Dissociation of Insulin Oligomers by Bile Salt Micelles and Its Effect on α-Chymotrypsin-Mediated Proteolytic Degradation

Yuping Li,[1] Zezhi Shao,[1] and Ashim K. Mitra[1,2]

Received January 2, 1992; accepted January 20, 1992

Bile salts have been found to be effective absorption promoters of insulin across mucosal barriers, i.e., nasal and gastrointestinal. One of the mechanisms proposed for absorption enhancement is the dissociation of insulin oligomers to monomers, rendering a higher insulin diffusivity. α-Chymotryptic degradation and circular dichroism studies were used to characterize such a transition. When zinc insulin (hexamers) and sodium insulin (dimers) were subjected to α-chymotryptic degradation, a 3.2-fold difference in the apparent first-order rate constants was observed (zinc insulin being slower than sodium insulin), representing the intrinsic difference in the concentration of total associated species in solution (three times). In the presence of a bile salt, sodium glycocholate (NaGC), the rate of degradation of both zinc and sodium insulin increased in an asymptotic manner. A maximum increase of 5.4-fold was observed for zinc insulin at a 30 m*M* NaGC concentration and a 2.1-fold increase was noted for sodium insulin at 10 m*M* NaGC, both values being close to the theoretical numbers of 6- and 2-fold as predicted by the complete dissociation of hexamers and dimers to monomers. The result indicates dissociation of insulin oligomers to monomers by bile salt micelles, probably by hydrophobic micellar incorporation of monomeric units. Circular dichroism studies also revealed progressive attenuation of molecular ellipticities at negative maxima of 276, 222, and 212 nm for zinc insulin solution in the presence of NaGC. Therefore, both α-chymotryptic degradation and circular dichroism studies have consistently demonstrated that the bile salts may be capable of dissociating insulin oligomers to monomers, a fact which may play an important role in enhancing insulin bioavailability.

KEY WORDS: insulin; dissociation; bile salt; circular dichroism; degradation; α-chymotrypsin.

INTRODUCTION

Insulin molecules are known to undergo self-association in aqueous solution, thus forming oligomers of different aggregation numbers. This balance of association–dissociation was found to depend largely on a number of factors such as insulin concentration, pH, solvent composition and dielectric properties, ionic strength, and additives (1,2). Association of insulin molecules not only hinders the physical stability for long-term therapeutic preparations (3) but also fosters permeability problems with regard to its *in vivo* absorption. Insulin hexamers are regarded as being unable to penetrate capillary pores (4) and thereby becoming nonbioavailable from nonparenteral pathways (5). Therefore, studies were initiated to overcome insulin self-association by means of additives such as aspartic acid, ethylenediaminetetraacetic acid (EDTA), glutamic acid, bicarbonate buffer, lysine, Tris buffer, etc. (1). These additives were shown to improve insulin solubility substantially and minimize its aggregation, which was further attributed to either the chelation effect or hindrance of the insulin–insulin interaction.

In order to facilitate insulin bioavailability, recently various absorption enhancers have been employed and investigated. Among various promoters evaluated, bile salts were found to improve insulin absorption significantly while exhibiting minimal toxic effects (6,7). The mechanisms by which bile salts increase insulin bioavailability were postulated to be a combination of several modes such as the alteration of biological membrane integrity, inhibition of protease activity, and dissociation of molecular aggregates through micellar solubilization (7,8). The main contributing mechanism, however, still remains unknown. In order to understand further the underlying mechanism(s), this investigation was undertaken to study the dissociation characteristics of insulin oligomers by bile salt micelles both qualitatively and quantitatively.

The present study also investigates the transitions of insulin aggregation by both the enzyme kinetic method and an independent spectroscopic (circular dichroism) measurement. Primary interest in studying α-chymotrypsin-mediated degradation stems from our previously reported investigation on intestinal mucosal transport and metabolism of insulin with the goal of designing oral insulin delivery systems (9). α-Chymotrypsin has been found to be the primary proteolytic enzyme responsible for initial cleavage and unfolding of insulin globular structure, exposing the molecule to subsequent attack by brush border and enterocytic enzymes (10). The smaller the aggregation number of insulin, the higher will be its diffusivity across a mucosal barrier (nasal, gastrointestinal routes) or capillary endothelial barrier (subcutaneous route). However, for the same unit mass, the smaller species (monomers, dimers, etc.) will present a correspondingly larger number of exposed peptide linkages susceptible to proteolytic enzyme attack (11). Therefore, a balance must be created between the rate of insulin absorption and its biodegradation at the site of absorption. It is important to know whether the presence of absorption enhancers such as bile salts would alter the rate of insulin degradation by pancreatic luminal enzymes. A quantitative mathematical relationship between rate constant of enzymic degradation and insulin dissociation was previously reported from this laboratory (11). Therefore, it is desirable to extend that line of investigation to bile salt-mediated insulin dissociation. α-Chymotrypsin, a model enzyme, was selected due to its high proteolytic activity (10). Both zinc insulin (hexamers) and sodium insulin (dimers) have been studied by adding various concentrations of a bile salt, sodium glycocholate. The interconversion behavior of insulin hexamer/dimer/monomer has also been confirmed by an independent spectroscopic method, i.e., circular dichroism (CD).

