Identification of ^1H Resonances from the Bait Region of Human α_2-Macroglobulin and Effects of Proteases and Methylamine†

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ABSTRACT: The ^1H NMR spectrum of human α_2-macroglobulin, M_r 716 000, consists of predominantly extremely broad unresolved resonances but also has nine relatively sharp ($\Delta\nu_{1/2} < 25$ Hz) resonances from aromatic residues. By treatment of α_2-macroglobulin with methylamine, chymotrypsin, and subtilisin, it has been shown that eight of these resonances arise from bait region residues. More specifically, assignment has been made of resonances at 6.80 and 7.11 ppm to the ortho and meta protons, respectively, of tyrosine-685 and tentative assignment of a resonance at 7.29 ppm to the aromatic protons of phenylalanine-684. C2 proton resonances from five histidine residues are also visible. Four of these are attributed to residues in the bait region or immediately adjacent to this, at positions 675, 694, 699, and 704. The sharpness of resonances from bait region residues demonstrates the great flexibility of this region of the polypeptide. It is proposed that the flexible region extends from residue 675 to residue 710. These resonances are all affected by proteolytic cleavage in the bait region but are not influenced by the subsequent conformational rearrangement of the whole protein tetramer. The significance of these findings is discussed in relation to the current structural models of α_2-macroglobulin.

Plasma protease inhibitors constitute the third largest functional group of plasma proteins by weight, representing approximately 10% of the total protein (Travis & Salveson, 1983). Of these, α_2-macroglobulin is unique in its mechanism of inhibition in that it appears to trap proteases (Barrett & Starkey, 1973) rather than bind tightly at their active sites. Entrapment excludes large substrates and protease inhibitors while still allowing access of low molecular weight species to the active site of the trapped protease (Haveback et al., 1962; Eddeland & Ohlsson, 1978). This is proposed to arise from a conformational change of the α_2M[1] that has been likened to the closing of the jaws of a spring trap (Barrett & Starkey, 1973). There is both direct and indirect evidence for such a conformational change. Electron micrographs indicate a more compact shape for α_2M–protease complexes than for native α_2M (Schramm & Schramm, 1983) and small-angle X-ray scattering studies show a reduction of about 6 Å in the radius of gyration (Branegard et al., 1982; Österberg & Pap, 1983). On nondenaturing polyacrylamide gels the α_2M–protease complex migrates more rapidly than unreacted α_2M (Barrett et al., 1979; Nelles et al., 1980), has an altered frictional ratio (Björk & Fish, 1982; Dangott & Cunningham, 1982), an increased isoelectric point (Van Leuven et al., 1981), increased fluorescence intensity (Richman & Vepoorte, 1981), and changes in its visible and CD spectra (Dangott et al., 1983).

Barrett and Starkey (1973) first proposed that the trigger for this conformational change was proteolytic cleavage of one or more of the four subunits of α_2M by the protease. There is now extensive support for such cleavages (Harpel, 1973; Hall & Roberts, 1978; Barrett et al., 1979), which occur approximately in the middle of the polypeptide chain within a very restricted segment of about 30 amino acids (Sottrup-Jensen et al., 1981a; Mortensen et al., 1981b). This region has been accorded the title of bait region (Salvesen & Barrett, 1980).

A further consequence of the proteolytic cleavage of the bait region is the appearance of a maximum of one sulfhydryl group per subunit of the α_2M tetramer as a result of the scission of an internal β-cysteinyl-γ-glutamyl thiol ester between Cys-949 and Glx-952 (Sottrup-Jensen et al., 1980, 1983; Howard, 1981; Salvesen et al., 1981). Cleavage of this thiol ester and a conformational change of the human α_2M tetramer can also be produced (in the absence of protease) by reaction with small primary amines such as methylamine (Barrett, 1981; Salvesen et al., 1981; Sottrup-Jensen et al., 1981b; Travis & Salvesen, 1983; Österberg & Malmensten, 1984).

As part of a study aimed at characterizing the structural featues of α_2-macroglobulin and the changes that occur upon reaction with proteases or small nucleophiles such as methylamine, we have recorded the 400-MHz ^1H NMR spectrum of the protease inhibitor and observed the consequences of these perturbations on the resolvable resonances.

Materials and Methods

Purification of α_2M. Human α_2M was purified by zinc chelate chromatography and gel chromatography, as described previously (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 (Davis, 1964). SDS–polyacrylamide gel electrophoresis was carried out in 7.5% slab gels according to Laemmli (1970). α_2M concentrations were determined spectrophotometrically with $E^{1\%}_{280} = 8.9$ (Hall & Roberts, 1978; Barrett et al., 1979) and a molecular weight of 716 000 (Kristensen et al., 1984).

Preparation of NMR Samples. α_2M was transferred from H_2O/buffer to D_2O/buffer by several cycles of dilution with

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[1] Abbreviations: α_2M, α_2-macroglobulin; CPMG, Carr–Purcell–Meiboom–Gill; SDS, sodium dodecyl sulfate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; PAGE, polyacrylamide gel electrophoresis.

