NMR Solution Structure of the Receptor Binding Domain of Human α2-Macroglobulin*

(Received for publication, August 31, 1999, and in revised form, October 11, 1999)

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Human α2-macroglobulin-proteinase complexes bind to their receptor, the low density lipoprotein receptor-related protein (LRP), through a discrete 138-residue C-terminal receptor binding domain (RBD), which also binds to the β-amyloid peptide. We have used NMR spectroscopy on recombinantly expressed uniformly 13C/15N-labeled human RBD to determine its three-dimensional structure in solution. Human RBD is a sandwich of two antiparallel β-sheets, one four-strand and one five-strand, and also contains one α-helix of 2.5 turns and an additional 1-turn helical region. The principal α-helix contains two lysine residues on the outer face that are known to be essential for receptor binding. A calcium binding site (Kd ~ 11 mM) is present in the loop region at one end of the β-sandwich. Calcium binding principally affects this loop region and does not significantly perturb the stable core structure of the domain. The structure and NMR assignments will enable us to examine in solution specific binding of RBD to domains of the receptor and to β-amyloid peptide.

Human α2-macroglobulin (α2M) is a highly abundant, very high molecular mass (~720 kDa) plasma protein that is best known as a broad spectrum inhibitor of proteinases (1). α2M is also reported to bind to certain growth factors (2) and to be involved in binding and clearance of the 42-residue β-amyloid peptide (3, 4) that is thought to contribute to the etiology of Alzheimer’s disease through formation of fibrils. The essential nature of α2M is indicated by the failure to find any individuals with α2M deficiency (5). The means of inhibition and clearance of proteinases by α2M is through a unique proteinase-induced conformational change in the α2M that physically traps the proteinase within a cage-like interior cavity (6, 7). The massive extent of the conformational change and the nature of the sequestration have been likened to the action of a Venus fly trap closing around its prey. Because inhibition by α2M involves sequestration of the proteinase from the surrounding milieu rather than direct and specific binding to the proteinase active site, the inhibitor is not limited to inhibition of one type or class of proteinase, but can instead, uniquely, inhibit proteinases of all four mechanistic classes.

The trapping conformational change not only results in sequestration of the proteinase, but also exposure of a receptor binding region on α2M that is solely responsible for mediating binding to the clearance receptor LRP (low density lipoprotein receptor-related protein) (8). It has been shown that the receptor binding region is located within a discrete domain that comprises the last 138 residues of the α2M monomer (9, 10). This domain, termed the receptor binding domain (RBD), can be preparatively isolated from methylamine-transformed human α2M by limited digestion with papain (9) or endo-Lys proteinase (10). Cleavage by either proteinase occurs between lysine and glutamate in the highly charged sequence Glu-Lys-Glu-Glu, which suggests that this tetrapeptide is an exposed linker that may act as a hinge to permit movement of RBD upon conformational transformation of α2M and, hence, exposure of the LRP-binding epitope on the domain. Isolated RBD retains the ability to bind specifically to LRP (9) and to be taken up by the receptor (11) and, hence, represents an appropriate species for studying the interaction between α2M and its receptor. Although the affinity for LRP is reduced ~200-fold compared with intact α2M-proteinase complexes, from 0.5 to ~100 ns, this is likely to be because of the difference in higher ligand valency of tetrameric α2M compared with the monomeric RBD (11).

The receptor to which these α2M-proteinase complexes bind is LRP, a mosaic protein that is a member of the LDL receptor family. It contains clusters of epidermal growth factor (EGF)-like repeats and of complement-like repeats (abbreviated to CR) (12). The latter are thought to be the sites of binding of different protein ligands both in LRP and LDLR (8, 13). In LRP there are four such clusters, designated I-IV, containing 2, 8, 10, and 11 CR domains, respectively. α2M-proteinase complexes have been shown to bind to the second cluster of repeats—domains CR3–CR8 (14). Given both the relatively small dimensions of these repeats compared with the size of the protein ligands that bind, together with the very broad range of proteins that LRP is able to bind, it may be that a protein ligand binding site on LRP is composed of interactions with two or more CR domains, where each interaction is relatively weak but where together they represent a high affinity ligand binding site.