[1] Department of Industrial and Physical Pharmacy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, Indiana 47907.

[2] To whom correspondence should be addressed.

0724-8741/92/0700-0864$06.50/0 © 1992 Plenum Publishing Corporation

MATERIALS AND METHODS

Materials

Crystalline porcine sodium and zinc insulins were gifts from Eli Lilly and Company (Indianapolis, IN). Lyophilized α-chymotrypsin prepared from bovine pancreas (56 units/mg protein) was purchased from Sigma Chemical Company (St. Louis, MO). Acetonitrile (HPLC grade) was obtained from Baxter Health Care Corporation (Muskegon, MI). Phosphoric acid and triethylamine were obtained from Fischer Scientific (Fairlawn, NJ). Trifluoroacetic acid (TFA), sodium glycocholate (NaGC), and tris(hydroxy-methyl)aminomethane (Tris) were provided by Sigma Chemical Co. Deionized double-distilled water was used throughout the study. All other chemicals were of analytical reagent grade and were used as received.

HPLC Analysis of Insulin

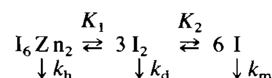
Insulin analysis was performed on a computer-controlled gradient high-pressure liquid chromatographic (HPLC) system (Rainin Instruments, Woburn, MA) equipped with a variable-wavelength ultraviolet/visible detector (Knauer, West Germany). The gradient system used in this study consisted of mobile phase A, triethylammonium phosphate (TEAP) solution prepared by adjusting the pH of 0.25 *N* phosphoric acid to 2.25 with triethylamine, and mobile phase B, 100% acetonitrile. The gradient system was programmed by increasing the proportion of mobile phase B from 22 to 40% within 32 min. Twenty microliters of the sample was injected onto a Rainin reversed-phase C-8 Microsorb column (250 × 4.6 mm) connected to a C-8 precolumn. The gradient mobile phase was run at a flow rate of 1 ml/min. The ultraviolet/visible detector was set at 220 nm; the recorded signal was analyzed with an electronic integrator (Model 3390 A, Hewlett-Packard Co., Avondale, PA). The chromatographic method as described previously (10) provides baseline separation of insulin from its metabolites.

α-Chymotryptic Degradation of Sodium and Zinc Insulin

Ten milliliters of 0.5 mg/ml sodium or zinc insulin solution was prepared in a buffer composed of 100 m*M* Tris and 1 m*M* $CaCl_2$ adjusted to pH 8.0. The solution was preequilibrated at 37°C for 15 min. Just prior to the addition of the enzyme, the solution was vortexed for 2 sec and a 100-μl sample was immediately taken as the zero-time sample. Then 50 μl of enzyme stock solution was added to the insulin solution to generate a final enzyme concentration of 0.5 μ*M*. Aliquots (100 μl) were withdrawn at 1, 2, 5, and 10 min and immediately added to 0.9 ml 0.2% TFA solution to arrest the reaction. The samples were subsequently stored in a freezer at −20°C until HPLC analyses were performed. Studies were performed in triplicate. This procedure was used throughout the enzymatic degradation study. In the case of micellar solutions, sodium glycocholate was added initially to the Tris buffer solution and sonicated for 5 min at room temperature prior to the addition of insulin.

Kinetics of Insulin Biodegradation Relative to Its Degree of Dissociation

The main species existing in aqueous solution of zinc insulin are hexamers. Following the addition of bile salts, the dissociation of hexameric species to dimer and progressively to monomer will proceed, resulting in two consecutive equilibria as shown in Scheme I. The constants K_1 and K_2 denote the dissociation equilibrium constants and were reported to be of the order of 10^{-4} M^2 and 10^{-6} *M*, respectively (13). The overall rate of degradation, therefore, can be expressed by Eq. (1).



