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Haem Accessibility in Monomeric Haemoglobins of *Glycera dibranchiata* and *Petromyzon marinus*, a Proton Magnetic Relaxation Study

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The temperature dependence of the longitudinal magnetic relaxation rates of water protons in solutions of differently liganded monomeric haemoglobins from *Petromyzon marinus* (fraction V) and *Glycera dibranchiata* (fraction III) was measured. The results were compared with horse and bovine myoglobins and interpreted according to the model of chemical exchange of water molecules. This exchange takes place between a site within the haem-pocket (but non-identical to the sixth-ligand position) and the bulk of the solvent.

Aquomethaemoglobin from *Glycera dibranchiata* only slightly enhances the relaxation rates of water protons between 0 °C and 40 °C and pH between 5.85 and 7.0. This finding is compatible with tight protein packing around the distal side of the haem.

In the solutions of aquomethaemoglobin from *Petromyzon marinus* up to 30 °C, the solvent-proton relaxation rates are determined by the rate of chemical exchange of water molecules. At higher temperatures the fast exchange mechanism takes place, an effect not observed in solutions of horse and bovine myoglobins. The distance of closest approach of water protons to the ferric ion of *Petromyzon* haemoglobin is at least 0.3 Å longer than in mammalian myoglobins.

Binding of fluoride to the ferric haem-irons of all the haemoglobins examined thus far enhances the proton relaxation rates relative to their aquomet forms, while in their nitrosyl complexes slightly lower rates were measured. These data indicate the sensitivity of the protein structure to the nature of the sixth ligand. The accessibility of the unpaired electron(s) in all the haemoglobins examined is in the order: NO- < aquomet- < fluoromet-forms.

From our previous and present data and from that found in literature, a scale of accessibilities of the ferric haem-irons for the exchangeable water molecules is compiled.

* Present address: National and University Library, Zagreb, P.O.B. 550, Yugoslavia. — S. M. recalls on this occasion with gratitude that Dr. Erich Herrmann introduced him to scientific research thirty years ago, through this Journal.

INTRODUCTION

The three-dimensional structures of five haemoglobins from different animal species are known to a resolution of 1.4 Å to 2.8 Å (for a review see ref 1). In spite of this knowledge, ligand binding in relation to the protein structure is nos yet fully understood. Additional insight into the structure and dynamics of the haemoprotein molecule in solution may be obtained by solvent proton magnetic relaxation (PMR). Because of the physical complexity of such a system, particularly in the case of tetrameric haemoglobins with two types of polypeptide chains, and because of the lack of a well-grounded theory for solvent proton relaxation induced by paramagnetic ions bound to macromolecules, a comparative study of differently liganded *monomeric* haemoglobins appears to be an useful approach.

Extensive PMR studies of mammalian and *Aplysia* myoglobins[2,3,4] and haemoglobins from *Petromyzon marinus*[5], *Chironomus thummi thummi*[3], *Lampetra fluviatilis*[6] and leghaemoglobin from *Lupinus luteus*[7] were completed in recent years.

In this paper we report additional results on differently liganded lampreyHb (*Petromyzon marinus* — fraction V) and the haemoglobin from *Glycera dibranchiata* (fraction III).

MATERIALS AND METHODS

Cyanomethaemoglobins from *Petromyzon marinus* (fraction V)[8] and *Glycera dibranchiata* (fraction III, the major monomeric fraction)[9] were prepared in the laboratory of Professor W. E. Love (Johns Hopkins University, Baltimore) by isoelectric focusing and subsequent crystallization in 50% ammonium sulphate. Horse aquometmyoglobin was kindly provided by Dr. K. Ruckpaul (Central Institute for Molecular Biology, Berlin-Buch) and bovine aquometmyoglobin was prepared according to Antonini and Brunori[10]. Crystals of cyanomet-derivatives were dissolved by extensive dialysis against 0.1 mol/dm^3 phosphate buffer, pH = 7.0, and then centrifuged for 30 minutes at 10000xg. Aquomethaemoglobins were prepared from the cyanomet-form by slight modification of the method of Dixon and McIntosh[11]: 0.2 ml of freshly prepared solution of sodium dithionite (10 mg/ml) was put at the top of a Sephadex G-25 column (1 × 12 cm), previously equilibrated with 0.1 mol/dm^3 phosphate buffer, pH = 7.0. The dithionite solution was led only to enter the column. Half a millilitre of cyanomethaemoglobin solution was then put at the top of the column. The red colour of the solution changed through the column, at first to violet (deoxyHb) and then to red of oxyHb. AquometHb was prepared by oxydation of oxyHb with concentrated solution of potassium ferricyanide, which was removed from the solution on the column described above. In both steps the final solutions of oxyHb and aquometHb were controlled spectrophotometrically, and the spectra were in good agreement with the known data[12].

