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Proximity of Thiol Esters and Bait Region in Human α_2-Macroglobulin: Paramagnetic Mapping†

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ABSTRACT: The two key structural features of α_2-macroglobulin (α_2M) involved in inhibitory caging of proteases are the thiol ester and the bait region. This paper examines the environment of the hydrolyzed thiol ester in methylamine-treated human α_2M and the separation between the bait region and the thiol ester and between the four thiol esters in the tetramer to try to further our understanding of how bait region proteolysis triggers thiol ester cleavage. The sulfhydryl groups of Cys-949, formed upon cleavage of the thiol ester by methylamine, were specifically labeled with the nitroxide spin-labels 3-(2-iodoacetamido)-PROXYL (iodo-I) (PROXYL = 2,2,5,5-tetramethylpyrrolidine-1-oxyl), 3-[2-(2-iodoacetamido)acetamido]-PROXYL (iodo-II), and 4-(2-iodoacetamido)-2,2,6,6-tetramethylpiperidine-1-oxyl (iodo-III). ESR spectra of these α_2M derivatives showed that label I is firmly held and label II has limited freedom of rotation consistent with location of the cysteine residue in a narrow cavity. Label III has much greater motional freedom. From the absence of dipole–dipole splittings in the ESR spectra, it is concluded that the four nitroxide groups in the tetramer are more than 20 Å apart for both label I and label II. Label I broadens ^{1}H NMR signals from one phenylalanyl, one tyrosyl, and four histidyl residues in the bait region. Separations of 11–17 Å are estimated between the nitroxide of label I and these residues. Label II is further away and only broadens resonances from one of the histidines. The bait region is thus shown to be quite close to the thiol ester in the methylamine-treated form of α_2M and in addition must have a compact structure. It is suggested that the bait region exists as a loop of two strands in β conformation in the native structure (as well as in the methylamine-modified form of α_2M) and that proteolytic cleavage disrupts the loop and triggers thiol ester cleavage.

Human α_2-macroglobulin (α_2M)[1] is a tetrameric protease inhibitor with a number of unusual features. Rather than binding to the active sites of proteases and thereby inhibiting them, α_2M traps enzymes in a cagelike manner (Barrett & Starkey, 1973; Crews et al., 1987) and thus restricts access to high molecular weight substrates and inhibitors. The trapping or caging occurs as a result of a large-scale conformational change (Branegard et al., 1982; Björk & Fish, 1982; Dangott & Cunningham, 1982; Dangott et al., 1983), which is triggered by proteolytic cleavage of one or more of the four subunits of α_2M by the protease. These cleavages occur in the middle of the polypeptide chain, within a restricted segment of at most 30 amino acid residues (Sottrup-Jensen et al., 1981; Mortensen et al., 1981) termed the bait region (Salvesen & Barrett, 1980). Different proteases cleave at different sites within the bait region, at positions consistent with their specificity (Sottrup-Jensen et al., 1984). A consequence of proteolytic cleavage is the scission of the internal β-cysteinyl-γ-glutamyl thiol ester between Cys-949 and Glx-952, 260 residues removed from the bait region (Sottrup-Jensen et al., 1984), and a subsequent conformational change that results in trapping of the protease (Sottrup-Jensen et al., 1980; Howard, 1981; Salvesen et al., 1981). Since a similar, if not identical, conformational change can be brought about by thiol

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[1] Abbreviations: α_2M, α_2-macroglobulin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); PROXYL, 2,2,5,5-tetramethylpyrrolidine-1-oxyl; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; CPMG, Carr–Purcell–Meiboom–Gill; SDS, sodium dodecyl sulfate; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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ester cleavage alone [using a potent nucleophile such as methylamine (Barrett, 1981; Salvesen et al., 1981; Sottrup-Jensen et al., 1981)] and since the thiol ester cleavage precedes the conformational change (Strickland & Bhattacharya, 1984) in the methylamine reaction, it seems likely that thiol ester opening is the event in the reaction with proteases necessary to precipitate the conformational change of α_2M. One explanation for the connection between proteolytic cleavage of the bait region and thiol ester hydrolysis might be a structural linkage of the bait region and thiol ester. Cleavage of the bait region at any point (determined by the reacting protease) might then result in a similar increase in accessibility of solvent to the inherently reactive thiol ester, through a localized conformational change.