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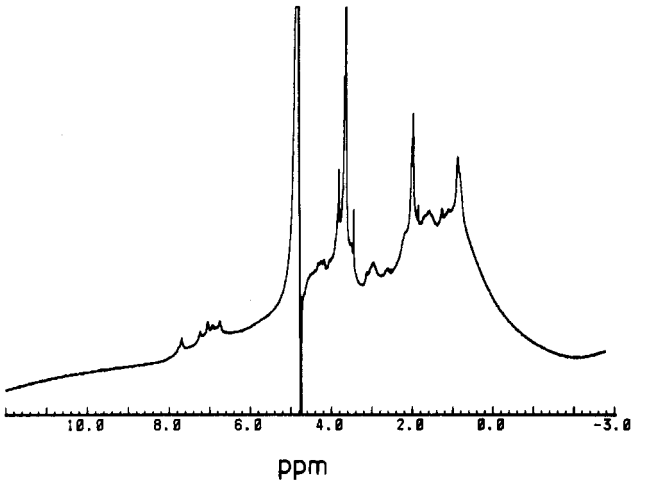

FIGURE 1: 400-MHz ^1H NMR spectrum of 22 μM human α_2-macroglobulin in D_2O containing 0.15 M NaCl, 10 mM Tris, pH 7.5. The HOD resonance and the Tris resonance at 3.5 ppm were reduced in intensity by presaturating pulses. This spectrum represents the average of 4400 scans.

D_2O and concentration in an ultrafiltration cell equipped with a PM30 membrane. Proteases were added from stock solutions made immediately prior to addition to α_2M to limit autodigestion.

NMR Measurements. ^1H NMR spectra were recorded on a 400-MHz Bruker AM400 narrow-bore spectrometer equipped with a 5-mm ^1H probe. The probe temperature was maintained at 298 K. Acquisition of normal spectra used a 70° pulse (9 μs), a sweep width of 6000 Hz, and a data block size of 8K points zero-filled to 16K points prior to Fourier transformation. In most cases it was necessary to reduce the residual HOD resonance and also that from the Tris buffer by irradiation at these frequences prior to the observation pulse. Some spectra were recorded with a Carr–Purcell–Meiboom–Gill pulse train $(90°_X-(\tau-180°_Y-\tau)_n)$ to reduce the intensity of the broad unresolved resonances relative to the sharper resonances in the spectrum (Meiboom & Gill, 1958). For these a τ value of 1 ms and $n = 6$ were usually used. *J*-modulated spectra were recorded with a $90°-\tau-180°-\tau$ pulse sequence and a value of τ of 30 ms. This is not sufficient to give complete inversion of a doublet with $J = 8$ Hz but is a compromise that achieves discrimination between singlets and doublets without too great a loss of signal intensity to permit observation (Campbell & Dobson, 1979). Chemical shifts are referenced relative to DSS at 0 ppm.

99.8 atom % D_2O and CD_3ND_2DCl were obtained from Sigma. Bovine α-chymotrypsin was purchased from Worthington Biochemical Corp. and subtilisin Carlsberg from Sigma.

RESULTS

^1H NMR Spectrum of α_2-Macroglobulin. The complete 400-MHz ^1H NMR spectrum of human α_2-macroglobulin at a concentration of 22 μM tetramer is shown in Figure 1. Despite its large size there are discrete resonances in the region between 6.6 and 8.0 ppm, which must arise from aromatic protons of histidine, tyrosine, phenylalanine, or tryptophan residues. Figure 2 shows only the aromatic region of the spectrum, obtained with a CPMG pulse sequence so that the very broad, unresolved resonances have been eliminated. Nine peaks are distinguishable, at 7.83, 7.77, 7.74, 7.29, 7.11, 6.98, 6.97, 6.92, and 6.80 ppm. Although a consequence of using a CPMG pulse sequence is to diminish resonance areas through T_2 relaxation and therefore to change relative areas of resonances with different T_2 values, the group of three resonances

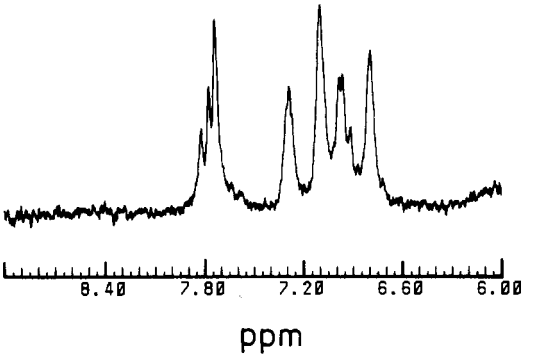

FIGURE 2: Carr–Purcell–Meiboom–Gill ^1H NMR spectrum of 22 μM human α_2-macroglobulin $(90°_X-(\tau-180°_Y-\tau)_n)$ with $n = 6$ and $\tau = 1$ ms. This is the average of 4000 scans. Only the aromatic region is shown.

centered on 7.77 ppm shows the same shape as in the normal spectrum given in Figure 1, reflecting similar relaxation times. If the resonance at 7.83 ppm is given the relative intensity of one proton, that at 7.77 ppm also corresonds to one proton, whereas that at 7.74 ppm represents three, or, less likely, two protons. At the pH used, 7.80, this chemical shift region is that expected for the C2 protons of histidine side chains. Lowering the pH does cause a downfield shift of these resonances (data not shown) while not affecting the resonances at 7.29, 7.11, and 6.80 ppm. This group of three resonances, at 7.83, 7.77, and 7.74 ppm, almost certainly arises from five histidine side chains. The corresponding C4 protons are at 6.98, 6.97, and 6.92 ppm; these are also affected by lowering the pH.

