some of the enzymes of a common function have evolved enough to require a slightly different structure in this region of the ubiquitin molecule.

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A Unique Pair of Zinc Binding Sites in the Human α_2-Macroglobulin Tetramer. A ^{35}Cl and ^{37}Cl NMR Study[†]

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ABSTRACT: ^{35}Cl NMR has been used to demonstrate that human α_2-macroglobulin tetramer possesses a unique pair of zinc binding sites. Zinc bound at these sites does not affect the ^{35}Cl NMR line width of free Cl$^-$. Additional lower affinity zinc sites exist that bind chloride weakly and cause broadening of the free chloride resonance through fast exchange with bound chloride. Using both ^{35}Cl and ^{37}Cl relaxation measurements it has been shown that chloride bound at these sites has an internal correlation time of 5.1 ns and a quadrupolar interaction, χ, of 4.2 MHz with zinc. Manganese binds to apo-α_2-macroglobulin analogously to zinc. α_2-Macroglobulin that has been reacted with methylamine still possesses two classes of zinc sites per tetramer, but their relative affinities differ more than for unreacted α_2-macroglobulin. These data are discussed with respect to possible models for the subunit arrangement in the tetramer.

α_2-Macroglobulin is present in human serum at a concentration of about 2.5 g L^{-1} and thus represents about 45% by weight of the major class of serum proteins that are characterized as proteinase inhibitors (Harpel & Brower, 1983). It also possesses bound zinc that has been quantitated as being in the range 3.9-9.2 mol of zinc/mol of α_2-macroglobulin (Parisi & Vallee, 1970). As a consequence it represents the second largest locus, after serum albumin, of zinc in the blood, accounting for 30-40% of the total (Parisi & Vallee, 1970; Vessel & Bearn, 1957). The zinc appears to be tightly bound, since exposure to ^{65}Zn does not lead to exchange with endogenous zinc (Parisi & Vallee, 1970) and therefore α_2-

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macroglobulin is entitled to the label of zinc metalloprotein (Vallee, 1955; Vallee & Wacker, 1970). More recently there have been reports of zinc binding capacity in excess of 100 mol/mol of α_2-macroglobulin (Gonias et al., 1984; Pratt & Pizzo, 1984). Despite this, no specific role has been determined for zinc in this protein. In part this may be due to the unknown function of α_2-macroglobulin, which, though it possesses a broad specificity antiprotease activity in vitro (Barrett & Starkey, 1973), has not been shown to be required in this role in vivo. As part of a study aimed at characterizing the structural features of α_2-macroglobulin and the changes that take place upon reaction with proteases and small nucleophiles, we have investigated the metal binding properties of apo-α_2-macroglobulin. We report here the detection of a unique pair of tight zinc binding sites as well as an undetermined number of weaker binding sites, using ^{35}Cl and ^{37}Cl NMR to follow the interaction of chloride ions with protein-bound metal ion.

THEORY

Calculation of χ and τ_c. ^{35}Cl and ^{37}Cl are both quadrupolar nuclei with $I = {}^3/_2$ and respective natural abundances of 75% and 25%. Their relaxation is normally dominated by the contributions from interaction of their quadrupole moments with time-dependent electric field gradients at the nucleus (Stengle & Baldeschweiler, 1966; Forsên & Lindman, 1981). For short correlation times, such as apply to small molecules or ions in solution, the relaxation rates are given by (Abragam, 1961)

$$\frac{1}{T_1} = \frac{1}{T_2} = \frac{2}{5}\pi^2\chi^2\tau_c \tag{1}$$

where χ is the quadrupolar coupling constant. For slow motion the three nuclear transitions no longer have the same relaxation properties and both longitudinal and transverse relaxations represent the sum of two decaying exponentials. If $\omega\tau_c < 1.5$, the following approximate equations give T_1 and T_2 (Bull, 1972; Bull et al., 1978):

$$\frac{1}{T_1} = \frac{2}{5}\pi^2\chi^2\left[\frac{0.2\tau_c}{1+\omega^2\tau_c^2} + \frac{0.8\tau_c}{1+4\omega^2\tau_c^2}\right] \tag{2}$$

$$\frac{1}{T_2} = \frac{2}{5}\pi^2\chi^2\left[0.6\tau_c + \frac{\tau_c}{1+\omega^2\tau_c^2} + \frac{0.4\tau_c}{1+4\omega^2\tau_c^2}\right] \tag{3}$$

In the presence of a macromolecule with which a small fraction of the total chloride ions present in solution interacts, the chloride NMR resonance will be affected in one of several ways, depending on the rate of exchange between solution and macromolecule environments. For very slow exchange, $\tau_{ex} \gg T_{1B}$, T_{2B}, where T_{1B} and T_{2B} represent the bound relaxation times, resonances due to free and bound chloride will be superimposed. The bound resonance, being of much lower intensity and also much greater line width, will go undetected. In the limit of rapid exchange, $\tau_{ex} \ll T_{1B}$, T_{2B}, only a single resonance will be observed, where the observed relaxation rates are given by

$$\frac{1}{T_{1\mathrm{obsd}}, T_{2\mathrm{obsd}}} = \sum P_i \frac{1}{T_{1i,2i}} \tag{4}$$

