The Conformation of the α-Helical Coiled Coil Domain of Macrophage Scavenger Receptor Is pH Dependent

Kazuo Suzuki,[‡] Takefumi Doi,[§] Takeshi Imanishi,[§] Tatsuhiko Kodama,[||] and Toshiki Tanaka*[,‡]

Biomolecular Engineering Research Institute, 6-2-3, Furuedai, Suita, Osaka 565, Japan, Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamadaoka, Suita, Osaka 565, Japan, and Research Center for Advanced Science and Technology, University of Tokyo, Tokyo 113, Japan

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ABSTRACT: Macrophage scavenger receptor is a trimerized membrane protein that binds ligands and undergoes internalization by endocytosis. The receptor releases the ligands in the endosome, and then is recycled. The mechanisms of the ligand release and the recycling of the receptor have not been clearly determined. We analyzed the structure of the α-helical coiled coil domain considered to be responsible for acid-mediated ligand dissociation, by chemical cross-linking, sedimentation equilibrium, Western blot, and circular dichroism analyses. This domain has 22 heptad repeats, which are characteristic of the sequence of an α-helical coiled coil structure, with a discontinuity in the middle. We prepared three peptides, corresponding to the entire α-helical coiled coil domain (α), its N-terminal half (α-N), and its C-terminal half (α-C), by expression of each gene in *Escherichia coli*. The α and α-N peptides show triple-stranded α-helical coiled coil structures, but in contrast, the α-C peptide shows a random structure. When connected to the N-terminus by a chemical ligation method, the α-C peptide also shows an α-helical coiled coil structure, but only at an acidic pH. These results suggest that the N-terminus of the α-helical coiled coil domain is responsible for the formation of a stable trimer and the C-terminus exhibits the pH-dependent conformational change that might be involved in the ligand release by the macrophage scavenger receptor.

Receptor-mediated endocytosis is an important cellular function. Ligands are internalized upon binding to their receptors at the cell surface, and most ligands are transported to lysosomes for digestion. However, several internalized receptors are not transported to lysosomes directly but are recycled many times. Examples include the low-density lipoprotein (LDL)[1] receptor (*1*, *2*), the insulin receptor (*3*), and other important receptors (*4*−*8*). The key to receptor recycling is ligand dissociation, which is affected by the acidic pH within the endosomal compartment. However, the molecular mechanisms of ligand dissociation are unclear. The macrophage scavenger receptor (MSR), a recycling receptor, binds modified LDL at the cell surface and is internalized into the cell by receptor-mediated endocytosis via coated-pit (*9*). The receptor releases the ligands in the acidic endosome. The released ligands are digested in the lysosome, and the receptor is routed to the cell surface again via the Golgi apparatus. The MSR is a suitable model to study ligand release in an acidic pH environment due to its structure described below.

The MSR exists as two types (types I and II). From the deduced amino acid sequence, the MSR is predicted to have six domains: cytoplasmic, membrane spanning, spacer, α-helical coiled coil, collagen-like, and C-terminal type specific domains (Figure 1). Both type I and type II MSR have the same amino acid sequence except for the C-terminal type specific domain (*10*, *11*). The collagen-like domain is critical to bind modified LDL (*12*, *13*). A synthetic collagen model corresponding to 18 residues of the C-terminus of the collagen-like domain binds Ac-LDL (*14*). On the other hand, the α-helical coiled coil domain is thought to be important for maintaining the trimeric structure. The functions of the other domains are for the most part undefined.

The α-helical coiled coil structure has a seven amino acid "heptad" repeat sequence, $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$, with hydrophobic residues at the *a* and *d* positions for interhelical association (*15*−*17*). Doi *et al.* reported that the substitution of His^{260} by Leu at the *a* position in the α-helical coiled coil domain sustained the ligand binding activity, but abolished the ability to release the ligands in the cells (*18*). This suggests that His^{260} in the α-helical coiled coil domain allosterically controls ligand release from the collagen-like domain. We, therefore, considered that a detailed analysis of the conformation of the α-helical coiled coil domain would provide further information about the mechanisms of the ligand release.

In the bovine MSR, the heptad repeats are between amino acids 109 and 271 containing a discontinuity as the three-residue deletion after Asn^{203} (*10*). A discontinuity often separates an α-helical coiled coil domain into two regions that have different functions or stabilities (*19*). In the present study, we prepared the three peptides through a discontinuity at Asn^{203}. The peptides correspond to amino acids 95−273, 95−208, and 204−273 in the α-helical coiled coil domain of the bovine MSR, which are designated here as α, α-N,

* To whom correspondence should be addressed. Telephone: +81-6-872-8208. Fax: +81-6-872-8219. E-mail: ttanaka@beri.co.jp.
[‡] Biomolecular Engineering Research Institute.
[§] Osaka University.
[||] University of Tokyo.
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[1] Abbreviations: LDL, low-density lipoprotein; MSR, macrophage scavenger receptor; Ac, acetylated; PCR, polymerase chain reaction; *E. coli*, *Escherichia coli*; IPTG, isopropyl 1-thio-β-D-galactopyranoside; EGS, ethylene glycol *O*,*O*′-bis(succinimidyl succinate); BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; CD, circular dichroism; GST, glutathione *S*-transferase; TFE, trifluoroethanol.

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and α-C, respectively (Figure 1). Further, we constructed a chemically ligated peptide, $(\alpha\text{-C})_3$, in which a three-branched linker was attached to the N-terminus of the α-C peptide, and analyzed the structures of these peptides. The α and α-N peptides had stable triple-stranded α-helical coiled coil structures, and the $(\alpha\text{-C})_3$ peptide was found to exhibit a pH-dependent conformational change.

