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Bait region-thiol ester mapping in human α_2-macroglobulin

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Abstract The separations between aromatic residues in the bait region and nitroxide spin labels attached to the thiol ester-forming residues (Cys^{949} and Gln^{952}) in human α_2-macroglobulin (α_2M) have been determined from paramagnetic broadening effects of the spin labels on bait region ^{1}H NMR signals. We found that both the Cys^{949} and Gln^{952} residues are within 11–17 Å of the aromatic residues in the bait region, with closer approach of some residues to the Gln^{952} spin label than to the spin label attached to Cys^{949}. A model of the location of bait regions and thiol esters within an α_2M half-molecule is proposed that places the bait regions in the central region of α_2M at the interface between the subunits.

Key words: α_2-Macroglobulin; Human; Spin label; Nitroxide; Paramagnetic mapping; Bait region; Thiol ester; NMR (^{1}H)

1. Introduction

Cleavage of the bait region in human α_2M by proteinase is the initiating event that results in the large-scale conformational changes that trap the proteinase and lead to binding of the α_2M-proteinase complex to receptor through exposure of the receptor binding region on the α_2M [1]. The mediator of the conformational changes is an internal thiol ester which stabilizes the native conformation of the α_2M. Bait region cleavage results in activation and rapid opening of the thiol ester [2]. Although an X-ray structure of the conformationally altered form of α_2M has recently been determined [3], the resolution is too low to permit tracing of the polypeptide chain and thus localization of particular regions of the protein. We therefore still do not have a good idea of the structural relationship of the bait region relative to the thiol ester. The only data that relate to this are from an earlier NMR study from this laboratory, which showed that certain aromatic residues in the bait region are close to the cysteine of the thiol ester [4]. In this paper we have extended this study by using spin labels attached to both residues that form the thiol ester, in order to provide a better map of the location of the bait region relative to that of the thiol ester-forming residues.

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Abbreviations: α_2M, α_2-macroglobulin; HO559, 3-(α-bromoacetyl)-2,2,5,5-tetramethyl-2,5-dihydropyrrol-1-yloxyl; PROXYL, 2,2,5,5-tetramethylpyrrolidine-1-oxyl; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl.

2. Materials and methods

2.1. Isolation of human plasma α_2M

Plasma α_2M was isolated from outdated pooled human plasma, obtained from Vanderbilt University Hospital Blood Bank, by affinity chromatography on zinc chelate resin using the procedure of Imber and Pizzo [5]. The α_2M was judged to be homogeneous by SDS-PAGE. α_2M concentrations were determined spectrophotometrically using an extinction coefficient of 640,000 $M^{-1} \cdot cm^{-1}$ at 280 nm [6].

2.2. Labelling of α_2M

α_2M was labeled at the Cys^{949} residues by first cleaving the thiol ester with 200 mM methylamine at pH 8.0 for 2 h at room temperature followed by removal of methylamine in an ultrafiltration cell and subsequent reaction of the free SH groups with a 10-fold molar excess of the iodo- or bromo- species for 2 h. The concentration of free SH groups was measured by DTNB assay [7] immediately prior to reaction with the labelling reagent and gave close to the theoretical value of 4 SH groups per α_2M tetramer. Covalent attachment of the amino-TEMPO label to the Gln^{952} side chain was by direct reaction of native α_2M with 500 mM amino-TEMPO, resulting in attack of the amino group on the thiol ester carbonyl. Because of the bulk of amino-TEMPO the reaction was very slow compared to reaction with the much smaller methylamine [8]. The progress of the reaction was therefore checked periodically by native gel electrophoresis, looking for conversion from slow to fast form. After 7 days the reaction was judged, by this criterion, to be >95% complete. All samples were dialyzed extensively to remove unreacted free nitroxide.

2.3. Polyacrylamide gel electrophoresis

Non-denaturing PAGE was carried out on 5% acrylamide slab gels according to the procedure of Davis [9]. SDS-PAGE was carried out on 7.5% slab gels according to the procedure of Laemmli [10].

2.4. EPR spectroscopy

EPR spectra were recorded at 277K on a Bruker ESP300 spectrometer operating at 9.8 GHz, using a field set of 3490 G, a scan range of 100 G and a modulation frequency of 100 kHz. Each spectrum was the average of 10 scans. Sample concentrations were between 14 and 23 μM α_2M tetramer.

