CROATICA CHEMICA ACTA CCACAA 46 (2) 145—155 (1974)

CCA-831

YU ISSN 0011-1643
547.96:577.1
Original Scientific Paper

A Proton Magnetic Relaxation Study of the Interaction between Methaemoglobin and Inositol Hexaphosphate

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Received June 3, 1974

Inositol hexaphosphate is the strongest allosteric effector even for the metform of haemoglobin. Its effects upon the quaternary structure of the tetramer have been studied in relation to the overall conformational state(s) of the haem-pockets in aqueous solutions of human haemoglobins. The method used, proton magnetic relaxation, yields information about the accessibility of solvent protons towards the haem-iron. No differences in the relaxation rates were detected by this method between the unstripped carbonmonoxyhaemoglobin and the phosphate-stripped sample in the presence and absence of IHP. There are considerable changes in those relaxation rates due to the paramagnetic haem-iron of aquomethaemoglobin when IHP is added to the stripped adult haemoglobin, but none is observed for the foetal haemoglobin, although a similar shift in the spin-state equilibrium is expected for both haemoglobins on addition of IHP. Neither was there any change with IHP in solutions of adult fluoromethaemoglobin. It is concluded that there is no tightening of the haem-pockets upon addition of IHP to solutions of any of the three haemoglobin samples. An increase in the accessibility of the haem-pockets is probable only for the aquometform of the adult haemoglobin. It is suggested that the structural aspect of ligand affinity, i. e. the haem-pocket conformation, is not as decisive in altering the affinity by IHP as is possibly the change in the haem-iron spin-state induced by IHP-binding.

INTRODUCTION

In a series of papers Perutz and his colleagues described the influence of globin structure on the state of the haem in deoxyhaemoglobin[1] and in methaemoglobin[2,3] as part of the elucidation of the allosteric mechanism. It has been stressed by Perutz *et al.*[1] that the affinity for oxygen in the tense, T-state quaternary structure »... becomes lower not simply because the haem pockets become too narrow to accommodate the oxygen molecule but because the globin somehow lowers the intrinsic ligand affinities of the haems themselves«. These two aspects of affinity have also been discussed by Greer[4] in relation to the quaternary/tertiary structure changes when the oxygen is bound or released. Perutz *et al.*[1,2,3] produced ample evidence about the effect of globin conformation on the haems, an effect which »lies at the heart of the problem of haem-haem interaction«. The experimental techniques they used were light absorption spectra, circular dichroism, sulphydryl reactivity, magnetic susceptibility, and high resolution nuclear magnetic resonance (nmr.)

in the aromatic region of the spectra. Except for the sulphydryl reactivity the other techniques yield information preponderantly about the electronic configurations of the haem and in particular about the spin-state of the iron-ion. The obtained results are influenced by the surrounding protein matrix as well, the nmr. spectra being most sensitive to it. The latter provided very convincing evidence as to the increased spin densities at the protons of the porphyrin when inositol hexaphosphate (IHP) binds to methaemoglobin. The spectrophotometric methods and the sulphydryl reactivity are also sensitive to the conformational changes at the α_1/β_2 subunit contacts produced by this most efficient allosteric effector among the phosphates. These data are therefore relevant for the protein part some distance away from the haem--pocket and the solvent.

We studied the other factor, the haem accessibility. More specifically, the aim was to find out to what extent the haem-pocket in methaemoproteins acquires an altered accessibility when IHP shifts the quaternary conformational equilibrium towards the T-state while there is no loss or acquisition of ligand. The proton magnetic relaxation (PMR) method is suitable in this respect because the »conformational probe« is in fact the water molecule next-neighbour to the one bound at the sixth site of the iron-ion[5]. The latter is a strong paramagnetic centre, so that owing to the magnetic interactions of the iron and proton spins the measurable parameter, the longitudinal relaxation time, T_1, depends on their mutual location, the »H_2O-probe« and the sixth ligand (H_2O) being present in the haem-pocket irrespective of the type of the qaternary structure. Because of a temperature dependent communication of H_2O-molecules between the haem-pockets and bulk solvent the method yields information on the overall dynamics of this segment of the protein structure looking at it from the solvent side, *i. e.* from the direction of ligand approach.

