Protein J (2011) 30:539–545
DOI 10.1007/s10930-011-9360-y

Bile Acids as Modulators of Enzyme Activity and Stability

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Published online: 2 October 2011

Abstract Bile acids deactivate certain enzymes, such as prolyl endopeptidases (PEPs), which are investigated as candidates for protease-based therapy for celiac sprue. Deactivation by bile acids presents a problem for therapeutic enzymes targetted to function in the upper intestine. However, enzyme deactivation by bile acids is not a general phenomenon. Trypsin and chymotrypsin are not deactivated by bile acids. In fact, these pancreatic enzymes are more efficient at cleaving large dietary substrates in the presence of bile acids. We targeted the origin of the apparently different effect of bile acids on prolyl endopeptidases and pancreatic enzymes by examining the effect of bile acids on the kinetics of cleavage of small substrates, and by determining the effect of bile acids on the thermodynamic stabilities of these enzymes. Physiological amounts (5 mM) of cholic acid decrease the thermodynamic stability of *Flavobacterium meningosepticum* PEP from 18.5 ± 2 kcal/mol to 10.5 ± 1 kcal/mol, while thermostability of trypsin and chymotrypsin is unchanged. Trypsin and chymotrypsin activation by bile and PEP deactivation can both be explained in terms of a common mechanism: bile acid-mediated protein destabilization. Bile acids, usually considered non-denaturing surfactants, in this case act as a destabilizing agent on PEP thus deactivating the enzyme. However, this level of global thermodynamic destabilization does not account for a more than 50% decrease in enzyme activity, suggesting that bile acids most likely modulate enzyme activity through specific local interactions.

Keywords Bile acids · Thermodynamic stability of proteins · Enzyme activity · Surfactants · Prolyl endopeptidase · Trypsin · Chymotrypsin · Celiac sprue

Abbreviations
PEP Prolyl endopeptidase
FM *Flavobacterium meningosepticum*
SC *Sphingomonas capuslata*)

1 Introduction

Celiac sprue is an autoimmune disease, triggered by dietary gluten, which manifests itself in inflammation of the upper intestine. Untreated celiac disease is associated with a major loss of intestinal surface area, which leads to malabsorption of nutrients, vitamins and minerals, and can cause serious complications associated with deficiencies in these substances. Currently the only treatment for celiac sprue is a difficult life-long gluten-free diet. Approximately 1% of the US population is estimated to be affected by celiac sprue, although the disease is currently under-diagnosed [14]. Glutenases, enzymes capable of cleaving toxic gluten-derived peptides, have been proposed as potential

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orally administered therapeutic agents for celiac sprue. Prolyl endopeptidases (PEPs) are a particularly promising class of gluten-cleaving proteases since gluten is unusually rich in prolines [24]. Ideally, PEPs and other enzyme-based therapeutics should be able to function by cleaving gluten in the digestive tract, specifically the upper intestine, which contains conjugated bile acids in the mM concentration range [12]. Unfortunately bile acids hinder the PEP activity by both lowering PEP resistance against cleavage by pancreatic enzymes, and by decreasing the enzymatic activity of PEPs [4]. This presents a problem in development of oral protein-based therapeutics, such as the proposed PEP-based therapy for celiac sprue [22, 24].

However, other proteases have increased activity in the presence of bile acids. The rate of cleavage of dietary proteins by pancreatic enzymes trypsin and chymotrypsin increases upon addition of bile acids [4]. This suggests a novel role of bile acids in aiding protein digestion. The enhancement of protein digestion is most significant above the critical micelle concentration (CMC) of bile acids, however, a smaller but significant increase in protein digestion rate has been observed at submicellar concentrations of bile acids [3]. This increase in the rate of digestion of dietary proteins could be a result of partial denaturation of large dietary substrates by bile acids. Even though bile acids appear to have opposite effects on PEPs and pancreatic proteases (increasing the rate of cleavage of dietary substrates by trypsin and chymotrypsin, and decreasing the rate of cleavage of gluten substrates by PEPs), the underlying mechanism is likely the same: bile acid-induced denaturation and destabilization of proteins. Denaturation of dietary protein substrates of trypsin and chymotrypsin makes them more amenable to cleavage by trypsin and chymotrypsin, hence increasing the proteolysis rate. Potential PEP denaturation could explain the observed decrease in PEP activity in the presence of bile acids [3, 6].

