A Proton Magnetic Relaxation Study of Methaemoproteins Bound to Monodisperse Polystyrene Latex Particles

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The influence of the binding of methaemoproteins to latex particles with respect to protein conformation was examined using the longitudinal magnetic relaxation rates of solvent protons. The temperature dependences of the paramagnetically induced molar relaxation rates in bound metmyoglobin samples as well as their absolute values suggest an opening of the haem-pocket. The decrease of the same parameter for the immobilized methaemoglobin points to a smaller haem-pocket accessibility with respect to that in the solution. The changes of the haem-pocket structure found for both methaemoproteins support the idea that proteins change their conformation by their binding to the solid surface, although in opposite ways.

INTRODUCTION

The possible conformational changes of proteins induced by their binding to solid surfaces are interesting both from theoretical (1–4) and practical points of view (5, 6). There are several methods usually applied in studies of such phenomena, but they give more or less indirect proofs of the conformational state of bound protein. These methods include kinetics, ligand binding equilibria (2–4), and the binding of protein molecules at interfaces (1, 7), all of which sense the functional rather than the structural changes. Spectropolarimetry, photometric, and fluorescent studies are normally impossible because the solid matrix is usually not transparent, but the possibility for reflectance studies remains. The direct method, high resolution nuclear magnetic resonance, cannot be applied successfully because the resonances of the bound-protein nuclei are broadened due to the very restricted motion of the protein molecules. These facts led us to apply the proton magnetic relaxation (PMR) method in the study of conformational changes of haemoproteins bound to monodisperse polystyrene latex particles.

There are several reasons for the choice of haemoproteins in our studies of structural changes induced by binding to the latex surface. Their ferric forms possess strong paramagnetic centers whose influence on solvent-proton relaxation in acid aqueous solutions is understood well in terms of the Solomon–Bloembergen theory (8). Myoglobin (Mb) is a monomer and this rules out the possible binding-induced changes due to subunit interactions in the quaternary structure. On the other hand, the haemoglobin (Hb) molecule is a very suitable model for the study of these complicated changes at the quaternary-structure level as reflected on the haem-environment.

We chose latex as the solid matrix because of its importance in immunochemical tests (5). Papers from other laboratories report studies on the structure and function of proteins bound to Sephadex resins. A strict

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comparison between effects of these two solid matrices is not permissible because the protein molecules are occluded in the polysaccharide Sephadex matrix (9), while the contact between the protein molecule and latex particle can occur only at the surface.

EXPERIMENTAL

Salt-free crystallized horse met(ferri)myoglobin (Calbiochem) and 6× crystallized lysozyme (Seikagaku Kogyo) were used without further purification. Solutions of human methaemoglobin *A* were prepared according to the method of Cameron and George (10). The monodisperse polystyrene latex 97-*T*, with an average particle diameter of 401.4 nm, polydispersity ratio $P = 1.008$, was prepared as previously described (11) and was extensively dialyzed against deionized water. The cyanmet and fluoromethaemoproteins were prepared by the addition of potassium cyanide and sodium fluoride, respectively (Merck).

The binding isotherms were determined by the method described elsewhere (1). The 0.05 *M* NaCl solutions of proteins and lattices were buffered close to pH 6 with 0.01 *M* *tris*-HCl buffer. The protein-latex equilibration was carried out in 1.04 wt% latex dispersions (density of latex $\rho = 1.06$ g/cm^3); in these dispersions the Mb concentrations ranged from 5×10^{-6} *M* to 17×10^{-6} *M* and the Hb concentrations from 4×10^{-6} *M* to 2.5×10^{-5} *M* (per haem concentration). The quantity of bound protein was determined from the difference between the free protein concentrations before and after binding.

The Mb and Hb concentrations in solution were determined as cyanomet complexes according to Zijlstra and Van Kampen's (12) method using the extinction coefficient of 11×10^3 cm^{-1} *M*$^{-1}$ (per haem) at 540 nm, while the lysozyme concentration was determined by the biuret reaction. The Hb concentrations will be referred as *per haem* concentrations throughout this paper.

