INTERNAL MOTION AT THE CHLORIDE BINDING SITES OF HUMAN SERUM ALBUMIN BY NMR RELAXATION STUDIES

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Received 12 November 1977

1. Introduction

Evidence is rapidly growing for the presence of very fast internal motion in biological macromolecules [1–5] and it seems reasonable to believe that the internal mobility is often involved in the functional role of the macromolecule. A rich variety of rapid motional phenomena in proteins is also a result of recent theoretical studies [6] and these authors stress the importance to have available methods which may in detail test the dynamic situation. There is, however, a pronounced lack of physico-chemical techniques which allow us to monitor these internal motion processes and, in particular, of methods which allow us to specifically study those internal motions which may be related to the biological function of the system, for example processes occurring at the active site of an enzyme. The purpose of this communication is to describe attempts to use ^{35}Cl NMR relaxation investigations for studying internal mobility phenomena at the anion binding sites of human serum albumin. Although a detailed quantitative interpretation of results of this type is quite difficult we will be able to deduce some qualitative features relating to the presence of internal motion.

2. Experimental

The longitudinal relaxation times (T_1) were measured using $\pi-t-\pi/2$ pulse sequences and the transverse relaxation times (T_2) with Carr-Purcell-Meiboom-Gill sequences, both on a Bruker BKr-322s spectrometer. The signals were time averaged on a Varian V-71 computer with home made interface to improve the signal-to-noise ratio. The measuring frequency was 8.8184 MHz and the magnetic field was held constant with an external proton lock operating at 90 MHz.

In each case, the relaxation times were determined by a least squares fit of the data to a single exponential. Although the relaxation is actually a sum of two exponentials [7], it can be shown that the analysis in terms of single exponentials gives accurate results as long as $\omega\tau \lesssim 1.5$, where τ is the apparent correlation time and ω is the resonance frequency. Furthermore, the breakdown of the single exponential approximation is always accompanied by a sharp increase in the standard deviation of the fit to the T_2 data. Consequently, we have carefully checked the T_2 fits to ensure the validity of our analysis.

Typically, the least squares fits for the T_1 measurements yielded a standard deviation of 2–4%, and the T_2 measurements yielded 2–5% standard deviations. The deviation between successive T_1 measurements was less than 4%; and for T_2 measurements, it was less than 6%.

The human serum albumin (Batch no. RbN 487) was a generous gift from AB KABI, Stockholm.

3. Results and discussion

The ^{35}Cl longitudinal and transverse relaxation times in aqueous solution of KCl and HSA were measured at temperatures ranging from 5–50°C, at pH 4, pH 7 and pH 11 and for Cl^- concentrations of 0.5 M and 0.15 M. In each case, the relaxation rates

were determined in the absence and in the presence of sodium dodecyl sulfate (SDS), with the difference between these rates yielding the relaxation rates for the sites excluded by the SDS (excess relaxation rates).

These excess relaxation rates (e.g., table 1) were then analyzed for the apparent correlation times and apparent quadrupole coupling constants (QCC), using the equations in [7]. The results of this analysis are shown in table 2. Also shown are the correlation times for the reorientation of the long axis of HSA. $\tau_\perp$, assuming the Debye-Perrin model [8] and either (i) that HSA is a prolate ellipsoid with dimensions 144 X 45 X 22 Å [9] or (ii) that the effective dimensions of HSA correspond to those claculated from the dielectric relaxation times [10].

Due to the poor excluding properties of SDS at pH 4 and the diminished number of binding sites at

Table 1
Effect of sodium dodecyl sulfate (SDS) on the $^{35}Cl^-$ longitudinal and transverse relaxations in solutions of human serum albumin (HSA) at pH 7.3

[HSA] · 10^4 (M)	[SDS]/[HSA]	temp. (°C)	$\Delta(1/T_1)$ s^{-1}	$\Delta(1/T_2)$ s^{-1}
0.72	11.5	5.5	47.4	137
0.72	11.5	18	41.8	113
0.73	12	28.5	43.4	98.5
0.73	12	39	65	136
0.74	12	50	57	123

Table 2
Correlation times (τ_c) and quadrupole coupling constants (χ) of Cl^- bound to human serum albumin evaluated as described in the text. Data refer to the high affinity sites

pH	[KCl] (M)	temp (°C)	τ_c (ns)	χ (MHz)	$\tau_\perp$[a] (ns)	$\tau_\perp$[b] (ns)
7.2	0.5	5.8	23	1.88	145	115
7.2	0.5	17.8	22	1.74	100	79
7.2	0.5	28.5	18	1.64	77	61
7.2	0.5	39.0	17	2.01	60	48
7.2	0.5	50.4	14	1.82	48	38
4.1	0.5	6.0	13	(4.2)[c]		
4.1	0.5	18.0	17	(3.5)[c]		
4.1	0.5	29.8	9	(3.1)[c]		
10.9	0.5	6.1	20	1.06		
10.9	0.5	18.0	20	0.96		
10.9	0.5	30.2	20	0.92		
7.5	0.15	30.5	19	1.25		
4.1	0.15	30.7	11	(2.7)[c]		
11.0	0.15	30.2	16	0.56		
11.0	0.15	30.9	10	0.45		

