THE COMPLEXATION OF ZINC IN INTACT HUMAN ERYTHROCYTES STUDIED BY ^{1}H SPIN-ECHO NMR

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1. Introduction

Nuclear magnetic resonance spectroscopy has proven to be a powerful technique for studying the binding of metal ions by biological molecules. Biological molecules generally possess a number of potential binding sites, giving rise to a variety of possible binding schemes. The main advantage of the NMR technique is that each site can be monitored at the molecular level; interactions at specific sites are detected, for example, by changes in chemical shifts of resonances for nuclei located near the sites. However, because of the complexity of cellular systems and the need for well-resolved resonances, it has been necessary to perform studies of this type with molecules in highly purified form, even though it is their coordination chemistry in cellular systems which is of interest. For example, cellular systems contain a multitude of potential ligands which might combine with metal ions to form mixed ligand complexes.

We report herein results which demonstrate for the first time that the binding of metal ions by molecules in an intact cellular system can be studied directly at the molecular level by ^{1}H NMR. Specifically, we report on the coordination chemistry of Zn^{2+} in intact human erythrocytes. Zn^{2+} is known to complex human hemoglobin and to increase its oxygen affinity [1–4]. However, the zinc-induced increase in oxygen affinity is considerably less for whole cells than for highly purified hemoglobin [2–4], which suggests that Zn^{2+} is also complexed by other molecules in whole cells.

Abbreviations: NMR, nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulphonic acid; GSH, reduced glutathione; SEFT, spin-echo Fourier transform; Hb hemoglobin

2. Experimental

The erythrocytes were prepared from venous blood which was collected in vacutainers (Becton, Dickinson and Co.). The whole blood was centrifuged at 5000 rev./min for 15 min; the packed cells were then washed 3 times with isotonic saline–glucose solution in D_2O.

^{1}H NMR spectra were measured at 400 MHz on a Bruker WH-400/DS spectrometer by the spin-echo Fourier transform technique ($90°-\tau_2-180°-\tau_2-$acquisition) [5–7]. A τ_2 of 0.015 s was used when hemoglobin resonances were being observed [5]; a τ_2 of 0.060 s was used when resonances from small molecules were being observed [6]. Spectra were measured at 25°C on ~0.5 ml packed or hemolyzed cells in 5 mm outer diameter NMR tubes. The free induction decay was collected in 8 K of data points with an acquisition time of 0.819 s. Quadrature detection was used with a spectral width of 5000 Hz, and 300 transients were collected for each spectrum. Chemical shifts are reported relative to the methyl resonance of DSS, based on the resonance for the α-CH_2 protons of the Gly residue of glutathione having a chemical shift of 3.76 ppm.

3. Results and discussion

The intracellular region of red cells contains a number of potential ligands for Zn^{2+}, including hemoglobin and small molecules such as glutathione (GSH) and ergothioneine. The standard ^{1}H NMR spectrum of red cells is dominated by a broad, rather featureless envelope due mainly to resonances from hemoglobin, with resonances from the smaller molecules buried in the envelope. The spin-echo FT

(SEFT) NMR technique was used to selectively eliminate the hemoglobin resonances on the basis of the shortness of their spin–spin relaxation times relative to those of resonances from the small molecules [6]. Fig.1A shows a portion of the 400 MHz ^{1}H SEFT NMR spectrum for intact cells, measured with a τ_2 of 0.060 s. Those resonances which have been assigned [6,8,9] to molecules known to form complexes with zinc and other metal ions in aqueous solution are:

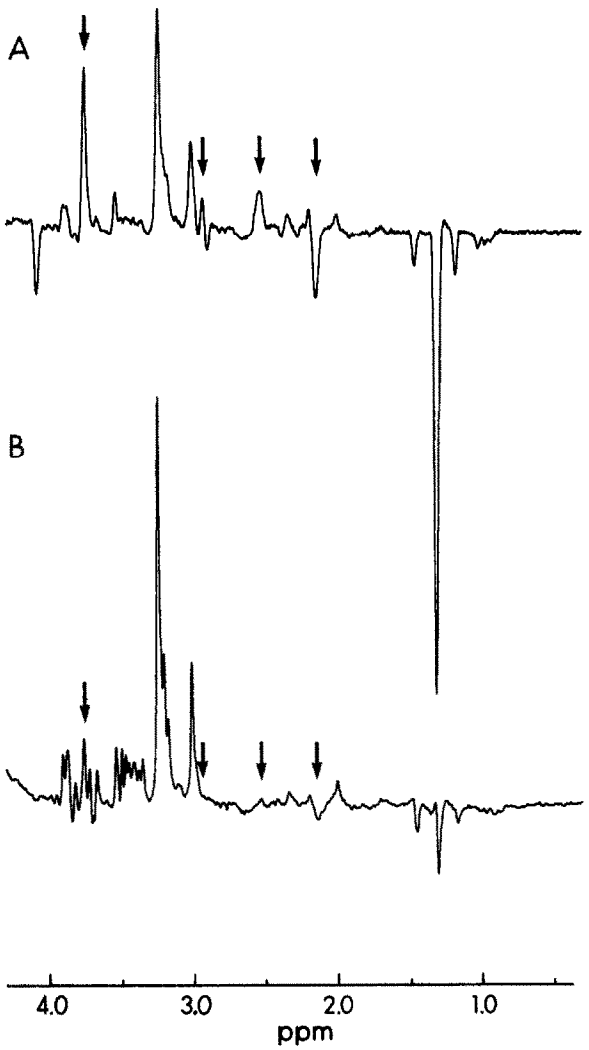

Fig.1. Portions of the 400 MHz SEFT ^{1}H NMR spectra of (A) packed intact red blood cells which had been washed 3 times with isotonic saline–glucose solution in D_2O and (B) packed intact red blood cells which had been washed 3 times as in (A) then incubated in an isotonic saline–glucose solution containing 2 mM $ZnCl_2$. A τ_2 of 0.060 s was used for each spectrum.