MATERIALS AND METHODS

Construction of Plasmid pGEX-3X-StuI. To generate a *Stu*I site after the codon for Arg within the coding sequence for Ile-Glu-Gly-Arg of pGEX-3X (Pharmacia Biotech), a polymerase chain reaction (PCR) was carried out between the *Bal*I and *Bam*HI sites of pGEX-3X, using two primers with 33 and 56 nucleotide lengths. The *Bal*I−*Bam*HI fragment of the PCR product was inserted into the large *Bal*I−*Bam*HI fragment of pGEX-3X to obtain pGEX-3X-StuI. The nucleotide sequence around Ile-Glu-Gly-Arg was changed from 5′-AAATCGGATCTGATCGAAGGTCGTGGGATCC-3′ (*Bam*HI site is underlined) to 5′-AACAACCTAGGGATCGAGGGTAGGCCTTGGATCC-3′ (*Avr*II, *Stu*I, and *Bam*HI sites, respectively, are underlined from the 5′-end). As a result, the amino acid sequence Asn-Asn-Leu-Gly, instead of Lys-Ser-Asp-Gln, is followed by Ile-Glu-Gly-Arg, and a *Stu*I site is generated after the Arg codon (AGG) of Ile-Glu-Gly-Arg.

Construction of Expression Plasmids. Using pXSR7, which has the intact bovine MSR cDNA, PCRs were carried out using the primers, 5′-CCGGGATCCTGAGTCCGGAAGGCAAAGGAAATGGC and 5′-CGGAAGCTTGAAGGTCTTCTGCATTTAAACAACAAGAGGAG (including a *Bam*HI site and an *Xmn*I site, underlined), and 3′-GTGAAATGAGGTTCCAGGA**ATT**CTTAAGGCC and 3′-GGTTCTCTTACGTAAATTTGTTGTT**ATT**CTTAAGGCC (including a stop codon, in boldface letters, and an *Eco*RI site, underlined). The PCR products were isolated by 10% agarose gel electrophoresis and ethanol precipitation, and were then treated with *Bam*HI−*Eco*RI or *Xmn*I−*Eco*RI. The fragments were ligated into the *Bam*HI−*Eco*RI or *Stu*I−*Eco*RI sites of pGEX-3X and pGEX-3X-StuI, respectively. The plasmids were transformed into *Escherichia coli* (*E. coli*) JM109. The nucleotide sequences were confirmed by DNA sequencing (ABI 373A DNA Sequencer).

Expression and Purification of the Protein. E. coli JM109 cells transfected with the reconstructed plasmids were cultured at 37 °C for 5 h in 500 mL of 2 × YT medium in the presence of 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG). The cells were harvested, resuspended in 4 mL of 50 mM Tris-HCl, pH 8, 10 mM EDTA, and 15% sucrose, and lysed by the addition of lysozyme (10 mg/mL) (1 mL) on ice for 15 min. Deoxyribonuclease I (10 mg/mL) (100 μL) and 0.5 M $MgCl_2$ (500 μL) were added, and the mixtures were incubated on ice for 15 min. After the addition of a detergent-containing buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1 M NaCl, and 10 mM Tris-HCl, pH 7.5) (3 mL) and an incubation on ice for 5 min, the fusion proteins were collected by centrifugation. The protein pellets were washed 3 times with 10 mM sodium phosphate buffer, pH 7.0, 150 mM NaCl, and 1 mM EDTA, were rewashed 3 times with 1.75 M guanidine hydrochloride, 1 M NaCl, and 1% Triton X-100, and were resuspended in 8 M urea. The supernatants were dialyzed against CAPS buffer, pH 10.7 [25 mM CAPS (DOJINDO), 1 mM EDTA, 50 mM NaCl, and 1 mM dithiothreitol], and against Factor Xa buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 2 mM $CaCl_2$). The fusion proteins were then treated with Factor Xa (New England Biolabs, Inc.) at 20 °C for 2 h. After centrifugation to remove precipitated proteins, the supernatants were loaded onto a Sephadex G-100 column [112 × 1 cm (i.d.)] and were eluted at a rate of 0.2 mL/min with 20 mM sodium phosphate, pH 7.0, 0.05 M NaCl, and 1 mM EDTA. Finally, the fractions were loaded onto a Q-Sepharose column (Pharmacia Biotech) (bed volume, 3.0 mL) in 10 mM sodium phosphate (pH 7.0) containing 1 mM EDTA. The columns were washed and then eluted with an NaCl gradient (0.1−0.4 M). The α, α-N, and α-C peptides were eluted at a 0.2−0.25 M NaCl concentration.

Cross-Linking. Cross-linking was performed by adding ethylene glycol *O*,*O*′-bis(succinimidyl succinate) (EGS) (Pierce Chemical Co., U.K.) to a 10 μM solution of each peptide in 25 mM sodium phosphate buffer (pH 8.8), to give final EGS concentrations ranging from 0 to 6000 μM. The reactions were carried out at 37 °C for 15 min and were stopped by adding glycine to a final concentration of 40 mM. Aliquots of the reactions were electrophoresed on either 10 or 15% SDS−polyacrylamide gels and were stained with Coomassie Brilliant Blue R-250.

Analytical Ultracentrifugation. Sedimentation equilibrium analysis was performed with a Beckman XL-A Optima Analytical Ultracentrifuge equipped with absorbance optics. The initial peptide concentration was 17.4 μM in 10 mM sodium phosphate, pH 7.0, and 150 mM NaCl. The sample was centrifuged at 18 000 rpm at 20 °C with the absorbance being monitored at 220 nm. The aggregation state was determined by fitting the data to a single species, as well as to cooperative monomer−*n*mer equilibria using Origin Sedimentation Equilibrium Single Data Set Analysis (Beckman). The partial specific volume used for the data analysis was 0.730 mL/g, calculated from the weighted average of the amino acid content using the method of Cohn and Edsall (*20*).