As an independent, though not highly accurate, estimate of the absolute number of protons represented by these aromatic resonances, a normal spectrum of α_2-macroglobulin, to which a known concentration of ammonium formate had been added, was recorded under fully relaxed conditions. The only ^1H resonance from this species that is present in D_2O solution is from the formate proton, which occurs at 8.45 ppm. This is a region of the α_2M spectrum that is devoid of narrow resonances and is reasonably flat, and thus a good estimate of the formate resonance base line and hence peak area could be made. The group of histidine C2 proton resonances occurs on the side of the substantial envelope of unresolved aromatic resonances, and so a true base line for the sharp histidine resonances alone is harder to establish. From the relative peak areas it was calculated that the group of three resonances centered at 7.77 ppm represents 4.2 protons per subunit of α_2M. The uncertainty in this estimation is at least one proton and thus does not distinguish clearly between four or five histidine resonances but does establish semiquantitatively the paucity of resolved aromatic resonances in the ^1H spectrum of α_2M. To estimate the number of protons in the remaining six sharp aromatic peaks, one must use a CPMG spectrum such as is given in Figure 2 to establish any sort of reasonable base line and bear in mind the artificial changes in relative peak areas arising from use of this pulse sequence (vide supra). With a value of five protons for the histidine C2 resonance group, the remaining six resonances correspond to 11.9 protons. This includes an anticipated contribution of five protons from histidine C4 positions. The residue of six protons is likely to be an underestimate due to the broader line width of the resonances at 7.29, 7.11, and 6.80 ppm compared with the histidine C2 protons and could readily be the nine required for one each of phenylalanine and tyrosine residues.

Effect of Reaction with Methylamine. A ^1H spectrum of α_2M was recorded with single-pulse observation. Deuterated

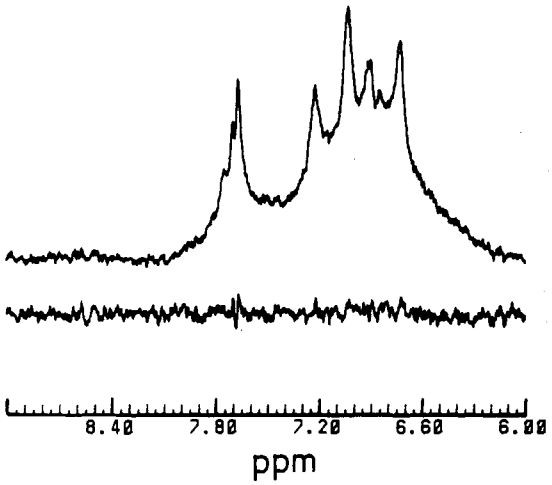

FIGURE 3: Effect of reaction of 22 μM human α_2-macroglobulin, pH 7.5, with 50 mM CD_3ND_2DCl for greater than 2 h. Top: aromatic region of ^{1}H NMR spectrum of α_2-macroglobulin, base line corrected to eliminate the effect of water shoulder and very broad protein resonances. Bottom: difference spectrum between reacted and unreacted α_2-macroglobulin. This was generated by direct subtraction and without any prior manipulation of the individual spectra. Each spectrum was the average of 2000 scans.

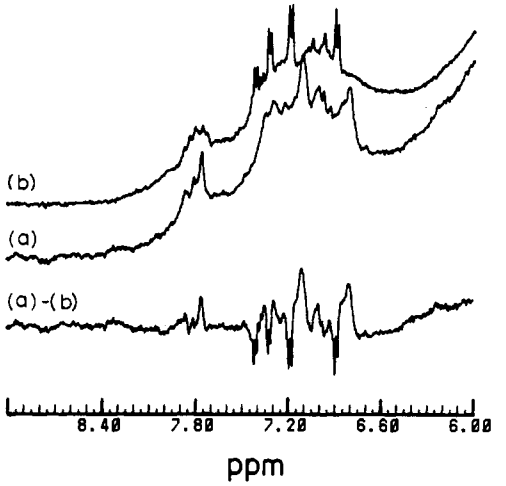

FIGURE 4: Effect of reaction with chymotrypsin on the ^{1}H NMR spectrum of α_2-macroglobulin. (a) Computer-generated sum of aromatic region spectra of 4000 scans each of 15 μM human α_2-macroglobulin and 30 μM bovine chymotrypsin, pH 7.5. (b) Aromatic region ^{1}H NMR spectrum of 15 μM human α_2-macroglobulin, 30 μM bovine chymotrypsin, pH 7.5, reacted together. Bottom trace is difference spectrum (a − b).