The samples for PMR measurements were prepared by equilibrating the protein, already liganded with relevant ligand, with latex overnight at 4°C in 0.05 *M* NaCl or 0.15 *M* NaF (in the case of fluoromethaemoproteins) buffered close to pH 6. The initial protein concentrations were adjusted to 1.5×10^{-5} *M* in the case of Mb and lysozyme and 1.7×10^{-5} *M* for Hb. The samples of proteins bound to latex particles were subsequently centrifuged at 18 000*g* at 4°C for 30 min. The supernatant was decanted and the sediment was transferred into a glass NMR tube (i.d. = 4 mm). The protein concentration in the sample was determined by weighing the wet and dry mass of the sample and by calculating the protein concentration from the known mass ratio of the bound protein per latex particle as determined by the binding isotherm.

The temperature enhancement of the proton longitudinal relaxation time T_1 was measured by a ¶$-t-$¶/2 pulse sequence using a phase-coherent spectrometer with digital readout operating at 24 MHz (Jožef Stefan Institute, Ljubljana). The actual T_1 values were calculated by a computer program (8).

RESULTS AND DISCUSSION

Binding of Myoglobin and Haemoglobin Molecules to Latex Particles

Over the whole range of initial Mb concentrations (5–17 $\times 10^{-6}$ *M*) in the latex suspension, no change in the amount of bound Mb per latex particle was observed. This constant value was 9600 ± 1000 Mb molecules/particle. Since the molecular weights (17 800 and 14 300, respectively) and the shapes of Mb and lysozyme are similar, we assumed the number of bound lysozyme molecules to be of the same magnitude. The calculated fraction of the latex surface occupied by Mb molecules under our experimental conditions is 0.38 (assuming a cross section of the native Mb molecule of 2×10^{-13} cm^2). With the initial Mb concentration of 1.5×10^{-5} *M* and with 1.04 wt% latex we found the protein concentration in the supernatant to be $\sim 1 \times 10^{-5}$ *M*. Since the latex sediment contained 36–61% of the liquid phase, the concentration of the protein

(Mb and lysozyme) bound to latex, calculated per free solvent volume (latex volume excluded) was 2.8 to 7.9 $\times$ 10^{-4} M. Hence, the concentrations of the free Mb and lysozyme in the interstitial liquid are about 1/15 of that of the bound protein.

The adsorption isotherm of haemoglobin to latex seems to be of the Langmuir-type with the plateau reached at the initial concentration of 1.0 $\times$ 10^{-5} M Hb (per haem). In the PMR-samples preparation the initial concentration of 1.7 $\times$ 10^{-5} M Hb was used (in 1.04 wt% latex) and the concentration in the equilibrium was found to be $\sim$6 $\times$ 10^{-6} M. The concentrations of the latex-bound Hb in the PMR-samples (per free solvent volume) were 1.1–1.4 $\times$ 10^{-3} M, and therefore the bound Hb/dissolved Hb ratio was even greater than that found for Mb samples. Since the quantity of Hb subunits bound to latex exceeds that of Mb molecules by a factor of about two, one must conclude that a significant fraction of these subunits is bound to latex indirectly, i.e., via their contacts with the directly bound protomers.

PMR Rates Induced by Bound Proteins

The logarithm of the molar longitudinal relaxation rate, $1/NT_1$, (N = molarity of protein) of solvent protons due to the presence of aquometMb, fluorometMb, cyanmetMb, and lysozyme bound to latex particles are plotted versus the reciprocal absolute temperature in Fig. 1. The $1/NT_1$ value due to the presence of the bound high-spin fluorometMb ($S = \frac{5}{2}$) increases from 5100 sec^{-1} M^{-1} at 40°C to 5700 sec^{-1} M^{-1} at 5°C. AquometMb ($S = \frac{5}{2}$) induces the molar relaxation rate