2.5. NMR spectroscopy

NMR spectra were recorded on a Bruker AM400 operating at 400 MHz for ^{1}H using a Carr–Purcell–Meiboom–Gill pulse train to select for the mobile bait region resonances [11], together with a solvent pre-saturation pulse. Spectra are the average of between 4500 and 7000 scans. The sample pH was 6.7. Chemical shifts are given relative to external dimethylsilapentane sulfonic acid.

2.6. Estimation of paramagnetic broadening of resonances

Since most of the resonances of interest overlapped with adjacent resonances, broadenings caused by proximity to paramagnetic spin labels were estimated from the reduction in resonance peak height in the presence of the paramagnetic label compared tot that in the diamagnetic control spectrum, together with an estimate of the initial linewidth estimated by deconvolution of the diamagnetic control spectrum or by direct measurement. Thus the initial unperturbed linewidths were estimated to be about 16Hz for the histidine C(2) proton resonances, 40 Hz for Tyr^{685} and 60 Hz for Phe^{684}. Reductions in peak height of as much as 85% were observed. However, it became apparent that this gave an

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overestimate of the broadening, which arose from the use of CPMG spectra rather than single pulse NMR spectra. The CPMG spectrum is heavily biased towards emphasis of narrow resonances. Thus broadening of a resonance in a CPMG spectrum would result in a decrease in peak height due both to the broadening and to a more rapid decay of the signal prior to sampling. Since the more upfield of the tyrosine resonances was well resolved, it was possible to estimate the linewidth of the resonance in both diamagnetic and paramagnetic spectra. From this it was estimated that peak height reduction used alone gave a 100% overestimate of the broadening for the broadest resonances, but only a 20% overestimate for the less perturbed histidine resonances. These scaling factors were therefore taken into account in estimating the actual resonance broadenings used subsequently in distance calculations.

2.7. Calculation of separation between nitroxide and bait region side chains

Distances between spin labels and bait region residues were calculated from the measured broadening of ^{1}H NMR signals using the Solomon–Bloembergen equation [12,13], with the assumption that the second, contact term was unimportant, which is valid given the calculated separations, which are an order of magnitude larger than those expected for contact interactions to be important. Although the estimates of the broadenings may be in error by as much as 50% given the poor Signal-to-noise ratio and resolution of individual resonances this should result in only about 7% error in distance because of the inverse sixth power relationship between separation and broadening. The correlation time appropriate for the calculations is that of the ^{1}H resonances, estimated previously to be about 10 ns for the highly mobile bait region, since the electron spin relaxation time (~12 μs [14]) and of the nitroxide held rigidly by α_2M (7×10^{-7} s [11]) are both much longer and not likely to contribute significantly to the electron spin-nuclear spin vector correlation time.

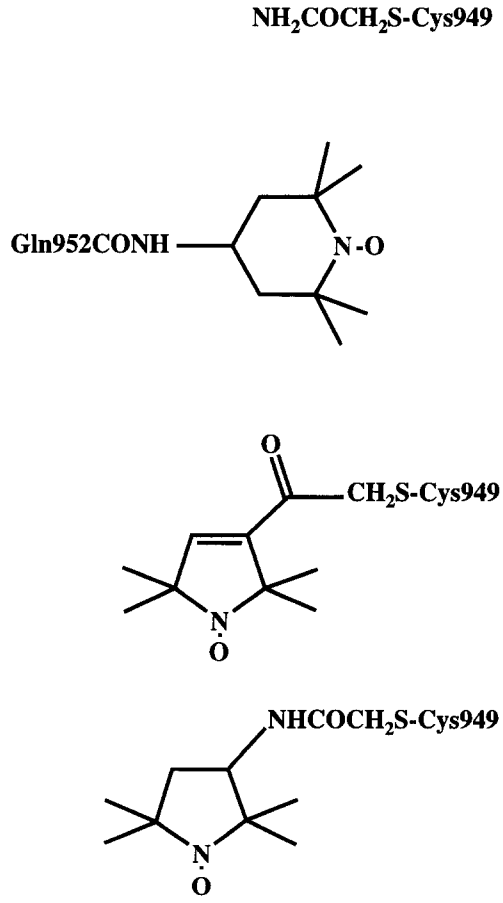

Fig. 1. Labelled α_2M species used in the study, showing the label used and the residue to which they became attached. (a) Control of acetamide; (b) Amino-TEMPO; (c) HO559; (d) acetamido-PROXYL. The acetamide and PROXYL labels were reacted as iodo-derivatives, HO559 was reacted as the bromo-derivative.