methylamine deuteriochloride was then added from 1 M stock solution to give a final concentration of 50 mM, and spectra were recorded every 30 min for 2 h. None of these spectra, though reflecting an average of fewer scans than the spectrum of the unreacted material, showed any discernible difference. After 2 h a spectrum of reacted α_2M was recorded under identical conditions to the initial spectrum. The aromatic region of the initial spectrum and the difference spectrum between reacted and unreacted α_2M are shown in Figure 3. Apart from a very small negative peak at 7.76 ppm, the two spectra are identical. This small perturbation of the histidine residues could easily be accounted for by the pH differences of 0.05 unit between initial and final values. Even in the aliphatic region (not shown), where subtraction of two much larger resonance areas is involved, there are only three peaks in the difference spectrum, one due to residual ^{1}H in the deuteriomethylamine methyl group. Under the conditions of the reaction, complete conformational change should have been accomplished within 90 min (Larsson & Björk, 1984; Larsson et al., 1985). A native polyacrylamide gel run shortly afterward showed that interconversion from slow to fast electrophoretic forms was essentially complete and a ^{1}H spectrum run subsequently again showed no discernible change.

Effect of Chymotrypsin. Chymotrypsin cleaves the bait region of α_2M at a unique site between residues Tyr-685 and Glu-686 (Mortensen et al., 1981) and is trapped by α_2M at a stoichiometry between 1 and 2 mol of chymotrypsin per mole of tetrameric α_2M (Pochon et al., 1978; Pochon & Bieth, 1982; Howell et al., 1983). The aromatic region ^{1}H spectra of a computer summation of 1 equiv of α_2M and 2 equiv of bovine chymotrypsin and of a 1:2 mixture of these proteins are shown in Figure 4, together with their difference. The changes are dramatic. In the histidine C2 proton region three histidines seem to be perturbed and change their chemical shift from 7.74 ppm to 7.72, 7.79, and 7.81 ppm so that all five of the histidine resonances are now resolvable. The three broad resonances at 7.29, 7.11, and 6.80 ppm have all disappeared, while five new resonances have appeared at 6.89, 7.21, 7.32, 7.40, and 7.43 ppm. These resonances all seem to exhibit resolved spin–spin coupling, indicating a very high degree of rotational freedom. To demonstrate that these changes are

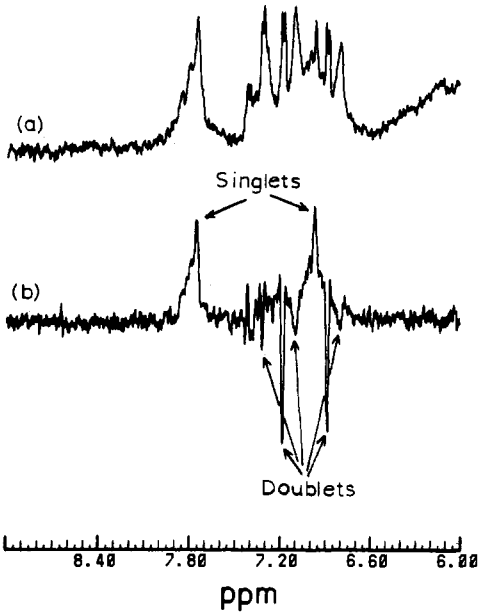

FIGURE 5: Aromatic region ^{1}H NMR spectra of 1:1 chymotrypsin: α_2-macroglobulin. The concentration of complex was 15 μM, pH 7.5. (a) CPMG spectrum with $n = 6$, $\tau = 1$ ms, 4000 scans. (b) Spin-echo spectrum obtained with $90°-\tau-180°-\tau$ pulse sequence and $\tau = 31$ ms. This is the sum of 8000 scans and required 8 h of data averaging.

due to stoichiometric cleavage by chymotrypsin at the bait region site rather than additional catalytic (i.e., not stoichiometric) cleavage at exposed tyrosine positions elsewhere, the reaction was repeated with 1 equiv of chymotrypsin per α_2M tetramer. This resulted in a spectrum composed of half of the original resonances and half of the new resonances. The aromatic region of the CPMG spectrum of this 1:1 complex is shown in Figure 5a. Figure 5b is a spin-echo spectrum of the same sample and clearly shows that the resonances previously ascribed to histidine C2 and C4 protons are the expected singlets and thus remain as positive peaks while the new resonances at 6.89 and 7.21 ppm are clearly inverted and are doublets (Campbell & Dobson, 1979). The new resonance at 7.32 ppm and the old resonances at 6.80 and 7.11 ppm are also inverted and are doublets, though the much lower intensity of their resonances makes this less clear. This lower intensity

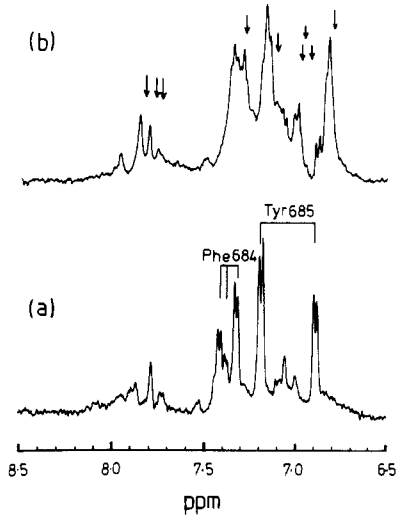

FIGURE 6: CPMG ^1H spectra of α_2-macroglobulin treated with chymotrypsin and subtilisin. (a) Spectrum of sample used for Figure 4b. τ = 1 ms, n = 6, 4000 scans. Resonances assigned to tyrosine-685 and phenylalanine-684 are indicated. (b) Spectrum of 17 μM α_2-macroglobulin reacted with 34 μM subtilisin. τ = 1 ms, n = 6, 11 000 scans. Arrows indicate resolved resonance positions in unreacted α_2M.

reflects shorter T_2 values than for the doublets at 6.89 and 7.21 ppm.