Before T_1-measurements the solutions were ultrafiltered in Visking dialysis tubings to concentrations between 1.5 and 3.6 mmol/dm^3.

Lamprey fluorometHb was obtained by addition of sodium fluoride up to a concentration of 0.2 mol/dm^3.

NitrosylMb was prepared by reducing the ferric sample with sodium dithionite in the presence of sodium nitrite.[13] Nitrosylderivatives of *Glycera* and lamprey haemoglobins were obtained by flushing the sample reduced with dithionite, first with argon and then with nitrogen oxide, in a specially constructed NMR tube. The stream of nitrogen oxide was led through pellets of potassium hydroxide.

Concentrations of haemoglobins were determined spectrophotometrically by the cyan-met method[14] assuming the millimolar extinction coefficient $\varepsilon_{1cm} = 11.0$ at 540 nm, except in the case of *Glycera* haemoglobin for which it is 12.5[12]. All the spectrophotometric analyses were performed by using a Perkin-Elmer 124 spectrophotometer.

T_1-measurements were done with an apparatus described earlier[3], at 24 MHz, using a π—t—$\pi/2$ pulse sequence and the diode or phase detection system.

The pH values were determined by an Iskra (Kranj, Yugoslavia) pH-meter equipped with Radiometer G222B and K4112 semimicroelectrodes.

RESULTS

The longitudinal relaxation rate of solvent protons in solutions of haemoproteins bearing paramagnetic centres can be described by standard theory for the solutions of inorganic complexes of transition metals[15,16,17]. The total relaxation rate measured in a solution is given by:

$$R_1 = R'_{1p} + R_{1d} + R_1^0 \tag{1}$$

where R'_{1p} and R_{1d} are the paramagnetic and diamagnetic relaxation contributions of the haemoprotein and R_1^0 is the relaxation of the solvent. The paramagnetic contribution consists of two terms:

$$R'_{1p} = R_{1p,\,exch} + R_{1p,\,out} \tag{2}$$

$R_{1p,exch}$ is due to magnetic dipole-dipole interaction between the magnetic moment of the paramagnetic centre and the nuclear spin of solvent proton(s) which exchange between a site close to the paramagnetic centre and the bulk solvent. $R_{1p,\,out}$ arises from the dipolar interaction between the electronic spin and all the solvent protons outside its immediate neighbourhood. The paramagnetic relaxation, normalized per haem concentration is given by:

$$\frac{R'_{1p}}{C} = R_{1p} = \frac{n}{111} \cdot \frac{1}{T_{1M} + \tau_M} + R_{1p,\,out} \tag{3}$$

where n is the number of the exchanging protons, with the residence time τ_M in proximity to the paramagnetic centre, while T_{1M} is the relaxation time of these protons during τ_M. According to Solomon[18], $1/T_{1M}$ is given by:

$$\frac{1}{T_{1M}} = \frac{2\,\mu^2_{eff}\,\beta^2\gamma_I^2}{15r^6}\left[\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_S^2\tau_c^2}\right] \tag{4}$$

ω_I and ω_S are the proton and electron Larmor frequencies, respectively; γ_I is the proton magnetogyric ratio, μ_{eff} is the effective magnetic moment, β is the Bohr magneton, τ_c is the correlation time for the dipolar interaction and r is the distance of closest approach of solvent proton(s) towards the paramagnetic ion. A fully quantitative estimation of structural parametres by this theory for macromolecular systems is of questionable value[19,20]. The term $R_{1p,\,out}$ in equation (3) can be treated only qualitatively[21] for haemoproteins.