P_i is the fraction of chloride in site i, and T_{1i} and T_{2i} are its respective relaxation times. For intermediate exchange, the residence time τ_{ex} affects the observed relaxation times and may enable an evaluation of τ_{ex} to be made. For no difference in chemical shift and only a small fraction of chloride ions bound to the macromolecule, the observed relaxation rates can be represented as the sum of contributions from free and bound ions. The difference between the observed rate and that for the free ion gives the contribution from the bound state. These are often termed the excess relaxation rates $1/T_{1,\mathrm{excess}}$ and $1/T_{2,\mathrm{excess}}$. One way to distinguish between rapid and intermediate exchange is to determine the excess relaxation rates for both ^{35}Cl and ^{37}Cl. For rapid exchange the excess relaxation rates do not depend upon τ_{ex}, and therefore the ratio of rates for ^{35}Cl and ^{37}Cl depends upon the second power of their quadrupole moments (5) (Falke et al., 1985). For much

$$\frac{T_{1,\mathrm{excess}}{}^{37}}{T_{1,\mathrm{excess}}{}^{35}}\left(\frac{^{35}\mathrm{Cl}_\chi}{^{37}\mathrm{Cl}_\chi}\right)^2 = 1.60 \tag{5}$$

slower exchange, but not the limit of slow exchange, the excess relaxation is proportional to the exchange rate and thus does not depend upon the nucleus. The ratio of T_1 values therefore has the value of 1. Intermediate rates will give ratios between 1 and 1.60 (Falke et al., 1985).

Binding Analysis. Consider an α_2M tetramer with two pairs of zinc binding sites A and B. Each A site is equivalent and independent as are the B sites. The microscopic binding constant of zinc to an A site is K_A and to a B site K_B. These are related by $K_B = nK_A$. From consideration of the 12 binding equilibria it can be shown that

$$\mathrm{Zn}_{\mathrm{total}} = \mathrm{Zn} + \left\{\left[\frac{2\mathrm{Zn}}{K_A} + \frac{2\mathrm{Zn}}{nK_A} + \frac{8\mathrm{Zn}^2}{nK_A{}^2} + \frac{2\mathrm{Zn}^2}{K_A{}^2} + \frac{2\mathrm{Zn}^2}{n^2K_A{}^2} + \frac{6\mathrm{Zn}^3}{n^2K_A{}^3} + \frac{6\mathrm{Zn}^3}{nK_A{}^3} + \frac{4\mathrm{Zn}^4}{n^2K_A{}^4}\right] \Big/ \left[1 + \frac{2\mathrm{Zn}}{K_A} + \frac{2\mathrm{Zn}}{nK_A} + \frac{4\mathrm{Zn}^2}{nK_A{}^2} + \frac{\mathrm{Zn}^2}{K_A{}^2} + \frac{\mathrm{Zn}^2}{n^2K_A{}^2} + \frac{2\mathrm{Zn}^3}{nK_A{}^3} + \frac{2\mathrm{Zn}^3}{n^2K_A{}^3} + \frac{\mathrm{Zn}^4}{n^2K_A{}^4}\right]\right\} P_{\mathrm{total}} \tag{6}$$

where $\mathrm{Zn}_{\mathrm{total}}$ is the total zinc added, Zn is the free zinc concentration, and P_{total} is the total α_2M concentration. If the broadening of Cl$^-$ bound to an A-site zinc is B_A and is B_B when bound to a B-site zinc, the observed broadening is given as a function of the free zinc concentration by 7.

$$\text{broadening} = \left[B_A P_{\mathrm{total}}\left(\frac{2\mathrm{Zn}}{K_A} + \frac{4\mathrm{Zn}^2}{nK_A{}^2} + \frac{2\mathrm{Zn}^2}{K_A{}^2} + \frac{4\mathrm{Zn}^3}{nK_A{}^3} + \frac{2\mathrm{Zn}^3}{n^2K_A{}^3} + \frac{2Zn^4}{n^2K_A{}^4}\right) + B_B P_{\mathrm{total}}\left(\frac{2\mathrm{Zn}}{nK_A} + \frac{4\mathrm{Zn}^2}{nK_A{}^2} + \frac{2\mathrm{Zn}^2}{n^2K_A{}^2} + \frac{2\mathrm{Zn}^3}{nK_A{}^3} + \frac{4Zn^3}{n^2K_A{}^3} + \frac{2Zn^4}{n^2K_A{}^4}\right)\right] \Big/ \left[1 + \frac{2\mathrm{Zn}}{K_A} + \frac{2\mathrm{Zn}}{nK_A} + \frac{4\mathrm{Zn}^2}{nK_A{}^2} + \frac{\mathrm{Zn}^2}{K_A{}^2} + \frac{\mathrm{Zn}^2}{n^2K_A{}^2} + \frac{2\mathrm{Zn}^3}{nK_A{}^3} + \frac{2\mathrm{Zn}^3}{n^2K_A{}^3} + \frac{\mathrm{Zn}^4}{n^2K_A{}^4}\right] \tag{7}$$

