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Structure and Function of the Recombinant Fifth Domain of Human β_2-Glycoprotein I: Effects of Specific Cleavage between Lys77 and Thr78[1]

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In order to elucidate the mechanism of binding of β_2-glycoprotein I (β_2-GPI) to cardiolipin (CL), we constructed a high-level expression system for the C-terminal domain (Domain V) of β_2-GPI using *Pichia pastoris* and studied its conformation and liposome-binding activity. Purified Domain V was found to have the native disulfide bonds. It had a compactly folded conformation, judging from the circular dichroism spectrum, and exhibited a cooperative unfolding transition induced by pH or urea. Also, it bound liposomes containing CL. Commercially available human β_2-GPI is known to be selectively cleaved between Lys 317 and Thr 318. We found that bovine factor Xa weakly but specifically cleaves the corresponding site of recombinant Domain V, *i.e.*, the peptide bond between Lys 77 and Thr 78. The conformation of the "nicked" Domain V, which was cleaved at this site, was examined by circular dichroism and fluorescence measurements, and concluded to be similar to that of the intact protein. The stability of the nicked Domain V to urea was slightly lower than that of the intact protein. Although both Domains V bound to liposomes containing CL, the affinity of the nicked Domain V was greatly reduced in comparison with the intact protein, indicating that the cleavage of the peptide bond between Lys 77 and Thr 78 controls the binding to CL. In addition, analysis of the fluorescence spectra in the presence and absence of CL liposomes indicated that Trp 76 is involved in the binding site. These results suggest that the region including Trp 76, Lys 77, and Thr 78 has a critical role in binding to CL.

Key words: anti-cardiolipin syndrome, autoimmune diseases, β_2-glycoprotein I, *Pichia pastoris*, protein-lipid interaction.

β_2-Glycoprotein I (β_2-GPI) is a highly glycosylated plasma protein consisting of a single polypeptide chain (326 amino acids) with a molecular mass of about 50 kDa. Although the function of β_2-GPI is still unclear, it is known to bind negatively charged substances such as DNA, heparin and dextran sulfate, and negatively charged phospholipids such as cardiolipin (CL) (*1-4*). This protein is composed of five repeated domains. Except for the fifth domain (Domain V), each domain has a common motif named the complement control protein (CCP) or Sushi-domain superfamily (*5-7*). Although Domain V has an additional disulfide bond, the location of the other two disulfide bonds is similar to that of the Sushi domain. The tertiary structures of the Sushi domains of human factor H have been established by NMR (*8-10*). The elements of secondary structure are predominantly β-sheets. The three-dimensional structures of the Sushi domains of β_2-GPI seem to be similar to that of factor H.

It has been established that β_2-GPI acts as a cofactor in the recognition of the phospholipid antigen CL by anti-CL antibodies present in patients with autoimmune diseases such as lupus erythematosus (*11-14*). Recent studies suggest that Domain V plays important roles in the binding to CL and expression of cofactor activity (*14-17*). Previously, we found that the N-terminal domain and Domain V have an important role in the interaction of the whole protein with CL liposomes (*18*). Steinkasserer *et al.* (*19*) reported that the recombinant human Domain V, which was

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Abbreviations: β_2-GPI, β_2-glycoprotein I; CAPS, cyclohexylaminopropanesulfonic acid; CD, circular dichroism; CHES, 2-(cyclohexylamino)ethanesulfonic acid; CL, cardiolipin; Domain V, C-terminal domain of β_2-glycoprotein I; HPLC, high-performance liquid chromatography; LUV, large unilamellar vesicles; MES, 2-(*N*-morpholino)ethanesulfonic acid; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; r-Domain V, recombinant Domain V; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UV, ultraviolet.

expressed in *Escherichia coli*, inhibited the interaction of the whole β_2-GPI molecule with CL, suggesting that the isolated Domain V can bind to CL. However, the mechanism of the binding of Domain V to CL and the tertiary structures of Domain V have been unclear. In addition, the location of one of the disulfide bonds was not determined in the recombinant Domain V reported by Steinkasserer *et al.* (*19*).

To clarify the mechanism of the interaction with CL in detail, we prepared the recombinant Domain V of human β_2-GPI using a methylotrophic yeast, *Pichia pastoris*. We found that the recombinant protein has the native disulfide bonds, a proper folded structure and CL membrane-binding activity. During the preparation of recombinant Domain V, we found that treatment of Domain V with bovine factor Xa specifically cleaved the peptide bond between Lys 77 and Thr 78 (Lys 317 and Thr 318 in the whole β_2-GPI molecule). The cleavage of this site was reported previously by Hunt *et al.* (*15*). Another cleavage site (Ala 314 and Phe 315) close to this peptide bond has been found in human and bovine β_2-GPI. Both cleavages critically reduce the binding affinity of β_2-GPI for CL (*15, 18*), suggesting the importance of the region around these sites for liposome binding. Comparison of the structure and affinity for CL of the "nicked" Domain V with those of the intact Domain V suggested that subtle structural change around the cleavage site critically decreases the affinity for CL.

MATERIALS AND METHODS

Construction of Expression Vectors and Transformation into Pichia pastoris—The pUC118-β_2-GPI plasmid containing the full-length human β_2-GPI was modified using site-directed mutagenesis so as to add a *Hin*dIII site to the hinge region between the fourth and fifth domains (*20*). The modified fifth domain was subcloned into *P. pastoris* expression vector, pPIC 9 (Invitrogen), with a linker containing the factor Xa recognition sequence, IEGRT. pPIC 9 carries the secretion signal from the *Saccharomyces cerevisiae* α-mating factor pre-pro peptide. This provided the expression plasmid pNPD5 (Fig. 1). DNA from the expression plasmid was integrated into the host cells, GS115 (Invitrogen), by transforming *his4 P. pastoris* cells with *Bgl*II-digested pNPD5.

For screening of the most efficient transformant, we picked one hundred His^+ transformants on a plate containing 0.5% methanol as a carbon source. After incubation at 30°C, we covered the plate with a nitrocellulose membrane and hybridized this membrane with a rabbit anti-human β_2-GPI antibody (SERBIO).