The terms R_1^0 and R_{1d} in equation (1) were determined experimentally as the relaxation rates of the solvent and cyanometHb solutions, respectively. The latter was used instead of the diamagnetic CO-form which is unstable for monomeric haemoglobins. The value of R_{1d} as measured on cyanometHb--solution, normalized per haem concentration, was 40—100 s^{-1} mol^{-1} dm^3 (from 40 °C to 0 °C). This is a low value usually obtained with the diamagnetic form. Namely, paramagnetic contribution to the PMR rates is negligible for the $S = 1/2$ iron ion with its extremely short electronic relaxation time[22,23]. There is, in addition, a steric hindrance from the CN^- ion for the water approach towards iron.

The temperature dependence of the paramagnetically induced relaxation rates between zero and 40 °C for aquometHb of *Glycera* and lamprey and also for the fluorometform of the latter are given in Figure 1. These rates are induced by the predominately high spin ($S = 5/2$) iron ion. The high spin form is deduced from the visible spectra. For *Glycera dibranchiata* this spin form has also been confirmed[24] by electron paramagnetic resonance at 1.6 K.

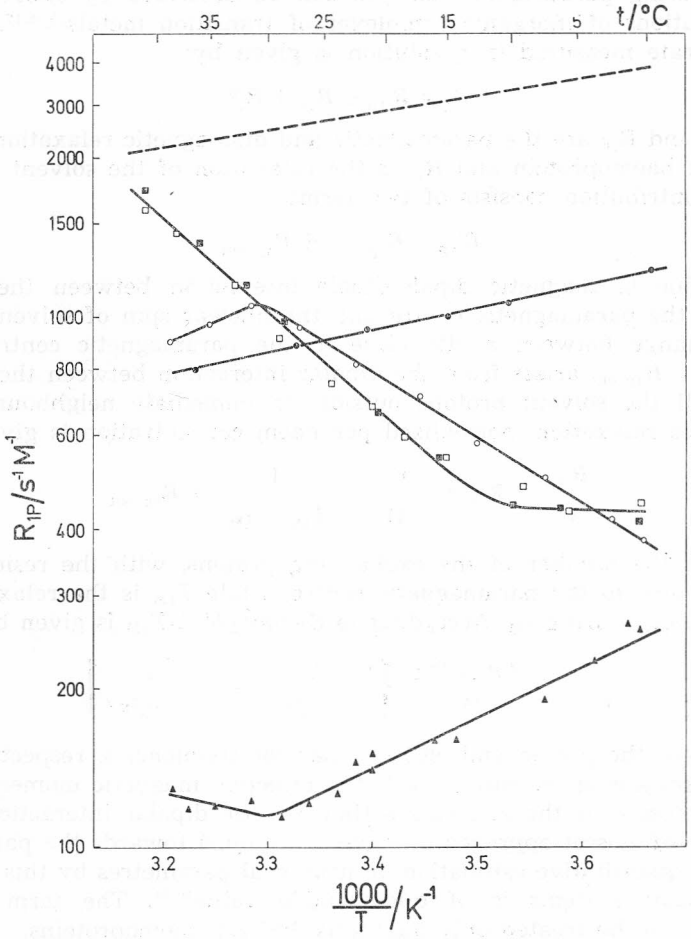

Figure 1.