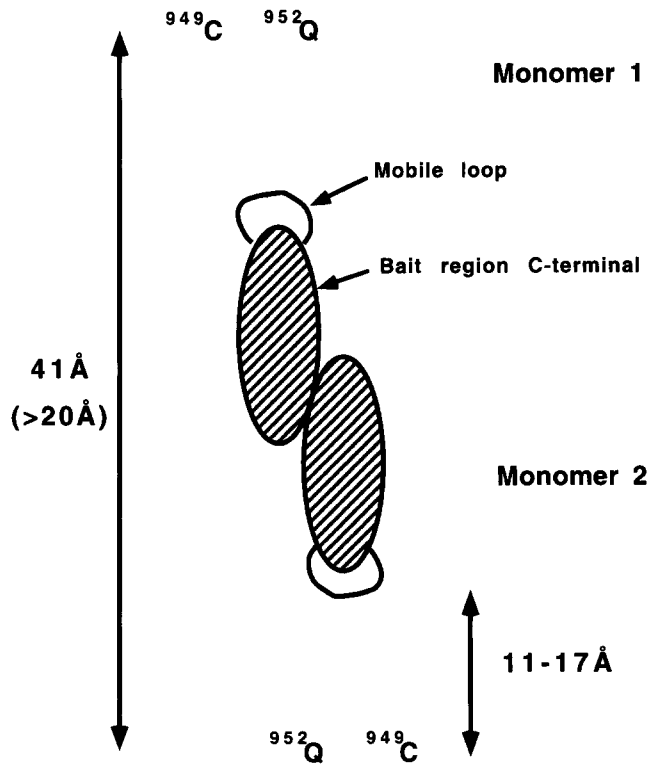

Fig. 2. EPR spectra of α_2M spin labeled at Cys^{949} or Gln^{952}. (a) Gln^{952}-labeled amino-TEMPO; (b) Cys^{949}-labeled HO559; (c) Cys^{949}-labeled acetamido-PROXYL.

2.8. Materials

Iodoacetamide, methylamine hydrochloride, and amino-TEMPO were from Sigma, iodoacetamido-PROXYL was from Molecular Probes. 3-(α-bromoacetyl)-2,2,5,5-tetramethyl-2,5-dihydropyrrol-1-yloxyl (HO559) was prepared as described [15].

3. Results

3.1. EPR spectra of spin-labelled α_2M species

The three spin labelled α_2M derivatives examined (Fig. 1) gave EPR spectra that reported nearly completely immobilized nitroxides as the major species in each case (Fig. 2). In addition, the HO559 derivative showed the presence of a minor component with smaller splitting, indicating some heterogeneity of labelling, perhaps involving one or more additional sites. Upon integration it was found that the extent of incorporation of HO559 label was greater (~1.4 labels per α_2M subunit) than that of the amino-TEMPO or acetamido-TEMPO, suggesting that a limited amount of secondary labelling had occurred in addition to reaction at Cys^{949}, probably as a result of using a less specific bromo-containing labelling reagent. However, since specific effects of the spin labels on the bait region NMR resonances are being considered in this study, only additional labelling close to the bait region would have any effect on the NMR spectrum. None of the EPR spectra showed any indication of dipole-dipole interaction between nitroxide spin labels

