Flexible Loop of β_2-Glycoprotein I Domain V Specifically Interacts with Hydrophobic Ligands[†]

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ABSTRACT: β_2-Glycoprotein I (β_2-GPI), which consists of four complement control protein modules and a distinctly folded fifth C-terminal domain, is an essential cofactor for the binding to phospholipids of anti-cardiolipin antibodies, isolated from patients with anti-phospholipid antibody syndrome, and its fifth domain has attracted attention as a specific phospholipid-binding site. We focused on the fifth domain of β_2-GPI (Domain V) and examined the interaction of intact Domain V, Domains IV−V, and nicked Domain V with various hydrophobic ligands, as a model molecule of phospholipid. We found that electrostatic and hydrophobic interactions are important for Domain V binding to the ligand molecules. We also found that, while Domain IV has no significant effect on the interactions with ligands, the nicked Domain V with cleavage in the flexible loop decreases the affinity, indicating that the flexible loop region is the binding site of the hydrophobic ligands. The synthetic peptide corresponding to the loop region was disordered and interacted with bis-ANS, confirming the critical role of the loop region. To clarify the nature of the interaction between the loop region and hydrophobic compounds, we prepared the reduced and alkylated Domain V, which was denatured but was assumed to be a collapsed state. Alkylation by iodoacetic acid decreased the interaction of Domain V with bis-ANS, probably because the protein net charge was decreased by the six introduced carboxyl groups and consequently the electrostatic interactions were decreased. In contrast, Domain V alkylated by iodoacetamide, therefore retaining a high positive net charge, bound bis-ANS more strongly than the intact Domain V. These results suggested that the interaction of Domain V with hydrophobic compounds through the flexible loop is similar to the binding of hydrophobic compounds to the protein folding intermediate.

Interactions of proteins with hydrophobic molecules, such as phospholipids and small hydrophobic compounds, are important issues in the effort toward understanding the mechanisms of protein folding, protein−ligand interactions, and protein−membrane interactions. β_2-Glycoprotein I (β_2-GPI),[1] also known as apolipoprotein H, is a plasma glycoprotein of about 50 kDa [326 amino acids with ∼20% (w/w) carbohydrate], which is associated with very-low-density lipoproteins, high-density lipoproteins, and chylomicrons and has been suggested to play a role in triacylglycerol metabolism (*1*, *2*). β_2-GPI has been shown to bind negatively charged substances such as DNA, heparin, and dextran sulfate, and negatively charged phospholipids such as cardiolipin (CL) (*3*−*6*). It has been identified as a cofactor in the recognition by a subset of anti-phospholipid antibodies in autoimmune diseases (*7*−*12*). Anti-phospholipid antibodies bind to β_2-GPI after complex formation with CL. However, the nature of this cofactor activity and the specific regions of the molecule involved in the interactions with phospholipids or with antibodies have not yet been determined.

Recently, the crystal structures of β_2-GPI isolated from human plasma have been reported by two groups, suggesting a specific phospholipid-binding site and the mechanism of interaction with anti-phospholipid antibodies (*13*, *14*). The structures revealed four complement control protein modules and a distinctly folded fifth C-terminal domain arranged like beads on a string to form an elongated J-shaped molecule. The observed spatial arrangement of the five domains suggests functional partitioning of protein adhesion and membrane adhesion over the N- and C-terminal domains, respectively. Domain V folds into a central β-spiral of four antiparallel β-strands with two small α-helices and an

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[1] Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic acid; β_2-GPI, β_2-glycoprotein I; bis-ANS, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid; CL, cardiolipin; DAUDA, 11-((5-dimethylaminonaphthalene-1-sulfonyl)amino)undecanoic acid; Domain V, the recombinant fifth domain of β_2-GPI; Domains IV−V, the recombinant fourth and fifth domains of β_2-GPI; GuHCl, guanidine hydrochloride; laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; NMR, nuclear magnetic resonance; RCAM-Domain V, reduced and carboxamidomethylated β_2-GPI Domain V; RCM-Domain V, reduced and carboxymethylated β_2-GPI Domain V; prodan, 6-propionyl-2-dimethylaminonaphthalene; SDS, sodium lauryl sulfate.

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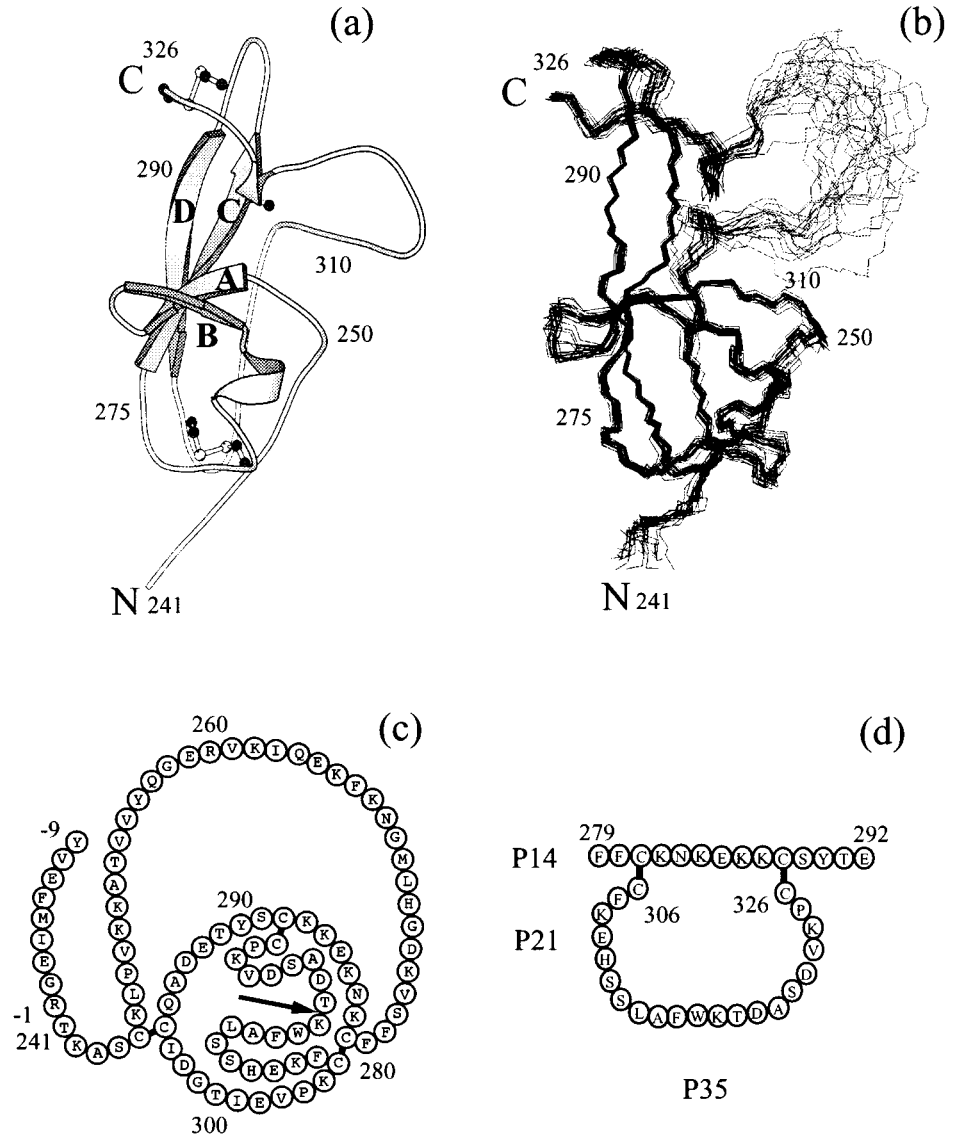