MATERIALS AND METHODS

Purification of α_2M.[1] Human α_2M was purified by Zn^{2+} chelate chromatography and gel filtration, as has been pre-

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[1] Abbreviations: α_2M, α_2-macroglobulin; apo-α_2M, α_2-macroglobulin with endogenous metal ions removed; α_2M-me, α_2-macroglobulin reacted with methylamine; SDS, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; T_1 and T_2, longitudinal and transverse relaxation times; τ_{ex}, bound lifetime; T_{1M} and T_{2M}, relaxation times for chloride bound to macromolecular zinc.

viously described (Dangott & Cunningham, 1982). The purity of each preparation was checked by electrophoresis under nondenaturing conditions and also in the presence of SDS. Nondenaturing polyacrylamide gel electrophoresis was performed in 5% acrylamide slabs according to Davis (1964). SDS–polyacrylamide gel electrophoresis was carried out in 7.5% slab gels according to Laemmli (1970). Samples for SDS electrophoresis were denatured by incubating with an equal volume of 2% SDS, 5% β-mercaptoethanol, and 1 mM PMSF for 45 min at 37 °C. Protein concentrations were determined spectrophotometrically with $E_{280}^{1\%} = 8.9$ (Hall & Roberts, 1978; Barrett et al., 1979) and a molecular weight of 716 000 (Kristensen et al., 1984).

Preparation of Apo-α_2M. Zinc-free α_2-macroglobulin was prepared by dialysis against 3 × 2 L of 20 mM Tris, 0.15 M NaCl, and 3 mM EDTA at pH 7.8 followed by dialysis against 3 × 2 L of the same buffer without EDTA. The buffer used was made metal free by extraction with 0.02% dithizone in CCl_4 (Vallee et al., 1960). Glassware was acid washed as described by Thiers (1957). Metal ion stoichiometries were determined by atomic absorption spectrometry on a Perkin-Elmer 403 spectrometer.

Methylamine-Treated α_2M. α_2M was converted from the slow to fast electrophoretic forms by reaction with 0.1 M methylamine at pH 8 and 25 °C for 3 h (Swenson & Howard, 1979).

NMR Measurements. ^{35}Cl and ^{37}Cl NMR spectra were recorded on a Bruker AM 400 narrow-bore spectrometer at 39.1 and 32.6 MHz, respectively, using a broad-band tunable probe; 2-cm^3 samples in 10-mm tubes were used. D_2O to a final concentration of 10% was added for the field-frequency lock. Data acquisition of normal spectra used a 90° pulse (25 μs), a 500-Hz sweep width and a data block of 1K points, zero-filled to 8K prior to Fourier transformation. Excess line widths were determined from a comparison of the line width at half-height of the sample with that of a sample of NaCl at the same concentration of Cl^-. A correction for the expected contribution of unbound zinc to the observed chloride line width is unnecessary, since, even if all added zinc remained unbound, which is not the case, the broadening at a stoichiometry of 4 mol of zinc per tetramer would be only 0.2 Hz (Ward & Happe, 1967). T_1 values were determined with an inversion–recovery pulse sequence of 180°–τ–90°–observe. The temperature used throughout was 298 K.

RESULTS

Titration of Zinc into Apo-α_2-macroglobulin. The excess broadening of the ^{35}Cl resonance of 0.15 M NaCl in the presence of 15 μM apo-α_2-macroglobulin as a function of added zinc is shown in Figure 1. At very low zinc:α_2M ratios (<1:1) there is almost no measurable broadening of the chloride resonance, while at ratios greater than 2.5:1 there is a linear increase in line width with zinc added. For intermediate ratios of zinc to protein there is curvature that was found to be reproducible in three separate determinations. The biphasic behavior is indicative of at least two classes of sites with different affinities for zinc, each of which has a different effect on the chloride line width. Considering only the first 4 equiv of zinc added to the α_2M tetramer, the chloride resonance broadening can be fitted with the model outlined under Theory of two classes of independent sites with different affinities for zinc and different broadening effects on chloride bound to or near each type of zinc. With the following parameters, the solid curve shown in Figure 1a is obtained: $K_A = 0.1$ μM, $n = 10$ (i.e., $K_B = 1$ μM), $B_A = 0$, and $B_B = 227$ kHz M^{-1} per site. The free zinc concentration at a stoichiometry of 4 Zn/α_2M was determined by ultrafiltration and atomic absorption analysis of the flowthrough. A value of less than 2 μM was obtained and so the slope of the experimental points in the linear region was used to estimate B_B.