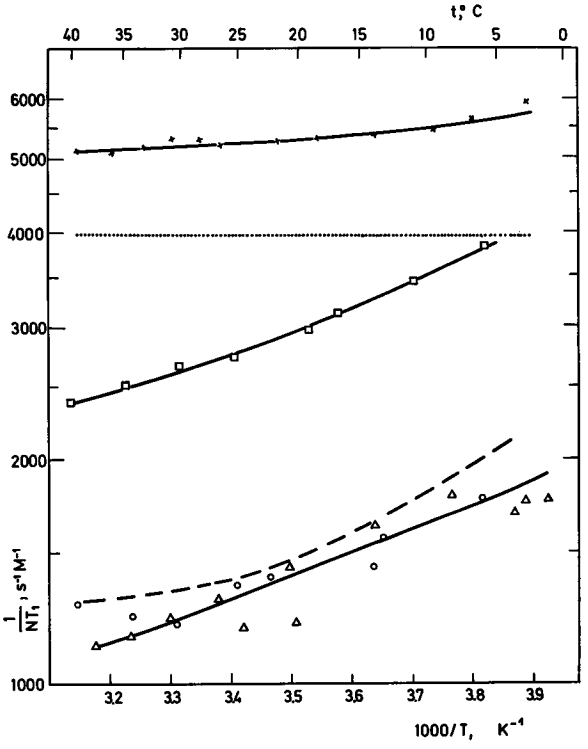

FIG. 1. The logarithms of the molar (per haem) proton longitudinal relaxation rates, $1/NT_1$, versus reciprocal absolute temperature. Crosses, the relaxation rates due to the presence of 3.1 $\times$ 10^{-4} M fluorometMb on latex; squares, 2.8 $\times$ 10^{-4} M aquometMb; circles, 7.9 $\times$ 10^{-4} M cyanmetMb; triangles, 5.2 and 5.9 $\times$ 10^{-4} M lysozyme. The dotted line refers to the paramagnetically induced $1/NT_1$ values in the presence of fluorometMb; the dashed line refers to those of aquometMb.

from 2400 sec^{-1} M^{-1} at 40°C to about 3900 sec^{-1} M^{-1} at 5°C. The low-spin cyanmet complex ($S = \frac{1}{2}$) and lysozyme (diamagnetic) produce rates between 1200 sec^{-1} M^{-1} and 1800 sec^{-1} M^{-1} in the same temperature range.

Note that the protein-free latex, either dried or dialyzed against heavy water, did not show any detectable PMR signal. This means that latex-particle protons do not contribute to the observed $1/NT_1$ values under our experimental conditions.

The temperature dependence of the relaxation rate induced by the latex bound aquometMb differs very much from that induced by the free metMb in acid aqueous solution (8). The significance of different temperature dependences of relaxation rates, as well as their molecular bases, have been discussed in detail elsewhere (8, 13).

The type of temperature enhancement of the proton $1/NT_1$ values in the case of bound fluorometMb does not differ from the relaxation rate observed in the fluorometMb solutions (8). However, the comparison of data from Fig. 1 in this paper and Fig. 3 in (8) indicates an approximately double relaxation rate in the presence of the bound fluoride complex compared to the dissolved one.

The longitudinal relaxation rate in a solution of protein bearing paramagnetic centers (i.e., ferric haem-iron in our case) is given, in a simplified form by

$$(1/NT_1)_{obs} = (1/NT_1)_P + (1/NT_1)_D \quad [1]$$

where $(1/NT_1)_P$ is the molar relaxation rate of solvent protons induced by the paramagnetic haem-iron; and $(1/NT_1)_D$ is the molar relaxation rate induced by dipolar interaction of solvent protons with the protein surface and proton mutual interaction in the free solvent molecules.

In the 40–5°C range in the free aquomethaemoprotein solution (pH 6) the relaxation is dominated by a thermally activated process of water–proton exchange between the haem-pocket and bulk solvent [see the schematic graph in (13)]. The comparison of the two terms on the right-hand side of Eq. [1] can give information about the type of relaxation process [*fast proton exchange* versus *no exchange* (8)], so that the diamagnetic term must be determined experimentally. Since we were not able to prepare the diamagnetic carbonmonoxyMb completely free of the paramagnetic metform, we measured T_1 of the stable low-spin cyanmetMb and simulated the diamagnetic Mb with lysozyme bound to latex.

