Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 67–73

BIOORGANIC & MEDICINAL CHEMISTRY

Alcohol-induced Denaturation of β-Lactoglobulin: A Close Correlation to the Alcohol-induced α-Helix Formation of Melittin

Nami Hirota-Nakaoka and Yuji Goto*

Institute for Protein Research, Osaka University, 3-2 Yamadaoka, Suita, Osaka 565-0871, Japan

Received 9 May 1998; accepted 14 September 1998

Abstract—Alcohols denature the native structure of proteins and induce α-helical structure. The potential of alcohols causing such effects varies substantially depending on the alcohol species. With β-lactoglobulin as a model protein, we compared the effects of various alcohols and observed the additive contribution of each group constituting the alcohol molecules. Whereas the hydrophobic hydrocarbon group promotes the transition according to their size, hydrophilic hydroxyl group suppresses the transition. Halogen groups promote the transition depending on their type and number. It has been known that alcohols induce the α-helical structure on the short peptides such as melittin. There is a close correlation between the potentials of alcohol in denaturing β-lactoglobulin and those in inducing the helical structure in melittin, indicating that the underlying mechanisms of the two phenomena are the same.

Introduction

Effects of alcohols on proteins and peptides have been studied extensively through the past few decades.[1–6] These studies are important because of the wide range applications of the alcohol effects in many fields. Previous studies on the effect of alcohols on proteins have focused mainly on their role as denaturants. It has been shown that various alcohols denature the rigid native state of proteins and, subsequently, stabilize the α-helical structure. Recently, alcohols, in particular, 2,2,2-trifluoroethanol (TFE, abbreviations used for alcohols are summarized in Table 1) have often been used to stabilize the α-helical structure in denatured proteins and their fragments.[6–8] The secondary structures stabilized in alcohols are suggested to be the initiation site of protein folding.[7] Recent extensive nuclear magnetic resonance studies by Dobson and co-workers[9] with ^{15}N-labeled hen egg-white lysozyme showed the structural and dynamic properties of the TFE-induced helical state and its relation to the native state, important to analyze the mechanism of protein folding. On the other hand, some alcohols such as 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) are used to dissolve aggregations which sometimes occur during peptide synthesis. Researchers investigating prion diseases[10,11] and Alzheimer's amyloid peptides[12] also use HFIP for the same purpose. Interestingly, it has been shown that HFIP has a much stronger potential than TFE in denaturing the protein native structure and also in inducing the helical structure.[13]

These effects of alcohols can be explained to some extent by the decreased polarity of the solvent.[13–16] In solvents of low polarity, hydrophobic interactions stabilizing the native structure are weakened, and simultaneously the local hydrogen bonds are strengthened, resulting in denaturation, and stabilization of the extended α-helical structures. However, the exact relation between the alcohol-induced denaturation of the native state and the alcohol-induced α-helix formation of proteins are still unclear. Luo and Baldwin[17] studied the mechanism of helix-induction by TFE. They analyzed the helix formation of the alanine based short peptides on the basis of the Lifson–Loig helix–coil transition theory.[18] At the same time, they measured the strength of the hydrogen bond in a model compound, salicylic acid, in TFE/water mixtures. They showed that the curve of hydrogen bond strength versus increasing TFE concentration matches both in shape and magnitude the increase in average helix propensity in TFE/water mixtures. Consequently, they concluded that strengthening the hydrogen bonds is responsible for the TFE effects on the helix formation of short peptides, suggesting that the mechanism of the alcohol-induced helix formation of peptides are distinct from that of the alcohol-induced denaturation of proteins in which hydrophobic effects arising from alcohol molecules are important.

In our previous study,[19] to address the mechanism of the alcohol-effects on proteins and peptides, we investigated the effects of various alcohols including TFE on melittin, a major component of honeybee venom. Melittin is made up of 26 amino acid residues, of which 5 are basic, none are acidic, and the C-terminal carboxyl

Key words: alcohol; β-Lactoglobulin; α-helix; melittin; protein folding.
*Corresponding author. Tel.: +81-6-879-8614; fax: +81-6-879-8616; e-mail: ygoto@protein.osaka-u.ac.jp

0968-0896/99/$ - see front matter
PII: S0968-0896(98)00219-3

Table 1. *m* Values of various alcohols for the alcohol-induced denaturation of β-lactoglobulin and the alcohol-induced helix formation of melittin: data for melittin on the basis of the two-state mechanism are from ref.19

