studia biophysica, Berlin, Band 55, 1976, Heft 3, S. 157 - 161
Printed in the German Democratic Republic

MATRIX-BOUND HAEMOGLOBIN AS SEEN BY PROTON-MAGNETIC RELAXATION

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The structural alterations induced by covalent binding as well as by adsorption of aquomethaemoglobin (Hb) to Sephadex-matrices are investigated by proton-magnetic relaxation. It is shown that the quaternary structure and inositol hexaphosphate binding ability are preserved in covalently bound Hb. Here the haem-pockets in a chains tighten, while adsorption increases the dynamics of solvent-protons in the haem-pockets, probably due to their loosening. It is concluded that the structure of an immobilized protein is dependent on the type of binding.

Introduction

Although the structural studies of matrix bound proteins are of considerable interest, the properties of the matrix itself severely restrict the choice of methods that can be applied to such studies. Proton-magnetic relaxation (PMR) has already been successfully used in a study of methaemoglobin and metmyoglobin derivatives adsorbed at the surface of monodisperse polystyrene latex /1/. The applicability of this method is based on the fact that the solvent-proton relaxation is induced by the relaxation of the electron-spin of the haem-iron, the latter being independent of the tumbling rate of the whole molecule /1/. On the other hand, the mechanisms of PMR in methaemoprotein solutions are rather well understood /2/, and the comparison of the PMR-data obtained in systems containing bound and free methaemoglobin can yield structural information complementary to ligand-binding studies already reported /3, 4/. It was concluded that the changes of the spin-state equilibrium /3/ as well as the decreased cooperativity of the reversible oxygenation /4/ may be due to the binding-induced diversity in the structures of haemoglobin subunits. The present

communication, therefore, deals with the haem-pocket structure both in the adsorbed and in the covalently bound human aquomethaemoglobin (H_2OmetHb) as well as with the effect of the allosteric effector inositol hexaphosphate.

Materials and methods

The preparation and binding of metHb both to Dialdehyde-Sephadex G-200 and CM-Sephadex C-50 were reported previously /3, 4/. The samples of adsorbed H_2OmetHb and cyanomethaemoglobin (CNmetHb) were buffered with 0.01 M phosphate, pH 6.2; the sample with the covalently bound protein contained 0.1 M phosphate, pH 6.0. The protein concentration refers to the haem content throughout this paper. In the samples prepared for the PMR-measurements it was 2.2 mM (Sephadex G-200) and 4.2 mM (CM-Sephadex C-50). To the former sample crystalline inositol hexaphosphate (IHP) was added up to a concentration exceeding four times that of haemoglobin.

The longitudinal proton-magnetic relaxation times, T_1, were measured at 24 MHz by the π-t-$\pi/2$ pulse sequence using a spectrometer built at "Jozef Stefan" Institute, Ljubljana, Slovenia, Yugoslavia. The data were evaluated by a computer programme as described previously /2/.

Results and discussion

The logarithms of the molar paramagnetically induced longitudinal solvent-proton relaxation rates, $1/NT_1$, due to the presence of H_2OmetHb bound to Sephadex-matrices are plotted v. reciprocal temperature in Fig. 1 with the linear temperature scale indicated as well. (N denotes the methaem concentration.) The circles refer to the adsorbed protein, while the triangles stand for the PMR values due to the covalently bound H_2OmetHb/without IHP (open symbols) or with this effector added (filled symbols). The squares represent the molar relaxation rates due to CNmetHb bound to CM-Sephadex. The values of $1/NT_1$ were obtained by subtracting the measured relaxation rates of swollen Sephadex without bound haemoglobin from the $1/T_1$ values in the presence of the bound protein; this difference is then divided by the methaem concentration in the sample. The pure paramagnetic relaxation rates for bound H_2OmetHb's (as given in Fig. 1 for ○, △, ▲) are calculated by subtracting the molar relaxation of bound CNmetHb from the total $1/NT_1$ value. The obtained molar relaxation rates due solely to the paramagnetic influence of bound H_2OmetHb

can yield useful stereochemical information, since they are influenced also by the structure of the haem-pocket /2/.

It will be shown in Ref. 1 that CNmetHb has a negligible paramagnetic relaxation due to its very rapid electron-spin relaxation /5/ and total electron spin S=1/2 as distinct from the H_2OmetHb with S=5/2. We used the stable CNmet-form to obtain the "Diamagnetic" relaxation correction because our experience was that carbonmonoxy-derivatives may be autooxydizable when immobilized /1/.