EXPERIMENTAL

The human adult haemoglobin was prepared according to Cameron and George[6]. In order to remove most of the originally present phosphates, and the ferro- and ferricyanide ions from the oxydation procedure, a part of the solutions was passed through a Sephadex G-25 (fine) column as described by Berman *et al.*[7]. The solutions of stripped methaemoglobin and the unstripped ones were submitted to the extensive final dialysis against 0.1 M sodium chloride, 5×10^{-2} M TRIS, 5×10^{-4} M EDTA, pH close to 6.

The carbonmonoxyhaemoglobin was prepared by blowing CO through the solutions either before or after the stripping procedure.

The solutions of the stripped methaemoglobin were deuterated by dialysis against 0.1 M NaCl in D_2O, pH (measured) 5.2, with 35% ethane diol, $C_2H_4(OD)_2$, the latter prepared as before[5].

The concentrations were determined by the cyanmet-method[8] and they ranged from 2.5 to 6.0 mM per haem.

Inositol hexaphosphate (Sigma, lot 72C-1180) was added in all cases in an equimolar (per haem) amount, except in one of the foetal haemoglobin preparations where the IHP/haem ratio was increased to 2.5.

The relaxation measurements (T_1) were performed as described before[5]. The $\pi/2 - t - \pi/2$ pulse sequence (Figs. 1 and 2) is liable to a small systematic error in determining T_1 as distinct from the $\pi - t - \pi/2$ sequence, but the comparisons with regard to the IHP-effect were always made with data obtained only by one of the two ways of measuring T_1.

Stripped fluoromethaemoglobin in the H_2O and D_2O solutions was obtained after the described dialysis by addition of sodium fluoride to a final concentration of 0.2 M.

The human foetal haemoglobin was prepared from the blood of 18—20 week old foeti. The blood was haemolysed by freezing. In one case the haemolysate was treated according to Kajita *et al.*[9] in order to obtain the pure HbF_{II} component. The other preparation was as for the adult haemoglobin, so that the final solution contained also a few percent of HbF_I and HbA components. However, in both cases the methaemoglobin solutions were passed through the Sephadex G-25 (fine) columns, with subsequent dialysis as for analogous preparations of HbA.

RESULTS AND DISCUSSION

The interpretation of proton magnetic relaxation (PMR) in methaemoglobin solutions with regard to the role of phosphates in the conformational state(s) of the haem-pockets must include the *diamagnetic* contribution of the protein matrix to the relaxation rates. The latter was examined using the diamagnetic carbonmonoxyhaemoglobin. As can be seen from Fig. 1 the temperature dependence of the relaxation rates induced by COHb is practically the same for solutions of COHb prepared in the ordinary way (two preparations), and for the phosphate-stripped COHb (two preparations), as well as for that solution of stripped COHb to which IHP was added. The absolute values of these relaxation rates (per haem) are less than 10% of those induced by methaemoglobin in solutions. Eley *et al.*[10] found somewhat larger diamagnetic contribut-

