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TRACER DIFFUSION COEFFICIENTS OF OXYHEMOGLOBIN A AND OXYHEMOGLOBIN S IN BLOOD CELLS AS DETERMINED BY PULSED FIELD GRADIENT NMR

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It is demonstrated that tracer diffusion coefficients can be determined for oxyhemoglobin A ($HbA\text{-}O_2$) and oxyhemoglobin S ($HbS\text{-}O_2$) in intact blood cells by means of pulsed field gradient NMR (PFG-NMR). This is possible because the method discriminates between both rapidly moving water molecules and molecules having small proton transverse relaxation times (T_2). The results indicate that only hemoglobin molecules contribute to the echo signals when large field gradients are used. The dependence of the measured diffusion coefficients on osmolarity and pH are attributed to changes in hemoglobin concentration resulting from changes in cell volume.

1. Introduction

Here we report a direct measurement of the translational diffusion coefficient of hemoglobin in intact blood cells [1]. This was possible through the use of a specially constructed pulsed field gradient NMR (PFG-NMR) system. The tracer diffusion coefficient which was determined in this experiment is known to have strong dependence on the hemoglobin concentration and the viscosity of the solvent mixture as well as on the degree of aggregation of the hemoglobin molecules. Thus, it is possible to obtain information concerning the internal state of the cell which was not previously available. This is particularly important in cases, e.g., sickle cell disease, where intermolecular interactions are crucial and where time-dependent processes must be monitored.

PFG-NMR is an established method for measuring diffusion coefficients and has been reviewed extensively in the literature [2–4]. The experiment as developed by Stejskal and Tanner [5] consists of measuring the amplitude of an NMR spin echo signal from nuclei in the molecule of interest in the presence and absence of a matched pair of magnetic field gradient pulses. The ratio of the amplitudes, denoted by R, is then related to the diffusion coefficient through the equation:

$$\ln R = -\gamma^2\delta^2G^2(\Delta - \delta/3)D \qquad (1)$$

where γ is the gyromagnetic ratio of the nuclei, G the amplitude of the field gradient pulses in G/cm, δ the duration of each pulse, Δ the separation of the starting points of the pulses, and D the tracer diffusion coefficient. In practice, the logarithm of the echo amplitudes rather than the ratio of amplitudes is plotted, but this change only affects the intercept. This method has been widely used for the measurement of diffusion coefficients of water molecules in various environments. The application to protein diffusion has been limited, since the small diffusion coefficients typically permit only very small amounts of echo attenuation to be observed. In our experiment this problem was circumvented by increasing the magnitude of G to compensate for the small magnitude of D. The spectrometer system is described elsewhere [6].

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In applying proton PFG-NMR to the study of hemoglobin in blood cells, we have taken advantage of the fact that this method can discriminate between both signals from rapidly moving water molecules and from molecules having restricted motion. The echo signal arising from water protons attenuates rapidly as G is increased because of the large value of D for water. Signals from macromolecules other than hemoglobin turn out to be unimportant either because of the small fraction of total protons which are contained in these molecules or because the protons in these molecules have small values of the transverse nuclear relaxation time T_2 [7]. Consequently, the echo amplitude for these molecules is negligible even in the absence of the gradient pulses.

In this report we illustrate the resolution of the hemoglobin echo signal, and we report the pH dependence of the diffusion coefficients for both oxy-HbA and oxy-HbS in blood cells. The primary effect is attributed to the dependence of mean cell volume on osmolarity and pH. The diffusion coefficients for oxy-HbS were found to be about 10% smaller than those for oxy-HbA in the cells which we studied.

2. Materials and methods

Normal blood required for whole red blood cell experiments was drawn from a single healthy donor by venipuncture into 25-ml tubes. Sickle cell blood was obtained with informed consent from a donor homozygous for the sickle gene. In both cases the blood was centrifuged to remove the plasma and buffy coat. The cells were then washed with appropriate cold buffers. The exchange with ^2H_2O was performed as follows. For normal blood the cells were suspended in buffer prepared in ^2H_2O, rolled gently for 3 min to permit equilibration, and centrifuged for 5 min at 2000 g. The procedure was repeated five times. For sickle blood the cells were allowed to equilibrate with the ^2H_2O buffer for 30 min and the procedure was repeated eight times. After the final centrifugation the packed cells were transferred to a 5-mm NMR tube which was then sealed with epoxy resin. The cells were stored at 4°C until required for use, and all samples were used within 2 days of preparation.

