^{1}H NMR STUDY OF THE BINDING OF 2,3-DIPHOSPHOGLYCERATE TO HUMAN OXYHEMOGLOBIN A IN INTACT RED CELLS

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1. Introduction

The ^{1}H NMR spectra of purified human hemoglobins have been widely studied in the region of paramagnetically shifted resonances [1–12]. In the aromatic region, 3 histidine C2 resonances have been assigned [13–14], some p*K*-values obtained [15–18] and relaxation times measured [14]. However, studies on intact red cells have been scarce [19–24,41]. Interest has been focused mainly on the binding of 2,3-diphosphoglycerate (DPG) to hemoglobin in the deoxy-state, while the binding to the oxy state has received much less attention. Conclusions regarding to the latter are contradictory, depending on the physical technique used in its study [25–36]. ^{31}P Studies on intact erythrocytes support the claim that 2,3-DPG binds to oxyhemoglobin [19].

Here we have studied 'in vivo' binding of 2,3-DPG to oxyhemoglobin by observing the changes in the histidine C2 resonances originated by an increase of the 2,3-DPG concentration inside the cell. The corresponding spectral region has been simplified by means of the spin-echo technique [37], which has proven to be useful in erythrocyte NMR studies [9,17,20–21,24].

2. Experimental

Blood from a local bank was washed twice with ^1H_2O Krebs/Ringer buffer [38] pre-gassed with an O_2/CO_2 mixture (19:1), and split into 15 ml samples (25% hemoglobin). An increase in the 2,3-DPG level was obtained by incubating at 37°C with 40 mM inosine, 50 mM P_i and 10 mM pyruvate [39]. A reference blood sample was incubated for 3 h without any addition. To avoid lysis of cells due to the growing of bacteria during the incubation and NMR measurements, the material was autoclave-sterilized, and buffer solutions filtered through millipore GS 0.22 μm filters. Blood samples were then washed again, once with ^1H_2O Krebs/Ringer buffer, and 5 times with ^2H_2O Krebs/Ringer buffer pre-gassed with the O_2/CO_2 mixture, so that substrates in excess were eliminated, ^1H_2O exchanged by ^2H_2O and hemoglobin kept in the oxy-state. [Hemoglobin] was ~80%. Aliquots were precipitated with trichloroacetic acid and subsequently analyzed for 2,3-DPG content by the enzymic method in [40]. Before the samples were transferred to NMR tubes, the pD was measured on a Radiometer pH meter. After immediate recording of the NMR spectra the pD was measured again, and no appreciable changes were observed.

Proton NMR spectra were recorded at 37°C, on a 300 MHz Bruker pulse spectrometer, equipped with quadrature detection, using the 90–τ–180–τ spin-echo pulse sequence, τ being equal to 10 ms. One hundred scans were sufficient to obtain adequate signal to noise ratio. Some broadening of the bands can occasionally occur, but this cannot be attributed to kinetic effects, because it disappears when the incubation is repeated again. Formation of met-hemoglobin or unexpected changes in the physical state of the blood sample, coagulation or sedimentation, are more probable reasons.

3. Results

The pD and the total [2,3-DPG] of the blood samples are shown in table 1, where it can be seen that the increase in 2,3-DPG level is approximately proportional to incubation time.

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Table 1
pD and [2,3-DPG] of blood samples obtained by different incubation times at 37°C with inosine (40 mM), inorganic phosphate (50 mM) and pyruvate (10 mM)

Sample no.	Time (min)	[2,3-DPG] mM (packed cells)	pD[a]
1[b]	180	1.2	7.44
2	30	6.8	7.62
3	60	9.5	7.57
4	120	13.3	7.50
5	180	17.4	7.44

[a] Uncorrected meter readings
[b] Blood sample used as a 'blank'; no substrates added