Western Blot Analysis. The α-helical coiled coil peptides (10 pmol) (1 μL) and bovine serum albumin (BSA) were spotted on a nitrocellulose filter. This filter was briefly soaked in TBST (50 mM Tris-HCl buffer, pH 8.0, 150 mM NaCl, and 0.05% Tween 20) and was then incubated with the blocking solution [TBST with 5% (w/v) non-fat dry milk] for 2 h. After three washes in TBST (5 min × 3), the filter was incubated with the monoclonal antibody IgG-D2. The bound IgG-D2 was detected by an incubation with peroxidase-conjugated anti-mouse IgG and an ECL detection system (Amersham).

N-Terminal Chemical Ligation. The α-C peptide (80 nmol) was oxidized by $NaIO_4$ (400 nmol) in 50 mM sodium phosphate, pH 7 (0.5 mL), for 5 min at room temperature, and was purified by reversed-phase HPLC. A tris(aminooxyacetyl) linker was synthesized by the previously described method (*21*), using aminooxyacetic acid instead of bromoacetic acid. The oxidized α-C peptide (45 nmol) was mixed with the tris(aminooxyacetyl) linker (12 nmol) in 0.5 mL of 3 M guanidine hydrochloride (pH 3). After 4 days at room temperature, the mixture was applied to a TSK gel G2000SWxl column, and the $(\alpha\text{-C})_3$ peptide was eluted with 45% CH_3CN at 50 °C. The peptide linkage was determined by SDS−polyacrylamide gel electrophoresis

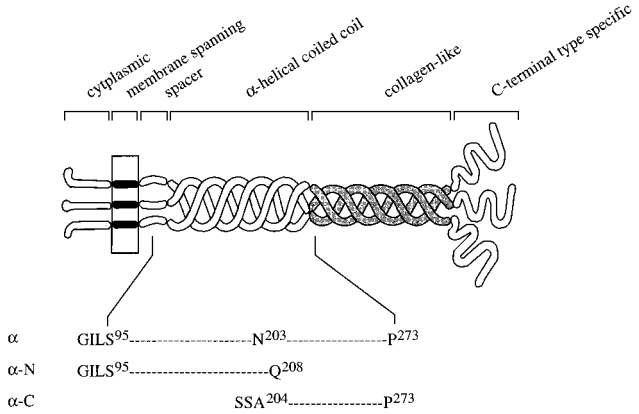

FIGURE 1: Structure of the type I bovine macrophage scavenger receptor. The collagen-like domain is critical for the substrate binding. There are five and two putative glycosylation sites on the α-helical coiled coil and the spacer domains, respectively. A deletion of three residues in the α-helical coiled coil domain exists after Asn^{203}. From the α-helical coiled coil domain, we prepared α, α-N, and α-C peptides that contained additional sequences.

(PAGE) and matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (PerSeptive Biosystems Voyager Elite).

Circular Dichroism (CD). CD measurements were carried out on a JASCO 720 spectrometer with the sample in a 2-mm path length cuvette. Except for the $(\alpha\text{-C})_3$ peptide, the spectra were obtained with 2.5−5.7 μM peptide solutions in 4 mM phosphate buffer containing 0.1 M NaCl and 0.4 mM EDTA. The mean residue ellipticity, $[\theta]$, is given in units of degrees centimeter squared per decimole. The percent α-helical content was estimated from the mean residue ellipticity at 222 nm, $[\theta]_{222}$ (*22*, *23*). Thermal transition curves were obtained by monitoring $[\theta]_{222}$ as a function of temperature with a 2 mm path length cuvette. The peptide concentrations were 5.7 μM and 2.5 μM for the α and α-N peptides, respectively, and the temperature was increased at a rate of 1 °C/min.

The spectra of the $(\alpha\text{-C})_3$ peptide were obtained with the 10 μM peptide solution in 10 mM phosphate buffer containing 0.1 M NaCl at 37 °C. The pH of the samples was measured directly with a microelectrode calibrated at two reference pHs.

RESULTS

Construction of the Recombinant Peptides. The coding regions corresponding to Ser^{95}−Pro^{273} (α) and Ser^{95}−Gln^{208} (α-N) were inserted into a glutathione *S*-transferase (GST) gene fusion vector, pGEX-3X, to generate GST-α and -α-N fusion proteins in *E. coli*. This construct adds a Gly-Ile-Leu sequence at the N-terminus of each peptide. The coding region encoding Ala^{204}−Pro^{273} (α-C) was inserted into pGEX-3X-StuI, as described under Materials and Methods; thus, the α-C peptide has an additional Ser-Ser sequence at the N-terminus (Figure 1). Upon IPTG induction of *E. coli* carrying each plasmid, a major protein band of approximately 44.5 for GST-α, 40 for GST-α-N, and 35 kDa for GST-α-C, respectively, appeared in the SDS−PAGE analysis (data not shown). All of the expressed fusion proteins existed in inclusion bodies. After refolding by dialysis, they were directly treated with Factor Xa. The α peptide, the α-N peptide, and the α-C peptide, respectively, were separated by gel filtration on Sephadex G-100 and purified by