The NMR experiments were performed at 17.1 MHz using a Spin-Lock Ltd. CPS-2 Coherent NMR Pulse Spectrometer and a Magnion 12-inch electromagnet with an FFC-4 field control unit. The spin echoes were digitized by a Biomation model 802 transient recorder which was interfaced with a Nuclear Data 812 minicomputer. Up to 100 echoes were averaged for each measurement. The field gradient pulses were provided by a current pulser, which was designed and built inhouse, and a quadrupole gradient coil [8]. Pulse durations of 0.5–10.5 ms and amplitudes up to 10 A were available with this arrangement. The amplitude of the gradient pulses were calibrated using n-decanol, which has a diffusion coefficient of 7.5×10^{-7} cm^2/s at 25°C [9]. A calibration constant of 34.9 G cm^{-1} A^{-1} was obtained with $\delta = 2.0$ ms, $\Delta = 8.9$ ms, and a separation $\tau = 6.2$ ms between the radio-frequency pulses. An error in the reported diffusion coefficient for decanol will of course cause a systematic error in our measurements, but will not affect relative measurements such as those reported here. The temperature was controlled to ±0.25°C over the range 0–40°C with a cold nitrogen gas flow system and a heater operated by a Leeds & Northrup CAT control unit.

3. Results

In fig. 1a, we show a plot of the echo attentuation factor R vs. the square of the field gradient (G^2) for packed cells in H_2O buffer. As indicated by eq. 1, the slope of this plot at large values of G^2 should be proportional to the diffusion coefficient for hemoglobin. However, the large values found for R when G is small and the curvature of the plot indicate interference from more rapidly diffusing components. Since eq. 1 applies to each component present, curvature arises when signals are detected simultaneously from molecules having different diffusion coefficients. For comparison we show in fig. 1b a plot of R for a filtered solution of HbA-CO, also in H_2O buffer, at a concentration of 32.9 g/dl. Our expectation that the proton NMR signal from H_2O will not interfere is confirmed by fig. 1b, but not by fig. 1a. The apparent

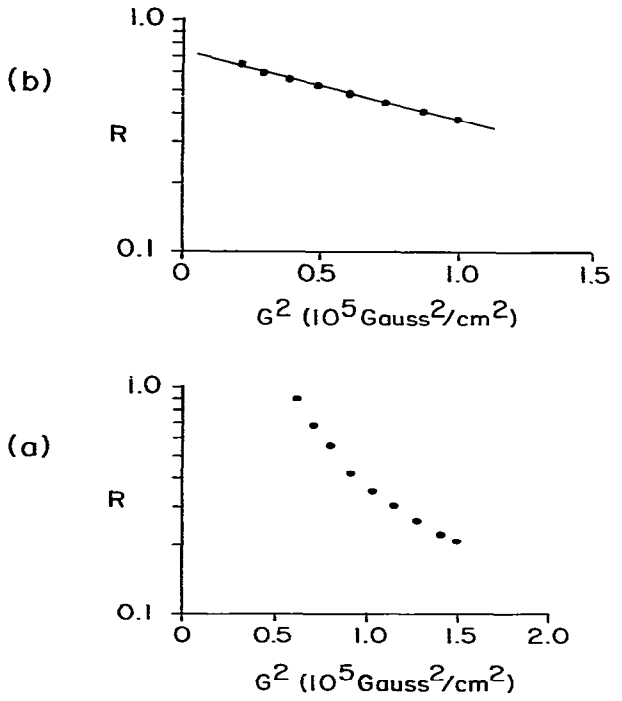

Fig. 1. Plots of the echo attenuation factor R vs. G^2. Experimental parameters $\delta = 2.40$ ms, $\Delta = 9.80$ ms, $\tau = 6.80$ ms. (a) Packed cells (suspending medium: 0.1 M NaCl, 0.01 M KH_2PO_4, pH 6.9, in H_2O). (b) HbA-CO in solution (0.1 M KCl, 0.01 M KH_2PO_4, pH 6.9, in H_2O) at a concentration of 32.9 g/dl.

diffusion coefficient for H_2O is considerably smaller in packed blood cells than in solutions for reasons which are discussed in the next section. In order to determine the diffusion coefficient for hemoglobin we reduced the interfering signal to an acceptable level by reconstituting the cells in a buffer prepared with ^2H_2O as described in the previous section.

