Role of the Buried Glutamate in the α-Helical Coiled Coil Domain of the Macrophage Scavenger Receptor

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ABSTRACT: The macrophage scavenger receptor exhibits a pH-dependent conformational change around the carboxy-terminal half of the α-helical coiled coil domain, which has a representative amino acid sequence of a $(defgabc)_n$ heptad. We previously demonstrated that a peptide corresponding to this region formed a random coil structure at pH 7 and an α-helical coiled coil structure at pH 5 [Suzuki, K., Doi, T., Imanishi, T., Kodama, T., and Tanaka, T. (1997) *Biochemistry 36*, 15140−15146]. To determine the amino acid responsible for the conformational change, we prepared several peptides in which the acidic amino acids were replaced with neutral amino acids. Analyses of their structures by circular dichroism and sedimentation equilibrium gave the result that the presence of Glu^{242} at the *d* position was sufficient to induce the pH-dependent conformational change of the α-helical coiled coil domain. Furthermore, we substituted a Glu residue for the Ile residue at the *d* or *a* position of a de novo designed peptide $(IEKKIEA)_4$, which forms a highly stable triple-stranded coiled coil. These peptides exhibited a pH-dependent conformational change similar to that of the scavenger receptor. Therefore, we conclude that a buried Glu residue in the hydrophobic core of a triple-stranded coiled coil has the potential to induce the pH-dependent conformational change. This finding makes it possible to elucidate the functions of natural proteins and to create a de novo protein designed to undergo a pH-dependent conformational change.

The macrophage scavenger receptor (MSR)[1] is a trimeric membrane protein that plays a key role in atherogenesis. The MSR binds ligands such as oxidized low-density lipoproteins. After the MSR-bound ligands are internalized by the cell, the MSR dissociates from the ligands in the acidic endosome and is recycled (*1*). The structure of the MSR consists of six domains: cytoplasmic, membrane-spanning, spacer, α-helical coiled coil, collagen-like, and Cys-rich domains (*2*). The Lys cluster of the collagen-like domain has been identified as a ligand binding site (*3*−*6*). The α-helical coiled coil domain was reported to participate not only in the trimerization of the MSR (*3*) but also in the dissociation of the ligands in the endosome (*7*).

The α-helical coiled coil structure has a seven-amino acid "heptad" repeat sequence, $(abcdefg)_n$, with hydrophobic residues at the *a* and *d* positions (*8*−*10*). In the bovine MSR, the heptad repeats are between amino acids 109 and 271, and contain a discontinuity as a three-residue deletion after Asn^{203} (*2*). This domain, therefore, can be separated into two parts at Asn^{203}, the N-terminal half (α-N) and the C-terminal half (α-C). His^{260}, which was reported to participate in the ligand release in the acidic endosome (*7*), is present at the *a* position in the α-C region. It has been suggested that His^{260} disrupts the coiled coil structure at an acidic pH through interhelical electrostatic repulsion (*2*) or rupture of hydrogen bonds (*7*). This conformational change is thought to influence the structure of the collagen-like domain, causing ligand release from the MSR.

We previously demonstrated that a peptide corresponding to the α-C region actually changed its conformation in a pH-dependent manner (*11*). However, the manner of the conformational change was different from that previously proposed (*2*, *7*). The peptide formed a random structure at pH 7 and an α-helical coiled coil structure at pH 5 (*11*). This result was supported by the electron microscopic image of the extracellular domain of the MSR (*12*). Therefore, we suggested that the α-C region of the MSR forms a different structure than an α-helix at the neutral pH on the cell surface and an α-helical coiled coil at the acidic pH in the endosome (*11*). In addition, we proposed that interhelical electrostatic repulsions by acidic residues in the α-C region might destabilize the coiled coil structure at neutral pH (*11*).