This study is aimed at determining the proximity of the thiol ester and bait region to one another and the separation of thiol esters in different subunits in the conformationally altered form of α_2M. The effects of nitroxide spin-labels, specifically attached to Cys-949, on ^{1}H NMR signals from bait region residues (Gettins & Cunningham, 1986; Arakawa et al., 1986) and on their own ESR signals are used to estimate distances between thiol esters and between thiol ester and bait region. It is shown that the ^{1}H resonances from several bait region residues are substantially broadened by a short spin-label attached to Cys-949 and that these bait region residues must be between 11 and 17 Å from the nitroxide. The broadening of resonances from several residues well separated in the amino acid sequence of the bait region supports a compact loop structure for the bait region. A longer spin-label broadens resonances from only one bait region residue. The bait region and thiol ester in modified α_2M are shown by these studies to be close together. The experiments presented here do not address the proximity of these groups in native α_2M, though they may also be close together. In contrast, no indication of electron–electron dipole–dipole interaction is evident in the ESR spectra, indicating that the nitroxides attached to the four thiol esters are probably a minimum of 20 Å apart.

Materials and Methods

Purification of α_2M. Human α_2M was purified from human plasma by zinc chelate and Cibachrome blue affinity chromatography, as described previously (Dangott & Cunningham, 1982). The purity of each preparation was checked by polyacrylamide gel electrophoresis both under nondenaturing conditions, using 5% acrylamide slabs (Davis, 1964), and also in the presence of SDS, in 7.5% slab gels, according to the procedure of Laemmli (1970). α_2M concentrations were determined spectrophotometrically by using $A^{1\%}_{280} = 8.9$ (Hall & Roberts, 1978; Barrett et al., 1979) and a molecular weight of 716 000 based on the primary structure (Sottrup-Jensen et al., 1984).

Preparation of α_2M Derivatives. Spin-labeled and iodoacetamide-labeled α_2M species were prepared by the same procedure. α_2M, in 0.1 M sodium phosphate at pH 8.0, was reacted with 200 mM methylamine for 1 h at room temperature. This results in cleavage of the internal thiol ester groups and generation of one free sulfhydryl per cleaved thiol ester (Sottrup-Jensen et al., 1980). Unreacted methylamine was quickly removed from the sample by ultrafiltration in an Amicon ultrafiltration unit equipped with an XM-100A membrane: This reduced the methylamine concentration to approximately 0.5 mM. The free sulfhydryl groups were then labeled with iodoacetamide or a spin-labeled iodoacetamide derivative by use of a 20-fold molar excess of reagent. The reaction was carried out at 4 °C for 3 h. The sulfhydryl-labeled α_2M was dialyzed against 1 L of 0.1 M sodium phosphate buffer, pH 7.0, with three changes of dialysate. Free SH concentrations were determined colorimetrically by using DTNB after initial reaction of α_2M with methylamine and during the subsequent reaction with iodoacetamide or iodoacetamide-linked spin-labels. Typically, 4 ± 0.2 SH groups were generated per α_2M tetramer upon reaction with methylamine, and complete labeling with iodoacetamide or iodoacetamido spin-labels was achieved.

ESR Measurements. Spectra were recorded on a Varian E-line ESR spectrometer operating at 9.41 GHz. The parameters employed were field set of 3350 G, sweep range of 100 G, modulation frequency of 100 kHz, modulation amplitude of 1 G, time constant of 0.032 s, microwave power of 10 mW, and scan time of 12 min. Samples were contained in a quartz flat cell of 370-μL volume (Wilmad, WG-813). The temperature of the E-238 cavity was regulated to within ±0.5 K by an E-257 VT unit by passing precooled nitrogen into the cavity through the optical port in the front. Signal averaging by an online PDP 11/73 microcomputer was necessary to achieve an acceptable signal to noise ratio. The number of scans is indicated in the figure captions.

NMR Measurements. ^{1}H NMR spectra were recorded on a 400-MHz Bruker AM400 narrow bore spectrometer equipped with a 5-mm ^{1}H probe. The probe temperature was maintained at 298 K. To remove very broad resonances arising from the bulk of the α_2M protons not associated with the bait region residues, spectra were recorded with a Carr–Purcell–Meiboom–Gill pulse train $[90°_x-(\tau-180°_y-\tau)n]$ (Meiboom & Gill, 1958). Values of $n = 6$ and $\tau = 1$ ms were used. Chemical shifts are referenced relative to external DSS at 0 ppm.

Samples for NMR spectroscopy were transferred from H_2O to D_2O buffer by five or six cycles of dilution with D_2O buffer and concentration in an Amicon ultrafiltration cell. Residual ^{1}H intensity from the HDO resonance was reduced further by a selective, low-power, saturating pulse of 0.5-s duration prior to data acquisition. Paramagnetic spin-labeled α_2M species were made diamagnetic by reduction with 3 mM sodium ascorbate. The reaction was allowed to proceed for 4–6 h at room temperature. Excess ascorbate was removed by dilution/reconcentration in an ultrafiltration cell. A nitrogen atmosphere was maintained above the samples to prevent oxidation back to the paramagnetic nitroxide.