In order to develop a PEP-based treatment for celiac sprue, we need to understand the scope and mechanism of bile acid-protein interaction. This understanding may allow a PEP to be engineered so that it is unaffected or even enhanced by bile salts. What is the molecular basis of the effect of bile acids on proteins? In a proteolytic reaction, such as the one depicted below, bile acids may be affecting any of the three players in the reaction: the substrate, the enzyme, or the final product.

$$\text{Protein substrate} \xrightarrow{\text{Protease}} \text{Cleaved protein products}$$

The most likely explanation for how bile acids affect the rate of enzyme catalysis involves a change in secondary or tertiary structures of one or more of the proteins [2, 7]. Such a change might be global, affecting the whole protein, or more local, affecting a certain region of the protein. This change in structure might be modulated by the amphipathic nature of bile acids. Because of their amphipathic nature, bile acids act as surfactants. Some surfactants preferentially bind to the unfolded state of the protein, thus shifting the equilibrium towards the unfolded state [18]. Alternatively, surfactants may bind preferentially to the native, folded state of the protein, thus stabilizing the protein [26]. Through these types of interactions, bile acids might promote unfolding or induce local conformational changes within the folded state of proteins.

In order to dissect the mechanism of bile acid-protein interaction, we have analyzed a reaction where the substrate is a small peptide, without higher orders of protein structure. This model allows us to eliminate potential higher order structural effects on the substrate and products, both of which are small peptides. Any observed effects of bile acid on the rate of enzymatic catalysis suggest that bile acids are affecting the protease itself.

One way in which bile acids may affect the activity of enzymes is by modulating the thermodynamic stability of proteins. To determine if this is the case, in this study we use urea-induced equilibrium denaturation monitored by fluorescence to investigate the effect of bile acids on the thermodynamic stability of two pancreatic proteases, trypsin and chymotrypsin, and two PEPs, one from the bacterium *Flavobacterium menningosepticum* (FM) and another from the bacterium *Sphyngomonas capsulata* (SC). We chose to focus on those two PEPs because of their potential for development of enzyme therapy for celiac sprue. Although previous studies, including structural characterization, have been done on *Myxococcus xantus* (MX) PEP, SC PEP may be a more exciting candidate for protease therapy because of its stability under low pH, suggesting that SC PEP might start detoxifying gluten as early as in the stomach [21]. SC has also been shown to be a good scaffold for improvement through protein engineering [22]. In our studies, FM was easier to work with, and behaved as a classical two-state protein. FM is also interesting because its ability to digest peptides longer than 30 amino acids [21].

Our study shows that bile acids act as mild denaturants for certain proteins, such as PEPs. This is surprising given the current classification of bile acids as non-denaturing surfactants. It remains to be seen whether bile-induced thermodynamic destabilization of proteins is common to a large set of proteins. This destabilization is not significant enough to fully account for the loss of enzymatic activity, suggesting other, more local mechanisms of bile acid enzyme deactivation. Interestingly, enzymes which normally function in the environment rich in bile acids are neither deactivated nor destabilized by bile. Further elucidation of mechanistic details of how bile acids affect various classes of enzymes will help

choose or engineer a better enzyme-based therapeutical agent for celiac sprue.

2 Materials and Methods

2.1 Materials

Trypsin and chymotrypsin were purchased from Sigma Aldrich (St. Louis, MO) and used without further purification. *Flavobacterium meningosepticum* (FM) PEP and *Sphyngomonas capusalta* (SC) PEP were expressed in *E. coli* and purified as previously described [1, 21]. Purity of PEPs was verified by SDS–PAGE, and the concentration of these proteins was determined spectrophotometrically in 20 mM sodium phosphate buffer (pH 7.0) containing 6.0 M guanidinium chloride by using $\varepsilon = 1.43\ \text{cm}^{-1}\ (\text{mg/mL})^{-1}$ at 280 nm. Ultra-pure urea and guanidinium hydrochloride were purchased from Sigma Aldrich (St Louis, MO).