▲ H_2OmetHb *Glycera dibranchiata*, pH = 7.0, 0.1 mol/dm³ phosphate buffer
○ H_2OmetHb *Petromyzon marinus*, pH = 7.0, 0.1 mol/dm³ phosphate buffer
□ H_2OmetMb horse, pH = 6.25, 0.1 mol/dm³ tris buffer, (from ref. 3)
■ Bovine H_2OmetMb, pH = 6.0, 0.1 mol/dm³ tris buffer
● F metHb *Petromyzon marinus*, pH = 7.0, 0.1 mol/dm³ phosphate buffer, 0.2 mol/dm³ NaCl
- - - F metMb horse (from ref. 3)

The *Glycera* aquometHb has an unusually low paramagnetic relaxation in comparison with many other haemoproteins. A decrease of such a low relaxation with temperature up to about 30 °C is consistent with the outer sphere relaxation

mechanism. A slight relaxation increase above 30 °C is suggestive of the onset of a thermally activated proton exchange process. Lowering pH from 7.0 to 5.85 has no influence on these relaxation rates.

The observed increase of the relaxation with temperature up to 30 °C in solution of lamprey aquometHb strongly indicates the mechanism of activated chemical exchange of water proton(s) ($\tau_M \gg T_{1M}$ in eq 3) between the haem pocket and bulk solvent, in good agreement with the results of Maričić and Rumen[5]. Only at the highest attainable temperatures (without protein denaturation) R_{1p} slightly decreases, which means that the fast exchange condition ($T_{1M} > \tau_M$) is fullfilled at these temperatures. This was not observed in ref. 5, but nearly the same effect was found with very similar aquometHb of *Lampetra fluviatilis*[6] for which a relaxation mechanism of this kind was also confirmed by stereochemical titration[3,6].

Addition of potassium fluoride to solutions of many different haemoproteins is known to cause abrupt change in the relaxation behaviour. The positive slope in the Arrhenius plot (Figure 1.) and a high absolute value of

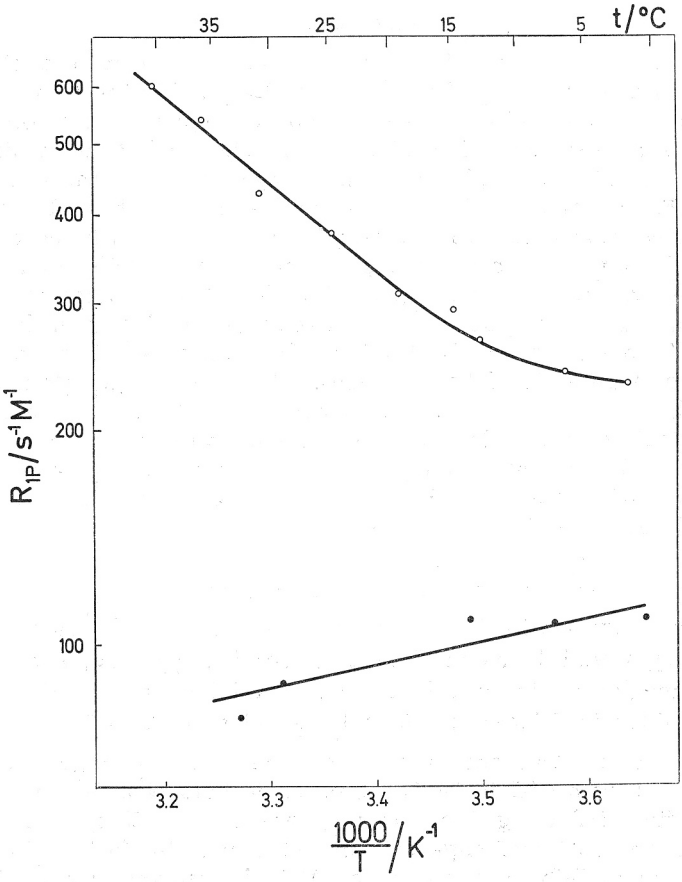

Figure 2.

○ NOMb horse, pH = 6.15, 0.1 mol/dm³ phosphate buffer
● NOHb *Petromyzon marinus*, pH = 7.0, 0.1 mol/dm³ phosphate buffer

the paramagnetic relaxation for lamprey fluorometHb are strong indications of the fast exchange mechanism. Addition of potassium fluoride to the solution of *Glycera* aquometHb up to very high concentrations enhanced the relaxation too, but because of the very high dissociation constant[12] the results cannot be interpreted quantitatively.

