A ^{81}Br NUCLEAR MAGNETIC RESONANCE STUDY OF BROMIDE ION BINDING TO PROTEINS IN AQUEOUS SOLUTION.

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Summary

The ^{81}Br nuclear magnetic resonance line width in aqueous bromide solutions is broadened upon the addition of various proteins. Comparison of metallo-enzymes with metal-free proteins gives evidence for bromide ion binding independent of metallic sites. The effect of temperature and pH on the ^{81}Br resonance has been studied. Some measurements on ^{85}Rb, ^{79}Br and ^{35}Cl resonances are included for comparison.

Since the work by Stengle and Baldeschwieler[1, 2] on haemoglobin interest in studies of chloride ion binding to metallic sites in macromolecules has steadily increased. Most of the work has been done on mercury-labelled proteins by measuring the ^{35}Cl nuclear magnetic relaxation in aqueous solutions of the macromolecules in the presence of sodium chloride.[3-8] It has been known for several decades that "non-specific" binding of anions to metal-free proteins can take place.[9] As will be outlined below quadrupole relaxation in nuclear magnetic resonance offers a possibility to also study this "non-specific" complexing of anions to non-metallic sites in proteins.

A Varian V-4200 nmr spectrometer equipped with a 12 inch V-3603 magnet was used for the measurements. The magnetic field was 13.75 kG in the case of ^{81}Br and 14.05 kG for the ^{79}Br and ^{85}Rb measurements. All measurements are line width measurements, the line width being taken as the distance between maximum and minimum slope of the absorption signals. Experimental details have been given elsewhere.[10] The error in the measured line

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widths is less than 10 % (considerably smaller for the narrow lines), and the error in the given temperatures is less than 1°C.

In each series of measurements the solutions contained potassium bromide in the concentration range 0.4 - 0.5 M (see Table 1). The pH was adjusted by 0.05 M TRIS-phosphate or acetate buffers. Metal contamination of the solutions was avoided by recrystallization of KBr and phosphates from EDTA-water/ethanol. The proteins used were lysozyme (LYSO) obtained from Boehringer u. Soehne, Mannheim, Germany, α-chymotrypsin (CHYM) obtained from Worthington, human serumalbumin (HSA) and human ceruloplasmin (CER) from KABI, Stockholm, Sweden, horse liver alcohol dehydrogenase (L-ADH) a gift from Dr. Åke Åkesson, Stockholm and human carbonic anhydrase, form B, (HCAB) a gift from Dr. Per Olof Nyman, Gothenburg. The commercially obtained proteins were used without further purification.

The line widths observed for solutions containing only buffer, protein and potassium bromide are given in Table 1. It may be seen that the ^{81}Br nmr signal broadens markedly when the proteins are added to the potassium bromide solution. This is interpreted as a change in binding for part of the bromide ions. It is interesting to note that the relaxation of cations is not affected. Thus when lysozyme is added to a 0.5 M rubidium bromide solution (final concentration 9.0 mg/ml) the ^{85}Rb line width does not change significantly, whereas in the same solution the ^{81}Br signal width is about four times that of the corresponding solution without lysozyme. These measurements show that the broadening of the ^{81}Br nmr signal is not due to an overall slowing down of the molecular motion in the solution. The same conclusion can be drawn from viscosity data. Thus a solution containing 12.5 mg/ml of ceruloplasmin, which gave one of the broadest ^{81}Br signals, had a viscosity less than ten per cent greater than that of the same solution without enzyme.

In order to investigate whether exchange processes contribute to the broadening effect, the ^{79}Br nmr signal (as well as the ^{81}Br signal) was recorded for some solutions (samples no. V, XV, XVIII and XIX). In the limit of rapid exchange the ratio of the line widths should be 1.545[10, 11] (^{79}Br gives the broader signal). Only in the case of the serum albumin solution (sample no. XVIII)

Table 1

Observed ^{81}Br nmr line widths.