The chromogenic substrate for PEPs (Suc-Ala-Pro-pNA Succinyl-L-Alanyl-L-Prolyl P-Nitroanilide(abbreviated Suc-Ala-Prop-pNA) was purchased from Bachem (Torrance, CA). Trypsin and chymotrypsin chromogenic substrates, Nα-Benzoyl-DL-arginine 4-nitroanilide (abbreviated Ben-Arg-pNA) hydrochloride and N-Benzoyl-L-tyrosine p-nitroanilide (abbreviated Ben-Tyr-pNA), were purchased from Sigma Aldrich (St Louis, MO).

2.2 Enzyme Activity Assays

All enzyme concentrations were determined by absorbance at 280 nm, as specified in Materials. Post-proline cleavage activity of PEPs was measured using the chromogenic substrate Suc-Ala-Pro-pNA. Activity of trypsin was evaluated by monitoring the cleavage of Ben-Arg-PNA; and the activity of chymotrypsin was monitored with Ben-Tyr-pNA. In order to obtain kinetic parameters, hydrolysis of eight different concentrations of chromogenic substrates (ranging from 0.05 to 1 mM) was performed in the presence of 0.02 μM enzyme, in PBS buffer (50 mM sodium phosphate, 300 sodium chloride, pH 7.0). Enzymes were equilibrated with bile acids for 12 h prior to each kinetic assay. Reactions were monitored for 2 min by following the absorbance at 410 nm. Initial velocities were plotted against substrate concentrations, and the data were fit to the Michaelis–Menten relationship, in order to calculate k_{cat}/K_M values.

2.3 Determination of Protein Stabilities by Intrinsic Fluorescence

FM and SC PEPs were diluted to the final concentration of 100 μg/mL in 20 mM Sodium Phosphate buffer (pH 7.0), containing 50 mM NaCl and various concentrations of urea. Trypsin and chymotrypsin were diluted to the final concentration of 100 μg/mL in 50 mM Sodium Acetate buffer (pH 3.0) containing 50 mM NaCl and various concentrations of urea. 3-mL samples were equilibrated at room temperature for at least 24 h. To check if equilibrium was reached in 24 h, three samples with urea concentrations closest to the midpoint, were incubated for 24, 47 and 72 h, and fluorescence scans were taken at each time point.

Trypsin and chymotrypsin stabilities were measured at low pH to prevent autocatalytic cleavage of these proteins at neutral pH[15, 27].Gel analysis and activity assays confirmed that autodigestion of PEP was not an issue, in agreement with previous observation that PEPs are most efficient at cleaving substrates smaller than 30 amino acids [5].

Fluorescence spectra of each sample were acquired on a Perkin Elmer LS 50 B fluorimeter. Excitation was set at 280 nm, and the emission spectra were recorded from 300 to 400 nm, with both slits at 4 nm. The center of mass (C_oM) of each spectrum was calculated using Eq. 1:

$$C_oM = \frac{\sum S_i \lambda_i}{\sum S_i} \quad (1)$$

where S_i is the signal at wavelength λ_i. C_oM versus urea concentration data were fit to the two state model in order to determine the free energy of unfolding (ΔG_{unf}) [20]. Exact urea concentrations were obtained from the refractive index measurements [29].

3 Results

3.1 Bile Acids Decrease the Rate of Cleavage of Small Peptide Substrates by PEPs

In this study we explore the effect of various concentrations of individual bile acids on the cleavage of a small peptide substrate by two PEPs, FM PEP, from the bacterium *Flavobacterium meningosepticum*, and SC PEP from the bacterium *Sphingomonas capsulata*.

Even sub-physiological concentrations of cholic acid drastically decrease the efficiency of cleavage of small chromogenic substrates by both PEPs, reflected in the k_{cat}/K_M value (Fig. 1). A similar effect is observed with conjugated bile acid (glycocholic acid). Equilibrating FM PEP with 5 mM amount of glycocholic acid resulted in k_{cat}/K_M of to $8{,}400 \pm 1{,}000\ M^{-1}s^{-1}$, whereas SC had a k_{cat}/K_M of to $23{,}000 \pm 5{,}100\ M^{-1}s^{-1}$. Regardless of whether the bile acid is conjugated or not, the enzyme activity is approximately three-fold lower in 1.5 mM bile compared to its value in the absence of bile acids.