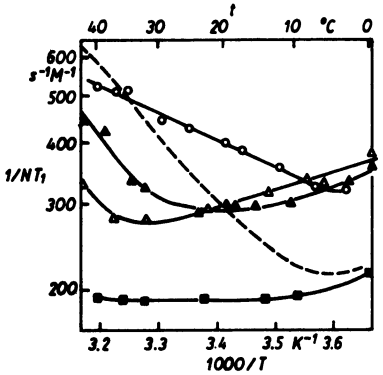

FIG. 1 The temperature enhancement of the molar (per haem) solvent-proton longitudinal magnetic relaxation rates induced by Sephadex-bound methaemoglobin derivatives. Circles: the paramagnetic relaxation rate induced by H_2OmetHb adsorbed to CM-Sephadex C-50. Triangles: the same rate in the presence of H_2OmetHb covalently bound to Dialdehyde-Sephadex G-200; open symbols: without IHP, filled symbols: with IHP. Squares: total molar relaxation rate induced by adsorbed CNmetHb. Dashed line: the paramagnetic relaxation in acid metHb solution, replotted from Ref. 6.

The purpose of this paper is (i) to demonstrate the integrity of immobilized haemoglobin tetramer as well as the preserved phosphate, binding ability (ii) We shall also consider the nature of the induced structural changes of subunits that are due to the immobilization. Such changes have already been postulated on the basis of ligand-binding data /3, 4/.

It has been shown /2/ that the temperature enhancement of the PMR rates in H_2OmetHb acid solutions above 5°C is due to the thermally activated interchange of water-protons between the haem-pocket and bulk solvent. Below some 5°C this exchange is too slow to affect the overall relaxation rates, and the paramagnetic term is then due solely to the "long-range" dipolar interaction called the "outer-sphere" relaxation /2/.

The thermally activated relaxation is greatly enhanced by IHP which "loosens" the haem-pocket structure /6, 7/ in haemoglobin-solution. We used this IHP-effect to solve the problem posed by BLANCK et al. /3/ whether the native tetrameric state of metHb covalently bound to Dialdehyde-Sephadex G-200 is

preserved. Inositol hexaphosphate binds to two β subunits. If any IHP-effect were observed, it should prove that the haemoglobin molecule exists as the tetramer. From curves Δ and $\blacktriangle$ (Fig. 1) it is clear that IHP increased the relaxation rates governed by the (water) proton exchange in bound Hb. This confirms unequivocally that the molecular integrity may not be significantly affected by covalent binding to the polysaccharide matrix, since the increase in relaxation rates observed upon IHP-binding is closely similar to that in a solution /6, 7/. We did not perform such experiments with H_2OmetHb adsorbed to CM-Sephadex C-50, since IHP would bind to the cation exchanger and the experiment could not be kept completely under control. Fortunately, the oxygenation-results reported by LAMPE and POMMERENING /4/ have shown that the tetrameric structure is preserved here, too.

The comparison of paramagnetic relaxation rates due to bound H_2OmetHb and to that in a solution (Fig. 1, dashed line, replotted from Ref. 6) indicates the existence of binding-induced structural changes in the immediate vicinity of the methaem-iron. It has been shown in a PMR-study dealing with valency-hybrids /7/ that α and β chains influence the solvent-proton relaxation differently, α chains having higher termally activated relaxation rates throughout the temperature range measured. On the other hand, β subunits induce only the outer-sphere relaxation up to some 25°C, similarly to the relaxation behaviour observed here for covalently bound H_2OmetHb. On the basis of this comparison we conclude that covalent binding tightens the α chain decreasing thus the solvent dynamics within the haem-pocket. This would mean that the α and β haem-pockets in bound subunits are more alike.

Thus far we have been concerned with the covalently bound Hb. The adsorption to CM-Sephadex C-50 leads to smaller differences compared with the native state than the covalent binding. Here it is not possible to deduce firm conclusions about the nature of distortion of the haem-iron vicinity since the lowering of the energy of activation of water-proton exchange might mean that β chains are loosened, becoming more similar to α chains or that α haems are accessible even more than for dissolved native haemoglobin. In any case, these induced changes show that the structure of a bound protein is dependent on the type of binding to the matrix.

We may conclude that: (i) the tetrameric structure as well as the flexibility necessary for the heterotropic allosteric IHP-effect in covalently bound

H_2OmetHb are preserved; (ii) covalent binding induces a tightening of the α pockets making them more similar to the β chain than they are in a solution; (iii) adsorption leads to the lowering of the energy of activation of proton-exchange between the haem-iron-vicinity and bulk solvent, most probably due to the loosening of β and/or α haem-pockets; (iv) binding-induced conformational changes depend on the type of forces involved in the binding to a particular matrix.

The authors thank Dr. S. MARIČIĆ for helpful discussions. The first two authors have been supported by the PL-480 grant 02-004-1.

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Изучаются изменения структури обусловленные ковалентной связю и адсорпцией аквометгемоглобина (Hb) на Sephadex пользуясь методом протонно магнитного резонанса. Показивается что структура тетрамера и способность связивания гексафосфата инозитола (IHP) сохраняются в гемоглобине связанном ковалентной связю. При этом карманы гема в α-цепях сужаются. Адсорпцией усиливается динамика протонов воды в кармане гема, возможно из-за их расширения. Приходится к выводу что структура связанного белка зависит от типа связи.

Eingegangen am 4. 9. 1975

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