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A PROTON MAGNETIC RELAXATION STUDY OF THE INTERACTION OF ISOLATED SHEEP METHAEMOGLOBINS A, B AND C WITH INOSITOL HEXAPHOSPHATE

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Abstract—1. Sheep aquomethaemoglobins A and B exert similar paramagnetic influence on the solvent-proton magnetic relaxation (PMR) rates as observed in human HbA, even in the presence of inositol hexaphosphate (IHP).

2. On the other hand, phosphate-free methaemoglobin C induces smaller PMR rates due to the restricted haem-accessibility.

3. Here IHP also binds, although with smaller affinity and with different structural consequences from the other two sheep haemoglobins. Some implications of these findings are discussed here.

INTRODUCTION

Four different haemoglobins (Hb's) can be isolated from the blood of adult sheep. Their presence is determined by genotype and certain physiological conditions (Harris & Warren, 1955; Blunt & Evans, 1963; Braend *et al.*, 1964; van Vliet & Huisman, 1964; Vaskov & Efremov, 1967). Three of these haemoglobins differ in β chains ($HbA = \alpha_2^A\beta_2^A$, $HbB = \alpha_2^A\beta_2^B$, $HbC = \alpha_2^A\beta_2^C$), while one differs in the α chains ($HbD = \alpha_2^D\beta_2^A$). The amino acid sequence of both α and β chains of sheep haemoglobins have already been reported (Dayhoff, 1969).

It has been shown that sheep haemoglobins are not able to bind 2,3-diphosphoglycerate anion (Antonini & Brunori, 1971; Blunt, 1972). In addition, the oxygen binding properties of the three haemoglobins also differ to a certain extent (Huisman & Kitchens, 1968). The aim of this study is to compare the sterical properties of the environments of the haems in the three β-chain variants. Inositol hexaphosphate (IHP), the most potent among allosteric polyanions (effectors) in solutions of various haemoglobins, influences the haem-environments differently, as was observed here by proton magnetic relaxation (PMR).

EXPERIMENTAL

Haemoglobins A, B and C were isolated by preparative chromatography (Dozy *et al.*, 1968) of haemolysate from Ovche pole cross breed sheep (Ovis aries). HbC was induced by severe bleeding of a sheep of HbA phenotype (Efremov & Braend, 1966). After oxydation with ferricyanide the solutions were extensively dialysed against 0.05 M Tris–HCl buffer containing 0.1 M NaCl and 5×10^{-4} M EDTA, pH 6.5. Inositol hexaphosphate (Sigma) was added from a buffered stock solution up to the molar ratio 1:1 regarding the haem. The Hb concentrations were determined spectrophotometrically by the cyanmet-method (Zijlstra & Van Kampen, 1960) using the absorbance coefficient $E_{540\,nm}^{1\,cm} = 11 \times 10^3\ M^{-1}$. The samples for PMR measurements were 3–4 mM per haem.

The temperature dependence of the molar solvent-proton longitudinal magnetic relaxation time, T_1, was measured by the π-t-$\pi/2$ pulse sequence at 24 MHz as previously described (Vuk-Pavlović *et al.*, 1974).

RESULTS AND DISCUSSION

The paramagnetically induced molar solvent-proton magnetic relaxation rates due to the presence of aquomet (=acid ferric) derivatives of sheep Hb's A, B and C, with and without IHP, are plotted against reciprocal absolute temperature in Figs. 1 and 2. Owing to the overall similarity in molecular shape of human and sheep tetramers, any difference in the diamagnetic interaction with solvent–protons is very unlikely. The rates in Figs. 1 and 2 were therefore calculated by subtracting the diamagnetic contribution measured for human HbA.

It is evident that in all the three phosphate-free metHb solutions (Figs. 1 and 2) the PMR rates are thermally enhanced and show an increase in the rate of proton fluctuation between the haem-pocket and bulk of the solvent (Vuk-Pavlović *et al.*, 1974). The PMR-behaviour in solutions of met forms of HbA and HbB is of a similar magnitude as measured for

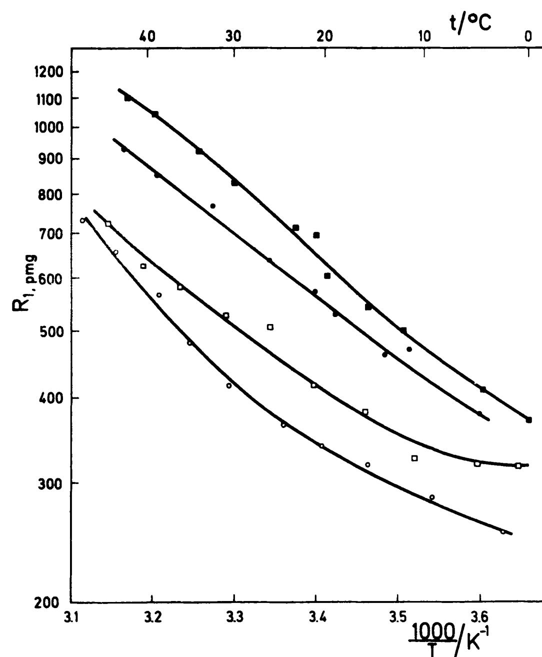

Fig. 1. The reciprocal temperature dependence of the paramagnetically induced solvent–proton magnetic relaxation rates in the acid solutions (pH 6.5) of sheep methaemoglobins A and B with and without inositol hexaphosphate (IHP). Circles: HbA; squares: HbB; open symbols: phosphate-free solutions; filled symbols: solutions containing an equimolar (per haem) concentration of IHP.