Polyacrylamide gels run under nondenaturing conditions confirmed that the 2:1 sample was entirely in the fast electrophoretic form whereas the 1:1 sample was approximately 50% in fast and 50% in slow forms. SDS–PAGE showed that reaction with chymotrypsin had resulted in the expected fragmentation of the monomer into two chains of molecular weights 85 000 and 95 000.

Spin-decoupling experiments were performed on the 2:1 chymotrypsin:α_2M sample, irradiating the resolved apparent multiplets at 6.89, 7.21, 7.32, 7.40, and 7.43 ppm. The CPMG spectrum of the 2:1 sample is shown in Figure 6a to show these multiplets more clearly than in the 1:1 sample spectrum of Figure 5a. Decoupling demonstrated that the 6.89 and 7.21 ppm doublets are coupled to one another and to no other aromatic protons and are therefore from a tyrosine residue. Their chemical shifts are also close to those of free tyrosine: 6.86 and 7.15 ppm for C3,5 and C2,6, respectively (Bundi & Wüthrich, 1979). The remaining three multiplets probably also arise from a single residue. Irradiation of the doublet at 7.32 ppm causes simplification of the resonance at 7.40 ppm and vice versa. However, the overlap of the 7.40 and 7.43 ppm multiplets makes it difficult to show the effects of decoupling one on the other. The existence of three spin–spin linked aromatic multiplets, one of which is a doublet, with chemical shifts close to 7.34 ppm (the chemical shift of the aromatic protons of phenylalanine (Bundi & Wüthrich, 1979), makes it very likely that they arise from a phenylalanine side chain.

Effect of Subtilisin. The known sites of cleavage in the bait region of α_2M by a number of proteases are shown in Figure 7, which is adapted from Sottrup-Jensen et al. (1984). To demonstrate that cleavage within the bait region, but at some distance from tyrosine-685, caused a perturbation of the same resolved aromatic region ^1H resonances, though in a different manner from chymotrypsin, α_2M was reacted with 2 equiv of subtilisin, and the normal and CPMG spectra were recorded. Subtilisin cleaves in two possible positions, on either side of Leu-697 and thus 11 or 12 residues removed from the site of cleavage by chymotrypsin. The spectrum of subtilisin-treated α_2M and the difference between it and unreacted α_2M are shown in Figure 8. Although the difference spectrum is not identical with that given in Figure 4 for the effects of reaction of α_2M with chymotrypsin, this is not unexpected given the different sites of cleavage. Importantly, the nine resolvable aromatic resonances in unreacted α_2M are again all perturbed and thus the changes in the two difference spectra result from distinct chemical shifts for the same ^1H resonances in the two reacted species. These positions for subtilisin-treated α_2M are more clearly seen in the CPMG spectrum shown in Figure 6b, above a CPMG spectrum of 2:1 chymotrypsin:α_2M for comparison. Also the positions of the nine resolvable α_2M resonances that are visible in unreacted α_2M are indicated by arrows. Four histidine C2 resonances are visible at 7.95, 7.85, 7.81, and 7.76 ppm, that at 7.81 ppm having an estimated line width of less than 8 Hz. The corresponding C4 resonances are probably those at 7.00, 7.02, 7.08, and 7.10 ppm. In addition there are major resonances at 6.83 and 7.18 ppm and a complex of overlapping resonances at 7.30–7.35 ppm. There is also a small doublet at 6.89 ppm. SDS–PAGE confirmed that α_2M had been cleaved in the expected region and nondenaturing PAGE showed that its conformation had changed to render it in the fast electrophoretic form.

Discussion

α_2M has a rotational correlation time of 720 ns (Pochon et al., 1978) and should, therefore, give ^1H NMR line widths of several hundred hertz for protons with no independent