Although there is good evidence for binding of the growth factor TGF-β to human α2M (2), such binding is not thought to involve the receptor binding region. In contrast, the noncovalent interaction of β-amyloid peptide with human α2M has been localized to the extreme C-terminal portion of the α2M mono-
NMR Structure of αM Receptor Binding Domain

Given the critical role of RBD in clearance of proteinases through binding of the exposed RBD to LRP, and its possible involvement in binding β-amyloid peptide, it is important to know not only the structure of this region of human αM but to have a means of examining the details of interactions between RBD and LRP or between RBD and β-amyloid peptide at the molecular level. Whereas crystallography in principle affords such a means, it requires the ability to crystallize not only RBD alone but also complexes formed with domains from LRP or with β-amyloid peptide. Probing the specificity of interaction of different CR repeats, where individual domains may bind only weakly, may be problematic because it may be hard to co-crystallize weak binding protein-protein complexes. For human αM, it has proved impossible to obtain satisfactory crystals even of RBD alone (16). NMR, however, provides a means of not only determining the structure of human RBD, but of subse-

**FIG. 1.** Schematic representation of the secondary structure of human RBD using the numbering of the isolated domain. The structure is composed of two sheets with four and five strands in β-conformation (arrows) and two regions of α-helix (filled cylinders). The location of the two cysteines that form the single disulfide (residues 16 and 131) are marked with a dot, and the single site of glycosylation (residue 88) is marked with an asterisk.

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**TABLE I**

| Constraints used in structure determination | RMSD from mean structure (Å) | Pairwise RMSD (Å) |
|-------------------------------------------|-----------------------------|-------------------|
| NOE constraints                           |                            |                   |
| Intra-residue                              | 1.70 ± 0.4                  | 1.84 ± 0.27       |
| Sequential (|i −|j| = 1)                             | 0.80 ± 0.6              | 2.81 ± 0.31       |
| Medium range (1 < |i −|j| < 5)                             | 0.05 ± 0.05             |                   |
| Inter-residue, long range (|i −|j| > 5)                             | 0                          |                   |
| Torsion angle constraints                   | 0.1 ± 0.1                  |                   |
| Mean number of violations of experimental restraints per structure | 125                        |                   |
| Distance restraints of 0.2–0.3Å            |                            |                   |
| Distance restraints of 0.3–0.4Å            |                            |                   |
| Distance restraints of 0.4–0.5Å            |                            |                   |
| Distance restraints >0.5Å                  |                            |                   |
| Angle violations >10°                      |                            |                   |
| Ramachandran analysis for non-Pro, non-Gly residues | 89.2%              | 10.8%            |
| S1–S9 and H1–H2                            |                            |                   |
| Most favorable regions                     | 71.8%                      | 25.0%            |
| Additionally allowed regions               | 3.2%                       |                   |
| Whole structure                            |                            |                   |
| Most favorable regions                     |                            |                   |
| Additionally allowed regions               |                            |                   |
| Generously allowed regions                 |                            |                   |
| RMSD of structures                         |                            |                   |
| Backbone (5–131)                           | 1.78 ± 0.31                | 1.84 ± 0.27       |
| Heavy atoms (5–131)                        | 2.73 ± 0.40                | 2.81 ± 0.31       |
| Ramsd from experimental distance constraints | 0.018 ± 0.005 Å          |                   |
| Ramsd from experimental torsion angle constraints | 0.23 ± 0.07 Å           |                   |

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**EXPERIMENTAL PROCEDURES**

Expression and Purification of Human RBD—Recombinant human RBD was expressed and purified as described previously (18). Briefly, RBD was expressed in E. coli as a 165 residue fusion protein that, in addition to RBD, contained an N-terminal 6-histidine tag, a thrombin cleavage site, and an extra 10 residues from αM at the N terminus that had been thought to be necessary for stability of RBD (19). These extra residues turned out to promote oligomerization of the refolded protein and so were removed with papain following refolding. The N terminus was confirmed by sequencing to be Glu-1314, using the numbering of the intact αM subunit (20). Plasma RBD, used for calcium binding experiments, was prepared by papain cleavage of methylamine-treated human plasma αM, as described previously (9). Residues of RBD are numbered consecutively from 1 to 138. This corresponds to residues 1314 to 1451 of the intact αM monomer.