Alcohol	Abbreviation used	*m* of β-lactoglobulin (± error) (kJ/mol/M)	*m* of melittin (± error) (kJ/mol/M)
Alkanols			
(1) Methanol	MeOH	1.97 (±0.03)	0.78 (±0.02)
		3.75 (±0.19)*	
(2) Ethanol	EtOH	3.92 (±0.07)	1.57 (±0.02)
		7.45 (±0.51)*	
(3) 1-Propanol	*n*PrOH	8.45 (±0.13)	3.68 (±0.10)
(4) 2-Propanol	*i*PrOH	6.01 (±0.14)	3.25 (±0.06)
(5) 2-Methy]-2-propanol	*t*BuOH	12.0 (±0.17)	4.75 (±0.12)
(6) 2-Butanol	*s*BuOH	13.0 (±0.18)	6.00 (±0.14)
Diols			
(7) 1,2-Ethanediol	$Et(OH)_2$	2.63 (±0.04)*	0.90 (±0.22)
(8) 1,2-Propanediol	$Pr(OH)_2$	2.75 (±0.07)	1.47 (±0.05)
		5.89 (±0.33)*	
(9) 1,4-Butanediol	$1,4Bu(OH)_2$	3.73 (±0.13)	1.42 (±0.03)
		6.52 (±0.16)*	
(10) 2,3-Butanediol	$2,3Bu(OH)_2$	7.63 (±0.18)*	2.86 (±0.13)
(11) 1,5-Pentanediol	$Pen(OH)_2$	5.15 (±0.26)	2.10 (±0.09)
		11.3 (±0.02)*	
(12) 2-Methyl-2,4-pentanediol	MPD	8.05 (±0.67)	4.44 (±0.28)
Triol			
(13) 1,2,3-Propanetriol	Glycerol	2.18 (±0.4)*	0.95 (±0.02)
Halogenols			
(14) 2-Fluoroethanol	FEtOH	3.16 (±0.14)	1.39 (±0.04)
(15) 2,2,2-Trifluoroethanol	TFE	9.15 (±0.11)	5.05 (±0.12)
(16) 1,1,1,3,3,3-Hexafluoro-2-propanol	HFIP	37.3 (±0.41)	18.2 (±1.19)
(17) 2-Chloroethanol	ClEtOH	8.23 (±0.22)	3.17 (±0.13)
(18) 3-Chloro-1-propanol	ClPrOH	16.7 (±0.26)	6.71 (±0.17)
		22.1 (±2.93)*	
(19) 2-Bromoethanol	BrEtOH	12.8 (±0.54)	9.04 (±0.16)

*Asterisks indicate data obtained in the presence of 20% ethanol.

group is amidated:[20] NH_3^+-G-I-G-A-V-L-K^+-V-L-T-T-G-L-P-A-L-I-S-W-I-K^+-R^+-K^+-R^+-Q-Q-$CONH_2$. Melittin is in a monomeric unfolded state under conditions of low salt at neutral pH and transforms into an α-helical tetramer under high-salt or high-acid conditions, at alkaline pH, or at high peptide concentrations.[21] Addition of alcohols also converts melittin into a monomeric α-helix.[22] A systematic approach was taken in order to understand the contribution of each group, for example, the hydrocarbon (CH) group, hydroxyl (OH) group and halogen group, to the alcohol effects on melittin (Fig. 1). Among alkanols, the effectiveness was proportional to the bulkiness of hydrocarbon groups, indicating that the hydrocarbon group contributes positively to the alcohol effects. Comparison of alcohols with the same hydrocarbon group but different number of hydroxyl groups showed that the hydroxyl groups contributes negatively to the alcohol effects. Comparison of several halogenols indicated that halogen increases the effectiveness in the order of $F < Cl < Br$. These results suggested that the effects of alcohol can be interpreted by the additive contributions of each of the constituent groups of the alcohol, which are proportional to the solvent-accessible surface area.

In order to improve our understanding of the mechanism of the alcohol effects, we examined the alcohol-induced denaturation of β-lactoglobulin systematically with various alcohol species. Bovine β-lactoglobulin is a major component of cow's milk and has a molecular mass of 18,400 (162 amino acid residues), containing two disulfide bonds and one free cysteine. It is a predominately β-sheet protein consisting of a β-barrel of eight antiparallel β-strands shaped into a flattened cone and one major α-helix.[23] Whereas it exists as a dimer at neutral pH, the native monomer is stable even at pH 2.[24,25] β-Lactoglobulin is a useful model to study the alcohol effects on proteins because it exhibits the marked conformational transition upon addition of alcohols from the native β-sheet to the α-helical structure.[26–28]

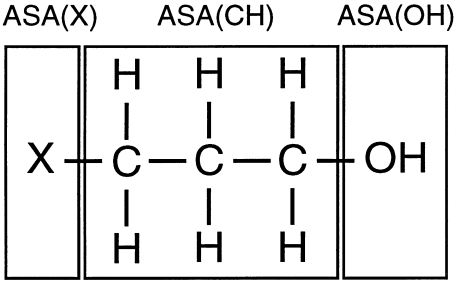

Figure 1. Group additive contributions to the alcohol effects on peptides and proteins. An alcohol molecule consists of hydrocarbon (CH), halogen (X), and hydroxyl (OH) groups. These groups contribute additively to the effect of alcohols. Hydrocarbon and halogen groups contribute negatively to the alcohol effects. In the case of the alcohol-induced α-helix formation of melittin, the *m* value can be expressed as the sum of the accessible surface area (ASA) of each group multiplied by a constant specific to each group: $m = a \times ASA(CH) + b \times ASA(OH) + c \times ASA(X)$, where a, b, and c are constants and $a > 0$, $b < 0$ and $c > 0$.