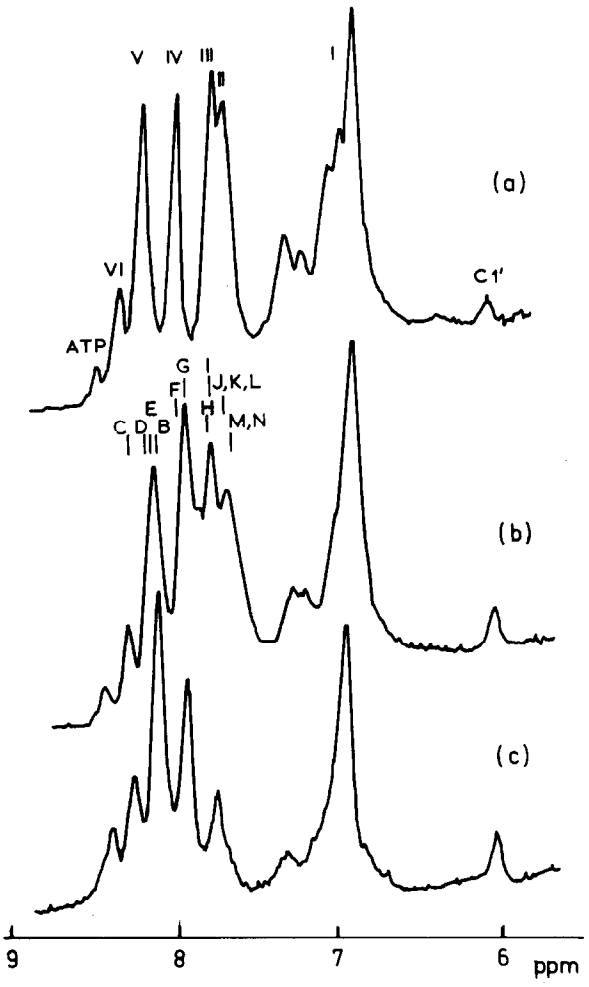

Fig.1. 300 MHz ^{1}H NMR spectra of intact oxygenated blood samples at 37°C, with different [2,3-DPG]: (a) 1.2 mM; pD 7.44; (b) 6.8 mM; pD 7.62; (c) 17.4 mM; pD 7.44. Peaks have been arbitrarily marked with roman numerals for convenience. Letters B–N mark the positions of C2 histidine protons in purified oxyhemoglobin [17].

Spin-echo ^{1}H NMR spectra of the blood samples studied are shown in fig.1 (aromatic region). The positions of the peaks observed in purified oxyhemoglobin [17] transformed to our pD (letters B–N) are also indicated in the figure, their labelling being maintained. Both spectra show a quite close correspondence, apart from the lower resolution found in intact erythrocyte spectra. Resonances from C1′ protons of sugar rings and C8 protons of purine rings (ATP,ADP) are visible on both sides of the spectra, their positions being independent of 2,3-DPG level.

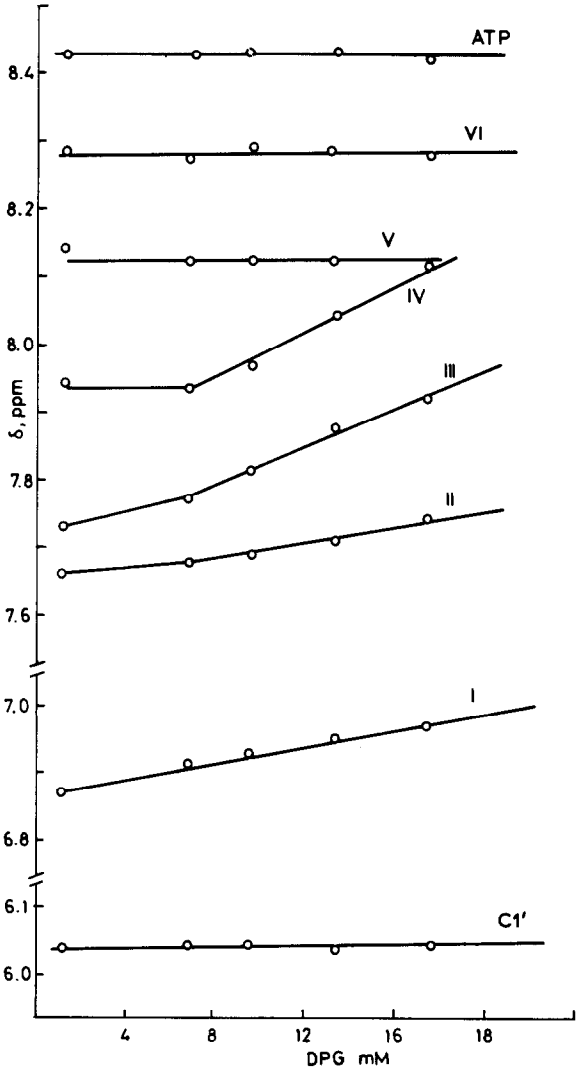

Fig.2. Chemical shifts of the bands observed in the ^{1}H NMR spectra of intact erythrocytes as a function of [2,3-DPG] (mM packed cells). The signal from HDO was used as internal reference and assigned a value of δ = 4.63 ppm (TSP) for purposes of comparison.