FIGURE 1: Primary and tertiary structural features of β_2-GPI Domain V. (a) X-ray crystal structure drawn by Molscript with Protein Data Bank file 1C1Z. The disulfide bonds connecting residues 245 and 296, 281 and 306, and 288 and 326 are shown. (b) Overlay of backbone traces of 20 NMR solution structures taken by Hoshino et al. (*21*). (c) Amino acid sequence and location of the disulfide bonds of recombinant Domain V of β_2-GPI. The arrow shows the specific cleavage (nicked) site. (d) Amino acid sequence of peptide fragments corresponding to the loop and short β-hairpin.

extended C-terminal loop region (Figure 1). The loop region, containing several hydrophobic residues, is highly flexible and has been suggested to be responsible for the interaction with negatively charged phospholipids.

Before the X-ray structures had been determined, Steinkasserer et al. (*15*) proposed that Domain V is responsible for the interaction of β_2-GPI with phospholipids based on the observation that the recombinant human Domain V, expressed in *Escherichia coli*, inhibited the interaction of the whole β_2-GPI molecule with CL. Hunt et al. (*16*) showed that cleavage of the peptide bond between Lys 317 and Tyr 318 in the flexible loop of Domain V, a potential thrombin cleavage site, reduced the binding affinity for CL, suggesting the importance of the flexible loop region for phospholipid binding. Hagihara et al. (*17*, *18*) showed by circular dichroism (CD) and fluorescence measurements that the conformation of the "nicked" Domain V, which was cleaved at that site, is similar to that of the intact Domain V and that the region including Trp316, Lys317, and Thr318 has a critical role in binding to CL. Mehdi et al. (*19*) also proposed that the hydrophobic sequence at positions Leu313−Trp316 is essential for the binding of β_2-GPI to anionic phospholipids because mutation of Trp316 to Ser disrupted the binding. On the other hand, Hunt and Krilis (*20*) reported that the peptide fragment derived from the highly positively charged sequence motif CKNKEKKC (Cys281−Cys288) inhibited the binding of whole β_2-GPI to CL, while peptides corresponding to other regions showed no such inhibitory activity, suggesting that this cluster of highly positively charged residues is also involved in phospholipid binding. The X-ray structures are consistent with these observations, indicating the importance of Domain V and the flexible loop regions.

More recently, Hoshino et al. (*21*) analyzed the structure and dynamics of recombinant Domain V expressed by *Pichia pastoris* using heteronuclear NMR approaches. They found by monitoring the changes in the $^1H-^{15}N$ heteronuclear single quantum coherence (HSQC) spectra that both the C-terminal flexible loop and the short β-hairpin between βC

and βD strands including the positively charged sequence motif specifically interact with SDS, a good model of phospholipid. The results directly demonstrated participation of the flexible loop and the short β-hairpin, as suggested by X-ray (*13*, *14*) and biochemical (*16*−*19*) studies. However, because β_2-GPI Domain V exhibited a marked propensity to aggregate at SDS concentrations above 0.2 mM, detailed analysis of the interactions, such as estimation of the binding constant, was difficult. Thus, small hydrophobic ligands that do not induce protein aggregation could provide useful information.

In this study, to further understand the mechanism of the interaction of human β_2-GPI with hydrophobic molecules, we studied the interaction of recombinant Domain V with various hydrophobic ligands by fluorescence measurements. To clarify the roles of other domains and the flexible loop structure, we also used recombinant Domains IV−V, nicked Domain V, and disulfide-reduced and alkylated Domain V. In addition, we used a 35-residue peptide consisting of the long C-terminal loop and the short β-hairpin between βC and βD of Domain V, connected by 2 disulfide bonds. Our results indicated that the flexible loop region is the binding site for hydrophobic ligands and that the interaction between Domain V and hydrophobic ligands has a resemblance to that observed between protein folding intermediates and hydrophobic ligands.

MATERIALS AND METHODS

Materials. Various hydrophobic ligands such as bis-ANS (ex. 385 nm, em. 500 nm), DAUDA (ex. 335 nm, em. 500 nm), laurdan (ex. 364 nm, em. 500 nm), and prodan (ex. 360 nm, em. 450 nm) were purchased from Molecular Probes (Eugene, OR), ANS (ex. 350 nm, em. 480 nm) was from Wako (Osaka, Japan), and *all-trans*-retinol (ex. 325 nm, em. 500 nm) was from Sigma (St. Louis, MO). The excitation and emission wavelengths of each ligand are indicated in parentheses. Bovine serum albumin, egg white lysozyme, and bovine β-lactoglobulin, isomer A, were purchased from Seikagaku Kogyo (Tokyo, Japan), Nacalai Tesque (Tokyo, Japan), and Sigma, respectively. The three peptides, P14 (F_{279}FCKNKEKKCSYTE_{292}), P21 (C_{306}FKEHSSLAFWKTD-ASDVKPC_{326}), and P35, comprised of P14 and P21 linked by disulfide bonds between Cys281 and Cys306, and between Cys288 and Cys326 (Figure 1), were obtained from Peptide Institute Inc. (Osaka, Japan), and their purity was greater than 95%.