NMR Sample Preparation—For the structure determination experiments, purified recombinant RBD was dialyzed into NMR buffer (100 mM phosphate, pH 5.1, 300 mM NaCl), to which 10% D2O was added. The final concentration for NMR studies was 0.9 mM for the 15N-labeled sample and 1.2 mM for the 13C/15N double-labeled sample.

NMR Spectroscopy—NMR experiments were carried out at 25 °C on a Bruker DIX600 at the University of Illinois at Chicago or a Bruker DMX600 at the University of Wisconsin, Madison. Both spectrometers were equipped with four channels and a pulsed-field gradient accessory. NMR data were processed and analyzed using Triad, Version 6.2 software (Tripos, Inc., St. Louis, MO).

Assignments—The backbone assignments were obtained according to standard procedures and have been published (18). Three-dimensional
Triple resonance HBHA(CO)NH, HNCACB, and CBCA(CO)NH were used for the Hb and Cb assignments. Three-dimensional 15N-edited TOCSY-HSQC and three-dimensional 13C-edited HCCH-TOCSY were used for other sidechain assignments. NOE constraints were obtained from the following two experiments: three-dimensional 15N-edited NOESY-HSQC with mixing time of 120 ms and three-dimensional 1H-13C-correlated NOESY-HSQC in D2O with mixing time of 120 ms. Sensitivity-enhancement gradient pulse sequences were employed for all experiments in which magnetization was detected on the amide HN. Resonance assignments have been deposited with the Protein Data Bank, code 1BV8.

Structure Calculations—Distance constraints were estimated using the criteria of small, medium, and large NOEs. Upper limits for backbone NOE correlations were set at 5.0, 4.0, and 3.0 Å, whereas values of 5.5, 4.5, and 3.5 Å were used for backbone-sidechain and sidechain-sidechain NOEs. These values were also the base values used to make pseudo-atom corrections for moieties where stereospecific assignments were not available. The input of this calculation used a total of 1142 upper distance constraints distributed as follows: 454 intraresidue, 331 sequential, 81 medium, and 276 long range correlations and 125 torsion angle constraints. Torsion angle restraints were derived from HNHA measurements of 3JH N Hα coupling constants (21) and chemical shift index-based assignment of secondary structure (22). Structure calculations were performed with the torsion angle dynamics annealing simulation program DYANA (23). The final ensemble of structures that represented the 20 best DYANA conformers from an input of 100 initial structures was analyzed, and figures were generated by MOLMOL (24).

Materials—(15NH4)2SO4 was from Cambridge Isotopes, 13C glucose was from Isotec, and D2O was from Sigma. Papain was from Roche Molecular Biochemicals. 99.997% CaCl2 and GdCl3 were from Johnson Matthey Inc.

RESULTS AND DISCUSSION

Secondary Structure of Human RBD—We have previously reported on the secondary structure of human RBD based on 1H and 13C chemical shift indices and some inter-strand NOEs (18). This showed the presence of eight major strands of β-sheet and one major α-helix of 2.25 turns. In completing the three-dimensional structure determination, we found evidence for an additional small ninth strand of β-sheet from residues 53 to 55 (S4) and a 1.25-turn α-helical region from residues 19 to 23 (H1). Neither of these additional regions was previously found from secondary structure predictions of human RBD or other RBDs from seven other α-macroglobulins (18). The complete secondary structure present in the final three-dimensional structure is presented schematically in Fig. 1. In addition to the two short α-helices, the protein consists of two antiparallel β-sheets. One β-sheet is composed of strands S1-(5–12), S2-(19–23), S3-(27–35), S4-(53–55), S5-(60–68), S6-(72–80), S7-(83–90), S8-(93–101), S9-(105–113), and S10-(116–130).