Materials. 4-(2-Iodoacetamido)-TEMPO, 3-(2-iodoacetamido)-PROXYL, and 3-[2-(2-iodoacetamido)acetamido]-PROXYL were purchased from Aldrich Chemical Co. D_2O (99.8 atom %) and methylamine hydrochloride were obtained from Sigma Chemical Co.

Results

Mobility of Nitroxide Labels Attached to Cys-949 of α_2M. α_2M species labeled at the sulfhydryl moiety of Cys-949 with three different nitroxide spin-labels (Figure 1) were examined by ESR spectroscopy. The spectra, recorded at 275 K, are shown in Figure 2. The line shape and separation between high- and low-field lines for the acetamido-PROXYL spin-labeled α_2M, 69 G, indicate a rigid glass limit spectrum (Figure 2A), implying a complete immobilization of the nitroxide relative to α_2M. The rotational correlation time of α_2M has been calculated to be 720 ns by fluorescence anisotropy depolarization measurements (Pochon et al., 1978). α_2M labeled with the longer PROXYL spin-label gives a spectrum with a smaller high- to low-field line separation, 65 G, indicating some independent motion of the spin-label relative to the protein (Figure 2B). Motion is, however, still considerably restricted since the peak separation corresponds to an apparent

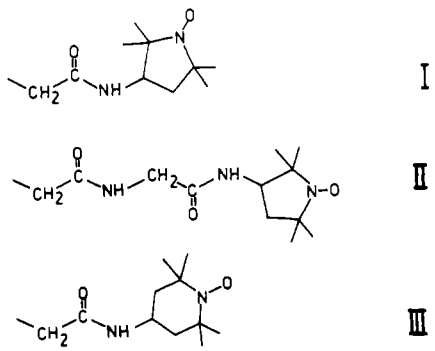

FIGURE 1: Spin-labels used in this study.

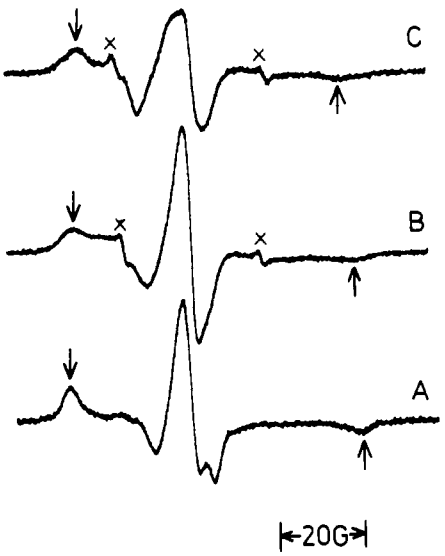

FIGURE 2: 9.4-GHz ESR spectra of methylamine-treated human α_2M spin-labeled at the sulfhydryl groups of Cys-949 at a stoichiometry of 4 ● 0.2 per tetramer of α_2M. Arrows indicate the positions of the high- and low-field peaks. The sharp peaks marked with an X are very minor contamination with mobile nonspecific label or free spin-label (<1%). (A) Spin-label I; 10.9 μM α_2M in 0.1 M sodium phosphate, pH 7.0. Four scans. (B) Spin-label II; 11.6 μM α_2M in 0.1 M sodium phosphate, pH 7.0. Four scans. (C) Spin-label III; 11.3 μM α_2M in 0.1 M sodium phosphate, pH 7.0. Four scans. All spectra were recorded at 275 K.

correlation time of approximately 40 ns by using the analysis of McCalley et al. (1972). In contrast to the immobile short PROXYL spin-label, the TEMPO spin-label, connected via the same acetamide linkage to the sulfhydryl, has a high-field to low-field peak separation of only 60.6 G (Figure 2C). This compares to a rigid glass limit value of 70 G for the same spin-label (Crews et al., 1987) and indicates an effective correlation time of only 9 ns.

Separation of Nitroxide Spin-Labels within the Tetramers. Dipole–dipole interaction between nearby nitroxides can cause splitting of the ESR transitions (Carrington & Maclaughlin, 1967; Beth et al., 1984). With the protonated ^{14}N spin-labels used here, the line widths may be too broad to resolve the fine structure present. Nevertheless, evidence for such dipolar interaction can be gauged from the half-height line width of the high- and low-field peaks of the spectrum. Line widths of 4.4 ± 0.2 G are found for spin-label I, which are similar to those for isolated spin-label under rigid glass limit conditions. The splitting, or apparent broadening for unresolved lines, depends upon the orientation of the two nitroxide coordinate axis systems relative to one another. By use of the program MENO developed by Eaton et al. (1983), spectra were simulated for different nitroxide orientations and separations. For both labels oriented at the magic angle (54° 44′) relative to the