β_2-GPI Domain V and Domains IV−V. β_2-GPI Domain V with signal peptide, Tyr-Val-Glu-Phe-Met-Ile-Glu-Gly-Arg-Thr, added at the amino-terminal Lys242 of human β_2-GPI was expressed in methylotrophic yeast *P. pastoris*, and then purified by anion-exchange chromatography, as described previously (*18*). The amino acid sequence of Domain V, including the locations of the disulfide bonds, is shown in Figure 1c. To facilitate comparison, the numbering of the amino acid residues is the same as the whole β_2-GPI molecule (see Figure 1c).

Wild-type Domains IV−V expressed in *P. pastoris* was not homogeneous because of the linked carbohydrates. To overcome this problem, the pUC118-β_2-GPI plasmid containing the full-length human β_2-GPI was first modified using PCR-based site-directed mutagenesis to introduce mutation of Asn234 to Ala. This mutation disrupts the consensus sequence for addition of carbohydrate chain in Domain IV. The region from Arg182 to Cys326 was amplified by PCR using the primers: 5′-cctctcgagaaaagagaagtaaaatgcccattccc-atcaag and 5′-aaagaattcaccttagcatggctttacatcgg (*22*). The modified DNA was digested with *Xho*I and *Eco*RI and ligated into the *P. pastoris* expression vector pPIC 9 (Invitrogen), resulting in pNPD45. DNA from the expression plasmid was integrated into host cells, GS115 (Invitrogen), by transforming his4 *P. pastoris* cells with pNPD45 digested with *Sal*I. Expressed protein was purified by the same method as described for Domain V. We confirmed that the recombinant Domains IV−V had the folded native structure at neutral pH by CD and differential scanning calorimetry (DSC) measurements (Hagihara and Goto, manuscript in preparation).

Nicked and Reduced Domains V. Nicked Domain V was prepared by enzymatic reaction performed in 0.1 M Tris-HCl (pH 8.0) containing 20 mM NaCl and 0.3 mM $CaCl_2$ at 37 °C, as described previously (*23*). Domain V was reacted with plasmin for 16 h at a molar ratio of enzyme to substrate of 1:100. Separation of the nicked form of Domain V from the intact Domain V was carried out using a HiTrap Heparin column (Amersham Pharmacia Biotech, Uppsala, Sweden). Proteins were eluted with a linear NaCl gradient from 0 to 1.0 M.

Reduction of three disulfide bonds in Domain V was carried out in 10 mM sodium phosphate buffer (pH 6.0) containing 5 M urea as described previously (*24*). About 1 mM Domain V was allowed to react with 10 mM dithiothreitol for 1 h at room temperature. The reduction of the disulfide bonds was confirmed by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and by reversed-phase high-performance liquid chromatography. Alkylation of thiol groups of the reduced Domain V was performed at pH 8.0 by addition of an excess of iodoacetamide or iodoacetic acid (about 0.1 M) over the thiol groups. The reaction was continued for 1 h, and then the alkylated Domain V was separated from residual reagents on a column of Sephadex G-25.

Fluorescence Measurements. Fluorescence spectra were measured with a Hitachi fluorescence spectrometer, model F-4500, at 20 °C. Equilibrium fluorescence emission spectra were obtained for the hydrophobic ligand alone or the ligand in the presence of proteins after 10 min incubation with the solution preparation. Binding constants (K_b) were determined from the change in fluorescence emission spectra of ligand upon addition of protein at various concentrations. The ligand concentration was 2 μM unless otherwise stated. Protein concentrations were varied between 10 and 300 μM or above.

Assuming that the protein binds to the ligand at a molar ratio of 1:1 (eq 1), the binding constant at equilibrium is defined by eq 2:

$$P + L \rightleftarrows PL \quad (1)$$

$$K_b = [PL]/[P][L] \quad (2)$$

where [P], [L], and [PL] are the concentrations of protein, ligand, and complex of protein and ligand, respectively. It is noted that the assumption of 1:1 binding will be valid under the experimental conditions of $[L_0] \ll [P_0]$, where

$[P_0]$ (=[P]+[PL]) is the total protein concentration and $[L_0]$ (=[L]+[PL]) is the total ligand concentration. In the titration experiment using the fluorescence of the ligand, the fraction of complex ($[PL]/[P_0]$) is expressed as

$$[PL]/[P_0] = (F - F_0)/(F_\infty - F_0) \quad (3)$$

where F_0 and F_∞ are the initial and final fluorescence intensities, respectively, and F is the observed fluorescence intensity. By rearranging eq 3 with eq 2, we can obtain observed F given by

$$F = F_0 + (F_\infty - F_0) \times \frac{[PL]}{[P_0]} = F_0 + (F_\infty - F_0) \times \frac{([P_0] + [L_0] + 1/K_b) - \sqrt{([P_0] + [L_0] + 1/K_b)^2 - 4[P_0][L_0]}}{2[P_0]} \quad (4)$$

The values of K_b and F_∞ were estimated by curve-fitting analysis of the plot of fluorescence intensity vs protein concentration using the nonlinear least-squares procedure employing the IGOR Pro data analysis program (WaveMetrics, Inc.).

CD Measurements. CD measurements were performed with a Jasco spectropolarimeter, model J-720, using quartz cells with a 1 mm path length. The temperature was controlled with a water-circulating cell-holder at 20 °C. The data were expressed as molar residue ellipticity, [θ], as described (*25*).

RESULTS

Interactions of Domain V with Various Hydrophobic Ligands. One of the best methods to evaluate the interactions between proteins and hydrophobic ligands is to use the fluorescence of the protein or ligand itself. We examined the interactions by monitoring the changes in fluorescence of the ligand. Binding of hydrophobic ligands such as ANS and bis-ANS etc. to accessible hydrophobic pockets in the native proteins or those in an intermediate state such as the molten-globule structure is followed by a marked increase in fluorescence emission of these ligands (*26*−*30*). Figure 2 shows the fluorescence emission spectra of bis-ANS and prodan as a function of Domain V concentration. In the case of bis-ANS, a negatively charged hydrophobic ligand, the fluorescence increased markedly with increasing concentration of Domain V. The maximal emission at 480 nm indicated that bis-ANS was bound to the hydrophobic site. In contrast, in the case of the neutral hydrophobic ligand prodan, no changes in the fluorescence spectrum were observed. Prodan, however, bound to the hydrophobic hole of β-lactoglobulin showed an increase in fluorescence with a maximum at around 450 nm (Figure 2b). Therefore, we concluded that prodan does not interact with Domain V. These observations indicated that the negatively charged hydrophobic ligand was bound to β_2-GPI Domain V more strongly than the neutral ligand and suggested that both the electrostatic and hydrophobic interactions are involved in the interaction between bis-ANS and Domain V.

