Chloride Ion Binding to Human Plasma Albumin from Chlorine-35 Quadrupole Relaxation†

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ABSTRACT: The ^{35}Cl nuclear magnetic quadrupole relaxation enhancement on binding of chloride ions to human plasma albumin (HPA) has been studied under conditions of variable temperature, pH, ionic strength, protein, and sodium dodecyl sulfate concentration. A small number ($\lesssim 10$) of chloride ions, most of which are bound to the primary detergent binding sites, contribute a major portion of the relaxation enhancement ($\gtrsim 80\%$ at neutral pH). A comparison of the pH dependence of the relaxation rate with the hydrogen ion titration curve, which was determined and analyzed, identified ten lysyl and arginyl residues as being involved in the chloride ion binding. These data, in conjunction with $NaDodSO_4$ titrations at different pH values and the amino acid sequence of HPA, suggest that the high-affinity chloride-binding sites are doubly cationic at neutral pH. An irreversible dimerization at acidic pH and 5×10^{-5} *m* HPA was detected. The data also indicate the presence of internal modes of motion in the expanded forms of the HPA molecule, probably an independent reorientation of domains. The rate of exchange of chloride ions was shown to be much higher than the corresponding intrinsic relaxation rate in the temperature range 2–26 °C and pH values ranging from 4.0 to 10.5. No indications of protein–protein interaction could be found up to the physiological concentration of ca. 6×10^{-4} *m* HPA at either neutral or alkaline pH. The mechanistic basis for HPA's exceptional capacity for binding of inorganic anions was discussed.

It is a well-established fact that the interaction of proteins with inorganic ions can be of structural as well as of functional importance. This interaction can be of a direct nature, as in the stoichiometrically well-defined site binding, or of an indirect nature, as in conformational transformations due to electrolyte-induced changes in the properties of the medium. Despite great experimental and theoretical efforts, the detailed mechanisms underlying the binding process and the essential features of the ligand and binding site are not well understood. Due mainly to its exceptional capacity for ion binding, plasma albumin has often been the system of choice for the study of ion–protein interactions. Several reviews on plasma albumin are available (e.g., Peters, 1975). The present investigation deals with these questions for the particular case of chloride-ion binding to human plasma albumin (HPA[1]).

In recent years the technique of nuclear magnetic quadrupole relaxation has proved useful for studying ion binding to proteins (Lindman and Forsén, 1976). The observed relaxation rates are determined by the distribution of nuclei, in the present case chloride ions, over different environments, by the exchange rate between the environments and by the intrinsic relaxation rates in the environments. The wide applicability of the method is due to the fact that most macromolecular systems are characterized by fast exchange (with a rate constant much greater than the corresponding intrinsic relaxation rate) between environments with greatly differing intrinsic relaxation rates (a factor of ca. 10^5 in the case of HPA). Under these circumstances, marked effects on the observed relaxation rate, representing a population average of the intrinsic relaxation rates in the various environments, are obtained even at low protein concentrations.

The aim of the present study has been to gain information on the nature of the interaction of human plasma albumin with chloride ion, by observing the ^{35}Cl transverse relaxation rate as a function of temperature and pH as well as of the concentration of protein, chloride ion, and competitive anions. The pH dependence of the ^{35}Cl relaxation rate is particularly informative in that it may reveal, in conjunction with the hydrogen ion titration curve, the identity of the amino acid residues involved in binding. To date, only one such study, namely, that of hemoglobin (Chiancone et al., 1972), has been reported. Previous work on chloride ion binding to HPA in this laboratory (Norne et al., 1975) has supplied some results pertinent to the present work and will be referred to in the text. [Except for the study by Norne et al. (1975), there are also other reports on halide ion quadrupole relaxation in solutions of plasma albumin (Zeppezauer et al., 1969; La Force and Forsén, 1970; Magnuson and Magnuson, 1972; Norne et al., 1973)]. It should be noted that the Fourier transform technique employed in the present study has considerably reduced the experimental uncertainties associated with the earlier wide-line spectrometer measurements.

Experimental Procedure

Materials. Human plasma albumin, a generous gift from AB KABI (Stockholm), was obtained as a freeze-dried preparation (batch no. RbN 487) and was used without further purification. This preparation contains 95% HPA, 0.3–0.6 equiv of fatty acid, and 0.6–0.7 equiv of free sulfhydryl (R. Einarsson, personal communication). The molar mass of HPA was taken as 66.5 kg mol^{-1} (Meloun et al., 1975). Protein solutions were prepared by weight using deionized, quartz-distilled water. No buffers were used. All measurements were performed within 24 h of solution preparation. pH was adjusted with HCl and KOH solutions and, in addition, the HPA solutions contained KCl. All chemicals used were of the finest grade available.

pH Measurements. A Radiometer PHM 63 digital pH meter equipped with either of the combination electrodes GK 2302C or GK 2322C was used. All pH measurements were

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[1] Abbreviations used are: HPA, human plasma albumin; NMR, nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate.

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carried out at 20–22 °C. The accuracy was estimated to be ±0.02 pH unit.

Relaxation Measurements. ^{35}Cl NMR spectra were recorded at 9.80 MHz (2.35 T) using a modified Varian XL-100-15 spectrometer operating in the Fourier transform mode. The probe temperature was maintained by a stream of dry thermostated nitrogen gas and is accurate to ±0.2 °C. The excess transverse relaxation rates $R_{2,ex}$ were calculated from the line width at half height of the NMR absorption curves according to $R_{2,ex} = \pi(\Delta\nu_{obsd} - \Delta\nu_F)$, where $\Delta\nu_{obsd}$ is the observed line width and $\Delta\nu_F$ that obtained in the absence of protein.

The pH dependence of the relaxation rate was also studied at 5.78 MHz (1.38 T) using a Varian V-4200 wide-line NMR spectrometer equipped with a 12-in. Varian V-4013A magnet. The magnetic-field modulation frequency was 400 Hz. The probe temperature was maintained at 25.0 ± 0.1 °C by passing thermostated water through the jacket of the sample tube. $R_{2,ex}$ was calculated as described above.

