Anion Binding Properties of Human Serum Albumin from Halide Ion Quadrupole Relaxation†

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ABSTRACT: The nuclear magnetic quadrupole relaxation enhancement of $^{35}Cl^-$, $^{81}Br^-$, and $^{127}I^-$ anions on binding to human serum albumin has been studied under conditions of variable protein and anion concentration and also in the presence of simple inorganic, amphiphilic, and complex anions which compete with the halide ions for the protein anion binding sites. Two classes of anion binding sites with greatly different binding constants were identified. Experiments at variable halide ion concentration were employed to determine the Cl^- and I^- binding constants. By means of ^{35}Cl nuclear magnetic resonance (NMR) the relative affinity for different anions was determined by competition experiments for both the strong and the weak anion binding sites. Anion binding follows the sequence $SO_4^{2-} < F^- < CH_3COO^- < Cl^- < Br^- < NO_3^- < I^- < ClO_4^- < SCN^- < Pt(CN)_4^{2-} < Au(CN)_2^- \ll CH_3(CH_2)_{11}OSO_3^-$ for the high affinity sites, and the sequence $SO_4^{2-} \simeq F^- \simeq Cl^- < CH_3COO^- < NO_3^- < Br^- < I^- < ClO_4^- < SCN^-$ for the low affinity sites. These series are nearly identical with the well-known lyotropic series. Consequently, those effects of anions on proteins described by the lyotropic series can be correlated with the affinities of the anions for binding to the protein. The data suggest that the physical nature of the interaction is the same for both types of binding sites, and that the differences in affinity between different binding sites must be explained in terms of tertiary structure. Analogous experiments performed using $^{127}I^-$ quadrupole relaxation gave results very similar to those obtained with $^{35}Cl^-$. A comparison between the Cl^-, Br^-, and I^- ions revealed that, as a result of the increasing affinity for the weak anion binding sites in the series $Cl^- < Br^- < I^-$, Cl^- is much more useful as a probe for the specific anion binding sites than the other two halide ions. The findings with human serum albumin in this and other respects are probably of general relevance in studies of protein–anion interactions. In addition to competition experiments, the magnitude of the relaxation rate is also discussed. Line broadening not related to anion binding to the protein is found to be small. A comparison of transverse and longitudinal ^{35}Cl relaxation rates gives a value for the quadrupole coupling constant of the high affinity sites in good agreement with a calculated coupling constant assuming anion binding to arginine.

Since the days of Hofmeister the interaction of neutral salts with biological macromolecules has been a matter of great interest, but, in spite of great experimental and theoretical efforts, the physical mechanisms underlying the perturbation of macromolecular structure by anions and cations are not well understood. In 1969 a critical review of this field was presented by von Hippel and Schleich (1969) who emphasized the complexity of the interactions between macromolecules, ions, and the solvent water. It is generally accepted that exposed groups on the macromolecular surface experience ion–ion or ion–dipole interactions. It is also accepted that other than mere electrostatic effects seem to be responsible for the so-called lyotropic action of ions. This action leads to either a stabilization or destabilization of the native structures as manifested by changes in the thermal transition temperatures, solubilities, enzymatic activities, optical properties of proteins, and changes in association–dissociation equilibria. It is often discussed to what extent the lyotropic action of ions is due to direct binding and interaction of the ions with certain groups on the macromolecules, and to what extent it is brought about by changes of the solvent structure. Site-directed interactions are characterized by large affinity constants and well-defined stoichiometries whereas indirect interactions will be characterized by low or undetectable levels of affinity. Apart from the principal difficulties of distinguishing between extremely low affinities and indirect effects, there is a shortage of analytical methods which allow the study of weak interactions between macromolecules and simple ions.

In recent years the technique of nuclear magnetic quadrupole relaxation has become a potent tool for the study of ion binding to proteins. (For a recent review see Dwek, 1973.) This method allows the study of both strong and weak anion and cation binding sites using nuclei like $^{35}Cl^-$, $^{37}Cl^-$, $^{79}Br^-$, $^{81}Br^-$, $^{127}I^-$, $^{23}Na^+$, and others as chemical probes. By applying certain criteria it is possible to discriminate between metallic and nonmetallic binding sites (Norne et al., 1973). Simple anions, e.g. Cl^-, Br^-, or I^-, can interact with the binding sites of negatively charged substrates such as the large group of biological phosphoric acid esters and can therefore be useful to characterize binding sites of functional importance (Lindman et al., 1972b; Ward and Happe, 1971; Ward and Cull, 1974; Norne et al., 1974; Springgate et al., 1973). ^{127}I nuclear magnetic resonance (NMR) has only rarely been utilized for studies of fluid systems partly because, for covalent environments, the ^{127}I NMR signal is generally broadened beyond detection. The iodide ion, on the other hand, may be conveniently studied and its NMR signal has been used in some cases to study ionic interactions (see, e.g., Itoh and Yamagata,

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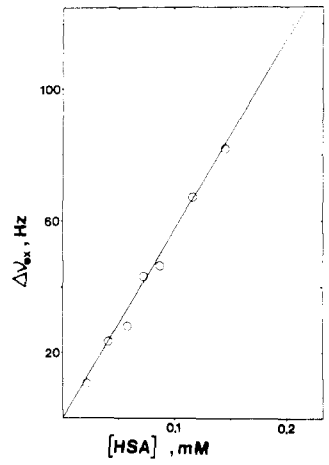

FIGURE 1: Variation of ^{35}Cl excess line width, $\Delta\nu_{ex}$, with human serum albumin concentration. In addition to the protein, the solutions contained 0.5 *M* KCl and 0.05 *M* Tris-HCl buffer (pH 7.4); temperature, 24°.