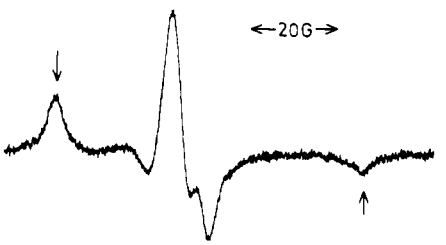

FIGURE 3: 9.4-GHz ESR spectrum of 9.3 μM methylamine-treated human α_2M spin-labeled at the sulfhydryl groups of Cys-949 with spin-label II. The spectrum was recorded at 265 K in 50% w/w glycerol and 0.05 M sodium phosphate, pH 7.0.

interelectron axis, which results in minimum sensitivity to dipolar interaction in the extreme regions of the spectrum, it was estimated that an approach of approximately 16 Å or less would be readily detectable in broadening of the lines. For the N–O axes oriented orthogonal to, and with the nitrogen P_z orbitals oriented along, the interelectron axis (a geometry that results in maximum dipolar interaction in the extreme regions of the spectrum), an approach of 24 Å or less would be readily detectable since a separation of 25 Å would result in half-height line widths of 5.3 G. In the absence of a knowledge of the relative orientation of the nitroxides to one another, an average value of the above maximum distances of closest approach consistent with the experimental absence of broadening will be taken as a close approximation to the true value of the minimum separation. This average value is 20 Å.

To permit the same type of analysis to be applied to the longer spin-label II, it was necessary to slow its motion from 40 ns to a value closer to that necessary to give a rigid limit spectrum. This was done by changing the solvent to 50% w/w glycerol and recording the spectrum at 265 K. The ESR spectrum is shown in Figure 3 and corresponds to rigid glass limit conditions. As with spin-label I, the high- and low-field peak line widths are approximately 4 G and thus show no evidence for dipole–dipole interaction. The nitroxides are therefore also greater than 20 Å apart in this derivative.

Perturbation of Bait Region ^{1}H NMR Signals. It has previously been shown that six aromatic residues within the bait region, the short stretch of polypeptide that contains the sites of proteolytic cleavage, give rise to resolvable, narrow ^{1}H NMR signals (Gettins & Cunningham, 1986; Arakawa et al., 1986). These residues are phenylalanine 684, tyrosine 685, and histidines 675, 694, 699, and 704, although the assignment to specific histidines within the group has not been made. The resonances from these residues afford a ready means of estimating the separation between the nitroxide-labeled SH from the cleaved thiol ester and the bait region by determining if there is any broadening of the resonances by the paramagnetic nitroxide spin-label. Such broadening is strongly distance dependent, falling off as the inverse sixth power of the separation. Figure 4A shows the aromatic region of the CPMG ^{1}H NMR spectrum of α_2M labeled with iodoacetamide. C(2) and C(4) resonances from the four bait region histidines, and one other histidine, and from Phe-684 and Tyr-684 are clearly seen [there is some overlap of C(2) ^{1}H resonances at this pH and the C(4) resonances are never completely resolved from one another]. Figure 4B shows a comparable spectrum of α_2M labeled with the paramagnetic 3-(2-iodoacetamido)-PROXYL. With the exception of the C(2) and C(4) proton resonances of one histidine, all the resonances are broadened. The extent of broadening differs for each resonance and may be approximately assessed from a difference spectrum between the spectra of iodoacetamide and nitroxide-labeled α_2M samples.

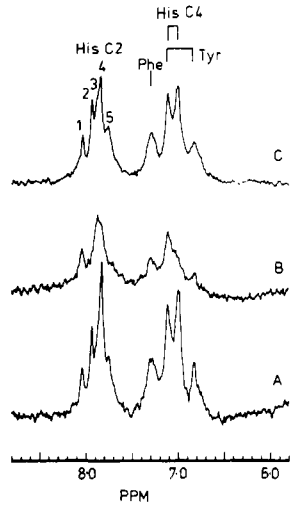

FIGURE 4: Effect of nitroxide spin-label I on the 400-MHz ^{1}H NMR spectra of methylamine-treated human α_2M, recorded by using a CPMG pulse sequence, showing the aromatic region resonances from the bait region. The histidine C2 resonances are numbered 1–5 for ease of reference. Spectra were recorded at 298 K. (A) Control spectrum of α_2M labeled at SH with iodoacetamide. 18 000 scans, 23.0 μM α_2M. (B) Spectrum of 20 μM α_2M labeled with I. 18 000 scans. (C) Sample B treated with 3 mM ascorbate. 18 000 scans.

