THE ClO_4^- ION AS A PROBE IN NMR STUDIES OF PROTEIN ANION BINDING SITES

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1. Introduction

Since the function of many proteins involves their interaction with negatively charged ligands, methods which permit a characterization of macromolecular anion binding sites are of great interest in protein chemistry [1]. One n.m.r. method which has been widely applied is the halide ion probe technique introduced by Stengle and Baldeschwieler [2]. This method is concerned with the determination of the effect of a protein on the nuclear magnetic relaxation rates of Cl, Br or I nuclei, (for a review see [3]). One important reason for the usefulness of the halide probe method is to be found in the great difference in the halogen quadrupole relaxation rate between free and protein-bound ions. For a monoatomic ion the magnitude of the quadrupole coupling constant is of intermolecular origin and, therefore, contains information on the environment of the ion which may, for example, be an anion binding site of a protein. For a polyatomic ion, on the other hand, the quadrupole coupling constant, being due to intramolecular effects such as the electron distribution in covalent bonds, will in general be relatively unaffected even by considerable changes in the binding state of the ion. The situation will be different for the case where the quadrupolar nucleus resides in a place of tetrahedral or octahedral symmetry such as for $^{35}ClO_4^-$. $R_4^{14}N^+$, $^{75}AsF_6^-$, $^{11}BR_4^-$ etc. Then due to symmetry the quadrupole coupling constant vanishes for the unperturbed ion and its value in a given situation should be directly related to the degree of distortion of the ion.

In the present communication we report using the binding of the perchlorate ion to human serum albumin as an example to demonstrate the feasibility of determining the quadrupole coupling constant for symmetric polyatomic ions bound to a protein. The quadrupole coupling constant contains information on the degree of distortion of the ligand at the protein site. It will also be shown that the ClO_4^- ion may be used (in the same way as halide ions) as a probe for investigating certain problems in protein chemistry. By combining results obtained with, for example, ClO_4^- and Cl^- a discrimination between different types of anion sites may be possible.

2. Experimental

The human serum albumin was a generous gift from AB Kabi, Stockholm; and was the same as that used in [4]. The preparation of the solutions is as described in [4]. Protein solutions were studied at pH 7.4 in 0.05 M Tris–$HClO_4$ buffer.

The ^{35}Cl relaxation times were measured at 8.82 MHz on a Bruker BKr-322s spectrometer with home-made probes. The longitudinal relaxation times were measured using a $180°$-τ-$90°$ pulse sequence and the transverse relaxation times by means of the Meiboom-Gill modification of the Carr–Purcell sequence. All signals were averaged with a Varian 1024 CAT time averaging computer to obtain a signal-to-noise ratio of at least 10:1. The resulting errors are estimated to be about 10% or less. Each reported relaxation time is the average of at least two independent measurements. The probe temperature was maintained by a stream of dry, thermostated gas and is accurate to ± 0.5 K.

3. Results and discussion

The ^{35}Cl longitudinal (T_1) and transverse (T_2) relaxation times of aqueous $NaClO_4$ solutions were

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determined as a function of the temperature and the concentration. The relaxation times of ClO_4^- were found to be considerably longer than those of Cl^- in aqueous solution. For both ions, T_1 and T_2 were found to be equal within the experimental error. Extrapolation to infinite dilution gives a ^{35}Cl relaxation time at 25°C in ClO_4^- which is 270 ms while that of Cl^- is 41 ms. For both ions the increase in ^{35}Cl relaxation rate with increasing concentration is slow; for $NaClO_4$ and NaCl solutions the relative changes in the concentration range 0–3 M are approx. 20 and 40%, respectively. The relaxation times were found to increase with increasing temperature, and to have Arrhenius' activation energies of ca. 11.2 and 11.4 kJ/mol for $NaClO_4$ (1.2 M) and NaCl (1.0 M) solutions, respectively.