Results and Discussion

Basic Relaxation Theory. Since the general theory for quadrupolar relaxation of spin 3/2 nuclei undergoing chemical exchange in a macromolecular system has been presented elsewhere (Lindman and Forsén, 1976), only the final equations are included here. It is assumed that the excess transverse relaxation rate, i.e., the difference between the observed relaxation rate and that obtained in the absence of protein, can be expressed as:

$$R_{2,ex} = R_{2,obsd} - R_{2,F} = \sum_S P_S R_{2,S} \quad (1)$$

where the sum extends over classes of identical and independent chloride ion binding sites. The assumed identity of binding sites within each class is not limited to the association constants but applies to all parameters that influence the relaxation rate. The assumption of independence is motivated primarily by the experimental fact (presented below) that the relaxation rate depends almost exclusively on the state of protonation of specific cationic amino acid residues, rather than on the net charge of the albumin molecule. In the general case, i.e., in the presence of competitive ligands X, the average relative population of chloride ions in class S is given by (Steinhardt and Reynolds, 1969):

$$P_S = \frac{m_{HPA} N_S}{1/K'_{S,Cl} + m_{Cl}^F + \sum_X m_X^F K'_{S,X}/K'_{S,Cl}} \quad (2)$$

N_S is the number of chloride ion binding sites and $K'_{S,X}$ the invariant, stoichiometric association constant for class S; m denotes the molality of the indicated species, with superscript F referring to unbound species. The intrinsic transverse relaxation rate $R_{2,S}$ for class S may be expressed as:

$$R_{2,S} = \frac{2\pi^2}{5} \nu_{Q,S}^2 \tau_{cS}^* \quad (3)$$

The quadrupole coupling constant $\nu_{Q,S}$ for sites in class S is defined as:

$$\nu_{Q,S} = \left| \frac{e^2 q_S Q}{h} \right| \quad (4)$$

where eq_S is the maximal component of the diagonalized electric-field gradient tensor, eQ is the electric quadrupole moment of the ^{35}Cl nucleus, and h is Planck's constant. Dielectric measurements (Scheider et al., 1976) have shown that the hydrated HPA molecule is, to a good approximation, hydrodynamically equivalent to a prolate ellipsoid of revolution with principal axes 3.9 ± 0.6 and 14.4 ± 2.0 nm, yielding an axial ratio of 3.7 ± 0.6. The correlation time τ_{cS}^* appearing in eq 3 is, for the axially symmetric case, given by Woessner (1962):

$$\tau_{cS}^* = \frac{1}{4}(3\cos^2\phi_S - 1)^2 \tau_{cl,S}^{eff} + 3\sin^2\phi_S \cos^2\phi_S \tau_{c2,S}^{eff} + \frac{3}{4}\sin^4\phi_S \tau_{c3,S}^{eff} \quad (5)$$

where ϕ_S is the angle between the symmetry axes of the field gradient and of the ellipsoidal HPA molecule. (The reasonable assumption of an axially symmetrical field gradient, although not critical, simplified the equations considerably.) The effective correlation times $\tau_{cj,S}^{eff}$ are, in the transverse case, defined by:

$$\tau_{cj,S}^{eff} = \frac{\tau_{cj,S}}{10}\left[3 + \frac{5}{1+\omega^2\tau_{cj,S}^2} + \frac{2}{1+4\omega^2\tau_{cj,S}^2}\right] \quad (6)$$

where $j = 1$, 2, or 3, ω is the Larmor angular frequency, and the correlation times $\tau_{cj,S} = (1/\tau_{Rj} + 1/\tau_S)^{-1}$, τ_S being the average lifetime of a chlorine nucleus in a binding site in class S. The three generalized rotational correlation times τ_{Rj} are related to the rotational diffusion coefficients for rotation about the major ($\theta_\parallel$) and the minor ($\theta_\perp$) axes:

$$1/\tau_{Rj} = [6 - (j-1)^2]\theta_\perp + (j-1)^2\theta_\parallel \quad (7)$$

where $j = 1$, 2, or 3. The most critical assumptions inherent in the derivation of the above equations are discussed in the appropriate paragraphs of the following.

Temperature Dependence of ^{35}Cl Relaxation. The simple interpretation of the excess relaxation rate as a population weighted average of the intrinsic relaxation rates (eq 1) is valid only in the limit of fast exchange of chloride ions, i.e., when the exchange rates are much higher than the corresponding intrinsic relaxation rates. Although the total temperature dependence of the excess relaxation rate is quite complex, it can be shown (Lindman and Forsén, 1976) that a plot of ln $(R_{2,ex}/T)$ vs. $1/T$ has a positive slope in the fast-exchange region, while the slope is negative for slow exchange. Figure 1 shows the results obtained at three pH values in the temperature range 2–26 °C. It is seen that the chloride ion exchange is indeed fast under the conditions investigated, confirming the validity of eq 1 in the pH range of interest. These results agree with an earlier study (Norne et al., 1973), which demonstrated fast exchange conditions at pH 7.0.

Dependence of ^{35}Cl Relaxation on Protein Concentration. At high HPA concentrations, protein–protein interactions are expected to influence the excess relaxation rate. Previous work (Norne et al., 1975) indicates the absence of such effects up to 2×10^{-4} m HPA at pH 7.4. Partly in view of the fact that the physiological HPA concentration is $5.6–8.8 \times 10^{-4}$ m (Harper, 1975), it was of interest to extend these studies to higher concentrations. According to eq 1 and 2, $R_{2,ex}$ should be proportional to m_{HPA} (in the absence of protein–protein interaction). Thus, the results in Figure 2 do not indicate any significant effects of protein–protein interaction in the investigated concentration range (up to 6.5×10^{-4} m HPA) at either pH (due to experimental difficulties with the very broad signals obtained at low pH and high HPA concentrations, no data are reported for acidic pH). Since the macromolecular center to center distance in a 6×10^{-4} m solution is about the same as the length of the major axis of the (hydrated) HPA molecule at neutral pH (14.4 nm), hindered reorientation must