In fig. 2, we show a plot of R vs. G^2 for HbA-O_2 in packed blood cells suspended in ^2H_2O at pD = 6.90. The interfering signal is still evident at small values of G, but the linear portion of the curve is adequate for the determination of D for hemoglobin. As G^2 is increased the echoes from components which have large values of D, i.e. water, attenuate to negligible values; and the fact that the

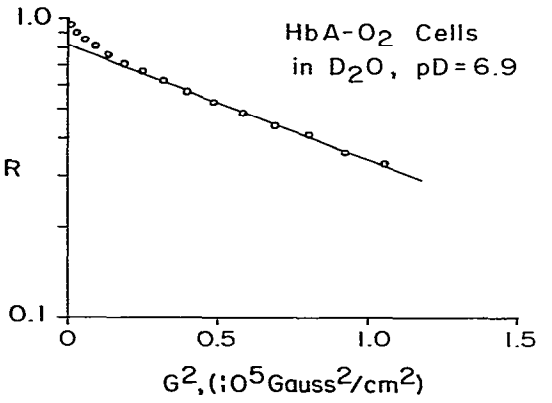

Fig. 2. Plot of the echo attenuation factor R vs. G^2 for packed cells (suspending medium: 0.1 M NaCl, 0.01 M KH_2PO_4, pH 6.9, in ^2H_2O). Experimental parameters are the same as in fig. 1.

plot becomes linear means that a single diffusion coefficient adequately describes the detected macromolecules. It is reasonable to attribute this diffusion coefficient to hemoglobin. Accordingly, we have used ^2H_2O suspensions for all of the measurements on hemoglobin in cells. We have also found that the magnitude of the interfering signal increases when the temperature of the packed cells is reduced from 37 to 18°C. Another finding of

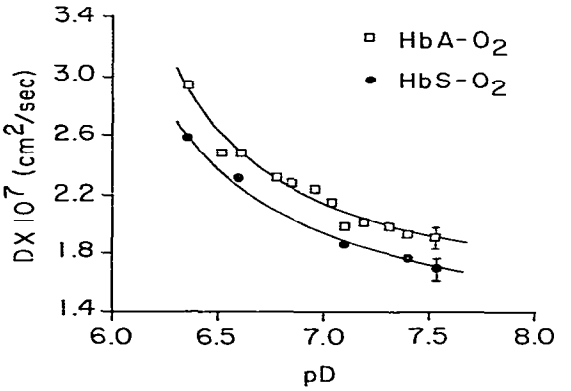

Fig. 3. Tracer diffusion coefficients of HbA-O_2 (□) and HbS-O_2 (●) plotted vs. the pH (pD) of the suspending medium (0.01 M KH_2PO_4, 0.1 M NaCl, $T = 37$°C). Experimental parameters are the same as in fig. 1.

possible importance is that cells containing HbS-O_2 require considerably more time to equilibrate with ^2H_2O than those containing liganded HbA. This could indicate a lower water permeability of the membrane of sickle cells, and we are currently using NMR relaxation measurements to investigate this point [10].

We have also investigated the dependence of the diffusion coefficient of hemoglobin in blood cells on the pH or pD of the suspending medium. In fig. 3, D is plotted vs. pD for both cells containing HbA-O_2 and HbS-O_2. In each case the osmolarity was 206 mosM. The vertical bars shown on the last data points represent typical standard deviations for these measurements. Additional measurements were performed for HbS-CO to ensure that HbS was totally liganded. We found no significant difference in the diffusion coefficients for HbS-O_2 and HbS-CO inside intact blood cells. Also, for comparison we measured the pH dependence of the diffusion coefficient of HbA-CO in solutions. Within experimental error no dependence was found.

4. Discussion

PFG-NMR provides a convenient method for determining diffusion coefficients for hemoglobin in solution, and as demonstrated by fig. 2 it is also applicable to hemoglobin in blood cells. It is important to note that this method measures the tracer diffusion coefficient and that with polydisperse samples it can provide (a) the number average of D in the limit of small attentuations, and (b) a measurement of D for large molecules only when G^2 is large [1]. In contrast to this, photon correlation spectroscopy (PCS) determines the mutual diffusion coefficient and provides the z-average of this quantity [11]. PFG-NMR offers a number of advantages over PCS for the study of hemoglobin, since NMR is not particulary sensitive to the presence of particulate matter and the optical absorbance of the sample is unimportant [12,13]. Applications of PFG-NMR to the concentration and temperature dependence of D for hemoglobin solutions are discussed elsewhere [6].