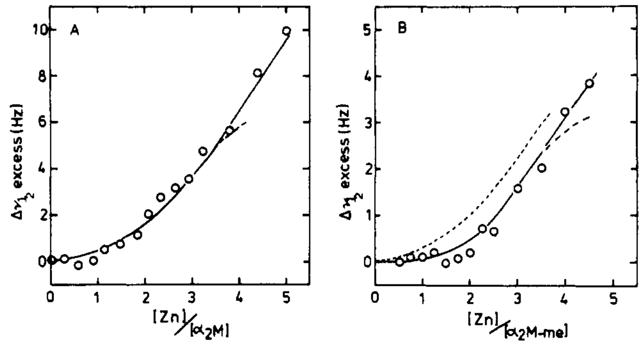

FIGURE 1: (A) Broadening of ^{35}Cl resonance of inorganic chloride by apo-α_2M as a function of added zinc. The initial concentration of α_2M was 15.2 μM in 10 mM Tris, pH 8.0, and a total Cl^- concentration of 0.15 M. Zn(II) was added in the same buffer. The broadenings are scaled to compensate for the change in α_2M:Cl^- ratio caused by dilution with the zinc stock solution. The solid curve up to a Zn:α_2M ratio of 3.6:1 is calculated for two pairs of zinc sites, the first pair having no broadening effect on chloride and the second pair broadening at the rate of 3.1 Hz per zinc site occupied for the conditions employed. This best fit was obtained with K_d values of 0.1 and 1.0 μM for the first and second pairs, respectively. The straight line beyond 3.6 Zn per α_2M is an extrapolation of the linear portion of the curve. (B) Broadening of ^{35}Cl resonance of inorganic chloride by methylamine-treated apo-α_2M as a function of added zinc. [α_2M] was 9.6 μM in 10 mM Tris, 0.15 M Cl^-, pH 8.0. The data are fitted to eq 7 with K_d values of 0.1 and 4 μM and a value of B_B the same as for Figure 1a. The dotted curve is the fit obtained with the parameters for Figure 1a.

Thus it is proposed that the tighter A site has no broadening effect on bulk chloride whereas the tenfold weaker B sites broaden it substantially. Since the model used considers only four binding sites, the theoretical curve shows plateauing out above Zn_{total}/α_2M values of 3.6 for the parameters given and will give constant broadening of 6.8 Hz at ratios >4:1. This is clearly not the case and so the solid line in Figure 1a above Zn_{total}/α_2M values of 3.6 is an extrapolation of the linear portion of the curve between values of 2.8 and 3.5, where added zinc is filling B sites almost exclusively, rather than the theoretical fit for this region. There is no doubt that there are more than four zinc binding sites in toto, but at the high α_2M concentrations used (and necessary to obtain accurate Cl^- broadenings) there is no discontinuity at 4 equiv of bound zinc, and it is therefore not possible to distinguish between more zinc sites equivalent to the B sites or different sites that are weaker but still tight enough to bind zinc under the experimental conditions employed. The distinction of the first two zinc sites from any others is, however, the most important finding here and has relevance to the structure of the tetramer and to subsequent exploitation for distance measurements.

While the experimental data could also be fitted to a model of four different zinc sites with broadenings increasing as the zinc affinity decreases, the model presented is the simplest one consistent with the data. Furthermore, the proposal of two identical A sites, rather than sites with different affinities and broadenings, is very attractive given the apparent antiprotease activity of the half-molecule. This is elaborated upon further under Discussion.

The absence of broadening by the more tightly bound zinc ions can be explained in two ways. First there may be tight binding of chloride to macromolecular zinc, resulting in slow exchange between bound and unbound chloride species and thereby resulting in a separate, extremely broad and therefore undetectable ^{35}Cl resonance from the bound chloride. Second, there could be an absence of coordination site for chloride on the first zinc ions or else the zinc could be sequestered from chloride, which then cannot gain access to bind. There is no ready means to distinguish between these possibilities. Nevertheless there seems to exist a pair of high affinity zinc sites in the α_2M tetramer that differs markedly from other weaker sites.

Effect of Methylamine Treatment on Zinc Binding. Methylamine and other small primary amines react with an internal thioester linkage in α_2M (Travis & Salveson, 1983; Barrett, 1981; Salveson et al., 1981; Sottrup-Jensen et al., 1981) and result in a conformational change similar to that effected by reaction with proteases (Dangott & Cunningham, 1982). To determine the effect of this shape change on the zinc binding sites, a titration of zinc into methylamine treated apo-α_2M was performed and followed by ^{35}Cl NMR. This α_2M species was prepared by reaction of native α_2M with 50 mM methylamine followed by removal of the endogenous zinc as described under Materials and Methods. Polyacrylamide gel electrophoresis under nondenaturing conditions readily distinguishes between the reacted and unreacted species (the latter being less mobile than the former (Barrett et al., 1979)) and was used to show that the sample used was completely in the reacted form. Figure 1b shows the resulting titration curve. This has the same general biphasic appearance as the unreacted α_2M shown in Figure 1a. The region up to 2 equiv of zinc per α_2M tetramer does seem to have less curvature, which indicates greater difference in affinities for the first and second pair of zinc sites, either from strengthening the affinity of the first or weakening of the second. If one uses the same K_A and B_B values as used for zinc binding to unreacted apo-α_2M, a good fit can be obtained by taking a value of n of 40. This corresponds to a fourfold weakening of the second pair of metal binding sites.