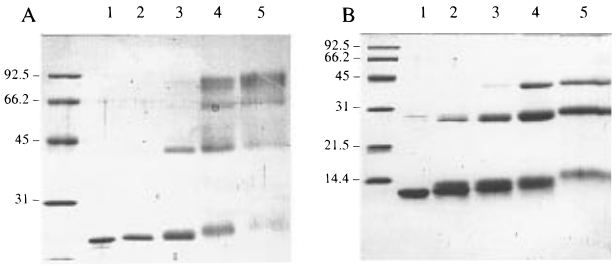

FIGURE 2: Chemically cross-linked α and α-N peptides fractionated by denaturing PAGE. (A) Cross-linking was performed by the addition of ethylene glycol *O*,*O*′-bis(succinimidyl succinate) (EGS) to the α peptide (10 μM). The samples were incubated for 15 min at room temperature, and the reactions were stopped by the addition of glycine to a final concentration of 40 mM. Lanes 1−5 contain 0, 20, 100, 500, and 1000 μM EGS, respectively. (B) The α-N peptide (8 μM) was cross-linked with EGS. Lanes 1−5 contain 0, 24, 120, 600, and 2400 μM EGS, respectively. The samples were run on a 10% (A) or 15% (B) SDS−polyacrylamide gel and were stained with Coomassie Brilliant Blue R-250.

Q-Sepharose column chromatography. The identities of the peptides were confirmed by sequencing of the N-terminal 10 amino acids and amino acid analyses.

Oligomerization State of the Recombinant Peptides. In order to determine the oligomerization state of the peptides, the peptides were cross-linked with various concentrations of a bifunctional protein cross-linker, EGS, and were analyzed by denaturing SDS−PAGE. As shown in Figure 2, peptide bands corresponding to 24, 44, and 65−80 kDa for the α peptide and 13, 26, and 39 kDa for the α-N peptide were seen. The cross-linked α peptide gave a smeared band between 65 and 80 kDa, since proteins cross-linked at different residues have slightly different electrophoretic mobilities on SDS−PAGE. The cross-linking of the α-N peptide was inefficient, probably due to the low content of Lys residues in the α-N peptide. A cross-linked product from the α-C peptide was not observed (data not shown).

We also used sedimentation equilibrium centrifugation for the α peptide (Figure 3). The apparent molecular size of the peptide at 20 °C was 54 422 daltons. Although this value is slightly lower than the calculated molecular size of the trimer (62 955 daltons), the fitting curve is closer to a theoretical curve for the trimer. Taken together with the cross-linking experiments, the major species of the α and α-N peptide are trimerized.

Trimerization of the peptides was further analyzed by Western blot analysis using an IgG-D2 monoclonal anti-bovine MSR antibody, which recognizes only the folded, trimeric form of the bovine MSR. Western blot analysis showed that the α peptide was recognized by the IgG-D2 antibody, indicating that the α peptide had the same trimerized structure as that of the natural receptor. Neither the α-N nor the α-C peptide was recognized by the IgG-D2 antibody. The α-N peptide is trimerized and forms an α-helical coiled coil structure described below. However, this antibody does not recognize a deletion mutant protein constructed from Glu^{209} to the N-terminus (*12*). Therefore, the α-N peptide is not considered to contain an epitope. The α-C peptide probably contains the epitope, but the α-C peptide does not form the trimeric α-helical coiled coil structure. Neither GST nor BSA was recognized by the IgG-D2 antibody.

Secondary Structure Analyses of the Recombinant Peptides. The CD spectra of the α and α-N peptides are

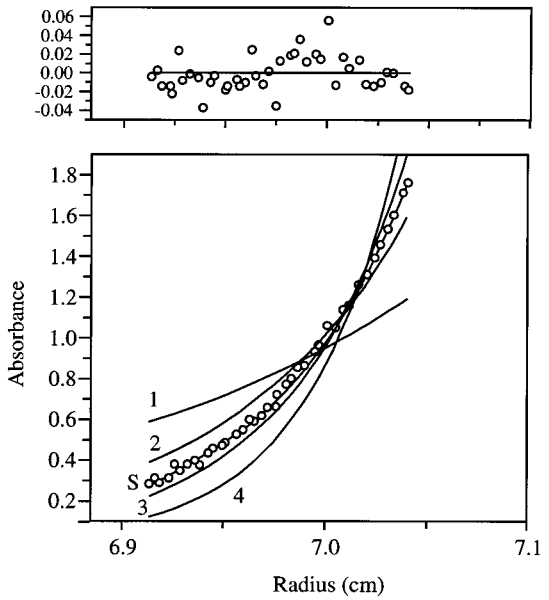

FIGURE 3: Sedimentation equilibrium analysis of the α peptide. The measurement was made using 17.4 μM peptide in 10 mM sodium phosphate and 150 mM NaCl, pH 7 at 20 °C. The circles represent the concentration distribution as a function of the radial position of the peptide at equilibrium after 18 h at 18 000 rpm. The data most closely fit a single ideal species with a molecular size of 54 422 daltons (curve S), which is closer to the calculated molecular size (62 956 daltons) for the trimer. For comparison, curves for monomer (curve 1), dimer (curve 2), trimer (curve 3), and tetramer (curve 4) are shown.