To estimate the binding constant, K_b, between Domain V and ligands, the normalized fluorescence intensities of various hydrophobic ligands were plotted against the concentration of Domain V (Figure 3). The K_b value was estimated by curve-fitting with eq 4, and the K_b values for various ligands under two pH conditions are summarized in Table 1. The K_b value of bis-ANS at pH 7.5 was 6×10^3 M^{-1}. This value was less than those between bis-ANS and bovine serum albumin (2.3×10^6 M^{-1}) or β-lactoglobulin (1.8×10^4 M^{-1}), both of which are known to bind various hydrophobic ligands tightly. Not only the negatively charged hydrophobic ligands but also retinol, a neutral hydrophobic compound, bound to Domain V with a K_b value of 8×10^3 M^{-1} at pH 7.5. However, the K_b value of ANS, a weakly

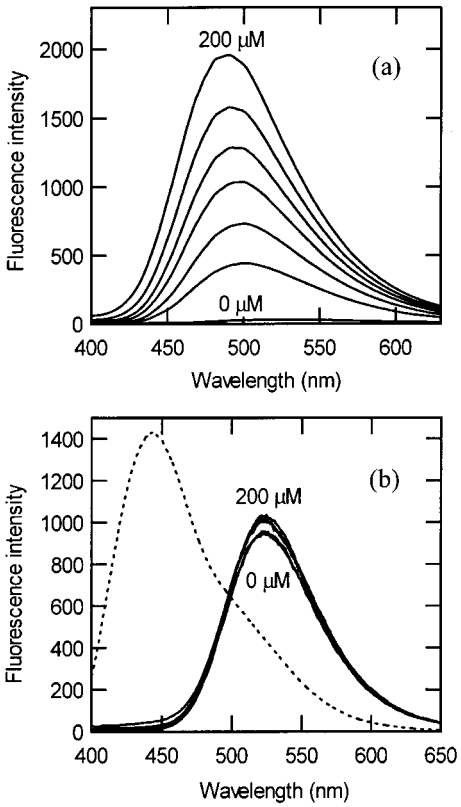

FIGURE 2: Fluorescence emission spectra of a 2 μM solution of hydrophobic ligands, (a) bis-ANS and (b) prodan, in 10 mM sodium phosphate buffer (pH 6.0) with and without β_2-GPI Domain V. The protein concentrations were as follows: (a) 0, 5, 10, 30, 50, 100, and 200 μM; (b) 0, 40, 80, 100, and 200 μM. The dotted line in (b) indicates the prodan spectrum measured in the presence of β-lactoglobulin (40 μM).

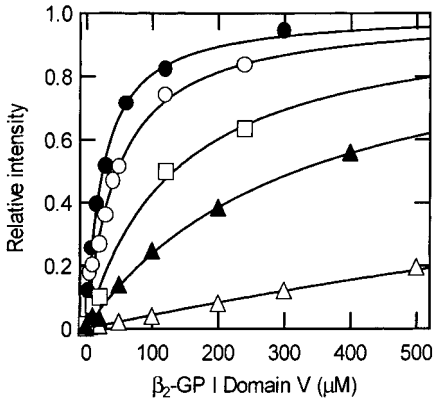

FIGURE 3: Relative fluorescence intensities for various ligands as a function of β_2-GPI Domain V concentration in 10 mM sodium phosphate buffer (pH 6.0). bis-ANS (●), retinol (○), DAUDA (□), laurdan (▲), and ANS (△).

Table 1: Binding Constants (K_b) of β_2-GPI Domain V with Various Hydrophobic Ligands and Net Charges of Ligands

ligands	K_b (±error) (M^{-1}) at pH 7.5	K_b (±error) (M^{-1}) at pH 6.0	net charge
bis-ANS[a]	$6.5 (\pm 1.4) \times 10^3$	$4.2 (\pm 0.5) \times 10^4$	−2
retinol	$8.2 (\pm 1.5) \times 10^3$	$2.2 (\pm 0.5) \times 10^4$	0
DAUDA	$4.2 (\pm 1.4) \times 10^3$	$7.6 (\pm 3.0) \times 10^3$	−1
laurdan	$7.9 (\pm 4.3) \times 10^2$	$3.2 (\pm 1.6) \times 10^3$	0
ANS	$5.0 (\pm 1.1) \times 10^2$	$5.6 (\pm 1.4) \times 10^2$	−1
prodan	NB[b]		0

[a] The K_b values of bovine serum albumin (pH 7.5), β-lactoglobulin (pH 7.5), and lysozyme (pH 6.0) for bis-ANS were $2.3 (\pm 0.5) \times 10^6$, $1.8 (\pm 0.2) \times 10^4$, and $1.4 (\pm 0.3) \times 10^4$ M^{-1}, respectively. [b] NB: no binding observed.

hydrophobic and negatively charged ligand, was not so large. The K_b value for the negatively charged bis-ANS was increased by lowering the pH from 7.5 to 6.0. The binding constants of neutral hydrophobic ligands such as retinol, DAUDA, and laurdan were relatively independent of pH. The increase in the K_b value for bis-ANS with decrease in pH was probably caused by the increase in the electrostatic attraction because the net charge of Domain V increases with decreases in pH (see below).

Interactions of Domains IV−V or Nicked Domain V with Bis-ANS. Domains IV−V and nicked Domain V were prepared to examine the roles of Domain IV and the flexible loop structure, respectively, on the binding of hydrophobic ligands. Figure 4a shows the titration curves of Domains IV−V and nicked Domain V with bis-ANS, compared with that of intact Domain V. The titration curve of Domains IV−V was similar to that of intact Domain V, showing that Domain IV has no significant role in the interactions with hydrophobic ligands. As summarized in Table 2, this behavior of Domains IV−V was the same as that with other ligands and pH conditions.

