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Proton Magnetic Relaxation in Ethane Diol—Water Solutions of Haemoglobin

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Ethane diol was added to aqueous solutions of bovine and human met(Fe^{III})- and CO-haemoglobin in order to extend the temperature range for nuclear magnetic relaxation measurements below freezing point. No significant difference in the relaxation rates was found on adding ethane diol except in the case of human met- and CO-haemoglobin, which may be ascribed to changes in the hydration sheath. The energies of activation derived from Arrhenius plots of the relaxation rates for bovine Hb are also independent (within ± 5%) of ethane diol. It is concluded that the gross conformation of the haem pocket is not altered by addition of ethane diol, so that measurements can be done down to — 30 °C.

INTRODUCTION

The temperature range within which proteins in solution can be studied in their native state is limited by denaturation and freezing. There are several reasons in favour of extending the lower temperature region for studies of haemoprotein solutions by proton magnetic relaxation: (a) The interpretation of the results in terms of stereochemical parameters requires extrapolation from low to higher temperature data. The accuracy of this procedure[1] would increase if more data were available at low temperatures. (b) An extension of measurements to temperatures below zero centigrades is sometime necessary for an unequivocal determination of the type of the temperature dependence of the relaxation rates. (c) The rate of exchange of protons between the haem pocket and bulk solvent may be easier to study when diminished by lowering the temperature. (d) A marker molecule with nonexchangeable protons which would not enter the haem pocket and be inert towards haemoproteins is needed also for magnetic susceptibility measurements by NMR[2] and for the elucidation of the molecular model of the proton relaxation in haemoprotein solutions[3].

It has already been found by spectroscopic[4] and other studies[5] that ethane diol has hardly any influence upon (haemo)protein molecules. The purpose of this paper is to discuss evidence in favour of using ethane diol for freezing point depression of haemoglobin solutions without perturbing significantly the haem environment.

EXPERIMENTAL

Materials

Bovine haemoglobin was prepared from freshly collected cattle blood, and human haemoglobin from the blood supplied by the local blood bank. The procedure

of Cameron and George[6] was followed as closely as possible. The oxyhaemoglobin dialysed against 0.1 M NaCl at pH = 6 was oxidized by addition of $K_3Fe(CN)_6$.

Three aliquots of the ferrihaemoglobin solution were subject to the final dialysis against 0.1 M NaCl + 5×10^{-5} M EDTA: (a) without addition of ethane diol; (b) with 10 vol. % ethane diol, and (c) with 25 vol. % ethane diol (which in case of human Hb was 35 vol. %). Ethane diol was analytical grade (»Merck«) and was used as supplied.

Carboxyhaemoglobin was obtained by bubbling CO through predialysed oxyhaemoglobin. The solutions for the final dialyses were also saturated with CO.

The absorption spectra in the visible range showed changes neither in respect to the ethane diol presence nor in comparisons before and after the relaxation measurements. The haem concentrations were calculated from the extinction coefficients given in the literature[7].

Methods

The proton magnetic longitudinal relaxation times, T_1, were measured with three pulsed spectrometers (made in the »Jožef Stefan« Institute, Ljubljana). The first one operating at 15 MHz was not phase-coherent and the free inducation decays after a $\pi/2 - \pi/2$ pulse sequence were read off the oscilloscope screen. Those operating at 32 and 24 MHz were phase-coherent, with a digital readout.

The sample temperature, achieved by thermostatted precooled nitrogen stream, was recorded with a Y. S. I. thermistor telethermometer directly immersed into the sample. The T_1 measurements were taken at 10—20 temperatures between — 30 and + 40 °C in two ways: (a) thermal equilibrium within 1 °C at any given temperature; (b) slow continuous heating from the lowest temperature. There was less than 5% difference between the (a) and (b) recording.

