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HYDRATION AND SELFASSOCIATION OF HAEMOGLOBIN IN SOLUTION

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SUMMARY

The hydration of haemoglobin was determined by the nmr-freezing technique. There is a hydration-threshold around 15 mM(haem)/L, with the maximum hydration of $\sim$ 0.46 g H_2O/g Hb. The phosphate buffered solutions show a completely different behaviour from the other ionic and deionized solutions. The results in ordinary water and in the presence of 80% ^2H_2O are indistinguishable. The hydration is neither a function of the extremes in the ionic double layer structure nor of the type of ions. The threshold Hb-concentration does not depend on the absolute amount of hydration and the type of ion. The results corroborate the selfassociation of haemoglobin in concentrated solutions.

INTRODUCTION

The rationale of this study was given in a previous paper (1). A biphasic proton magnetic relaxation (pmr) behaviour was observed in solutions with less than 25% ^1H_2O (i.e. >75% ^2H_2O) at concentrations above 12-13 mM in haem. This was ascribed to association of haemoglobin molecules. The fast relaxing phase of protons was postulated to be due to the additional haemoglobin hydration, namely to the water molecules encaged within the macromolecular associates.

A dielectric frequency dispersion study completed in the meantime (2) revealed more directly and quantitatively that 6-9 $(\alpha\beta)_2$-molecules of oxyhaemoglobin form chain-like associates in the most dense solutions (22-24 mM in haem). A change in several dielectric and viscosity parameters indicated the onset of a transition in solutions above 8 mM.

The aim of the present study was to seek answers to the following questions:

(i) Is it possible to verify in ordinary aqueous solutions the concentration-dependent hydration of haemoglobin inferred from pmr rates in $^2H_2O/^1H_2O$-solutions, but using also somewhat more direct method to determine the hydration?

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(ii) Is the hydration of haemoglobin, as observed by pmr in ionic solutions, interrelated with the macromolecular association evidenced by the dielectric data for deionized solutions?

We resorted to Kuntz's method (3/a,b,c) of recording the pmr signal intensity from the unfrozen part of water in an otherwise frozen aqueous protein solution. This is believed (3) to represent the amount of protein hydration commensurate with that in solution. As we are concerned here mainly with the relative change of hydration, the method served the purpose well, and affirmative answers were reached to the above two questions, though, as usual, other questions have arisen out of this study.

EXPERIMENTAL

The haemoglobin solutions were prepared from freshly drawn human blood, as before (1), followed by extensive dialyses against appropriate solvent: 0.1 M potassium phosphate buffer (in ^1H_2O and in 80% ^2H_2O), pH 7.0; 0.1 M KCl, pH 6.9, and 0.1 M LiCl, pH 7.0. The deionized oxyhaemoglobin solution was obtained by dialysis against deionized water, under electrolysis (6 V potential difference), untill the current dropped to a constant value. The specific conductivity of that solution was 4.2×10^{-5} $\Omega^{-1}cm^{-1}$ (it was between 4 and 2×10^{-6} $\Omega^{-1}cm^{-1}$ in ref. 2). No phosphate stripping of haemoglobin was done. The concentrations were determined by the cyanmet-method and the visible spectra were recorded before and after the pmr measurements as a control of oxyhaemoglobin.

A digital readout pulsed spectrometer ("J. Stefan" Institute, Ljubljana) in conjunction with a high resolution superstabilized magnet but without current shimms ("Bruker Physik" Karlsruhe) was used at 24 MHz. The nuclear magnetization, M_o, was measured from the free induction decay (fid) 200 μs after a $\pi/2$ pulse, in dependence on temperature between -15 and +10°C. The temperatures were kept constant to within 0.5° by a thermostated nitrogen stream, and monitored by a thermocouple placed outside, but close to the sample. Two examples of M_o-recordings are given in Fig. 1. The points above and below the freezing temperature are means of several fid-recordings after thermal equilibration, while those within the freezing transition were recorded consequtively with time. Though these curves were somewhat different in shape if the sample was rotated manually during thermal equilibration, or when the initially frozen solution was warmed up, the ratio of the magnetization in the frozen state at -15°, M_f, to that in solution at +5°C, $M_{l(iquid)}$, was constant for each solution. The standard for measuring the relative change in M_o was thus the identical solution (its M_l), a difference from the original (3) high-resolution mode of operation. This M_f/M_l-ratio is assumed to be directly proportional to the amount of unfrozen water and ascribed (3) to the hy-