Scheme I

$$-\frac{d[\mathrm{I}]_t}{dt} = k_h f_h[\mathrm{I}]_t + k_d f_d[\mathrm{I}]_t + k_m f_m[\mathrm{I}]_t = k_{obs}[\mathrm{I}]_t \quad (1)$$

$$k_{obs} = k_h f_h + k_d f_d + k_m f_m \quad (2)$$

where k_{obs} is the observed apparent first-order rate constant, while k_h, k_d, and k_m are referred to as the individual rate constants associated with hexamers, dimers, and monomers in the ternary system and f_h, f_d, and f_m refer to the corresponding fractions in solution. Addition of all three fractions should equal unity as shown in Eq. (3).

$$f_h + f_d + f_m = 1 \quad (3)$$

According to the collision theory, which is based solely on the number of hydrolyzable insulin species present in solution, Eq. (4) can be written to correlate various degradation rate constants (11):

$$k_m = 2k_d = 6k_h \quad (4)$$

By integrating Eqs. (3) and (4) into Eq. (2), Eq. (5) is obtained:

$$k_{obs} = k_m\left(1 - \frac{5}{6}f_h - \frac{1}{2}f_d\right) \quad (5)$$

It is conceivable from Eq. (5) that the maximum apparent first-order rate constant of degradation is k_m, in which case the fractions of hexamers and dimers are both negligible. The minimum rate of degradation, on the other hand, equals $k_m/6$ when insulin exists only in the hexameric form. Between these two extremes, k_{obs} is a function of the fractions of both hexamers and dimers.

The situation becomes much simpler for sodium insulin since $f_h = 0$. Therefore, k_{obs} depends only on the fraction of dimers in the system, with $(k_{obs})_{max} = k_m$ and $(k_{obs})_{min} = k_m/2$.

Circular Dichroism Studies

A CD spectropolarimeter (JASCO Model J600, Japan Spectoscopic Co., Tokyo) was utilized to illustrate the gradual changes associated with insulin dissociation, i.e., change

of hexameric form to dimeric and monomeric forms in the presence of NaGC. Zinc insulin solutions containing 0.5 mg/ml zinc insulin and 0, 5, 10, 20, 30, 40, and 50 m*M* NaGC were scanned from 300 to 250 nm and from 240 to 200 nm at a scanning speed of 5 nm/min, respectively. The temperature was controlled at 25°C. A 10- and a 1-mm-pathlength quartz cuvette (American Scientific Products, McGaw Park, IL) was used for the higher- and lower-wavelength regions, respectively, to obtain optimum resolution of the spectra. The generated ellipticity values were subsequently converted to molar ellipticities for the entire wavelength range with the help of a computer using the equation $[\theta]_\lambda = \theta_\lambda/(C \cdot l)$ (where θ_λ is the observed ellipicity at wavelength λ, C is the decimolar insulin concentration, and l is the pathlength in decimeters).

RESULTS AND DISCUSSION

Figure 1 depicts a representative HPLC chromatogram of intact insulin in the presence of its metabolites. The gradient change in acetonitrile composition during elution has also been included. A total of five metabolites was rapidly formed, with four of them being prominent. This result concurs with the report of Schilling and Mitra (10), where the major metabolite fractions were collected and identified. Amino acid sequencing indicated that the sites of cleavage occurred after B26-Tyr and A19-Tyr in a sequential manner, forming metabolites A and B (10). Metabolite A was additionally cleaved by α-chymotrypsin after A14-Tyr and B16-Tyr residues, to form metabolites C and D. Additional bonds were also reportedly cleaved after long-term exposure to the enzyme (12).

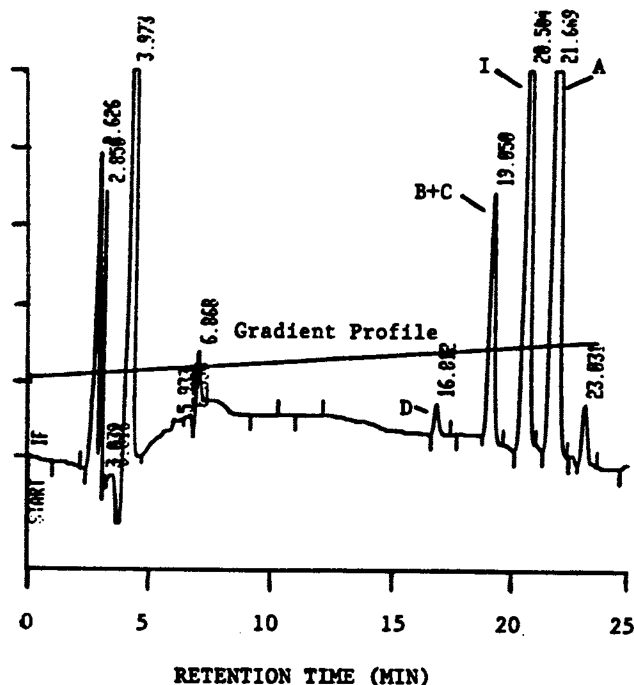


Fig. 1. HPLC chromatogram of insulin and its metabolites degraded by α-chymotrypsin. The molar ratio of insulin to α-chymotrypsin was 172:1. Peak I represents intact insulin, and A–D refer to insulin metabolites at 10 min without the presence of NaGC.