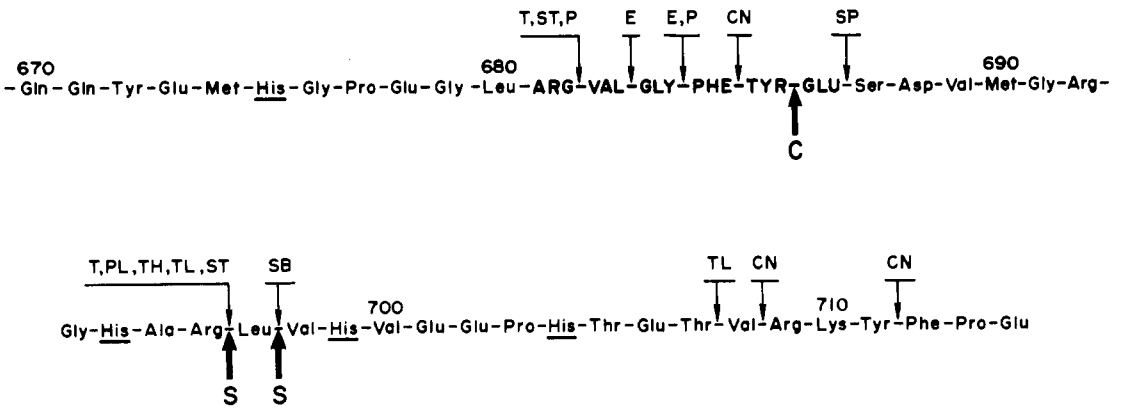

FIGURE 7: Amino acid sequence of human α_2-macroglobulin bait region and adjacent residues, including sites of cleavage by various proteases. This is adapted from Figure 9 of Sottrup-Jensen et al., 1984. The residues in the principal region of cleavage are shown in bold capitals, and the four histidine residues in this region are underlined. Arrows indicate primary and secondary sites of cleavage for specific enzymes. Abbreviations are T, bovine trypsin; ST, *Streptomyces griseus* trypsin; P, papain; E, porcine elastase; CN, calf chymosin; C, bovine chymotrypsin; SP, *Staphylococcus aureus* V8 protease; PL, human plasmin; TH, bovine thrombin; TL, thermolysin; S, subtilisin; SB, *Streptomyces griseus* protease B.

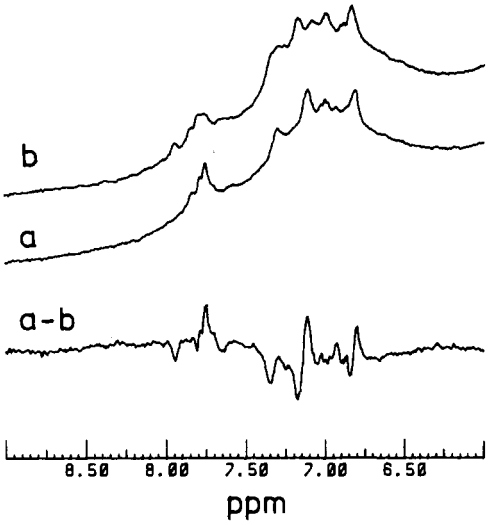

FIGURE 8: Effect of reaction with subtilisin on the ^{1}H NMR spectrum of α_2-macroglobulin. (a) Aromatic region spectrum of 17 μM α_2-macroglobulin at pH 7.5, 4000 scans. (b) Aromatic region of sample a reacted at 2:1 stoichiometry of protease to inhibitor with subtilisin, 4000 scans. The bottom trace is the difference spectrum (a − b).

motional freedom (Abragam, 1961), depending on the proximity of neighboring protons; a value of 600 Hz results for a proton relaxed by one other proton 2.5 Å away. The observation of nine resonances in the aromatic region of the ^{1}H NMR spectrum of α_2M with line widths in the range 15–20 Hz (though these also contain a contribution from spin–spin coupling and the true line width should be less for any of the resonances that are multiplets) requires that they arise from residues that occur in regions with a high degree of independent flexibility and thus faster motion. A correlation time of the order of 10 ns is required to account for the observed line widths, assuming a dipole–dipole relaxation mechanism.

This is not the first documented instance of well-resolved ^{1}H resonances arising from discrete domains or flexible regions of very large proteins and superimposed on an extremely broad background from the remaining resonances. Moreover in many of these cases the regions seem to have functions that require this greater mobility or flexibility. Thus the head piece of the lac-repressor protein (59 out of 360 residues per monomer and tetramer molecular weight of 148 000) contributes all of the resolvable aromatic resonances and also contains the DNA binding site (Wade-Jardetzky et al., 1979). In gene 32 protein from bacteriophage T4 (an oligomer of 33 500 Da subunits) the only sharp resonances have been shown to arise from small flexible N and C terminal domains (Prigodich et al., 1984) to both of which have been attributed specific functions (Williams & Konigsberg, 1981). In myosin, M_r 450000, it has been shown that the sharp ^{1}H NMR resonances arise solely from the C-terminal S1 region, with 22% of this component (150 amino acids) thought to contribute all the signals (Highsmith et al., 1979) and to constitute the actin binding site. Two other examples are the pyruvate dehydrogenase complex and immunoglobulins. The former has an even higher molecular weight than any so far considered ($(4.5–6) \times 10^6$ Da) and yet contains some sharp resonances in its ^{1}H NMR spectrum (Perham et al., 1981). They also arise entirely from a distinct functional region; the portion of polypeptide carrying the lipoic acid residues. Immunoglobulins possess a flexible hinge region between the Fab and Fc portions of the molecule that may serve a role in complement binding to the Fc subsequent to antigen binding to the Fab. This short region contributes sharp histidine resonances that have been characterized (Arata et al., 1980) as well as identifiable threonine, lysine, leucine, and glutamate resonances (Endo & Arata, 1985).