RESULTS

All the measurements were performed with solutions close to pH = 6. The concentrations (per haem) of six independent bovine ferrihaemoglobin preparations were between 2.8 and 6.6 mmol/l. The paramagnetic contribution to the relaxation rate ($1/T_1$) normalized to unit haem-concentration was derived

TABLE I

The molar (per haem) proton magnetic relaxation rates ($T_1^{-1}/s^{-1}\,mol^{-1}\,l$) due to dissolved bovine ferrihaemoglobin (in 0.1 M NaCl, pH = 6) with addition of ethane diol

ν_L/MHz	temp./°C	vol. % ethane diol		
		0	10	25
15	0	275, 394, 363 329 ± 20	366, 251, 459 359 ± 60	247, 174, 479 299 ± 92
	40	657, 606, 815 693 ± 60	896, 658, 935 829 ± 86	563, 714, 880 719 ± 94
	Δ	364 ± 60	470 ± 86	420 ± 94
32	0	290, 318, 334 314 ± 12	252, 368, 379 333 ± 40	444, 454, 418 439 ± 10
	40	755, 762, 1000 839 ± 80	629, 835, 849 771 ± 70	901, 800, 1025 908 ± 65
	Δ	525 ± 80	438 ± 70	469 ± 65

as already described[1]. The data from the low (0 °C) and high temperature range (40 °C) are collected in Table I together with the difference between the two which is related to the stereochemistry of the haem pocket.

The activation energies in Table II are obtained by assuming the Arrhenius relation for the thermally activated relaxation rates in the middle temperatures region.

TABLE II

The energies of activation (E_a/kcal mol^{-1}) for the thermally activated proton magnetic relaxation rates due to dissolved bovine ferrihaemoglobin (0.1 M NaCl, pH=6) *with addition of ethane diol.*

ethane diol vol. %	ν_L/MHz	
	15	32
0	5.78; 4.99; 3.80 4.85 ± 0.57	4.99; 3.46; 4.99 4.48 ± 0.44
10	3.84; 3.80; 4.23 3.96 ± 0.13	3.80; 3.46; 3.65 3.64 ± 0.10
25	4.11; 3.46; 4.88 4.15 ± 0.20	4.23; 4.23; 4.04 4.17 ± 0.02

As the results obtained with bovine ferrihaemoglobin did not reveal any systematic difference in relaxation rates on addition of ethane diol, measurements were done with human ferri- and CO-haemoglobin only with 35 vol. % ethane diol.

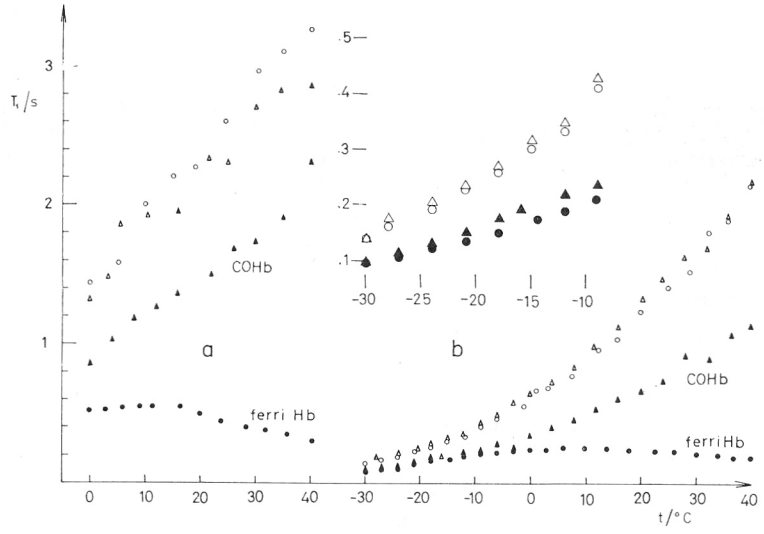

Fig. 1. The temperature dependence of longitudinal proton magnetic relaxation times, T_1 for aqueous solutions (0.1 M NaCl, pH = 6.4 ± 0.2) of human haemoglobin A without ethane diol (a), and with 35 v. % ethane diol (b). Circles and triangles refer to the solvents of the ferri-, and COHb solutions resp., which in turn are marked with the filled signs. The haemoglobin concentrations, mM per haem, were as follows: COHb — 5.1 (a) and 8.6 (b); ferri Hb — 5.1 (a) and 6.6 (b)

The T_1 data for human CO- and ferrihaemoglobin solutions and for the corresponding solvents are presented in Fig. 1. The relaxation rates normalized per haem concentration, $\Delta\frac{1}{T_1}$, in dependence on reciprocal temperature are given in Fig. 2. The finely drawn lines (either full or dotted for zero and 35 vol. % added $C_2H_6O_2$, resp.) are for the overall, ferrihaemoglobin or COHb contribution to the relaxation rates of solvent protons while the thickly drawn lines represent their relaxation rates due only to the presence of the paramagnetic haem iron.