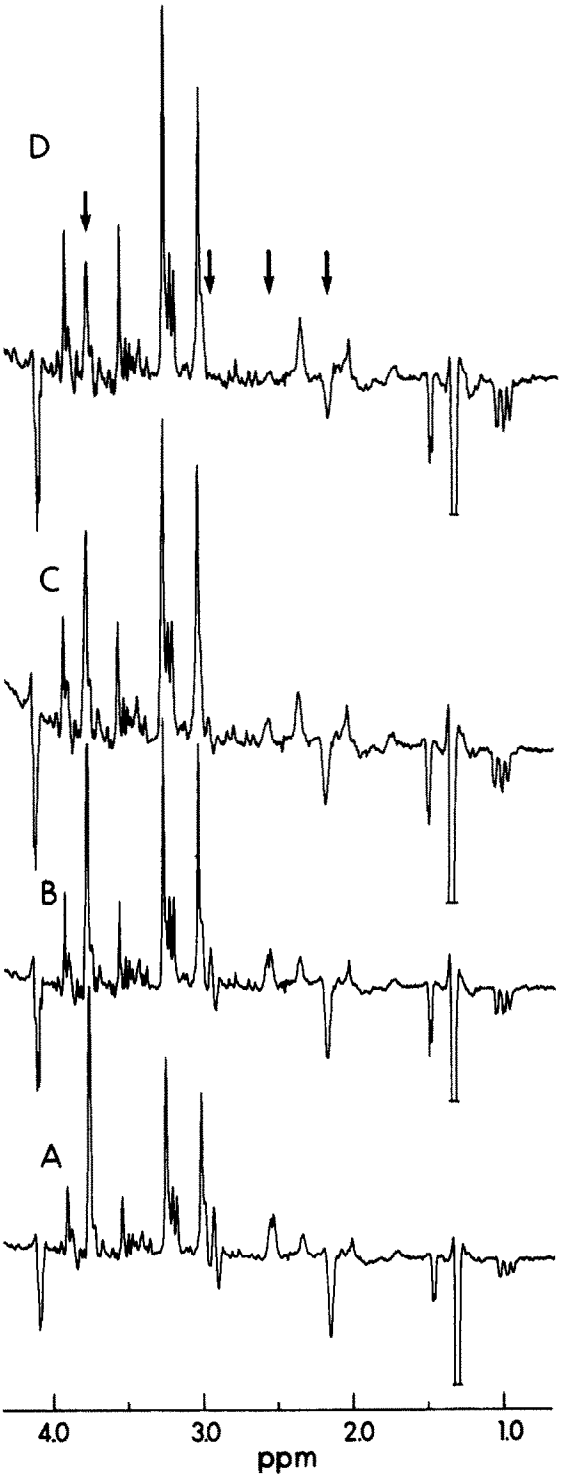

Fig.2. Portions of the 400 MHz SEFT ^{1}H NMR spectra of hemolyzed red blood cells to which $ZnCl_2$ had been added. The Zn^{2+} levels are: (A) 0 mM; (B) 1.46 mM; (C) 2.38 mM; (D) 4.55 mM. Spectra were measured with τ_2 = 0.060 s. The pH* was held constant at 7.18.

CH_3 of lactate, 1.28 ppm; CH_3 of alanine, 1.46 ppm; β-CH_2 and γ-CH_2 of glutamic acid residue of glutathione (GSH), 2.15 and 2.55 ppm; β-CH_2 of cysteine residue of GSH, 2.92 ppm; CH_2 of glycine, 3.54 ppm; CH_3 and CH_2 of creatine, 3.01 and 3.91 ppm. Fig.1B shows the same spectral region for cells which had been incubated in an isotonic saline–glucose suspension containing 2 mM $ZnCl_2$. The disappearance of the resonances for the carbon-bonded protons of GSH from fig.1B provides direct evidence for the binding of the Zn^{2+} by GSH. The other differences between the two spectra are in the relative intensities of the glucose and lactic acid resonances [6], which apparently indicates a reduced metabolic activity in the Zn^{2+}-containing cells.

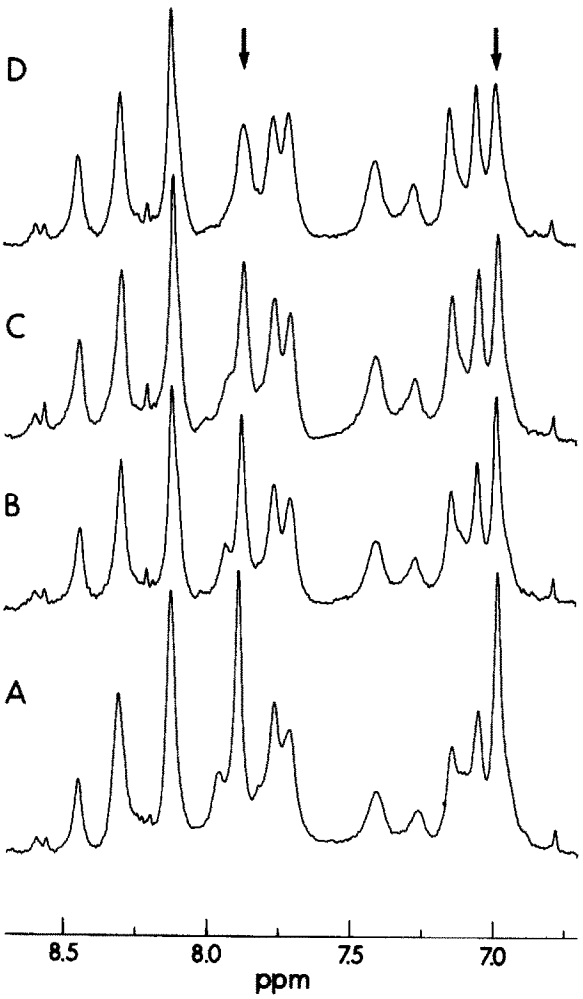

Fig.3. Portions of the 400 MHz SEFT ^1H NMR spectra for the same hemolyzed red blood cell samples as in fig.2. A τ_2 of 0.015 s was used.