Binding of Manganese to Apo-α_2-macroglobulin. Zinc is not a useful spectroscopic atom for probing the nature of its environment in macromolecules. It has no unpaired electrons to permit ESR studies, or an $I = {}^1/_2$ nucleus for NMR measurements, or a visible absorption spectrum. As a result many studies of zinc metalloproteins have resorted to substitution of the zinc with a transition metal ion for UV–visible or ESR studies or with ^{113}Cd for NMR studies. Ions such as Mn(II), Gd(III), and Cu(II) have long electron-spin relaxation times and are therefore good NMR relaxation probes (Dwek, 1973). They can be used, among other things, to measure distances of other, NMR-visible, nuceli from the paramagnetic center (Campbell et al., 1975). This is of potentially great usefulness in α_2M, since we have identified a number of ^{1}H NMR resonances in the spectrum of α_2M and attributed them to bait region protons (Gettins & Cunningham, 1986). If manganese can be shown to bind specifically and its sites identified with the corresponding native zinc sites, it should be possible to use the paramagnetic broadening effects of the metal on the identified bait region resonances to locate one with respect to the other. For this reason we followed the binding of manganese to apo-α_2M.

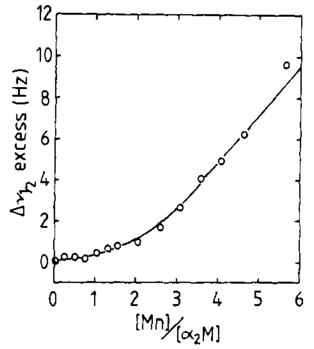

FIGURE 2: Broadening of ^{35}Cl resonance of inorganic chloride by α^2M as a function of added manganese. [α_2M] was 3.1 μM in 10 mM Tris, 0.15 M Cl$^-$, pH 8.0. The solid curve is fitted to eq 7 for two pairs of metal sites with K_d values of 0.1 and 1.0 μM and a broadening for the second sites of 2.4 Hz per zinc site occupied for the conditions employed.

The effect of manganese binding on the chloride line width is shown in Figure 2 and is similar in form to that obtained with zinc. The data were again fitted to two pairs of sites of $K_A = 0.1$ μM and $n = 10$. A value of 774 kHz M^{-1} was used for B_B and 0 for B_A. This value of B_B is approximately 3.4 times greater than for zinc. For the conditions employed of 3.1 μM α_2M and 150 mM Cl$^-$ it corresponds to a bound line width of 116 kHz. If the contribution to the bound line width from quadrupolar relaxation is the same as for zinc (34 kHz) the contribution from paramagnetic relaxation is 82 kHz. This value can be used to estimate the Mn–chloride separation by use of the Solomon–Bloembergen equation for bound line width (Solomon, 1955; Bloembergen, 1957), eq 8. Here γ_I,

$$\frac{1}{T_{2M}} = \frac{1}{15}\gamma_I^2 g^2 \frac{S(S+1)\beta^2}{r^6}\left(4\tau_c + \frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{13\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{1}{3}S(S+1)\left(\frac{A}{\hbar}\right)^2\left(\frac{\tau_e}{1+\omega_s^2\tau_e^2} + \tau_e\right) \quad (8)$$

g, S, β, and A have the usual meanings. τ_c is the correlation time, τ_e is the hyperfine correlation time, and ω_I and ω_S are the resonant frequencies for ^{35}Cl(nuclear) and Mn(electron), respectively. If the contribution from hyperfine interaction can be neglected, the separation calculated depends then only upon the value of τ_c used in the equation. Where τ_c is dominated by τ_s, the electron spin resonance time (as it does here with both τ_M, the chloride residence time, and τ_R, the rotational correlation time, having large values), it has been found to have values in the range 0.5–10 ns (Reuben & Cohn, 1970). These values give separations from 3.62 to 5.96 Å, which are too large for inner-sphere complexation but may represent a second sphere interaction of chloride with the metal ion. Inclusion of any hyperfine contribution would tend to increase these distances.