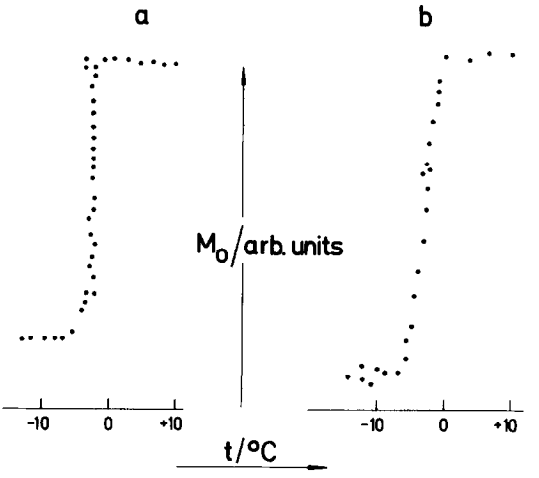

FIGURE 1: The temperature dependence of the proton magnetization, M_o (in arbitrary units), of (a) 19.6 and (b) 5.3 mM (in haem) aqueous oxyhaemoglobin solutions, 0.1 M phosphate buffer, pH 7. The values of M_o were recorded by decreasing the temperature (see Experimental).

dration shell of haemoglobin. The contribution to M_f from the ice-protons is definitely excluded, because of very fast fid-decay ($T_2 \sim 6$ µs (4)) compared to the read-off time (200 µs). The same pertains to the protons from haemoglobin: we were unable to record any signal in the frozen, completely deuterated solutions of any concentration. However, in the more concentrated liquid samples the protein protons contribute some 20% to M_1. The quantitative determination of this correction for M_1 was done in the following way. Firstly, M_0-values of weighed Hb-solution samples were measured at 10°C in dependence on Hb-concentration. The H_2O-content of each Hb-sample was calculated using the partial specific volume of Hb (0.74 g cm^{-3}). Under the same spectrometer setting and temperature M_0 of a weighed H_2O-sample was determined. This procedure resulted in a quantitative diminuation of M_1-values measured on the Hb-solutions by the amount of M_1 due to the protein-protons.

RESULTS AND DISCUSSION

Fig. 2 comprises all the data expressed either as the directly measured M_0-ratio (section A), or as the derived hydration, h, in g H_2O/g Hb (section B), in dependence on haemoglobin concentration. The hydration values were corrected for the M_0 due to the protons from haemoglobin. An inspection of the graphs reveals:

(i) the results with phosphate-buffered solutions in ordinary water and

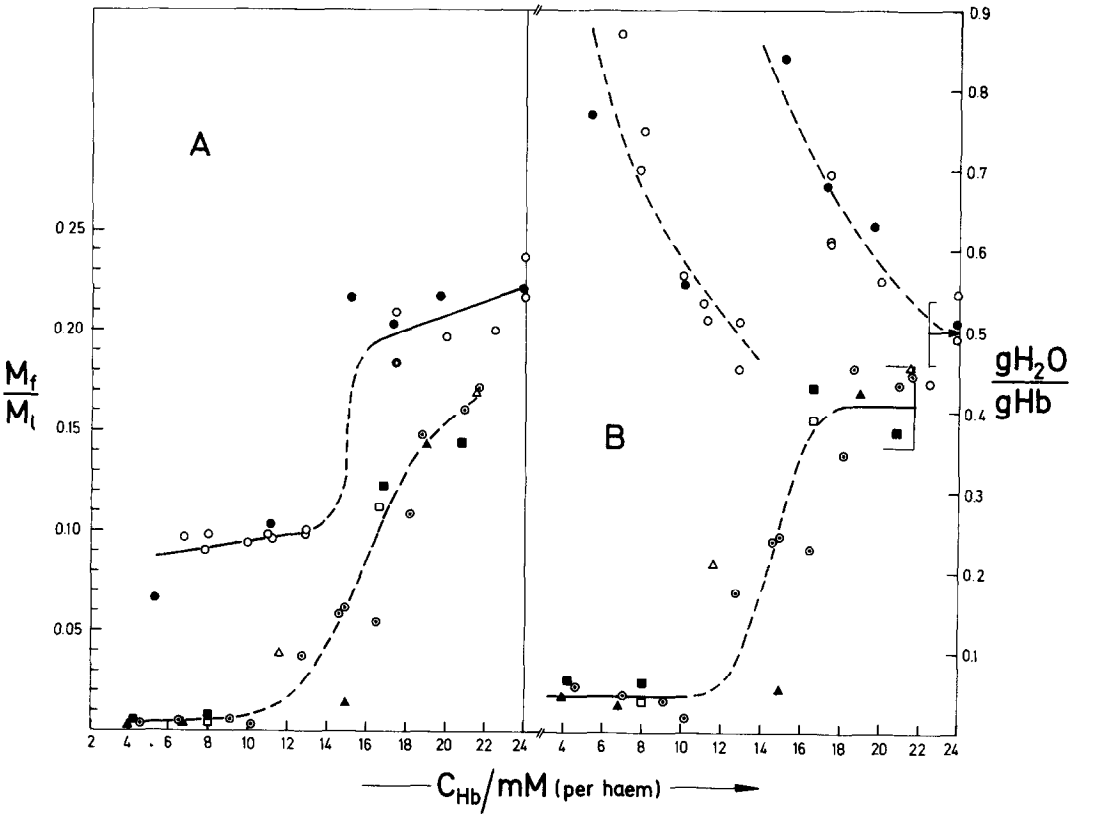

FIGURE 2: The concentration dependence of (A) the relative proton magnetization from the unfrozen water and (B) the specific hydration, in g H_2O per g protein, from various haemoglobin solutions - *oxyhaemoglobin* in 0.1 M phosphate buffer, pH 7, ^1H_2O (○); 0.1 M. phosphate buffer, pH 7, 80% ^2H_2O (●); 0.1 M LiCl, pH 7 (△); 0.1 M KCl, pH 6.9 (□); deionized ^1H_2O, pH? (⊖); *methaemoglobin* in 0.1 M LiCl, pH 7 (▲); 0.1 M KCl, pH 6.9 (■). The vertical lines with limiting bars indicate the standard deviation for the two mean values of the plateau-hydrations.

in the presence of 80% ^2H_2O are indistinguishable (the open vs. the full circles);

(ii) the concentration dependence of haemoglobin hydration is similar in the presence of 0.1 M monovalent cations (Li, K) with their common chloride anion, and in the deionized solution (see the legend of Fig. 2/a,b);

(iii) there is a completely different concentration dependence of haemoglobin hydration in potassium phosphate buffered solutions (Fig. 2/b);

(iv) common to both sets of ionic solutions (ii, iii) is the transition-range of concentrations centered around 14 mM per haem, but the higher concentration plateau appears to be attained only above some 20 mM, with 0.42 ± 0.05 g H_2O/g Hb in case of (ii) and 0.50 ± 0.04 g H_2O/g Hb for (iii).

The freezing method did not reveal whatever difference in hydration there may be on replacing ^1H_2O by ^2H_2O, but the results confirm the concentration--dependent hydration of haemoglobin (1). The answer to the second question posed in the Introduction is also affirmative: the same transition-range and the high-concentration plateau are observed here irrespective of the solvent ionic composition. It now seems warranted to discuss the hydration of haemoglobin as emerging from the pmr measurements in relation to its selfassociation deduced from the pmr results (1), and substantiated directly by the dielectric study(2).

Implicit in the above conclusions is the notion that the hydration measured by this method is proportional, if not equal, to that in the (unfrozen) solution. As regards the relative picture, i.e. the concentration dependence observed here and in (1), this is very likely so. However, the absolute amounts of hydration must be viewed with caution.

At lower Hb-concentrations, the hydration of haemoglobin in phosphate--buffered solutions is much larger compared to that in all the other solutions. Further, in distinction to all the other solutions, in the phosphate solution there is a reciprocal dependence of hydration on Hb-concentration. A plot of g H_2O/g Hb vs. $1/C_{Hb}$ (from Fig. 2/b) is consistent with straight lines having regression coefficients of 0.98 and 0.87 for the data above and below the concentration threshold.This suggests a binding equilibrium of the phosphate anion with haemoglobin (in the presence of the retained diphosphoglycerate anion). According to a direct study of inorganic phosphate binding to haemoglobin (5), the equilibrium in our case ought to have been shifted towards complete binding, at least for the lower Hb-concentrations. However, it is not possible to extrapolate from (5) in a straightforward manner to our frozen system. One has to determine first the actual ionic concentration within the hydration shell like it has been done in (6) for the ternary system KF-H_2O-protein. The concentrations of ions are expected to increase within the hydration shell on freezing owing to their exclusion from ice (6). This effect may partly explain the much larger hydration in phosphate solutions. Namely, while in other ionic solvents (without protein) no hydration at all could be observed on freezing, we recorded the M_f/M_1-ratios of 0.002, 0.004 and 0.018 for the 0.05, 0.1 and 0.2 M phosphate solvents, pH 7, respectively. This hydration of the phosphate anion alone could account for the observed large hydrations in phosphate+Hb solutions if it were concentrated more than tenfold within the hydration shell of Hb on freezing. This is not quite improbable, but it must be verified experimentally. On the other hand, because of the observed reciprocal dependence of hydration on Hb--concentration it is also likely that the (PO_4+Hb)-complex has an enhanced hydration compared to the sum of the components. However, if this is true the

