DRUG DEVELOPMENT AND INDUSTRIAL PHARMACY, 21(14), 1661-1669 (1995)

PHOTOPHYSICAL CHARACTERIZATION OF INSULIN DENATURATION AND AGGREGATION AT HYDROPHOBIC INTERFACES

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ABSTRACT

The peak III/I ratio of pyrene monomer fluorescence was used to characterize surface-induced insulin denaturation and aggregation. An indicator of the hydrophobicity of pyrene's microenvironment, the III/I ratio can be used to study self-association of amphipathic molecules. In sodium lauryl sulfate (NaLS), the III/I ratio was 0.71 below the critical micelle concentration (CMC) and increased to 0.98 when the concentration was above the CMC. When insulin was placed in a polystyrene cuvet for 2 days at 37°C, the III/I ratio decreased from 0.84 to 0.78. The decrease in III/I ratio corresponds to surface-induced denaturation of insulin which exposed pyrene to a more polar environment. Increasing the duration of incubation for up to 12 days increased the III/I ratio to 1.06. Increased hydrophobicity of pyrene's environment correlates with the self-association of insulin. Once the aggregates reached a critical size, they began precipitating as was evident by an increase in the turbidity (absorbance at 600 nm) of solution. The results of this study support previous hypothesis that the aggregation and precipitation of insulin at hydrophobic interfaces is initiated by adsorption and surface-induced denaturation.

INTRODUCTION

Recent advances in molecular biology and genetic engineering have made feasible the production of peptides and proteins as therapeutic and diagnostic agents. The successful development of protein-based pharmaceuticals requires preservation of the biological activity in all steps of development, purification, storage, and delivery of these agents [1,2]. On the basis of their physical and chemical properties, peptide and protein drugs are associated with unique formulation challenges. As reviewed by Manning et al. [3], protein in solution can

be degraded by physical and chemical pathways. The relatively fragile nature of peptides and proteins, such as insulin, presents an obstacle in the development of conventional therapeutic systems, long-term infusion devices, and controlled release drug delivery systems [4-7].

The tendency to self-associate in solution has led to serious difficulties in the use of insulin in long-term drug delivery systems [8-10]. Blockage of tubing, membranes, and pumps with macroscopic aggregates is a concern that requires replacement or flushing of the insulin reservoir in infusion devices. In addition, the altered insulin which is devoid of any therapeutic benefits, has been implicated to cause elevated serum amyloid proteins and could manifest diabetic amyloidosis [11]. The aggregation process is influenced by factors such as the presence of metal ions (Zn^{2+}, Cu^{2+}, Fe^{2+}), temperature, pH and ionic strength, motion, and the hydrophobicity of the contacting surface [8,9]. Attempts to minimize insulin aggregation usually involve the addition of stabilizers such as autologous serum [12], non-ionic surfactants [6,8], amino acids [13], and, more recently, cyclodextrins [14].

Although factors that promote insulin aggregation (e.g., temperature, motion, and surface hydrophobicity) have been identified for some time, the mechanism of insulin aggregation remained entirely speculative. A model of the processes involved in insulin aggregation was reported by Sluzky et al. [15,16]. The authors suggested that the aggregation process at air-liquid and solid-liquid interfaces is initiated by the adsorption of insulin monomer, followed by surface-induced denaturation. The denatured insulin monomer, initiating nucleation, can combine with other unfolded species to form aggregates. Consequently, the growth process continues as increasingly more native insulin is removed from solution. The formed insulin aggregates are thought to be stabilized mainly by hydrophobic interactions [17]. Once a critical size has reached, the aggregates can no longer remain in aqueous solution and thus begin to precipitate. The precipitation is clearly observed by an increase in the turbidity ("milkiness") of insulin solution.

In the present study, we have utilized the peak III/I ratio of pyrene monomer fluorescence to probe the molecular mechanism of insulin denaturation and aggregation at hydrophobic interfaces. Being a hydrophobic compound with low aqueous solubility (~0.3 μM), pyrene is known to localize preferentially in the hydrophobic domains of amphipathic molecules. In insulin, pyrene could bind to the hydrophobic surface or cavity of the native molecule. Pyrene monomer emission is associated with fine vibronic structures whose intensities correspond with the polarity of pyrene's microenvironment [18]. In polar solvents such as water, there is an enhancement in the intensity of peak I (at 372 nm), whereas no effect is observed on the intensity of peak III (at 384 nm) [19]. The III/I ratio, therefore, is used to characterize the change in polarity of pyrene's environment upon association of amphipathic molecules in aqueous solution [20]. The III/I ratio is particularly suited for measurement of the critical micelle concentration (CMC) of various surfactants [21].

