs.2a and 2b. The average pK_a of the curves in fig.2a is 6.9 whereas in fig.2b it is 7.15. Inspection of the curves suggests that there are a considerable number which undergo a shift in pK_a. However, a detailed cross correlation between the oxy and deoxy curves must be made before these effects can be properly quantified since account must be taken of the fact that two fewer curves are characterisable in the deoxy state than in the oxy state. (Presumably two of the histidines are near enough to the iron to be broadened or shifted beyond detection in the paramagnetic deoxy state).

The most obvious method for correlating resonances in the two states is to examine in detail the changes in the n.m.r. spectra as the relative concentration of liganded to unliganded haemoglobin is changed. An example of this type of experiment is shown in fig.3., where the positions of the histidine resonances are

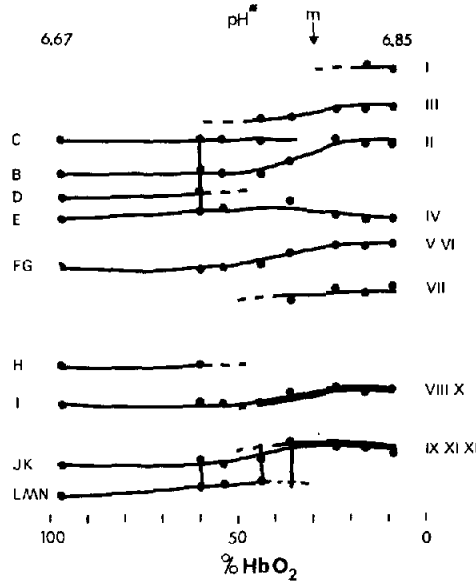

Fig.3. Plot of the chemical shift positions of the histidine, labelled as in fig.2, as a function of pO_2. The pH of each solution was measured after the spectrum was accumulated and the points were corrected to the oxyhaemoglobin value (6.67) using the curves of fig.2a or 2b depending on whether the points be on the oxy or the deoxy side of the pH median (indicated by the letter m).

plotted as a function of pO_2, with the pH corrected to the oxyhaemoglobin value. It is evident that while correlation is possible for some resonances, others are difficult to follow because of broadening or discontinuities in the resonance positions (see also fig.4). This behaviour can be explained by a simple chemical exchange model which can be described briefly as follows (see for example [12]).

When a nucleus (e.g. from a C(2) proton) can exist in two environments which are characterised by resonance positions X and Y then the observed behaviour depends on the rate of exchange, $k(S^{-1})$, between the environments and the chemical shift difference between X and Y, $\Delta\upsilon$ (Hz). (In general the exchange rate from X to Y is not the same as from Y to X, but for the purpose of this discussion k can be considered as the mean of the two rate constants). When $k > 2\pi\Delta\upsilon$, a weighted average is observed and the resonance titrates between the two extreme positions as the relative populations of X and Y change. An example of this is the exchange between the ionised and non-ionised forms of histidine in a pH titration. In cases where $k \sim 2\pi\Delta\upsilon$, the resonances X and Y broaden, with a maximum effect when the populations of the 'X' and 'Y' environments are equal. When $k < 2\pi\Delta\upsilon$, the resonances remain at X and Y, the intensity of each being proportional to the population in that environment. In this case it has been shown that magnetisation can be transferred from one site to another by selectively irradiating X

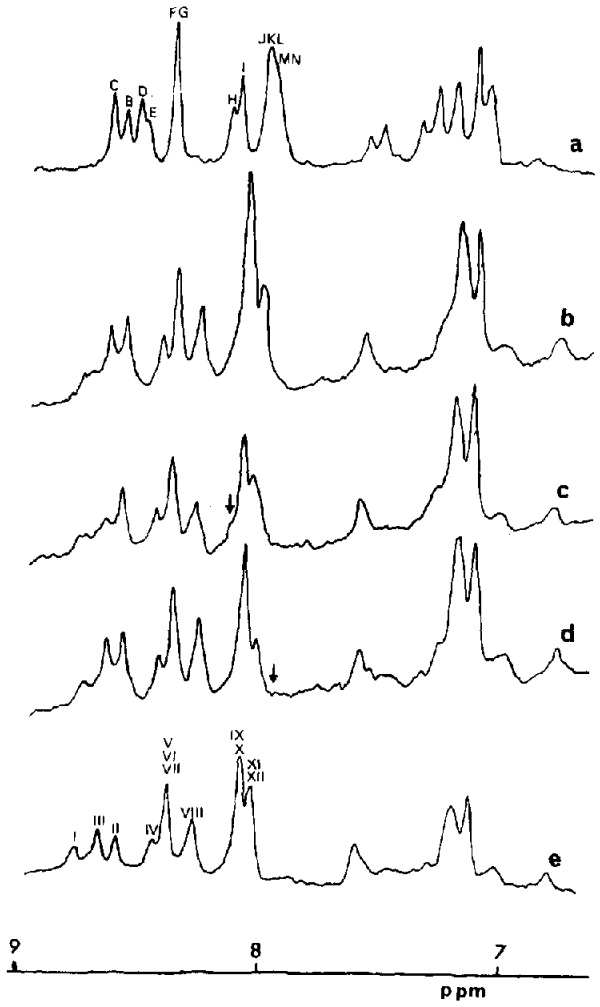

Fig.4. Illustration of a double resonance experiment correlating resonance H of oxyhaemoglobin with resonance III of the deoxy form spectrum of (a) oxy, pH 6.4, (b) a 4:1 mixture of deoxy to oxy, without irradiation; (c) as (b) with irradiation at the position of peak H in the oxy spectrum; (d) as (b) with irradiation at the position indicated by the arrow; (e) deoxy.