Production of Domain V in a High-Cell-Density Fermentor and Purification of the Product—The production of Domain V by batch-type fermentation was examined. The culture growth was performed in a 2-liter Mituwa Biosystem fermentor, model KMJ-2C, equipped with monitors and controllers for pH, dissolved oxygen, agitator speed, temperature, and air flow. Inocula for the fermentor run were grown in a 500-ml baffled flask containing 100 ml of BM medium [yeast extract (Difco) 10 g/liter; meat peptone (Sigma) 20 g/liter; yeast nitrogen base (Difco) 6.7 g/liter; D-biotin (Nacalai Tesque) 0.4 mg/liter; 1 M potassium phosphate buffer (pH 6) 100 ml/liter] containing 2% glycerol. The culture was started in growth medium (4% glycerol-BM medium). At 14 h after inoculation, the medium was changed from growth medium to a one-third volume of induction medium (3% methanol-BM medium), and the culture was continued for 13 h to allow efficient expression of the product.

After removal of cells by low-speed centrifugation, the supernatant was diluted in a sixfold volume of buffer containing 50 mM Na acetate (pH 4.5), 50 mM NaCl, and 1 mM EDTA. A sample was applied to a column of CM-Sepharose CL-6B (Pharmacia-LKB Biotechnology), which had been equilibrated with 50 mM Na acetate (pH 4.5) containing 50 mM NaCl and 1 mM EDTA. After the column had been washed, the protein was eluted by a linear salt gradient formed from 300 ml each of the equilibration buffer and the buffer containing 0.8 M NaCl. The peak fractions were collected and concentrated by ultrafiltration using a PM 3 membrane (Amicon) and the concentrate was dialyzed against 1 liter of distilled H_2O for 1 day. Then the dialysate was purified by reversed-phase HPLC using a Cosmosil $5C_{18}$-AR column (20×250 mm: Nacalai Tesque) with an acetonitrile gradient in 0.05% trifluoroacetic acid. Two peaks were obtained. The N-terminal sequences of the major peak (r-Domain V*, see "RESULTS") and the minor peak (r-Domain V, see "RESULTS") were determined to be YVEFMIEG and TKASXKLP, respectively, using a protein sequencer model 473A (Applied Biosystems).

Determination of Disulfide Bond Positions—For determination of the positions of the disulfide bonds, 1 mg of the

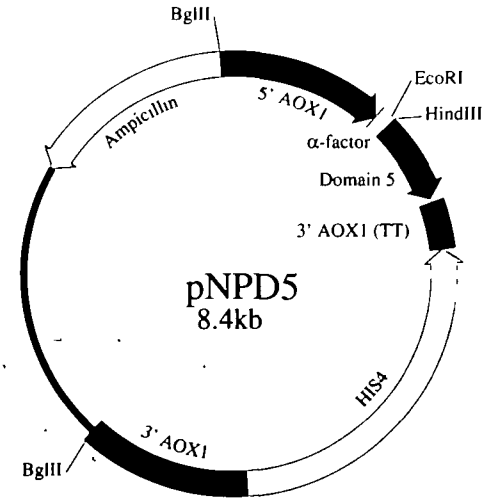

Fig. 1. **Construction of the fifth domain of the human β_2-GPI (Domain V) expression plasmid, pNPD5.** Plasmid pNPD5 contains the gene for Domain V inserted between the *AOX1* promoter (5′ *AOX1*) and the *AOX1* terminator (TT) for methanol-regulated expression of Domain V in *Pichia pastoris*. This plasmid also contains the *HIS4* gene from *P. pastoris* which serves as a selection marker in the transformation of GS115, and the 3′ *AOX1* DNA fragment which contains the 3′ noncoding portion of *AOX1*. The 3′ *AOX1* sequence together with 5′ *AOX1* is necessary for site-directed integration of the linear DNA (derived by *Bgl*II digestion of pNPD5) containing the Domain V expression cassette and *HIS4* into the *P. pastoris AOX1* locus. This plasmid contains the *S. cerevisiae* α-factor sequence and the linker sequence with the factor Xa recognition sequence (IEGRT). The double-stranded linker,

5′-AAT TCA TGA TCG AAG GAA GAA CAA A-3′
3′ -GT ACT AGC TTC CTT CTT GTT TTC GA-5′

was formed by two synthetic oligonucleotides.

purified protein was incubated with 100 μg of *Staphylococcus aureus* V_8 protease (ICN Immuno Biologicals) in 20 mM Na acetate (pH 4.0) containing 20 mM NaCl at 37°C for 24 h. When the peptides had more than one disulfide bond, they were further digested by protease Asp-N (Takara Shuzo) in 50 mM Na phosphate (pH 8) at 37°C for 24 h. The weight ratio of Asp-N to the peptides was 1:50. Isolation of the peptides was performed using Cosmosil $5C_{18}$-AR (4.6×250 mm; Nacalai Tesque) with an acetonitrile gradient in 0.05% trifluoroacetic acid. The N-terminal sequences of all the digested peptides were determined by the protein sequencer.

Removal of the N-Terminal Pro-Sequence—In order to remove the N-terminal pro-sequence including the secretion signal sequence and factor Xa recognition sequence, *i.e.*, YVEFMIEGR, from r-Domain V*, the domain was digested by bovine factor Xa in reaction buffer (60 mM HEPES, 1.3 mM $CaCl_2$, 0.1 M NaCl, 0.1% Na azide, pH 8) at 37°C for 9 h. The weight ratio of factor Xa to r-Domain V* was 1:50. The digest was purified by reversed-phase HPLC, as in the case of the crude recombinant Domain V.

We found that a longer reaction time or a higher concentration of factor Xa caused an unexpected cleavage between Lys77 and Thr78. This cleavage site is also a "nicked" site of human β_2-GPI (*15*). In order to prepare the nicked r-Domain V, 10 mg of r-Domain V* was incubated with 4 mg of immobilized human factor Xa in the reaction buffer at 30°C for 1 week. The samples were then purified by reversed-phase HPLC as described above.

The purity of each sample was determined by a protein sequencer and SDS-PAGE. SDS-PAGE was performed in a 15% slab gel essentially as described by Laemmli (*21*). The position of the nick was confirmed by the protein sequencer.

Liposome Preparation and Binding Assay—CL/PC (1:1)-LUV were prepared in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl by the reversed-phase evaporation method described by Yoshimura and Sone (*22*), and then filtered through a polycarbonate membrane of 0.1 μm pore size. The liposome concentration was determined by the method of Bartlett (*23*). The binding of proteins to liposome membranes was determined by the centrifugation procedure described by Yoshimura *et al.* (*24*). The mixtures of protein and liposomes were incubated in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl at 25°C for 30 min, and then centrifuged at 120,000×g for 60 min. The amount of protein in the supernatant was determined by the method of Bradford (*25*).