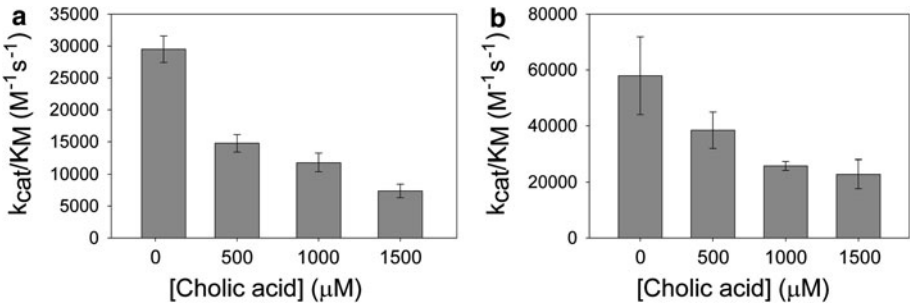

Fig. 1 Catalytic efficiency (k_{cat}/K_M) of two PEPs, FM (**a**) and SC (**b**) in cholic acid. Assays were done using small chromogenic substrate SucAlaProPNA, with 0.02 μM of PEP in PBS buffer, at pH 7.0. All experiments were done in triplicates, with error bars representing standard deviation. The same experiment was also performed with 5 mM glycocholic acid (conjugated bile acid), resulting in similar deactivation effect on both FM and SC PEPs. FM k_{cat}/K_M of 8,400 ± 1,000 M^{-1} s^{-1} and SC k_{cat}/K_M of 23,000 ± 5,100 M^{-1} s^{-1} were recorded in the presence of 1.5 mM glycocholic acid

Table 1 Effect of conjugated and unconjugated cholic acids on cleavage of small chromogenic substrates by trypsin and chmyotrypsin

	Trypsin k_{cat}/K_M (M^{-1} s^{-1})	Chymotrypsin k_{cat}/K_M (M^{-1} s^{-1})
No bile acid	19,600 ± 2300	18,800 ± 1000
Cholic acid	19,200 ± 4200	17,500 ± 900
Glycocholic acid	17,000 ± 3000	18,000 ± 2000

Assays were done with 1.5 mM bile acids, 0.02 μM of enzyme in PBS buffer, pH 7.0. All experiments were done in triplicates, with variation represented by standard deviation. Student t-test was performed to compare each bile sample with no-bile reaction, and *p* values >0.05 were obtained in all cases, suggesting that the results were not significantly different

3.2 Bile Acids Do Not Affect the Catalytic Efficiencies of Native Pancreatic Enzymes Trypsin and Chymotrypsin

As a control, we examined the effect of bile acids on the catalytic efficiencies of two pancreatic enzymes, trypsin and chymotrypsin. Neither unconjugated cholic nor conjugated glychocholic acid have a significant effect on the rate of cleavage of small chromogenic substrates by trypsin and chymotrypsins (Table 1). The catalytic efficiencies, reflected in k_{cat}/K_M of these two pancreatic enzymes, are comparable with and without bile acids (*t*-test *p*-values > 0.05) at bile acid concentrations where the effect was significant for the two PEPs. This is in contrast to how bile acids affect the cleavage of large dietary substrates by trypsin and chymotrypsin. Bile acids actually increase the rate of that reaction [4].

3.3 Bile Acids Decrease the Thermodynamic Stabilities of PEPs

Equilibrium denaturation of PEPs by urea was monitored using intrinsic tryptophan fluorescence. Equilibrium denaturation curves of FM PEP exhibit a single, cooperative transition, suggestive of two-state unfolding (Fig. 2a). To ensure equilibrium was reached, three samples with urea concentrations closest to the midpoint were incubated for 24, 47 and 72 h. There was no difference in the fluorescence signal over time suggesting that a 24-h incubation was sufficient. FM denaturation curves were fit using the linear extrapolation model [20].