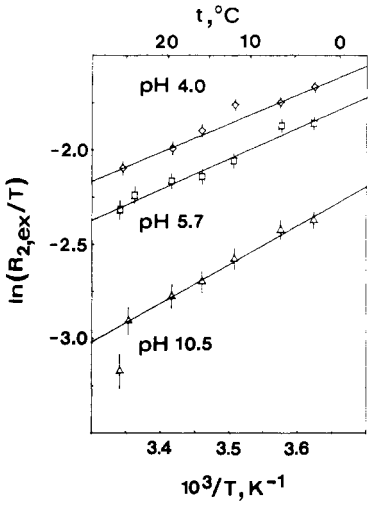

FIGURE 1: Temperature dependence of the ^{35}Cl excess relaxation rate, $R_{2,ex}$, at indicated pH values for solutions containing 2.54×10^{-5} *m* HPA and 0.5 *m* KCl. Error bars are based on estimated uncertainties (±1–3%) in line-width measurements.

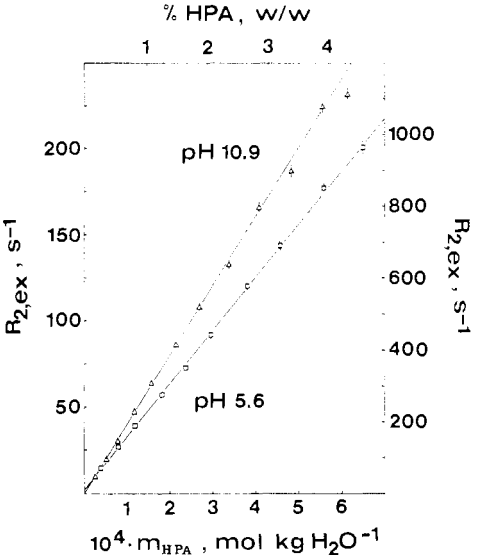

FIGURE 2: Variation of the ^{35}Cl excess relaxation rate, $R_{2,ex}$, with human plasma albumin concentration at 26 °C and indicated pH values. Left scale refers to pH 10.9 (△), right scale refers to pH 5.6 (□). Error bars are based on ±2% uncertainty in line-width measurements.

occur at slightly higher concentrations. Water proton relaxation measurements (Oakes, 1976) on solutions of bovine plasma albumin reveal, however, a linear concentration dependence up to about 15×10^{-4} *m* (neutral pH). In this as in the present case, however, the interpretation is complicated by the possibility of compensating effects of protein–protein interaction on the observed relaxation rate (e.g., slower protein reorientation combined with reduced binding). It should also be mentioned that a hindered reorientation affecting only $\theta_\perp$ may have very little effect on the relaxation rate.

Hydrogen Ion Titration. To our knowledge, only one complete hydrogen ion titration curve of human plasma albumin (Tanford, 1950) has been reported in the literature. Since these data are not sufficiently accurate for our purposes (and also because of possible differences in the quality of the protein preparations), the complete hydrogen ion titration curve (pH 2–12) of HPA in 0.15 *m* KCl was redetermined (Figure 3). The average "isoionic" net charge Z_H was calculated by the standard procedure (Tanford, 1959) using activity coefficients for protein-free solutions. The interpretation of the titration curve, however, was not based on the conventional Linderstrøm-Lang equation (Nozaki and Tanford, 1967), which relies on several doubtful assumptions. Instead, the experimental data were computer fitted, using a modified Marquardt algorithm (Bevington, 1969), to the equation:

$$Z_H = \sum_S \frac{z_S N_S}{1 + 10^{z_S(pH - pK_S')}} \qquad (8)$$

where the interactions (local coulombic interactions, hydrogen bonds, "salt bridges", etc.) between different groups are implicitly taken into account by dividing the prototropic groups into a number of classes S, each consisting of N_S identical (potential charge z_S and semistoichiometric dissociation constant K_S') and independent groups. These classes are not necessarily identical to the "chemical" classes of prototropic amino acid residues. Local interactions, rather than the net charge of the protein molecule, are thus assumed responsible for the cooperativity in the prototropic multiple equilibria. It should be stressed that neither model is capable of describing the cooperativity that results from conformational transformations.

The first fit (dashed curve in Figure 3), which was based on five classes (COOH, His, Lys, Tyr, Arg) using known N_S

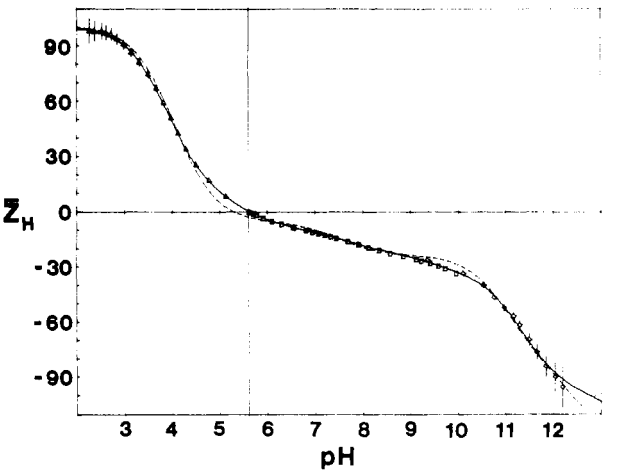

FIGURE 3: Hydrogen-ion titration curve of human plasma albumin (9.64×10^{-5} *m*) at 22 °C and 0.15 *m* KCl. Different symbols refer to different protein solutions. Error bars are based on uncertainties of ±0.02 pH unit and ±2% in titrant volumes. The curves are least-square fits of eq 8 for five (dashed curve) or eight classes (solid curve) of prototropic groups (see text for details).