Circular Dichroism Measurements—CD spectra were measured at 20°C with a Jasco spectropolarimeter, model J-720A and J-500A, equipped with an interface and a personal computer. The instruments were calibrated with ammonium *d*-10-camphorsulfonate. The results were expressed as the mean residue ellipticity $[\theta]$, which is defined as $[\theta]=100\times\theta_{obs}/lc$, where θ_{obs} is the observed ellipticity, c is the concentration in residue moles per liter, and l is the length of the light path in cm. Measurements were performed at a protein concentration of 10 μM with a 1-mm cell from 255 to 195 nm and a 10-mm cell from 345 to 245 nm. The temperature was controlled using a thermostatically controlled cell holder and measured by a BAT-12 microprobe (Sensortex).

The buffers used for pH titration were Na acetate (pH 3-5), MES (pH 5-7), Tris-HCl (pH 7-9), CHES (pH 9-10), and CAPS (pH 10-11). The buffer concentration was 10 mM. The measurements of urea-induced denaturation were carried out in 10 mM Na acetate at pH 5.5.

Fluorescence Measurements—Fluorescence spectra were measured with a Hitachi fluorescence spectrophotometer, model F-4500, at 20°C. Tryptophan fluorescence was measured with excitation at 295 nm at a protein concentration of 5 μM. A microcell with a 5-mm light path was used and the spectra were normalized using the same concentration of *N*-acetyltryptophanamide.

Other Methods—Ion-spray ionization mass spectrometry was carried out using a Perkin-Elmer SCIEX API-III quadrupole mass spectrometer equipped with ion-spray ion sources, which was calibrated with ammonium adducts of poly(propyleneglycols) at room temperature.

The concentrations of the r-Domain V* and r-Domain V were calculated from their extinction coefficients of 10,185 and 8,735 at 276 nm, respectively (*26*).

RESULTS

Expression and Characterization of Human Recombinant Domain V—P. pastoris GS115 transformed with plasmid pNPD5 was grown to a cell density of about A_{600}= 60. Then, the culture medium was changed to induction medium containing 3% methanol. The expression of Domain V was started 6 h after induction and reached a maximum level after about 14 h. The proteins secreted into the medium were collected and then purified by a series of chromatography steps. After reversed-phase HPLC, two protein fractions with a content ratio of 9:1 were obtained. On the basis of the N-terminal sequence and molecular weight determined by ion-spray mass spectrometry (see below), the major peak was identified as Domain V consisting of the amino acid residues from −9 to 86 (r-Domain V*). The amino acid sequence of r-Domain V*, including the location of the disulfide bonds, is shown in Fig. 2. On the other hand, the minor peak was identified as Domain V, consisting of the amino acid residues from 1 to 86 (r-Do-

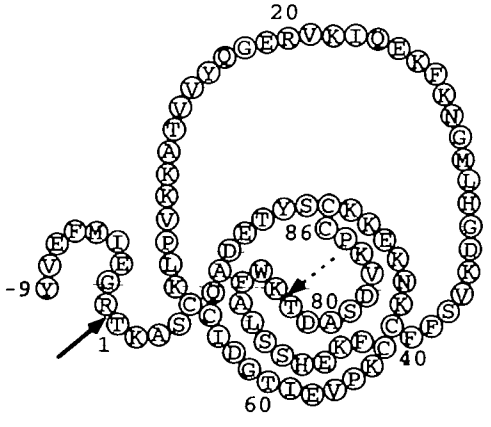

Fig. 2. **Amino acid sequence and location of the disulfide bonds of recombinant Domain V of human β_2-GPI.** The solid arrow shows the cleavage site for removing the secretion signal sequence by factor Xa. The dashed arrow shows the specific cleavage (nicked) site. The amino acid sequence of r-Domain V corresponds to 1-86 and that of r-Domain V* corresponds to −9-86.

main V). This shows that r-Domain V is a product cleaved at the factor Xa recognition site, which is indicated by the solid arrow in Fig. 2 (see below). The yield of purified r-Domain V* was more than 100 mg per liter of growth medium.

The molecular weights of r-Domain V* and r-Domain V were determined by ion-spray mass spectrometry to be 10,856.4 and 9,731.6, respectively. These are consistent with the calculated values (10,855 for r-Domain V* and 9,730 for r-Domain V).

To determine the positions of the disulfide bonds, protease digestions were carried out. Then, the peptides containing only one disulfide bond were isolated and their N-terminal sequences were determined. r-Domain V* was treated with V_8 protease at pH 4, and the digest was subjected to reversed-phase HPLC (Fig. 3A). Six major peaks were isolated, and their N-terminal amino acid sequences were determined. Peak 6 was found to be a precursor of peaks 4 and 5, in which the peptide bond located at the C-terminal side of Glu 45 was not cleaved (Fig. 3A, peak 6). Indeed, treatment of the isolated peak 6 with V_8 protease for a long period resulted in the separation of peak 6 into peaks 4 and 5 (data not shown). From a comparison between the N-terminal amino acid sequencing data and the available sequences of human β_2-GPI, we identified the peptides contained in each of the peaks (Fig. 4) (*7, 20, 27*). Peak 4 consisted of two peptides, each containing one Cys residue, showing that the disulfide bond was formed between Cys 48 and Cys 86 (Fig. 4). On the other hand, the amino acid sequences of the peptides in peak 5 showed that three peptides were connected by two disulfide bonds. Therefore, in order to separate the peptides containing only one disulfide bond, further digestion was necessary.

Peak 5 was digested with Asp-N at pH 8 and the digests were subjected to reversed-phase HPLC, resulting in the separation of three peaks, a, b, and c (Fig. 3B). Peaks b and c contained two peptides and each of them had one Cys residue. The amino acid sequences show that the disulfide bonds are formed between Cys 5 and Cys 56 (for peak b), and between Cys 41 and Cys 66 (for peak c) (Fig. 4). Two peptides, which appeared at about 27 min, as shown in Fig. 3B, came from the unexpected cleavage of peak a at the N-terminal of Asn 29, judging from the amino acid sequences (data not shown).

Chromatography of peptides treated with V_8 protease and Asp-N showed that there was no significant peptide fraction with another disulfide bond. Similar results were obtained for r-Domain V. These results indicate that almost all the recombinant Domain V secreted by *P. pastoris* has the same disulfide bond pairs as the intact bovine β_2-GPI (*6*), *i.e.*, native disulfide bonds.