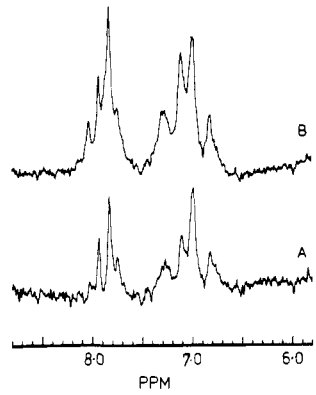

FIGURE 5: Difference spectrum between spectra of paramagnetic and diamagnetic α_2M samples, shown in parts A and B of Figure 4. (A) Difference spectrum. (B) Same as Figure 4a for comparison.

This difference is shown in Figure 5.

Since the pulse sequence used to obtain these spectra emphasizes narrow resonances over broad ones, it is not possible to use reduction in peak height as an accurate indicator of increase in line width. Nevertheless, the comparatively small delay between the initial 90° pulse and data sampling (12 ms) makes change in peak height a rough approximation to change in line width for these narrow resonances, which should not be in error by more than 50%. Such an error in the line width would result in only a 7% error in r because of the r^{-6} relationship between the two. The estimated line widths, changes in line width, and separations are given in Table I. In calculating values for r, the Solomon–Bloembergen equation was used (Solomon, 1955; Bloembergen, 1957), with the assumption that the second, contact, term is unimportant; this assumption is almost certainly valid here given the values found for r. The only other unknown is the correlation time for the electron spin-nuclear spin vector. As discussed above, the rotational correlation time appropriate to α_2M and thus to a nitroxide group held rigidly by α_2M is 7.2×10^{-7} s. The electron spin relaxation time is even longer ($\sim$12 μs) (Fajer et al., 1986). The bait region residues, however, possess greater freedom of movement than the bulk of α_2M. It can be estimated that a rotational correlation time of 10 ns is needed to account for the narrow ^{1}H NMR signals observed from these residues (Gettins & Cunningham, 1986). This correlation time will thus dominate the electron spin-nuclear spin vector correlation time and is used in estimating the values of r given in Table I.

Table I: Broadening of ^{1}H Resonances of Bait Region Residues by Nitroxide Label I

resonance	line width (Hz)[a]	Δ peak height[b]	Δ line width (Hz)[c]	r[d] (Å)
His-1 C(2)	16	0.30	7	17
His-2 C(2)	16	0.62	26	14
His-3 C(2)	18	0	0	>23
His-4 C(2)	18	0.62	29	13
His-5 C(2)	26	0.62	42	12
Phe	60	0.48	55	12
Tyr-m[e]				
Tyr-o	40	0.56	50	12

[a] Half-height line width in diamagnetic control CPMG spectrum. The error is estimated to be ±2 Hz. [b] Fractional reduction in peak height in CPMG spectrum of α_2M labeled with I. [c] Increase in line width in paramagnetic spectrum estimated from reduction in peak height and subject to an error of ≤50%. [d] Separation between nitroxide and proton calculated from Solomon–Bloembergen equation. The error is estimated to be no more than 7%. [e] Resonance overlaps with histidine C(4) proton resonances.

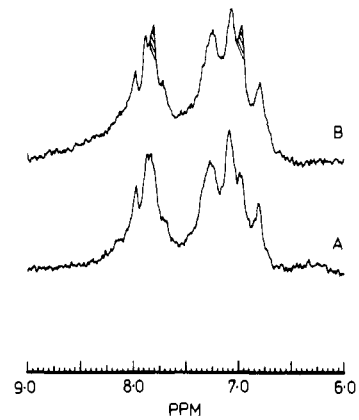

FIGURE 6: Effect of spin-label II on the 400-MHz ^{1}H NMR CPMG spectrum of α_2M. Each spectrum is 40 000 scans and was recorded at 298 K. The resonances from histidine 4 that are broadened are shaded. (A) α_2M, 20.2 μM, labeled with II. (B) Sample A treated with 3 mM ascorbate.

To confirm that the perturbation of the ^{1}H resonances is due only to the paramagnetic broadening by the nitroxide, the nitroxide was reduced with 3 mM ascorbate and the ^{1}H NMR spectrum recorded. This is shown in Figure 4C. The spectrum obtained shows almost complete recovery of intensity as judged by comparison with the spectrum of iodoacetamide-labeled α_2M (Figure 4A). There are small differences between parts A and C of Figure 4, notably in a smaller relative peak height for His-4 C(2) and the tyrosine resonance at 6.8 ppm. These differences are most likely due to incomplete reduction of the spin-label. Experimental limitations precluded simultaneous ESR and NMR observations on the sample to prove this unambiguously.

In contrast to the pronounced effect that spin-label I has on the ^{1}H NMR spectrum of the bait region, the longer spin-label affects only one of the histidine residues. The ^{1}H spectrum of α_2M labeled with this nitroxide is shown in Figure 6A and that of the same sample treated with ascorbate is shown in Figure 6B. Only the C(2) and C(4) proton resonances of histidine 4 are broadened.