values (Meloun et al., 1975) and the spectrophotometrically determined value of $pK_{Tyr}' = 11.7$ (Tanford, 1950), is unsatisfactory in three regions: near pH 5, 7, and 9.5. To improve the fit, the carboxyl and histidyl classes were each divided into two (apparent) classes, while the combined lysyl and arginyl classes were converted into three classes, one of which includes groups with "anomalously" low pK' values. Least-squares fitting of the 11 resulting parameters to the 57 data points yielded the solid curve in Figure 3. The original discrepancies around pH 5 and 7 can be ascribed to the familiar N–F and N–B transformations (Sogami and Foster, 1968; Leonard et al., 1963), which expose previously unaccessible carboxylate and histidyl groups, respectively. In the pH range 9–10, where the third discrepancy appeared, no conformational change is known to occur, the sulfhydryl-catalyzed isomerization to the A form being completely negligible at the present ionic strength (Stroupe and Foster, 1973). The results of the final fit indicate that this part of the titration curve is due to ten groups (7–10

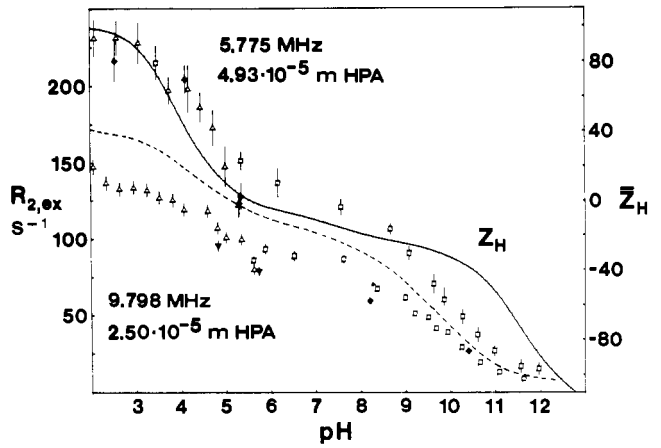

FIGURE 4: Variation of the ^{35}Cl excess relaxation rate, $R_{2,ex}$, with pH at 25 °C, 0.5 *m* KCl, and indicated HPA concentrations and resonance frequencies. All data have been normalized to 4.93×10^{-5} *m* HPA. Symbols refer to titration toward lower (△) or higher (□) pH, measurement after exposure to extreme pH (▽), and control measurements on separate samples (♦). Error bars represent 90% confidence interval (5.78 MHz) or are based on ±2% uncertainty in line-width measurements (9.80 MHz). The hydrogen-ion titration curve (solid curve, right scale) from Figure 3 is included for comparison. The dashed curve represents 25% frequency correction (see text for details).

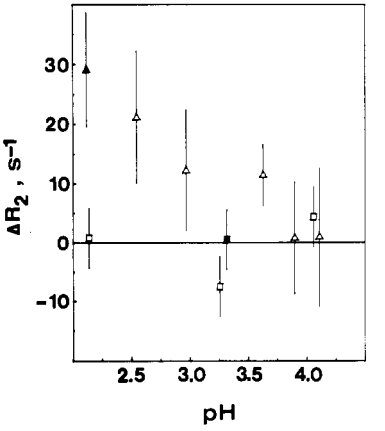

FIGURE 5: Effects of exposure of HPA solutions to low pH on the ^{35}Cl excess relaxation rate. The residual relaxation rate, ΔR_2, is the difference in relaxation rate at isoelectric pH, 0.5 *m* KCl, and 25 °C due to exposure to low pH. All data have been normalized to 4.93×10^{-5} *m* HPA. Symbols refer to measurements at 2.18×10^{-5} *m* HPA and 9.80 MHz (squares) and at 4.93×10^{-5} *m* HPA and 5.78 MHz (triangles). Filled symbols indicate longer duration of exposure to low pH. Error bars as in Figure 4.

Lys and 3-0 Arg) with pK' = 9.4. Low-titrating lysyl residues in plasma albumin have previously been observed in fluorescence studies (Halfman and Nishida, 1971) and in studies of the reactivity toward fluorodinitrobenzene (Green, 1963). The behavior of the lysyl and arginyl groups is of paramount interest in connection with anion binding and will be further elaborated upon in the concluding discussion.

pH Dependence of ^{35}Cl Relaxation. A comparison of the hydrogen-ion titration curve with the pH dependence of the excess ^{35}Cl relaxation rate can, in principle, yield information on the identity of the amino acid residues involved in anion binding. Unfortunately, this approach is somewhat complicated by the numerous pH-induced conformational changes in the albumin molecule. Variations in the relaxation rate with pH may thus have other causes (e.g., changes in the rate of protein reorientation) than an altered chloride ion affinity. Figure 4 shows the results of two series of measurements at different resonance frequencies and at different protein concentrations. The data for the lower concentration have been normalized to the higher concentration. For convenient comparison, the hydrogen-ion titration curve has also been included (solid curve). Separate measurements demonstrated that $R_{2,F}$ is independent of pH.

For nonexponential relaxation of bound chloride ions, $R_{2,ex}$ depends on the resonance frequency (eq 6). Using the equations given above together with Perrin's expressions for the rotational diffusion coefficients of a prolate ellipsoid (Koenig, 1975), it can be shown that the frequency effect cannot exceed 30% (irrespective of the values of τ_S and ϕ_S). The dashed curve in Figure 4 shows the effect of 25% correction (this value is based on reliable estimates of the relevant parameters). Clearly, the frequency dependence alone cannot explain the observed difference in relaxation rate between the two sets of data.

The remaining difference can, however, be rationalized on the basis of sedimentation studies of plasma albumin (Williams and Foster, 1960), which show that dimerization occurs at acidic pH via "irreversible" formation of disulfide bonds between albumin molecules in the F conformation. It has been demonstrated (Sogami et al., 1972) that the dimerization is catalyzed by trace amounts of Cu(II), the presence of which cannot be ruled out in the present work. The observed excess relaxation rate in the presence of dimer is $R_{2,ex} = (1 - \alpha) \cdot R_{2,ex}^{mono} + \alpha R_{2,ex}^{di}$, where α is the degree of dimerization. Assuming that the dimerization affects only the reorientation rate (i.e., $N_S^{di} = 2N_S^{mono}$) and using data for the shape and dimensions of the dimer (Squire et al., 1968), it was estimated that the 25–35% difference between the high-concentration curve and the dashed curve corresponds to α = 0.6–0.9. Since nearly all the relaxation rate measurements in the high-concentration series were performed on the same protein solution going from isoelectric to acidic and then to alkaline pH, the difference persists over the entire pH range; i.e., the irreversible dimerization gives rise to hysteresis in the pH dependence of the relaxation rate. Figure 4 also shows that an abrupt change from pH 5.3 to 2.55 results in a lower $R_{2,ex}$ compared to a gradual pH reduction. This finding conforms with the observed maximum at pH 3.3 in the dimerization rate (Williams and Foster, 1960). The observed hysteresis effect, due to the irreversibility (on the experimental time scale) of the dimerization process, is even more clearly demonstrated in Figure 5. Dimerization is seen to occur at pH <4 for the higher protein concentration, while no change is visible at the lower concentration.