In the α-C regions of the MSRs from human, mouse, rabbit, and bovine sources, several acidic residues are conserved at or near the hydrophobic core. In the bovine MSR, for example, Glu^{242} is present at the *d* position. Glu^{236} and Asp^{257} are present at the *e* positions, and Glu^{238}, Glu^{259}, and Asp^{252} are at the *g* positions. These residues might participate in the pH-dependent conformational change of the α-C region. Therefore, we prepared several mutant peptides corresponding to the α-C region in the bovine MSR and analyzed the effects of these acidic amino acids on the pH-dependent conformational change. We identified Glu^{242} as the amino acid primarily responsible for the pH-dependent conformational change. Furthermore, we report here that one Glu residue in the hydrophobic core is sufficient to induce

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[1] Abbreviations: MSR, macrophage scavenger receptor; PCR, polymerase chain reaction; GST, glutathione *S*-transferase; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; Fmoc, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; CD, circular dichroism.

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the conformational change of a designed peptide, from a random coil at neutral pH to an α-helical coiled coil at an acidic pH. Thus, a buried Glu residue in the hydrophobic core of a triple-stranded coiled coil, such as Glu^{242} of the MSR, induces the pH-dependent conformational change. This finding should be useful not only in elucidating the function of the natural proteins but also in creating de novo proteins designed to change their conformations in response to pH.

MATERIALS AND METHODS

Construction of Expression Plasmids. The H260L α-C (parent) peptide gene plasmid was constructed as follows. The H260L MSR gene plasmid (*7*) was used as a template. Polymerase chain reaction (PCR) was carried out using the primers 5′-CCGGGATCCGTGTCCAAGAGAATGCATT-TAAACAAC-3′, which is upstream of the *Xba*I site, and 3′-GTGAAATGAGGTTCCAGGA**ATT**CTTAAGGCC-5′(including a stop codon, in bold 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 *Xba*I−*Eco*RI. The fragments were ligated into the *Xba*I−*Eco*RI sites of the plasmid expressing the wild-type α-C peptide fused to glutathione *S*-transferase (GST) (*11*).

The genes for the other mutants were prepared by PCR, using the parent peptide gene plasmid as a template. The PCR primers were 5′-TCAACAGTTTCATTACCCCTTTTA-3′ and 5′-TAAAAGGGGTAATGAAACTGTTGA-3′ (including the mutation site, underlined) for the E242V [*d*(L)] mutant, 5′-CCCTTTTATTTGCTGTTGCAAATA-3′ and 5′-TATTTGCAACAGCAAATAAAAGGG-3′ (including the mutation site, underlined) for the E236Q/E238Q [*eg*(L)] mutant, and 5′-AGAGAGCTGCCAATTCTTCAGCCTCA-GATTATTAGT-3′ and 5′-ACTAATAATCTGAGGCTGAA-GAATTGGCAGCTCTCT-3′ (including the mutation site, underlined) for the D252N/D257N/E259Q [*geg*(L)] mutant. The PCR products were treated with *Avr*II−*Eco*RI, and the fragments were ligated into the *Avr*II−*Eco*RI sites of the GST gene fusion vector, pGEX-3X-StuI (*11*).

The plasmids were transformed into *Escherichia coli* JM109 cells. The nucleotide sequences were confirmed by DNA sequencing (ABI 373A DNA Sequencer).

Expression and Purification of Peptides. The GST fusion peptides were expressed and were treated with Factor Xa (New England Biolabs, Inc.) as described previously (*11*). The obtained peptides were applied to a YMC-Pack ODS-A column (1.0 cm × 25 cm, 120 Å, 5 μm, YMC Inc.) and were eluted with a linear gradient of 35 to 60% CH_3CN/H_2O containing 0.1% TFA over the course of 30 min. The corresponding peak was collected, concentrated, and lyophilized. The peptides were identified by MALDI-TOF mass spectroscopy (PerSeptive Biosystems Voyager Elite).