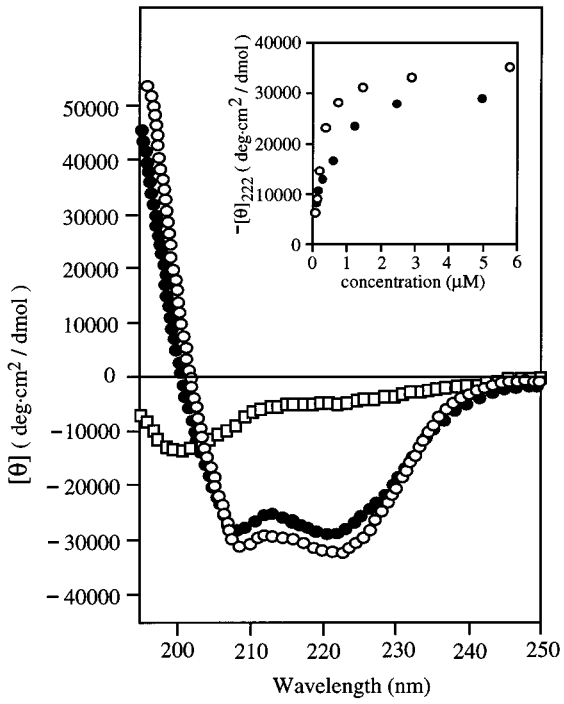

FIGURE 4: Circular dichroism spectra of the α, α-N, and α-C peptides. CD spectra of 5.7 μM α (open circles), 2.5 μM α-N (closed circles), and 5.7 μM α-C (open squares) peptides were measured in 4 mM sodium phosphate, pH 7.0, 0.1 M NaCl, and 0.4 mM EDTA at 20 °C. Inset: negative molar ellipticities at 222 nm versus peptide concentration. Open circles and closed circles represent the α- and α-N peptides, respectively.

characteristic of an α-helical structure, with minima at 222 nm and 208 nm (Figure 4). $[\theta]_{222}$ was dependent on the peptide concentration and did not significantly change with peptide concentrations above 2.5 μM. The ratio of $[\theta]_{222}$ and $[\theta]_{208}$ was 1.03 for both peptides, indicating that they form an α-helical coiled coil structure (*24*−*26*). Using a $[\theta]_{222}$ of 33 000 deg·cm^2·dmol^{-1} for the α peptide at a 5.7 μM concentration and 20 °C, the α peptide has an almost complete α-helical structure. The α-N peptide has a $[\theta]_{222}$ of 29 000 deg·cm^2·dmol^{-1} at a 2.5 μM concentration, showing a fractional helical content of 88%. On the other hand, the CD spectrum of the α-C peptide showed a random structure with a minimum at 201 nm. This spectrum did not change until a 50 μM peptide concentration at 4 and 20 °C.

The CD spectra of the α, α-N, and α-C peptides were compared in an aqueous solution and in 50% trifluoroethanol (TFE), a solvent that induces helicity in a single-chain peptide with potential α-helical content (*27*). As shown in Figure 5, $[\theta]_{222}$ values of the α and α-N peptides were the same in both buffer and 50% TFE. In 50% TFE, however, the ratio of $[\theta]_{222}$ and $[\theta]_{208}$ is less than 1, which is characteristic of a single-stranded α-helix. This result also indicates that they have a nearly complete α-helical coiled coil structure in aqueous buffer. The α-C peptide showed a random structure in buffer, but a typical α-helical structure in 50% TFE, showing that the α-C peptide has the potential to form an α-helical structure. The pH dependence of the spectra of the α and α-N peptides could not be monitored, due to precipitation of the peptides. The α-C peptide had a disordered structure even at an acidic pH (data not shown).

Due to the presence of discontinuity in the α-helical coiled coil domain, it was expected that the α peptide might have two parts with different stabilities, one from the α-N region and one from the α-C region. We analyzed the thermal denaturation of the α and α-N peptides (Figure 6). The thermal transition curves were obtained by monitoring $[\theta]_{222}$ as a function of temperature. However, both the α and α-N peptides exhibited sharp thermal transitions with midpoints of 56 and 50 °C, at peptide concentrations of 5.7 μM for the α peptide and 2.5 μM for the α-N peptide, respectively. These transitions were reversible.

Secondary Structure Analyses of the Chemically Ligated (α-C)$_3$ Peptide. As described above, the α peptide had a trimerized and almost complete α-helical coiled coil structure, indicating that the α-C region in this peptide also formed an α-helical coiled coil. The α-C peptide, however, showed a monomeric, random structure. To determine the conformation of the α-C region in the MSR in further detail, we constructed an (α-C)$_3$ peptide, in which the N-termini of three α-C peptides were connected with a three-branched linker by chemical ligation (Figure 7). Oxidization of the N-terminal Ser by $NaIO_4$ generates an aldehyde group, which reacts with an aminooxy group (*28*). Thus, we designed the α-C peptide to have an N-terminal Ser. The α-C peptide was treated with $NaIO_4$ to generate the aldehyde group and was reacted with the tris(aminooxyacetyl) group of the linker molecule to give the (α-C)$_3$ peptide.

The (α-C)$_3$ peptide did not precipitate at an acidic pH, in contrast to the α and α-N peptides. Therefore, the pH-dependent conformational change of the peptide could be examined. The spectrum of the peptide was dramatically dependent on the pH at 37 °C (Figure 8). This peptide forms an α-helical coiled coil structure only at an acidic pH, and not at a neutral pH. The spectrum at pH 5 shows a $[\theta]_{222}$ of 25 000 deg·cm^2·dmol^{-1} at a 10 μM concentration of each