MATERIALS AND METHODS

Preparation of Solutions

Crystalline porcine insulin, received from Eli Lilly and Company (Indianapolis, IN), was used without further purification. Filtered deionized distilled water (NANOpure®, Barnsted/Thermolyne, Dubuque, IA) was used exclusively to prepare all aqueous solutions. Insulin was dissolved in 0.1 M TRIS base (Sigma Chemical Company, St. Louis, MO) and the pH of the solution was

adjusted to 7.2 using 0.1 M hydrochloric acid. The final concentration of insulin was maintained at 1.0 mg/ml. In some cases, urea (Sigma) was added to induce insulin denaturation in aqueous solution. The final concentration of urea was in the range of 0.5 M to 5.0 M.

Sodium lauryl sulfate (NaLS), purchased from Sigma, was dissolved in deionized distilled water to prepare solutions with the concentration ranging from 0.10 mg/ml to 10 mg/ml.

Pyrene Fluorescence Studies

Pyrene (99% pure) was purchased from Aldrich Chemical Company (Milwaukee, WI) and was further purified by repeated recrystallization in methanol. Purified pyrene, dissolved in methanol, was added to NaLS or insulin solution to give a final concentration of 2.0 μM. Previously, we have found that there is no interference from pyrene excimer emission at this low concentration [22]. In addition, the pyrene to insulin molar ratio of 1:80 and the volume of methanol added were found not to induce any structural alterations on insulin in solution.

A polystyrene cuvet (10 x 10 x 55 mm, Sigma) was filled with pyrene-containing insulin solution for the aggregation studies. The sealed cuvet was incubated in a shaking water-bath (Precision Scientific Inc., Chicago, IL) at 65 r.p.m. and 37°C. At pre-determined time intervals, insulin-containing cuvet was removed from the water-bath and the emission spectrum of pyrene was obtained using a Perkin-Elmer LS-50B (Norwalk, CT) fluorescence spectrophotometer. Pyrene in solution was excited at 343 nm and the emission spectrum was collected in the range of 360 nm to 500 nm at an integration time of 1.0 nm per second. The excitation and emission slit widths were 15 nm and 2.5 nm, respectively. The intensities of peak III (at 384 nm) and peak I (at 372 nm) was used to calculate the III/I ratio. The data represent mean ± S.D. from four independent experiments.

Turbidity Measurements

In a separate set of experiments, insulin was placed in a polystyrene cuvet and incubated at 65 r.p.m and 37°C as described above. In addition, polyethylene oxide/poly(propylene oxide)/poly(ethylene oxide) triblock copolymer (Pluronic® F108, BASF Corporation, Parsipanny, NJ) was added to insulin solution at a final concentration of 0.05 mg/ml to prevent aggregation and precipitation. The precipitation of insulin was monitored by measuring the change in the visible absorbance at 600 nm using a Shimadzu UV-160U (Columbia, MD) spectrophotometer. The data represent mean ± S.D. from four independent experiments.

RESULTS AND DISCUSSION

Pyrene Fluorescence Study of NaLS Self-Association

NaLS is an amphipathic compound capable of self-assembly into organized structures in aqueous solution above the critical micelle concentration (CMC). Figure 1 shows the emission spectra of pyrene in NaLS below and above the CMC. The III/I ratio increased from 0.71 at lower concentrations of NaLS (0.5 mg/ml) to 0.98 when the NaLS concentration was above the CMC (5.0 mg/ml). The III/I ratio of 0.98 corresponds to pyrene being localized in a relatively hydrophobic microenvironment in the interior of the micellar structure [21]. In addition, above the CMC, as pyrene was localized in an organized medium, there was an enhancement in the emission intensities due to less fluorescence quenching by dissolved oxygen.