The relaxation rates induced by the presence of bound aquometMb and cyanmetMb can be analyzed in terms of the Solomon–Bloembergen equation (see later Eqs. [2] and [3]). With the fast proton exchange condition fulfilled (8), assuming the longitudinal electronic relaxation time ratio (15, 16) $\tau_{\frac{5}{2}}:\tau_{\frac{1}{2}} \simeq 75$ and proton-to-iron interspin distance to be equal, the paramagnetic term in Eq. [1] for the cyanmet complex must be about 1/100 of that for the aquometMb. ($\tau_{\frac{5}{2}}$ and $\tau_{\frac{1}{2}}$ denote the electronic relaxation time for spins $S = \frac{5}{2}$ and $S = \frac{1}{2}$, respectively). Therefore, it is not surprising that the measured relaxation rates for both immobilized diamagnetic lysozyme and slightly magnetic cyanmetMb closely resemble each other within the limits of experimental uncertainties.

The pure paramagnetically induced relaxation rates for the high-spin ferric complexes can be obtained by subtracting the relaxation rate induced by bound diamagnetic protein (or the cyanmet in this case, as explained above) from the total observed relaxation rate in presence of the fluoromet or aquomethaemoprotein on latex.

Two properties of the paramagnetic relaxation rate due to the aquometMb (dashed line in Fig. 1) must be emphasized: (i) the slope of the temperature dependence of this parameter clearly shows that the transition from no proton exchange to fast exchange in this temperature region does not occur; and (ii) the dominant relaxation mechanism cannot be any other than the fast proton exchange, simply because the paramagnetic term in Eq. [1] is even larger than for the aquometMb in solution (8, 14), i.e., without latex, at the highest temperature.

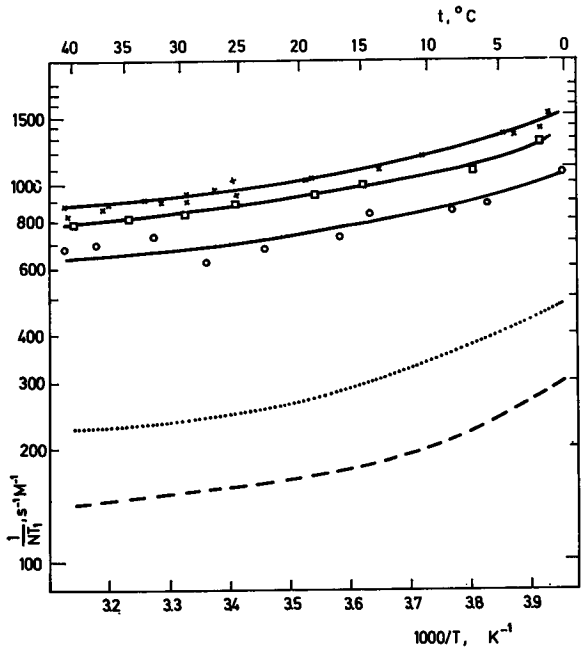

FIG. 2. Coordinates as in Fig. 1. Crosses, 1.1 and 1.2 $\times$ 10^{-3} M bound fluorometHb; squares, 1.3 $\times$ 10^{-3} M aquometHb; circles, 1.4 $\times$ 10^{-3} M cyanmetHb. The dotted line refers to the paramagnetically induced $1/NT_1$ value by fluorometHb; the dashed line refers the same for aquometHb.

This paramagnetically induced solvent protons relaxation rate points to a higher accessibility of the ferric haem-iron of the bound aquometMb with respect to that in a solution. That this is indeed the case was verified by our marker-method (8), in which the relaxation rates due to the aliphatic ethane diol protons (in D_2O) were practically identical to those for aquometMb as given in Fig. 1.

The temperature dependences of the $1/NT_1$ values due to the presence of the bound cyanmetHb, aquometHb, and fluorometHb are plotted in Fig. 2. The high-spin fluorometHb ($S = \frac{5}{2}$) induces $1/NT_1$ values from 900 sec^{-1} M^{-1} to 1300 sec^{-1} M^{-1} (normalized per haem concentration) between 40°C and 5°C. In the same temperature range the effect of the aquometHb ($S = \frac{5}{2}$) varies from 800 sec^{-1} M^{-1} to 1100 sec^{-1} M^{-1}, and for cyanmetHb ($S = \frac{1}{2}$) from 700 sec^{-1} M^{-1} to 900 sec^{-1} M^{-1}.