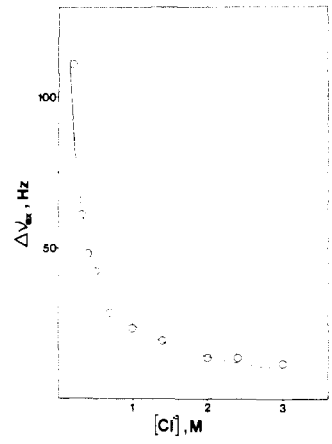

FIGURE 2: Variation of ^{35}Cl excess line width, $\Delta\nu_{ex}$, with chloride concentration at a constant human serum albumin concentration of 0.72×10^{-4} *M*. The solutions contained 0.05 *M* Tris-HCl buffer at pH 7.4 and the experimental temperature was 24°. The solid curve is calculated assuming $K_{A,Cl} \gg C_{Cl}$, $K_{B,Cl} = 0.95\ M^{-1}$, $n_A\Delta\nu_A = 1.97 \times 10^5$ Hz, and $n_B\Delta\nu_B = 2.66 \times 10^5$ Hz. These quantities were obtained by computer least-squares fits as described in the text, where their definition is also given.

1958; Hertz et al., 1974; O'Reilly et al., 1963; Wennerström et al., 1971). Since no reports are given in the literature on studies of macromolecular systems it was of interest to explore the possibility of using $^{127}I^-$ NMR for probing into the anion binding sites of proteins.

The present work was carried out with the aim of using $^{35}Cl^-$ and $^{127}I^-$ as probes for the anion binding properties of human serum albumin and, by competition experiments, to find out the relative binding strength of various anions at both strong and weak anion binding sites. As will be shown the NMR experiments reveal that both classes of sites respond similarly to the lyotropic series.

Experimental Procedure

Materials. Human serum albumin was obtained from AB KABI (Stockholm) and used without further purification. Samples dialyzed exhaustively against triply distilled water gave identical results. Albumin solutions were prepared either by appropriate dilution of stock solutions with buffer containing the salts or by dialysis against saline buffers. Tris(hydroxymethyl)aminomethane was obtained as the Ultra Pure grade from Mann Research Laboratories. All other chemicals were of the highest grade available. Triply distilled water was used in all experiments.

Methods. ^{35}Cl NMR line widths were measured at half-height of the NMR absorption curves and the experimental conditions were as previously described (Norne et al., 1974). ^{81}Br NMR line widths were measured between inflection points of the NMR absorption curve as described earlier (Lindblom et al., 1973).

The ^{127}I NMR signals were recorded using a Varian V-4200 NMR spectrometer and a 12-in. Varian V-3603 magnet at a magnetic field of 1.40 T. The magnetic field modulation frequency was 80 Hz. Broadening resulting from the use of a finite modulation amplitude and saturation broadening are estimated to be 1–2%. The ^{127}I NMR line width was taken as the distance between maximum and minimum in the recorded absorption mode derivative signal.

All line widths are reported as excess line widths, $\Delta\nu_{ex}$, where $\Delta\nu_{ex} = \Delta\nu_{obsd} - \Delta\nu_0$, $\Delta\nu_{obsd}$ being the observed line width and $\Delta\nu_0$ that obtained in the absence of protein. $\Delta\nu_0$ was observed to be ca. 16 Hz for ^{35}Cl and ca. 980 Hz for ^{127}I. For ^{35}Cl there is a small contribution to $\Delta\nu_0$ resulting from magnetic field inhomogeneity. The $\Delta\nu_0$ value obtained for ^{127}I is close to that selected by Hertz (1973).

All spectra were recorded at 24 ± 1°. The reported line widths constitute the averages of 5–10 spectra. The error in the measurements is estimated to be generally ca. 5% in the case of ^{35}Cl. For low Cl^- concentrations and for ^{127}I the error is somewhat greater.

For ^{127}I NMR studies of aqueous iodide solutions, even quite small amounts of I_2 may produce a substantial line broadening as a result of chemical exchange of ^{127}I between I^- and I_2. In I_2, ^{127}I relaxation is extremely rapid due to the great quadrupole coupling constant. In order to minimize the influence of this effect all solutions were investigated freshly prepared. Furthermore, it was established that the addition of a small amount of sodium thiosulfate did not significantly affect the line width.

Results

Different types of experiments were carried out in order to study the influence on the $^{35}Cl^-$ or $^{127}I^-$ NMR line widths of protein concentration, anion concentration, and of the presence and concentration of anions competing for the halogen ion binding sites. As a rule the potassium salts were utilized. The results are given in terms of the excess NMR line width, $\Delta\nu_{ex}$, defined as the difference between observed line width and line width in the absence of protein. Line widths are related to the transverse relaxation times, T_2, by $\Delta\nu = 1/\pi T_2$ in the case of ^{35}Cl where line width is measured at half-height of the NMR absorption curve and by $\Delta\nu = 1/\pi 3^{1/2} T_2$ in the case of ^{81}Br and ^{127}I where line width is measured between the inflection points of the NMR absorption curve.

Protein Concentration. The ^{35}Cl line width increases linearly with protein concentration as shown in Figure 1. As is apparent from the discussion below, this implies that the intrinsic relaxation rate of protein-bound chloride ions and the number of chloride binding sites do not change with protein concentration. No effect of protein self-association is indicated in our data.

Anion Concentration. Figure 2 shows the variation of the ^{35}Cl NMR line width with chloride concentration in the

Table I: Effect on the ^{35}Cl NMR Excess Line Width, $\Delta\nu_{ex}$, of Addition of Anions in the Lyotropic Series to Solutions Containing Human Serum Albumin, Potassium Chloride, and 0.05 *M* Tris-HCl Buffer (pH 7.4); Temperature, 24°.