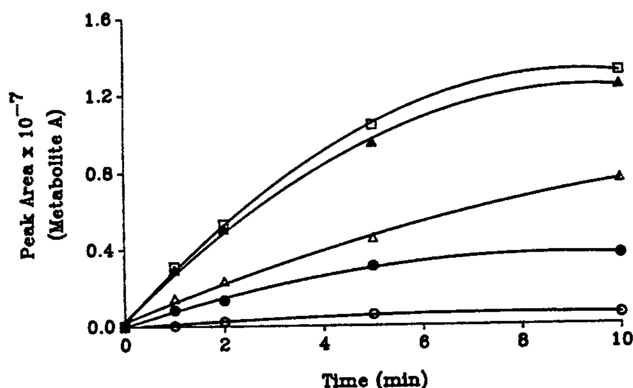


Fig. 2. The area–time profiles of the appearance of metabolite A as a function of NaGC concentration. (○) 0 m*M* NaGC; (●) 5 m*M* NaGC; (△) 10 m*M* NaGC; (▲) 20 m*M* NaGC; (□) 30 m*M* NaGC.

In the presence of sodium glycocholate the appearance of metabolite A was greatly augmented. The reaction rate became so rapid that metabolite A appeared within seconds after the mixing of α-chymotrypsin and insulin. Since metabolite A is the first and main degradation product, its appearance represents direct degradation of intact insulin. Figure 2 illustrates the integrated area of metabolite A as a function of digestion time.

In agreement with Liu *et al.* (11), the biodegradation of insulin by α-chymotrypsin appeared to follow an apparent first-order process. Figures 3 and 4 illustrate the semilogarithmic plots of zinc and sodium insulin disappearance as a function of NaGC concentration. Interestingly, the degradation of both zinc and sodium insulin was found to be dependent on the concentration of NaGC. The apparent first-order rate constants at various NaGC concentrations were then calculated and plotted against NaGC concentration as shown in Fig. 5.

In the absence of NaGC, the zinc insulin degradation rate constant was calculated to be 0.03822 ± 0.00389 min^{-1} (mean ± SD; n = 3), representing the hexameric degradation rate constant, k_h. With the addition of NaGC the rate of degradation increased gradually; this was evident even at a

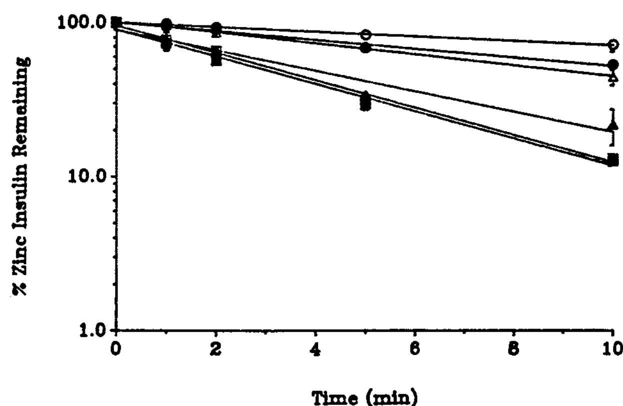


Fig. 3. Semilogarithmic plots of zinc insulin biodegradation by α-chymotrypsin as a function of NaGC concentration. Values represent means ± SD (n = 3). (○) 0 m*M* NaGC; (●) 5 m*M* NaGC; (△) 10 m*M* NaGC; (▲) 20 m*M* NaGC; (□) 30 m*M* NaGC; (■) 40 m*M* NaGC.

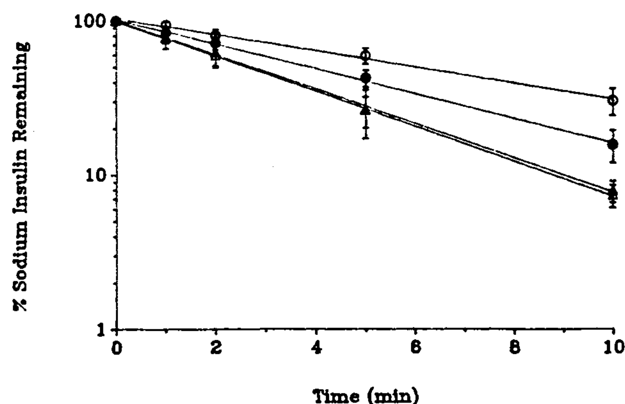


Fig. 4. Semilogarithmic plots of sodium insulin biodegradation by α-chymotrypsin as a function of NaGC concentration. Values represent means ± SD (n = 3). (○) 0 mM NaGC; (●) 5 mM NaGC; (△) 10 mM NaGC; (▲) 20 mM NaGC.