As shown in fig.1 addition of human serum albumin to a solution containing ClO_4^- ions leads to marked decreases in both T_1 and T_2 demonstrating that both relaxation times may be used to follow the interaction of ClO_4^- with proteins. The excess relaxation rates,

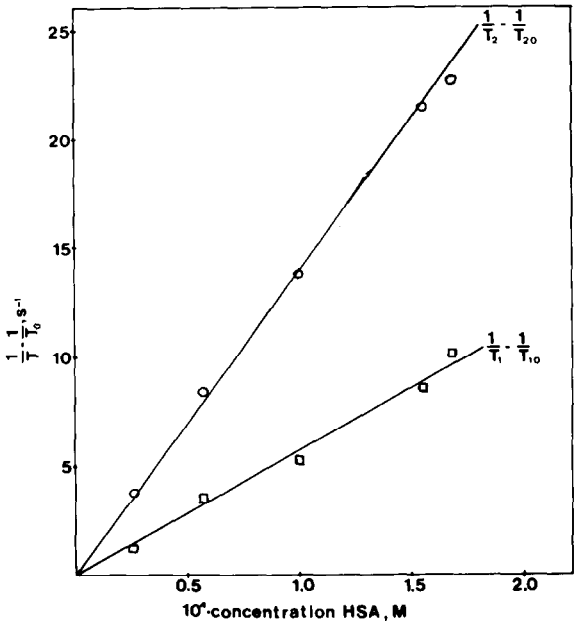

Fig.1. Variation of longitudinal (T_1^{-1}, □) and transverse (T_2^{-1} ○) ^{35}Cl relaxation rates of the ClO_4^- ion with concentration of human serum albumin (HSA). T_{1_0} and T_{2_0} denote values obtained in the absence of protein. The pH was 7.4 and the temperature was 22°C.

i.e. the differences between the relaxation rates in the presence and in the absence of protein, are proportional to the serum albumin concentration. The excess longitudinal relaxation rate may be written approximatively as [5]

$$\left(\frac{1}{T_1}\right)_{ex} = \frac{1}{T_1} - \frac{1}{T_{10}} = \sum_i \frac{2\pi^2}{5} p_i \nu^2 Q_i \tau_{ci} \left[\frac{0.8}{1 + 4\omega^2\tau_{ci}^2} + \frac{0.2}{1 + \omega^2\tau_{ci}^2}\right] \quad (1)$$

when the life-time of a ClO_4^- ion at the protein is much shorter than its ^{35}Cl relaxation time. A similar expression holds for the excess transverse relaxation rate. T_1 and T_{10} are the relaxation times in the presence and absence of protein, respectively; p_i is the probability of a ClO_4^- ion to be found at a site i on the protein; $\nu_Q = \frac{e^2qQ}{h}$ is the quadrupole coupling constant; τ_c is the correlation time characterizing the time-dependence of the field gradients; $\omega = 2\pi\nu$ is the Larmor frequency. In the case of slow exchange, T_1 and T_2 depend also on the rate of chemical exchange; expressions may be found in [5].

By investigating the dependence of $1/T_1$ on the ClO_4^- concentration at a constant protein concentration it could be established, in the same way as for Cl^- [4], that at least two classes of ClO_4^- binding, sites on serum albumin have to be accounted for. Addition of small amounts of sodium dodecyl sulfate (SDS) leads to increases in the relaxation times due to the displacement of the strongly bound ClO_4^- ions by dodecyl sulfate ions. SDS titration curves are analogous to those obtained previously in studies of the Cl^- ion [4], i.e. with a slope alteration at a SDS-to-protein molar ratio of about 9. Investigations of the competition between ClO_4^- and the halide ions gave the sequence $F^- < Cl^- < Br^- < I^- < ClO_4^-$ of increasing affinity for both the high and low affinity binding sites.

The temperature dependence of relaxation was investigated both for solutions containing ClO_4^- and serum albumin and for the corresponding solutions containing SDS in a concentration sufficient for approximatively total elimination of the contribution to relaxation from the high affinity binding sites.