(or Y) provided that $k > 1/T_{1y}$ (or $1/T_{1x}$) [7,12] where T_{1y} is the spin lattice relaxation time of the Y site.

In mixtures of oxy- and deoxyhaemoglobin, each histidine changes its environment to some extent as the limited number of oxygen molecules exchange between the haemoglobin molecules causing quaternary structure changes. A rough estimate of the rate constant in this experiment may be obtained from measurements of the off-rate of oxygen from the fully ligated tetramer, 17 sec^{-1} at pH 7 [13]. This suggests that if the shift varies between a few Hz to a few hundred Hz then all the exchange limits defined above could be observed.

Returning to fig.3, therefore, it is clear that the chemical exchange model explains why some resonances are discontinuous while others 'titrate' between the extremes of oxygen concentration. This model also predicts the observed behaviour, namely, a critical dependence on temperature and pH since k and $\Delta\upsilon$ are sensitive to these parameters.

In the case when $k < 2\pi\Delta\upsilon$, cross correlation can be achieved using double resonance. In fig.4c it may be observed that irradiation at the position of the oxy peak causes substantial collapse of a peak in the mixture spectrum which corresponds to deoxy peak III. This transfer of saturation effect could be observed at concentrations of oxy between about 10% and 30% when there is, presumably, and adequate concentration of sites at the irradiation frequency and sufficient intensity in the deoxy spectrum to observe the collapse. On increasing the concentration of oxy to around 70% the experiment could then be performed in reverse with irradiation at the position of peak III causing partial collapse of peak H. These experiments are consistent with resonances III and H arising from one chemical group exchanging between two environments at a rate $k < 2\pi\Delta\upsilon$.

The work of Kilmartin et al. [5] shows that resonance III corresponds to the peak assigned to the C(2) resonance of His β146. The pK_a value observed in that study was 8.0 which compares well with the value of 7.9 obtained here (Kilmartin et al. made the measurements at 30°C). The oxy peak H has a pK_a of 6.8 which is somewhat lower than the value obtained by Kilmartin et al. for β146 in CO liganded haemoglobin. This could be due to small differences between the CO and oxy forms of haemoglobin.

It may be observed in fig.3 that resonances other than H and III are discontinuous. One other cross saturation pair has been observed and this will be discussed in a later publication when the resonances have been properly characterised and assigned.

4. Conclusions

The methods illustrated promises to be very useful for cross correlating resonances from the oxy and deoxy forms of haemoglobin. A complete cross correlation will give very detailed information about the origin of the Bohr effect and should assist in the task of assigning all the observed histidine resonances. The observed chemical exchange effects on ligand binding could also be interpreted in detail to give information about the cooperative binding process. There are distinct advantages in a physical technique which allows a study of an unmodified, functioning haemoglobin molecule.

Acknowledgements

This is a contribution from the Oxford Enzyme Group which is supported by the Science Research Council. F.F.B. is supported by the Medical Research Council. We also wish to thank R. Edwards and P. Bateman for their help in the supply and preparation of the haemoglobin samples, and Dr R. E. Richards and Dr M. Halsey for their advice and encouragement.

References

[1] Edelstein, S. J. (1975) Ann. Rev. Biochem. 45, 209–231.
[2] Baldwin, J. W. (1975) Progress in Biophys and Mol. Biol. 29, 225–320.
[3] Shulman, R. G., Hopfield, J. J. and Ogawa, S. (1975) Quart. Rev. in Biophys 8, 325–420.
[4] Perutz, M. F. (1970) Nature 228, 726–739.
[5] Kilmartin, J. V., Breen, J. J., Roberts, G. C. K. and Ho, C. (1975) Proc. Nat. Acad. Sci. USA 70, 1246–1249.
[6] Kilmartin, J. V. and Rossi-Bernardi, L. (1969) Nature 222, 1243–1246.
[7] Redfield, A. G. and Gupta, R. K. (1971) Cold Spring Harbor Symp. Quant. Biol. 36, 405.

[8] Brown, F. F., Halsey, M. and Richards, R. E. (1976) Proc. Roy. Soc. B. in the press.
[9] Campbell, I. D., Dobson, C. M., Williams, R. J. P. and Wright, P. E. (1975) FEBS Lett. 57, 96–99.
[10] Campbell, I. D., Dobson, C. M. Jeminet, G. and Williams, R. J. P. (1974) FEBS Lett. 49, 115–119.
[11] Greenfield, N. J. and Williams, M. N. (1972) Biochim. Biophys Acta. 257, 187–197.
[12] Hoffman, R. A. and Forsen, S. (1966) in: Progress in N.m.r. Spectroscopy (Emsley, J. W., Feeney, J. and Sutcliffe, L. H. eds.) p. 15. Pergamon Press, Oxford.
[13] Olsen, J. S., Anderson, M. E. and Gibson, Q. H. (1971) J. Biol. Chem. 246, 5919–5923.