In the remainder of this section, the results for the lower HPA concentration in Figure 4 will be discussed. As pH is reduced from 5.6 to 3.8 (the pH range of the N-F transformation), $R_{2,ex}$ increases by 35%. Presumably, this increase does not reflect a decreased reorientational rate, since the N–F transformation does not appreciably affect the hydrodynamic properties of the albumin molecule (Hilak et al., 1974) but rather an increased number of accessible chloride-ion binding sites. This does not imply that nonspecific electrostatic effects are important (z_H increases from 0 to nearly +60 in this pH range), since it is known that the N–F transformation exposes a number of cationic residues (in hydrophobic clefts between the domains), which in the N form are engaged in "salt bridges" with carboxylate groups (Vijai and Foster, 1967).

In the region of the acid expansion (pH <3.8), only a slight increase in $R_{2,ex}$ is observed (8% down to pH 2.5). This is surprising, since the expansion ought to decrease the reorientational rate considerably. The expansion effect can be estimated using the equations given here and Perrin's equations

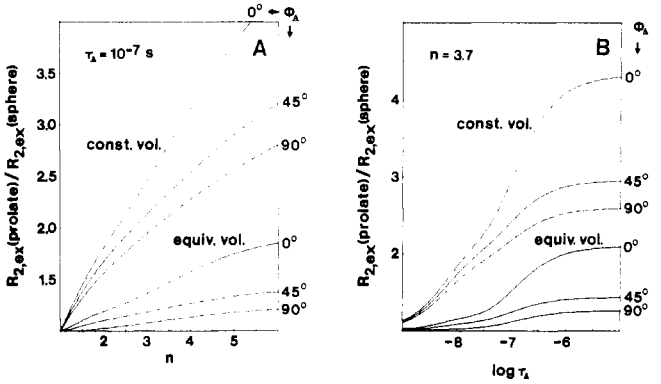

FIGURE 6: (A) Calculated effect of expansion of the HPA molecule (axial ratio, n) on the ^{35}Cl excess relaxation rate, $R_{2,ex}$, relative to that for a sphere of constant volume (radius = a, the minor semiaxis of the prolate) or of equivalent volume (radius = $n^{1/3}a$). Only one class (A) of binding sites was used in the calculations. Numerical data used: $\tau_A = 10^{-7}$ s, a = 1.95 nm, T = 298 K, $\eta = 8.82 \times 10^{-4}$ kg ms^{-1}. The symbols are defined in the text. (B) Calculated effect of the average lifetime, τ_A, of a chloride ion in a binding site on the ^{35}Cl excess relaxation rate, $R_{2,ex}$, relative to that for a sphere of constant or equivalent volume. The axial ratio n = 3.7 and other parameters are the same as in A.

for the rotational diffusion coefficients. According to the domain model of the albumin molecule (Bloomfield, 1966; Zurawski et al., 1975), the expansion should affect only the length (major axis) of the molecule. The effect on the reorientational rate of such an expansion (see the curves labeled "constant volume" in Figure 6A) is due to a larger effective molecular volume as well as an increased molecular anisotropy, the latter part of the effect being displayed separately in Figure 6A ("equivalent volume"). Figure 6B shows how these effects are influenced by the chloride ion exchange rate (or the lifetime τ_A of a chloride ion in a binding site). The value of τ_A can be estimated from the demonstrated condition of fast exchange (Figure 1) and the fact that τ_A cannot be much smaller than τ_{Rj}, since then the relaxation rate would be independent of the protein reorientational rate. Using intrinsic relaxation rates obtained from the $NaDodSO_4$ blocking experiments (to be described) and rotational correlation times derived from dielectric measurements (Scheider et al., 1976), we obtain $10^{-8} \lesssim \tau_A \lesssim 10^{-6}$ s. Since the axial ratio of the expanded albumin molecule (at pH 2.3) is about 9 (Harrington et al., 1956), it is clear from Figure 6A that the anticipated expansion effect greatly exceeds the observed 8% increase in $R_{2,ex}$ (irrespective of the value of ϕ_A).

Inherent in the derivation of the equations presented above is the assumption of a rigid macromolecule; i.e., internal modes of motion are ignored. It is, however, well known that such motions do occur in globular proteins (Careri et al., 1975). The presence of fast internal motion (e.g., rotations around carbon–carbon bonds in lysyl or arginyl side chains) in the chloride ion binding sites of HPA has recently been demonstrated by investigating the temperature dependence of the longitudinal and transverse ^{35}Cl relaxation rates (Bull et al., 1978). It seems highly probable that yet another mode of internal motion is operating in the expanded conformations of the albumin molecule, namely, independent reorientations around the short peptide segments connecting the three separate domains of the molecule. A simple model for this kind of motion is a rotational diffusion of the field gradient around the molecular symmetry axis at constant ϕ_S, independently of the reorientation of the molecule as a whole. It has been shown (Woessner, 1962) that the effect of this idealized internal motion is to reduce the apparent rotational correlation times, thereby also reducing the relaxation rate. The insignificant increase in $R_{2,ex}$ in the pH range of the acid expansion can thus be explained by increasing domain reorientation nearly compensating for the reduction in the overall reorientational rate. Similar considerations should apply to the analogous alkaline expansion above pH 10 (Wallevik, 1973). The hypothesis of independent reorientation is further supported by fluorescence polarization studies (Harrington et al., 1956; Sogami et al., 1975) showing a decreasing (harmonic mean) rotational relaxation time with decreasing pH below 3.5–4.