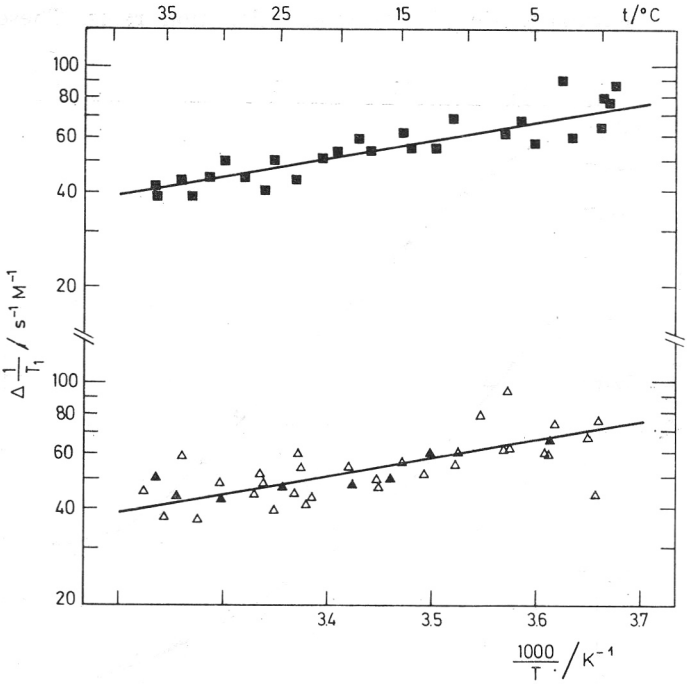

Fig. 1. The temperature dependence of the longitudinal PMR rates induced by the interaction of solvent protons with human (A) carbonmonoxyhaemoglobin in 0.1 M NaCl aqueous solutions, normalized per haem concentration. The symbols refer to the following samples:

		pH	C_{COHb}/mM
unstripped,	■	6.5, 7.0	5.1, 5.4
stripped,	△	6.4, 6.4	5.5, 5.2
△+ IHP,	▲	6.9	5.4

The least-squares straight line is the same for both sets of data.

ion of COHb, which might possibly be due to methaemoglobin admixture. It has been shown by Gray and Gibson[11] that IHP, as distinct from DPG, binds firmly to the liganded forms of haemoglobin and not only to deoxyhaemoglobin. It also binds to COHb[12,2]. One would thus expect that owing to the binding of IHP to COHb the R→T shift in the quaternary conformational equilibrium may possibly alter the interaction of water with the (changed) protein surface and hence the diamagnetic contribution to the solvent proton relaxation rates. The magnitude of such an effect, if it exists for COHb at all, is not large enough to be observed by the PMR method as we use it, which is revealed in Fig. 1. Therefore, whatever changes there may be in the relaxation rates due to methaemoglobin after the addition of IHP, such data may indeed be discussed purely in terms of conformational changes around the haem-pocket. These are sensed by the dipole-dipole interaction of solvent protons with the *paramagnetic* haem-iron, an effect three orders of magnitude greater for equal spin separation than is the diamagnetic effect discussed above.

The mean-straight-line from Fig. 1 was used in subtracting the contribution of the diamagnetic relaxation rates which are inherently measured in those induced by methaemoglobin, too. The results in Figs. 2 and 3 represent the remaining paramagnetic dipole-dipole relaxation rates. These will now