As it was shown for human haemoglobin, nitric oxide (NO) bound to the haem iron is a good probe in this type of experiments[25], in spite of considerable delocalization of the unpaired electron spin density[26,27]. The results for horse NO-myoglobin and lamprey NO-haemoglobin are shown in Figure 2. Binding of NO to *GlyceraHb* did not change the relaxation rate compared to the ferriHb solution. As it is clearly evident, the relaxation in the solution of nitrosylMb is determined by the thermally activated exchange mechanism, except in the low temperature range where the outer sphere relaxation is predominant. The much lower relaxation of the lamprey nitrosylHb is consistent with an outer sphere relaxation mechanism, especially in view of the higher rates observed with the ferri-form (Figure 1).

DISCUSSION

The results for solutions of various haemoproteins can be explained self-consistently by the exchange of water molecules outside the first coordination sphere of iron ion (but within the haem pocket) and the bulk solvent[21]. This statement is supported by the results of stereochemical PMR titration[3,7,28] for various methaemoglobins and by the measurements on horse-radish peroxidase[29] and catalase[30]. In corroborating this view, recently it has also been independently shown that there is no water molecule at the sixth coordination site of the haem in horse-radish peroxidase[31]. The efficient PMR enhancement for the fluoride- and nitrosyl-complexes of different haemoproteins eliminates completely the role of the haem-iron liganded water molecule in the relaxation mechanism. A comparison of the PMR results with those of the recent X-ray crystal structure analysis of fluorometHb[32] also supports the proposed model. Namely, in both α- and β-pockets of haemoglobin there is a water molecule in close contact with the fluoride ion. The distance of 4.3 Å between the oxygen atom and the centre of the haem is in excellent agreement with the corresponding distance estimated from the PMR dispersion data[33]. Hence, the experimental results will be discussed in view of the model described above.

An almost complete outer sphere relaxation mechanism in the temperature range from 0 °C to 40 °C for *Glycera* aquometHb (Figure 1), as well as the same paramagnetic relaxation in the nitrosyl-complex strongly indicate a very tight protein folding around the haem. Compared with horse or bovine myoglobin and lamprey haemoglobin, this could partly be explained by the different distal aminoacid residue in *GlyceraHb* (leucyl instead of histidyl)[9].

Lamprey aquometHb shows a faster chemical exchange (Figure 1) of water molecules from 0 °C to 30 °C compared to horse and bovine myoglobins (especially after deducting the outer sphere contribution), and, at higher temperatures, the fast exchange sets in. Although the fast exchange is not observed in aquometMb solutions up to 40 °C, the relaxation rate at this temperature is higher than for lampreyHb. Assuming one exchangeable water molecule in both cases, T_{1M} in aquometMb is less than one half that for

lampreyHb. This means that the water molecule approaches the ferric ion in myoglobin closer than it does in lampreyHb, by more than 0.3 Å. A similar conclusion can be drawn from the difference in the relaxation rates in fluoride complexes where myoglobin shows approximately twice as high relaxation rates with respect to lampreyHb (see the dashed curve in Figure 1.). This is most likely due to a 0.3 Å — 0.4 Å shorter distance of closest approach (r in equation 4) in fluorometMb, because the correlation time, τ_c, should be very similar if not equal in both cases. Additional independent support for this conclusion can be derived from the different temperature dependences of the relaxation rates for the nitrosylderivatives of horse myoglobin and of lamprey haemoglobin (see Figure 2.) In these two cases the actual relaxation mechanism is different: for horse NO-Mb there is a chemical exchange as for its aquomet form, whereas for the lampreyNO-Hb there is the outer sphere relaxation, as completely distinct from aquomet lampreyHb.