Calculation of the Quadrupolar Coupling Constant and Correlation Time from ^{35}Cl/^{37}Cl Relaxation Measurements. The T_1 and T_2 relaxation times of chloride were determined at 298 K in the presence of apo-α_2-M and Zn$_4$ α_2M for both the ^{35}Cl and ^{37}Cl isotopes. T_1 values were determined with an inversion–recovery pulse sequence as described under Materials and Methods and then by plotting $\log_e (A_\infty - A_\tau)$ against τ, where A_∞ is the maximum peak height and A is the height for a delay of between 180° and 90° pulses. The slope is equal to $-T_1^{-1}$. These are shown in Figure 3. T_2 values were determined from the line width at half-height. Since the protein and Cl$^-$ concentrations for the two samples were identical, the contribution to the chloride T_1 and T_2 from interaction specifically with macromolecule-bound zinc could be found by subtraction of $T_{1,2,\mathrm{obsd}}^{-1}$ for the apoprotein from $T_{1,2,\mathrm{obsd}}^{-1}$ for the Zn$_4$ protein. This can then be converted to T_{1M} and T_{2M} by assuming two chloride binding sites per α_2M tetramer that contribute to bulk chloride relaxation, one for each of the two zinc ions that cause broadening of chloride in Zn$_4$ α_2M. These data are given in Table I. The accuracy of T_1 measurements is to within 0.1 ms so that the $^{37}T_1/^{35}T_1$

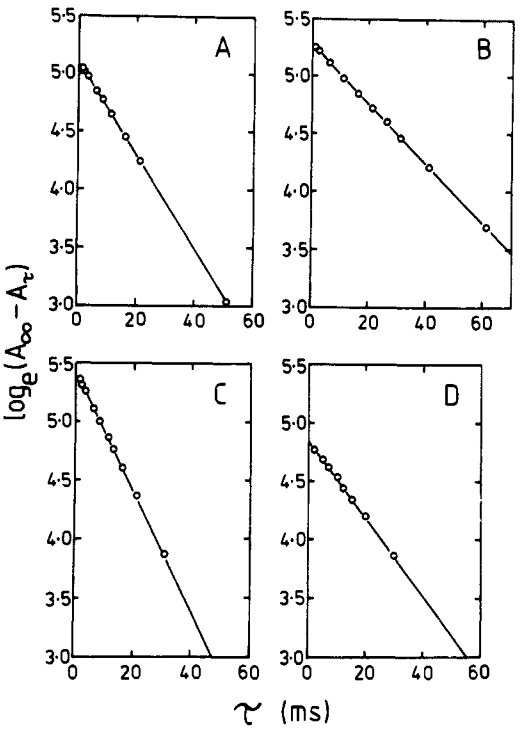

FIGURE 3: ^{35}Cl and ^{37}Cl T_1 relaxation of Cl^- in the presence of apo-α_2M and Zn_4 α_2M, obtained from inversion-recovery spectra. Both [apo-α_2M] and [Zn_4 α_2M] were 11.5 μM in 10 mM Tris and [Cl^-] of 0.15 M. τ is the delay between the 180° inversion pulse and the 90° observation pulse: (a) ^{35}Cl, apo-α_2M; (b) ^{37}Cl, apo-α_2M; (c) ^{35}Cl, Zn_4 α_2M; (d) ^{37}Cl, Zn_4 α_2M.

Table I: ^{35}Cl and ^{37}Cl Relaxation Times, T_1 and T_2, for Cl^- Bound to Apo-α_2M and Zn_4 α_2M

	T_1 (μs)		T_2 (μs)		$^{37}T_{1M}/^{35}T_{1M}$[a]	$^{37}T_{2M}/^{35}T_{2M}$
apo-α_2M	3.80	5.97	3.76	5.09	1.68 ± 0.12	1.34 ± 0.2
Zn_4 α_2M	3.05	4.85	2.17	2.94		

[a] The T_1 and T_2 values used are for Cl^- bound specifically to zinc and are obtained from the relaxation rates for apo-α_2M ($T_{1,2\mathrm{apo}}^{-1}$) and Zn_4 α_2M ($T_{1,2\mathrm{Zn}}^{-1}$) using the expression $T_{1M,2M}^{-1} = T_{1,2\mathrm{Zn}}^{-1} - T_{1,2\mathrm{apo}}^{-1}$.

ratio is 1.68 ± 0.12, and, therefore, according to eq 5, rapid exchange of chloride holds for the second pair of zinc sites. With the $^{35}T_{1M}$ and $^{35}T_{2M}$ values for zinc-bound chloride, eq 2 and 3 enable τ_c and $^{35}\chi$ to be calculated. Values of 5.1 ns and 4.2 MHz, respectively, are obtained. Since fast exchange obtains for these binding sites $\tau_{\mathrm{exchange}} \ll T_1, T_2$, therefore $k_{\mathrm{off}} \gg 2 \times 10^5\ \mathrm{s}^{-1}$. Assuming diffusion control of binding this gives an upper limit for K_a of $K_a \ll 2 \times 10^3\ \mathrm{M}^{-1}$.