NaGC concentration of 5 mM, which is below its critical micellar concentration (CMC) of 9 mM (13). This observation could be explained by the fact a bile salt can partially form premicellar aggregates even below its CMC. The rate of degradation plateaus at a 30 mM NaGC concentration, with a degradation rate constant of 0.2051 ± 0.0083 min^{-1}. Further increases in the bile salt concentration did not enhance the proteolytic degradation rate constant to any significant extent.

It was therefore postulated that bile salt micelles probably caused complete dissociation of zinc hexamers to monomers at or near a 30 mM concentration, due in part to the solubilization of insulin aromatic amino acid residues and the hydrophobic side chains. According to Eq. (5), the plateau rate constant should be six times higher than the hexamer degradation rate constant when complete dissociation of hexamers to monomers occurs. Indeed, a 5.4-fold difference was actually observed, a value very close to the theoretical prediction. Therefore, the data strongly indicate that 30 mM NaGC completely dissociated 0.5 mg/ml zinc insulin from hexamers to monomers.

Since insulin hexamers were completely dissociated by NaGC, it was then hypothesized that similar behavior would

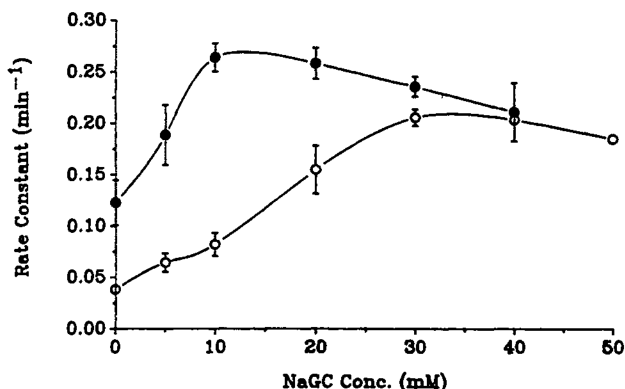


Fig. 5. The effect of various NaGC concentrations on zinc and sodium insulin biodegradation by α-chymotrypsin. Values represent means ± SD (n = 3). (○——○) Zinc insulin; (●——●) sodium insulin.

also be observed if dimers were used instead of hexamers. Therefore, sodium insulin (mainly dimers) was then used in the degradation experiments. The apparent first-order rate constants were plotted against NaGC concentration as presented in Fig. 5. In the absence of NaGC the rate of degradation of sodium insulin was found to be 0.1227 ± 0.0221 min^{-1} (n = 3), a 3.2-fold increase over that of zinc insulin, a value which is in close agreement with the theoretical ratio of 3.0 and very consistent with our previous report (11). Furthermore, the incorporation of NaGC in the system further increased the rate of sodium insulin degradation process as expected. At a NaGC concentration of 10 mM, a plateau rate constant of 0.2635 ± 0.0137 min^{-1} was obtained, which represents a 2.1-fold increase over that of insulin dimers. This value, again, is very close to the predicted value of 2-fold. With known k_{obs}, the fraction of monomers in a solution of sodium insulin in the presence of NaGC can be calculated by using the simplified form of Eq. (5). For example, at a 5 mM NaGC concentration, a f_m value of 0.429 can be obtained knowing that the average k_{obs} is 0.1883 min^{-1}. Therefore, at a 5 mM NaGC concentration almost half of the sodium insulin molecules are in the form of monomers.

Being comprised of two interlinked peptide chains, insulin molecules coil as α-helices involving both A and B chains arranged in a distorted form. Meanwhile, the aromatic residues in the B chain are involved primarily in the formation of an intermonomer antiparallel β structure (14). These three-dimensional structures exhibit optical activity as a function of the extent of aggregation, resulting in several main Cotton effects and a few minor bands on the ultraviolet circular dichroic spectra (14). Dissociation of insulin oligomers will certainly be accompanied by ellipticity changes at the respective maxima.

Figure 6A displays the CD spectral differences between insulin hexamers and dimers, both performed at a total insulin concentration of 0.5 mg/ml. The negative maxima at 276 nm in zinc insulin is assigned to the contribution of the B23–28 aromatic residues in the form of the antiparallel β structure. Attenuation of this band could, therefore, be correlated with deaggregation, while a strengthening of this band could be associated with enhanced association of monomers. The effect of incorporation of sodium glycocholate on the magnitude of this band is shown in Fig. 6B. The gradual addition of the bile salt to the insulin solution caused a progressive decrease in molecular ellipticities, suggesting dissociation of insulin hexamers into smaller units, i.e., dimers and monomers. Below a 30 mM NaGC concentration, the change in molar ellipticity was easily noticeable, while above 30 mM the decreasing trend nearly stopped. Besides, the addition of NaGC to sodium insulin solution (dimers) did not bring up any significant change at this maximum. Therefore, in this wavelength range the dissociation of insulin dimers into monomers could not be properly characterized.