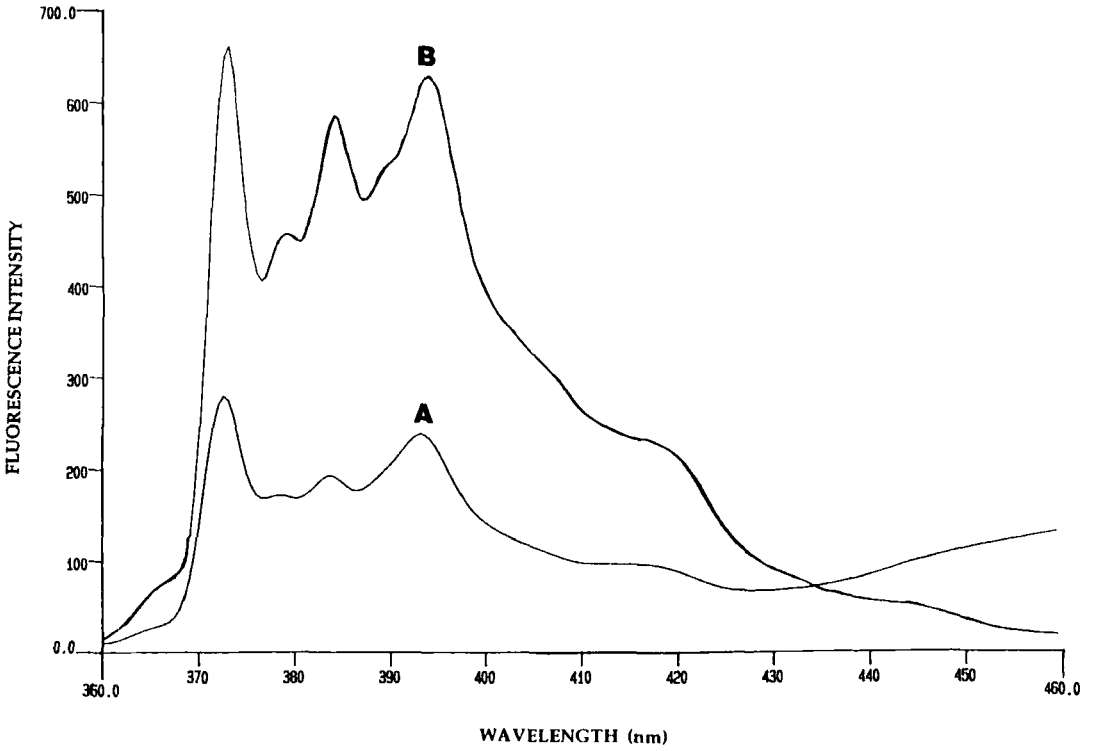

FIGURE 1
Pyrene monomer emission spectra in aqueous sodium lauryl sulfate (NaLS) solution at concentrations below (A) and above (B) the critical micelle concentration. Pyrene was dissolved in NaLS at a final concentration of 2.0 μM.

Figure 2 shows the change in the III/I ratio as a function of NaLS concentration in aqueous solution. In pure water and at lower NaLS concentrations, the III/I ratio was in the range of 0.50 to 0.60. The III/I increased with increasing NaLS concentration up to 2.30 mg/ml (shown by the dashed line in Figure 2). Cabane showed that NaLS forms well-defined micellar structures above the CMC (i.e., 2.30 mg/ml) with an average aggregation number of about 60 [23]. Above the CMC, the III/I ratio remained at 0.98 as pyrene was now confined to the interior of the NaLS micellar structure whose hydrophobicity remains relatively constant. These experiments in NaLS provide reasonable assurance on the utility of the III/I ratio as a sensitive indicator of the self-assembly of amphipathic molecules in aqueous solution.

Pyrene Fluorescence Study of Insulin Denaturation and Aggregation

Figure 3 shows the change in III/I ratio of pyrene and the absorbance at 600 nm in insulin solution as a function of the duration of incubation at 65 r.pm. and 37°C. In native insulin, pyrene is expected to bind preferentially to the hydrophobic

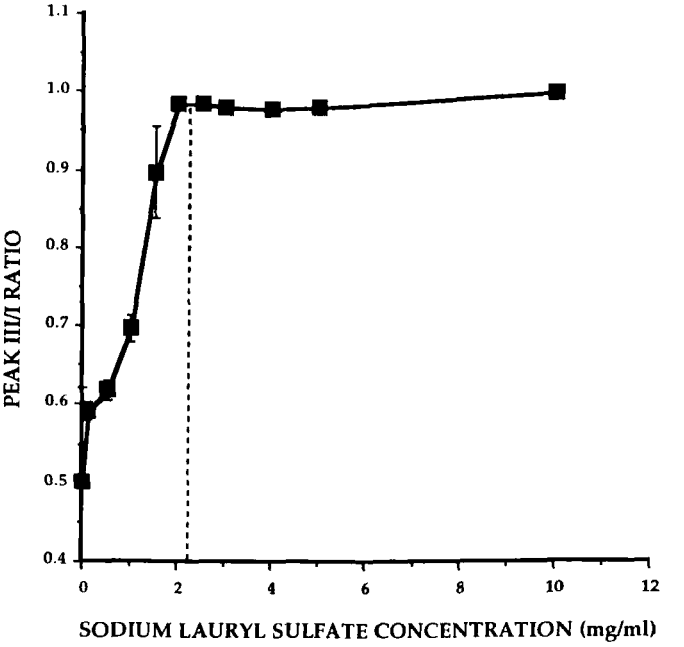

FIGURE 2
The peak III/I ratio of pyrene as a function of the sodium lauryl sulfate concentration. The dashed line corresponds to the critical micelle concentration (i.e., 2.30 mg/ml) of sodium lauryl sulfate in aqueous solution.