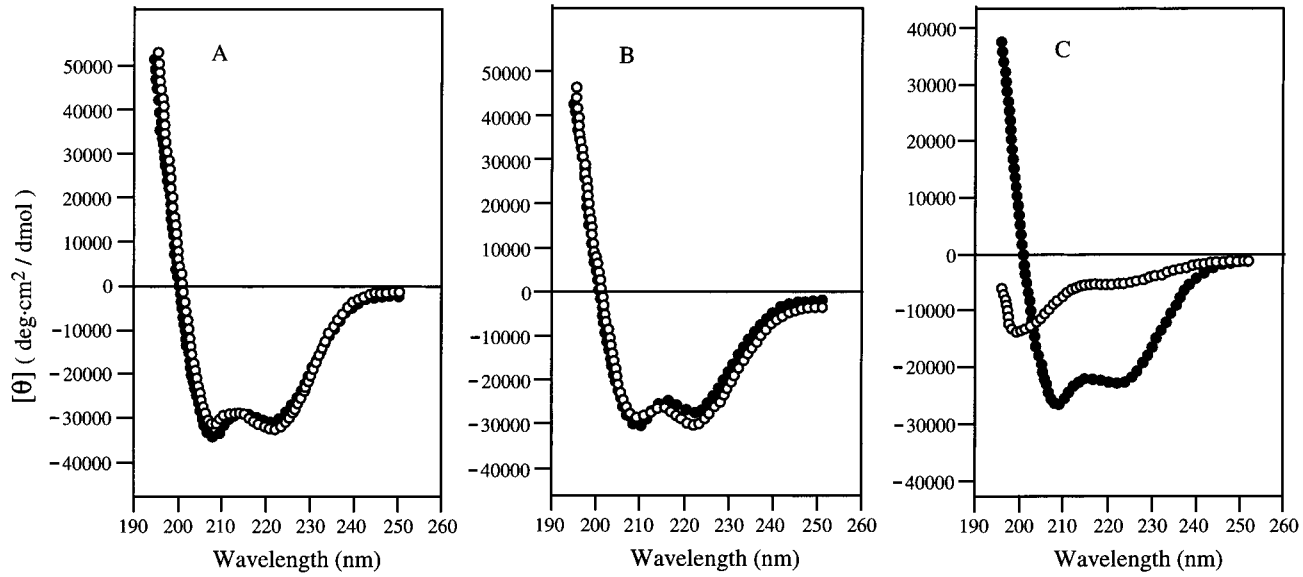

FIGURE 5: Effect of trifluoroethanol on the circular dichroism spectra of the α, α-N, and α-C peptides. CD spectra of the α (A), α-N (B), and α-C (C) peptides in the absence (open circles) or presence (closed circles) of 50% TFE were measured in sodium phosphate at concentrations of 5.7 μM for the a peptide, 2.5 μM for the α-N peptide, and 5.7 μM for the α-C peptide and 20 °C.

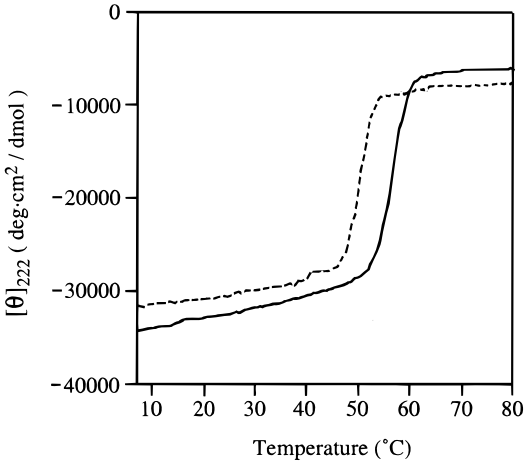

FIGURE 6: Thermal melting curves of the α and α-N peptides. Mean residue ellipticity at 222 nm of the α (solid line) and α-N (dashed line) peptides as a function of temperature. The CD spectra were recorded in 4 mM sodium phosphate, pH 7.0, 0.1 M NaCl, and 0.4 mM EDTA. Peptide concentrations are 5.7 μM for the α peptide and 2.5 μM for the α-N peptide.

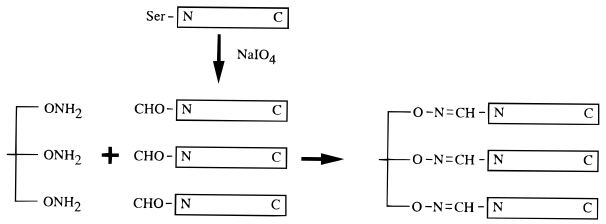

FIGURE 7: Synthetic protocol to generate the $(\alpha\text{-C})_3$ peptide by chemical ligation. The α-C peptide was oxidized to generate the aldehyde group and was reacted with the tris(aminooxyacetyl) group of a linker molecule, which was synthesized by the previous method (*21*) using aminooxyacetic acid instead of bromoacetic acid. The open box indicates the α-C peptide.

strand and is the same in both buffer and 50% TFE (data not shown).

DISCUSSION

A ligand binding site of the MSR is known to exist in the collagen-like domain (*12*, *13*). A nine residue peptide from the C-terminus of the collagen-like domain is reported to be destabilized under acidic conditions (*29*). This suggests that the collagen-like domain may play an important role in ligand release by the MSR. To study the interactions of the receptor with ligands, we have constructed a collagen structure containing 18 residues of the C-terminal collagen-like domain (*14*). This peptide includes the nine residue peptide and is stable, even at pH 4. Binding of Ac-LDL to the collagen-like domain should further stabilize the structure of the collagen-like domain, since Ac-LDL is large enough to cover all of the collagen-like domain. Therefore, we consider that the collagen-like domain is not the main factor mediating ligand dissociation under acidic conditions. On the other hand, His^{260} in the α-helical coiled coil domain was reported to influence ligand release (*15*). Therefore, we speculated that the dramatic conformational change in the α-helical coiled coil domain is critical for ligand release by the MSR and investigated the conformations of various fragments of the MSR.

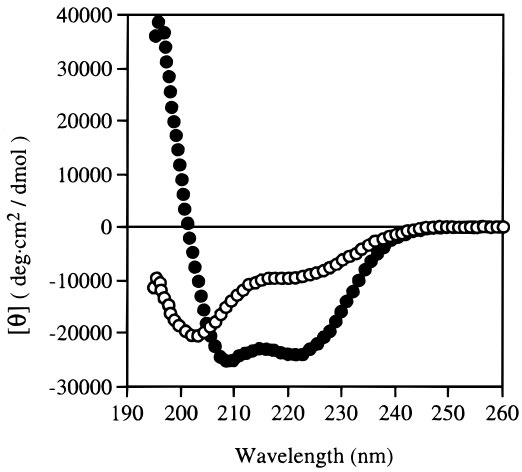

FIGURE 8: Circular dichroism spectra of the $(\alpha\text{-C})_3$ peptide. Measurements were performed in 10 mM sodium phosphate containing 0.1 M NaCl, pH 7 (open circles) or pH 5 (closed circles), at 37 °C. The peptide concentration was 3.3 μM.