The effects of label III on the ^{1}H NMR spectrum of α_2M were not examined, since this label appears to be the most

mobile of the three and may have a poorly defined position.

Discussion

Four main conclusions can be drawn from the results presented in this paper. They concern (1) the environment of the cysteine residue formed upon hydrolysis of the thiol ester linkage, (2) the separation between the four such cysteines formed in the α_2M tetramer, (3) the proximity of the bait region and thiol ester in methylamine-treated α_2M, and (4) the structure of the bait region.

Environment of Cys-949. Spin-label I attached to Cys-949 of α_2M is held firmly by the protein, as judged by the appearance of its ESR spectrum (Figure 2), even though there are three bonds about which free rotation might occur, not including the methylene group of the cysteine. This suggests that the free cysteine occurs at the bottom of a cavity deep enough to hold the pyrrolidyl moiety (~9 Å). Extension of the spacer group by 4 Å, as in label II, places the nitroxide ring in a less restricted environment and results in greater motional freedom for this terminal moiety, though its motion is still somewhat restricted, as shown by its correlation time of 40 ns. Another explanation for the rigidity of I is a specific interaction on a more exposed surface. This is considered less likely since there is such a large difference in mobility between the chemically nearly identical labels I and III.

Location of the thiol group at the bottom of a narrow cavity, as suggested here, is consistent with recent fluorescence studies (Larsson et al., 1987), which concluded that an *N*-[(acetylamino)ethyl]-8-aminonaphthalene-1-sulfonic acid group attached to Cys-949 in methylamine-treated human α_2M is partially buried, though still accessible to solvent.

Somewhat surprising is the difference in mobilities between labels I and III attached to α_2M. These are very similar in length and chemical composition, and yet III is moderately mobile while I is rigid. This may reflect subtle differences in allowed conformation for the 5- and 6-membered spin-labels resulting from interactions with the walls of the cavity. It should be pointed out that labeling reagent I was racemic, though no indication of two distinct bound species was seen in the ESR spectrum. Thus, it is not known whether there is preferential reaction of α_2M with one stereoisomer or whether both stereoisomers react and are held equally firmly.

Separation of the Four Thiol Groups. The absence of any indication of dipole–dipole interaction between spin-labels in the same α_2M tetramer labeled with four such paramagnetic groups (Figures 2 and 3) requires that the nitroxides be more than about 20 Å apart. Since spin-label I is firmly held by α_2M and spin-label II can be immobilized by an increase in solvent viscosity, it is likely that the labels have well-defined locations rather than being able to probe a complete sphere of radius equal to the dimensions of the label. Given the approximate length of label I (9 Å), the extreme values for separation of pairs of SH groups are between >2 Å—for nitroxides pointed away from one another—and >38 Å—for nitroxides pointed toward one another (Figure 7). These values are consistent with an earlier fluorescence energy transfer experiment (Tourbez & Pochon, 1986) that determined a separation of 20 Å between donor and acceptor fluorophores linked to SH groups within the "half-molecule" of α_2M. This value also indicates a closer or greater separation of the SH groups depending on whether the fluorophores are between the SH groups or outside them. The value of 20 Å obtained by these authors is based upon a value of K^2 (used in calculating R_0, the separation necessary for 50% efficiency of energy transfer) of $^2/_3$. This value is valid when donor and acceptor are freely rotating (Förster, 1959). In view of the present finding of the rigidity with which label I is held and also the finding of Larsson et al. (1987) that a different fluorophore attached to the same sulfhydryl is partly buried, the assumed value of K^2 and hence of the interfluorophore separation may be in error. If the maximum value of K^2 of 4 is used instead, the calculated separation is, however, only increased to 27 Å. A value of K^2 between $^2/_3$ and 4 and hence an interfluorophore separation between 20 and 27 Å might better accommodate the ESR data with the longer label II, since it is likely that the nitroxides point more toward one another than away from one another within the half-molecule. This orientation is considered likely on the basis of the expected location of caged proteases within the hollow α_2M molecule (see below) and the ability of proteases to form covalent cross-links between the glutamyl residues generated from the thiol esters of two α_2M subunits and lysine residues of the protease (Sottrup-Jensen et al., 1983). For such an orientation the nitroxides in α_2M labeled with II might be as much as 7.6 Å closer together, yet they must still be greater than 20 Å apart to account for the absence of dipole–dipole interaction.