Table II

| Region | RMSD |
|--------|------|
| Sheet 1 (S1, S2, S7, S4) | 1.21 |
| Sheet 2 (S5, S6, S3, S8, S9) | 1.05 |
| Helix 1 | 0.59 |
| Helix 2 | 0.31 |
| All secondary structure | 1.77 |
| Whole structure | 2.91 |

Comparison is only for backbone atoms of the 130 residues that are visible in both structures.
Terminal regions, which lie at opposite ends of the molecule, are

The family of twenty best structures obtained by simulated annealing is shown in Fig. 2, and a ribbon representation of the mean structure is shown in Fig. 3. The extreme N- and C-terminal regions, which lie at opposite ends of the molecule, are relatively poorly defined. Such poor definition for the N terminus is as expected from it being the region that links RBD to the central body of the α2M tetramer (6) and that is susceptible to specific proteolytic cleavage (9, 10). The poor definition of the C terminus parallels the behavior of the bovine RBD in the crystal, in which there is sufficient disorder that the last six residues cannot be traced. The quality of the structures in regions of secondary structure is good, with RMSD of 0.41–0.52 Å for regions of secondary structure backbone atoms. Overall the backbone RMSD is 1.78 Å for residues 5–131 (i.e. excluding the disordered N and C termini) (Table I). For heavy atoms, the RMSDs are 1.36–1.57 Å for regions of secondary structure and 2.73 Å for all heavy atoms for residues 5–131 (Table I). There are few distance constraint violations, with an average of 2.55 per structure for violations >0.2 Å. Ramachandran analysis shows that, for regions in defined secondary structure (S1–S9 and H1–H2), 89.2% of residues are in the most favored region, and 100% of residues are within either this region or the additional allowed region. For the whole structure, these percentages fall to 71.8 and 96.8%, respectively.

The structure consists of a sandwich of two β-sheets of 4 and 5 strands, edged by one α-helix of 2 turns and with a second 1-turn α-helix between strands S1 and S2. The major α-helix contains two lysine residues, 57 and 61, on the outward-facing flank with their terminal ε-amino groups no more than 11 Å apart. These lysines have been shown by others to be required to define this site. The study used phage display to identify possible discontinuous epitopes that might form part of the binding interface to specific proteolytic cleavage (9, 10). The poor definition of the C terminus parallels the behavior of the bovine RBD in the crystal, in which there is sufficient disorder that the last six residues cannot be traced. The quality of the structures in regions of secondary structure is good, with RMSD of 0.41–0.52 Å for regions of secondary structure backbone atoms. Overall the backbone RMSD is 1.78 Å for residues 5–131 (i.e. excluding the disordered N and C termini) (Table I). For heavy atoms, the RMSDs are 1.36–1.57 Å for regions of secondary structure and 2.73 Å for all heavy atoms for residues 5–131 (Table I). There are few distance constraint violations, with an average of 2.55 per structure for violations >0.2 Å. Ramachandran analysis shows that, for regions in defined secondary structure (S1–S9 and H1–H2), 89.2% of residues are in the most favored region, and 100% of residues are within either this region or the additional allowed region. For the whole structure, these percentages fall to 71.8 and 96.8%, respectively.

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Three-dimensional Structure of RBD—Standard three-dimensional NMR experiments on doubly labeled human RBD were used to assign the backbone resonances and most of the side chain resonances and to obtain NOE constraints and some backbone torsion angle constraints (Table I). A total of 1142 NOE constraints and 125 torsion angle constraints were used. The family of twenty best structures obtained by simulated annealing is shown in Fig. 2, and a ribbon representation of the mean structure is shown in Fig. 3. The extreme N- and C-terminal regions, which lie at opposite ends of the molecule, are relatively poorly defined. Such poor definition for the N terminus is as expected from it being the region that links RBD to the central body of the α2M tetramer (6) and that is susceptible to specific proteolytic cleavage (9, 10). The poor definition of the C terminus parallels the behavior of the bovine RBD in the crystal, in which there is sufficient disorder that the last six residues cannot be traced. The quality of the structures in regions of secondary structure is good, with RMSD of 0.41–0.52 Å for regions of secondary structure backbone atoms. Overall the backbone RMSD is 1.78 Å for residues 5–131 (i.e. excluding the disordered N and C termini) (Table I). For heavy atoms, the RMSDs are 1.36–1.57 Å for regions of secondary structure and 2.73 Å for all heavy atoms for residues 5–131 (Table I). There are few distance constraint violations, with an average of 2.55 per structure for violations >0.2 Å. Ramachandran analysis shows that, for regions in defined secondary structure (S1–S9 and H1–H2), 89.2% of residues are in the most favored region, and 100% of residues are within either this region or the additional allowed region. For the whole structure, these percentages fall to 71.8 and 96.8%, respectively.