Figure 7A illustrates the CD spectra of both zinc and sodium insulin (0.5 mg/ml) in the wavelength range from 240 to 200 nm. In agreement with a previous report (14), two adjacent bands were observed at 222 and 212 nm. The 222-nm band was assigned in large part to the β structure, which is a predominant feature of the dimer. Both zinc and sodium

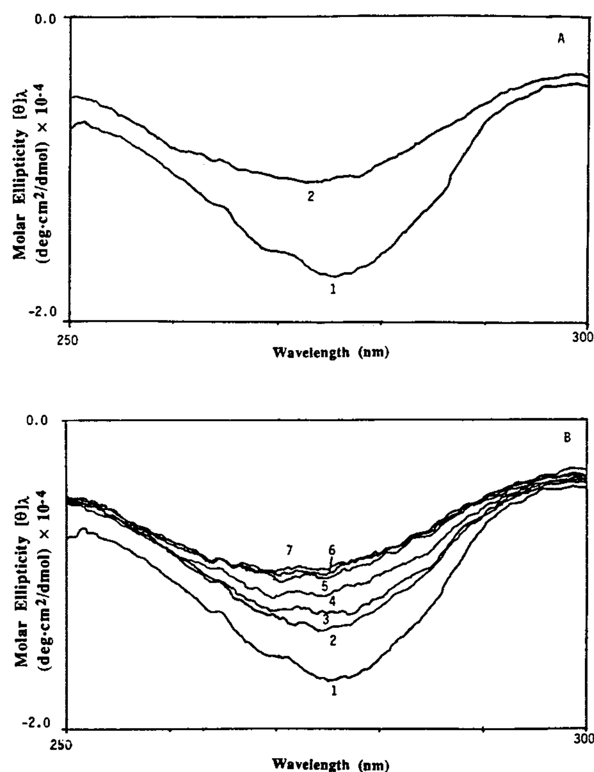


Fig. 6. (A) The circular dichroic spectra of 0.5 mg/ml zinc and sodium insulin. (1) 0.5 mg/ml zinc insulin; (2) 0.5 mg/ml sodium insulin. (B) The circular dichroic spectra of 0.5 mg/ml zinc insulin containing various concentrations of NaGC. (1) 0.5 mg/ml zinc insulin; (2) 1 with 5 m*M* NaGC; (3) 1 with 10 m*M* NaGC; (4) 1 with 20 m*M* NaGC; (5) 1 with 30 m*M* NaGC; (6) 1 with 40 m*M* NaGC; (7) 1 with 50 m*M* NaGC.

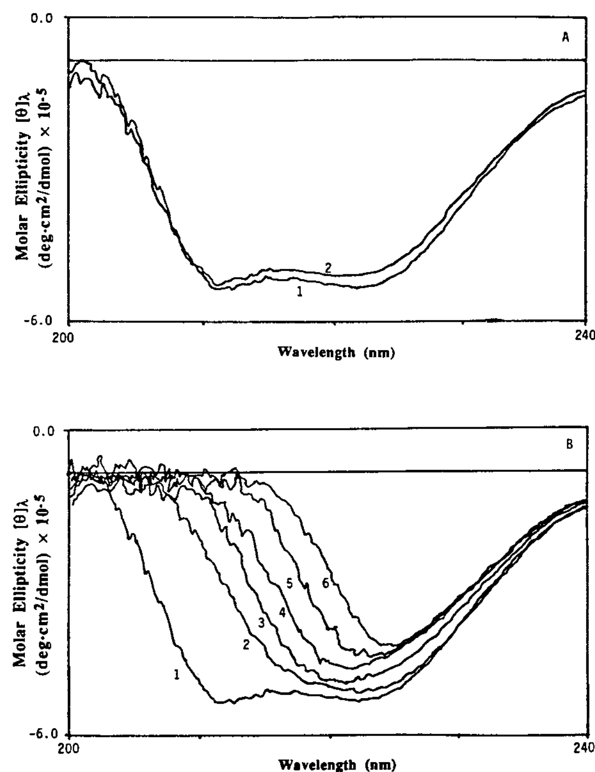


Fig. 7. (A) The CD spectra of 0.5 mg/ml zinc and sodium insulin. (1) 0.5 mg/ml zinc insulin; (2) 0.5 mg/ml sodium insulin. (B) The CD spectra of 0.5 mg/ml zinc insulin containing various concentrations of NaGC. (1) 0.5 mg/ml zinc insulin; (2) 1 with 10 m*M* NaGC; (3) 1 with 20 m*M* NaGC; (4) 1 with 30 m*M* NaGC; (5) 1 with 40 m*M* NaGC; (6) 1 with 50 m*M* NaGC.

insulin exhibited this band, with only a minor difference in intensity. On the other hand, the 212-nm band was proposed to be attributed largely to the α-helical structure which is a characteristic feature of the monomer. The presence of this band involves residues B10–19, A2–6, and A13–19, which are arranged in distorted helices. Zinc and sodium insulin displayed a similar pattern at this maximum as shown in Fig. 7A.