Peptide Synthesis. Peptides were synthesized by the solid-phase peptide synthesis method with Fmoc chemistry on an ABI 430A peptide synthesizer. Deprotection and cleavage were performed by treatment with TFA containing 5% ethanedithiol and anisole (1/3, v/v) for 1.5 h. Peptide purifications were carried out on a YMC-Pack ODS-A column with a linear gradient of 30 to 50% CH_3CN/H_2O containing 0.1% TFA over the course of 30 min. The final products were characterized by analytical HPLC and MALDI-TOF mass spectrometry.

Circular Dichroism (CD). CD measurements were carried out on a JASCO 720 spectrometer with the sample in a 1 mm path length cuvette. The spectra of the α-C mutants were obtained with 10 μM peptide solutions in 10 mM phosphate buffer containing 0.1 M NaCl (pH 7 or 4.5) at 20 °C. The spectra were recorded five times for each sample with a bandwidth of 2 nm and a step resolution of 2 nm, using a J-720 series spectropolarimeter system (JASCO). The mean residue ellipticity, $[\theta]$, is given in units of degrees cm^2 $dmol^{-1}$. To determine the effect of pH on α-helical content, the $[\theta]_{222}$ values of the synthetic peptides were monitored as a function of pH from pH 3.2 to 8.6 in the same buffer at 20 °C.

Sedimentation Equilibrium Ultracentrifugation. Sedimentation equilibrium analysis was performed with a Beckman XL-I optima analytical ultracentrifuge equipped with absorbance optics. The initial peptide concentrations were 50 μM for both the H260L parent peptide and the *d*(L) mutant, 40 μM for the *eg*(L) mutant, 20 μM for the *geg*(L) mutant, and 100 μM for the synthetic peptides, in 10 mM phosphate buffer containing 0.1 M NaCl. The α-C mutants were centrifuged at 15 000 rpm, except for the *d*(L) and *geg*(L) mutants at pH 7, which were centrifuged at 17 000 rpm. The synthetic peptides were centrifuged at 25 000 rpm. Measurements were performed at 20 °C with the absorbance being monitored at 280 nm. The aggregation state was determined by fitting the data to a single species, using Origin Sedimentation Equilibrium Single Data Set Analysis (Beckman). The partial specific volumes were 0.741 and 0.740 mL/g for the *geg*(L) mutants and the other α-C mutants, respectively, and 0.768 mL/g for the synthetic peptides, which were calculated from the weighted average of the amino acid content using the method of Cohn and Edsall (*13*).

RESULTS

Analysis of the Sequences of the α-C Regions of the MSRs. In the previous study, we proposed that interhelical electrostatic interactions by acidic residues might control the pH-dependent conformational change of the α-C region (*11*). To predict the amino acid residues responsible for the conformational change, we compared the amino acid sequences of the α-C regions from various MSRs (Figure 1). We considered that important acidic residues for the interhelical electrostatic interactions would exist at or near the hydrophobic interface, the *a*, *d*, *e*, and *g* positions, and that these residues would be highly conserved among the MSRs of various species.

As shown in Figure 1, there are three sets of residues that satisfy these requirements, Glu^{242}, Glu^{236}/Glu^{238}, and Asp^{252}/Asp^{257}/Glu^{259}. Glu^{242} at the *d* position might be centered in the hydrophobic core of the α-helical coiled coil. Glu^{236} at the *e* position might face Glu^{238} at the *g* position of the neighboring strand. Asp^{252}, Asp^{257}, and Glu^{259} at the *g*, *e*, and *g* positions, respectively, might form an acidic cluster. These sets of acidic residues, therefore, could destabilize the coiled coil structure at neutral pH through the interhelical electrostatic repulsions. At first, we analyzed the effect of His^{260}, which was previously proposed to influence the conformation of the α-helical coiled coil domain. We then determined the amino acid responsible for the pH-dependent conformational change among the above three sets of acidic residues.