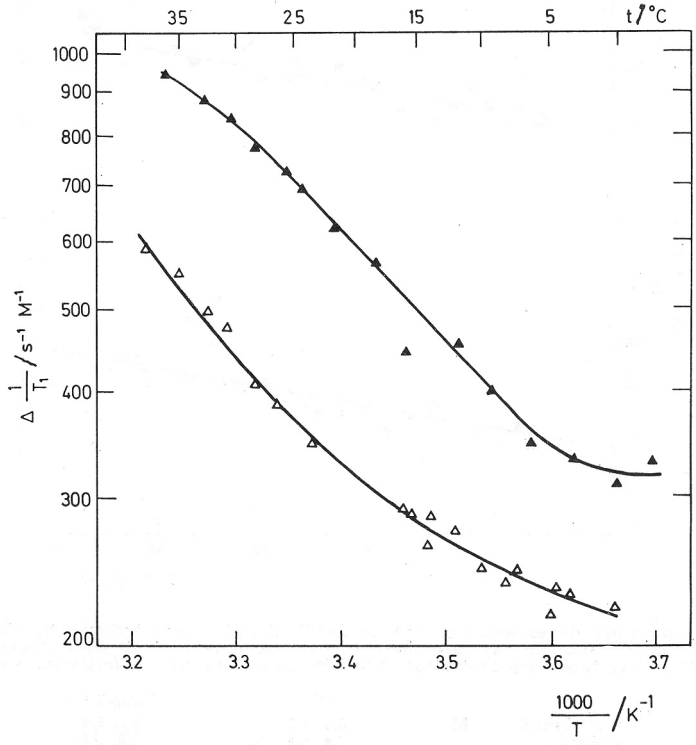

Fig. 2. The temperature dependence of the PMR rates due to the presence of the paramagnetic haem-iron in 0.1 M NaCl, pH = 6.2, aqueous solutions of human (A) aquomethaemoglobin (c = 4.5 mM); △ = stripped sample; ▲ = △ + IHP.

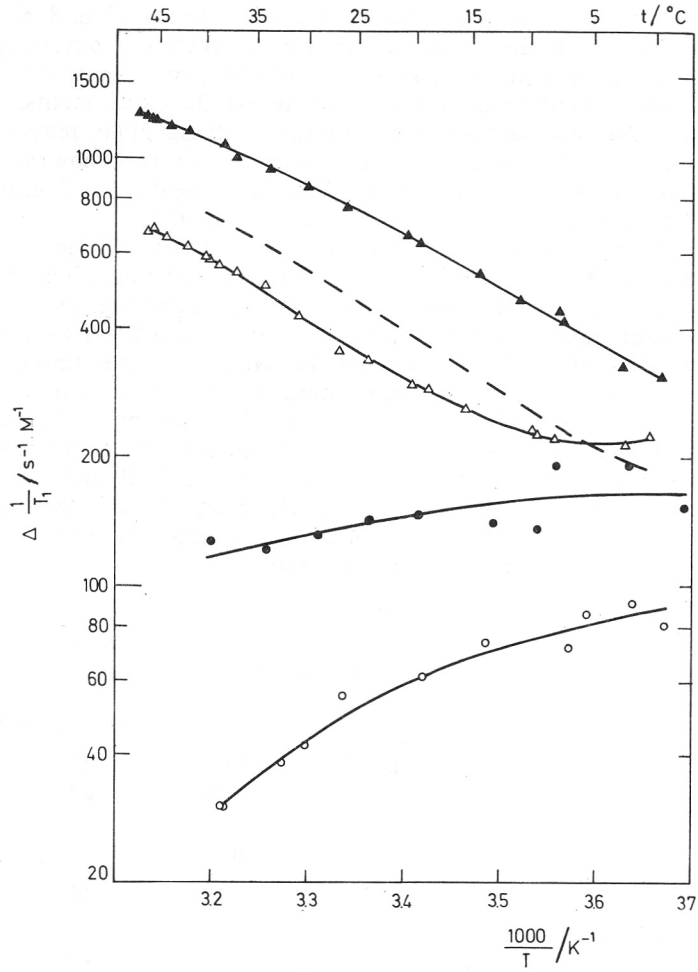

Fig. 3. The temperature dependence of the PMR rates as in Fig. 2, but from independent preparations and the $\pi - \pi/2$ pulse sequence (see text):

		pH (measured)	C/mM
unstripped,	- - -	6.0	4.4
stripped,	△	6.0	5.1
△ + IHP,	▲	6.0	5.1
stripped »D_2O«,	○	5.1	2.9
○ + IHP,	●	5.1	2.9

be discussed in terms of the relaxation by proton exchange between the haem-pocket and bulk solvent, a mechanism which has recently been verified[5].

There is no doubt that the relaxation rates approximately double in the presence of IHP. Those at low temperatures near 0 °C are not influenced by the proton exchange mechanism because the rate of this exchange diminishes below that of the PMR relaxation itself. The magnitudes of the relaxation rates around 0 °C in Figs. 2 and 3 are directly related to the (water)proton — (iron)electron spin interaction, usually called the »outer-sphere« relaxation. This outer-sphere relaxation was also determined directly and independently

by our marker method[5] and the results are denoted by ○ and ● in Fig. 3. As explained in ref. 5 the slight difference between the outer-sphere relaxation determined in ordinary aqueous solutions from the low temperature data, which will hereafter be referred to as »H_2O« experiments, and those »D_2O« in D_2O-solutions containing the marker, ethane diole, may possibly be ascribed to the different solvents. The important point is, however, that for both, »H_2O and »D_2O«, measurements there is a significant increase in the outer-sphere relaxation caused by the addition of IHP.