In the presence of bile salt, the two negative maxima were attenuated significantly as shown in Fig. 7B. Both zinc and sodium insulins exhibited similar spectral transitions and therefore only zinc insulin is shown. Incorporation of NaGC significantly attenuated the 222-nm band, while the 212-nm band totally disappeared in the presence of 50 m*M* NaGC. It must be noted that the position of the negative maxima at 222 nm shifted to 225 nm in the presence of 50 m*M* NaGC. The molar ellipticities at 276, 222, and 212 nm are summarized in Table I for easy comparison. In conclusion, the molar ellipticity change in the two wavelength ranges (300 to 250 and 240 to 200 nm) characterized the fractional changes of insulin aggregation from hexamers to dimers and from dimers to monomers, respectively.

The enhanced nasal absorption of insulin by bile salts was postulated to be due to a combination of several factors. First, the detergent-like properties of bile salts can directly perturb the mucosal barrier of the nasal membrane. Recently, Shao and Mitra (15) quantitatively evaluated nasal protein release from the rat nasal cavity using a variety of bile salts. Their findings revealed that nasal protein release depends on both the hydrophobicity and the concentration of bile salts. Nasal morphological changes introduced by bile salts were also confirmed by Tengamnuay and Mitra (5). A moderate damaging effect ranging from hydropic degeneration to nuclear pycnosis was observed even using NaGC, an enhancer known to be much safer than other bile salts.

Micellar hydrophobic cores can increase the solubility of lipophilic compounds, increasing the juxtamembrane concentration of solute. The mechanism was initially proposed

Table I. Molar Ellipticities ($[\theta]_\lambda$) at 276, 222, and 212 nm as a Function of NaGC Concentration

NaGC conc. (m*M*)	$[\theta]_\lambda$, 276 nm $[(\deg \cdot cm^2/dmol) \times 10^{-4}]$	$[\theta]_\lambda$, 222 nm $[(\deg \cdot cm^2/dmol) \times 10^{-5}]$	$[\theta]_\lambda$, 212 nm $[(\deg \cdot cm^2/dmol) \times 10^{-5}]$
0	−1.67	−5.23	−5.23
5	−1.34	—	—
10	−1.23	−4.97	−2.58
20	−1.13	−4.63	−1.32
30	−1.01	−4.34	−0.77
40	−0.99	−4.06	−0.27
50	−0.96	−3.35	0

by Gordon *et al.* (8) to explain the enhanced insulin nasal absorption caused by bile salts. Nevertheless, controversial results were also reported (16). The mucosal transport of several lipophilic compounds was shown to decrease considerably as a result of micellar solubilization. This was attributed to the decreased free drug concentration in the intestinal lumen, which, in turn, caused a decrease in the diffusional gradient of free drug species.

The presence of bile salts was also thought to exert influences on the mucosal enzyme activity, as proposed by Hirai *et al.* (6). Sodium glycocholate was found to cause a marked inhibition of the leucine aminopeptidase activity. This protective effect might be due to the low activity and high selectivity of leucine aminopeptidase (LAP) as described previously (17). LAP hydrolyses only zinc-free insulin at an extremely slow rate. Besides, LAP attacks insulin only at its N-terminus. Therefore, solubilization of insulin side chains may prevent its hydrolysis by LAP. On the other hand, α-chymotrypsin possesses a high activity and cleaves insulin at various sites (10). Schilling and Mitra (10) have in fact reported that all five amino acid residues in insulin which chymotrypsin initially cleaves are all exposed to the surface of the molecule and therefore accessible to attack. The presence of NaGC, in fact, did not hinder the biodegradation of insulin as evidenced by the constant rate of degradation in the range of 30 to 50 m*M* NaGC as shown in Fig. 5.

The actual interaction between insulin molecules and bile salt micelles is still unclear. It is known, however, that insulin molecules aggregate into oligomers through both hydrogen bonds and hydrophobic interactions (18). There are four hydrogen bonds in a pleated sheet between two B chains, and primarily aromatic residues are known to contribute to the hydrophobic intermolecular forces. Therefore, any displacement of these groups from self-associated insulin B chains into the micelles will most likely break down the oligomer structure. Two NaGC molecules can pack back to back, forming a primary micelle. The 3α-, 7α-, and 12α-trihydroxy groups on each NaGC molecule face the outside aqueous environment, forming strong hydrogen bonds with water molecules. Therefore, the hydroxy groups may form hydrogen bonds with insulin monomers, thus replacing the intermonomeric forces. The hydrophobic cores in the bile salt micelles, on the other hand, will probably solubilize the aliphatic and aromatic side chains. One or both of the mechanisms may play an important role in dissociating insulin oligomers. Figure 5 also demonstrated a difference in zinc and sodium insulin dissociation characteristics. Zinc insulin needs 30 m*M* NaGC to dissociate completely, while only 10 m*M* NaGC is needed for sodium insulin. Therefore, the presence of zinc in the solution may require excess bile salt molecules for complexation to occur.