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                204      211      218      225      232      239      246      253      260      267
                 a  de g a  de g a  de g a  de g a  de g a  de g a  de g a  de g a  de g a  de
human :    TFKQQEE ISKLEER VYNVSAE IMAMKEE QVHLEQE IKGEVKV LNNITND LRLKDWE HSQTLRN ITLIQGP
mouse :    TAKQQED ISKLEER VYKVSAE VQSVKEE QAHVEQE VKQEVRV LNNITND LRLKDWE HSQTLKN ITFIQGP
rabbit:    TLKGQEE ISKLKER VHNASAE IMSMKEE QVHLEQE IKREVKV LNNITND LRLKDWE HSQTLRN ITLIQGP
bovine:    AFKQQEE MRKLEER IYNASAE IKSLDEK QVYLEQE IKGEMKL LNNITND LRLKDWE HSQTLKN ITLLQGP

α-C   : SS------- ------- ------- ------- ------- ------- ------- ------- ------- -------
H260L : SS------- ------- ------- ------- ------- ------- ------- ------- L------ -------
d(L)  : SS------- ------- ------- ------- ------- ---V--- ------- ------- L------ -------
eg(L) : SS------- ------- ------- ------- ----Q-Q ------- ------- ------- L------ -------
ege(L): SS------- ------- ------- ------- ------- ------- ------N ----N-Q L------ -------
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FIGURE 1: Sequences of the α-C regions of the MSRs from human, mouse, rabbit, and bovine sources. Conserved acidic residues at the *a*, *d*, *e*, and *g* positions are in boldface. Numbers on the top indicate the positions of the first residues of the heptad repeats in the bovine MSR.

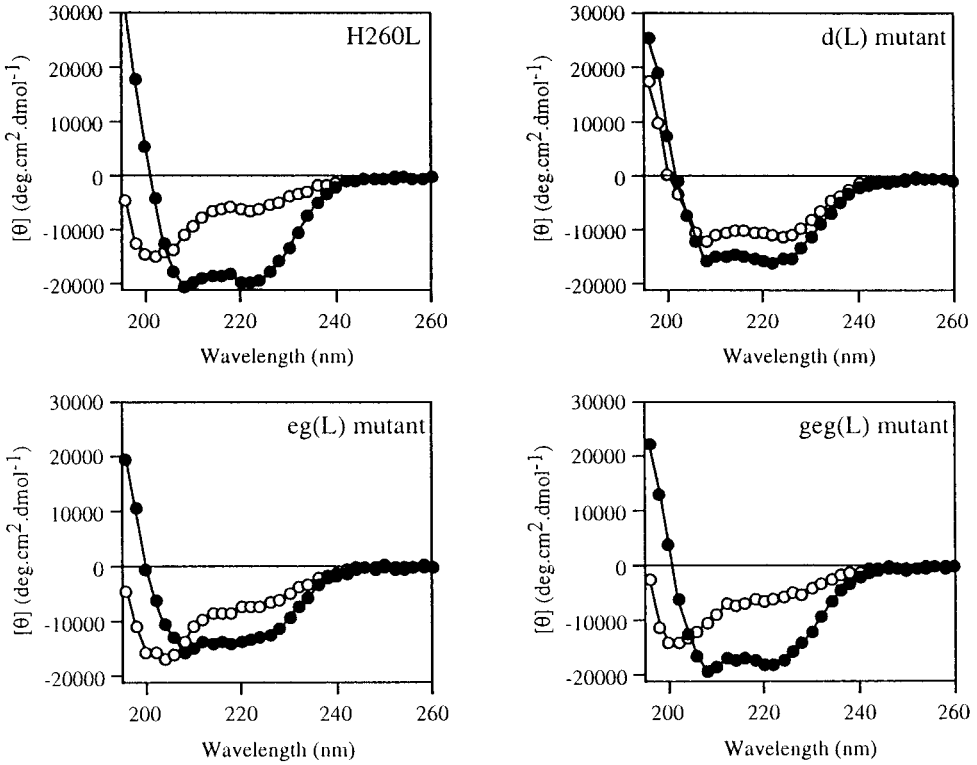

FIGURE 2: Circular dichroism spectra of the H260L, *d*(L), *eg*(L), and *geg*(L) peptides. Measurements were performed in 10 mM sodium phosphate and 0.1 M NaCl at pH 7.0 (○) and 4.5 (●). The peptide concentrations were 10 μM.