The most interesting change observed in the spectra is the downfield shifting of some bands as [2,3-DPG] increases (see fig.2). Bands III and IV move faster than band II, while bands V and VI do not move at all. Band I also moves downfield, but no information on bonded histidines can be obtained, due to the complexity of this band (resonances from histidine C4 protons and aromatic protons are included in it).

4. Discussion

Although there are slight pD differences among the samples (see table 1), they cannot be responsible for the large shifts observed, because, according to reported titration shifts [17], none of the peaks is in its maximum variation point, so that they should experience only slight downfield shifts. Changing the pD of the 'blank' blood sample from 7.6–7.4, caused +6, +4 and +5 Hz shifts in bands VI, V and IV, respectively, while bands III, II and I remained, within experimental error, at the same positions.

The presence of the deoxy-form could shift downfield the ^{1}H NMR lines of the oxy-form, through a rapid exchange between oxy- and deoxy-forms, the corresponding lines of the latter being at lower field. However, the oxygenated buffer maintains a high partial pressure of O_2, and even at a high [2,3-DPG] inside the cell, the fraction of deoxy-form must be very low and therefore, the downfield shifts negligible.

The possibility that phosphorylated metabolites of increasing concentration other than 2,3-DPG, could bind to the hemoglobin, must be considered. Only a moderate level of P_i is reached in the cells (~4 mM) [39], and this level seems not to affect the binding of 2,3-DPG to the hemoglobin. In fact, no shifts were observed in the 2,3-DPG ^{31}P NMR lines when P_i was added to model solutions [19], and the P_i line itself did not shift in intact erythrocytes when the state of the hemoglobin changed from oxy to deoxy. The small increase of the adenosine triphosphate (ATP) level originated by the incubation (~0.2 mM) [39], together with the small percentages of binding of oxyhemoglobin to either ATP or ATP–Mg^{2+} complex [41], exclude any significant contribution of this compound to the observed shifts. Even assuming that other minor metabolites, such as, adenosine diphosphate (ADP), inosine monophosphate (IMP), 2-phosphoglycerate (2-PG), nicotinamide dinucleotide (NADH), bind to oxyhemoglobin with binding constants of the same order of magnitude as the 2,3-DPG–oxy constant, only a small contribution to the shifts is expected, because of their low concentration (<1 mM).

From what we have shown above, it follows that the main contribution to the observed shifts must be the binding of 2,3-DPG to the oxyhemoglobin. Evidence of that binding by other techniques has been reported [25–27,30–34]. A rapid exchange between the oxy-form and the 2,3-DPG–oxy complex could explain the existence of downfield shifts as the 2,3-DPG level rises in the cell. When 2,3-DPG is 1–7 mM, only one band is clearly moving downfield, namely band III. When 2,3-DPG is 7–18 mM, in addition to band III, bands IV and II start to move downfield, but with different slopes (fig.2). This suggests that there is one preferential binding site in oxyhemoglobin, accessible at low 2,3-DPG levels. Other binding site(s) involving several histidines are also accessible at high levels of 2,3-DPG, in agreement with calorimetric studies [32].

As far as we know, peaks in the ^{1}H NMR spectrum of hemoglobin in the oxy-state, have not been assigned to individual histidines, so it is not possible to know where the binding sites are located within the protein. But in any case, it is clear that at least 2 histidines are involved in the binding (corresponding to bands III and IV), because they exhibit severe downfield shifts. At least another histidine (band II) is also affected by the binding but to a lesser extent. Assuming that $\beta 2$ and $\beta 143$ C2 histidine resonances in the oxy-state lie near to their positions in the deoxy-form [14] (band III), our ^{1}H NMR spectra support the fact that these 2 histidines are involved in binding to the preferential site.

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