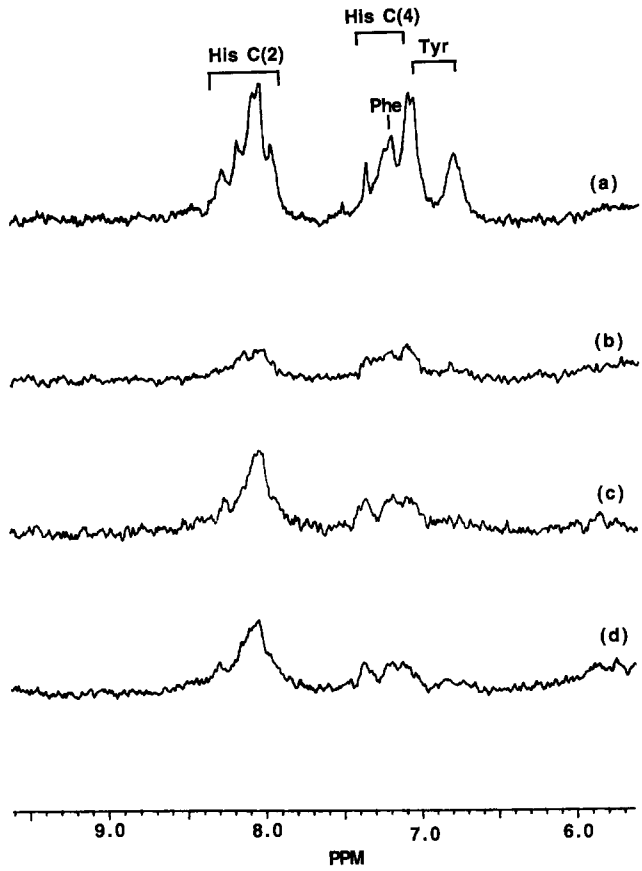

Fig. 3. Paramagnetic broadening of ^{1}H NMR resonances from α_2M bait region aromatic residues caused by proximity to spin labels attached to Cys^{949} or Gln^{952}. (a) Control spectrum of α_2M labeled with acetamide (diamagnetic); (b) Gln^{952}-amino-TEMPO; (c) Cys^{949}-HO559; (d) Cys^{949}-acetamido-PROXYL.

attached to separate Cys^{949} sulfhydryls within the α_2M tetramer. The absence of such dipole-dipole interaction has previously been shown to require a separation of at least 20 Å between pairs of nitroxides within α_2M [4].

3.2. *Broadening of α_2M bait region aromatic resonances*

The diamagnetic control spectrum of α_2M labelled at the four Cys^{949} SH groups with acetamide showed the expected sharp resonances (Fig. 3a) from the four histidines, one tyrosine and one phenylalanine that occur in the mobile bait region of α_2M and one additional unidentified histidine [11]. The corresponding NMR spectra of the three paramagnetically labelled α_2M species showed significant broadening of these resonances, though with different degrees of broadening for the resonances in a given spectrum and different broadening of the same resonance by different labels (Fig. 3b–d). HO559 produced very similar, though not identical, broadening to acetamido-PROXYL (Fig. 3c and d). Greater differences were seen with amino-TEMPO, attached to the side chain of Gln^{952} (Fig. 3b). This spin label caused much greater broadening of the histidine resonances. We have previously shown with acetamido-PROXYL-labelled α_2M that the broadening of the bait region resonances is specifically due to paramagnetic broadening, by restoring the sharpness of the resonances upon reduction of the paramagnetic nitroxide to a diamagnetic moiety with ascorbate [4]. From estimates of the broadening of the bait region aromatic residues, separations between the nitroxides and these side chains were calculated and found to be in the range 11–17 Å (Table 1).

4. Discussion

The appearance of the EPR spectra of Cys^{949}- and Gln^{952}-nitroxide labelled α_2M species and the broadening effect of these nitroxide spin labels on NMR resonances from aromatic side chains within the bait region have provided additional information on the location of the two mechanistically critical structural features in human α_2M, the bait region and the thiol ester. Thus the absence of dipole-dipole interaction between pairs of nitoxides within the same α_2M tetramer, whether attachment of the nitroxide was to Cys^{949} or to Gln^{952} shows that the separation between such nitroxides must be >20 Å, which is in keeping with other measurements from this laboratory of the separation between pairs of Cys^{949} residues made using fluorescence resonance energy transfer [16,17].