human metHbA (Maričić *et al.*, 1966). The addition of IHP to these two sheep Hb's enhances the PMR rates; this effect is more pronounced at higher temperatures and for HbB than for HbA. On the other hand, the increase of the PMR rates in acid ferric HbC with temperature is smaller and the IHP-influence diminishes with increasing temperature, becoming almost negligible at about 40°C.

The differences in the PMR rates in the phosphate-free solutions of HbA and HbB are minor and not very informative. On the other hand, IHP induces in them effects similar in magnitude to that in human HbA (Pifat *et al.*, 1974). This confirms that IHP binds to HbA and HbB, though diphosphoglycerate does not, as mentioned in the Introduction. It is plausible to assume that IHP binds to an analogous, though not equivalent, place as in human HbA, i.e. between the β chains (Arnone & Perutz, 1974), because IHP requires lower sterospecificity of the binding site due to its more negative charge than that of diphosphoglycerate. The IHP-induced changes of the haem-pocket structure may be related to the hypothesis of Asakura and Sono (1974), namely that the conformation of the haem-vinyls may modulate the iron–oxygen affinity. It would be interesting to measure the oxygen-dissociation curves of sheep Hb's A and B in the presence of IHP. It may be predicted on the basis of the present results that IHP should influence the oxygenation similarly as observed for human haemoglobin by lowering the affinity, i.e. by increasing $p_{1/2}$.

We also took the difference spectra in the visible range (using a Cary 17 spectrophotometer) with and without IHP at room temperature for each of the three Hb's. For HbA and HbB these spectra are practically identical to those reported by Perutz *et al.* (1974) for human adult haemoglobin, and therefore they are not reproduced here. Surprisingly, contrary to our PMR-evidence for IHP-effect, in HbC no peak in the difference spectrum was observed.

The PMR rates in the phosphate-free HbC are less enhanced by the increase of temperature than for HbA and HbB. Its visible electronic spectrum at pH 6.5 indicated no signs of more low-spin species than typical for acid methaemoglobins. Therefore, the PMR rates of HbC reflect the dynamics of the solvent as modulated by the haem-pockets which appear less accessible than in other two sheep haemoglobins. If comparisons with human HbA may be drawn, then this should be ascribed mainly to α chains, since β affect the PMR rates only slightly (Markovska *et al.*, 1975).

The IHP-effect in the solution of HbC merits special comment. Due to a deletion the β^C chains lack four amino acid residues (compared to β^A and β^B chains) at the N-termini (Dayhoff, 1969), and therefore they cannot be expected to bind IHP at the same site. Yet the observed increase of the PMR rate upon the addition of IHP suggests that it does interact with HbC tetramer. This effect is most evident at lower temperatures, where the dominant relaxation mechanism is the outer-sphere relaxation (Vuk-Pavlović *et al.*, 1974). It is due to the long-range effect of the (iron) electronic spin on the nuclei outside the site of closest approach to the iron (inside the haem-pocket). The increase of the PMR rates in the outer

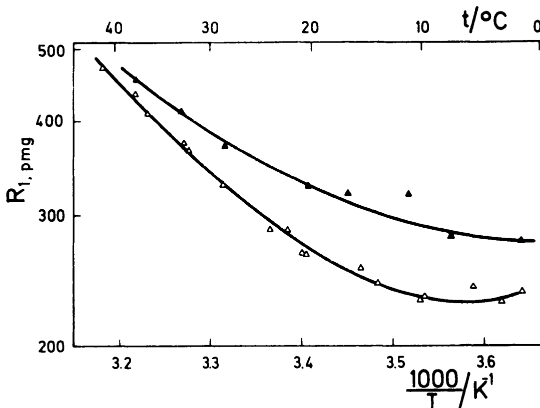

Fig. 2. The same as in Fig. 1, but for sheep methaemoglobin C. Open triangles: phosphate-free solution; filled triangles: equimolar amount (per haem) IHP added.

sphere' due to IHP may be interpreted in terms of closer contact of the paramagnetic centre and the bulk of the solvent, i.e. as a loosening of the part of the protein matrix that forms the haem-pocket. The unexpected 'damping' of the thermal enhancement of the PMR rate may be rationalized by non-specific and weak binding site(s) for IHP. Due to weak interaction with the protein, IHP may dissociate at higher temperatures, so that the PMR rates become similar to those measured in the absence of IHP. From the data in Fig. 2 it is not possible to determine the exact nature of the interaction between HbC and IHP. It may bind (somewhere) between β chains like in human haemoglobin (Arnone & Perutz, 1974), but the possibility of existence of yet another low-affinity binding site cannot be excluded *a priori*. If this is the case, such 'low-affinity' sites of interaction with highly charged anions may exist also in other haemoglobins, but the consequences of such binding might be 'masked' by the effect of those allosteric molecules which bind 'specifically' and strongly.