Added Salt	$\Delta\nu_{ex}$ (Hz)		
	a	*b*	*c*
None	47.9	33.5	17.6
K_2SO_4	46.9	32.7	16.8
KF	46.5	33.5	16.9
KCl	42.0	30.0	16.8
CH_3COONa	40.5	31.2	16.2
KBr	32.2	27.1	13.8
KNO_3	28.5	26.8	14.4
KI	19.5	20.9	10.8
$NaClO_4$	18.7	19.1	10.0
KSCN	13.6	16.1	9.0

[a] Addition of 0.1 *M* competing anion to solutions containing 0.4 *M* KCl and 0.72×10^{-4} *M* human serum albumin. [b] Addition of 0.6 *M* competing anion to solutions containing 2.4 *M* KCl and 1.8×10^{-4} *M* human serum albumin. [c] Addition of 0.1 *M* competing anion to solutions containing 0.4 *M* KCl, 1.03×10^{-4} *M* human serum albumin, and 13.4×10^{-4} *M* sodium dodecyl sulfate.

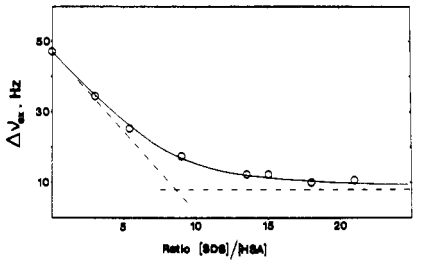

FIGURE 3: Effect of addition of sodium dodecyl sulfate (SDS) on the ^{35}Cl excess line width, $\Delta\nu_{ex}$, for solutions containing 0.4 *M* KCl, 0.72×10^{-4} *M* human serum albumin, and 0.05 *M* Tris-HCl buffer at pH 7.4; temperature, 24°.

range 0.2–3.0 *M* at a constant protein concentration of 0.72×10^{-4} *M*. The curve shows a continuous decrease of the excess line width, $\Delta\nu_{ex}$, even at high concentrations of chloride and fits closely to a theoretical curve calculated on the assumption of two classes of chloride binding sites with high and low affinities, respectively (see below).

Competition Experiments with Anions of the Lyotropic Series. If a protein contains two types of anion binding sites, one can test the relative affinities for these sites of different anions by three types of experiments. The halogen NMR line width is measured at constant protein concentration in solutions containing the halogen and the competing anions in fixed ratios: (i) at low absolute halogen ion concentration; under these conditions the effect of the high affinity sites is dominant; (ii) at high absolute anion concentrations; here the former sites are fully occupied and the influence of the low affinity sites comes into account; (iii) in the presence of stoichiometric amounts of strongly binding halide-competitive ligands; here the influence of the high affinity sites is eliminated. In Table I are given experimental ^{35}Cl line-width results referring to the three cases i, ii, and iii. In all cases the solutions contained chloride and competing anions in the ratio 4:1. The total electrolyte concentration was 0.5 *M* in the experiments of types i and iii and 3.0 *M* in the experiments of type ii. The salts are ordered in Table I according to their increasing efficiency of competition. The order corresponds clearly to the lyotropic series for both the high and the low affinity sites.

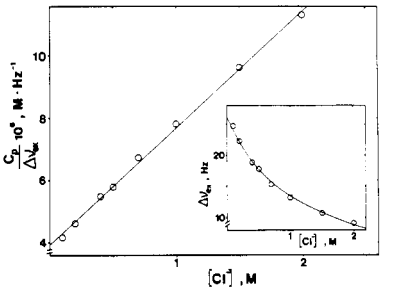

FIGURE 4: Variation of the ^{35}Cl excess line width, $\Delta\nu_{ex}$, with chloride concentration for solutions containing 1.03×10^{-4} *M* human serum albumin, 13.4×10^{-4} *M* sodium dodecyl sulfate, and 0.05 *M* Tris-HCl buffer at pH 7.4; temperature, 24°. In the main figure is given a plot of protein concentration divided by $\Delta\nu_{ex}$ vs. chloride concentration whereas in the insert $\Delta\nu_{ex}$ is given as a function of chloride concentration. The full-drawn curve in the insert was calculated using the values $K_{B,Cl} = 0.95\ M^{-1}$ and $n_B\Delta\nu_B = 2.66 \times 10^5$ Hz which were obtained by computer least-squares fits. (The symbols are defined in the text.)

Table II: Effect on the ^{35}Cl NMR Excess Line Width, $\Delta\nu_{ex}$, of Addition of Various Complex Anions to Solutions Containing 0.72×10^{-4} *M* Human Serum Albumin, 0.5 *M* Potassium Chloride, and 0.05 *M* Tris-HCl Buffer (pH 7.4).[a]

Added Salt	$\Delta\nu_{ex}$ (Hz)	Added Salt	$\Delta\nu_{ex}$ (Hz)
None	40.0	KSCN	29.6
$Na_3(Co(NO_2)_6)$	45.8	KPF_6	29.1
KBr	38.0	$CH_2(CH_2)_4CHNHSO_3Na$	23.0
KNO_3	37.7	$KAsF_6$	22.1
$K_4Fe(CN)_6$	37.0	$NH_4(Cr(NH_3)_2(SCN)_4)$	14.5
KI	36.3	$K_2Pt(CN)_4$	18.2
$K_3Fe(CN)_6$	36.0	$KAu(CN)_2$	16.1
$Na_2NH_4Fe(CN)_5NH_3$	35.4	$CH_3(CH_2)_{11}SO_4Na$	10.0
$NaClO_4$	32.0		

[a] Temperature, 24°; concentration of added anions was 6×10^{-3} *M*. As a comparison some anions of the lyotropic series were studied under these conditions.

