NITROSYL HAEMOGLOBIN: THE *NO*-SPIN AS A RELAXATION PROBE IN THE SOLVENT-PROTON MAGNETIC RESONANCE EXPERIMENT DEMONSTRATING THE PHOSPHATE-INDUCED WIDENING OF THE HAEM-POCKET

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This communication presents evidence that nitric oxide bound to ferrous haem-iron of haemoglobin is a powerful relaxation probe in the solvent-proton magnetic resonance experiments. When such an experiment is applied to the interaction of human haemoglobin with inositol hexaphosphate, it shows that phosphate increases the solvent-dynamics in the haem-pockets, consistent with their widening. Some implications of these findings are discussed here.

While the high-resolution nuclear magnetic resonance method in macromolecular solutions yields specific information regarding the environment of a particular nucleus or group of nuclei, magnetic relaxation rates of solvent-nuclei may provide complementary stereochemical data about the immediate environment of paramagnetic centres in metalloproteins (1). In the haemoglobin (Hb) solutions such a paramagnetic probe is present when the haem-iron is in the ferric high-spin form (2). Interpretations of the recent solvent-proton magnetic relaxation (PMR) experiments in methaemoglobin solutions are not completely consistent, especially regarding the sterical effects of the binding of inositol hexaphosphate (IHP; refs. 3-5): similar experimental data are interpreted differently – both as the IHP-induced opening and tightening of the methaem-pockets (3,4,8). Results obtained by other methods do not clearly discriminate between these alternatives in methaemoglobin (6,7). The relevance of data obtained with ferric derivatives for the understanding of haemoglobin function is at present not completely clear, since doubts have been cast on the reliability of the extrapolation of conclusions from ferric to ferrous haemoglobin (8,9). Therefore, direct demonstration of the sterical effects inside the haem-crevice induced by binding of IHP to ferrous haemo-

globin would provide data which may be relevant also to the elucidation of the particular role of the protein environment of the haem in regulating the haemoglobin-oxygen affinity.

It has recently been shown by various spectroscopic methods (spectrophotometry, infra-red spectroscopy, electron-spin resonance) that the conformation of the nitrosyl derivative of human haemoglobin is very sensitive to the presence of IHP (10) due also to the weakening of the bond between the iron-ion and the proximal histidine (11). However, it is very difficult to interpret such results in terms of relative changes of the haem-accessibility, since they sense mostly the properties of the ligand-haem complex itself rather than those of the protein matrix. We have approached the problem of haem-accessibility by the solvent-proton magnetic relaxation using the unpaired electron of the liganded nitric oxide as a relaxation probe, an approach similar to the use of the spin label covalently bound to the macromolecule (1,12). We demonstrate here (i) that IHP induces substantial changes in the tertiary structure increasing the haem-accessibility as seen from the solvent side, and (ii) the potentialities of the nitric oxide-ferrous haem complex as a relaxation probe in similar structural studies of haemoproteins by solvent-proton magnetic relaxation.

Materials and Methods

Human haemoglobin was prepared according Cameron and George (13). The protein solutions were dialysed against 0.1 M NaCl containing 0.05 M Tris and 5×10^{-4} M EDTA, pH 6.2. The nitric oxide derivatives were obtained by reducing ferric samples with sodium dithionite in the presence of sodium nitrite (14). Inositol hexaphosphate and sodium nitrite were added prior to reduction as freshly prepared buffered solutions. The temperature dependence of the longitudinal magnetic relaxation time, T_1, was measured by the π-t-$\pi/2$ pulse sequence at 24 MHz using the equipment as previously (15).

Results and Discussion

The logarithms of the molar paramagnetically induced solvent-proton magnetic relaxation rates, R_{pmg}, are plotted v. reciprocal absolute temperature in Fig. 1. These rates are obtained by subtraction of the molar relaxation