Whether haxameric insulin dissociates directly into monomers or dissociates via the dimers as the intermediates is still unknown at the present time. We are currently developing a size exclusion chromatographic method to understand these dissociation equilibria further. The effects of other dihydroxy and unconjugated bile salts as well as other surfactants on insulin aggregation are also under investigation.

ACKNOWLEDGMENTS

This investigation was supported in part by a Merck Faculty Development Award (A.K.M.) and in part by a Young Investigator Award from the American Association of Pharmaceutical Scientists (A.K.M.). Z.S. is a David Ross Research Fellow. The authors wish to thank Mr. Ramesh Krishnamoorthy for his help in obtaining the CD spectra.

REFERENCES

1. R. Quinn and J. D. Andrade. Minimizing the aggregation of neutral insulin solution. *J. Pharm. Sci.* 72:1472–1483 (1983).
2. S. Sato, C. D. Ebert, and S. W. Kim. Prevention of insulin self-association and surface adsorption. *J. Pharm. Sci.* 72:228–232 (1983).
3. A. M. Alibisser, W. Longheed, K. Perlman, and A. Bahoric. Nonaggregating insulin solutions for long-term glucose control in experimental and human diabetes. *Diabetes* 29:241–243 (1980).
4. E. Mosekilde, K. S. Jensen, C. Binder, S. Pramming, and B. Thorsteinsson. Modeling absorption kinetics of subcutaneous injected soluble insulin. *J. Pharmacokin. Biopharm.* 17:67–87 (1989).
5. P. Tengamnuay and A. K. Mitra. Bile salt-fatty acid mixed micelles as nasal absorption promoters of peptides. II. In vivo nasal absorption of insulin in rats and effect of mixed micelles on the morphological integrity of the nasal mucosa. *Pharm. Res.* 7:370–375 (1990).
6. S. Hirai, T. Yashiki, and H. Mima. Mechanisms for the enhancement of nasal absorption of insulin by surfactants. *Int. J. Pharm.* 9:173–184 (1981).
7. M. D. Donovan, G. L. Flynn, and G. L. Amidon. The molecular weight dependence of nasal absorption: The effect of absorption enhancers. *Pharm. Res.* 7:808–815 (1990).
8. G. S. Gordon, A. C. Moses, R. D. Silvers, J. S. Flier, and M. C. Carey. Nasal absorption of insulin: Enhancement by hydrophobic bile-salts. *Proc. Natl. Acad. Sci. USA* 82:7419–7423 (1985).
9. R. J. Schilling and A. K. Mitra. Intestinal mucosal transport of insulin. *Int. J. Pharm.* 62:53–64 (1990).
10. R. J. Schilling and A. K. Mitra. Degradation of insulin by trypsin and α-chymotrypsin. *Pharm. Res.* 8:721–727 (1991).
11. F-y. Liu, D. O. Kildsig, and A. K. Mitra. Insulin aggregation in aqueous media and its effect on alpha-chymotrypsin-mediated proteolytic degradation. *Pharm. Res.* 8:925–929 (1991).
12. A. Ginsburg and H. K. Schachman. Studies on the enzymatic breakdown of proteins. I. Action of chymotrypsin on insulin. *J. Biol. Chem.* 235:108–114 (1960).
13. P. Tengamnuay. *Nasal Absorption of Selected Amino Acids and Peptides in Rats,* Ph.D. thesis, Purdue University, West Lafayette, IN, 1989.
14. J. Goldman and F. H. Carpenter. Zinc binding, circular dichroism, and equilibrium sedimentation studies on insulin (bovine) and several of its derivatives. *Biochemistry* 13:4566–4574 (1974).
15. Z. Shao and A. K. Mitra. Membrane protein and enzyme release by bile salts and bile salt–fatty acid mixed micelles—Correlation with facilitated nasal drug transport. *Pharm. Res.* (in press).
16. F. G. J. Poelma, R. Breas, and J. J. Tukker. Intestinal absorption of drugs. IV. The influence of taurocholate and L-cysteine on the barrier function of mucus. *Int. J. Pharm.* 64:161–169 (1990).
17. E. L. Smith, R. L. Hill, and A. Borman. Activity of insulin degraded by leucine aminopeptidase. *J. Biol. Chem.* 29:207–208 (1958).
18. D. Crowfoot, D. C. Hodgkin, E. Dodson, G. Dodson, and C. Reynolds. Structure and function of proteins and nucleic acids. *Biochem. Soc. Trans.* 11:411–417 (1983).