Concerning type iii it has been shown that the high affinity sites can be blocked by adding stoichiometric amounts of detergent anions (Gillberg-La Force and Forsén, 1970). This has been confirmed in this work using sodium dodecyl sulfate (see Figure 3). The stoichiometry corresponds to the strong binding of 9 ± 1 dodecyl sulfate ions per serum albumin molecule and about 13 mol of sodium dodecyl sulfate/mol of serum albumin is sufficient to decrease the ^{35}Cl NMR line width to a constant level. At this molar ratio of detergent to protein the chloride concentration was varied at a constant protein concentration. The results are plotted in Figure 4 as the ratio of protein concentration, C_p, over excess line width vs. chloride concentration. The plot is linear as expected for a simple model (see Discussion below).

Competition by Complex Anions. Kinetically stable complex anions of varying charge and size should offer many possibilities for probing and labeling the high affinity sites. If they possess suitable physical properties, they can be studied separately by different spectroscopic techniques. Table II shows the effect on the ^{35}Cl NMR line width of a few complex anions.

It should be noted that the complex anions reduce the $^{35}Cl^-$ line width at very low concentrations. The only exception is hexanitrocobaltate(III), which increases the line width. Most probably, the nitro groups are exchanged rapidly and the cobalt becomes part of a metallic chloride binding site in close analogy to the action of metal complexes of

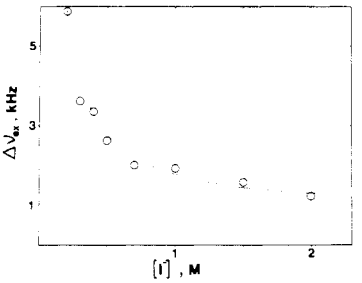

FIGURE 5: Variation of ^{127}I excess line width, $\Delta\nu_{ex}$, with iodide concentration at pH 7.4 and 24° and at a constant human serum albumin concentration of 0.72×10^{-4} M. The curve is calculated assuming $K_{A,Cl} \gg C_{Cl}$, $K_{B,I} = 6$ M^{-1}, $n_A\Delta\nu_A = 6 \times 10^6$ Hz, and $n_B\Delta\nu_B = 22 \times 10^6$ Hz. These values were obtained by computer least-squares fits as described in the text, where the symbols are also defined.

Table III: Effect on the ^{127}I NMR Excess Line Width, $\Delta\nu_{ex}$, of Addition of 0.2 M Anions in the Lyotropic Series to Solutions Containing 0.5 M KI and 0.72×10^{-4} M Human Serum Albumin; Temperature, 24°; pH 7.4.

Added Salt	$\Delta\nu_{ex}$ (kHz)
None	2.62
KBr	2.52
KNO_3	2.37
$NaClO_4$	2.27
KI	2.01
KSCN	1.94

serum albumin (Norne et al., 1973; Sudmeier and Pesek, 1971).

^{127}I NMR Measurements. In order to test the relative affinities of strong and weak anion binding sites with iodide as a probe, the variation of $\Delta\nu_{ex}$ of ^{127}I with the iodide concentration was investigated (Figure 5). Blocking of the high affinity sites with dodecyl sulfate yielded a plot of $C_p/\Delta\nu_{ex}$ vs. iodide concentration which was analogous to the results obtained for ^{35}Cl shown in Figure 4. The action of different salts (0.2 M at 0.5 M KI, 0.72×10^{-4} M protein) in competition experiments analogous to those reported for $^{35}Cl^-$ again followed the lyotropic series as shown in Table III. These data can be used to evaluate the relative usefulness of Cl^-, Br^-, and I^- as chemical probes of anion binding sites (cf. Figure 6 and Discussion section).

^{81}Br NMR Measurements. Some determinations of $\Delta\nu_{ex}$ of ^{81}Br were made in the absence and presence of dodecyl sulfate to obtain the parameters used in constructing Figure 6.

Discussion

Evaluation of Binding Constants. The observable NMR relaxation rates or line widths are determined by the distribution of the nuclei studied over different environments, by the rate of exchange between the different environments, and by the intrinsic relaxation rates of the environments. From these intrinsic relaxation rates it is possible to obtain information on field gradients and molecular mobility of the sites. The discussion of these matters will be deferred to the end of this section. First the distribution of the anions over different binding sites will be considered.

It has been shown earlier that, in the case of serum albumin, the rate of exchange of chloride ions between protein binding sites and the solvent exceeds the nuclear magnetic relaxation rate of the different sites (Norne et al., 1973).

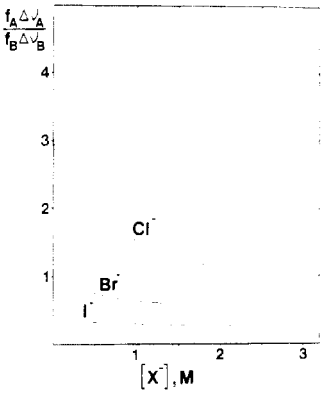

FIGURE 6: The ratio of the broadenings of Cl^-, Br^-, and I^- NMR signals of the high and low affinity binding sites of human serum albumin as a function of halide ion concentration. The following values were used in constructing the curves: for $^{35}Cl^-$, $n_A\Delta\nu_A = 1.97 \times 10^5$ Hz, $n_B\Delta\nu_B = 2.66 \times 10^5$ Hz, and $K_{B,Cl} = 0.95$ M^{-1}; for $^{81}Br^-$, $n_A\Delta\nu_A = 1.7 \times 10^6$ Hz, $n_B\Delta\nu_B = 3.3 \times 10^6$ Hz, and $K_{B,Br} = 4.5$ M^{-1}; for $^{127}I^-$, $n_A\Delta\nu_A = 6 \times 10^6$ Hz, $n_B\Delta\nu_B = 22 \times 10^6$ Hz, and $K_{B,I} = 6$ M^{-1}. The ratio was calculated by use of the expression in eq 3c and assuming $K_{A,X}$ to be much greater than the inverse halide ion concentration.