Removal of the N-Terminal Signal Sequence and Introduction of the Nick in the C-Terminal Loop—In order to remove the signal sequence after the expression of r-Domain V*, we designed an insertion for the factor Xa recognition site (*i.e.*, IEGRT) between the C-terminal of the signal sequence and the N-terminal of Domain V (see "MATERIALS AND METHODS") (*28*). Unexpectedly, r-Domain V cleaved at the factor Xa recognition site was secreted into the medium as a minor component (10% of the total).

Approximately 90% of the product (*i.e.*, r-Domain V*) had 9 extra amino acid residues at the N-terminal. In order to convert r-Domain V* into r-Domain V, the former was reacted with factor Xa at 37°C. After 9 h of incubation, the signal sequence was removed from most of the molecules, the N-terminal sequence being identical to that of r-Domain V (*i.e.*, T-K-A-). Under reducing conditions, the SDS-PAGE mobility of the factor Xa-treated r-Domain V* was the same as that of r-Domain V (Fig. 5), further confirming the correct cleavage.

Surprisingly, we found that a longer reaction time or higher concentration of factor Xa caused a nick between Lys77 and Thr 78 (Fig. 2). The position of the nick was the same as that reported by Hunt *et al.* (*15*). In order to prepare the nicked r-Domain V, we treated r-Domain V* with immobilized factor Xa for 1 week at 30°C. As a result, almost 100% of the molecules were transformed to the nicked r-Domain V. By N-terminal amino acid sequencing, the absence of other cleavages was confirmed. SDS-PAGE revealed that the mobility of the reduced form of the nicked r-Domain V was greater than that of the intact r-Domain V because of the lack of 9 residues in the C-terminal region (Fig. 5). Since Domain V has the disulfide bond between Cys 86 (*i.e.*, the C-terminal amino acid) and Cys 48, the C-terminal peptide between Thr 78 and Cys 86 should not be removed by this cleavage. Indeed, the molecular mass of the nicked r-Domain V was determined by ion-spray mass spectrometry to be 9,749.1. This was identical to the calculated value, 9,748, within the error of measurement, confirming that no amino acid was lost by the introduction of the nick. Thus, the nicked r-Domain V differs from the intact protein only by the presence of cleavage between

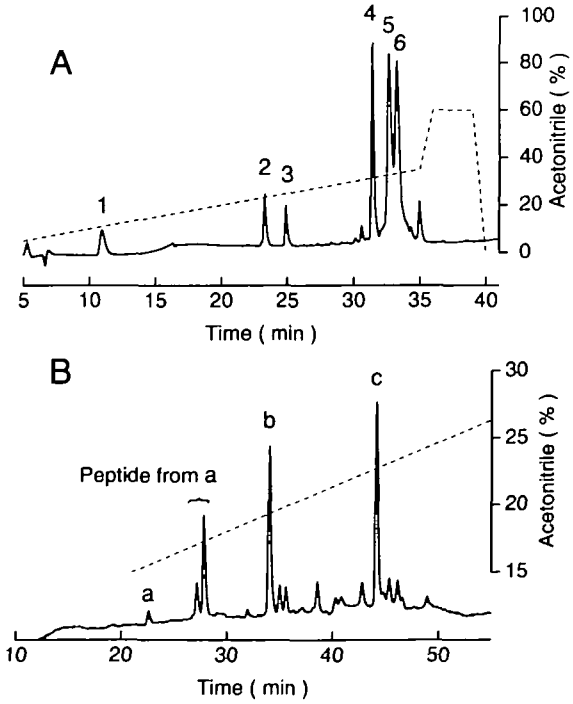

Fig. 3. **Reversed-phase HPLC patterns of V_8 protease-(A) and Asp-N-(B) treated fragments of r-Domain V*.** A: 1 mg of r-Domain V* was treated with V_8 protease and the digest was subjected to reversed-phase HPLC. B: About 50 μg of peak 5 was treated with Asp-N and the digest was subjected to reversed-phase HPLC.

Peptide	Position	Amino acid sequence									
1	-6 - -3	Phe	Met	Ile	Glu						
		4	N.D	N.D	N.D						
2	-9 - -7	Tyr	Val	Glu							
		1386	1778	438							
3	20 - 25	Arg	Val	Lys	Ile	Gln	Glu				
		380	2788	3369	3654	2308	1452				
4	46 - 52	Lys	Lys	(Cys)	Ser	Tyr	Thr	Glu			
		3210	3493		169	784	241	351			
	70 - 86	His	Ser	Ser	Leu	Ala	Phe	Trp	Lys	Thr	Asp
		1794	273	125	819	702	689	194	175	128	138
		Ala	Ser	Asp	Lys	Pro	(Cys)				
		336	37	150	74	112					
5	-2 - 19	Gly	Arg	Thr	Lys	Ala	Ser	(Cys)	Lys	Leu	Pro
		746	60	255	736	998	98		334	496	318
		Val	Lys	Lys	Ala	Thr	Val	Val	Tyr	Gln	Gly
		905	481	884	319	110	341	428	251	231	241
		Glu									
		183									
	26 - 45	Lys	Phe	Lys	Asn	Gly	Met	Leu	His	Gly	Asp
		503	811	628	358	1127	947	776	512	622	399
		Lys	Val	Ser	Phe	Phe	(Cys)	Lys	Asn	Lys	Glu
		507	778	64	382	889		532	303	375	175
	53 - 69	Asp	Ala	Gln	(Cys)	Ile	Asp	Gly	Thr	Ile	Glu
		237	836	539		896	411	686	228	531	416
		Val	Pro	Lys	(Cys)	Phe	Lys	Glu			
		905	350	884		889	757	111			
a	26 - 34	Lys	Phe	Lys	Asn	Gly	Met	Leu	His	Gly	
		47	68	45	36	65	74	26	N.D	10	
b	-2 - 19	Gly	Arg	Thr	Lys	Ala	Ser	(Cys)	Lys	Leu	Pro
		244	22	71	91	155	25		77	126	49
		Val	Lys	Lys	Ala	Thr	Val	Val	Tyr	Gln	Gly
		63	50	109	64	25	72	85	50	45	67
		Glu									
		18									
	53 - 57	Asp	Ala	Gln	(Cys)	Ile					
		94	184	104		69					
c	35 - 45	Asp	Lys	Val	Ser	Phe	Phe	(Cys)	Lys	Asn	Lys
		121	153	325	39	266	260		239	100	190
		Glu									
		55									
	58 - 69	Asp	Gly	Thr	Ile	Glu	Val	Pro	Lys	(Cys)	Phe
		121	192	83	209	78	134	75	239		98
		Lys	Glu								
		127	72								

Fig. 4. **Amino acid sequences of the peptides obtained from the digestion of r-Domain V* by V_8 protease and Asp-N.** Each value under the amino acid residue identified shows the amounts of PTH-amino acid (in picomoles) recovered using a gas-phase sequencer.