Fig. 1 raises interesting questions concerning differences between hemoglobin solutions and packed blood cells. It is possible that the viscosity inside the blood cell is greater than in a solution having the same hemoglobin concentration, or that water trapped between the packed cells is somehow different from bulk water. Also, protons bound to small mobile proteins may contribute to the interfering signal. However, we believe that signals from components other than hemoglobin and water are relatively unimportant. It is well known that mature human blood cells contain neither nuclei nor mitochondria, and that metabolism proceeds at a very low level [14]. The cell primarily contains a solution of hemoglobin with a concentration of roughly 33 g/dl under isotonic conditions, and there is little evidence for severe restrictions on the motion of either water [15] or hemoglobin molecules [16].

A partial explanation for the effect seen in fig. 1a is that the apparent diffusion coefficient for water in blood cells, as measured by PFG-NMR in our experiment, is at least an order of magnitude smaller than for bulk water. This effect arises because PFG-NMR responds to the distance which a molecule moves along a magnetic field gradient rather than to the short time diffusion coefficient. According to elementary diffusion theory, the displacement x in a given direction is related to the lapsed time through the equation, $\langle x^2 \rangle = 2Dt$. With $D = 2.5 \times 10^{-5}$ cm^2/s for water and $t = 10$ ms we find that x is approx. 7 μm. This should be compared with the reported diameter and minimum thickness of discocytes which are 7.6 and 1.4 μm, respectively [17]. The restrictions placed on the range of movement of water molecules by the cell membrane reduces the apparent diffusion coefficient of water to the point that interference with hemoglobin measurements is expected [18]. This effect has been verified by Cooper et al. [19], who found that the ratio of the apparent diffusion coefficient for water in blood cells to that for pure water ranges from 0.22 at $\Delta = 2.2$ ms to 0.08 at $\Delta = 16$ ms. The exchange of ^2H_2O for H_2O does not affect this process, but simply reduces the proton NMR signal to an acceptable level. The internal viscosity of the cell cannot be ruled out as a contributing factor in reducing the effective diffusion coefficient of water in view of the results of

Cooper et al., at low values of Δ and the fact that we have found a significant temperature dependence of the magnitude of the interfering signal.

Fig. 3 shows a decrease of approx. 50% in the diffusion coefficients of HbA-O_2 and HbS-O_2 as the pD of the medium is increased from 6.4 to 7.5 under hypotonic conditions. In both cases the calculated osmolarity was 206 mosM. Under isotonic conditions (300 mosM) the change in D over the same pD range was found to be less than 30%. The important factor here is the concentration of hemoglobin in the cell. For hemoglobin solutions the predicted tracer diffusion coefficient D (cm^2/s), corrected to water at 20°C, is related to the concentration C (g/dl) by eq. 2.

$$D = 6.750 - 0.3822C + 0.01134C^2 - 1.895\times10^{-4}C^3 + 1.353\times10^{-6}C^4 \quad (2)$$

The coefficients in this equation were obtained by combining the phenomenological diffusion equation with data on activity coefficients from sedimentation and viscosity experiments [20] and mutual diffusion coefficients from PCS [21]. Eq. 2 is in reasonable agreement with all reported tracer diffusion coefficients for hemoglobin in solution including those obtained using PFG-NMR. It remains to be seen whether this equation is appropriate for hemoglobin inside blood cells, but the predicted changes in D are in qualitative agreement with the reported dependence of cell volume on osmolarity and pH [22,23]. The strong dependence of D on C indicates that PFG-NMR is potentially a convenient and accurate method for the determination of hemoglobin concentrations in blood cells and changes in cell volume. This possibility of currently being explored.

Fig. 3 also presents preliminary results which compare diffusion rates for HbA-O_2 and HbS-O_2 in cells under similar conditions. Significant differences have been found throughout the range of osmolarities and pD values which we have studied. Typical values of D for HbS-O_2 were 10% lower than those found for HbA-O_2. We have not previously detected such differences in PCS studies of hemoglobin solutions, and it is possible that differences in the PFG-NMR results can be attributed to variations in mean hemoglobin concentrations in the cells. However, we point out that differences have been reported for rotational correlation times for HbA-O_2 and HbS-O_2 both in concentrated solutions [24] and in cells [25]. The solubilities are also known to be different [26].

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