The observed line width is then a weighted average of the line widths of the different sites according to eq 1:

$$\Delta\nu_{obsd} = f_0\Delta\nu_0 + f_1\Delta\nu_1 + \ldots = \sum_{i=0}^{n} f_i\Delta\nu_i \quad (1)$$

where f_i designates the probability of the ion occurring in site i and $\Delta\nu_i$ is the characteristic line width of this site. Since the chloride concentration in our experiments exceeds the protein concentration by a factor of ca. 10^3, f_0, the fraction of free ions, may be approximated to unity. $\Delta\nu_0$ is the line width in the absence of protein. If we let $\Delta\nu_{ex}$ be the excess line width observed in the presence of protein, then we obtain:

$$\Delta\nu_{ex} = \Delta\nu_{obsd} - \Delta\nu_0 = \sum_{i=1}^{n} f_i\Delta\nu_i \quad (2)$$

At constant chloride ion concentration, $\Delta\nu_{ex}$ will increase proportionally to the protein concentration as shown in Figure 1. This observation is consistent with, but not a proof of, the validity of the fast exchange model cited above, which was confirmed through investigations of the temperature dependence of the line width.

If the protein possesses only one type of binding site, A, characterized by an affinity constant, $K_{A,Cl}$, eq 2 reads:

$$\Delta\nu_{ex} = f_A\Delta\nu_A \quad (3a)$$

where

$$f_A = C_p n_A K_{A,Cl}/(1 + K_{A,Cl}C_{Cl}) \quad (3b)$$

n_A is the number of binding sites per protein molecule and C_{Cl} is the total chloride concentration. If there are two classes of binding sites, A and B, on the protein, characterized by the binding constants $K_{A,Cl}$ and $K_{B,Cl}$, respectively, eq 2 becomes:

$$\Delta\nu_{ex} = f_A\Delta\nu_A + f_B\Delta\nu_B$$

or

$$\Delta\nu_{ex} = C_p\left[\frac{n_A\Delta\nu_A K_{A,Cl}}{1 + K_{A,Cl}C_{Cl}} + \frac{n_B\Delta\nu_B K_{B,Cl}}{1 + K_{B,Cl}C_{Cl}}\right] \quad (3c)$$

n_A and n_B are the number of binding sites of classes A and B, respectively. To distinguish between classes of strong and weak binding sites, the chloride concentration may be var-

ied at a constant protein concentration. If eq 3a and 3b are valid and if K is large, $\Delta\nu_{ex}$ will approach zero as the chloride concentration is increased. From Figure 2 it is apparent that eq 3a cannot be used to rationalize the experimental data. It has been shown earlier by different authors that serum albumin contains at least two different kinds of anion binding sites (Steinhardt and Reynolds, 1969; Lindman et al., 1972a; Muller and Mead, 1973; Tanford, 1968), $K_{A,Cl}$ being much greater than $K_{B,Cl}$. With increasing chloride concentration, interactions with the low affinity sites come into account, giving an additional contribution to $\Delta\nu_{ex}$. Attempts were made to fit the observed data to a theoretical curve corresponding to eq 3c and in this way determine the quantities $n_A\Delta\nu_A$, $n_B\Delta\nu_B$, and $K_{B,Cl}$. The value for $K_{A,Cl}$ has been taken to be much greater than $1/C_{Cl}$. In such cases the actual value has no influence on plots like that in Figure 2. In the study by Tauja-Chareyre (1972), chloride binding constants of 100–400 M^{-1} were obtained, which is in agreement with our assumption. $n_A\Delta\nu_A$ was determined by blocking the strong binding sites with dodecyl sulfate anions. From Figure 3, we obtain by extrapolation $n_A = 9 \pm 1$, which is in accordance with previous findings (Gillberg-La Force and Forsén, 1970; Steinhardt and Reynolds, 1969; Lindman et al., 1972a; Muller and Mead, 1973). From the same diagram we obtain $f_A\Delta\nu_A$, the contribution of the strong binding sites to the line width. Since f_A equals the ratio between the concentration of strong sites and the total chloride concentration, i.e. n_AC_p/C_{Cl}, we get $n_A\Delta\nu_A = f_A\Delta\nu_A$ and $C_{Cl}/C_p = 2.0 \times 10^5$ Hz. The binding of dodecyl sulfate to site A eliminates the contribution of the high affinity sites to the ^{35}Cl line width and eq 3c simplifies to:

$$\Delta\nu_{ex} = C_p[n_B\Delta\nu_BK_{B,Cl}/(1 + K_{B,Cl}C_{Cl})] \quad (4)$$

or

$$C_p/\Delta\nu_{ex} = 1/n_B\Delta\nu_BK_{B,Cl} + C_{Cl}/n_B\Delta\nu_B \quad (4a)$$

From a plot of $C_p/\Delta\nu_{ex}$ vs. C_{Cl} (see Figure 4) it is seen that the experimental observations conform closely to the predicted behavior. For the evaluation of $n_B\Delta\nu_B$ and $K_{B,Cl}$ it was found to be more appropriate to use eq 4. Computer least-squares fits were used to find the best agreement between experimental and theoretical titration curves (see insert of Figure 4). In this way we obtained $n_B\Delta\nu_B = (2.66 \pm 0.30) \times 10^5$ Hz and $K_{B,Cl} = 0.95 \pm 0.20$ M^{-1}. In Figure 2 is included a titration curve calculated on the basis of the derived parameters for the strong and weak binding sites.