Localization of Resolved ^{1}H Resonances. From which residues do the sharp resonances in human α_2M arise and are they in any way structurally related? Although there is no reason a priori why all of these mobile residues occur in the same region, the demonstration that the two proteases, chymotrypsin and subtilisin, affect all of the resolved aromatic resonances, with a possible exception of one or two of the five C2 histidine resonances, requires that they are influenced by the well-defined changes brought about by these proteases. Both cleave in the bait region (see Figure 7) and cause a conformational change of α_2M that results in trapping of the protease. For the 1:1 and 2:1 chymotrypsin-treated α_2M and the subtilisin-treated α_2M samples used in the NMR studies we have confirmed by gel electrophoresis that only the expected cleavages of the α_2M polypeptide and the subsequent conformational change did take place. Furthermore the stoichiometric changes caused by 1:1 and 2:1 additions of chymotrypsin reinforce the conclusion that the ^{1}H NMR changes are a direct consequence of one or both of these bait-region cleavage and conformational change events, rather than an unassociated cleavage elsewhere.

Reaction of α_2M with methylamine results in the same conformational change as is produced by reaction with proteases (Barnett et al., 1979; Björk & Fish, 1982; Gonias et al., 1982; Dangott et al., 1983). However, Figure 3 shows that this conformational change causes no perturbation of the resolvable ^{1}H resonances of α_2M. It can, therefore, be concluded that these aromatic resonances at 7.83, 7.77, 7.74, 7.29, 7.11, 6.98, 6.97, 6.92, and 6.80 ppm (Figure 2) arise from aromatic residues in the bait region of α_2M.

Assignment of Aromatic Resonances. In chymotrypsin-treated α_2M the resonances that were at positions 6.80, 6.92, 6.98, 7.11, and 7.29 ppm prior to reaction have disappeared and been replaced by even narrower resonances at 6.89, 6.99, 7.05, 7.21, 7.32, 7.40, and 7.43 ppm (see Figure 6a). These have small enough line widths that spin–spin coupling can be resolved in five of these. The resonances at 6.89 and 7.21 ppm constitute the four protons of a tyrosine residue, as shown by spin decoupling, while the three resonances at 7.32, 7.40, and 7.43 ppm probably arise from a single phenylalanine residue, based on chemical shift and spin decoupling of the resonance at 7.32 ppm. Chymotrypsin cleaves α_2M at a unique site, between tyrosine-685 and glutamate-686 such that the C-terminal sequence of the N-terminal half-chain is Gly-Phe-Tyr-CO_2^-. The only other tyrosine residues in or close to the bait region are 14 and 26 residues removed from this cleavage site at positions 672 and 711, respectively. Given the dramatic increase in resolution achieved upon cleavage of the polypeptide adjacent to tyrosine-685, which is to be expected for an already mobile interior residue that becomes a terminal residue and therefore even less constrained, it is almost certain that the two doublets at 6.89 and 7.21 ppm arise from tyrosine-685 and the three multiplets at 7.32, 7.40, and 7.43 ppm arise from the adjacent phenylalanine residue at position 684 (only one other phenylalanine occurs in this region, at position 712, i.e., well removed from the site of cleavage). The resonances at 6.99 and 7.05 ppm are histidine C4 protons and therefore singlets.

Without resolvable spin–spin coupling in unreacted α_2M it is not possible to make assignments with the same confidence as in the chymotrypsin-treated species. Also the CPMG spectra distort relative intensities according to T_2 values. Despite this, a reasonable interpretation of the spectrum shown

in Figure 2 is that the resonances at 6.80 and 7.11 ppm arise from tyrosine-685 (both were inverted in the spin-echo spectrum of 1:1 chymotrypsin:α_2M shown in Figure 5b and are therefore doublets; their low intensity reflects their broader line width, i.e., shorter T_2, and consequent loss of intensity relative to the sharper resonances). The resonance at 7.29 ppm is intense enough, given its broadness, to arise from all five of the aromatic protons of phenylalanine-684. The remaining two groups of three resonances centered on 6.95 and 7.75 ppm and exhibiting the behavior expected of singlets in a spin-echo spectrum (Figure 5b) are undoubtedly histidine C4 and C2 protons, respectively.

The CPMG spectrum of α_2M treated with subtilisin (Figure 6b), while showing perturbation of these same bait region resonances, is different in three main respects from that of chymotrypsin-treated α_2M. First, the chemical shifts of these tyrosine, phenylalanine, and histidine residues are different. Second, narrowing of the phenylalanine and tyrosine line widths seems to be less pronounced. Third, there seems to be greater complexity to the spectrum, in that more resonances are apparently represented. The first difference is indeed to be expected from the different reported sites of cleavage for chymotrypsin and subtilisin (see Figure 7), such that in the latter case tyrosine-685 is 11 or 12 residues remove from the sites of cleavage. The relationship of histidines-694 and -699 relative to the subtilisin cleavage sites (1, 2, or 3 residues removed) is also quite different from the chymotrypsin cleavage site (9 or 14 residues removed). These differences could also explain the second point, in that the increased freedom of movement of tyrosine-685 and phenylalanine-684 would be less upon subtilisin treatment than upon treatment with chymotrypsin. The reverse situation should hold with regard to histidines-694 and -699, and in fact there are three very sharp C2 histidine resonances in Figure 6b vs. only one in Figure 6a. It is tempting, on this basis, to assign the resonances at 7.85 and 7.95 ppm in Figure 6b to histidines (-694, -699), though this does not shed light on their assignments in unreacted α_2M. The third point may well be due to heterogeneity in the sites of cleavage. Subtilisin has not been reported to cleave in the primary cleavage region between residues 681 and 686 but rather between residues 696 and 697 and between 697 and 698 (Mortensen et al., 1981b). This heterogeneity would not be expected to cause much difference to tyrosine-685 or phenylalanine-684. However, given the observed cleavage between arginine-696 and leucine-697, it is expected that cleavage should also occur, though possibly to a lesser extent, between arginine-681 and valine-682 in the primary cleavage region. Cleavage here might thus readily account for the small, but obvious, doublet at 6.89 ppm, which in chymotrypsin-treated α_2M has been assigned to tyrosine-685. Its partner at 7.21 ppm, if present, is obscured by a larger overlapping resonance.