The major change in the excess relaxation rate occurs in the pH range 8–10.5, coinciding with the deprotonation of the anomalous lysyl and arginyl residues. This interesting finding will be further commented upon in the concluding discussion. Since experimental pH values cannot be interpreted quantitatively for solutions with significantly higher ionic strength (and liquid junction potential) than the standard buffers (Bates, 1964), the hydrogen ion titration curve was determined at lower ionic strength (0.15 m KCl) than the pH dependence of the ^{35}Cl relaxation rate (0.5 m KCl). A comparison is still possible, since the hydrogen ion titration curve is only slightly dependent on the ionic strength above 0.15 m. Thus, the difference in pK' values between 0.15 and 0.5 m should not exceed 0.03 unit (Tanford, 1962), which is negligible compared to the experimental uncertainty in the relaxation measurements.

Titration with Dodecyl Sulfate. The physiologically important role of plasma albumin in the transport of fatty acids through the blood has motivated a large number of studies of its interaction with various detergents. It is thus known (Steinhardt and Reynolds, 1969) that plasma albumin at neutral pH binds 8–10 equiv of dodecyl sulfate anions with extremely high affinity ($K'_{NaDodSO_4} = 1.2 \times 10^6$ M^{-1}). These primary $NaDodSO_4$ binding sites are believed to consist of hydrophobic clefts (some of which may be located between the domains) containing one or more cationic groups. Several studies indicate that the primary $NaDodSO_4$ binding and the chloride ion binding are, at least partially, competitive (Norne et al., 1975; Markus et al., 1964). Since the highest reported K'_{Cl} is 2500 M^{-1} (Steinhardt and Reynolds, 1969), it follows that $K'_{NaDodSO_4} \gg K'_{Cl}$, so that chloride ions are virtually completely excluded from competitive sites by $NaDodSO_4$. The simplest binding model for HPA thus includes one class (A) of chloride-ion binding sites with $NaDodSO_4$ competition and one class (B) without. (It is assumed that each molecule of bound $NaDodSO_4$ excludes one, and only one, chloride ion.) Equations 1 and 2 then yield:

$$R_{2,ex} = m_{HPA} \times \left[\frac{N_A R_{2,A}}{1/K'_{A,Cl} + m_{Cl}{}^F + m_{NaDodSO_4}{}^F K'_{A,NaDodSO_4}/K'_{A,Cl}} + \frac{N_B R_{2,B}}{1/K'_{B,Cl} + m_{Cl}{}^F} \right] \quad (9)$$

The $NaDodSO_4$ titration curve, i.e., $R_{2,ex}$ vs. equivalents of $NaDodSO_4$ added to a chloride ion – HPA solution, should thus decrease until N_A equiv of $NaDodSO_4$ have been added, at which point the first term in eq 9 vanishes and thereafter remain constant.

The results of $NaDodSO_4$ titrations at three pH values are shown in Figure 7. From the "equivalence points" at neutral and alkaline pH, we obtain N_A (pH 7.2) = 7 ± 1 and N_A (pH 10.9) = 4 ± 1 (account has then been taken of the small amount of unbound $NaDodSO_4$ and of the 0.3–0.6 equiv of fatty acid present in the protein preparation). The slightly higher primary $NaDodSO_4$ binding at neutral pH (8–10 equiv

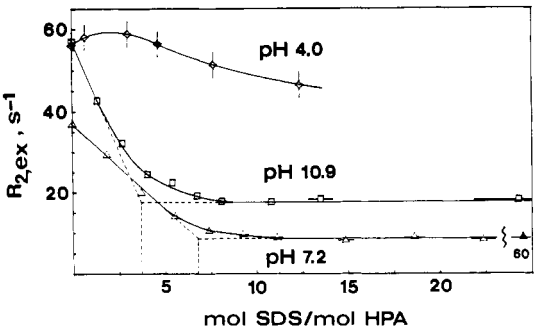

FIGURE 7: Effect of the addition of sodium dodecyl sulfate (SDS) on the ^{35}Cl excess relaxation rate, $R_{2,ex}$, at 26 °C, 0.5 *m* KCl, and indicated pH values. The solutions contained 2.15×10^{-5} *m* HPA (pH 4.0), 2.72×10^{-5} *m* HPA (pH 7.2), or 15.11×10^{-5} *m* HPA (pH 10.9). Filled symbols indicate an appreciable amount of undissolved $NaDodSO_4$ (▲) or the addition of $NaDodSO_4$ at pH 7.5 (♦). Error bars are based on estimated uncertainties in line-width measurements and titrant volumes.

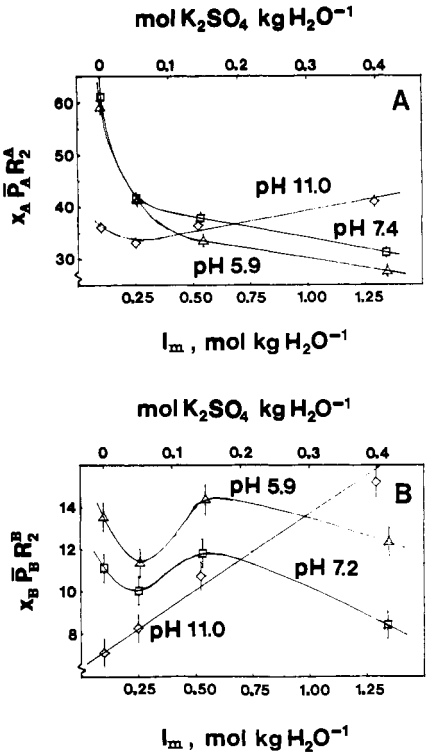

FIGURE 8: (A) Effect of ionic strength, I_m, on the contribution, $P_A R_{2,A}$, to the ^{35}Cl excess relaxation rate from the $NaDodSO_4$ competitive anion binding sites (class A) at 26.5 °C. In addition to 0.1 *m* KCl and varying amounts of K_2SO_4 (upper scale), the solutions contained 10.98×10^{-5} *m* HPA (pH 5.9), 1.54×10^{-5} *m* HPA (pH 7.4), or 1.50×10^{-5} *m* HPA (pH 11.0). Error bars are based on ±3% uncertainty in line-width measurements. (B) Effect of ionic strength, I_m, on the contribution, $P_B R_{2,B}$, to the ^{35}Cl excess relaxation rate from the $NaDodSO_4$ noncompetitive anion binding sites (class B) at 26.5 °C. Concentrations and error bars as in A.