The effect of IHP on PMR in the solution of MetHbC with none on the visible spectra points to the conclusion that the haem-iron spin-state and the globin conformation in HbC are not necessarily linked. Pifat *et al.* (1974), Hensley *et al.* (1975) and Pennely *et al.* (1975) using different techniques came to the similar conclusions regarding human and carp haemoglobins, respectively.

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REFERENCES

ANTONINI E. & BRUNORI M. (1971) *Hemoglobin and Myoglobin in their Reaction with Ligands*, pp. 248–249. North-Holland, Amsterdam.

ARNONE A. & PERUTZ M. F. (1974) Structure of inositol hexaphosphate–human deoxyhaemoglobin complex. *Nature, Lond.* **249**, 34–36.

ASAKURA T. & SONO M. (1974) Optical and oxygen binding properties of spirographis, isospirographis, and 2,4-diformyl hemoglobins. *J. biol. Chem.* **249**, 7087–7093.

BLUNT M. H. (1972) Hemoglobin function in the developing goat and sheep. *Anim. Blood Grps biochem. Genet.* **3**, 211–217.

BLUNT M. H. & EVANS J. V. (1963) Changes in concentration of potassium in the erythrocytes and in haemoglobin type in Merino sheep under a severe anaemic stress. *Nature, Lond.* **200**, 1215–1216.

BRAEND M., EFREMOV G. & HELLE O. (1964) Abnormal haemoglobin in sheep. *Nature, Lond.* **204**, 700.

DAYHOFF M. O. (1969) *Atlas of Protein Sequence and Structure*, Vol. 4, pp. D 219–220. Natn. Biomed. Res. Found., Silver Spring, MD.

DOZY A. M., KLEIHAUER E. F. & HUISMAN T. H. J. (1968) Studies on the heterogeneity of hemoglobin—XIII. Chromatography of various human and animal hemoglobin types on DEAE-Sephadex. *J. Chromatog.* **32**, 723–727.

EFREMOV G. & BRAEND M. (1966) Haemoglobin N of sheep. Age, breed and seasonal distribution. *Anim. Prod.* **8**, 161–169.

HARRIS H. & WARREN F. L. (1955) Occurrence of electrophoretically distinct haemoglobins in ruminants. *Biochem. J.* **60**, xxix.

HENSLEY P., EDELSTEIN S. J., WHARTON D. C. & GIBSON Q. H. (1975) Conformation and spin state in methemoglobin. *J. biol. Chem.* **250**, 952–960.

HUISMAN T. H. J. & KITCHENS J. (1968) Oxygen equilibria studies of the hemoglobins from normal and anemic sheep and goats. *Am. J. Physiol.* **215**, 140–146.

MARIČIĆ S., RAVILLY A. & MILDVAN A. S. (1966) Proton relaxation measurements of dissolved and crystalline methemoglobin and metmyoglobin. In *Hemes and Hemoproteins* (Edited by CHANCE B., ESTABROOK R. W. & YONETANI T.) pp. 157–170. Academic Press, N.Y.

MARKOVSKA B., EFREMOV G. D., VUK-PAVLOVIĆ S., BENKO B. & MARIČIĆ S. (1975) Structural non-equivalence of the α- and β-heme-pockets in human methemoglobin. A proton magnetic relaxation study in solution. *Archs Biochem. Biophys.* **171**, 337–342.

PENNELLY R. R., TAN-WILSON A. L. & NOBLE R. W. (1975) Structural states and transitions of carp hemoglobin. *J. biol. Chem.* **250**, 7239–7244.

PERUTZ M. F., FERSHT A. R., SIMON S. R. & ROBERTS G. C. K. (1974) Influence of globin structure on the state of haem—II. Allosteric transitions in methaemoglobin. *Biochemistry* **13**, 2174–2186.

PIFAT G., BENKO B., MARIČIĆ S. & VUK-PAVLOVIĆ S. (1974) A proton magnetic relaxation study of the interaction between methaemoglobin and inositol hexaphosphate. *Croat. Chem. Acta* **46**, 145–155.

VASKOV B. & EFREMOV G. (1967) Fourth haemoglobin type in sheep. *Nature, Lond.* **216**, 593–594.

VAN VLIET G. & HUISMAN T. H. J. (1964) Changes in the haemoglobin types of sheep as response to anaemia. *Biochem. J.* **93**, 401–409.

VUK-PAVLOVIĆ S., BENKO B. & MARIČIĆ S. (1974) The molecular mechanism of the temperature enhancement of proton magnetic relaxation rates in methaemoprotein solutions. *Biophys. Chem.* **2**, 359–368.

ZIJLSTRA W. G. & VAN KAMPEN E. J. (1960) Standardization of hemoglobinometry—I. The extinction coefficient of hemiglobincyanide at $\lambda = 540$ nm: E^{540}_{HiCn}. *Clin. chim. Acta* **5**, 719–726.