DISCUSSION

The absence of broadening of the ^{35}Cl resonance of Cl^- upon binding of the first 2 mol of zinc to the apo-α_2M tetramer, but not for subsequently bound zinc ions, establishes that the first pair of metal ions to bind to the tetramer has different properties from subsequently occupies weaker sites. Although the tetrameric species is composed of identical monomeric species, there is much evidence to support a model in which the individual polypeptides are arranged to form a functional dimeric species. Limited reduction and alkylation has produced half-molecules of molecular weight 360 000 that still possess the protease-inhibiting properties of native α_2M (Gonias & Pizzo, 1983a,b). There are also α_2-macroglobulins from fish (Starkey & Barrett, 1982; Starkey et al., 1982) and from grass frog (Feldman & Pizzo, 1986) that exist solely as the 360 000 Da dimer. Finally the stoichiometry of binding of proteases to α_2-macroglobulin is in the range from 1 per tetramer to 2 per tetramer depending upon the size of the protease but also on other factors such as the rate of reaction (Travis & Salvesen, 1983). Stoichiometries of protease to α_2M tetramer of greater than 2:1 have not been observed. Feldman and co-workers (Feldman et al., 1985) have proposed a model for α_2M that rationalizes these observations in that the molecule is arranged as two equivalent dimers, each composed of two monomer chains related by a twofold rotation axis. The whole structure had D_2 symmetry. It is therefore very likely that the pair of zinc sites that represents the sites of highest affinity for zinc arises from equivalent sites in each half-molecule. This could occur in two distinct ways. Either the zinc site is located on the twofold rotation axis that relates the two chains of the half-molecule, with both monomers presumably contributing ligands, or the half-molecule must have an asymmetric arrangement of subunits so that a unique metal site results. Our present data do not distinguish between these possibilities. Both models do, however, place such a zinc binding site at or in the vicinity of the monomer–monomer interface and may thus account for the absence of broadening of the Cl^- ^{35}Cl resonance, through an absence of interaction with chloride resulting from burial of the zinc rather than tight binding of chloride and consequent slow exchange with free Cl^-. Alternatively, these two zinc sites could lie along one of the remaining twofold rotation axes that relate the two half-molecules and could thus be involved in holding the half-molecules together. It will be particularly interesting to investigate the zinc binding properties of the half-molecule (either from fish or produced chemically) to see if zinc is required to hold the half-molecules together.

The next two zinc ions to bind to α_2M have one-tenth the affinity of the first pair, as determined from fitting the data in Figure 1a, are accessible to the solvent, and interact with chloride, causing a broadening of the observable ^{35}Cl resonance through fast exchange averaging between the free and bound environments, as demonstrated by the ratio of the bound T_1 relaxation times for ^{37}Cl and ^{35}Cl being equal to 1.6. The rotational correlation time calculated for Cl^- bound to these zinc sites is 5.1 ns, to be compared with the rotational correlation time of 720 ns for the whole protein that was calculated by triplet probe depolarization (Pochon et al., 1978). The former value is not in conflict with the latter, since it has been found for many other proteins, with molecular correlation times from 30 to 161 ns, that the correlation time relevant to bound Cl^- is in the range 0.7–6.7 ns and arises from considerable independent motion of the chloride, modeled as an ion bound at the end of a rigid rod that can move within a cone of half-angle ranging from 53° to 90° (Bull et al., 1978). The quadrupolar coupling constant, χ, has a value considerably lower than might be expected for covalent binding to zinc. Although there are no available data for such coupling constants to zinc in macromolecules, a value of χ = 15–18 MHz has been determined in the solid state for Na_2ZnCl_4 (Irion et al., 1978) and a larger value of 45 MHz determined for ^{35}Cl in an HgCl bond in $HgCl_2$ (Biedenkapp & Weiss, 1964). Using an electrostatic model to calculate χ for chloride binding to an NH_3^+ group, Bull et al., (1978) estimated a value of 3.6 MHz. Our value of 4.2 MHz, which results specifically from zinc binding to α_2M, probably indicates an outer-sphere chloride–zinc complex with consequently greater Zn–Cl separation and smaller electric field gradient at the chlorine nucleus than would result from a normal Zn–Cl single bond. The calculated Mn–Cl distance of 3.62–5.96 Å is also consistent with this explanation.

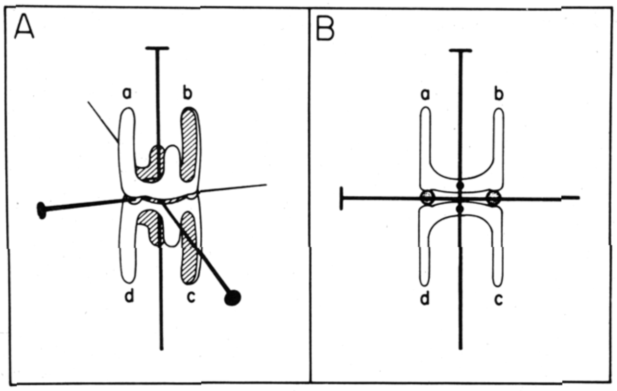

FIGURE 4: (A) Model of α_2M, based on that of Feldman et al. (1985), showing the twofold rotation axes. (B) Cross section of the α_2M tetramer through points a, b, c, and d, showing one possible arrangement of two pairs of zinc binding sites located along the twofold rotation axes. Since Feldman's model is hollow in the middle and therefore has no protein density along the vertical twofold axis, we have modified the model by making the base of each half-molecule a solid disk. The vertical C_2 axis now passes through protein and makes it possible to locate zinc sites *within* each half-molecule rather than solely between half-molecules.