As was previously indicated,[13] the effectiveness of alcohols in denaturing the native state of β-lactoglobulin varies substantially depending on the alcohol species. To compare quantitatively the effect of alcohols, we approximated the alcohol-denaturation by a two-state mechanism between the native and α-helical states. We also assumed the linear dependence of the free energy change (ΔG_D) of denaturation upon alcohol molarity and obtained the *m* value, the slope of the linear dependency, as a measure of the effectiveness of an alcohol. Similar to the case of melittin, the effects of alcohol can be interpreted by the additive contributions of each of the constituent groups of the alcohol. Consistent with this, a high correlation was observed between the effectiveness of various alcohols on β-lactoglobulin and melittin, suggesting that a common mechanism explains the two contrasting phenomena. We consider that direct interactions between the hydrophobic groups of alcohols with those of proteins are responsible for the alcohol effects on peptides and proteins and that the aggregation of alcohol molecules is an essential factor for understanding the marked effects of TFE and HFIP.

Results

Alcohol-denaturation of β-lactoglobulin

The native structure of β-lactoglobulin is stable as a monomer even at pH 2.[24] In the absence of alcohol in 20 mM HCl, the far-UV circular dichroism (CD) showed a spectrum with a minimum at 218 nm, consistent with the abundance of β-sheets (Fig. 2). The fine negative peaks in the near-UV region indicate the unique packing of aromatic side chains (not shown). Upon addition of EtOH more than 4 M, the far and near-UV peaks of the native state were disrupted and simultaneously the far-UV spectrum representative of the α-helical structure was formed. The helical structure in the presence of high concentration of EtOH had no significant tertiary structures as evidenced by the monotonous near-UV CD spectrum (data not shown). The maximal ellipticity at 222 nm is −26,000 and the helical content was estimated to be about 90% by the method of Chen et al.[29] These results were consistent with those reported previously, showing the high helical propensity of β-lactoglobulin in alcohols.[26–28,30]

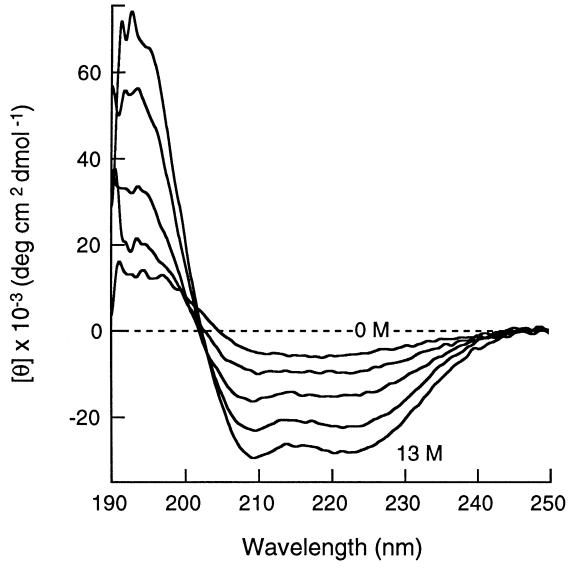

Figure 2. Far-UV CD spectra of β-lactoglobulin in the presence of various concentrations of EtOH in 20 mM HCl and 20 °C. EtOH concentrations of the spectra from top to bottom at 222 nm: 0, 4.8, 5.3, 7.3 and 13 M.

Group additive contribution

We compared the effectiveness of various alcohols by using the transition curves measured by the ellipticity at 222 nm. Important groups constituting an alcohol molecule are CH, halogen, and OH groups (Fig. 1).

Hydrocarbon group. First, in order to close up the role of the CH group, we compared the effects of various alkyl alcohols (alkanols). Figure 3(a) shows the results for MeOH, EtOH, *n*PrOH, *i*PrOH and *s*BuOH. The maximal ellipticity was similar independent of the alcohol species, as was the case of alcohol-induced transition of melittin (see Fig. 2 of Hirota et al.[19]). It is evident that the alcohol concentration required to induce the transition decreases as the size of alcohol molecule becomes larger (MeOH < EtOH < *n*PrOH < *i*-PrOH < *t*BuOH < *s*BuOH, Table 1). This result is consistent with Uversky et al.,[30] who reported an order of MeOH < EtOH < *n*PrOH. The similar order was reported previously for the thermal denaturation of lysozyme[3–5] or ribonuclease A.[1,3] This order is also the same as the order observed for the alcohol-induced α-helix formation of melittin.[19]

Halogen group. High effectiveness of TFE suggested that F atom has a high potential for inducing the alcohol effects. On the contrary, our previous study with melittin demonstrated that F atom has the least effect among halogen atoms.[19] We compared the effects of various halogenols (Table 1, Fig. 3(b)). The ellipticity of the helical state was again independent of the alcohol species. For all the cases, the effects of halogenols are higher than those of the corresponding alkanols (Table 1). It is clear that the order of effectiveness for halogen groups is: H < F < Cl < Br. In other words, effectiveness of halogen groups is proportional to its bulkiness. This order is the same as that for melittin[19] and confirms that isolated F atom has only a weak potential to induce the alcohol effects, although the presence of multiple F atoms as in TFE or HFIP increases the effects markedly.[13] Jackson and Mantsch[31] also reported a similar order for the alcohol-induced helix formation of concanavalin A, a β-sheet protein.