as compared to the 7 ± 1 $NaDodSO_4$ competitive chloride ion binding sites) suggests that there are 1-3 $NaDodSO_4$ binding sites that do not contribute significantly to the ^{35}Cl relaxation rate. Since the ^{35}Cl relaxation rate decreases considerably upon addition of the very first equivalents of $NaDodSO_4$ (Figure 7), these noncompetitive $NaDodSO_4$ binding sites must have lower affinity than the chloride ion competitive $NaDodSO_4$ sites (class A); i.e., $K'_{NaDodSO_4} \lesssim K'_{A,NaDodSO_4}/K'_{A,Cl}$. This could be the case if the noncompetitive sites are devoid of positively charged residues at neutral pH, thus eliminating the electrostatic, but not the hydrophobic, contribution to the free energy of detergent binding. In a similar titration at pH 7.4, $N_A = 9 \pm 1$ was obtained (Norne et al., 1975). The small discrepancy with the present result at pH 7.2 can probably be ascribed to a higher uncertainty in the extrapolation from higher $NaDodSO_4$ concentration in the earlier study.

The titration curve at pH 4.0, however, does not exhibit an equivalence point. This may seem surprising in view of the findings that $NaDodSO_4$ binding isotherms in the pH range 3.8-7.5 are virtually identical within the primary binding region (Reynolds et al., 1970). It should be noted, however, that the identity of the binding isotherms does not imply that the binding sites are the same at different pH values. Indeed, the occurrence of the N-F transformation within this pH range makes such an assertion highly improbable. The appearance of the $NaDodSO_4$ titration curve at pH 4.0 may indicate that the competition is masked by conformational changes (induced by $NaDodSO_4$ binding) exposing new noncompetitive chloride ion sites and/or decreasing the protein reorientational rate.

Ionic Strength Dependence of ^{35}Cl Relaxation. Any investigation of the effect of the ionic strength on ion-protein interactions faces the problem of distinguishing nonspecific electrostatic effects from specific competition effects. In a comprehensive study (Norne et al., 1975) of anion binding to HPA, the sulfate ion was found to have the lowest affinity among the studied anions. In order to suppress competition effects, potassium sulfate was consequently chosen as the ionic strength regulating electrolyte (in addition, all solutions contained 0.1 *m* KCl). Relaxation measurements were performed at three pH values with and without blocking of the A sites by the addition of ca. 10 equiv of $NaDodSO_4$. It was thus possible to separate the contributions from the two classes of chloride ion binding sites ($R_{2,ex} = P_A R_{2,A} + P_B R_{2,B}$). The results are displayed in Figures 8A and B.

Several studies have shown that the albumin conformation is independent of ionic strength above 0.1 *m* in the pH range 4-8 (Raj and Flygare, 1974; Squire et al., 1968). The intrinsic relaxation rates at neutral pH should therefore be independent of ionic strength. [The possibility of concentration-dependent exchange rates was excluded on the basis of the previous data on the chloride ion concentration dependence of the relaxation rate (Norne et al., 1975).] The manner in which P_A decreases (Figure 8A) at neutral pH indicates a polydispersity of the ratio $K'_{A,SO_4}/K'_{A,Cl}$ within the class of $NaDodSO_4$ competitive anion binding sites. (If the ratio $K'_{A,SO_4}/K'_{A,Cl}$ were the same for all anion sites in class A, a plot of P_A vs. ionic strength should yield a rectangular hyperbola (cf. eq 2), while $1/P_A$ should be a linear function of the ionic strength. The data in Figure 8A show that this is not the case.) The data are consistent with the presence of one site with comparable affinity for the two anions and six other sites, whose affinity is about 40 times higher for chloride than for sulfate (assuming the same intrinsic relaxation rate for all A sites). At pH 11 (Figure 8A,B), the competition effect is masked by one or more processes that tend to increase the relaxation rate with increasing ionic strength. Two plausible explanations are: (1) increasing ionic strength suppresses the alkaline expansion, thereby reducing the rate of domain reorientation; (2) chloride ion binding sites in the hydrophobic clefts between the domains are weakened as the expansion progresses with decreasing ionic strength. The minima in $P_B R_{2,B}$ (Figure 8B) at neutral pH probably reflect the superimposed effects of several processes and are thus hard to interpret unambiguously.

Concluding Discussion

The hydrogen-ion titration curve (Figure 3) indicates the presence in the HPA molecule of about ten anomalous lysyl

and arginyl residues with $pK' = 9.4$. It is suggested that this low pK' value results from local coulombic interaction with neighboring cationic groups. Since only lysyl and arginyl groups are positively charged in the pH range 8.5–10.5, it seems reasonable to associate these findings with the presence of eight pairs of vicinal lysyl and/or arginyl residues in the amino acid sequence of HPA (Meloun et al., 1975). (Depending on the precise tertiary structure, sequentially nonvicinal cationic groups may also be in close proximity, thereby increasing the number of centers with a high local positive charge density.) The substantial reduction of the relaxation rate in the same pH range, 8.5–10.5 (Figure 4), strongly suggests that these lysyl and arginyl residues are involved in the anion binding. At least three of these doubly cationic sites consist of vicinal lysyl residues, both of which should be deprotonated at pH 10.9, accounting for the observed reduction of the number of high-affinity chloride ion sites at this pH (Figure 7). The remaining sites (Lys–Arg and Arg–Arg) are only singly charged at pH 10.9 and thus have lower association constants and lower field gradients, both factors leading to a reduced relaxation rate. The reduced field gradients should result in lower effective quadrupole coupling constants (eq 4) at alkaline pH, which is amply verified by a recent relaxation study (Bull et al., 1978).