The α-helical coiled coil domain of the MSR has 22 heptad repeats, characteristic of the amino acid sequence of an

α-helical coiled coil structure, with discontinuity in the middle of the domain (*10*). We prepared the entire α-helical coiled coil domain (α), the N-terminal half (α-N), and the C-terminal half (α-C) (Figure 1), and their structures were analyzed by various methods. The α-N peptide, which has the 13 heptad repeats, has a triple-stranded α-helical coiled coil structure at a 2.5 μM concentration and pH 7 (Figure 4). On the other hand, the α-C peptide, although it contains the nine heptad repeats, has a disordered structure at a 50 μM concentration and 4−20 °C. The α peptide, containing the α-C peptide plus the α-N peptide, forms a complete α-helical coiled coil structure at a 5 μM concentration (Figure 4), suggesting that the structure of the α-C peptide is influenced by the presence of the α-N peptide. In spite of the discontinuity in the heptad repeats, the α peptide shows a single transition for thermal denaturation (Figure 6), suggesting that the α-N and α-C regions form an α-helical structure without a disordered structure between them. The stabilities of the α and α-N peptides were nearly the same, 56 °C *versus* 50 °C for the thermal denaturation (Figure 6), suggesting that the α-C region does not contribute to the stability of the α-helical coiled coil structure. We could not observe the pH-dependent conformational change of the α and α-N peptides, since the peptides precipitated at around pH 5. In the case of the natural MSR, it has seven potential N-linked glycosylation sites on the α-helical coiled coil domain. The glycosylation may contribute to increasing the solubility.

For further analysis of the conformation of the α-C region and, in particular, its pH dependence, we constructed the $(\alpha\text{-C})_3$ peptide, in which three α-C peptides were linked at their N-termini (Figure 7). The spectrum of the peptide dramatically depends on the pH at 37 °C (Figure 8). This peptide forms an α-helical coiled coil only at an acidic pH, and not at neutral pH. This result suggests that the structure of the α-C region in the MSR could be flexible at neutral pH. An electron microscopic analysis by Resnick and co-workers revealed that the MSR formed a rod-like shape, with a 0° bending angle around the α-C region at neutral pH, whereas the α-helical coiled coil and the collagen-like domains were extended with a hinge angle of 20−180° between them at pH 4.3 (*30*). This suggests that the α-C region adopts a more rigid structure at an acidic pH than at a neutral pH. These results are consistent with our results that the $(\alpha\text{-C})_3$ peptide has a disordered structure at pH 7, but an α-helical coiled coil structure at pH 5. At a neutral pH, such as that on a cell surface, the MSR might bend around the α-C region, forming a different structure than an α-helical structure. At the acidic pH in the endosomal compartment, the receptor may have an extended structure with the α-helical coiled coil structure around the α-C region. On the other hand, we demonstrated that the α-C region in the α peptide had a stable α-helical coiled coil at pH 7 (Figure 4). How can this discrepancy be explained? The MSR might be expressed with a bent form around the α-C region during the folding process or by the aid of a chaperone. Therefore, the α-C region in the MSR could form a structure different from the thermodynamically stable conformation. There might be intramolecular interactions to retain the bent form between the collagen-like domain and the α-helical coiled coil or the spacer domains.

Doi *et al.* suggested that His^{260} in the α-helical coiled coil domain influenced a certain conformational change in a pH-dependent manner (*15*). We now propose additional mechanisms in which acidic residues may cause the pH-dependent conformational change of the α-C region. In the α-C region, there are nine acidic residues and two basic residues at the *e* and *g* positions, adjacent to the hydrophobic core of the heptad repeat, and therefore there is a net charge of −7 (Figure 9). These negative-rich sequences may cause electrostatic repulsions that destabilize the coiled coil structure of the α-C region at a neutral pH. At an acidic pH, however, the protonation of the acidic residues alleviates the electrostatic repulsions because of the removal of negative charges. The HA2 loop region of influenza hemagglutinin (*31*), the Fos leucine zipper homodimer (*32*), and the model peptide E/E35 (*25*) display pH dependencies similar to that of the $(\alpha\text{-C})_3$ peptide. They also have negative-rich sequences at the *e* and *g* positions. Furthermore, Glu^{242} exists at the *d* position in the hydrophobic core in the α-C region (Figure 9). And this residue is conserved among bovine, human, mouse, and rabbit. The electrostatic repulsions by Glu^{242} in the hydrophobic core might also contribute to the conformational change.

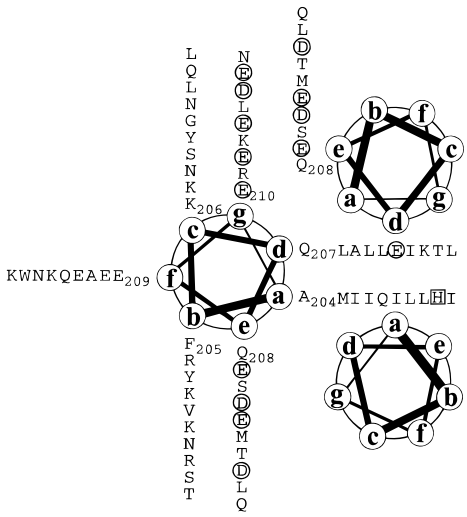

FIGURE 9: Helical wheel diagram of the α-C region. The sequence corresponding to the α-C region of bovine macrophage scavenger receptor (residues 204−271) has a heptad pattern, $(abcdefg)_n$. Encircled are acidic residues at the *e* and *g* positions or the *d* position, and boxed is His^{260} which was reported to influence ligand release (*15*).