In contrast, the nick in the loop decreased the interaction of Domain V with the ligand. The K_b value of the nicked Domain V was about half of the value for intact Domain V, indicating that, although flexible, the tertiary structure as observed in the intact Domain V is important for the interaction with hydrophobic ligands. This result was consistent with the observation that the affinity of nicked Domain V to liposomes was reduced in comparison with the intact protein (*18*, *23*). We also examined the effects of salt concentration on binding of intact Domain V and nicked Domain V to bis-ANS (Figure 4b). The affinity of both intact Domain V and nicked Domain V decreased with increases in NaCl concentration, whereas the K_b values of the nicked Domain V were always smaller than those for the intact Domain V. These results were again consistent with the interaction between intact Domain V or the whole β_2-GPI with liposomes, confirming the participation of attractive electrostatic interactions (*18*, *23*).

Disulfide-Reduced and Alkylated Domain V. Hydrophobic ligands such as ANS and bis-ANS are often bound to the intermediate states of protein folding (*26*−*30*). To determine the relationship between ligand binding to the intact Domain V and binding to the folding intermediates, we studied the effects of disulfide bond reduction of Domain V. Figure 5a compares the CD spectra of the intact Domain V, RCM-Domain V, and RCAM-Domain V. While the CD spectrum

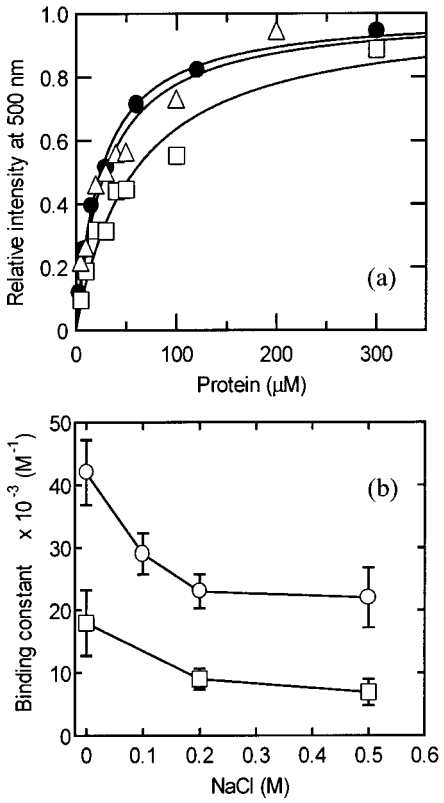

FIGURE 4: Interactions of Domains IV−V and nicked Domain V with bis-ANS. (a) Relative fluorescence intensities of bis-ANS at 500 nm as a function of the concentrations of the intact Domain V (●), nicked Domain V (□), and Domains IV−V (△) in 10 mM sodium phosphate buffer (pH 6.0). (b) Effects of NaCl concentration on the binding constants of the intact Domain V (○) and nicked Domain V (□) with bis-ANS.

Table 2: Binding Constants (K_b) of β_2-GPI Domain V, Domains IV−V, and Nicked Domain V with Hydrophobic Ligands

ligands	proteins	K_b (±error) (M^{-1}) at pH 7.5	K_b (±error) (M^{-1}) at pH 6.0
bis-ANS	Domain V	$6.5 (\pm 1.4) \times 10^3$	$4.2 (\pm 0.5) \times 10^4$
	Domains IV−V	$7.0 (\pm 1.2) \times 10^3$	$3.6 (\pm 1.0) \times 10^4$
	nicked Domain V		$1.8 (\pm 0.5) \times 10^4$
retinol	Domain V	$8.2 (\pm 1.5) \times 10^3$	
	Domains IV−V	$9.5 (\pm 2.8) \times 10^3$	

of the intact Domain V showed no obvious peak in the far-UV region (*18*), those of RCM-Domain V and RCAM-Domain V exhibited the typical spectra of a random coil conformation. The near-UV CD spectrum of the intact Domain V showed positive ellipticity at around 260−280 nm, indicating a unique tertiary structure (*18*). For RCM-Domain V and RCAM-Domain V, however, there were no fine peaks, indicating that the proteins were substantially disordered although they were also different from that of the GuHCl-induced unfolded state.

Then, we examined the interactions of RCM-Domain V and RCAM-Domain V with bis-ANS. The fluorescence intensities of the ligand were plotted against the protein concentrations (Figure 5b). While the affinity of RCM-Domain V with bis-ANS was decreased as compared with that of intact Domain V, the affinity of RCAM-Domain V was increased markedly. This result is interesting because these two modified proteins have similar structures as shown