We chose identical ligands to the ferric haem-iron in metHb (CN^-, F^-, H_2O) for the same reasons as explained for the case of bound metMb, and the theory, discussed above, for the bound Mb holds for the immobilized Hb samples as well.

The absolute values of the paramagnetically induced molar relaxation rates in the presence of bound fluorometHb (dotted line in Fig. 2) and aquometHb (dashed line in Fig. 2) differ essentially from these rates found for the analogous Mb samples. The significant decrease of this parameter compared to the values in solution points to the reduced accessibility of the haem-iron in the metHb samples; it shows that the paramagnetic contribution to the $1/NT_1$ value resembles the *outer sphere* relaxation, i.e., the solvent protons *sense* the paramagnetism of the haem-iron only through long-range interaction without any contribution from the proton-exchange mechanism.

The Conformational States of the Haem-Pockets of the Metmyoglobin and Methaemoglobin Molecules Bound to the Latex Surface

To explain the increase of about 2000 sec^{-1} M^{-1} in the relaxation rates on binding fluoro-

metMb to latex, other factors must be considered in the Solomon–Bloembergen equation, which, abbreviated, reads (for a constant Larmor frequency):

$$1/NT_1 = nK\mu_{eff}^2 r^{-6} F(\tau_c) \qquad [2]$$

where n is the number of (water) protons taking part in the exchanging mechanism; K comprises numerical factors and the basic nuclear magnetic constants; μ_{eff} is the effective magnetic moment of the paramagnetic haem-iron, and $F(\tau_c)$ is a function of the correlation time τ_c for the unpaired electrons and proton dipole–dipole interaction. It has been shown (16) that $\tau_c \equiv \tau_S$, the electron relaxation time, of the order of 10^{-10} sec. $F(\tau_c)$ is thus proportional to τ_S up to 10^{-8} sec, so that

$$1/NT_1 = nK\mu^2_{eff} r^{-6} \tau_S. \qquad [3]$$

The fluoromet complex is known to be almost completely in the high-spin ($S = \frac{5}{2}$) form (17). As already quoted (15), τ_S for the low-spin form ($S = \frac{1}{2}$) of methaemoproteins is smaller by an order of magnitude than τ_S for the high-spin complexes. Whatever change of the haem-iron spin state there may be due to a change in the ligand-field symmetry in the latex-bound fluorometMb, μ_{eff} may only become smaller, i.e., the low-spin states may become more populated, but then τ_S also should diminish. Hence, the increase in the relaxation rates cannot be brought about by any change either in μ_{eff} or τ_S. Neither could the iron–proton distance, r, become smaller than it is already with the sixth ligand position occupied by F^- or H_2O. The increased relaxation rates may only be understood by an increase in n (see Eq. [2]), i.e., by postulating a relaxation mechanism with more than one water molecule simultaneously exchanging very fast between the immediate neighborhood of the iron–ion and bulk solvent. Such a mechanism may not be possible in the fluorometMb solution because of another shape of the haem-pocket. The only explanation for the increased relaxation rate of the bound fluorometMb is the opening of the haem-pocket induced by the binding of metMb molecule to the latex surface. On the other hand, if τ_S became shorter for the bound fluorometMb than for fluorometMb in solution (with, or without a lower μ_{eff}), n would have to be even larger, suggesting a profound unfolding of the haem-pocket, so that in no way could these data be understood otherwise than to imply an opening of the haem-pocket.