Principally on the basis of the assignments from chymotryptic cleavage, the assignments of major resonances in the CPMG spectrum shown in Figure 6b for subtilisin-cleaved α_2M are of the peaks at 6.83 and 7.18 ppm to tyrosine-685 and the group of resonances between 7.30 and 7.35 ppm to phenylalanine-684. The asymmetry of the resonances at 6.83 and 7.18 ppm compared with their proposed equivalent resonances in Figure 6a, at 6.89 and 7.21 ppm, respectively, is probably attributable to the heterogeneity of products arising from cleavage on either side of leucine-697. Although this does not cause enough of a difference in environment for tyrosine-685 in the two products to give resolvable resonances for each species, it does account for the distinct shoulders in each resonance from overlap of the two peaks. The smaller change in chemical shift for the tyrosine ortho and meta protons upon cleavage of the bait region by subtilisin (0.03 and 0.06 ppm, respectively) compared with cleavage by chymotrypsin (0.09 and 0.10 ppm, respectively) is also consistent with the greater separation between tyrosine-685 and the cleavage site in the former case and presumably smaller perturbation, since in the latter case Tyr-685 and Phe-684 becomes terminal and penultimate residues, respectively. Similarly the group of phenylalanine resonances at 7.29 ppm in unreacted α_2M moves only between 0.01 and 0.06 ppm upon subtilisin treatment but between 0.03 and 0.14 ppm upon reaction with chymotrypsin.

Structural Consequences for the Bait Region. At a minimum, residues 683–700 in α_2M must constitute a highly flexible surface loop. This segment includes tyrosine-685, phenylalanine-684 and the two histidine residues, -694 and -699, that become even more mobile upon cleavage in their vicinity. It is likely, however, that this flexible exposed region extends further than this. In addition to positions 694 and 699, histidine residues occur at positions 675 and 704, in adjacent segments of the polypeptide, with the next closest in sequence being at position 623 on the N-terminal side and position 823 on the C-terminal side (Sottrup-Jensen et al., 1984). Since four of the (5?) resolved histidine C2 resonances are perturbed by chymotrypsin or subtilisin cleavage, histidines-675 and -704 may be better limits for the flexible loop. Further toward the carboxy terminus, a tyrosine–phenylalanine pair occurs at residues 711 and 712, but does not seem to contribute sharp resonances to the proton spectrum, and this position is in fact the furthest point of protease cleavage reported (Sottrup-Jensen et al., 1984). Extending the region five residues to the N-terminal side of residue 675 includes the two positions of transglutamination (Mosher, 1976; Mortensen et al., 1981a; Van Leuven et al., 1981), which presumably must at least be surface residues, but also includes an additional, ^{1}H NMR-invisible and therefore not independently mobile, tyrosine at position 672. We conclude, therefore, that residues 674 to 710 or 711 constitute an exposed flexible region of α_2M that contains all of the major sites of protease cleavage, through inclusion of a wide range of dipeptide sequences. Out of these 38 residues, 16 are charged and the hydropathic index (Kyte & Doolittle, 1982) of this segment is −0.5, consistent with its being exposed. Secondary structure predictions indicate possible β-structure from residues 679 to 686 and from residues 695 to 701 (Welinder et al., 1984).

With regard to the symmetry of the α_2M tetramer, it should be noted that the simplicity of the aromatic region of the CPMG spectrum both before and after reaction with chymotrypsin requires that the bait region environments of all four subunits are indentical. There may still be an asymmetric arrangement of the remainder of the polypeptide chain with respect to that of the other monomer within a given half-molecule, but this must be such as not to influence the bait region. This region is, however, not overly sensitive to the overall gross conformation of α_2M or to the existence of the internal thiol ester, as demonstrated by the absence of intensity in the difference spectrum upon reaction with methylamine (Figure 3).

Significance of Assignments. The importance of being able to see resonances from residues in the bait region and furthermore to assign some to specific residues lies with our broader goals of determining the spatial relationships of the bait region, the thiol ester site, possible protease binding sites, and the zinc binding sites in α_2M both before and after con-

formational changes have occurred. This is with the aim of both explaining the mechanism of trapping and perhaps shedding light on the true role in vivo of α_2M with regard to specific protease inhibition. We have demonstrated that α_2M has a unique pair of zinc sites that can also be substituted with the paramagnetic metal ion manganese (Gettins and Cunningham, 1986). With the identification of ^{1}H resonances from the bait region, we now have a means of measuring distances from specific residues to these unique metal sites by way of paramagnetic broadening effects. These studies are currently under way.

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