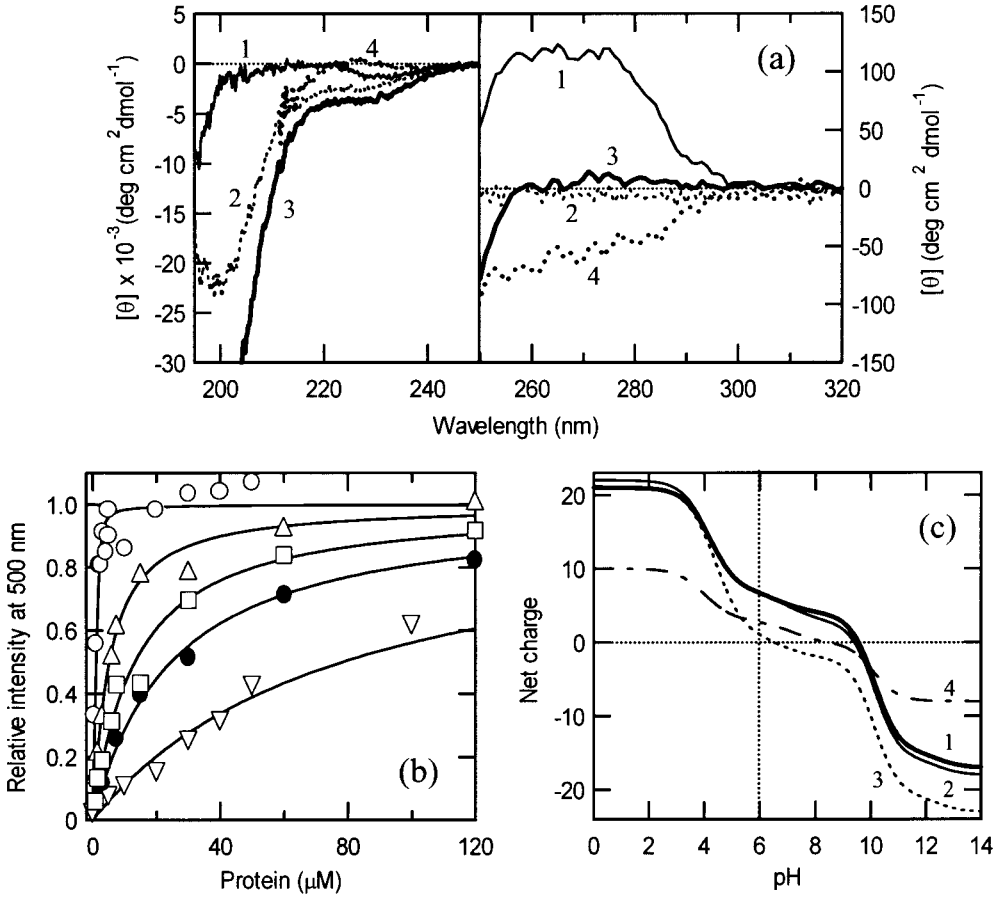

FIGURE 5: Properties of the disulfide-reduced and alkylated Domain V. (a) Far- and near-UV CD spectra for the intact Domain V (fine continuous line, 1), RCM-Domain V (dotted line, 2), and RCAM-Domain V (boldface continuous line, 3) at pH 6.0 in 10 mM sodium phosphate buffer. The boldface dotted line (4) shows the 8 M GuHCl-induced unfolded state of intact Domain V. (b) Relative fluorescence intensities of bis-ANS at 500 nm as a function of the concentrations of the intact Domain V (●), RCAM-Domain V (○, 0 M; △, 0.1 M; □, 0.25 M NaCl), and RCM-Domain V (▽) in 10 mM sodium phosphate buffer (pH 6.0). The K_b values for RCAM-Domain V and RCM-Domain V were 7.5×10^6 and 3.0×10^3 M^{-1}, respectively, in the absence of NaCl. (c) Dependencies of protein net charges against pH values. Intact Domain V (boldface continuous line, 1), nicked Domain V (fine continuous line, 2), RCM-Domain V (dotted line, 3), and P35 (dash and dotted line, 4).

by the CD spectra (Figure 5a). It is noted that the far-UV CD spectra of RCAM-Domain V did not change notably upon interaction with bis-ANS (data not shown), indicating the absence of significant ligand-induced conformational change.

For RCM-Domain V, the decrease in affinity may be due to the reduction of protein net charge by the six added carboxyl groups, resulting in a decrease in the electrostatic attraction between the positively charged Domain V and the negatively charged bis-ANS. We calculated the net charges of the intact Domain V and its modified forms assuming independence of titratable groups based on the pK_{int} values used by Matthew (*31*), and they were plotted against pH (Figure 5c). The titration curve indicated that intact Domain V has a p*I* value of 10 because of the high content of basic amino acid residues. The increase in net charge with decrease in pH explains the larger binding constant at pH 6 than at pH 7.5 (Table 1). The curve for RCM-Domain V was shifted to the lower pH region in comparison with the intact Domain V, and its p*I* value was about 6. This decrease in p*I* results in a reduction of the electrostatic attraction between RCM-Domain V and bis-ANS at pH 6. The titration curve for RCAM-Domain V was similar to that of intact Domain V, and, therefore, the increase in binding affinity of RCAM-Domain V cannot be explained by the change in protein net charge. Similarly, the titration curve for the nicked form was not markedly different from that for intact Domain V, although its interaction with bis-ANS was less than that of the intact form.

Figure 5b also shows that the interaction between RCAM-Domain V and ligand decreased markedly with increasing salt concentration, as found for the intact and nicked forms (Figure 4b). These salt effects indicate the participation of electrostatic interactions even for the disulfide-reduced proteins.

Effects of Urea on the Interaction between Domain V and Bis-ANS. The results described above for reduced Domain V further suggested that the intermediate conformational state of Domain V is responsible for the binding of hydrophobic ligands, as observed for the protein folding intermediate (*26*−*29*). Thus, we expected that the destabilization of Domain V by the addition of denaturant might increase the ligand binding. We examined the effects of urea on the binding of bis-ANS using the fluorescence change of ligand (Figure 6). The fluorescence intensities of bis-ANS in the presence of the intact Domain V decreased immediately with increases in the concentration of urea. Figure 6 also shows the urea-induced unfolding transitions of the intact Domain V detected in terms of the ellipticities at 213 and 276 nm, taken from Hagihara et al. (*18*). The agreement of the transition curves

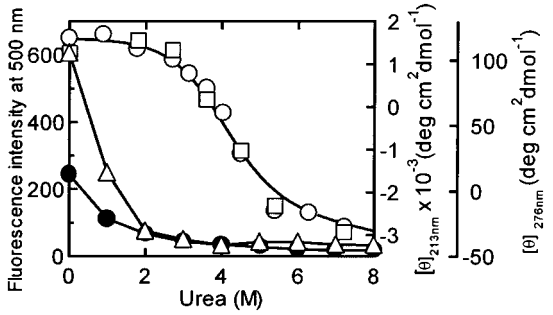

FIGURE 6: Effects of urea concentration on the bis-ANS fluorescence intensities at 500 nm in the presence of intact Domain V (●) and RCAM-Domain V (△). The protein concentration was 15 μM. Dependencies on urea concentration of the ellipticity of the intact Domain V at 213 (○) and 276 (□) nm are also indicated. The CD data are taken from Hagihara et al. (*18*).

measured by far-UV CD and near-UV CD suggested that the unfolding transition can be approximated by a two-state transition. These results indicated that the intermediate conformational state is not involved in bis-ANS binding to the intact Domain V and that bis-ANS binding occurs only to the native form through the flexible loop region.

The strong binding of bis-ANS to RCAM-Domain V was also prevented by the addition of urea. It is likely that RCAM-Domain V assumes a collapsed conformation with exposed hydrophobic clusters and that this collapsed conformation is transformed to the highly disordered random coil-like structure by the addition of urea, resulting in the dissociation of bis-ANS. The similarity of the transition curves of RCAM-Domain V and intact Domain V suggested that the regions of intact Domain V responsible for bis-ANS binding assume a conformation similar to that of RCAM-Domain V, i.e., a flexible but collapsed conformation.