Effect of His^{260} on the pH-Dependent Conformational Change. We previously prepared the α-C peptide, corresponding to the α-C region of the MSR, which did not form an α-helical structure under either neutral or acidic conditions (*11*). Only when the N termini of the three peptide chains were chemically cross-linked, the peptide exhibited the pH-dependent conformational change (*11*).

When His^{260} of the α-C peptide was replaced with Leu, the peptide (H260L) showed an α-helical structure, with minima at 222 and 208 nm at pH 4, while it showed a random structure at pH 7 (Figure 2). The apparent molecular weight of the peptide, determined by sedimentation equilibrium analysis, indicated that it was trimerized at the acidic pH and monomeric at neutral pH (Figure 3 and Table 1). Thus, the H260L peptide showed the same conformational change as the α-C region of the natural MSR. These results suggest that the substitution of Leu for His^{260} stabilized the coiled coil structure of the α-C region, and that His^{260} might not be crucial for the pH-dependent conformational change of the α-C region. The H260L peptide is suitable as a parent peptide for the following mutation analyses, since the peptide, without the N-terminal cross-linking, showed the same structural properties as the natural MSR, leading to the easy preparation of the various mutants.

Table 1: Sedimentation Equilibrium Analyses of the α-C Mutants

		apparent MW	
	calculated MW	pH 7.0	pH 4.5
H260L	8482	9014 (1.1)[a]	24608 (2.9)
d(L)	8481	29005 (3.4)	24087 (2.8)
eg(L)	8480	–	26757 (3.2)
geg(L)	8479	9780 (1.2)	25937 (3.1)

[a] Apparent MW/calculated MW.

Effect of Acidic Residues on the pH-Dependent Conformational Change. As described above, we used the H260L peptide as the parent peptide and prepared three peptide variants, in which the sets of acidic amino acids on the

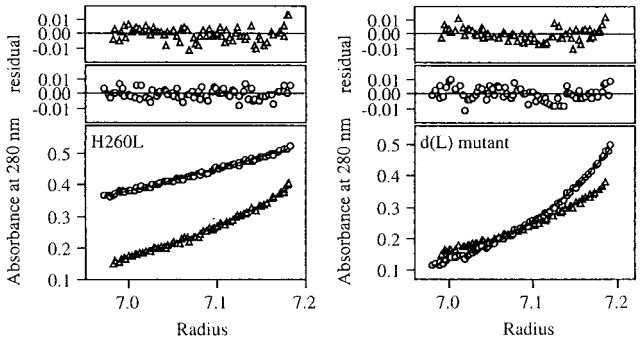

FIGURE 3: Sedimentation equilibrium analyses of the H260L and *d*(L) peptides at pH 7.0 (○) and 4.5 (△). The experimental procedures are described in Materials and Methods. The data are shown fit to a single ideal species. The residuals are random and centered around zero, indicating that both the H260L and *d*(L) peptides sediment as a single homogeneous species. The data for the *eg*(L) and *geg*(L) peptides, which also fit to a single ideal species, gave similar residuals (data not shown).

H260L peptide were mutated to neutral amino acids. Glu^{242} at the *d* position was mutated to Val, and was designated *d*(L), because a Val residue often appears at the *d* position of a triple-stranded coiled coil (*14*). Then, Glu^{236} at the *e* position and Glu^{238} at the *g* position were both mutated to Gln, and the mutant was designated *eg*(L). Finally, Asp^{252}, Asp^{257}, and Glu^{259} at the *g*, *e*, and *g* positions, respectively, were mutated to Asn for Asp^{252} and Asp^{257} and Gln for Glu^{259}, and the mutant was designated *geg*(L).