Because of the lack of spherical symmetry of the magnetic moment interaction of solvent protons with the haem-iron it is not possible[13] to derive an unequivocal distance of the closest proton-to-iron approach from the outer--sphere relaxation rates. Anyway, these data are still a good overall measure of the accessibility of the haem-iron for the solvent protons under conditions of »no-exchange« between the two environments. With this in mind the incremental outer-sphere relaxation rate caused by IHP binding to aquomethaemoglobin (Table I) may be interpreted by an increase in the accessibility of the haem-iron for solvent protons. This means that the IHP induced change of the quaternary structure results in a modification of the tertiary protein conformation at the inlet of the haems such that the solvent approaches the haem-iron closer than in the absence of IHP.

TABLE I

The outer-sphere relaxation rates, in $s^{-1} M^{-1}$ per haem, determined in two ways, in dependence on IHP

	from Fig. 2 »H_2O«, at 5 °C	from Fig. 3		
		»H_2O«, at 5 °C	»D_2O«, at 5 °C	»D_2O«, at 40 °C
with IHP	344	376	163	115
no IHP	230	215	81	30
the incremental rates due to IHP-binding	114	161	82	85

The increase of the paramagnetic relaxation rates with temperature (see Figs. 2 and 3) is due to the thermally activated exchange of solvent protons between the haem-pocket and bulk solvent[5]. From the Arrhenius plots in these figures the energies of activation for this exchange were calculated (see Table II). There are no significant differences caused by the presence of IHP. From this point of view, therefore, the haem-pocket is not altered in the presence of IHP. However, the increase in the rate of water exchange between the two environments in the presence of IHP, an increase from 1.6×10^4 s^{-1} to 3.5×10^4 s^{-1} at 20 °C is quite significant and points again towards an easier communication between the haem-pocket and bulk solvent.

For reasons discussed elsewhere[5] it is not possible to derive with sufficient certainty the distance, r, between the haem-iron and the exchanging proton(s) in aquomethaemoproteins from mammals. It is unfortunate that this important stereochemical information pertaining to the conformation inside the haem-pocket is not available for the further elucidation of the effects of IHP.

TABLE II

Energies of activation, E_a, for the proton exchange

	Stripped Hb		Stripped Hb + IHP	
	(Fig. 2)	(Fig. 3)	(Fig. 2)	(Fig. 3)
E_a/kcal mol^{-1}	6.2	6.3	6.0	5.4

The relaxation rates discussed here have all been calculated per haem, assuming tacitly an average rate of relaxation for all four of the haems. On the basis of the data presented here it is not possible to discern (a) whether the relaxation rates for the α and β chains differ, (b) whether the IHP induced change of the PMR rates is confined to the β chains where IHP binds[14], or (c) whether this change affects the α chains as well.

In spite of the enumerated limitations in the evaluation of the presented PMR results, the conclusion is reached, on balance, that the presence of IHP in solutions of human aquomethaemoglobin A increases the communication of the haem-pocket with the bulk solvent. This cannot be ascribed to the IHP-induced increase in the magnetic susceptibility, because the change of the effective magnetic moment of haem-iron[3] is too small.