hydration enhancement for the (PO_4+Hb)-complex must be very large because the concentration of phosphate-free Hb changes only by a few percent within the whole range of Hb-concentrations.

From 13 to 15 mM Hb (in haem) in the presence of phosphate, the hydration of haemoglobin doubles from the more or less "normal" value of some 0.5 gH_2O/gHb (3,6,7) to attain it again at the highest Hb-concentrations. The effect is being studied now in dependence on the PO_4/Hb-ratio and c_{Hb}.

At high Hb-concentrations in other ionic solvents our value of Hb-hydration is close to that observed by others, but with more dilute Hb solutions (3/a,7). Neither in the difference in sample preparation (liophylized Hb in refs. 3/a and 7), nor in the measuring technique (high resolution spectra and separate H_2O-standard in refs. 3/a and 7), could we find any possible source of this discrepancy in the amount of hydration <u>below</u> the Hb-concentration threshold. As no pH-value was given in the quoted two references this point cannot be discussed here. Moreover, Eagland (8) has pointed out that "the role of salts in the hydration process is, at least, ambiguous". This author quoted evidence that indicates an increase of macromolecular hydration up to a maximum value in the presence of salt at an ionic strength of 0.1-0.15, and that marked dehydration sets in at high ionic strength, with the ionic double layer maximally supressed. The solvent in (3/a) was 0.001 M KCl and 0.01 M KCl in (7), while we had 0.1 M KCl, but the hydrations are just opposite to what would be expected according to (8). In view of quite different kind of experiments (adsorption of water vapour by dry protein) summarized in (8) and because of the uncertainty as to the actual ionic concentrations in the freezing experiments, a further systematic study is necessary.

Notwithstanding the enumerated problems, and setting aside the specific PO_4^{3-}-effects discussed above, two further conclusions may be drawn here: (a) the hydration of haemoglobin is neither a function of the extremes in the ionic double layer structure (8, p. 425) nor of the type of monovalent ions: Li^+, a "water-structure maker" (9) and a potent "dehydrating agent" (8, p. 424) in contrast to the "neutral" KCl. (b) The threshold Hb-concentration does not depend on the absolute amount of hydration and the type of monovalent ion.

There are two Hb-concentration regions within which the specific hydrations are constant, the phosphate solutions excluded (Fig. 2/B). This corroborates our deduction (1) that below 8 mM (in haem) the normal $(\alpha\beta)_2$-haemoglobin molecules exist in solution, whereas above 18 mM there are well defined molecular entities, too, but larger ones - according to the dielectric study (2), associates of 6 to 9 Hb-molecules. The latter range of Hb-concentrations is of physiological importance. Further, as 0.9% NaCl-solution (≡0.15 mM NaCl) is isotonic for the red cell, the ionic concentrations used in the present study (0.1 M) is of the

same order of magnitude as that in the red cell. Hence, the association behaviour of normal oxyhaemoglobin and its hydration must be taken into account in various structure/function relationships (1).

The abnormal, sickling red cell, has attracted much attention in the past few years. A recent paper (10) concludes that not only deoxyhaemoglobin, but that oxyhaemoglobin in the sickling red cell is also likely to associate. We may now add that the selfassociation of oxyhaemoglobin (and of metHb) appears to be an intrinsic property of the normal molecule, whereas the aminoacid substitution in the sickle cell haemoglobin enhances the effect. Measurements of partial specific volume of bovine serum albumin (BSA) and haemoglobin (11), and proton magnetic relaxation studies (at very low frequencies) of apotransferrin (12), haemoglobin (13) and BSA (14) in dependence on protein concentration indicate that selfassociation phenomenon may be common for globular proteins, though a pmr-frequency dispersion study of BSA did not reveal it (15).

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