Hydroxyl group. The transitions induced by alkanediols and alkanetriol (glycerol) were also examined (Fig. 3(c)). This is to reveal how OH groups contribute to the effectiveness of an alcohol. Our studies with melittin showed that alkanediols have a profoundly weaker potential than alkanols in inducing α-helices, indicating that OH groups contribute negatively to the alcohol

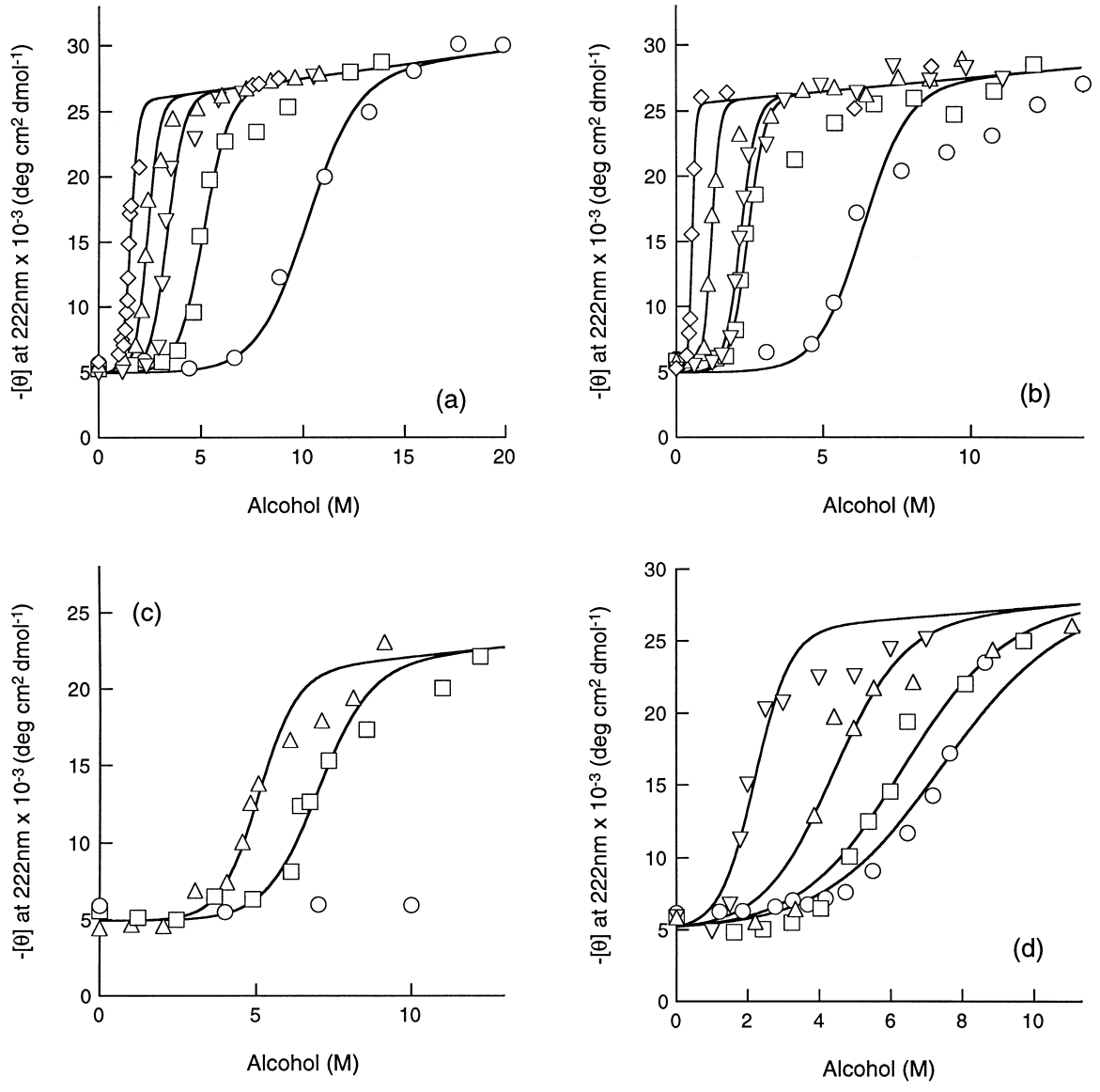

Figure 3. Conformational transition of β-lactoglobulin induced by various alcohols measured by the ellipicity at 222 nm at 20 °C. The curves were drawn assuming a linear dependence of ΔG_D upon alcohol concentrations with the *m* values shown in Table 1. (a) Alkanols: ○, MeOH; □, EtOH; ▽, *i*PrOH; △, *n*PrOH; ◇, *s*BuOH. (b) Halogenols: ○, FEtOH; □, ClEtOH; ▽, TFE; △, ClPrOH; ◇, HFIP. (c) Alkanediols: ○, Glycerol; □, $Pr(OH)_2$; △, 1:4BU$(OH)_2$. (d) Alkanediols in the presence of 20% (~3 M) EtOH: ○, Glycerol; □, $Et(OH)_2$; △, MeOH; ▽, 2,3Bu$(OH)_2$.