The conformation and the oligomerization states of the peptides at pH 7 and 4.5 were determined by CD spectra (Figure 2) and sedimentation equilibrium (Figure 3 and Table 1) analyses. The results for the *eg*(L) peptide and the *geg*(L) peptide were similar to that of the parent peptide, which showed the monomeric random structure at pH 7 and the trimerized α-helical structure at pH 4.5. However, the *d*(L) peptide showed the α-helical structure at pH 7 as well as at pH 4.5 (Figure 2). The peptide was trimerized under both conditions (Table 1). Thus, the *d*(L) peptide did not exhibit the pH-dependent conformational change. These results suggest that Glu^{242} is mainly responsible for the pH-dependent conformational change of the MSR.

Effect of a Buried Glutamate in the Hydrophobic Core of a Designed Triple-Stranded α*-Helical Coiled Coil.* To determine if this role of the buried Glu residue is a general rule in triple-stranded coiled coils, we introduced a Glu residue at the *a* or *d* position of a designed coiled coil peptide and analyzed its structure. We have prepared a de novo-designed α-helical coiled coil peptide, IZ, which has the amino acid sequence $I^dE^eK^fK^gI^aE^bA^c$ with four repeats of the heptad (*15*) (Figure 4). The IZ was already shown to have a parallel triple-stranded coiled coil structure with native-like folding, and the structure was stable toward heat denaturation, with a T_m of >80 °C (*15*). We substituted Glu for the Ile at the *d* or *a* position in the second heptad repeat of the IZ, and designated the mutants IZ-2*d*E and IZ-2*a*E, respectively (Figure 4). These substitutions dramatically destabilized the coiled coil structures at neutral pH. The IZ-2*d*E and IZ-2*a*E peptides did not show any α-helical properties in the CD spectra at pH 7 and 20 °C. On the contrary, they showed a typical α-helical structure at pH 4.5 (data not shown). The pH titration curves of the IZ, IZ-2*d*E, and IZ-2*a*E peptides were obtained by monitoring the $[\theta]_{222}$ values. As shown in

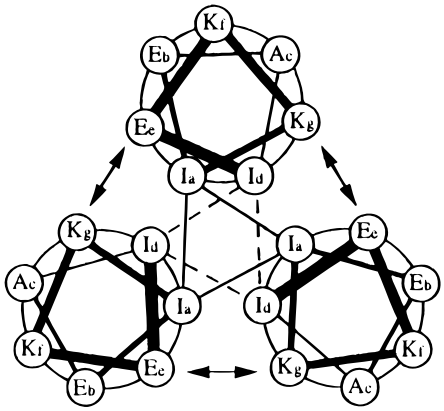

FIGURE 4: Helical wheel representation of the heptad pattern of the IZ peptide, viewed from the N to the C terminus, and the sequences of the IZ variants. The IZ peptide has a parallel triple-stranded coiled coil structure with a native-like folding pattern (*16*). In the IZ-2*d*E and IZ-2*a*E peptides, single Glu residues were substituted for the Ile residues at the *d* and *a* positions, respectively, in the second heptad repeat of the IZ.

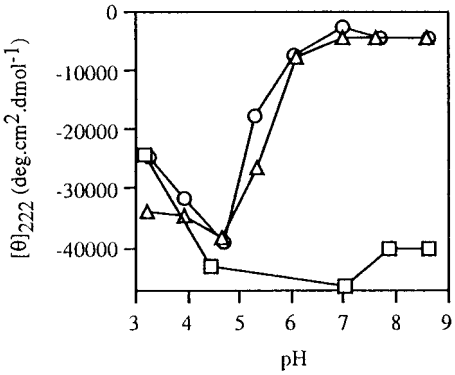

FIGURE 5: pH dependence of the IZ variants. Measurements were performed in sodium phosphate and 0.1 M NaCl under various pH conditions. The peptide concentrations were 20 μM: IZ (□), IZ-2*d*E (△), and IZ-2*a*E (○).