surface or cavity of the monomers, dimers, and hexamers. Pyrene localization in the hydrophobic region of insulin molecule was confirmed by the III/I ratio of 0.84, indicating a non-polar microenvironment (Figure 3-A). After 2 days of incubation, the III/I ratio decreased to 0.78. The decrease in III/I ratio is most probably due to surface-induced conformational change in insulin which may allow pyrene to partition from a non-polar environment of the native insulin molecule to a more polar environment as the insulin molecule partially unfolds. As the duration of incubation continued, there was a dramatic increase in the III/I ratio for up to 12 days. After 12 days of incubation the III/I ratio increased to 1.06. The increase in the III/I ratio which is related to an increase in the hydrophobicity of pyrene's microenvironment suggests the possibility of self-association of partially unfolded insulin to form aggregates.

After 12 days of incubation at 37°C, the formed aggregates were still soluble in aqueous medium since no change in the turbidity of insulin solution, as measured by the absorbance at 600 nm, was observed (Figure 3-B). After 14 days of incubation, however, the absorbance at 600 nm increased significantly indicating formation of insoluble insulin aggregates. The absorbance continued to increase with increasing duration as more insulin aggregates were formed. Although there was evidence of precipitation, the III/I ratio did not change significantly suggesting that the hydrophobicity of pyrene's environment was not affected.

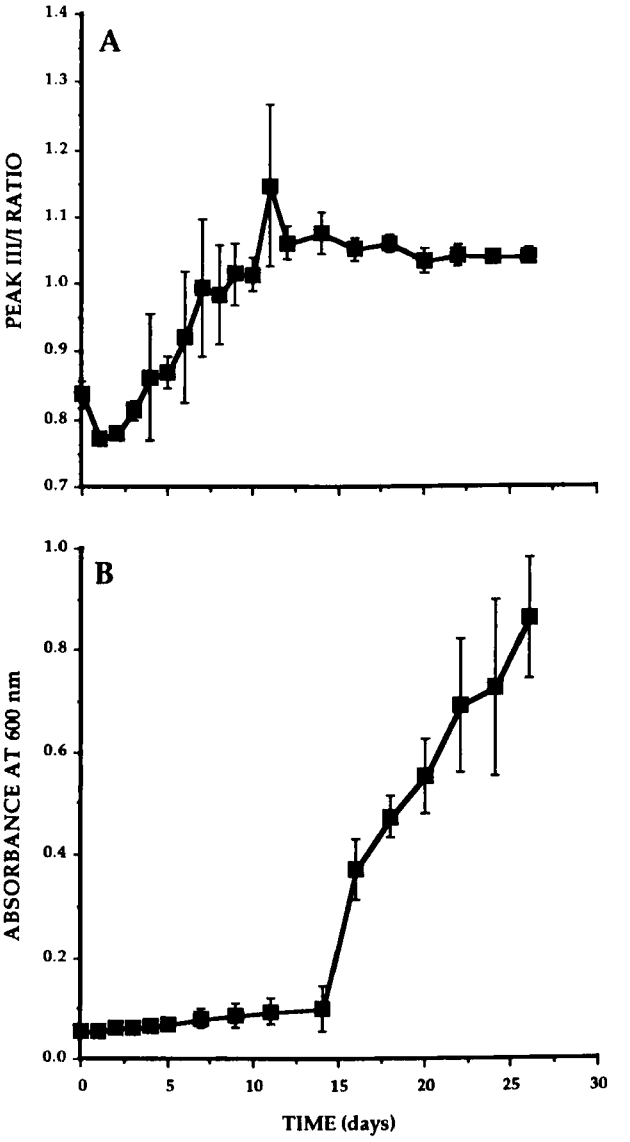

FIGURE 3
The change in peak III/I ratio of pyrene (A) and the absorbance at 600 nm (B) of insulin solution as a function of time. Insulin at 1.0 mg/ml concentration was incubated in a shaking water-bath at 37°C, 65 r.p.m.