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### Table I

| Method | RMSD (Å) |
|--------|----------|
| Backbone | 1.36–1.57 |
| Heavy atoms | 2.73 |

Fig. 4. Primary structure alignments of human and bovine RBDs. There are two sites of glycosylation in bovine RBD (human numbering 67 and 88), but only one in human RBD (itali-

Fig. 5. [1H,15N]HSQC NMR spectra of recombinant 15N-labeled human RBD in the absence and presence of calcium. RBD in the absence of Ca2+ (blue) and in the presence of 20 mM Ca2+ (green). Spectra were recorded at 600 MHz for 1H on a sample of 50 mM RBD in 50 mM Tris buffer, pH 7.4, in 90% H2O. The temperature was 298 K.
LRP binding site on RBD (30). The two discontinuous regions identified map to loop regions of RBD at opposite ends of the molecule. One segment, DEPK, is located between S1 and H1 (including the first residue of H1), whereas the second segment, SRS, is ~50 Å away at the far end of the molecule, between S2 and S3. Because the identification of these regions was from a heptapeptide that included both portions of the proposed binding site, the two epitopes should be contiguous in space, which clearly they are not. It is therefore questionable whether either portion of the epitope represents a part of the binding site for LRP.

A comparison was made between the mean NMR structure of human RBD and the crystal structure of bovine RBD (17), using coordinates generously provided by Dr. Nyborg (Protein Data Bank code 1AYO). Overall, there is high similarity between the two structures (Table II and Fig. 3), with one difference being in the orientation of helix H2 relative to the body of the protein. This may be because of the presence of an extra residue (histidine 68) in the loop that links the C-terminal end of the helix to the body of the protein in human RBD (Fig. 4). Whereas the C-terminal seven residues were not visible in the crystal structure, they do show some NOEs to define their position in the NMR structure. This high similarity between the two structures occurs despite the structure of bovine RBD being determined at higher pH, in the presence of 20 mM Ca$^{2+}$, and on a naturally glycosylated form. The lack of effect of Ca$^{2+}$ is consistent with our present findings that calcium binds to a loop region and has no structural effect on the core of the domain (see below). Similarly, the lack of effect of carbohydrate is consistent with our previous findings of the invariance of conformation-sensitive regions of the one-dimensional $^1$H spectra in H$_2$O of glycosylated human plasma $\alpha_2$M-derived RBD and nonglycosylated recombinant human RBD (18).

**Characterization of the Calcium Binding Site**—Calcium is known to be essential for binding of $\alpha_2$M-proteinase complexes and other ligands to their receptor LRP (12). While this is thought to result primarily from the presence of calcium binding sites in each of the complement-like repeats of LRP (31), the finding that there is a calcium ion shared between the two molecules of bovine RBD present in the asymmetric unit of the crystal structure pointed out the need to establish whether such a calcium binding site exists in solution in the receptor binding domain of human $\alpha_2$M and, if it does, whether it is of sufficient affinity to be physiologically significant and what effect it has on the structure of RBD. The latter point is also important because the three-dimensional structure of RBD we had determined was in the absence of calcium.