effects.[19] In other words, although OH groups are essential for defining alcohol molecules, they are important only for dissolving the hydrophobic CH and halogen groups and it is not responsible for the alcohol effects. Similar results were reported from the effect of various alcohols on the heat-denaturation of lysozyme and ribonuclease A.[3]

Similar to the case of melittin, effectiveness of alkanediols (e.g. $Et(OH)_2$) was much less than the corresponding alkanols (e.g. EtOH). It is noted that the ellipticities of the helical states induced by alkanediols are evidently less than those of alkanols and halogenols. The decreased helical intensity for alkanediols were also observed for melittin,[19] probably reflecting the low potential of alkanediols as protein denaturant and helix-inducer.

With glycerol, an alkanetriol, we did not see a clear denaturing effect on β-lactoglobulin (Fig. 3(c)). Nevertheless, it was intriguing to examine further the effects of glycerol, because glycerol induces the helical structure of melittin.[19] We expected that glycerol also destabilizes the native state of β-lactoglobulin, even though its potential is weak. Because glycerol is conventionally used as a solvent stabilizing the protein native structure,[3,32] our expectation for glycerol as a helix inducer is contrary to the expectation as a protein stabilizer.

To detect weak alcohol effects, we first destabilized the native β-lactoglobulin by adding EtOH at 20% (v/v). Then, the effects of several alcohols including glycerol were examined in the presence of 20% (v/v) EtOH (Fig. 3(d)). Now, it is clear that glycerol denatures the native state and induces the helical structure. This

demonstrates that, although weaker than other alcohols, glycerol also has a potential to destabilize the native protein structures.

Quantification of the alcohol effects

The transition curves measured by the ellipticity at 293 nm were highly cooperative (data not shown). On the other hand, the transition curves measured by the ellipticity at 222 nm consisted of two phases, that is, the initial cooperative phase and the subsequent gradual phase with a small amplitude (Fig. 3). The initial change occurred at the same EtOH concentrations where the transition at 293 nm occurred. Therefore, we considered that the transition curves can be approximated by a two-state mechanism assuming the baselines as shown in Figure 3 (see Discussion). Then, the *m* values specific to each alcohol were obtained.

In these analysis, we assumed a unique value for the ΔG_D (20.0 kJ/mol) in the absence of alcohol. The estimated value is consistent with a value (23.3 kJ/mol) determined from calorimetry at pH 2.0 and 20 °C.[33] The *m* values for various alcohols are summarized in Table 1, showing that the effectiveness of alcohols varies substantially depending on the alcohol species. The *m* values in the presence of 20% EtOH were generally consistent with those in the absence of EtOH, although the former has a tendency to be slightly larger than the latter. This suggests that the added alcohols effect additively in decreasing the stability. Theoretical curves based on the two-state mechanism are shown in Figure 3, in reasonable agreement with the experimental data.

Correlation of the *m* values with those of melittin

We examined the correlation between the *m* values for β-lactoglobulin with those for melittin (Fig. 4). As can be seen, a marked correlation is evident, indicating that an alcohol with a high helix-inducing potential has a correspondingly high potential to denature the native state of β-lactoglobulin. HFIP has the highest potential in both denaturing the native state of β-lactoglobulin and inducing the helical structure in melittin. The slope of the linear correlation is about 2.0. In the former case, the alcohols fold the unfolded melittin to the helical structure. In the latter case, the critical step of the alcohol effect is the destabilization of the closely packed native structure. The higher *m* values of the latter case mean that the induction of helical structure is coupled with the rate-limiting denaturation of the native structure.

