PROTON MAGNETIC RESONANCE STUDIES OF INTRACELLULAR WATER IN SICKLE CELLS

Adam ZIPP and I. D. KUNTZ
School of Pharmacy, Department of Pharmaceutical Chemistry

and

S. J. REHFELD and S. B. SHOHET
School of Medicine, Department of Clinical Pathology and Medicine, University of California, San Franciso, Calif. 94143, USA

Received 19 April 1974

1. Introduction

Sickle cell disease is characterized by a change in erythrocyte shape from a biconcave disk to a crescent-like structure. This shape change results from aggregation of the sickle hemoglobin (HbS) molecules into parallel filaments [1–5]. Studies of this protein aggregation process have provided much useful information about the molecular mechanism of sickling [1,2,4,6–9]. However, the red cell also contains 80% water and one might anticipate that an understanding of the sickling process would be facilitated by a knowledge of the role of water. For example, irreversibly sickled cells which may be related to the clinical severity of the disorder are apparently dehydrated [10]. In addition, recent studies have suggested that a loss of 10% of the mean cell water occurs in the sickling process [11]. Therefore, in order to learn more about water–HbS interactions, we have studied the properties of water in sickle erythrocytes by Nuclear Magnetic Resonance (NMR) spectroscopy. A preliminary report has appeared elsewhere [12].

2. Experimental

Spectral measurements were carried out with a Varian model A60-A NMR spectrometer and a JEOL model JNM-4H-100 instrument for the 60 MHz and 100 MHz spectra, respectively. Water samples doped with 10^{-4} M Mn^{2+} to give a linewidth of 2 Hz were always used as reference standards. Spectra were recorded at the operating temperature of the instruments, 37°C for 60 MHz and 25°C for 100 MHz.

Whole blood was obtained from normal individuals and patients both homozygous and heterozygous for sickle cell anemia. Heparin or EDTA were used as the anticoagulants. No effects attributable to the anticoagulants were observed. The hemoglobin S content was determined by microzone electrophoresis.

Oxygenated wet packed cells were prepared by filling an NMR tube with whole blood and centrifuging at 2000 *g* for 30 min. For deoxygenated samples, whole cells were treated with a 2% sodium metabisulfate solution for 20 min or subjected to a stream of 95% N_2, 5% CO_2 (~ 10 min). All de-gassing was done in a glove bag under nitrogen. After deoxygenation was complete, the erythrocytes were transferred to a nitrogen purged NMR tube, capped with mineral oil, and centrifuged as described above.

Whole cell hemolysates were prepared by plunging the NMR tubes containing packed cells into liquid nitrogen and allowing the samples to thaw at room temperature. Polyethylene inserts were used to prevent the NMR tubes from breaking as a result of the freeze–thaw cycle [13]. Each sample was subjected to at least two freeze–thaw cycles.

To prepare cell-free hemoglobin, erythrocytes were

washed with 0.9% reagent grade NaCl and hemolyzed both by freeze–thawing and osmotic shock (10 vol distilled H_2O to 1 vol cells). The lysate was centrifuged at 12 000 g to remove the stroma and the resulting hemoglobin solution was concentrated by ultrafiltration through an Amicon P30 membrane to 30 g %. Hemoglobin concentrations were determined by converting the oxy Hb to cyanomethamoglobin and measuring the absorbance at 540 nm.

3. Results and discussion

When erythrocytes from individuals with sickle cell disease were deoxygenated the NMR water proton linewidth of the wet packed cells broadened considerably (12–20 Hz). Similar results were observed whether deoxygenation was achieved by 2% sodium metabisulfite treatment or with 95% N_2, 5% CO_2. The water line broadening of the deoxygenated samples was reversed with oxygenation. Broadening of the water proton line was also observed when cell-free preparations of hemoglobin S were deoxygenated with 95% N_2, 5% CO_2 (15 Hz). These results are in general agreement with a preliminary report of other investigators [14].

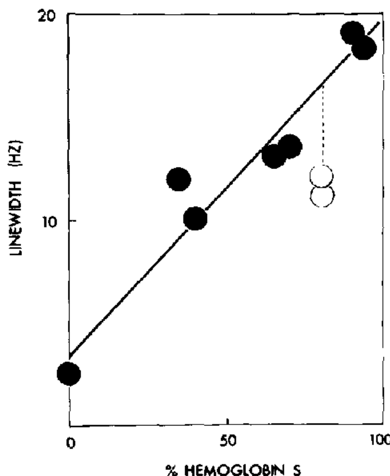

Fig. 1. Water proton linewidth as a function of % sickle hemoglobin in various samples of packed whole cells. Each experimental point represents a different subject. Cells sickled with 2% sodium metabisulfite. Temperature, 37°C. 60 MHz NMR frequency. Closed circles patients with HbS and HbA in various proportions. Open circles patients with HbS and 18% fetal Hb.