According to the results given above there is a competition for sites A and B between chloride ions and various other anions and this can be used to obtain information on the relative binding strength of the competing anions. The situation can be formally described by eq 5 (see Steinhardt and Reynolds, 1969):

$$\Delta\nu_{ex} = C_p\left[\frac{n_A\Delta\nu_AK_{A,Cl}}{1 + K_{A,Cl}C_{Cl} + K_{A,X}C_X} + \frac{n_B\Delta\nu_BK_{B,Cl}}{1 + K_{B,Cl}C_{Cl} + K_{B,X}C_X}\right] \quad (5)$$

In the case where X = dodecyl sulfate, $K_{A,X}$ equals $(1.0–1.2) \times 10^6$ M^{-1} (Steinhardt and Reynolds, 1969) and the first term within brackets in eq 5 vanishes even at low dodecyl sulfate concentrations. After rearrangement we get, in the presence of dodecyl sulfate:

$$\frac{C_p}{\Delta\nu_{ex}} = \frac{1}{n_B\Delta\nu_BK_{B,Cl}} + \frac{C_{Cl}}{n_B\Delta\nu_B} + \frac{K_{B,X}C_X}{n_B\Delta\nu_BK_{B,Cl}} \quad (6)$$

Table IV: Binding Constants Relative to Chloride of Anions (X) in the Lyotropic Series.[a]

(a) High Affinity Sites	
Anion, X	$K_{A,X}/K_{A,Cl}$
SO_4^{2-}	0.05
F^-	0.1
CH_3COO^-	0.8
Br^-	2.3
NO_3^-	3.7
I^-	7.8
ClO_4^-	8.1
SCN^-	15
(b) Low Affinity Sites	
Anion, X	$K_{B,X}/K_{B,Cl}$
SO_4^{2-}	1.0
F^-	1.0
CH_3COO^-	1.7
NO_3^-	3.8
Br^-	4.7
I^-	10
ClO_4^-	12
SCN^-	15

[a] The binding constants were obtained from the ^{35}Cl line-width data as described in the text. A and B denote high and low affinity sites, respectively.

In the absence of competing anions other than the stoichiometric amount of dodecyl sulfate blocking the A sites, i.e. $C_X = 0$, we have $(C_p/\Delta\nu_{ex})_{C_X=0} = (1 + C_{Cl}K_{B,Cl})/n_B\Delta\nu_BK_{B,Cl}$. In the presence of a competing ligand this is modified to eq 7 and one obtains for the ratio of the binding constants:

$$\frac{K_{B,X}}{K_{B,Cl}} = \frac{n_B\Delta\nu_B}{C_X}\left[\frac{C_p}{\Delta\nu_{ex}} - \left(\frac{C_p}{\Delta\nu_{ex}}\right)_{C_X=0}\right] \quad (7)$$

Table IV gives the numerical values of $K_{B,X}/K_{B,Cl}$ obtained for the most common anions. (It should be mentioned that since our main interest was in establishing the sequence of anion binding strength, no detailed titrations were performed in order to obtain precise binding constants.) As already mentioned, the relative affinity of the anions for binding to B sites corresponds to the lyotropic series. Knowledge of $K_{B,X}/K_{B,Cl}$ enables us to calculate $K_{A,X}/K_{A,Cl}$ from the experimental ^{35}Cl line widths obtained in the absence of dodecyl sulfate and eq 5 which is written as:

$$n_A\Delta\nu_AC_p/(\Delta\nu_{ex} - C_p\beta) = 1/K_{A,Cl} + C_{Cl} + (K_{A,X}/K_{A,Cl})C_X \quad (8)$$

where $\beta = n_B\Delta\nu_BK_{B,Cl}/(1 + K_{B,Cl}C_{Cl} + K_{B,X}C_X) = f_B\Delta\nu_B/C_p$. Using the data given above (Table I) the values of $K_{A,X}/K_{A,Cl}$ given in Table IV were calculated for different anions assuming that $1/K_{A,Cl} \ll C_{Cl}$ (cf. Tauja-Chareyre, 1972).

The principles for evaluation of the data described for ^{35}Cl apply equally well for ^{81}Br and ^{127}I. Thus, using the procedures outlined above we obtained for $^{81}Br^-$, $n_A\Delta\nu_A = 1.7 \times 10^6$ Hz and $n_B\Delta\nu_B = 3.3 \times 10^6$ Hz, and for $^{127}I^-$, $n_A\Delta\nu_A = (6 \pm 1) \times 10^6$ Hz, $n_B\nu_B = 22 \times 10^6$ Hz, and $K_{B,I} = 6$ M^{-1}. From these data, the relative contributions to the total excess line width from sites A and B was determined. Figure 6 shows, as a function of halide concentration, the ratio between the line broadening caused by the strong binding sites and that due to the weak ones for the three halide ions. It is obvious from Figure 6 that the relative impor-

tance of the B sites increases from chloride to iodide. This difference between the halide ions can be referred to differences in both the ratios $n_B\Delta\nu_B/n_A\Delta\nu_A$ and in the binding constants of the low affinity sites. As a consequence of the stronger binding of I^- ions to the low affinity sites, contributions to the line width from these sites become significant at much lower ligand concentrations than for Cl^-. This means that measurements performed to obtain information about the strong anion binding sites must be carried out at much lower halide concentrations in the case of iodide; for example, to obtain a relative contribution of 80% to $\Delta\nu_{ex}$ for the A sites one has to measure at 0.2 *M* Cl^-, 0.03 *M* Br^-, and 0.007 *M* I^-. There are indications that these findings concerning the relative contributions of high and low affinity sites apply also to other proteins (Norne et al., 1973; Csopak et al., 1970; J.-E. Norne et al., manuscript in preparation). In such cases it is apparent that Cl^- ions are much more useful for probing into specific high affinity sites than Br^- or I^- ions.