Discussion

To understand the mechanism producing the variation of alcohol effects on proteins depending on the alcohol species, we used β-lactoglobulin as a model protein, because it exhibits a dramatic β to α conformational transition upon addition of alcohols.[26] The results demonstrated the additive contribution of each group constituting alcohol molecules, the same as the results observed for melittin (Fig. 1). Assuming a two-state

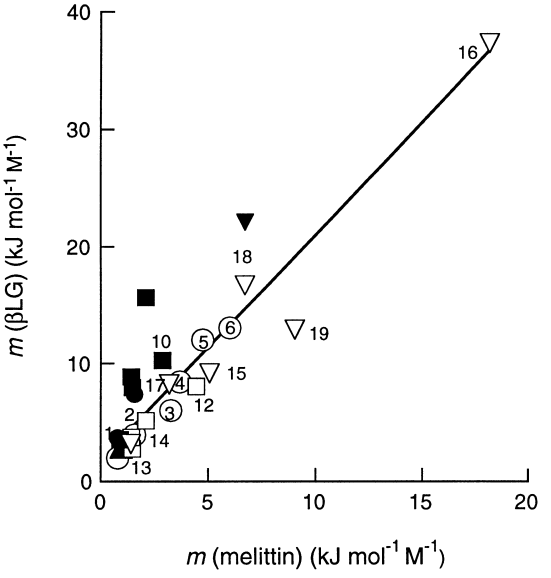

Figure 4. Correlation between the *m* values of β-lactoglobulin and melittin. The solid line is the best correlation for the data, with the intercept at 1.82 and the slope = 1.91. Data point are for alkanols (○), halogenols (▽), alkanediols (□) in the absence (open symbols) and presence (closed symbols) of 20% EtOH. Data point for glycerol (▲) is in the presence of 20% EtOH.

transition and a linear dependence of ΔG_D upon alcohol concentration, we analyzed the transition curve measured by the ellipticity at 222 nm and obtained the *m* value for each alcohol (Table 1). For some alcohols, we also obtained the *m* value in the presence of 20% (v/v) EtOH.

Uversky et al.[30] examined the effects of several organic solvents (i.e. MeOH, EtOH, *i*PrOH and dimethylformamide, on the structure of β-lactoglobulin at pH 2. They compared the transition curves measured by several probes including near and far-UV CDs, tryptophan fluorescence, and fluorescence decay of 8-anilinonaphthalene-1-sulfonate (ANS). From the disagreement of the transition curves measured by these probes, they proposed the accumulation of the intermediate (I_H) with disordered side chains but with increased helical content, before formation of the highly helical denatured state (H): $N_\beta \rightleftarrows I_H \rightleftarrows H$. Our results were basically consistent with theirs: the $N_\beta \rightarrow I_H$ is a major transition accompanied by both the near and far-UV CD changes and the subsequent $I_H \rightarrow H$ is accompanied mostly by the gradual increase in the far-UV CD intensity. However, the analysis on the basis of the three-state mechanism was not straightforward becuase the $I_H \rightarrow H$ transition is gradual and its amplitude is small. Therefore, although phenomenological, we assumed a two-state mechanism to compare the effectiveness of various alcohols. The advantage of the two-state approximation is the simplicity: when this approximation was used, the *m* value is related by the C_m value of the alcohol-induced transition by $\Delta G_D = m \cdot C_m$.

Alcohol-induced denuration of proteins has been considered to arise from the low polarity of the solvent, which decreases the hydrophobic interactions stabilizing the compact native structure of proteins.[3,15–17] Therefore,

it is plausible to expect that variation of the effectiveness of alcohol may be related to the polarity of the solvent. In fact, Uversky et al.[30] showed a high correlation between the extent of conformational transition with the relative dielectric constant of several organic solvents and concluded the importance of the solvent polarity. On the other hand, in our previous paper[19] with melittin, we proposed that, although the polarity of the solvent is an important factor determining the alcohol effect, the markedly high potential of some alcohols such as TFE or HFIP cannot be explained by the low polarity of the solvent alone. The high correlation of the *m* values for melittin and β-lactoglobulin indicates that the high potential of TFE or HFIP against the native proteins cannot be explained by the low polarity of the solvent alone.

As described in the Introduction, Luo and Baldwin[17] studied the mechanism of helix-induction by TFE and concluded that the strengthening of the hydrogen bonds is responsible for the TFE effects on the helix formation of short peptides. On the other hand, they consider hydrophobic effects arising from alcohol molecules are responsible for denaturing the native structure, the mechanism being distinct from the hydrogen bond-driven α-helix formation of peptides. However, the high correlation between the effectiveness of various alcohols in denaturing β-lactoglobulin and in inducing helical structure in melittin strongly suggests that the underlying mechanism is the same and seems not to be the strengthening of hydrogen bonds.

Kurpin et al.[34] studied alcohol/water mixtures, including HFIP, by small-angle X-ray scattering and reported that HFIP has a high tendency to form micelle-like assemblies with a maximum at about 30% (v/v). Other alcohols also tend to form micelle-like assemblies but to a much lesser extent. In aqueous solution of water-miscible alcohols, alcohols associate so as to minimize their contact with water, which results in the formation of micelle-like assembly with the hydrophobic groups inside, although no macroscopic phase separation takes place.[35] We consider that this characteristic of HFIP or TFE may be the reason for their unexpectedly high potential in stabilizing α-helices and destabilizing the native structure of proteins.