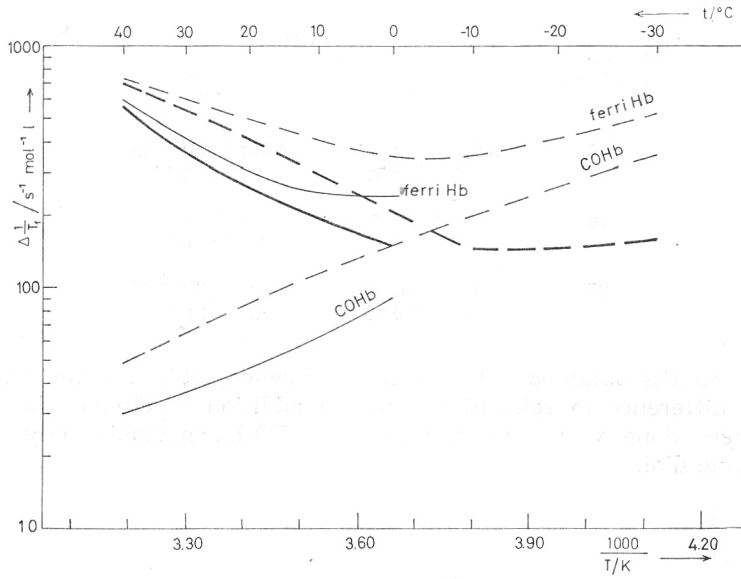

Fig. 2. The Arrhenius plot of the proton magnetic relaxation rates normalized per unit haem concentration, as derived from Fig. 1. The dashed lines refer to the solutions with 35 v. % ethane diol and the others to those without ethane diol. The finely drawn curves depict this relationship for the indicated solutions while the thickly drawn ones are obtained by subtracting the COHb relaxation rates from the ferri-Hb data, thus obtaining the relaxation rates induced solely by the presence of the paramagnetic haem iron in ferri Hb-solutions.

DISCUSSION

In the following discussion neither of the two existing molecular models[3] for the relaxation mechanism will be involved. Nor will the data be quantitatively evaluated by the existing theory, because the full interpretation of this kind of experiments is being treated elsewhere[1,3,8,9]. We are only interested here in the relative differences induced by the presence of ethane diol. It is thus sufficient to note that the low temperature relaxation rates as well as the difference between them and those at 40 °C are related to the distance from the haem iron and the relaxing proton(s). Hence, any changes in haem environment caused by addition of ethane diol would be reflected in the proton magnetic relaxation rates.

Comparison of the data presented in Table I shows no consistent dependence of the relaxation rates on ethane diol at constant frequency; they are equally spread around a mean value. The same statement holds for the rates

measured at 0 °C and 40 °C as well as the difference between them. The mean values of the subtractions are 420 ± 90 s^{-1} mol^{-1} l for 15 MHz and 480 ± 80 s^{-1} mol^{-1} for 32 MHz. All the single measurements are within the region of one σ. The difference in values at the two frequencies is within one σ as well.

The T_1 data on human haemoglobin (Fig. 2.) corroborate our conclusion that on the whole the haem surrounding is almost unaltered even in the presence of 35 vol. % of $C_2H_6O_2$. In the formerly discussed case of bovine ferrihaemoglobin (Table I) we had no correction of the diamagnetic contribution to the relaxation rates measured in solutions of the paramagnetic ferrihaemoglobin. It is not large, but it does influence the shape of the temperature dependence of the relaxation rates as shown in Fig. 2. where we subtract the CO-curve from the ferri-curve. The difference in the relaxation rates between 40 °C and those at low temperatures is 400 ± 20 s^{-1} mol^{-1} l for the H_2O solution and 540 ± 10 s^{-1} mol^{-1} for the solution with 35 vol. % of $C_2H_6O_2$. If this difference truly reflects some change in the haem environment (otherwise undetected by visible spectrophotometry) it is a slight one, because the distance of the interacting spins, r, is related to these experimental parameters by r^{-6}.