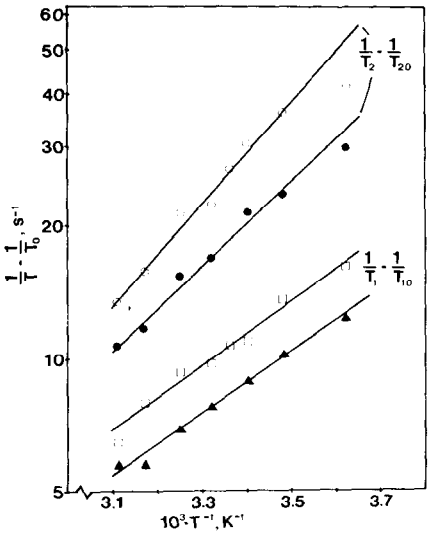

Fig.2. Temperature dependence of excess ^{35}Cl relaxation rates (Eq. 1) of the ClO_4^- ion in the presence of human serum albumin. The protein concentration was $2.2 \cdot 10^{-4}$ M. (•) and (▲) give the excess relaxation rates after addition of sodium dodecyl sulfate to $2.6 \cdot 10^{-3}$ M.

According to the results, which are shown in fig.2, both the strong and weak binding sites appear to give contributions to the relaxation rates which decrease with increasing temperature. This suggests that rapid exchange conditions may be assumed for both types of binding sites.

For the characterization of a ligand binding site on a protein both the correlation time and the quadrupole coupling constant are informative. The correlation time gives information on the rigidity of the binding site. For a monoatomic ion like Cl^- the quadrupole coupling constant is affected by nearby charges and dipoles and by coordination of the ion to metal ions while for the ClO_4^- ion it is mainly the degree of distortion from tetrahedral symmetry which comes into play. Assuming rapid exchange conditions it is possible, as described in [5] and [6] to obtain τ_C and ν_Q from a comparison of T_1 and T_2. For both the high and low affinity binding sites we find τ_C to be approx. 20 ns around room temperature which is in the range expected for protein reorientation. The ^{35}Cl quadrupole coupling constant of ClO_4^- bound at a high affinity site was calculated to be 1.5 ± 0.4 MHz assuming the number of these sites to be 9 (cf. above and [4]). For two reasons, the precision of this quadrupole coupling constant is reduced. First, as a result of a relatively strong (compared to Cl^-, see [4]) association of ClO_4^- with the low affinity sites the relative contribution to the relaxation rates of the high affinity sites is rather small at manageable ClO_4^- concentrations. Second, comparative studies using ^{37}Cl n.m.r. suggest that the exchange contribution to the relaxation may be important at room temperature and below.

These preliminary ^{35}Cl n.m.r. results suggest two main types of applications. One is to use the ClO_4^- ion in competition experiments to study the binding of auxiliary ligands in the same way as with the halide probe technique. In this way information on the stoichiometry and affinity of ligand binding as well as on the nature of the binding sites may be obtained analogously as by studies of the Cl^- ion [3,4,6]. However, because of their different chemical nature, the ClO_4^- and Cl^- ions are expected to interact differently with anion binding sites in proteins. Association involving dispersion forces, such as with binding sites composed of positively charged and non-polar amino-acid residues, should be more important for the more polarizable ClO_4^- ion. On the other hand, metal-coordinative binding, which important with Cl^-, should not be significant in the case of ClO_4^-. (The interaction of the ClO_4^- ion with most metal ions is generally considered to be very slight compared to other anions [7]). Both these ideas are supported by our experimental results. For example, addition of Zn^{2+} ions to a solution containing serum albumin and ClO_4^- gave no significant change in either T_1 or T_2 of ClO_4^- while considerable effects have been noted for Cl^- [8,9]. Therefore, studies using Cl relaxation of Cl^- and ClO_4^- are complementary and comparisons of the results obtained for the two ions should help in the discrimination between different types of protein–anion interactions.

The second type of application concerns the use of τ_C and ν_Q to elucidate the mechanism of ligand–protein interactions. The quadrupole coupling constant should be very sensitive to the distortion of the symmetric ligand and it seems that the present method provides a unique possibility of probing into the distortion of ligands on binding to proteins. Two different paths may be followed to investigate these problems. First, by comparisons of quadrupole coupling constant of different proteins an insight into the rela-

tion between the composition of the anion binding sites and the degree of ligand distortion may be gained. Second, at least for small ions it should be possible to calculate the relation between the geometrical distortion and the quadrupole coupling constant. Work along these lines will be attempted.

Acknowledgement

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