Chemical Nature of Protein–Anion Interactions. The experiments described above illuminate several points of interest, both special and general ones. For the special case of serum albumin, the NMR quadrupole relaxation method has confirmed the existence of two classes of anion binding sites which has been inferred earlier from many different kinds of measurements (Steinhardt and Reynolds, 1969; Lindman et al., 1972a). Since the interaction of anions with the weak binding sites can be described in terms of binding constants, the anions must interact directly with the protein and not by modification of the solvent only. This is important for the halogen quadrupole relaxation work with other proteins, where specific and nonspecific line broadening has been described in many cases. The latter one has often been referred to as viscosity effects. Our results, however, suggest that the nonspecific line broadening observed after blocking of functional anion binding sites could also result from direct halogen ion binding to low affinity sites which are remote from, e.g., active centers or effector binding sites.

The background for attributing nonspecific broadening to a viscosity increase is that simple hydrodynamic theory predicts the correlation time to be proportional to macroscopic viscosity. While this theory fails in predicting correlation times of the correct order of magnitude, the proportionality between NMR relaxation rate and viscosity has been observed to hold approximately in a number of cases where solute and solvent molecules are of similar size. For protein solutions, which are markedly microdynamically heterogeneous, the viscosity relation may be expected to apply approximately to the protein motion but to fail completely for ions or molecules which are not bound to the protein molecules.

The viscosity increase on addition of protein to an aqueous solution is predominantly due to the presence of the large molecules. The effect of the protein molecules on the microdynamical behavior can be assumed to be significant only in the close neighborhood of the protein and, therefore, even if the viscosity is affected by the protein, solutes which are not very close to the protein should be virtually unaffected in their motion. A number of experiments performed to illuminate these problems tend to show that nonspecific line broadening connected with macroscopic viscosity is unimportant. For example, we have observed the quadrupole relaxation of ions not binding to the macromolecule to be unaffected by viscosity increases due to the macromolecule (Zeppezauer et al., 1969; Lindqvist and Lindman, 1970; Lindman and Danielsson, 1972; cf. also Lindman et al., 1972a). Quite striking in this connection are some observations made for amphiphilic liquid crystals. Thus it has, for several surfactant systems, been observed that in spite of viscosity alterations by orders of magnitude, at most only small changes in alkali or halide ion quadrupole relaxation accompany transitions from amorphous isotropic solutions to different types of liquid crystals (Lindblom and Lindman, 1971, 1973; Lindblom et al., 1973). Recently, a theoretical study has been presented which demonstrates that there is no connection between the viscosity increase due to a macromolecule and the mobility of small ions (Turq et al., 1973).

In order to further illustrate these problems some experiments were performed with agarose gels. These gave direct support for the conclusions drawn. Thus, for example, for a gel containing 1% agarose and 0.5 *M* KBr the ^{81}Br line width is 850 Hz which is only ca. 2.5 times that of a 0.5 *M* KBr solution. The viscosities in the two cases, of course, differ by orders of magnitude.

Another point of general importance is the series of relative anion affinities which govern the interactions with the two types of binding sites. The series are, according to our competition experiments, nearly the same for both the high-affinity and the low-affinity sites and are nearly identical with the well-known lyotropic or Hofmeister series. The lyotropic series orders anions according to their polarizability which is decisive for their ability to respond to dispersion forces. The weak anion–protein interactions will necessarily occur at many places on the surface where most of the positively charged side groups are exposed and can be assumed to be freely accessible to ion–ion interactions. Despite this fact our observations indicate that dispersion forces are governing the relative strength of the interaction of anions with the exposed positive groups. This is unexpected at first glance and would mean either that the anions are in contact with nonpolar groups at the surface or that dispersion forces are operating at larger distances than previously expected (Parsegian, 1973).

Clearly, the same kind of forces may, according to our findings, determine the binding of anions to the high-affinity sites as well. It remains to be shown why the strength of interaction differs by orders of magnitude in terms of a binding constant. Since we lack structural information about serum albumin, we shall consider what is known about anion-binding sites in those few other proteins which have been studied by halogen quadrupole relaxation. A feature which they have in common is that a large part of the relaxation enhancement is due to the interaction of anions with *functional sites*, i.e. the coenzyme site in horse liver alcohol dehydrogenase (Ward and Happe, 1971; Bull et al., 1975; Lindman et al., 1972c), the site of diphosphoglycerate and ATP in hemoglobin (Chiancone et al., 1972), the catalytic site in carbonic anhydrase (Ward, 1969, 1970), the 1,6-fructose bisphosphate site in aldolase (J.-E. Norne, unpublished experiments), and the phosphate binding site in alkaline phosphatase (Norne et al., 1974). All these substrates carry negative charges. It is evident from the results of X-ray analyses that functional sites are in most cases formed in cavities of the protein molecules. Even if single side chains inside these cavities may possess a certain individual mobility, the overall shape of the functional clefts must be substantially maintained as long as the tertiary structure of the protein is intact. Changes in the tertiary

structure due to the interaction with substrates or effectors may alter the affinity for anions considerably. We have found that large, complex anions seem to be accommodated by the functional sites of all proteins tested so far (J.-E. Norne et al., manuscript in preparation). Anions like $Au(CN)_2^-$, $Pt(CN)_4^{2-}$, and $Cr(SCN)_4(NH_3)_2^-$ are highly polarizable and relatively weakly hydrated due to their large surface-to-charge ratios. Their binding into a large cleft will be favored by an increase in entropy if hydration water is released and if the structure-stabilizing effect exerted by nonpolar groups on the water lattice is reduced. In fact, there is some evidence that positively charged groups are embedded between nonpolar groups in functional regions, e.g. in aldolase (Morse and Horecker, 1968). In carbonic anhydrase, where a zinc ion liganded by three imidazole rings is responsible for the chloride relaxation enhancement under slow exchange conditions (Ward and Cull, 1972; J.-E. Norne et al., manuscript in preparation), one-half of the active site has "hydrophobic character" (Liljas, 1971; Vaara, 1974; Notstrand, 1974). We have, in line with this, observed a considerable reduction in the $^{35}Cl^-$ line width upon binding to carbonic anhydrase of $Au(CN)_2^-$ (J.-E. Norne, unpublished experiments) which is not expected to be a good ligand of the zinc ion.