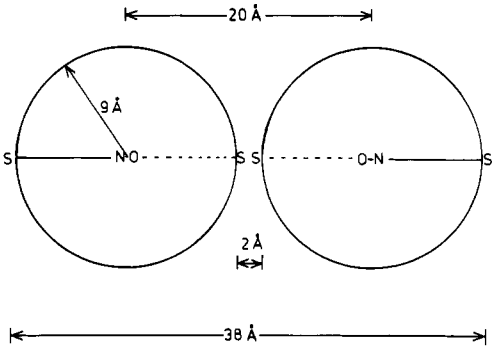

FIGURE 7: Schematic representation of maximum and minimum separation of pairs of SH groups consistent with an inter-nitroxide separation of 20 Å, the *minimum* expected nitroxide separation. The SH may be anywhere along the circumference of the circle centered on the nitroxide.

The distances calculated here apply to separations between SH groups both within the same half-molecule and in different halves of the molecule. While our findings do not disagree with those of Tourbez and Pochon (1986) that SH groups in *different* half-molecules may be 80 Å apart, we would like to point out that their calculations, involving energy transfer between labeled SH and labeled trapped chymotrypsin, were based upon a unique and static location for the protease and hence for the fluorophore in the enzyme's active site. Recently, we demonstrated that trapped chymotrypsin is loosely caged by α_2M and is free to rotate within the cage (Crews et al., 1987). If this finding were taken into account, the value of 80 Å might have to be substantially reduced.

Proximity of the Bait Region to Cys-949. The substantial broadening of ^{1}H resonances from the bait region of α_2M by label I attached to the SH moiety of Cys-949 demonstrates the closeness of these two crucial structures in the protein. Although the calculated values of r may be in error from inaccurate estimations of the broadenings or from the effective correlation time not being precisely 10 ns, the error in r is unlikely to be more than 10–20% because of the r^{-6} relationship with τ_c and $1/T_{2M}$. In a protein the size of α_2M—estimated from electron micrographs to be a hollow barrel 170 Å long and 60 Å in diameter (Schramm & Schramm, 1982)—a separation of 11–17 Å (Table I) represents a relatively close approach of the two groups. This is the first demonstration that these two groups are close together in any form of α_2M.

Structure of the Bait Region. It is of great significance for the structure of the bait region that all six aromatic amino acid

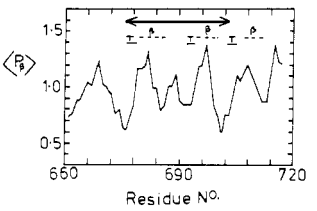

FIGURE 8: Chou and Fasman secondary structure prediction for the region of human α_2M that includes the bait region. The heavy bar indicates the bait region. β indicates β strand, and T signifies β turn. Note the pronounced troughs on either side of the two-strand β structure in the bait region corresponding to the sequences His-Gly-Pro-Glu and Glu-Glu-Pro-His, which contain three and four β breaker or strong β breaker residues, respectively.

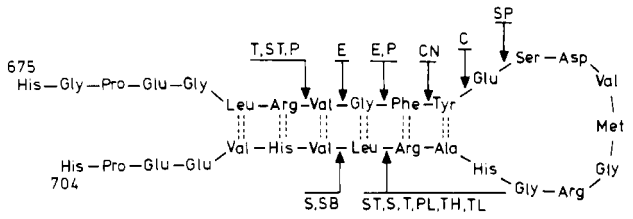

FIGURE 9: Proposed secondary structure of the bait region loop of human α_2M showing the sites of proteolytic cleavage of various enzymes. The cleavage sites are taken from Sottrup-Jensen et al. (1984). Abbreviations: T, bovine trypsin; ST, *Streptomyces griseus* trypsin; P, papain; E, porcine elastase; 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.

side chains are within 11–17 Å of the nitroxide. These marker residues are distributed over a 26-residue region in α_2M. Their narrow line widths in both native and methylamine-treated α_2M indicate high mobility and a location in a flexible loop of polypeptide (Gettins & Cunningham, 1986). If such a stretch of polypeptide were extended, the first and last of the four histidines might be as much as 110 Å apart. However, that they are all in a narrow spread of distances from the rigid nitroxide label I strongly suggests that the exposed flexible bait region is a relatively compact loop. In addition, the observed chemical shifts are not those of random coil residues (Gettins & Cunningham, 1986) and support a folded structure. Figure 8 shows the results of a Chou and Fasman (1978) secondary structure prediction on the bait region of human α_2M and its flanking sequences. There is a strong prediction of two stretches of β secondary structure with an intervening β bend. The two stretches of β structure are terminated by tetrapeptides containing effective β breakers.