Fig. 1 shows a plot of the percentage of cellular HbS vs. water proton linewidth for deoxygenated packed cell preparations from various homo- and heterozygotes. The non-sickle hemoglobin in the cell was either A or F (fetal). Packed cells from normal individuals were used to obtain the initial point in fig. 1. Four subjects were used, each contributing at least two different samples. Each HbS point refers to a different experimental subject and is the average of 3–5 replicate spectra. With the exception of 2 points, the water proton linewidth was linear with the HbS concentration. The two experimental points (at 80% HbS) that fall somewhat below the best straight line in fig. 1 (lease squares fit) are from cells that contained 18% fetal hemoglobin. Since fetal hemoglobin is believed to inhibit sickling this deviation is not surprising [15,16].

To obtain some insight into the source of the line broadening in deoxygenated sickle cells, the water proton linewidth was also studied at 100 MHz. The results are shown in table 1. The rationale used here is that all effects directly related to water mobility would act to give an unchanged or a narrower line at 100 MHz than at 60 MHz, while effects related to the static magnetic environment (e.g., chemical shifts, bulk susceptibility effects) or to water proton exchange would give a wider line at 100 MHz. According to the Solomon–Bloembergen equation for the frequency dependence of the relaxation time [17], the predicted ratio of linewidths is 0.8 to 1.0

Table 1
Water proton linewidth (Hz) as a function of NMR spectrometer frequency for packed normal and sickle cells

Sample	60 MHz	100 MHz	Ratio of LW at 100 MHz to 60 MHz[c]
Normal cells (oxy)	5.1	7.6	1.5
	3.9	6.8	1.7
Sickle cells (deoxy)	12.1[a]	15.8[a]	1.3
	17.3[a]	17.3[a]	1.0
	18.1[b]	19.4[b]	1.1

[a] Deoxygenated with 2% sodium metabisulfite.
[b] Deoxygenated with 95% CO_2, 5% N_2.
[c] Estimated error ± 0.2.

for pure motional effects; and 1.67 for pure field effects. Table 1 gives values of this ratio ranging from 1.0 to 1.3 for packed sickle cells and from 1.5 to 1.7 for packed normal cells. These results suggest that for deoxygenated sickle cells both sources (water motion and magnetic environment) are important in the line broadening.* On the other hand, for the packed normal cells the water proton line broadening (compared to pure water) arises largely from static field dependent effects.

The most important field effects are chemical shift differences for intracellular and extracellular water, proton exchange between water protons and 'exchangeable' protons of hemoglobin, relaxation by paramagnetic ions, and bulk magnetic susceptibility effects (e.g., cell shape effect). Chemical shift, proton exchange and paramagnetic relaxation effects are all expected to be temperature dependent. If these effects contributed significantly to the observed line broadening for sickle cells, we would expect the water linewidths to be temperature dependent. For deoxygenated sickle cells the linewidth changed by less than 1 Hz (17 ± 1 Hz to 16.2 ± 1 Hz) on cooling from 36° to 10°C suggesting little contribution to the broadening from proton exchange or chemical shifts. The paramagnetism of deoxy hemoglobin is also probably of little importance since we observed no broadening with deoxy HbA which has the same paramagnetic qualities (due to the Fe^{+2} atom) as hemoglobin S.

There are significant differences in bulk magnetic susceptibility between water and red blood cells. It has been recently shown [18] that in heterogeneous aqueous systems, these susceptibility differences could lead to significant broadening of the water proton resonance. On a cellular level, we are concerned with the magnetic field inhomogeneities caused by the presence of the intact erythrocytes. However, for both normal cells and sickle cells (oxy or deoxy), the water proton linewidth differences between packed and lysed cells amount to at most 3 Hz. This small difference suggests that bulk susceptibility effects at a *cellular* level are not a significant source of broadening for the deoxy vs. oxy sickled cells. Therefore, static field effects do not contribute significantly to the water proton line broadening upon sickling.

This leaves the role of water mobility. Generally, any effect that decreases water mobility will broaden the water proton line. Our data suggest that significant changes in water mobility occur when SS cells sickle. It is of interest to consider the following possible causes of the altered water mobility: viscosity, gross cellular dehydration, and water hemoglobin binding.