The hydrophobic groups of alcohols can associate with each other, burying their hydrophobic surface. It is likely that hydrophobic groups of proteins or peptides also take part in such association. An analogy is the denaturation of proteins by sodium dodecyl sulfate (SDS). We assume that SDS is similar to alcohol since it consists of a hydrophobic hydrocarbon group and hydrophilic carboxyl group. Although monomeric SDS can interact with proteins and peptides, the interaction is not extensive. Once SDS forms micelles above critical micelle concentration (cmc), the interaction becomes marked because micelles provide extensive sites of interaction (i.e. the increase in the effective concentration of the interacting sites). This results in the formation of the extended and persistent helical structures. This situation may be resembling the marked alcohol effects induced by HFIP.

Conclusion

The results described in this and previous papers[13,19] strongly indicate that a common mechanism may explain the alcohol effects on proteins and peptides. Although the low polarity of alcohols is an important factor determining the alcohol effects, it does not explain the marked effects of halogenated alcohols such as HFIP or TFE. We consider that the direct interaction of the hydrophobic groups of alcohols with the hydrophobic groups of proteins or peptides is responsible for the alcohol effects. The marked effects of HFIP arise because of the high propensity of this alcohol to form micelle-like aggregates which then promote the interaction between the alcohols and peptides or proteins.

Experimental

Materials

MeOH, EtOH, *n*PrOH, *i*PrOH, *t*BuOH, TFE, HFIP, $Pr(OH)_2$, $Pen(OH)_2$, $Hex(OH)_2$ and glycerol were purchased from Nacalai Tesque. *s*BuOH, FEtOH, ClEtOH, ClPrOH, $Et(OH)_2$, $1,4Bu(OH)_2$, $2,3Bu(OH)_2$ and glycerol were purchased from Wako Pure Chemical. BrEtOH purchased from Tokyo Kasei Kogyo was dehydrated with magnesium sulfate and then distilled in vacuum. β-Lactoglobulin (isomerA) was purchased from Sigma and used without further purification. The protein concentration was determined from the absorption at 280 nm using the absorption coefficient of $E_{278} = 9.6$.[36]

CD measurements

CD measurements were done with a Jasco spectropolarimeter, model J-720, at 20 °C. Far-UV CD spectra were obtained using a cell with 1-mm or 0.1-mm light path depending on the absorption of the solvent. The temperature was controlled with a water-circulating cell holder. The data were expressed as molar residue ellipticity [θ], which is defined as $[\theta] = 100\theta_{obs}/lc$, where θ_{obs} is the observed ellipticity in degrees, *c* is the concentration in residue moles per liter, and *l* is the length of the light path in centimeters. Typically, 50 μL of protein solution at a protein concentration of 1 mg mL^{-1}, dissolved in deionized water, was mixed with 450 μL of 20 mM HCl (pH 1.8) containing various concentrations of alcohols. We did not correct the apparent shift in pH upon addition of alcohols. Changes in peptide concentration did not affect the CD spectra. The alcohol-induced transitions were reversible.[26]

In order to analyze the effects of alcohols on β-lactoglobulin, we have approximated the transition by a two-state transition between the native state (N) and the α-helical state (H): $N \rightleftarrows H$. The apparent equilibrium constant for folding, K_D, is defined by $K_D = [H]/[N]$, where [N] and [H] are the concentrations of the native and helical states, respectively, and the free energy change of the denaturation, ΔG_D, is calculated by: $\Delta G_D = -RT\ln K_D$, where *R* is the gas constant and *T* is

the temperature in Kelvin. We assumed a linear dependence of ΔG_D upon alcohol concentration, [Alcohol].

$$\Delta G_D = \Delta G_0 - m\,[\text{Alcohol}] \quad (1)$$

where ΔG_0 is the ΔG_D value in the absence of alcohol and m is a measure of the dependence of ΔG_D on alcohol concentration.

After assuming the common linear baselines for the native and helical states, each of the transition curves as shown in Figure 3 was analyzed by a least-squares curve fitting program in order to obtain the ΔG_0 and m values. Although it was possible to include the native and denatured baselines into the fitting parameters, reliability of the denatured baselines seemed low for the alcohols which require high concentrations to induce the transition. Thus, we chose to estimate the baselines manually. For the alcohols with a high potential like *n*PrOH or *i*PrOH, the manually estimated baselines were consistent with those determined by the least-squares curve fitting (data not shown). Because we considered that the ΔG_0 value is independent of alkanol species, we fixed the common ΔG_0 value to the average of the obtained values: $\Delta G_0 = 20.0$ kJ/mol. We then determined the m values by a least-squares method using the average ΔG_0 and baselines (Table 1). Least-squares curve fitting was done using the IGOR Pro data analysis program (WaveMetrics, Inc.).

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. We thank Dr. K. Mizuno (Fukui Univ.) for valuable discussion.