[a] Correlation time of HSA for reorientation around the short axis. Calculated from dielectric relaxation data [10] assuming proportionality between $\tau_\perp$ and viscosity divided by absolute temperature

[b] Correlation time of HSA for reorientation around the short axis. Calculated from the Debye-Perrin model [8] and the assumed dimensions 144 X 45 X 22 Å [9]

[c] Quadrupole constant multiplied by the square root of the number of sites given since the competition experiments were not conclusive as regards the stoichiometry

pH 11, the errors in these data are quite large. However, there are no dramatic changes in the apparent correlation times at these pH values; and the slightly smaller apparent QCC at pH 11 is probably a reflection of the decreased binding at this pH. Furthermore, the decrease in the apparent QCC at 0.15 M KCl indicates that the binding constant for the Cl^- is small, particularly at pH 11.

For the data at pH 7, however, the errors seem to be sufficiently small to warrant a further analysis. Two important features of the apparent correlation times can be seen from table 2. First, the correlation time is the same for the 0.5 M KCl solution as for the 0.15 M KCl. This indicates that the analysis of the 0.5 M KCl data should be applicable to solutions containing physiological salt concentrations. Second, all of the apparent correlation times are considerably smaller than those calculated theoretically or derived from the dielectric relaxation times.

These small correlation times have usually been interpreted as resulting from internal motion of the Cl^- binding group relative to the protein; and in one case [11] very strong experimental evidence for this interpretation has been presented. Furthermore, a simplified theory for describing the effects of internal motion has been presented [12]. Basically, this theory assumes that the internal motion can be described as the diffusion of a rod in a conical hole embedded in the protein, with the end of the rod being attached at the apex of the cone. The unknown parameters involved in the calculations are the half angle of the cone, the correlation times for the internal plus overall diffusion, plus the orientation of the cone relative to the molecular symmetry axis.

Using the theory for internal rotation, we have tried to fit the experimental correlation times (i) while assuming the Debye-Perrin overall correlation times given in table 2 and (ii) taking the overall correlation times derived from the dielectric relaxation data [10]. For case (i) we were unable to fit the experimental data. However, for case (ii) we found a series of solutions which are in excellent agreement with the experimental results. Two such fits are shown in fig.1, corresponding to internal correlation times that are 17 and 10^4 times smaller than $\tau_\perp$ and with conical holes having half angles of 50° and 81°, respectively.

Clearly the experimental correlation times alone do not enable us to distinguish these fits. However,

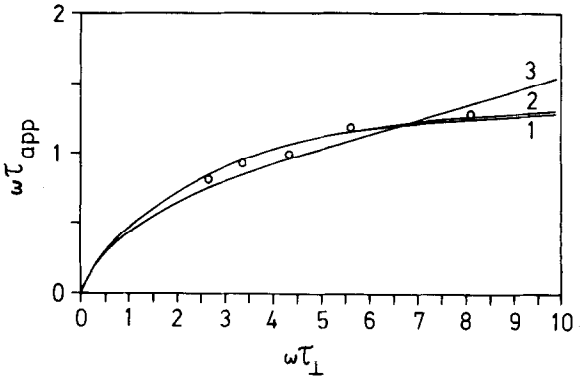

Fig.1. The apparent correlation time multiplied by the resonance frequency, $\omega\tau_{app}$ versus $\omega\tau_\perp$ obtained as described in the text. The curves correspond to the theoretical relation with (1) $\tau_\perp/\tau_i = 10^4$, $\psi = 86°$; (2) $\tau_\perp/\tau_i = 118$, $\psi = 63.5°$, and (3) $\tau_\perp/\tau_i = 17$, $\psi = 48°$. The circles correspond to the experimental data for HSA bound chloride at pH 7.2 and temperatures from 5.8–50.4°C.

with an apparent QCC of 1.6 MHz, the two fits correspond to actual QCC values of 2 MHz and 23 MHz, respectively. Consequently, an independent determination of the quadrupole coupling constant for the Cl^- bound to HSA should allow us to choose one set of self-consistent parameters.

In the absence of an independent value, we have estimated [13,14] the QCC for Cl^- bound to lysine to be about 3.6 MHz. This, in turn, leads to a correlation time for the internal motion $\tau_{int} = 7 \cdot 10^{-10}$ s at 28°C and to a half angle for the conical hole of about 65°.

Although these values should not be taken too literally, they do indicate that the chloride-binding sites have a great deal of freedom to move about relative to the protein structure as a whole. Furthermore, they show that the chloride relaxation times are dominated by these internal motions and, consequently, that they should be quite sensitive to substrate binding and/or protein conformational changes that affect this mobility.

It seems that ^{35}Cl quadrupole relaxation studies along the lines presented here for the particular case of human serum albumin provide an interesting novel possibility of exploring internal mobility phenomena in proteins. The method is complementary to other experimental approaches and has the advantage of being capable of specificity for certain anion binding

sites of a protein. Studies in progress have given results demonstrating the presence of rapid internal motion at the active sites of a number of enzymes. These results add to the recent ideas of a general occurrence of internal mobility phenomena in proteins.

Acknowledgement

The project 'NMR Studies of Ion Binding to Proteins' is supported by a grant from the Swedish Natural Sciences Research Council.

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