Since average water motions should be slowed by increasing the overall or 'macroscopic' intracellular viscosity, any increase in viscosity should broaden the water proton line. However, we are most concerned here with 'microscopic' viscosity in the sickle cell where we want to know the local environment of water molecules adjacent to sickled and non-sickled hemoglobin. Theories that relate viscosity to molecular motion and hence to linewidth provide a poor description of such solvent microscopic motion [16]. It is in this regard that gross cellular dehydration and changes in water–hemoglobin binding come into play.

Sickling may result in a net loss of water (10%) from the red cell [11]. Since this loss may affect the mobility of the water molecules remaining in the cell by increasing the HbS concentration, cellular dehydration may be important in the water proton line broadening. Cell hydration was varied by altering the osmolarity of the suspending plasma. Reducing the salt concentration swelled the cells and reduced the water linewidth of packed normal cells to 2–3 Hz. Conversely, increasing the salt concentration of the plasma by 15% decreased the hematocrit by 10% [19] and broadened the water line by not more than 1.5 Hz for both normal and sickle cells. These results suggest that dehydration of the SS cells upon deoxygenation does not contribute significantly to the water proton line broadening.

It appears, therefore, that of all the sources of linewidth broadening, increased HbS–water interactions upon sickling appear to have the dominant effects. In particular, changes in the amount and the mobility of the water 'bound' to the protein may be important.*

* Preliminary relaxation measurements indicate a possible 2-fold decrease in the spin–lattice relaxation time upon sickling. This decrease is consistent with altered water mobility.

* Preliminary low temperature (−20 to −40°C) experiments suggest that the amount of water 'bound' to the HbS molecule decreases by about 10% upon sickling. The method is described elsewhere [20].

Additional spin–lattice [13], spin–spin, and low temperature experiments are underway in an effort to define the molecular mechanism of this effect. In addition, such studies may offer some insight into the sickling process itself.

Acknowledgement

We wish to thank Dr. H. F. Lokin for use of the clinical laboratories and Dr. T. L. James for useful discussions. Support from NIH (IDK, SBS) is gratefully acknowledged.

References

[1] Finch, S. T., Perutz, M. F., Bertles, J. F. and Döbler, J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 718.
[2] White, J. C. and Heagan, B. (1970) Am. J. Path. 58, 1.
[3] White, J. G. (1968) Blood 31, 561.
[4] Muryama, M. M. (1967) Clin. Chem. 14, 578.
[5] Döbler, J. and Bertles, J. F. (1968) J. Exp. Med. 127, 711.
[6] Magdoff-Fairchild, B., Swerdlow, P. H. and Bertles, J. F. (1972) Nature 239, 217.
[7] Williams Jr., R. C. (1973) Proc. Natl. Acad. Sci. U.S. 76, 1506.
[8] Freedman, M. L., Weissmann, G., Gorman, B. D. and Cunningham-Rundles, W. (1973) 22, 667.
[9] Bookchin, R., Nagel, R., Jaffe, E. (1971) Clin. Res. 19, 411.
[10] Nathan, D. G. and Shohet, S. B. (1970) Sem. in Hemat. 1, 381.
[11] Masys, D. R., Bromberg, P. A., Balcerzak, A. (1973) Clin. Res. 21, 873.
[12] Zipp, A., McComas, D. B., Kuntz, I. D. and Shohet, S. B. (Dec. 1973), Proceed. of the 16th. Annual Meet. of Am. Soc. of Hemat. Chicago, 114.
[13] White, J. P., Kuntz, I. D. and Cantor, C. R. (1972) J. Mol. Biol. 14, 511.
[14] Cameron, B. F. and Block, R. E. (1973) Am. Chem. Soc. Abs. 166, 3336.
[15] Bookchin, R. M. and Nagel, R. C. (1971) J. Mol. Biol. 60, 263.
[16] Ranney, H. M. (1972) Biochimie 54, 633.
[17] Carrington, A. and McLachlan (1967) Introd. to Magnetic Resonance, Harper and Row, New York, pp. 187–194.
[18] Glasel, S. A. and Lee, K. H. (1974) J. Am. Chem. Soc. 96, 970.
[19] Harris, J. W. and Kellermeyer, R. W. (1970) The Red Cell, Harvard Univ. Press, Cambridge, Mass., p. 544.
[20] Kuntz, I. D., Brassfield, T. S., Law, G. D. and Purcell, G. V. (1969) Science 163, 1329.