It was shown by X-ray crystal structure analysis[15] that in aquomethaemoglobin with IHP, *i. e.* when the protein is in the T, r-conformational state, the iron-ion is shifted approximately halfway between its position in the T, t(deoxy) and R, r(aquomet without IHP) states. There is no similar information for fluoromethaemoglobin. The dissociation constant for the IHP+fluorometHb system has not yet been determined, but there is no reason to expect it to differ much from that for aquomethHb, because the two haemoproteins are identical except for the 6th iron ligand. Perutz *et al.*[3] concluded from the spectroscopic evidence that there must be a similar iron shift also in fluoromethaemoglobin when IHP is added. If this were so, then the fluoride ion would be expected to move in the same direction, but to a lesser degree because of the repelling action of the d_{z^2}-orbital. The next-neighbour water molecule which is the structural probe in our PMR measurements, would probably have to follow the same shift of the iron and fluoride ion due to the hydrogen bonding. In such a case the relative iron-to-proton(s) distance should increase with a consequent diminution of the inner-sphere relaxation rates. On the other hand, the expected penetration of this water molecule deeper into the haem-pocket must involve an overall increase in the accessibility of the solvent toward the haem-iron. Hence an increase of the outer-sphere relaxation should be observed in the presence of IHP.

There is no obstacle to relating the inner-sphere relaxation to the distance, *r*, for fluorometHb, because the temperature dependence of the relaxation rates is a clear-cut case of the fast-exchange condition[5] as required by the theory for calculating *r*. In addition, »D_2O« measurements with fluorometHb yield the outer-sphere relaxation in a wide temperature range. The purely paramagnetic inner-sphere relaxation rate is obtained by subtracting the »D_2O« from the »H_2O« relaxation rates at any temperature. Such measurements

with fluorometHb are presented in Fig. 4, and no change in either of the two relaxation rates was observed with addition of IHP. These data strongly suggest that there is no conformational change of the haem-pocket in fluorometHb A if IHP is added to the solution of a phosphate-stripped sample. If there are any changes at all in the iron-to-proton distances within the haem-pocket, they are not larger than 0.05 Å. The relaxation rates for both »H_2O« and »D_2O« solutions do not change on addition of IHP within a considerable temperature range. Such a result would be possible in case of the proposed[3] iron-shift mechanism only by a fortuitous and very delicate cancellation of the changes in interspin distances (inner-sphere, r, and outer-sphere, d) — a highly improbable case indeed. We are therefore led to the conclusion that there are no IHP-induced changes in the tertiary structure of fluorometHb A related to the haem-pocket, while certain »loosening« in this part of the structure very likely occurs with the addition of IHP to aquometHb A solutions.

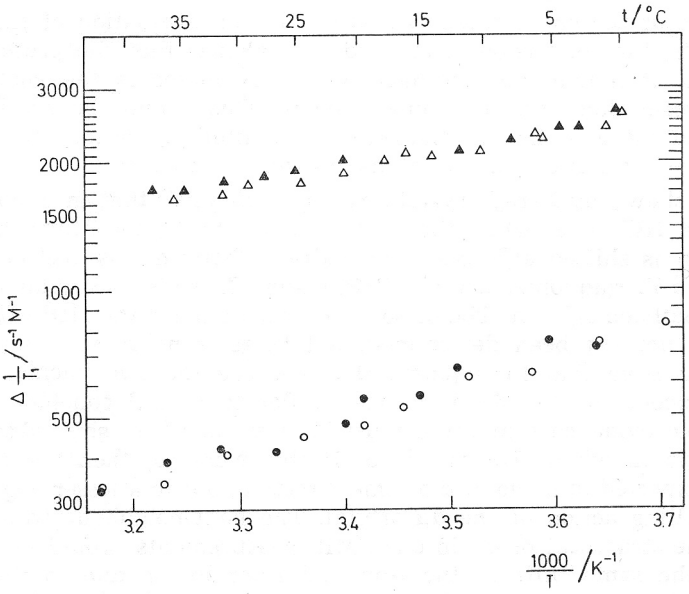

Fig. 4. The PMR rates as in Fig. 3, but for fluoromet HbA: pH 6.2 and C = 5.9 mM for △ and ▲; pH_{meas} = 5.3 and C = 2.6 mM for ○ and ●.