References

1. Schrier, E. E.; Ingwall, R. T.; Scheraga, H. A. *J. Phys. Chem.* **1965**, *69*, 298.
2. Brandts, J. F.; Hunt, L. *J. Amer. Chem. Soc.* **1967**, *89*, 4826.
3. Gerlsma, S. Y.; Stuur, E. R. *Int. J. Peptide Protein Res.* **1972**, *4*, 377.
4. Parodi, R. M.; Bianchi, E.; Ciferri, A. *J. Biol. Chem.* **1973**, *248*, 4047.
5. Velicelebi, G.; Sturtevant, J. M. *Biochemistry* **1979**, *18*, 1180.
6. Nelson, J. W.; Kallenbach, N. R. *Proteins Struct. Funct. Genet.* **1986**, *1*, 211.
7. Dyson, J.; Wright, P. *Curr. Opin. Struct. Biol.* **1993**, *3*, 60.
8. Yang, J. J.; Buck, M.; Pitkeathly, M.; Kotik, M.; Haynie, D. T.; Dobson, C. M.; Radford, S. E. *J. Mol. Biol.* **1995**, *252*, 483.
9. Buck, M.; Schwalbe, H.; Dobson, C. M. *J. Mol. Biol.* **1996**, *257*, 669.
10. Gasset, M.; Baldwin, M. A.; Lloyd, D. H.; Gabriel, J. M.; Hotzman, D. M.; Cohen, F.; Fletterick, R.; Prusiner, S. B. *Proc. Natl. Acad. Sci. USA* **1992**, *89*, 10940.
11. Zhang, H.; Kaneko, K.; Nguyen, J. T.; Livshits, T. L.; Baldwin, M. A.; Cohen, F. E.; James, T. L.; Prusiner, S. B. *J. Mol. Biol.* **1995**, *250*, 514.
12. Wood, S. J.; Maleeff, B.; Hart, T.; Wetzel, R. *J. Mol. Biol.* **1996**, *256*, 870.
13. Hirota, N.; Mizuno, K.; Goto, Y. *Protein Sci.* **1997**, *6*, 416.
14. Nozaki, Y.; Tanford, C. *J. Biol. Chem.* **1971**, *246*, 2211.
15. Thomas, P. D.; Dill, K. A. *Protein Sci.* **1993**, *2*, 2050.
16. Liu, Y.; Bolen, D. W. *Biochemistry* **1995**, *34*, 12884.
17. Luo, P.; Baldwin, R. L. *Biochemistry* **1997**, *36*, 8413.
18. Rohl, C. A.; Chakrabartty, A.; Baldwin, R. L. *Protein Sci.* **1996**, *5*, 2623.
19. Hirota, N.; Mizuno, K.; Goto, Y. *J. Mol. Biol.* **1998**, *275*, 365.
20. Haberman, H. *Science* **1972**, *177*, 314.
21. Hagihara, Y.; Kataoka, M.; Aimoto, S.; Goto, Y. *Biochemistry* **1992**, *31*, 11908.
22. Bazzo, B.; Tappin, M. J.; Pastore, A.; Harvey, T. S.; Carver, J. A.; Campbell, I. D. *Eur. J. Biochem.* **1988**, *173*, 139.
23. Brownlow, S.; Cabral, J. H. M.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C. T.; Sawyer, L. *Structure* **1997**, *5*, 481.
24. Futage, R. G.; Song, P.-S. *Biochim. Biophys. Acta* **1980**, *625*, 28.
25. Kim, T.-R.; Goto, Y.; Hirota, N.; Hoshino, M.; Kuwata, K.; Denton, H.; Wu, S.-Y.; Sawyer, L.; Batt, C. A. *Prot. Eng.* **1998**, *10*, 1339.
26. Shiraki, K.; Nishikawa, K.; Goto, Y. *J. Mol. Biol.* **1995**, *235*, 180.
27. Hamada, D.; Segawa, S.; Goto, Y. *Nature Struct. Biol.* **1996**, *3*, 868.
28. Hamada, D.; Goto, Y. *J. Mol. Biol.* **1997**, *269*, 479.
29. Chen, Y. H.; Yang, J. T.; Martinez, H. M. *Biochemistry* **1972**, *11*, 4120.
30. Uversky, V. N.; Narizhneva, N. V.; Kirschstein, S. O.; Winter, S.; Löber, G. *Folding & Design* **1997**, *2*, 163.
31. Jackson, M.; Mantsch, H. H. *Biochim. Biophys. Acta* **1992**, *1118*, 139.
32. Timasheff, S. N.; Arakawa, T. In *Protein structure, a practical approach*; Creighton, T. E., Ed.; IRL: Oxford, 1989; pp 331–345.
33. Griko, Y. V.; Kutyshenko, V. P. *Biophys. J.* **1994**, *67*, 356.
34. Kurpin, S.; Grauslund, A.; Ehrenberg, A.; Koch, M. H. J. *Biochem. Biophys. Res. Comm.* **1995**, *217*, 1151.
35. Mizutani, Y.; Kamogawa, K.; Kitagawa, T.; Shimizu, A.; Taniguchi, Y.; Nakanishi, K. *J. Phys. Chem.* **1991**, *95*, 1790.
36. Byler, D. M.; Susi, H.; Farrell, Jr. M. M. *Biopolymers* **1983**, *22*, 2507.