Thus, a proximity of positive and nonpolar groups seems to have a favorable effect upon anion binding. A necessary condition seems to be a suitable three-dimensional arrangement of these groups which is dependent on the intact tertiary structure of the whole protein. Also, in serum albumin, peptide sequences built up by nonpolar and positively charged amino acids have been inferred as part of the binding sites of large anions (Jonas and Weber, 1971a,b; Swaney and Klotz, 1970). It is evident from Figure 3 and from $^{81}Br^-$ NMR relaxation studies (Gillberg-La Force and Forsén, 1970) that the strong binding of halogen and surfactant anions occurs at the same sites. Together with the findings presented above, we might characterize the strong halogen binding sites as rigid cavities of relatively hydrophobic character containing positive charges and being large enough to accommodate anions of considerable size.

The intrinsic relaxation rate of the protein-bound halide ions is determined by the quadrupole coupling constant (e^2qQ/h, where eq is the field gradient and eQ is the quadrupole moment) characterizing the site at the protein, and by the correlation time (τ_c) of the motion causing the time variation of the field gradients. The correlation time may be determined by the tumbling of the whole protein molecule, by internal motions at the anion binding site, or by very rapid halide exchange. As shown by Bull (1972), the correlation time may, for long τ_cs, be determined by a comparison of the transverse (T_2) and longitudinal (T_1) relaxation times or by investigating the frequency dependence of T_1. In order to obtain τ_c and e^2qQ/h for the high-affinity sites we have determined T_1 (π-t-$\pi/2$ pulse sequence) and T_2 (Meiboom-Gill modification of the Carr-Purcell pulse sequence) of $^{35}Cl^-$ both in the presence and in the absence of sodium dodecyl sulfate. These experiments were performed at 8.82 MHz using a Bruker BKr-322s spectrometer. Analyzing these data using the method of Bull et al. (1975), assuming $n_A = 9$, we obtain $\tau_c = 11.4$ nsec and $e^2Q/h = 1.2$ MHz. This value is in the range obtained by simple estimates for general anion binding sites in a large number of proteins (Norne et al., 1973; J.-E. Norne et al., manuscript in preparation). We recently suggested comparisons between experimental quadrupole coupling constants and those obtained from a simple electrostatic model (Bull et al., 1975). (Unfortunately, eq 14 in the paper by Wennerström et al. (1974) has been found to lack the factor ½, resulting in too high a calculated quadrupole coupling constant in the paper by Bull et al. (1975).) According to the arguments presented by Bull et al. (1975) we calculate then, using a simple electrostatic model, a quadrupole coupling constant of 1.4 MHz for Cl^- binding to arginine. The good agreement between this value and the experimental one shows that our observed relaxation rates are consistent with anion binding to arginine groups in the case of the high affinity sites. It is interesting in this connection to note that chemical modification experiments on albumin lead to a marked decrease in the binding affinity for a hydrophobic anion if the modification involves arginine residues but not if it involves lysine residues (Jonas and Weber, 1971a).

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Purification and Chemical Characterization of the Major Neurotoxin from the Venom of *Pelamis platurus*†

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ABSTRACT: A major toxin was isolated from the venom of the sea snake *Pelamis platurus* (yellow-bellied sea snake) by Sephadex G-50 and carboxymethylcellulose column chromatography. The LD_{50} of the pure toxin (*Pelamis* toxin a) was 0.044 μg/g in mice representing a tenfold increase in toxicity after purification. The toxin was homogeneous in acrylamide disc gel electrophoresis and eluted as a single peak after isoelectric focusing in a sucrose density gradient column. The isoelectric point was 9.69; thus it is a highly basic protein. The toxin contained 55 amino acid residues with four disulfide linkages. When all disulfide linkages were reduced and alkylated, the toxic action of the pure toxin disappeared leading to the conclusion that the disulfide bonds of the neurotoxin were essential for toxic action.

Venoms from the sea snake family (Hydrophiidae) contain potent neurotoxic proteins which bind strongly to the acetylcholine receptor sites of the neuromuscular junction (Tu, 1974). Toxins from a number of the sea snake species which inhabit the coastal waters of Asia have been studied in detail. However, the venom of *Pelamis platurus* (yellow-bellied sea snake), a truly pelagic sea snake found in the Indian and Pacific Oceans and the only species known to inhabit the Pacific Coast of Central and South America, has not been examined in detail. The isolation and characterization of the major toxin from the venom of *P. platurus* is described in this report.

Materials and Methods

Venoms. Sea snakes, *Pelamis platurus,* were captured on the Pacific coast of Costa Rica, Central America, in 1973. Venom was extracted from the venom glands of 3069 snakes. The extraction was done by the method described previously (Tu and Hong, 1971).

Isolation Procedure. Crude venom (1.5 g) dissolved in buffer was loaded on a Sephadex G-50 column (3.5 × 110 cm) previously equilibrated with 0.01 *M* potassium phosphate buffer (pH 6.5) containing 0.1 *M* NaCl. The toxin was eluted with the same buffer at a flow rate of 18 ml/hr. The eluate, collected in 3-ml aliquots, was monitored at 280 nm with an ISCO Model UA-2 dual beam ultraviolet analyzer equipped with a recorder. For the accurate measurement of absorbance, each tube was determined again at 280 nm using a Beckman DB-G spectrophotometer. The tubes from each protein peak were pooled and lyophilized. The samples were then desalted by passage through a Sephadex G-10 column (2.5 × 40 cm) and each fraction was tested for toxicity.

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