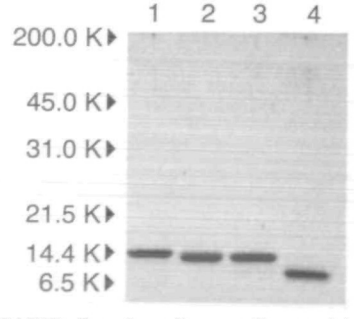

Fig. 5. **SDS-PAGE of various forms of recombinant Domain V.** One microgram of each r-Domain V* (lane 1), the intact r-Domain V purified from the crude Domain V by reversed-phase HPLC (lane 2), the intact r-Domain V obtained from r-Domain V* by treatment with factor Xa (lane 3), and the nicked form of r-Domain V obtained by treatment of r-Domain V* with immobilized factor Xa for 1 week (lane 4) was subjected to 15% SDS-PAGE under reducing conditions.

Lys77 and Thr78. As mentioned by Hunt *et al.* (*15*), Lys77 and Thr 78 would satisfy the criteria for a thrombin cleavage site. However, bovine and human thrombins did not cleave this site (data not shown).

Secondary and Tertiary Structures of the Intact and Nicked r-Domains V—Figure 6 shows the far-UV CD spectra of the intact and nicked r-Domains V. At pH 7.5, the spectra of both proteins showed no obvious peaks. The spectra shown here are different from those of either α-helix or β-sheet structures. However, the urea- and acid-induced unfolded states of the intact r-Domain V exhibited the typical spectra of the unfolded proteins. The nicked protein also showed similar spectra in the unfolded condition (data not shown). These results suggest that both r-Domains V have a unique secondary structure under the native conditions. Figure 6 also shows the near-UV CD spectra of the intact and nicked r-Domains V. Domain V of human β_2-GPI has a Trp residue at position 76, two Tyr

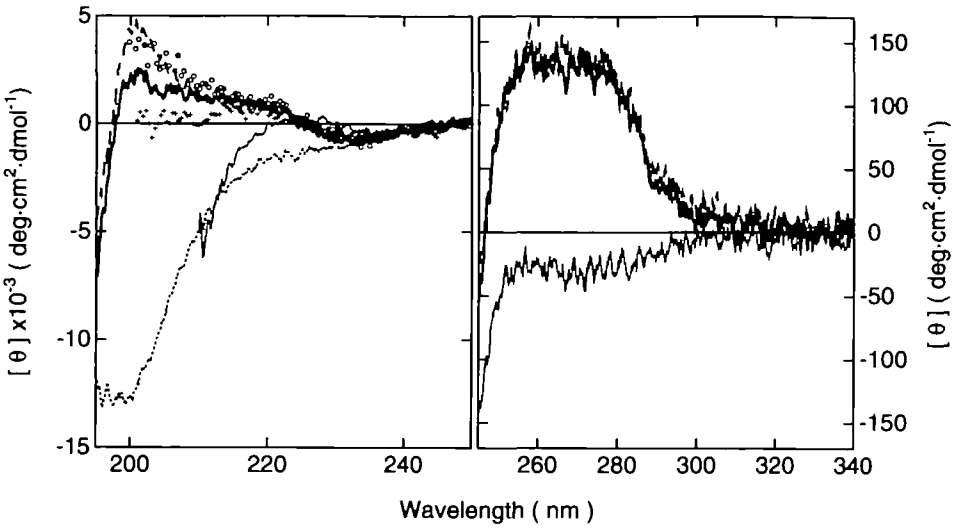

Fig. 6. Far- and near-UV CD spectra of the intact and nicked r-Domains V. CD spectra for the intact r-Domain V (10 μM) at pH 7.5 in 10 mM NaCl (bold continuous line) and in the presence of 280 μM CL/PC (1:1)-LUV in 0.41 M NaCl (++++), and for the nicked r-Domain V (10 μM) at pH 7.5 in 10 mM NaCl (bold broken line) and in the presence of 280 μM CL/PC (1:1)-LUV in 0.41M NaCl (○○○○) were measured. The fine continuous line shows the urea-induced unfolded state of the intact r-Domain V (10 μM) at pH 5.5 by 7.2 M urea. The dotted line shows the acid-induced unfolded state of the intact r-Domain V at pH 2.0 in 10 mM HCl.

residues at positions 16 and 50, and three disulfide bonds. The spectra showed positive ellipticity at around 260 to 280 nm, indicating that both proteins have a unique tertiary structure.

Next, we performed pH titration and urea-induced unfolding measurements of the intact and nicked r-Domains V detected in terms of the ellipticities at 213 and 276 nm (Fig. 7). The secondary and tertiary structures of both proteins were cooperatively destroyed by acidic pH and urea, strongly suggesting that both r-Domains V are compactly folded and adopt a native conformation under physiological conditions.

It is remarkable that both the far- and near-UV CD spectra of the nicked r-Domain V were very similar to those of the intact protein. In contrast, the stability of the nicked r-Domain V to urea was slightly less than that of the intact protein (Fig. 7B). To compare the difference quantitatively, we analyzed the unfolding transition assuming a two-state mechanism and linear dependence of the free energy change of unfolding (ΔG_U) on urea concentration ([urea]):

$$\Delta G_U = -RT\ln\frac{[\theta]-[\theta]_N}{[\theta]_U-[\theta]} = \Delta G_U(H_2O) - m[\text{urea}], \quad (1)$$

where $\Delta G_U(H_2O)$ is the value of ΔG_U in the absence of urea, m is a measure of the dependence of ΔG_U on urea concentration, R is the gas constant, T is the temperature in Kelvin, and $[\theta]$, $[\theta]_N$, $[\theta]_U$ are the observed ellipticities at a urea concentration of [urea], and for the native and unfolded states, respectively. Using this equation, we estimated the $\Delta G_U(H_2O)$ values of the intact and nicked r-Domains V at pH 5.5 to be 13.6 and 11.9 kJ/mol, respectively. On the other hand, the stabilities of these proteins to pH were apparently indistinguishable (Fig. 7B). It is likely that the acid unfolding transitions are too sharp to allow detection of a slight difference in conformational stability.