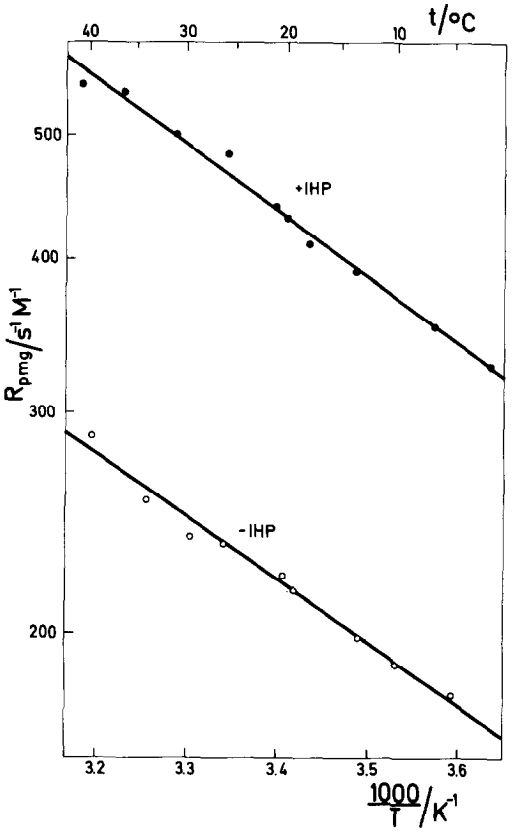

Fig. 1. Paramagnetically induced solvent-proton molar (per haem) longitudinal magnetic relaxation rates, R_{pmg}, in NoHb solutions plotted v. reciprocal absolute temperature. Open circles: phosphate-free solution; full circles: solution containing equimolar (per haem) IHP. Concentration: 5.3 mM (per haem), pH 6.2.

rate measured in the solution of diamagnetic carbonmonoxiHb from the molar relaxation rate measured in the solution of NO complexes. Therefore, R_{pmg} is due solely to the (dipole-dipole) interaction of the NO-electron and solvent--proton spins. Under our experimental conditions the PMR rates in a protein--free dithionite-reduced nitrite solution were equal to those measured in oxygen-equilibrated buffers.

From the slope of the temperature dependence of R_{pmg} in the Arrhenius graph (Fig. 1) it is obvious that here the exchange-limited mechanism takes place (16). According to the theory (16) the increase of R_{pmg} with increasing

temperature results solely from the thermally enhanced interchange of protons between bulk solvnet and the sites of their interaction with electron spin: $R_{pmg} \propto \tau_M^{-1}$. This rate of proton-fluctuation, τ_M^{-1}, is modulated by the sterical properties of the haem-pockets (5,15,17). This statement is fairly well illustrated by the effect of IHP on the R_{pmg}; here the twofold increase of the relaxation rate with respect to the phosphate-free sample can be rationalized only in terms of more intensive fluctuation of protons at the site of interaction of spins.

The IHP-induced increase of R_{pmg} is consistent with higher accessibility of the unpaired electron-spin of the NO-haem complex, namely to a widening of the haem-pocket. From the present data it is not possible to quantify the sterical contribution of the haem-propionyls and the immobilized water molecules (18-20) as well as to assign this effect to one or more particular amino acids, but we may consider here a plausible possibility. The electron--spin resonance (ESR) experiments in solutions of NO-haemoglobin have emphasised the role of sterical relations in the proximity of the bound NO in determining the intensity of the hyperfine structure of ESR lines (21). Considering the data obtained by the same method it was proposed that it was the distal histidine (E7) which in acid solutions of ferric phosphate-free haemoglobin might approach the sixth-ligand water molecule and thus induce a certain fraction of low-spin complex (22). However, inositol hexaphosphate prevents its formation, probably by a withdrawal of the E-helix, together with the distal histidyl, from the haem-iron (22). These findings are consistent with the present results as well as with those obtained in methaemoglobin solutions by the PMR technique (3,5), supporting the idea that the increased accessibility of the electron-spin is due to a movement of the distal histidine as a result of IHP-binding. Recent infra-red experiments concerning IHP--effect in NOHb are also consistent with and IHP-induced change of the steric relations between NO and distal histidyl, though quantitative conclusions were not possible (11). The present results show that, although the spin-state

and position of the iron-ion are not significantly changed by IHP, the conformation of the haem-environment may be altered. This may add some weight to the hypothesis on the role of distal histidine in haemoglobin function (22) and evolution (23).

Our results demonstrate also for the first time the potentialities of the use of NO-haem complex as a relaxation probe in *solvent*-proton magnetic relaxation technique. These potentialities rest mainly on the comparative experiments monitoring allosteric effects or comparing different haem-environments. Such experiments are now underway in our laboratory.

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