Since both fluoromet and aquometMb are high-spin, their overall globin conformational states must be very similar. There is no reason, therefore, to assume either a different type of binding to the latex particles or a different conformational change of the haem-pocket induced by this binding. The same opening of the haem-pocket, postulated for bound fluorometMb, explains the observed type of PMR rates in the presence of the bound aquometMb. The opened haem-pocket cleft is not a barrier any more for the free exchange of water molecules coming from the solvent side. The observed paramagnetically induced relaxation rate in the sample containing bound aquometMb indicates that even here the sixth-coordinated water molecule, observed in the X-ray diffraction studies of metMb molecule, does not exchange with bulk solvent. If it were exchanging, the $1/NT_1$ value would be at least of the magnitude observed in the sample containing the fluoromet complex. For these reasons we extend the exchange mechanism postulated for the bound fluorometMb to the case of the bound aquocomplex. The difference in the absolute values of the paramagnetically induced PMR rates can be ascribed to different positions and orientations of water molecules in the neighborhood of differently liganded haem-irons (H_2O versus F^-).

Thus far we have not been concerned with the problem of the orientation of the Mb molecules when located on the latex surface. This surface bears hydrophobic and hydrophylic groups (1). Since the protein surface is essentially built of hydrophylic aminoacids, we assume that hydrophobic interactions between such groups on the latex surface and the hydrophobic interior of the haem-pocket

buried in the protein matrix is very unlikely. In any case, if any haem-pocket is oriented towards the latex surface, it does not contribute significantly to the observed relaxation rate because the water molecules cannot approach close enough to the iron–ion for their relaxation rates to be affected by its presence. Since all the results are normalized per total Mb concentration, the partial loss of the paramagnetic centers would require a correction that would increase the finally calculated molar relaxation rates; however, the absolute $1/NT_1$ values suggest that the number of inefficient paramagnetic centers, if there are any, is not appreciable.

The small paramagnetic relaxation rates induced by the bound methaemoglobin can be rationalized by the following mechanisms or their combinations: (i) some of the haemoglobin protomers may adhere to the latex surface by their haem-mouthpieces, thus preventing fast proton exchange; (ii) the adsorbed protomers may induce a tightening of the haem-pocket(s) oriented towards the solvent by a quaternary structure change; or (iii) any conformational change such as that envisaged in (i) and (ii) may lead to formation of the low-spin haemochromogen by inducing distal–histidine binding in place of the sixth ligand. Anyone of these possible mechanisms induces a closing of the haem-pockets, i.e., a decreased accessibility of the bound methaemoglobin-iron for the solvent protons. The data suggest that essentially all the haem-irons are inaccessible.

The results of binding experiments (1, 7) suggest partial unfolding of the polypeptide chains in contact with the latex surface. The present results do not contradict this view because the method applied monitors the conformational changes in the close vicinity of the haems, which may not necessarily be influenced by partial protein unfolding some distance away.

SUMMARY

I. Myoglobin molecules bind to the polystyrene latex 97-T particles by occupying a calculated fraction of the latex surface similar to that found for other globular proteins.

II. The convenient low-spin cyanmetMb and Hb can serve in similar studies as a substitute for the diamagnetic carbon-monoxyhaemoproteins, which are susceptible to autooxydation.

III. The high $1/NT_1$ value in the sample with bound ferric high-spin fluorometMb with respect to $1/NT_1$ in solution of such liganded protein indicates opening of the haem-pocket induced by the binding of Mb to the latex surface. This opening enables the simultaneous fluctuation of more than one water molecule between the opened haem-pocket and bulk solvent.

IV. The molar PMR rate of the bound aquometMb indicates similar opening of the haem-pocket as in the case of the fluorometMb. The water molecule at the sixth ligand site does not exchange, but the other water molecules exchange with bulk solvent and fulfill the fast exchange condition throughout the temperature range measured. The difference in the paramagnetically induced PMR rates observed between fluoromet and aquomet Mb can be ascribed to different locations of the water molecules in the immediate neighborhood of differently liganded iron ions.

V. The small paramagnetically induced $1/NT_1$ values in bound Hb samples are due to the smaller haem-iron accessibility possibly caused by: (i) some of the haem-pocket mouthpieces facing directly the latex surface; and/or (ii) induced closing of the haem-pocket cleft in the protomers pointing towards solvent.

VI. The experiments reported in this paper prove that metmyoglobin and methaemoglobin change their conformation in opposite ways on binding to a polystyrene latex surface.

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