The conformation and stability of r-Domain V* were very similar to those of r-Domain V as judged from the CD spectrum and the stability to urea and acidic pH (data not shown). Therefore, the N-terminal signal sequence and factor Xa recognition sequence do not affect the structure and stability of Domain V.

Figure 8A shows the fluorescence spectra of the intact

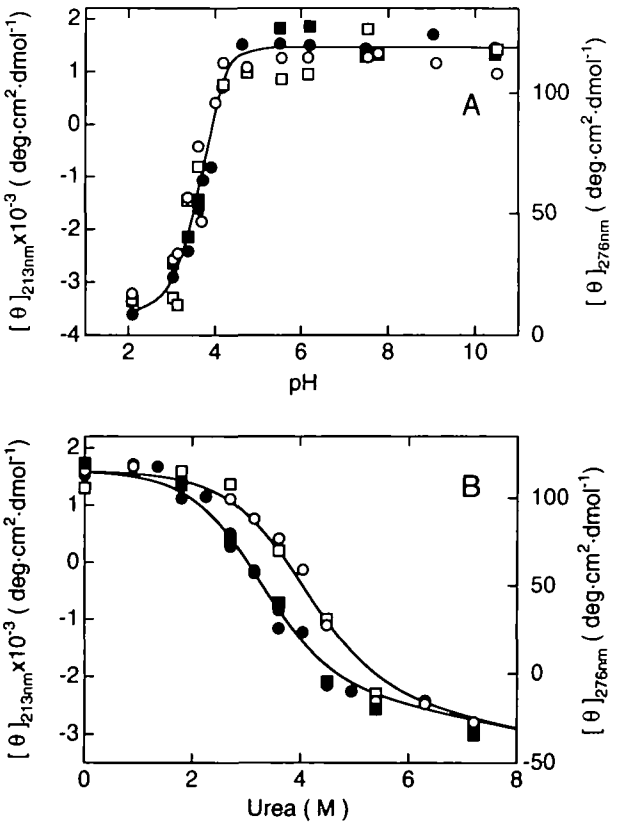

Fig. 7. Dependence on pH (A) and urea concentration (B) of the ellipticities of intact r-Domain V at 213 (○) and 276 (□) nm and the ellipticities of nicked r-Domain V at 213 (●) and 276 (■) nm at 20°C. The buffers used for pH titration were 10 mM Na acetate (pH 3-5), MES (pH 5-7), Tris-HCl (pH 7-9), CHES (pH 9-10), and CAPS (pH 10-11). The measurements of urea-induced denaturation were carried out in 10 mM Na acetate at pH 5.5. The solid lines in panel B were calculated with Eq. (1) using the following m and $\Delta G_U(H_2O)$ values. The intact r-Domain V, m=3.43, $\Delta G_U(H_2O)$=13.6. The nicked r-Domain V, m=3.75, $\Delta G_U(H_2O)$=11.9.

and nicked r-Domains V in the presence and absence of 7.7 M urea. The fluorescence intensity was normalized relative to the same concentration of *N*-acetyltryptophanamide. The maximum emission intensities of the native and unfolded states of intact r-Domain V were 1.15 at 345 nm and 0.63 at 346 nm, respectively. Those of the nicked proteins were 1.08 at 347 nm and 0.71 at 348 nm, respectively. Therefore, the introduction of a nick shifts the maximum slightly to a longer wavelength. For both proteins, upon unfolding, the fluorescence intensity decreased with a negligible shift of maximum wavelength. These results suggest that Trp 76 in the native states of the intact and nicked r-Domains V is substantially exposed to the solvent.

Binding of the Intact and Nicked r-Domains V to CL Liposomes—We examined the interactions of the intact and nicked r-Domains V with CL/PC at 25°C in 10 mM Tris-HCl containing 0.1 M NaCl (Fig. 9A). Both the intact and nicked Domains V bound CL/PC (1:1)-LUV. However, the affinity of the nicked r-Domain V for the liposomes was lower than that of intact r-Domain V. In order to confirm this difference, we examined the effect of salt on the interactions of the r-Domains V with CL/PC (1:1)-LUV (Fig. 9B). The bindings of the intact and nicked r-Domains V to the liposomes were strongly dependent on the concentration of NaCl. The binding of the nicked r-Domain V was more sensitive than that of intact r-Domain V, *i.e.*, the amount of bound nicked r-Domain V was drastically reduced with an increase in NaCl concentration. Previously, we reported the NaCl-dependencies of the intact and nicked forms of bovine β_2-GPI, which was cleaved at the peptide bond between Ala 314 and Phe 315 (Ala 74 and Phe 75 in r-Domain V) (*18*). For comparison, the data for these two proteins are shown in Fig. 9B. It should be noted that the NaCl-dependencies of both forms of Domain V were similar to those of the respective forms of the whole molecule of bovine β_2-GPI.

Next, we examined the CD spectra of the intact and nicked r-Domains V in the presence of CL/PC (1:1)-LUV under conditions where significant binding was observed