![Selective broadening of resonances in $[^1$H,$^{15}$N]HSQC spectrum of human RBD upon binding Gd$^{3+}$. Panel A, no Gd$^{3+}$; panel B, 1.8 mM Gd$^{3+}$; and panel C, 3.6 mM Gd$^{3+}$. Resonance assignments that are underlined are from backbone or side chain amide nitrogens that are most strongly broadened by Gd$^{3+}$. Where broadening is so strong that the resonance is no longer visible, a box represents the location of the resonance. Note that only a portion of the whole spectrum is shown, to illustrate the selective broadening. Spectra were recorded on 50 mM recombinant $^{15}$N-labeled human RBD, at pH 7.4 in 50 mM Tris buffer in 90% H$_2$O and temperature of 298 K.](image1)

![Location of Gd$^{3+}$-broadened resonances in RBD, indicating the location of the Gd$^{3+}$/Ca$^{2+}$ binding site.](image2)

![NMR Structure of $\alpha_2$M Receptor Binding Domain](image3)
The HSQC spectrum of $^{15}$N-labeled human RBD at pH 7.4 showed that, while calcium does cause some perturbation of backbone resonances (Fig. 5), the magnitudes of the perturbations are relatively small ({$<0.09$ ppm for $^{1}$H and $<0.52$ ppm for $^{15}$N}) and involve relatively few of the cross peaks. Although the resonance assignments used above in the structure determination were made at pH 5.1 whereas the present spectra were recorded at physiological pH, there is sufficient similarity in the appearance of the spectra that we can assign with reasonable confidence the resonances in the pH 7.4 spectrum that are most strongly perturbed by calcium. We found that those resonances with significant perturbations arose from the loop regions at the “bottom” of RBD (orientation of Fig. 2), whereas the core of the molecule, which contains all of the secondary structure, as well as the “top” loop regions were very little affected. This was reinforced by the effect of Ca$^{2+}$ on the one-dimensional proton spectrum. Although there are few significant effects on the main part of the aliphatic region of the spectrum (not shown), indicating no major conformational change, the most sensitive regions of the spectrum, i.e. the upfield methyl region and the aromatic region, show some small perturbations (not shown). In particular, the presence of a cluster of aromatic side chains in the calcium-sensitive 115–122 loop results in one of the resolved aromatic resonances undergoing a readily measurable shift. This resonance was used to monitor calcium binding and gave a saturable change in chemical shift of $\sim0.04$ ppm, indicating a specific binding interaction. This titration could be well fitted to a single site binding process and gave a $K_D$ for the calcium site of $\sim11$ mM. Such an affinity is sufficiently high for there to be at least partial occupancy under physiological conditions, even if the presence of the remainder of the $\alpha_2M$ polypeptide does not enhance the affinity. In addition to showing that calcium binds to human RBD, these results show that the core secondary structure is not affected by calcium, so that the structure determined in the absence of calcium should have equivalent secondary and tertiary structure in the core region as when calcium is bound. Finally, the results suggest that any specific calcium binding site is probably located in the bottom loop region that connects the strands of the $\beta$-sandwich.

To better localize this low affinity calcium site, we used the distance-dependent paramagnetic broadening effect of Gd$^{3+}$ and the fast exchange conditions that apply to the calcium site. HSQC spectra of RBD were recorded at physiological pH in the absence or presence of low concentrations of added Gd$^{3+}$ and the fast exchange conditions that apply to the calcium site. Such an affinity is sufficiently high for there to be at least $K_D$; 0.09 ppm for $^{1}$H and 0.09 ppm for $^{15}$N.

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The NMR Structure of $\alpha_2M$ Receptor Binding Domain

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**Acknowledgments**—We thank Esper Boel for the $\alpha_2M$ expression plasmid p1167 and Jens Nyborg for making the coordinates of the crystal structure of bovine RBD available. The Bruker DRX600 NMR spectrometer was funded by a grant from the NSF Academic Research Infrastructure Program and matching funds from the University of Illinois at Chicago. The DMX600 at the University of Wisconsin-Madison is supported by National Institutes of Health Grant RR02301 from the Biomedical Research Technology Program and contains equipment purchased with funds from the University of Wisconsin, the NSF Biological Instrumentation Program (DMB-8415048), the NSF Academic Research Infrastructure Program (BIR-9214394), the NIH Biomedical Research Technology Program (RR02301), the NIH Shared Instrumentation Program (RR02781), and the United States Department of Agriculture.

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