Interactions of Loop Peptides with Bis-ANS. To directly demonstrate the roles of the loop regions and the positively charged sequence motif (Cys281−Cys288), we examined the interactions of three peptides, P14, P21, and P35, with bis-ANS. The amino acid sequences of these peptide fragments are shown in Figure 1c. The far-UV CD spectra of these peptides were typical of random coils (Figure 7a). The near-UV CD spectra of all peptides showed no fine peaks (data not shown). Thus, the three peptides had no persistent secondary or tertiary structures.

The fluorescence spectra of bis-ANS (2 μM) in the presence of these peptides (100 μM) were measured (Figure 7b). While peptide P14 with four Lys residues showed no interaction with bis-ANS, bis-ANS was weakly bound to peptides P21 and P35. Therefore, the isolated peptide corresponding to the loop region can interact with the hydrophobic ligand, confirming that the flexible loop region (P21) is mostly responsible for the binding of small hydrophobic ligands. In the presence of bis-ANS, the CD spectrum of peptide P21 was similar to that of the peptide alone (data not shown), indicating the absence of significant conformational change upon binding of bis-ANS.

We tried to estimate the binding constant for P35 and P21 peptides. However, P35 in the presence of bis-ANS exhibited a high propensity to aggregate at peptide concentrations above 30 μM. Accordingly, we could not determine the binding constants for P35. Assuming that the binding of P35 or P21 and bis-ANS results in the same fluorescence increase as that of intact Domain V, the binding constants were 1 order of magnitude smaller than that of intact Domain V.

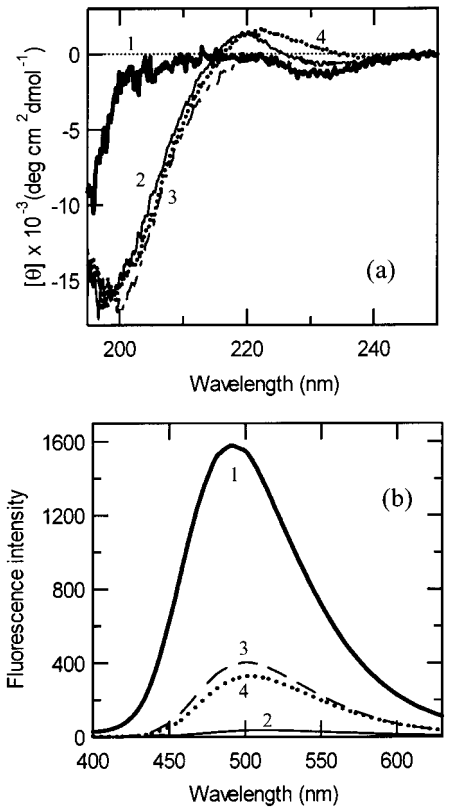

FIGURE 7: Interactions of loop peptides with bis-ANS. (a) Far-UV CD spectra of intact Domain V (boldface continuous line, 1), P14 (fine continuous line, 2), P21 (dash and dotted line, 3), and P35 (boldface dotted line, 4) in 10 mM sodium phosphate buffer (pH 6.0). (b) Fluorescence emission spectra of bis-ANS in the presence of the intact Domain V (boldface continuous line, 1), P14 (fine continuous line, 2), P21 (dash and dotted line, 3), and P35 (boldface dotted line, 4) in 10 mM sodium phosphate buffer (pH 6.0). The concentrations of peptide and bis-ANS were 100 and 2 μM, respectively.

DISCUSSION

While the four N-terminal domains of β_2-GPI have a typical short consensus motif fold, similar to each other and to other domains of this superfamily, Domain V with an additional two cysteines shows strong deviation from the other domains (*13*, *14*). Within Domain V, the exposed C-terminal loop (Ser311−Lys317) is highly mobile as it was not visible in the final electron density map (*13*), and its temperature factors were very high (*14*). NMR analysis of the isolated Domain V confirmed the high flexibility of the loop region (*21*). This flexible loop has been suggested to be inserted into the lipid layer when Domain V binds to the lipid membrane. The importance of Domain V and its flexible loop region in the function of β_2-GPI is now becoming evident. However, the mechanism by which the loop region interacts with phospholipids, or other negatively charged compounds, is not clear. To address this problem, we studied the interaction of Domain V and its modified forms with various small hydrophobic compounds. The results clarified several basic features of the binding of hydrophobic compounds through the flexible loop, which will be common to the interaction with biologically important phospholipids.

Critical Role of the Flexible Loop of Domain V. X-ray structural analyses suggested that domains of β_2-GPI are independent structural units without significant interactions between them (*13*, *14*). Consistent with this, the binding constants of Domains IV−V were similar to those of Domain V (Figure 4a), confirming that Domain V is responsible for the interactions with ligands.

Kamboh and co-workers demonstrated that two naturally occurring missense mutations in the β_2-GPI Domain V, Trp316Ser and Cys306Gly, disrupted the binding of native β_2-GPI to phosphatidylserine (*32*), and the binding of the Leu313Gly and Phe315Ser mutations to cardiolipin was reduced to 25% and 13%, respectively, of that of the wild-type (*19*). These results suggested that the integrity of the highly conserved hydrophobic amino acids at positions 313−316 is essential for the binding of β_2-GPI to anionic phospholipids. Our findings with the nicked Domain V are consistent with this idea. The nicked form of Domain V showed reduced affinity for bis-ANS with a binding constant half that of the intact Domain V, supporting the important role of the loop structure in the interaction of β_2-GPI with hydrophobic ligands. In the recently published X-ray crystallographic (*13*, *14*) and NMR solution (*21*) structures of β_2-GPI Domain V, this loop region was considered to assume a highly flexible structure (Figure 1). Hoshino et al. (*21*) showed that, while introduction of the nick in the loop increased the flexibility of the loop regions measured by the nuclear Overhauser effect (NOE) and order parameters, the conformation and dynamics of the rest of the Domain V molecule are the same as those of intact Domain V. The appropriate flexibility of the loop region is thought to be important for binding of hydrophobic ligands. It is also possible that the charges introduced by the nick contribute to decreases in the interactions.