In the absence of bile acids, FM has ΔG_{unf} of 18.0 ± 2.0 kcal $mole^{-1}$ and an m value of 5.3 kcal $mole^{-1}$ M^{-1}. Equilibrium denaturation performed in the presence of a cholic acid shows that FM PEP is thermodynamically destabilized by cholic acid in the millimolar concentration range (Table 2; Fig. 2a).

The urea denaturation curves of SC PEP had a shallow, gradual, not very cooperative transition, suggesting a more complex unfolding mechanism, and were not analyzed quantitatively (data not shown). Despite our inability to analyze SC PEP fluorescence denaturation data with the two-state model, we observed that the addition of cholic acid completely abolished the sigmoidal transition in the equilibrium unfolding curve of SC PEP, suggesting a destabilizing effect (data not shown).

3.4 Bile Acids Do Not Affect the Thermodynamic Stabilities of Pancreatic Enzymes Trypsin and Chymotypsin

Equilibrium denaturations of trypsin and chymotrypsin were carried out at low pH, where there is no significant autolysis [28]. The proteins remain intact during the incubation at pH 3.0, as confirmed by visualization on SDS PAGE (data not shown). Both proteins exhibit a cooperative unfolding transition, suggestive of a two-state mechanism of unfolding.

The ΔG_{unf} of trypsin does not change significantly as a result of addition of 5 mM cholic acid. The ΔG_{unf} of