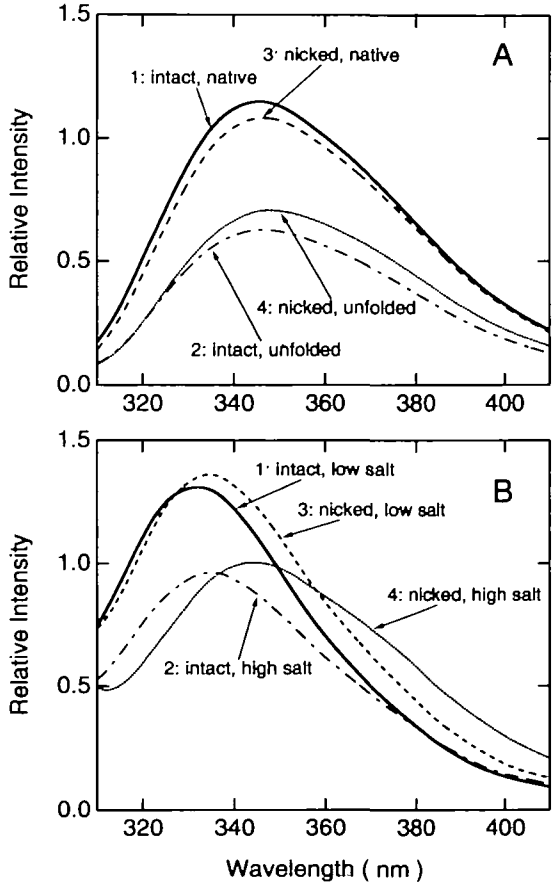

Fig. 8. **Fluorescence spectra of the intact and nicked r-Domains V.** The measurements were carried out at a protein concentration of 5 μM. The ordinate represents the fluorescence relative to that of the same concentration of *N*-acetyltryptophanamide. A: The native states of the intact (1) and nicked (3) r-Domains V in 10 mM Tris-HCl (pH 7.5) containing 10 mM NaCl; the unfolded states of the intact (2) and nicked (4) r-Domains V in 10 mM acetate (pH 5.5) containing 7.7 M urea. B: The intact r-Domain V with 200 μM CL/PC (1:1)-LUV in 10 mM Tris-HCl (pH 7.5) containing 10 mM (1) or 0.41 M NaCl (2); the nicked r-Domain V with 200 μM CL/PC (1:1)-LUV in 10 mM Tris-HCl (pH 7.5) containing 10 mM (3) or 0.41 M NaCl (4).

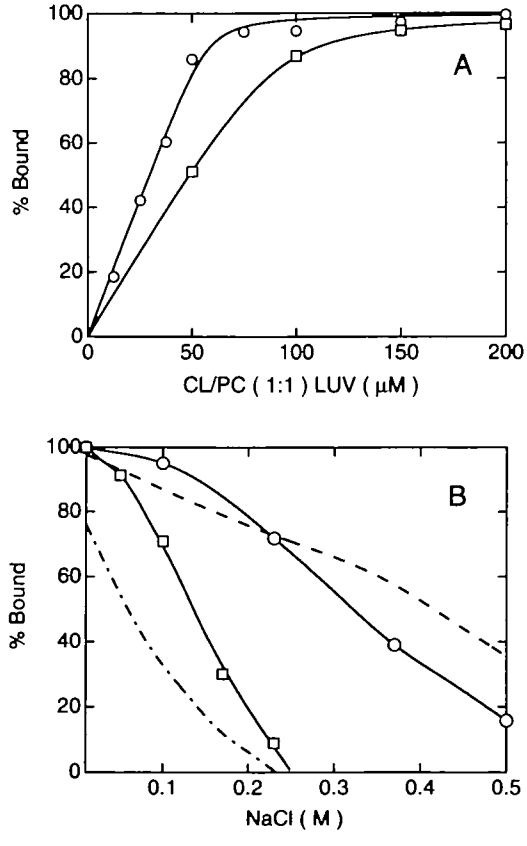

Fig. 9. **The amount of the intact and nicked r-Domains V associated with CL/PC (1:1)-LUV as a function of the phospholipid concentration (A) and effects of the NaCl concentration on the binding of the intact and nicked r-Domains V to CL/PC (1:1)-LUV (B).** A: The intact (○) or nicked (□) r-Domain V (2.5 μM) was incubated with LUV at 25°C in 10 mM Tris-HCl (pH 7.5) containing 0.1 M NaCl. B: 3.0 μM of the intact (○) or nicked (□) r-Domain V was incubated with 100 μM LUV at 25°C in 10 mM Tris-HCl (pH 7.5) containing NaCl of the indicated concentration. For comparison, the NaCl concentration-dependencies of binding of the whole molecule of intact bovine β_2-GPI (0.9 μM) (- - -) and nicked bovine β_2-GPI (1.0 μM) (·····) are shown.

(Fig. 6A). No change in the far-UV CD spectra of the intact and nicked r-Domains V in the presence of the liposomes was observed. This indicates that the secondary structure of Domain V does not change notably upon binding to the liposomes. It was difficult to measure the near-UV CD spectra of the intact and nicked proteins in the presence of liposomes because of the large absorption.

We also followed the interaction using the fluorescence spectra (Fig. 8B). In 0.01 M NaCl, where both the intact and nicked r-Domains V bound the CL/PC (1:1)-LUV (Fig. 9B), a significant blue shift (10 nm) of the maximum emission wavelength was observed in both r-Domains V, suggesting that Trp 76 is buried upon interaction. In 0.41 M NaCl, where about 30% of the intact Domain V still bound the liposomes, but the nicked r-Domain V did not, a blue shift was observed only in the spectrum of the intact r-Domain V. These results indicate that the membrane binding buries Trp 76, which is located on the protein surface of the intact and nicked r-Domains V.

DISCUSSION

Structure of Domain V of β_2-GPI—The three-dimensional structure of the Sushi domains of factor H was established by two-dimensional NMR (*8-10*). The structure of Domain V was modeled using the structure of the 16th domain of factor H (*19*). From this model, the secondary structure of Domain V was predicted to be predominantly β-sheet. However, Domain V has an additional disulfide bond that forms a loop between Phe 67 and Cys 86. The structure of this loop was not constructed in this model. In addition, Domain V has a large insertion in the "hypervariable loop region" (Gln 17-Gly 30) compared with the Sushi domain of factor H. According to the secondary structure prediction, this region may have α-helical structure (*19*).

The far-UV CD spectra of the intact and nicked r-Domains V were unusual for a β-structure (Fig. 6). This might arise from the influence of the ellipticity of the aromatic residues. Thus, it is very difficult to estimate the secondary structure content on the basis of the CD spectrum. However, the ellipticities at 222 and 208 nm were clearly positive, indicating that Domain V has little α-helix. Very fine near-UV CD spectra were observed for the native states of both the intact and nicked r-Domains V (Fig. 6). These spectra clearly show that the proteins have a specific tertiary structure. The Trp fluorescence spectra of the native states of the intact and nicked r-Domains V have maxima at 346 and 347 nm, respectively. The maximum emission wavelength did not change drastically upon unfolding, suggesting that the Trp residue in Domain V is exposed to the solvent (Fig. 8A).

The urea- and pH-induced transitions of the intact r-Domain V measured by far-UV CD were in good agreement with those obtained by near-UV CD (Fig. 7) and the same was true for the nicked r-Domain V. These results suggest that, for both proteins, the unfolding transitions can be approximated by a two-state transition. The thermal unfolding transitions of both forms of r-Domains V were also well approximated by a two-state mechanism (data not shown). The m value, a measure of the cooperativity of the denaturant-induced unfolding, is known to be proportional to the difference in solvent-accessible surface area between the folded and unfolded states (*29*). The m values of the intact and nicked proteins are 3.43 and 3.75 kJ/mol·M, respectively. These values are slightly smaller than those for other proteins of similar size, *e.g.* the m values of *B. subtilis* HPr (88 residue) and barstar (89 residue) are 4.4 and 5.2 kJ/mol·M, respectively (*30, 31*). However, disulfide bonds are known to reduce the m value because of the compactness of the unfolded state (*29*). HPr and barstar have no disulfide bonds. Therefore, the m value of r-Domain V is appropriate for the native structure. These results confirm that both the intact and nicked r-Domains V have a specific folded structure.