We did not titrate zinc into α_2M much in excess of 4 equiv per tetramer since this could readily give rise to high free zinc concentrations if binding is substantially weaker than for the first four equivalents. Under such conditions the protein aggregates, as has been shown by small-angle X-ray scattering studies in which α_2M was dialyzed against a constant free zinc concentration of 12.5 μM (Österberg & Malmensten, 1984). Four equivalents of zinc per tetramer is, however, a relevant stoichiometry, given the tetrameric nature of α_2M and also the finding by Carson (1984) that the tetramer has four metal sites with K_d values tighter than 1.5 μM. His data, following the binding of ^{109}Cd(II) to apo-α_2M, show sufficient scatter that he could not readily distinguish between four equivalent sites or, as we propose, two sets of distinct sites with relative affinities of 10:1. Nevertheless his finding of four binding sites rather than six, when taken with our finding of two unique sites per tetramer, is imporant with regard to the two models discussed above. A tetramer with D_2 symmetry in which a pair of unique zinc sites could be located along one of the three C_2 rotation axes has four identical subunits and should therefore bind further zinc ions in multiples of 4 mol per tetramer, unless the binding sites all lie on one or more of the three twofold rotation areas. In contrast, an α_2M tetramer composed of two equivalent dimers, with monomers asymmetrically disposed within the half-molecule, might have only four metal sites, consisting of two distinct pairs. A model of α_2M with D_2 symmetry, based on that of Feldman et al. (1985) but modified so that the base of each half-molecule is a solid disk rather than an annulus, is shown in Figure 4, together with possible locations of two pairs of zinc binding sites on the twofold rotation axes.

It should be emphasized that our data alone do not distinguish between a total of six zinc sites consisting of the first pair and a subsequent equivalent set of four sites or binding of distinct pairs to a stoichiometry of four zincs per tetramer followed by weaker binding that also affects the chloride line width. Figure 1a clearly shows zinc binding beyond a stoichiometry of 4:1 Zn:α_2M, but under the conditions employed of 15 μM α_2M substantially weaker metal sites than those considered for the first four metal ions would bind zinc. To discriminate between two pairs of sites and a pair plus a quartet, binding analysis at much lower α_2M concentrations would be necessary. Such studies by equilibrium dialysis are under way. The most important conclusion, however, is that the first two sites are different from any subsequently occupied sites.

The data obtained with manganese binding to apo-α_2M appear very similar to those obtained with zinc and can be fitted with the same binding constants as for zinc binding, with the exception of the magnitude of the broadening caused by the second class of sites, which is, as expected, greater for manganese. This establishes that manganese has similar relative affinities for the two types of site as zinc.

α_2-Macroglobulin that has been treated with methylamine and has thus undergone a conformation change as a result of cleavage of the internal thiol esters still possesses the unique pair of high affinity zinc sites. The only difference in the effect of zinc on the ^{35}Cl line width of the Cl$^-$ resonance compared with unreacted α_2M is that the ratio of the affinities of the first class of sites to the second class of metal ions is increased. Carson (1984) found that the affinity of the first 2 equiv of metal bound were similar in α_2M and methylamine-treated α_2M but that only two metal ions bound to the latter under his experimental conditions (up to 0.3 μM α_2M). It thus seems that the conformational change does not affect the highest affinity zinc sites but weakens the second class. This is also the conclusion reached from the Cl$^-$ broadening data on native or methylamine-treated α_2M. In the former case the data are well represented by classes of sites with affinities of 0.1 and 1.0 μM, whereas the latter data can be fitted to sites with affinities of 0.1 and 4.0 μM. The affinity of the first pair is unchanged, whereas that of the next class is substantially diminished.

Whereas the presence of zinc does not seem to be a prerequisite for the antiprotease activity of α_2-macroglobulin,[2] the high affinity of zinc for the protein may result in increased stability and may well affect the rates of protease inhibition through indirect effects on conformational stabilization. This may be particularly relevant since protease inactivation requires large-scale conformational change to trap the protease (Pochon et al., 1981; Barrett & Starkey, 1973) rather than chemical reaction with it. We intend to investigate the effect of the presence and absence of zinc on the kinetics of protease- and methylamine-induced conformation changes in α_2M to help elucidate the roles of these distinct high-affinity zinc sites. In this regard it is particularly useful that manganese (and probably other metal ions) binds to apo-α_2M analogously to zinc and can thus be used for spectroscopic studies to establish the nature of these metal sites.

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Registry No. Zn, 7440-66-6; Cl$^-$, 16887-00-6; Mn, 7439-96-5.

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[2] Trypsin and methylamine both react with apo-α_2M and result in identical changes in electrophoretic mobility from "slow" to "fast" to those seen with native α_2M (L. W. Cunningham, unpublished results).

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