Sample no.	Protein	Protein conc. mg/ml	Approximate protein molarity	KBr conc. moles/liter	pH	Temp. °C	Line-width, gauss
I	-	-	-	0.50	7.5	4	0.33
II	-	-	-	0.50	7.5	26	0.22
III	L-ADH	1.84	$2.3 \cdot 10^{-5}$	0.45	7.5	4	0.46
IV	L-ADH	2.76	$3.4 \cdot 10^{-5}$	0.41	7.5	4	0.66
V	L-ADH	3.68	$4.6 \cdot 10^{-5}$	0.40	7.5	4	0.80
VI	L-ADH	2.76	$3.4 \cdot 10^{-5}$	0.41	4.25	4	0.71
VII	L-ADH	2.76	$3.4 \cdot 10^{-5}$	0.41	8.6	4	0.61
VIII	L-ADH	2.76	$3.4 \cdot 10^{-5}$	0.41	8.9	4	0.64
IX	L-ADH	2.76	$3.4 \cdot 10^{-5}$	0.41	9.25	4	0.65
X	HCAB	3.0	$1.0 \cdot 10^{-4}$	0.50	7.5	4	0.49
XI	LYSO	5.0	$3.5 \cdot 10^{-4}$	0.50	7.5	4	0.92
XII	LYSO	5.0	$3.5 \cdot 10^{-4}$	0.50	7.5	17	0.59
XIII	LYSO	10.0	$7.1 \cdot 10^{-4}$	0.50	7.5	17	1.13
XIV	CER (native)	12.5	$1 \cdot 10^{-4}$	0.375	6.35	4	0.81
XV	CER (native)	10.3	$0.8 \cdot 10^{-4}$	0.42	7.5	26	0.40
XVI	CER (reduced)	12.5	$1.0 \cdot 10^{-4}$	0.375	6.35	4	1.16
XVII	CER (reduced)	10.3	$0.8 \cdot 10^{-4}$	0.42	7.5	26	0.57
XVIII	HSA	4.9	$0.7 \cdot 10^{-4}$	0.50	7.5	26	0.71
XIX	CHYM	4.9	$2.0 \cdot 10^{-4}$	0.50	7.5	26	0.37

was a significant deviation from this figure obtained. Except for this solution the observed line widths should be given by the following expression (for arguments see e.g. ref. 10 and 11)

$$\Delta B_{obsd} = \Sigma p_i \Delta B_i + p_o \Delta B_o \qquad (1)$$

Here p_i is the probability for the bromine to be located in a site $\underline{i}$ on the protein and ΔB_i is the ^{81}Br line width characterizing this site. p_o is the mole fraction of unbound bromide ions and ΔB_o the corresponding line width.

For the 0.5 % solution of lysozyme, measurements were made at two temperatures. The calculated apparent energy of activation for the relaxation process is 5-6 kcal/mole which is considerably higher than that observed in aqueous solutions of alkali bromides[12] (cf. also ref. 11).

We now turn to a discussion of the ^{81}Br line widths obtained for the various protein solutions. For L-ADH the line width increases strongly with increasing enzyme concentration when the KBr concentration is kept approximately constant, whereas the line width is only slightly dependent on pH within the pH-range investigated. Addition of even fairly large amounts of substances believed to block at least some of the zinc atoms, such as the coenzymes NAD (nicotine adenine dinucleotide) and NADH, as well as oxyquinoline, had no detectable influence on the line width. This indicates that bromide ion binding to those zinc atoms blocked does not contribute significantly to the observed line broadening effects. Similarly, addition of an aromatic sulfonamide to a HCAB solution did not influence the line width. This is in striking contrast to the behaviour of the ^{35}Cl resonance (unpublished measurements, see also ref. 13).

In the case of ceruloplasmin, a ^{81}Br line-broadening is observed, which is enhanced upon reduction with ascorbate. Addition of 3,7-dimethylphenanthroline to the reduced enzyme causes no further change in the ^{81}Br resonance. The ^{35}Cl resonance (measured in a 0.5 M RbCl solution) is, however, not significantly affected by reduction. The ^{81}Br line broadening of ceruloplasmin seems to be enhanced after denaturation in 6 M urea.

We are investigating these phenomena in more detail.

The considerable ^{81}Br line broadening by lysozyme, α-chymotrypsin, and especially serum albumin, is direct evidence for bromide binding independent of metallic sites. The mechanism involved in the anion binding is not yet understood. In the case of lysozyme the X-ray structure[14] shows that some hydrophobic side chains as well as the polar side chains of all lysine and arginine residues are present on the "outside" of the enzyme. The alkyl chains may modify the surrounding water lattice[15, 16] and bring about a more efficient quadrupole relaxation of the anions coupled to the modified lattice as compared to ordinary aqueous solutions[10, 11]. Ion-pair type bonding of the anion to the guanidyl side chain of arginine or the $\varepsilon\text{-}NH_3^+$ group of lysine residues - some of which are close to each other on the enzyme surface and allow a chelating type of anion binding similar to that in strongly basic ion exchangers - should also result in a more efficient quadrupole relaxation of ^{81}Br and ^{79}Br. One way of determining the mechanism for bromide ion binding is to investigate the change in anion binding on modifying the protein structure and to compare measurements on protein solutions with those on polyamino acid solutions. Work along these lines is in progress in our laboratories.

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