In fig.2 are shown NMR spectra for the titration of hemolyzed red cells with $ZnCl_2$ solution. As $ZnCl_2$ is added, the GSH resonances decrease in intensity in a step-wise fashion, with the complete disappearance of several of the GSH resonances from spectrum D. The $ZnCl_2$ causes no detectable effect on any of the other resonances from small molecules, indicating that, of the small molecules which can be observed, Zn^{2+} is binding selectively to the GSH.

The spectra in fig.3 show, however, that Zn^{2+} is simultaneously being complexed by hemoglobin. These spectra were obtained on the same samples as in fig.2. Resonances in the 6.85–7.5 and 7.5–8.5 ppm regions are due to the C-4 and C-2 hydrogens of some of the imidazole side chains of hemoglobin. Of interest in fig.3 is the step-wise decrease in intensity of the resonances at 6.98 and 7.87 ppm as the hemolyzed red cells are titrated with $ZnCl_2$. Previous studies have shown that human hemoglobin has two particularly strong zinc binding sites per hemoglobin tetramer, and that these are the oxygen-linked binding sites [3]. The amino acid residues involved in the binding have yet to be firmly established, but histidines β-143 and β-146 and cysteine β-93 have all been proposed [3,4]. The results in fig.3 clearly indicate that at least one histidine residue is involved. The stepwise decrease in intensity is consistent with a strong binding, and indicates further a considerable decrease in the local mobility of the particular imidazole side chain upon complexation.

The results in fig.2 and 3 indicate that the added Zn^{2+} is being complexed by GSH and by hemoglobin. Both the GSH resonances and the hemoglobin resonances reappear when the zinccontaining hemolyzed cells are titrated with EDTA, indicating that the complexation reactions are reversible. GSH has a high affinity for Zn^{2+}, with the sulfhydryl group the predominant binding site at physiological pH [10]. However, the effect of Zn^{2+} binding on the NMR properties of GSH is considerably different for GSH in red cells and in aqueous solution. Titration of 2 mM GSH in pH 7 aqueous solution with $ZnCl_2$ causes changes in the chemical shifts of some of the GSH resonances in the SEFT NMR spectrum, however they do not disappear as in fig.2, which indicates that the binding of Zn^{2+} by GSH in red cells is different from that in aqueous solution. The disappearance of the GSH resonances suggests a significant decrease in their spin–spin (T_2) relaxation times when GSH is complexed by Zn^{2+}. One mechanism by which this

could happen is through the formation of a ternary complex involving GSH, Zn^{2+} and a macromolecule, e.g., hemoglobin:

$$GSH + Zn^{2+} + Hb \rightarrow GSH{-}Zn{-}Hb$$

In the ternary complex, GSH would have motional characteristics, and thus T_2 values, similar to those of protons of the macromolecule. Consistent with this, the GSH resonances also decrease in intensity and then disappear from the ^1H SEFT NMR spectra of an aqueous solution containing purified hemoglobin and glutathione as it is titrated with Zn^{2+}.

Several explanations have been proposed to account for the difference in the magnitude of the zinc-induced increase in oxygen affinity of purified hemoglobin and of whole cells, including a substance in the cells which inhibits the increase in oxygen affinity due to zinc [3]; the results in fig.1,2 indicate that, of the more abundant small molecules in red cells, GSH has the highest affinity for zinc.

Acknowledgements

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