The near rigid limit EPR spectra for both Cys^{949}-linked and Gln^{952}-linked nitroxides is also in keeping with other data that suggest that the thiol ester is located at the bottom of a relatively narrow hydrophobic cleft, to which access is limited to small molecules in native α_2M. Thus the reactivity of the thiol ester towards nucleophilic amines shows a strong inverse correlation to the size of the amine [7]. Also the fluorescence properties of 8-[(*N*-acetylamino)ethyl] naphthalene-1-sulfonic acid attached to Cys^{949} report on a hydrophobic environment [18].

It is clear from the broadening of all of the bait region aromatic resonances by each of the spin labels used in the present study that at least a portion of the bait region is close to both residues that are involved in forming the thiol ester. The similar broadening caused by HO559 and acetamido-PROXYL, despite the additional bond between the nitroxide and the Cys^{949} SH for the latter may be due to different orientations of the nitroxide ring, resulting from the planarity of the

Table 1
Characterization of paramagnetic α_2M derivatives by EPR and NMR spectroscopies

Label	Derivative	ΔH (G)*	^{1}H-nitroxide separation (Å)**		
			Phe^{684}	Tyr^{685}	Histidines***
TEMPO	Gln^{952}	68	12	12	11–12
HO559	Cys^{949}	68.5	12	12	12–17
PROXYL	Cys^{949}	67	12	12	12–17

*Splitting between high field and low field peaks in EPR spectrum at 277K.
**Distance between bait region residues and nitroxide estimated from paramagnetic broadening of ^{1}H resonances in NMR spectrum, using the Solomon–Bloembergen equation.
***Five histidines give visible resonances in a CPMG spectrum. Four occur in the bait region. Specific assignments have not been made.

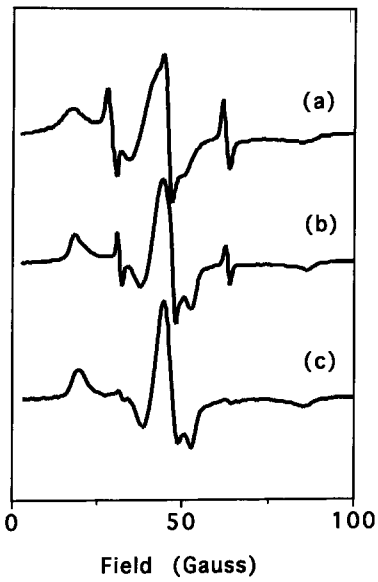

Fig. 4. Model of the bait regions and thiol esters within a half molecule of α_2M showing possible location of aromatic bait region residues relative to residues that form the thiol ester (Cys^{949} and Gln^{952}). The model represents a transverse section across the α_2M tetramer that contains the two pairs of thiol ester-forming residues, 17 Å above the central plane (based on previous fluorescence energy measurements which place thiol esters in *different* half molecules ~35 Å apart). The aromatic residues that give rise to the sharp NMR signals that are broadened by the nitroxides are located in the 'mobile loop' portion of the bait region, 11–17 Å from C^{949} and Q^{952}, whereas the C-terminal portion of the bait region is thought, from mutagenesis experiments involving bait region truncations [19] to be involved in the non-covalent interface between the two halves of the α_2M tetramer. The separation between pairs of thiol esters is shown as 41 Å based on previous fluorescence resonance energy transfer measurements [16] and >20 Å based on present EPR measurements.

C=C–C=O and CONH groups and the consequent restriction in location of the nitroxide ring. The greater broadening caused by amino-TEMPO attached to Gln^{952} indicates an asymmetric placement of the bait region with respect to Cys^{949} and Gln^{952}. These findings permits a very approximate triangulation to be carried out that places the bait region at the apex of an acute angled triangle with the other vertices formed by the Cys^{949} and Gln^{952} side chains.

This model can be extended to include bait regions and thiol esters from both of the monomers that constitute a proteinase binding site, using recent findings from this laboratory that showed that variants of human α_2M in which parts of the bait region have been deleted are no longer able to associate non-covalently to form tetramers [19], which suggested a direct role for parts of the bait region in dimer-dimer interactions. Taken together with the distances estimated between bait region residues and the thiol ester and between pairs of thiol esters within one half of an α_2M tetramer, a model of the bait region and thiol ester within one half of the α_2M tetramer can be proposed in which the bait regions from non-covalently associated subunits may be in contact with one another and lie between pairs of thiol ester residues (Fig. 4).

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