Very similar far- and near-UV CD and fluorescence spectra were observed for the native states of the intact and nicked r-Domains V (Figs. 6 and 8). These spectral similarities suggest that the secondary and tertiary structures of the nicked r-Domain V are similar to those of the intact r-Domain V. In contrast, the conformational stability of the nicked r-Domain V was lower than that of the intact r-Domain V by 1.7 kJ/mol. However, this difference is smaller than that expected from the conformational entropic effects of the cleavage, assuming that the native state is rigid. The calculated difference in the loop entropy of the unfolded state between the intact and nicked r-Domains V is 69 J/mol/K, which corresponds to 20 kJ/mol in free energy of unfolding at 20°C (*32*). Indeed, in the case of acid-induced unfolding, the difference was within the experimental error (Fig. 7B). As mentioned above, Trp 76 is exposed to solvent in both the intact and nicked r-Domains V. Thus, a possible explanation is that the region around Trp 76 fluctuates even in the native state, so that the effects of cleavage upon the conformation and stability are small.

Interaction of the Intact and Nicked r-Domains V with CL Liposomes—The site of β_2-GPI binding to CL has been studied by several groups using synthetic peptides. Lauer *et al.* (*17*) suggested that His 273 of the whole molecule of β_2-GPI (which corresponds to His 33 in r-Domain V) is important for binding to CL. Hunt and Krilis (*16*) showed that the peptides containing the sequence between Cys 281 and Cys 288 (which corresponds to the sequence from Cys 41 to Cys 48 in r-Domain V) inhibited the binding of β_2-GPI to CL. These findings suggest the importance for the binding of the linear sequence of the above regions rather than their tertiary structure. In addition, Kertesz *et al.* (*33*) reported that the reduced/alkylated form of human β_2-GPI bound to a CL-coated polystyrene microtiter plate more strongly than the intact form, further suggesting that the native tertiary structure is not required for the binding to CL.

To examine the interaction of Domain V with CL in detail, we measured the affinity of the intact and nicked r-Domains V for CL/PC (1:1)-LUV under physiological conditions. Our results suggested that the region containing Lys 77 and Thr 78 is important for the binding of Domain V to CL. The binding curve of the intact r-Domain V was similar to that of the whole molecule of intact β_2-GPI in 10 mM and 0.3 M NaCl (data not shown), confirming that Domain V is largely responsible for the binding of β_2-GPI. The affinity of the nicked r-Domain V for CL was notably reduced, compared with that of the intact protein.

The dependence of the binding on NaCl concentration emphasizes the importance of the region around the nick

(Fig. 9B). The amount of the r-Domain V associated with liposomes decreased with increasing NaCl concentration, demonstrating the participation of electrostatic attraction between the positively charged r-Domain V and the negatively charged CL. On the other hand, the affinity of the nicked r-Domain V for liposomes was more sensitive to NaCl concentration, indicating that factors other than the electrostatic interactions critically modulate the interaction. We noted that the dependence on NaCl of binding of the nicked r-Domain V was similar to those of the N-terminal domain of bovine β_2-GPI and the nicked form of the whole molecule of bovine β_2-GPI (Fig. 9B, see also Fig. 5 in Ref. *18*). On the other hand, the NaCl-dependence of the intact r-Domain V was similar to that of the intact form of the whole molecule of bovine β_2-GPI, further suggesting that Domain V, in particular the region around the nick, plays a critical role in the binding of β_2-GPI.

When the intact and nicked r-Domains V bound CL/PC (1:1)-LUV, a marked blue shift in the maximum wavelength of the fluorescence spectra was observed. This indicates that Trp 76 is buried in the hydrophobic environment upon binding, irrespective of the presence or absence of the nick. The far-UV CD spectra were not changed upon binding to liposomes, indicating that the secondary structures were not affected by the binding. However, the change in tertiary structure upon binding to liposomes is unclear.

The nicked forms of human and bovine β_2-GPI have lower affinity for CL (*15, 18*). However, it was unknown whether the introduction of a nick caused a simultaneous loss of the amino acid residues beside the N-terminal side of the nick, *i.e.*, it was unclear whether the observed loss of activity arose from the loss of a short peptide or the cleavage of a single peptide bond. Using N-terminal amino acid sequencing and ion-spray mass spectrometry, we confirmed that no amino acid residue was lost in the nicked r-Domain V. Therefore, we can exclude the former possibility. From these binding, CD and fluorescence experiments, it is clear that the region containing Trp 76, Lys 77, and Thr 78 plays an important role in binding to CL, although the exact binding site could not be determined. It is noteworthy that this region has been suggested to be important for binding to CL, because it is completely conserved in available sequences of β_2-GPI from several species (*19, 33*).

Using the methods we employed here, we were unable to observe any notable structural difference between the intact and nicked r-Domains V. Thus, neither a large conformational change nor denaturation by introduction of the nick may be responsible for the decrease in affinity for CL. This seems to leave the following two possibilities to explain the decreased affinity. (1) The peptide bond between Lys 77 and Thr 78 is critical for binding. (2) The cleavage induces a conformational change, which is critical for binding, but cannot be detected in the CD or fluorescence spectra. It is unclear at present which mechanism is responsible for the decrease in affinity. To elucidate the mechanism of binding to CL and the role of the nick, more precise analysis of r-Domain V, such as site-directed mutagenesis and structural analysis by X-ray crystallography or NMR, would be useful.

CONCLUSIONS

1. r-Domain V expressed in *Pichia pastoris* has specific native structure with the correct disulfide bonds.

2. As judged from CD and fluorescence measurements, the specific cleavage of a peptide bond between Lys 77 and Thr 78 does not significantly affect the secondary and tertiary structures of r-Domain V. However, this cleavage critically reduces the affinity of r-Domain V to CL.

3. Based on a comparison of the affinity to CL and the change in fluorescence spectra upon binding to CL of the intact and nicked proteins, the region including Trp 76, Lys 77, and Thr 78 is suggested to be important for the interaction with CL.

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