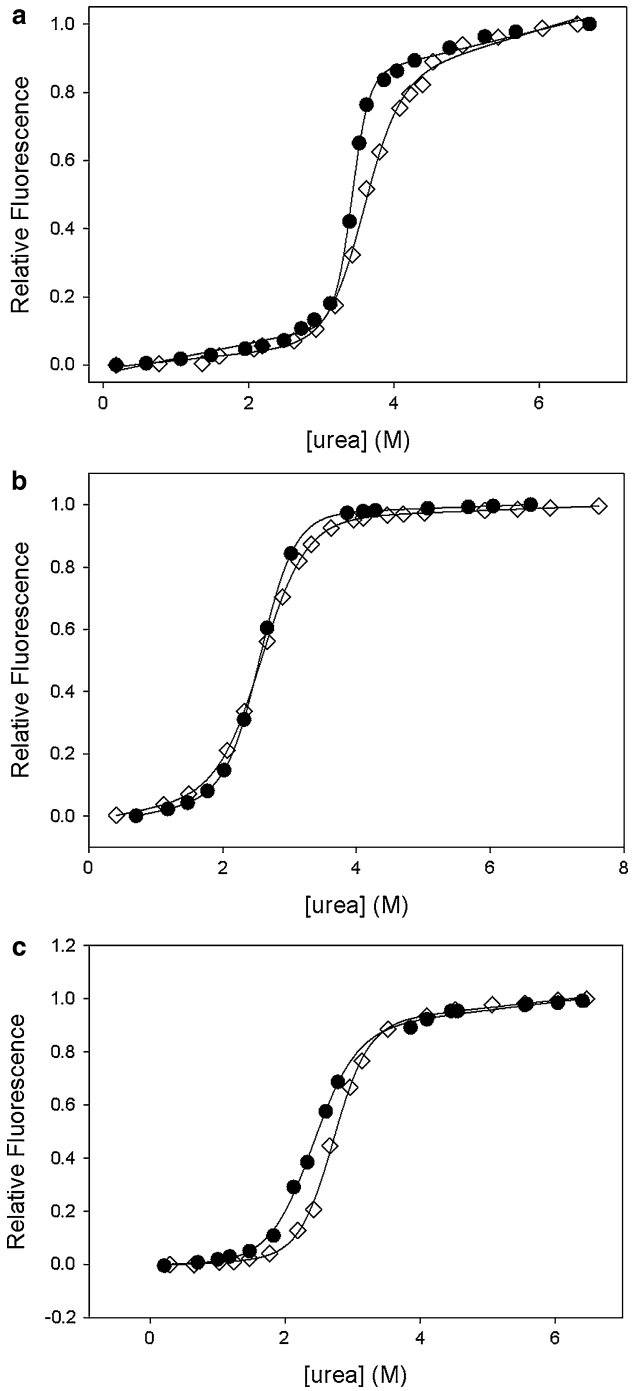

Fig. 2 Equilibrium unfolding of FM PEP (**a**), trypsin (**b**) and chymotrypsin (**c**) in the absence (*filled circle*) and presence (*open diamond*) of 5 mM cholic acid, monitored by fluorescence. FM PEP samples were incubated in various concentration of urea, at pH 7.0, for 24 h. Trypsin and chymotrypsin samples were equilibrated in various concentration of urea, at pH 3.0 for 24 h. Data were fit to the two-state model. Fit parameters are shown in Tables 1 and 2

trypsin at pH 3.0 is 5.8 ± 0.5 kcal mol^{-1} in the absence of bile, and 6.1 ± 0.3 kcal mol^{-1} in the presence of 5 mM cholic acid (Fig. 2b). The m values were also not affected significantly by bile (2.2 ± 0.5 kcal mol^{-1} M^{-1} and 2.5 ± 0.6 kcal mol^{-1} in the absence and presence of 5 mM cholic acid respectively). Chymotrypsin stability was similarly not affected by the addition of 5 mM bile acids (Fig. 2c).

4 Discussion

Bile acids, amphipathic steroidal surfactants, are well known as being central to the digestion and absorption of lipids in the intestine. Bile acids may also have a role in protein processing. In vitro experiments show that conjugated bile acids enhance the digestion of dietary proteins. Cleavage of certain dietary proteins by pancreatic enzymes trypsin and chymotrypsin occurs faster in the presence of bile acids [4]. However, the activity of trypsin and chymotrypsin towards small peptides is not affected by bile acids (Table 1). This suggests that the observed increase in the rate of cleavage of dietary proteins most likely stems from the effect of bile acids on the dietary protein substrates. Bile acids may facilitate the cleavage of large protein substrates by destabilizing or partially unfolding these protein substrates, thus making them more easily accessible to cleavage by trypsin and chymotrypsin. Because small substrates do not have any higher orders of structure, the rate of their cleavage is unaffected by the bile acids. This also suggests, that the enzymes, trypsin and chymotrypsin, are not directly affected by the presence of bile acids.

Similar to dietary proteins, PEPs are also affected by bile acids (Table 2; Fig. 2). Enzymatic activity of both PEPs is two to three-fold lower in the presence of milimolar amounts of bile acids (Table 1; Fig. 1). This deactivation rate is similar to previously reported rates of deactivation of subtilisin in the presence of comparable amounts of various surfactants [19]. Fluorescence-monitored equilibrium unfolding experiment shows that cholic acid, at a concentration as low as 1.5 mM, destabilizes FM PEP by 5.5 kcal mol^{-1} (Table 2). Though significant, this level of destabilization does not account for a more than twofold decrease in activity. Under physiological

Table 2 Thermodynamic stabilities of FM PEP in the presence of cholic acid

[Cholic acid] (mM)	ΔG_{unf} (kcal mol^{-1})	m value (kcal $mole^{-1}$ M^{-1})
0	18.1 ± 2.0	5.3 ± 0.5
1.5	12.5 ± 1.7	4.1 ± 0.5
5	11.0 ± 1.5	3.8 ± 0.4

The values were obtained by fitting intrinsic fluorescence data to a two-state model of unfolding

Table 3 Accessible surface area (ASA), including polar and non polar accessible surface) areas, were calculated for all studied proteins for which high resolution structures are available

PDB code	Protein name	Isolectric point	Polar ASA (Å)	Non-polar ASA (Å)	Ratio of polar/nonpolar ASA
1YR2	SC PEP	6.42	10618	18698.2	0.57
2ZHD	Trypsin	8.4	3704	5262.5	0.70
1YPH	Chymotrypsin	8.5	7078.8	10429.4	0.68

GetArea program, available at http://www.curie.utmb.edu/getarea.html was used for calculation of accessible surface area

conditions, most of the PEP molecules will still be fully folded in the presence of 1.5 mM bile acids. This suggests that the deactivation mechanism likely includes some specific local interactions between the enzyme and bile acids, rather than being caused by global destabilization and/or unfolding of the whole protein.