In this study, we could not show clear evidence that the ligand release by the MSR arose from the conformational change of the α-helical coiled coil domain. However, we could show the pH-dependent conformational change of the α-C region. This may lead to the structural change of the ligand binding site in the collagen-like domain, which then causes ligands to dissociate from the MSR. Alternatively, there might be an unknown, second ligand binding site in the α-helical coiled coil or spacer domains, since the collagen-like domain is in close proximity to these domains. In the endosome, the α-C region may be extended. As a result, it prevents the ligand from interacting with both binding sites, and finally the ligand is dissociated.

The studies of the structures and functions of the site-directed mutants should provide further information concerning the role of electrostatic charges and His^{260} in the conformational change of the α-helical coiled coil domain and the ligand release by the MSR.

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REFERENCES

1. Anderson, R. G. W., Brown, M. S., Beisiegel, U., and Goldstein, J. L. (1982) *J. Cell Biol. 93*, 523−531.
2. Brown, M. S., Anderson, R. G. W., and Goldstein, J. L. (1983) *Cell 32*, 663−667.
3. Krupp, M. N., and Lane, M. D. (1982) *J. Biol. Chem. 257*, 1372−1377.
4. Wall, D. A., Wilson, G., and Hubbard, A. L. (1980) *Cell 21*, 79−93.
5. Hopkins, C. R., and Trowbridge, I. S. (1983) *J. Cell Biol. 97*, 508−521.
6. Dunn, W. A., and Hubbard, A. L. (1984) *J. Cell Biol. 98*, 2148−2159.
7. Geuze, H. J., Slot, J. W., Strous, G. J. A. M., Peppard, J., von Figura, K., Hasilik, A., and Schwartz, A. L. (1984) *Cell 37*, 195−204.
8. Woods, J. W., Doriaux, M., and Farquhar, M. G. (1986) *J. Cell Biol. 103*, 277−286.
9. Krieger, M. (1992) *Trends Biochem. Sci. 17*, 141−146.
10. Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P., and Krieger, M. (1990) *Nature 343*, 531−535.
11. Rohrer, L., Freeman, M., Kodama, M., and Krieger, M. (1990) *Nature 343*, 570−572.
12. Doi, T., Higashino, K., Kurihara, Y., Wada, Y., Miyazaki, T., Nakamura, H., Uesugi, S., Imanishi, T., Kawabe, Y., Itakura, H., Yazaki, Y., Matsumoto, A., and Kodama, T. (1993) *J. Biol. Chem. 268*, 2126−2133.
13. Acton, S., Resnick, D., Freeman, M., Ekkel, Y., Ashkenas, J., and Krieger, M. (1993) *J. Biol. Chem. 268*, 3530−3537.
14. Tanaka, T., Nishikawa, A., Tanaka, Y., Nakamura, H., Kodama, T., Imanishi, T., and Doi, T. (1996) *Protein Eng. 9*, 307−313.
15. Cohen, C., and Parry, D. A. D. (1990) *Proteins: Struct., Funct., Genet. 7*, 1−15.
16. Betz, S. F., and DeGrado, W. F. (1996) *Biochemistry 35*, 6955−6962.
17. Harbury, P. B., Zhang, T., Kim, P. S., and Alber, T. (1993) *Science 262*, 1401−1407.
18. Doi, T., Kurasawa, M., Higashino, K., Imanishi, T., Mori, T., Naito, M., Takahashi, K., Kawabe, Y., Wada, Y., Matsumoto, A., and Kodama, T. (1994) *J. Biol. Chem. 269*, 25598−25604.
19. Oas, T. G., and Endow, S. A. (1994) *Trends Biochem. Sci. 19*, 51−54.
20. Cohn, E. J., and Edsall, J. T. (1943) in *Proteins, Amino Acids and peptides as Ions and Dipolar Ions*, pp 370−381, Reinhold Publishing Corp., New York.
21. Tanaka, T., Wada. Y., Nakamura, H., Doi, T., Imanishi, T., and Kodama, T. (1993) *FEBS Lett. 334*, 272−276.
22. Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) *Biochemistry 11*, 4120−4131.
23. Peteranderl, R., and Nelson, H. C. M. (1992) *Biochemistry 31*, 12272−12276.
24. Kohn, W. D., Kay, C. M., and Hodges, R. S. (1995) *Protein Sci. 4*, 237−250.
25. Graddis, T. J., Myszka, D. G., and Chaiken, I. M. (1993) *Biochemistry 32*, 12664−12671.
26. Zhou, N. E., Kay, C. M., and Hodges, R. S. (1994) *Protein Eng. 7*, 1365−1372.
27. Lau, S. Y. M., Taneja, A. K., and Hodges, R. S. (1984) *J. Biol. Chem. 259*, 13253−13261.
28. Geoghegan, K. F., and Stroh, J. G. (1992) *Bioconjugate Chem. 3*, 138−146.
29. Anachi, R. B., Siegel, D. L., Baum, J., and Brodsky, B. (1995) *FEBS Lett. 368*, 551−555.
30. Resnick, D., Chatterton, J. E., Schwartz, K., Slayter, H., and Krieger, M. (1996) *J. Biol. Chem. 271*, 26924−26930.
31. Carr, A. M., and Kim, P. S. (1993) *Cell 73*, 823−832.
32. O'Shea, E. K., Rutkowski, R., and Kim, P. S. (1992) *Cell 68*, 699−708.

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