In NMR titration experiments with the anionic surfactant SDS, Hoshino et al. (*21*) showed that this highly mobile loop was directly involved in the interaction with the surfactant. Hunt and Krilis (*20*) suggested that the short β-hairpin of the positively charged sequence motif CKNKEKKC (Cys281−Cys288), located near the loop in the tertiary structure, is also involved in phospholipid binding (Figure 1). The participation of this β-hairpin was also supported by the results of NMR analysis (*21*). To directly demonstrate the regions responsible for binding, we prepared peptides corresponding to the loop region (P21) and the short β-hairpin (P14) and the linked peptide (P35) of P21 and P13. While P14 did not bind to bis-ANS, P21 showed the interaction with bis-ANS, confirming that the loop region interacts with the hydrophobic ligands (Figure 7b). Contrary to our expectations, the affinity of bis-ANS to P35 was the same as that of P21, indicating no significant role of P14 even in the linked peptide. However, the precise role of the positively charged short β-hairpin in Domain V was not clear from our experiments, because the conformation of the peptide is different from that in intact Domain V. It is likely that the rigid tertiary structure of the β-hairpin is important for its participation in binding.

Nature of Interaction. From the interaction of β_2-GPI Domain V with various hydrophobic ligands monitored by the changes in fluorescence spectra of ligands (Figures 2 and 3), the negatively charged ligands were shown to exhibit more strong interactions than the neutral ligand, consistent with the observation that β_2-GPI interacts preferentially with negatively charged phospholipid (*6*, *17*, *18*, *33*). The results argue the critical role of electrostatic interactions between the positively charged Domain V and the negatively charged ligands. These results also emphasized the importance of hydrophobic interactions, as shown by the tight binding of retinol and DAUDA. However, the values of K_b of Domain V for the sufficiently negatively charged and hydrophobic ligands were of the order of 10^4, not so large as compared with other proteins known to bind hydrophobic ligands tightly (Table 1). These results also suggested that the interaction is not so specific that various compounds with hydrophobic regions and negative charges can be bound to the β_2-GPI Domain V. This is consistent with the observations that the β_2-GPI Domain V interacts with a variety of negatively charged compounds such as DNA, heparin, and dextran sulfate (*3*−*5*), although the biological significance of these interactions is unknown.

We noticed the similarity between the binding of hydrophobic compounds to Domain V and those to the protein folding intermediates. The molten-globule states specifically interact with ANS or bis-ANS because of their exposed hydrophobic surfaces (*26*−*29*). The importance of electrostatic interaction in the binding of ANS to the molten-globule state has also been suggested (*34*). The binding is often negligible for the native state and the fully unfolded states, except in cases in which the native state has a specific binding pocket for the hydrophobic ligands as observed for apomyoglobin (*35*) or β-lactoglobulin (*36*, *37*). The X-ray and NMR structures showed no hydrophobic pocket in the native state of Domain V, although these experiments did show the exposed flexible and hydrophobic region (Figure 1). Thus, we considered that Domain V in solution may assume a molten-globule conformation even in the absence of denaturants. We anticipated that the population of the intermediate was increased by the addition of a low concentration of urea before global unfolding starts at around 2 M urea (Figure 6). However, the denaturation experiments indicated that bis-ANS binding occurs only to the native state of Domain V in the absence of denaturant. Then, we found that bis-ANS is bound to RCAM-Domain V. Interestingly, the K_b value of RCAM-Domain V was 2 orders of magnitude larger than that of intact Domain V (Figure 5b). Although the RCAM-Domain V assumed the disordered structure measured by CD, it is likely to be a compact collapsed state that can interact with bis-ANS.

On the basis of these observations, we consider that the intact Domain V consists of a rigidly folded region, which does not interact with bis-ANS, and a flexible molten-globule-like region, which interacts with bis-ANS through the hydrophobic and electrostatic interactions. The reduction of the three disulfide bonds converts the whole of the molecule to the collapsed molten-globule-like conformation. The molten-globule-like conformation of RCAM-Domain V is definitely more disordered than the typical molten-globule state (*28*, *29*), considering the absence of persistent secondary structures. Although the refolding of protein structure upon binding of a hydrophobic ligand like ANS has been reported (*34*), the absence of notable change in the CD spectra suggested no significant conformational change in our cases. Thus, the intact Domain V assumes a unique bipartite structure comprised of the compactly packed native region

and the flexible collapsed region. This bipartite structure causes interactions with hydrophobic compounds, including the negatively charged phospholipids. The unique tertiary structure of the loop region surrounded by several positive charges of the short β-hairpin (Cys281−Cys288) is likely to determine the specificity toward biologically important ligands, although the present results do not clarify the specificity of ligand recognition. This specific interaction with negatively charged phospholipids results in the conformational changes of other domains of β_2-GPI and/or aggregation of β_2-GPI, which enhances the antigenicity of β_2-GPI against anti-phospholipid antibodies. We assume that the aggregation of β_2-GPI plays an important role in the biological function of β_2-GPI. Instead of the exposed hydrophobic region, if the binding site of β_2-GPI was the hydrophobic pocket as observed for apomyoglobin (*35*) or β-lactoglobulin (*36*, *37*), the ligand binding would not induce the aggregation of β_2-GPI, and consequently biological activities of β_2-GPI would not take place.

Concluding Remarks. Domain V has a bipartite structure consisting of a rigidly packed core region and a disordered region. The two regions are independent of each other so that even cleavage of the disordered loop does not affect the conformation and stability of the rigid region. The disordered region contains many hydrophobic residues resulting in formation of the collapsed structure. This collapsed structure can interact with hydrophobic compounds, similar to the observation that the molten-globule intermediates interact with ANS or bis-ANS. The positively charged short β-hairpin surrounding the flexible loop may promote the binding of negatively charged compounds even if they are not directly involved in the interaction. The short β-hairpin may also be important for determining the specificity of the interaction. The binding of hydrophobic compounds to the protein folding intermediate often results in aggregation of protein. This is also observed with Domain V or the loop peptide, and it is likely that the ligand-induced aggregation of β_2-GPI is important for the biological activities of this protein. Thus, the interaction of Domain V with hydrophobic compounds is intriguing not only for understanding the function of β_2-GPI but also for understanding the binding of hydrophobic compounds to the non-native conformational states of proteins.

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