Most of the bile acids normally present in the small intestine are conjugated. Fluorescence stability studies were performed with the unconjugated bile acid cholic acid because a highly concentrated protein stock solution (necessary for making equilibrium unfolding samples) precipitated in the presence of glycocholic acid. Encouragingly, the kinetics of both FM and SC PEP enzymatic activity were not significantly different between conjugated glycocholic and unconjugated cholic acid.

The concentration of cholic acid at which FM PEP is significantly deactivated and destabilized (1. 5 mM) is lower than the physiological bile acid concentration [10, 11].This concentration is also lower than the critical micellar concentration (CMC) of cholic acid, reported at 15–20 mM [16, 23]. Most of the published studies of interactions of proteins and bile acids have focused on the interactions with micelles [13, 25]. Our results suggest that even sub-micelle-forming levels of bile acids may also have a significant effect on both enzyme activity and thermodynamic stability.

Interestingly, the destabilization is not achieved by a decrease in c_m, the midpoint of the denaturation curve, but rather by a decrease in m value. The m value is correlated with a change of accessible surface area upon protein unfolding [17], and one potential explanation for a lower m value is a bile-induced increase in the compactness of the unfolded state ensemble in the presence of bile acids. The m value of FM PEP unfolding (5.3 kcal mol^{-1}) is close to, but lower than the m value predicted from the size of the protein (predicted value of 7.5 kcal mol^{-1} based on the size of 705 amino acids) [17]. An even lower m value of 3.8 kcal mol^{-1} is observed in the presence of bile acid. Perhaps, by binding to the unfolded state, bile acids limit its conformational freedom. This hypothesis remains to be experimentally investigated.

If bile acids do indeed preferentially interact with unfolded state or unfolded regions of the protein, bile acids could be considered to be protein denaturants. However, bile acids are not typically viewed as denaturants. To the contrary, bile acids are classified as anionic non-denaturing surfactants [13]. Their non-denaturing property is one of the reasons bile acids are often used as a surfactant of choice in solubilization of membrane proteins [9, 25].

It remains to be seen why certain proteins, such as trypsin and chymotrypsin, and possibly some membrane proteins, are not destabilized by bile acids, while other proteins, such as PEPs, are. Pancreatic enzymes that evolved to function in the presence of bile could have acquired a multitude of sequence and structural features which render them insensitive to destabilization and deactivation by bile acids. Both the overall charge and the available hydrophobic surface area of the protein play a role in destabilization by SDS, another surfactant [8, 13]. The two pancreatic enzymes, trypsin and chymotrypsin, have a higher ratio of polar to non-polar accessible surface area (Table 3). Because of their higher isolectric points, trypsin and chymotrypsin carry more positive charge on the surface area than PEP under physiological conditions, in addition to having a somewhat higher proportion of polar surface area. These factors may play a role in the mechanism of interaction of proteins with bile acids, which contain both polar and non-polar features (Table 3). The finely tuned interplay of hydrophobic and electrostatic interactions involving both the polar and apolar sides of the bile acid is yet to be explored.

Results of this study provide a motivation for further exploration of such mechanistic details. A clearer understanding of mechanisms of interaction of proteins with bile acids and other surfactants will have implications for design of protein-based therapeutics, such as PEP treatment for celiac sprue, as well as for choosing optimal surfactants for purification of membrane proteins. It remains to be seen whether bile-induced deactivation and destabilization of proteins might be common to a large set of proteins. Our results suggest that in further protein engineering efforts aimed at designing gut-active proteases we should consider enzyme activity profile in the presence of bile acids as one of the dimensions in the selection process.

Acknowledgments We thank Chaitan Khosla for providing us with plasmids encoding FM and SC PEP, Ruth Riter (Agnes Scott College, Department of Chemistry) for allowing us to use the fluorimeter, and Giulietta Spudich and Chiwook Park for critical reading of the manuscript.

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