The two curves for CO-Hb, *i. e.* with and without $C_2H_6O_2$ are different. These relaxation rates are due to the interaction of solvent protons with those mainly at the surface of the diamagnetic protein (COHb), and therefore, the larger the rates the greater the interaction of solvent molecules with the protein surface. Hence, the interaction of solvent protons with the protein surface is more pronounced in the solution with 35 vol. % $C_2H_6O_2$ than in the one without it, so that it seems that ethane diol influences the structure of the hydration layer around haemoglobin and, perhaps through it, to a small degree the conformation of the haem pocket, but this conclusion has to be verified by deuterium and oxygen-17 relaxation measurements.

The activation energies (for the relaxation rates), closely related to the dynamics of the haem pocket conformation, are another test upon the similarity of the haem environment in the haemoglobin solutions with and without ethane diol irrespective of which molecular model is used to explain them. Changes in activation energies with addition of ethane diol would indicate differences in the conformational states of the haem environment, although nothing definite could be said about their nature.

The mean value for all the activation energies data presented in Table II, for bovine ferrihaemoglobin, which are not corrected for the diamagnetic contribution is 4.2 ± 0.2 kcal mol^{-1} (1 kcal = 4.184 kJ). The value is frequency independent which is to be expected and shows no tendency of changing with ethane diol addition. All the obtained values are within the experimental error except the data for samples with no ethane diol at 15 MHz and with 10 v. % ethane diol, at 32 MHz. Their deviation could be due to random error as the mean does not change with their rejection.

The two curves for human ferrihaemoglobin corrected for the diamagnetic (COHb) contribution have $E_a = 6.2$ kcal mol^{-1} (no $C_2H_6O_2$) and $E_a = 5.2$ kcal mol^{-1} (with 35 vol. % $C_2H_6O_2$).

The comparison of activation energy data supports the conclusion that ethane diol has no effect on the conformational state of the haem pocket which would result in changes of the relaxation process.

The low temperature part in Fig. 2. merits comment. The COHb correction becomes important at these temperatures and is in fact about more than half the value of the relaxation rates for the (uncorrected) ferriHb solution. With this correction, the paramagnetically induced relaxation rates are practically constant in this range of temperatures. As pointed out in ref. 1, no unique stereochemical parameter can be derived from the low temperature data because of the shape of the haem pocket. The relaxation rates at low temperatures are an overall measure of the accessibility of the haem pocket for solvent molecules and so Fig. 2. suggests that the conformational state of the haem pocket does not change from about — 10 °C down to at least — 30 °C. This means that for all practical purposes, when working without $C_2H_6O_2$, the measurement close to zero centigrades should be corrected by the corresponding value of the COHb — relaxation rate. This corrected low temperature value can then be subtracted directly from the uncorrected relaxation rate for the ferriHb solution at 35—40 °C where the COHb correction is of the same magnitude (~ 7%) as the experimental eror in T_1 determination. However, because the COHb correction changes E_a and because the minimum for the ferriHb curves is not always well defined above zero centigrade the most accurate way of doing all these measurements is to extend them below 0 °C and correct fully for COHb. Even if it involves some departure from the »standard« native state of the haemoglobin molecule in solution, this procedure may eliminate other systematic errors.

As for the possible overall perturbation of the native Hb conformation on addition of ethane diol our results are in agreement with the spectrophotometric[4] and other studies[5]. The proton magnetic relaxation measurements are complementary to the difference spectrophotometry[4] in that they yield information about the gross conformational state of the haem surrounding.

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IZVOD

Protonska magnetska relaksacija hemoglobina u vodenim otopinama s etan diolom

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Etandiol je bio dodavan vodenim otopinama goveđeg i ljudskog met(Fe^{III})- i CO-hemoglobina da bi se područje mjerenja nuklearne magnetske relaksacije proširilo na temperature ispod točke smrzavanja. Nije bilo značajnije razlike u brzinama relaksacije uz dodatak etandiola osim u slučaju ljudskog met- i CO-hemoglobina, što bi se moglo pripisati razlikama u hidratacijskoj ovojnici. Energije aktivacije dobivene iz Arrheniusovih grafova za brzine relaksacije goveđeg Hb također su (unutar ± 5%) nezavisne o etandiolu. Zaključuje se da se opće konformacijsko stanje džepa hema ne mijenja dodatkom etandiola, tako da se može mjeriti i do — 30 °C.

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