This difference in behaviour between the two forms of methaemoglobin A may be explained by of either the following two factors (or by their combination):

Firstly, the overall accessibility of the fluoromet haem-pocket to solvent molecules is much greater than for the aquometform. The final effects upon the haem-pocket tertiary structure induced by IHP must therefore be stronger in the fluorometform before they could be detected by the PMR rates. On the other hand it must be pointed out that were the tightening of the haem-pocket such an effect it would be even easier to detect it by PMR for fluorometHb than it is for aquometHb.

Secondly, if there are any conformational consequences for the haem--pocket with addition of IHP, these may be much smaller in fluorometHb than in the aquometform because in the former there is practically no change in the iron-spin state. This, of course, holds true if one assumes the transmission of all the structure effects from the quaternary to the tertiary level to go only via the haem-iron.

The human foetal aquometHb served to test the last two possibilities. This haemoglobin shows the type of temperature enhancement of PMR rates similar to that of human adult aquometHb. The change in the iron-spin state upon addition of IHP is significant though smaller than for the adult Hb as inferred from the difference spectra published by Perutz et al.[2] The two haemoglobins may therefore be compared with regard to the factors discussed above.

Fig. 5 contains the results obtained with aquomet HbF_{II} for the same type of experiments (»H_2O« and »D_2O«) as in Figs. 3 and 4. In order to avoid

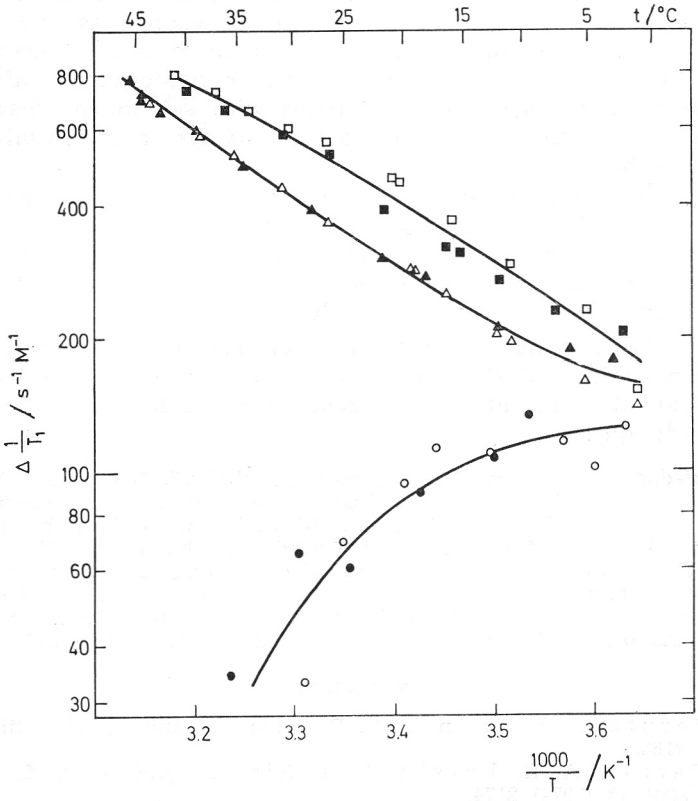

Fig. 5. The temperature dependence of the PMR rates due to the presence of the paramagnetic haem-iron of the human foetal aquomethaemoglobin in 0.1 M NaCl solutions, normalized per haem concentration. The sybols refer to the following samples:

		$pH_{meas.}$	C_{HbF}/mM	[IHP]/[$Hb_{tetramer}$]
foetal haemolysate stripped,	□	6.0	2.1	
□ + IHP,	■	6.0	2.1	40
HbF_{II} stripped, »H_2O«	△	6.1	3.8	
△ + IHP,	▲	6.1	3.8	4
HbF_{II} stripped, »D_2O«	○	5.0	2.6	
○ + IHP,	●	5.0	2.6	4

any misinterpretation because of differences in K_{diss} for IHP binding[2] to the adult and foetal aquomethaemoglobins, measurements with two independent preparations of stripped HbF were performed by the addition of IHP in a 4 : 1 and 40 : 1 molar ratio of IHP :.Hb (tetramer). Calculation shows that more than 99% of HbF-molecules would have IHP bound to them even with the lower IHP—Hb ratio. There is no doubt as to the complete absence of any IHP-effects upon the paramagnetic relaxation rates in Fig. 5. Therefore, no changes induced by IHP in the overall conformational state(s) of the haem--pockets in foetal aquometHb could be sensed by this experimental technique although it was sensitive enough in this respect for the adult aqumoetHb.