The emerging picture is one of a small number of chloride ions, bound to the functional anion sites of HPA, contributing the major portion of the excess relaxation rate. Thus, it can be deduced from Figure 7 that seven chloride ions account for 80% of $R_{2,ex}$ at neutral pH. The extensive low-affinity association of chloride ions to HPA, which has been demonstrated in several studies employing equilibrium dialysis or anion selective electrodes (Carr, 1953; Scatchard et al., 1957; Scatchard and Yap, 1964), appears to have little, or even negligible, effect on the ^{35}Cl relaxation rate.

The presented results also shed some light on the mechanistic basis for the exceptional binding capacity of plasma albumin for inorganic anions. The fact that most of the high-affinity chloride ion binding sites are also strong detergent binding sites suggests that a hydrophobic environment may be an essential feature of the chloride ion sites. This is reasonable, since dielectric shielding (Schellman, 1953) and saturation effects strongly reduce the local effective dielectric constant in a hydrophobic cleft, thereby enhancing electrostatic interactions. It may also be noted that the presence of two flexible cationic side chains in a chloride site permits the formation of "chelated ion triplets", which should result in an entropic contribution to the free energy of association through the liberation of water of hydration (Cotton and Wilkinson, 1972). This mechanism is in accord with the appreciable positive entropy changes on chloride binding to HPA that have been reported (Scatchard and Yap, 1964).

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Electronic and Resonance Raman Spectra of Iron(III) Complexes of Enterobactin, Catechol, and *N*-Methyl-2,3-dihydroxybenzamide†

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ABSTRACT: Resonance Raman electronic absorption and circular dichroism spectra and pH titration curves are reported for the trianionic ferric complexes of enterobactin, catechol, and *N*-methyl-2,3-dihydroxybenzamide (MDHB). The spectral signatures of the enterobactin and MDHB complexes are virtually identical and differ from those of the catechol complex in ways that reflect the influence of the amide group on the electronic structure. Excitation in either the visible charge-transfer bands or the near-ultraviolet $\pi-\pi^*$ bands enhances Raman bands associated with benzene ring modes, although the relative enhancements differ markedly in the two regions. The data strongly support a structural model in which iron is bound exclusively to the phenolate oxygen atoms in all three complexes.

Blood diseases such as sickle cell anemia and β-thalasemia major are treatable only by transfusion therapy on a continued basis. Virtually all the iron administered as erythrocytes is retained and accumulated in the liver, heart and pancreas. Lacking an effective means of iron secretion, these tissues are subject to progressive fibrotic changes resulting in organ failure and death (Walker & Williams, 1974). Hence, an active interest is being taken in low molecular weight compounds capable of sequestering iron and rendering it in a form excretable by the body. Prime candidates are the bacterial iron transport chelators, which include desferrioxamine (Barry et al., 1974) and rhodotorulic acid (Grady et al., 1976). Rather high toxicity together with the required mode of administration (daily intramuscular injections) render these compounds less than ideal (Barry et al., 1974; Grady et al., 1976; Hwang & Brown, 1964). Another chelator, enterobactin, has received considerable attention (Smith, 1964; Gerwitz et al., 1965). This cyclic ester trimer of 2,3-dihydroxybenzoyl-1-serine (see Figure 1) is secreted by *Escherichia coli, Salmonella typhimurium,* and many other bacteria, and is 100 times more effective in sequestering iron and aiding in its transport than its monomer (Brot et al., 1966; O'Brien, 1966). In an effort to elucidate the structure and mode of iron binding in this complex we present here detailed spectroscopic studies of ferric enterobactin, together with two proposed model compounds, ferric tris(catecholate), first suggested by Webb and co-workers (Anderson et al., 1976), and a new complex, ferric tris(*N*-methyl-2,3-dihydroxybenzamide) (MDHB).[1]

Experimental Section

Enterobactin was obtained as previously described (Llinas et al., 1973). The ferric complex was prepared by extracting the ligand from ethyl acetate into a suspension of ferric chloride, the pH of which was maintained at 7.5 by the addition of 1 M NaOH. The dark red-brown layer containing the complex was then centrifuged and filtered. The ligand *N*-methyl-2,3-dihydroxybenzamide was prepared from the acid chloride of 2,3-dihydroxybenzamide and methylamine. Five grams of the free acid was suspended in 25 mL of thionyl chloride purified by distillation from quinoline, followed by distillation from linseed oil. To this suspension was added 0.4 mL of dry dimethylformamide, and the mixture was stirred overnight under a gentle stream of nitrogen. During this time the acid chloride formed and dissolved in the excess thionyl chloride. The thionyl chloride was removed by distillation under reduced pressure, and the residue was washed with dry benzene which was also removed by vacuum distillation. The crude acid chloride was recrystallized from dry acetone. One gram of the purified acid chloride was dissolved in 50 mL of dry acetone. This solution was added dropwise over a 30-min period to a stirred 40% aqueous solution of methylamine. Argon was vigorously bubbled through the amine solution for 15 min prior to, and during, the addition of the acid chloride, and for 15 min thereafter. The solution was then cautiously adjusted to pH 5.5 with concentrated HCl, chilled to 0 °C and extracted twice with 1-volume portions of ether. The ether fractions were pooled, dried over anhydrous sodium sulfate, filtered, and stripped to dryness under reduced pressure. The crude amide was recrystallized from acetone–water. Anal. Calcd for $C_8H_9NO_3 \cdot H_2O$: C, 51.26; H, 5.92; N, 7.25. Found: C, 51.70; H, 5.23; N, 6.46. The ferric complex of this ligand was prepared in a manner similar to that of enterobactin, or by addition of a standardized solution of ferric chloride to a known quantity of the ligand. Crystalline ferric tris(catecholate) was a generous gift of Dr. John Webb.

Electronic absorption spectra were obtained with a Cary 118

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[1] Abbreviations used: MDHB, *N*-methyl-2,3-dihydroxybenzamide; CD, circular dichroism; NMR, nuclear magnetic resonance; RR, resonance Raman.

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