Figure 5, the IZ-2*d*E and IZ-2*a*E peptides exhibited pH-dependent conformational changes with transition midpoints at pH 5−6, whereas the IZ peptide did not change its conformation around that pH. Sedimentation equilibrium analyses showed that the IZ-2*d*E and IZ-2*a*E peptides were trimerized at the acidic pH and were monomeric at neutral pH (Figure 6 and Table 2). These results suggest that, like the α-C region of the MSR, the buried Glu residues in the designed triple-stranded coiled coil peptide induce the pH-dependent conformational change through interhelical electrostatic interactions.

DISCUSSION

The MSR releases the ligands in the acidic endosome, whereas the His^{260} mutant of the MSR lost the ability to release the ligands (*7*). It was proposed that protonation of His^{260} perturbed the structure of the α-C region, which causes the ligand release from the MSR (*2*, *7*). However, our previous results and the electron microscopic study suggested that the α-C region formed a coiled coil structure at an acidic

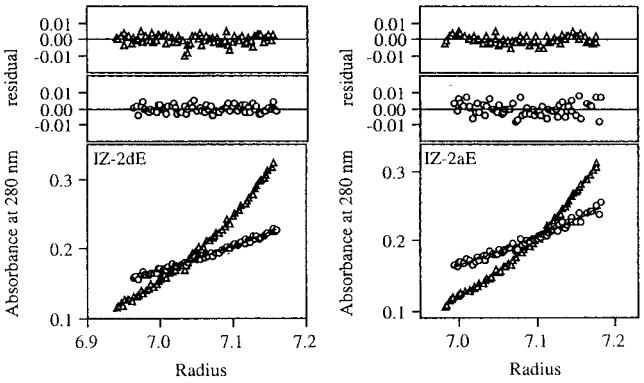

FIGURE 6: Sedimentation equilibrium analysis of the IZ-2*d*E and IZ-2*a*E peptides at pH 7.0 (○) and 3.3 (△). The concentrations of the peptides were 100 μM. The samples were centrifuged at 25 000 rpm at 20 °C. The partial specific volume used for the data analysis was 0.768 mL/g. The data fit to a single ideal species, and the residuals are random and centered around zero.

Table 2: Sedimentation Equilibrium Analyses of the Synthetic Peptides

		apparent MW	
	calculated MW	pH 7.0	pH 3.3
IZ[a]	3542.22	10736 (3.0)[b]	–
IZ-2dE	3558.18	4036 (1.1)	11726 (3.3)
IZ-2aE	3558.18	4860 (1.4)	11424 (3.2)

[a] From ref *16*. [b] Apparent MW/calculated MW.

pH and a disordered structure at neutral pH (*11*, *12*). Thus, the following question is raised. How is His[260] involved in this pH-dependent conformational change?

To address this question, we substituted Leu for His[260] in the peptide corresponding to residues 204−273 of the bovine MSR. The substitution stabilized the triple-stranded coiled coil structure at an acidic pH, but not at neutral pH (Figure 2 and Table 1). Thus, the H260L peptide showed a pH-dependent conformational change similar to that of the natural MSR, suggesting that His[260] does not play a major role in the pH-dependent conformational change. In another experiment, we substituted His for one Ile at the *a* position of the de novo-designed IZ peptide, which was already shown to form a triple-stranded coiled coil structure (*15*). This substitution dramatically destabilized the coiled coil structure of the peptide but did not induce the pH-dependent conformational change (unpublished data). Therefore, we speculate that His[260] might reduce the stability of the coiled coil structure to make the α-helical coiled coil domain more susceptible to the conformational change by the pH change. Alternatively, the primary role of His[260] might be to participate in another process, rather than in the pH-dependent conformational change.