We propose that the bait region of α_2M forms an exposed loop of polypeptide in antiparallel β conformation (Figure 9) projecting into solution and accessible to proteases from all sides. Since there is no change at all in the ^{1}H NMR signals from the bait region before and after treatment with methylamine (Gettins & Cunningham, 1986), the structure proposed here would also apply to native α_2M. The absence of change in the ^{1}H spectrum, despite a large conformational change in α_2M, supports a distinct and stable structure for the bait region. The sites of cleavage by different proteases are indicated, and all but one fall within the proposed β-sheet region. A mechanism for triggering the conformational change in α_2M might then be as follows. Cleavage of the bait region at any of the positions within the β sheet results in introduction of a charged carboxyl and a charged amino group. Such groups might be expected to disrupt the small β sheet. If the bait region connects two domains of the protein, such a disruption of structure in the connecting region could readily cause a local conformational rearrangement. Since the thiol ester is close by, its accessibility to solvent and to proteases could easily be increased so that reaction with protease or with solvent water could occur. The last step is the crucial one for the trapping reaction. This mechanism explains how different proteases, cleaving at quite different sites within the bait region, could result in identical trapping reactions.

Summary. The Cys-949 residues that form the thiol ester groups in human α_2M are located in a constricted cavity in the methylamine-induced conformationally altered form of α_2M. Spin-labels attached to the four Cys-949 residues in the tetramer are at least 20 Å apart and show no dipole–dipole interaction with one another. The functionally important bait region is very close to the thiol ester group and may well form a compact loop of two strands of β sheet. We propose that proteolytic cleavage anywhere is this loop disrupts the loop and is the common trigger for cleavage of the thiol ester and trapping of the protease.

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Bovine Plasminogen Activator Inhibitor 1: Specificity Determinations and Comparison of the Active, Latent, and Guanidine-Activated Forms†

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ABSTRACT: The plasminogen activator inhibitor 1 (PAI-1) synthesized and released by cultured bovine aortic endothelial cells is present in conditioned medium in a latent form that can be activated by guanidine hydrochloride [Hekman, C. M., & Loskutoff, D. J. (1985) *J. Biol. Chem. 260*, 11581–11587]. The purified, guanidine-activated PAI-1 was shown to inhibit both plasmin and trypsin in a dose- and time-dependent manner. Second-order rate constants for these interactions were calculated to be 6.6×10^5 and 7.0×10^6 M^{-1} s^{-1} for plasmin and trypsin, respectively. Experiments were conducted to compare the inherently active and the guanidine-activated forms of PAI-1. The two active forms had similar kinetic parameters for interaction with urokinase (K_d, 0.3 pM; k_{assoc}, 1.5×10^8 M^{-1} s^{-1}) and were both inactivated upon treatment with acid or base and by incubation at 37 °C. The latent form was relatively stable when incubated under similar conditions. The decrease in PAI-1 activity upon incubation at 37 °C was partially restored by a second treatment with guanidine hydrochloride. However, the degree of recovery decreased as a function of incubation time at 37 °C. These data suggest that active and guanidine-activated PAI-1 represent a single form of PAI-1. Incubation of this form at 37 °C yields two distinct populations of inactive PAI-1, one capable of reactivation and another that appears to be irreversibly inactivated.

The specific lysis of fibrin is catalyzed by the protease plasmin that exists in plasma as the inactive zymogen, plasminogen (Collen, 1980). The generation of plasmin occurs through the limited cleavage of plasminogen by the plasminogen activators, urokinase (UK),[1] and tissue plasminogen activator (tPA). In addition to their role in fibrinolysis, the plasminogen activators (PA's) have been implicated in various other biological processes including ovulation (Beers et al., 1975; Reich et al., 1985; Ny et al., 1985), cell migration (Ossowski et al., 1975), epithelial differentiation (Henrickson & Astrup, 1967), and tumor invasion (Dano et al., 1985). Thus, precise regulation of PA activity is essential to the maintenance not only of hemostasis but also of other biological processes. This control is accomplished through several different mechanisms including the formation and resolution of fibrin (Collen, 1980; Korninger & Collen, 1981), the controlled synthesis and secretion of the PA's (Loskutoff, 1986), and the presence of specific PA inhibitors (PAI's). These recently discovered PAI's have been detected in tissues (Holmberg et al., 1978), cells (Loskutoff & Edgington, 1977; Dosne et al., 1978; Coleman et al., 1982; Emeis et al., 1983; Levin, 1983; Loskutoff et al., 1983; Badenoch-Jones et al., 1985; Laug, 1985; Kruithof et al., 1986; Vassalli et al., 1984), and plasma and serum (Chmielewska et al., 1983; Kruithof et al., 1984; Verheijen et al., 1984a; Thorsen et al., 1984; Erickson et al., 1986). Recent reports correlating high plasma PAI levels with increased incidence of deep vein thrombosis and myocardial

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[1] Abbreviations: PA, plasminogen activator; PAI, plasminogen activator inhibitor; tPA, tissue plasminogen activator; UK, urokinase; PBS, phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; p-APMSF, *p*-amidinophenylmethanesulfonyl fluoride; NPGB, *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); Tris, tris(hydroxymethyl)aminomethane; IgG, immunoglobulin G.

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