Fluoro-, aquo and nitrosyl-complexes of horse myoglobin and lamprey and human haemoglobins[3,25] show marked differences in the relaxation mechanism with respect to the particular ligand. According to equations 3 and 4, these different relaxations cannot be attributed to the change of the physical parameters such as magnetic moment and correlation times. In fluorometMb the fast exchange condition ($T_{1M} \gg \tau_M$) is fulfilled at — 20 °C [3] whereas at the same temperature in the aquometMb the relaxation is determined by the outer sphere relaxation mechanism (very long τ_M). Assuming an activation energy of 10 kcal/mol for water exchange in aquometMb[3,17] in the whole temperature range from 40 °C to — 20 °C, this difference can be explained by more than a hundred-fold increase of the water exchange rate after addition of fluoride ion to an aquometMb solution. A qualitatively similar conclusion can be drawn for lamprey and human haemoglobins. In the nitrosyl complexes of all the three haemoproteins the rate of water exchange is significantly lower than in the corresponding aquometderivatives. The general conclusion is therefore that binding of different ligands induces a significant conformational change in the protein structure on the distal side of the haem[34].

With respect to all PMR results for various and differently liganded haemoglobins and myoglobins the accessibility of the ferri ion for exchangeable water molecules rise in the following order:

Glycera dibranchiata(III) ≃ *Aplysia*[4] < β-chains of HbA[35] < α-chains of HhA[35] < *Petromyzon marinus* (III, IV, V)[5] ≃ *Lampetra fluviatilis*[6] < horse[17], seal[2] and bovine Mb < *Chironomus thummi*(III)[3] ≃ *Petromyzon marinus*(II) < < legHb[7].

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SAŽETAK

Pristupačnost hema u monomernim hemoglobinima *Glycera dibranchiata* i *Petromyzon marinus* određena protonskom magnetskom relaksacijom

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Mjerena je temperaturna zavisnost brzine longitudinalne magnetske relaksacije protona vode u otopinama različito ligandiranih monomernih hemoglobina vrsta *Petromyzon marinus* (frakcija V) i *Glycera dibranchiata* (frakcija III), a rezultati su uspoređeni s onima dobivenim na mioglobinima konja i goveda. Pri interpretaciji

rezultata poslužio je model prema kojemu se utjecaj paramagnetskog centra širi na cijelo otapalo putem kemijske izmjene molekule vode izvan prve koordinacijske ljuske željeznog iona, ali još uvijek unutar džepa hema.

Akvomethemoglobin vrste *Glycera dibranchiata* vrlo malo utječe na brzinu relaksacije protona vode u temperaturnom području od 0 °C do 40 °C te između pH = 5,85 i pH = 7,0, što ukazuje na vrlo zatvorenu proteinsku strukturu s distalne strane hema. Suprotno tome, relaksacija protona vode u otopinama akvomethemoglobina vrste *Petromyzon marinus* određena je sve do 30 °C brzinom njihove kemijske izmjene, nakon čega, za razliku od mioglobina konja i goveda, dolazi do brze izmjene. Unatoč tome, udaljenost na koju može prići molekula vode veća je nego kod mioglobina za najmanje 0,3 Å. Vezivanje fluorida na željezni ion hema uzrokuje znatan porast brzine relaksacije pa čak i promjenu relaksacijskog mehanizma od aktivirane u brzu izmjenu u gotovo svih do sada ispitivanih hemoglobina, što se može objasniti otvaranjem proteinske strukture s distalne strane hema. Vezivanje dušikova monoksida na fero-hem različitih hemoglobina smanjuje brzinu relaksacije protona vode u odnosu na akvomet-formu, što je posljedica zatvorenije strukture proteina oko šestog liganda.

Uspoređujući rezultate protonske magnetske relaksacije na različitim i različito ligandiranim hemoglobinima i mioglobinima pristupačnost molekula vode željeznom ionu hema raste ovim redom: *Glycera dibranchiata* (III) $\approx$ *Aplysia* $<$ β-lanac HbA $<$ $<$ α-lanac HbA $<$ *Petromyzon marinus* (III, IV, V) $\approx$ *Lampetra fluviatilis* $<$ Mb konj, tuljan i govedo $<$ *Chironomus thummi thummi* (III) $\approx$ *Petromyzon marinus* (II) $<$ $<$ legHb.

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