In contrast, the conformational change, from a random coil at neutral pH to an α-helical coiled coil at an acidic pH, implies the participation of acidic residues at the interface of the coiled coil. Therefore, we analyzed the effects of the acidic residues on the coiled coil formation. In MSRs, eight acidic residues are conserved at the *a*, *d*, *e*, and *g* positions in the α-C region (Figure 1). The Glu or Asp residue at position 210 and the Glu at position 224 are not thought to participate in the pH-dependent conformational change, because no partners for interhelical repulsion are conserved around these positions. On the other hand, Glu[242], Glu[236] and Glu[238], and Asp[252], Asp[257], and Glu[259] could cause interhelical electrostatic repulsions. Therefore, we prepared three mutants, *d*(L), *eg*(L), and *geg*(L), in which Glu[242], Glu[236] and Glu[238], and Asp[252], Asp[257], and Glu[259] were replaced with neutral amino acids, respectively. The *eg*(L) and *geg*(L) mutants underwent a conformational change similar to that of the natural MSR. However, only the *d*(L) mutant could form the triple-stranded coiled coil under neutral conditions (Figure 2 and Table 1). These results suggest that Glu[242] plays a crucial role in the pH-dependent conformational change. The possibility that the other residues partially contribute to the conformational change cannot be ruled out. However, the single substitution of Glu for Ile at the *d* or *a* position of the IZ peptide induced a pH-dependent conformational change similar to that of the MSR (Figure 5 and Table 2). Therefore, it can be concluded that only one Glu residue in the hydrophobic core can accommodate the pH-dependent conformational change of the triple-stranded coiled coil.

The residues at the *e* and *g* positions of coiled coils are often charged (*8*−*10*), and the interhelical interactions between these residues have been well characterized (*16*−*18*). The crystal structures of leucine zipper peptides showed the existence of interhelical Glu−Lys salt bridges between the *g* and the succeeding *e*′ positions (*19*−*21*). In the case of designed triple-stranded peptides, the salt bridges formed both between the above positions and between the *e* and *g*′ positions within the same heptad (*22*, *23*). On the other hand, when several Glu residues were aligned at both the *e* and *g* positions, they caused interhelical repulsion. In this case, the conformations of both double-stranded (*24*−*26*) and triple-stranded (*27*) coiled coils changed in a pH-dependent manner, similar to that of the MSR. In the MSR, however, Glu[236]/Glu[238] and Asp[252]/Asp[257]/Glu[259] do not seem to contribute much to the pH-dependent conformational change. The net electrostatic contribution per Glu−Glu repulsion between the *e* and *g* positions of the double-stranded coiled coil was estimated to be 0.5 kcal/mol (*18*). These results suggest that a single interhelical electrostatic interaction might be too weak to induce the conformational change.

The effects of polar residues in the hydrophobic core are also well characterized (*28*−*32*). A Gln residue at the *a* position favors the formation of a triple-stranded coiled coil (*30*, *31*). The substitution of Gln for Asn at the *a* position of the GCN4 leucine zipper allowed the peptide to form both a dimer and a trimer (*32*). In the crystal structure of the Gln mutant dimer, the Gln side chains adopted extended conformations in the direction away from the hydrophobic core (*32*). In the Gln mutant trimer crystal structure, however, the Gln side chains were oriented toward the inside of the hydrophobic core and were buried more than in the dimer (*32*). Although the orientation of the Gln side chain at the *d* position is still unknown, the orientation of the side chains of the Glu residues at both the *d* and *a* positions of the peptides in the present study might also be toward the inside of the hydrophobic core. In these cases, the side chains of the buried Glu residues might repel each other or lose their solvation in the putative coiled coil structure at neutral pH, thereby destabilizing the coiled coil structure. On the other hand, the protonated Glu residues at pH <4 would eliminate the unfavorable interactions and stabilize the coiled coil structure. A comparison of the dimer and the trimer of the Gln-substituted GCN4 leucine zipper suggests that the buried

Glu residue in the triple-stranded coiled coil might be more influential in that conformation than in the double-stranded one. Actually, the c-Myc leucine zipper peptide, which has two Glu residues at the *a* positions, showed a slightly pH-dependent conformational change (*33*, *34*).

Here we have provided evidence that the buried Glu residue can control the pH dependence of the triple-stranded coiled coil. Other natural proteins with a Glu residue at the *d* or *a* position of the triple-stranded α-helical coiled coil should be considered as being potentially able to undergo a conformational change in response to pH. Our results are also useful for the design of de novo proteins with a conformational change around pH 4−5.

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