The following conclusions may now be drawn:

(i) No tightening of the haem-pokets is observed by PMR irrespective of whether IHP increases the high-spin-state of the haem-iron like in the adult aquometHb and (possibly) in ts foetal species, or does not change the spin-state at all like in the adult fluoromethaemoglobin A.

(ii) An increased accessibility of the haems is most probable when IHP binds to the adult aquomethaemoglobin, but no change has been revealed by PMR for the foetal aquometHb although there is an increase in the high-spin-state in both cases. Hence, whatever quaternary conformational rearrangement takes place when IHP binds with subsequent change of the haem-iron spin-state, the latter effect does not always result in conformational changes at the haem inlet.

(iii) The structural aspect of the haem-iron affinity cannot therefore play any decisive role in decreasing the affinity due to the T-quaternary state imposed by IHP. Rather, it is probably the change of the iron-spin-state which is more important as far as one is allowed to extrapolate from the metHb T→R transition to that of oxy—deoxy.

(iv) The PMR revealed no changes larger than 0.05 Å in the proton-iron distance(s) on addition of IHP to fluorometHb A. These results therefore do not corroborate the shift of the iron relative to the haem plane as inferred by other techniques[3].

Acknowledgements. The work was done under the P. L. 480 contract No. 02-004/1 between N. I. H. and The University Institute of Biology, with partial support from the Research Fund of the S. R. Croatia. B. B. and S. V.-P. thank the latter fund and The Institute of Biology for postgraduate scholarships. The authors are grateful to Dr. D. Šerman of the Medical Faculty, Zagreb, for making available the foetal blood, and to Mrs. V. Bračika for excellent technical cooperation. The analysis of the results was greatly facilitated by the fact that Dr. M. Perutz kindly sent to the authors the three papers of his and his colleagues well in advance of publication.

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SAŽETAK

Studij interakcije methemoglobina i inozitolheksafosfata metodom protonske magnetske relaksacije

Greta Pifat, B. Benko, S. Maričić i S. Vuk-Pavlović

Inozitolheksafosfat (IHP) najjači je alosterički efektor čak i za metformu hemoglobina. Njegovo djelovanje na kvarternu strukturu tetramera ispitivano je sa stanovišta konformacijskog stanja džepa hema u vodenim otopinama hemoglobina. Upotrijebljena metoda daje informacije o pristupačnosti željeznog iona hema za protone otapala. Nije bilo razlike u mjerenim brzinama relaksacije između karbonmonoksi hemoglobina sa i bez fosfata. Znatne razlike u relaksacijskim brzinama uzrokovane paramagnetskim željezom hema akvomethemoglobina nastaju kada se IHP doda adultnom hemoglobinu, ali nikakvih razlika nema u slučaju fetalnog hemoglobina, iako bi se u oba slučaja očekivala slična promjena ravnoteže spinskih stanja zbog dodatka IHP. Nije bilo promjena dodatkom IHP niti kod otopina adultnog fluoromethemoglobina. Zaključeno je da nema sužavanja džepa hema dodatkom IHP ni u jednomu od ta tri uzorka hemoglobina. Povećanje pristupačnosti džepa hema vjerojatno je samo za akvometformu adultnog hemoglobina. Prema tome strukturni aspekt afiniteta hemoglobina za ligande, tj. konformacija džepa hema, ne bi bila dominantna pri promjeni afiniteta dodatkom IHP, vec intrinsički aspekt veze željezo—ligand zbog promjene spinskog stanja željeza hema.

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Primljeno 3. lipnja 1974.