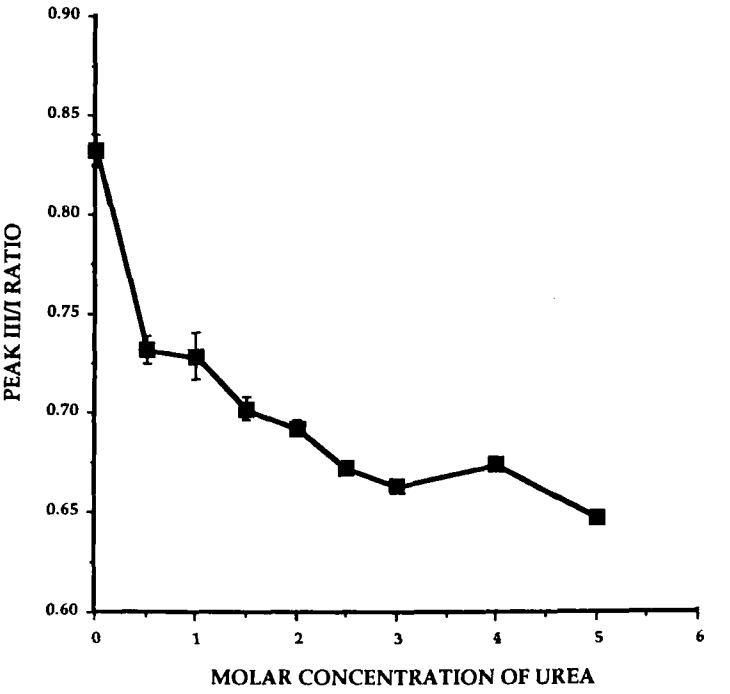

FIGURE 4
The change in peak III/I ratio of pyrene in insulin (1.0 mg/ml) as a function of increasing concentrations of urea.

In order to confirm that the decrease in III/I ratio after 2 days of incubation was due to surface-induced denaturation, we examined the change in III/I ratio in insulin in the presence of urea, a known denaturing agent. Various analytical techniques have been used to measure the degree of unfolding of proteins in the presence of urea [24]. In most cases, urea concentration of up to 5.0 M is found sufficient to completely denature proteins from a three-dimensional folded structure to a random coil structure [25]. Figure 4 shows the change in III/I ratio in insulin upon addition of different concentrations of urea. The decrease in III/I ratio with increasing urea concentration suggests that upon unfolding of insulin, pyrene molecules did experience a more polar environment. Depending on the exact location of pyrene in insulin, the unfolding process in the presence of urea could enhance the interactions between pyrene and surrounding water molecules. At 5.0 M urea concentration, the III/I ratio decreased to 0.65 as insulin gradually unfolded into a random coil structure.

In this study, the likelihood of insulin denaturation at the air-liquid interface is much greater than at the solid-liquid interface because of the mobility of this boundary which allows for the creation of a new surface each time the protein is agitated. Microscopic air bubbles in insulin solution provide a massive surface area for adsorption and surface-induced denaturation. Insulin denaturation and

TABLE 1
Effect of Pluronic® F-108 Surfactant on Insulin Aggregation and Precipitation.

Incubation Time (days)	Absorbance at 600 nm	
	Without Additive	With Pluronic® F-108*
0	0.06	0.06
5	0.07	0.06
10	0.07	0.07
16	0.37	0.07

* The final concentration of Pluronic® F-108 was 0.05 mg/ml.

aggregation could be minimized by addition of non-ionic surfactants to occupy the air-liquid interface and prevent insulin denaturation in aqueous solution [26]. Furthermore, insulin denaturation at the solid-liquid interface can be minimized also, if the surfactant adsorbs preferentially to the hydrophobic solid surface due to higher affinity for the surface than the protein. Table 1 shows a decrease in the aggregation and precipitation of insulin in the presence of Pluronic® F-108, a poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide) triblock copolymer, containing 129 residues of ethylene oxide and 56 residues of propylene oxide. The absorbance at 600 nm after 16 days of incubation decreased from 0.37 to 0.07 in insulin containing 0.05 mg/ml of Pluronic® F-108. Non-ionic surfactants, such as Pluronic® F-108, with the right proportion of hydrophilic and hydrophobic segments, can preferentially adsorb onto the hydrophobic surface even in the presence of proteins [27].

In summary, we have utilized the III/I ratio of pyrene monomer fluorescence to study the mechanism of insulin aggregation. The results confirm previous hypothesis that the denaturation and aggregation of insulin at hydrophobic interfaces is initiated by adsorption and surface-induced denaturation.

ACKNOWLEDGMENTS

This study was supported in part by the Biomedical Research Support Grant SO7-RR-05830-12.

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