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# Characterization of physiochemical and biological properties of an insulin/lauryl sulfate complex formed by hydrophobic ion pairing

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**Abstract**

An insulin/lauryl sulfate complex was prepared by hydrophobic ion pairing (HIP). The physiochemical and biological properties of the HIP complex were characterized using octanol/water partition measurement, isothermal titration calorimetry (ITC), ultraviolet-circular dichroism (UV-CD) and Fourier transform infrared spectroscopy (FTIR). Sodium dodecyl sulfate (SDS) bound to the insulin in a stoichiometric manner. The formed complex exhibited lipophilicity, and its insulin retained its native structure integrity. The in vivo bioactivity of the complex insulin was evaluated in rats by monitoring the plasma glucose level after intravenous (i.v.) injection, and the glucose level was compared with that for free insulin. The pharmacodynamic study result in rats showed that the complex insulin had in vivo bioactivity comparable to free insulin.


*Keywords:* Hydrophobic ion pairing; Insulin complex; Protein structure; In vivo bioactivity

## 1. Introduction

The hydrophobic ion pairing (HIP) technique has been receiving increasing interest in the field of protein/peptide delivery (Quintanar-Guerrero et al., 1997; Meyer and Manning, 1998). With HIP, the complex formed is much more hydrophobic than the corresponding free protein/peptides, thereby leading to a large increase in the partition coefficient (Adjei et al., 1993; Matsuura et al., 1993; Powers et al., 1993; Paradkar and Dordick, 1994b). The remarkably enhanced hydrophobicity of the HIP complex enables scientists to dissolve more protein/peptides in nonaqueous solvents, and to permit homogeneous mixing of the complex with water–insoluble polymers for encapsulating protein/peptides (Falk et al., 1997; Yoo et al., 2001). Consequently, the HIP technique can greatly increase drug encapsulation efficiency and drug loading, as well as minimize the undesirable burst effect (Yamakawa et al., 1992; Niwa et al., 1993, 1994; Falk et al., 1997; Yoo et al., 2001). In addition, HIP has enhanced drug permeability (Lee et al., 1991; Wang et al., 1994) and drug transport across membranes (Anderberg and Artursson, 1993; Anderberg et al., 1993), and has improved oral bioavailability (Lee et al., 1991; Aungst and Hussain, 1992).

There is always a concern over protein denaturation and unfolding during encapsulation in organic solvents. As previously reported, HIP complexes are more stable than free proteins in organic solvents (Matsuura et al., 1993; Meyer et al., 1996; Kendrick et al., 1997; Wangikar et al., 1997; Yoo et al., 2001), and the complex proteins seem to maintain their native-like structure in organic solvents (Matsuura et al., 1993; Powers et al., 1993; Meyer et al., 1995). These complex proteins in solvents, however, are still much less active than free proteins in an aqueous solution (Paradkar and Dordick, 1994a; Meyer et al., 1995; Sergeeva et al., 1997; Wangikar et al., 1997). These previous studies provide a great deal of important information about the structure and activity of the proteins in the complex. However, these studies were mainly focused on the protein structure and in vitro enzyme activity in organic solvents due to the interests in applications of the protein complex in nonaqueous media. There is little information regarding the structure of the protein complex in the solid state or in the aqueous environment where the protein complex may be used in other applications. In addition, these studies were only concerned with the in vitro enzymatic activity of the complex in the organic solvent, and few of these studies directly evaluated the in vivo bioactivity of the complex proteins. Therefore, there is a need to study the

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structure of the protein complex in the solid state or in an aqueous medium, and to evaluate directly the in vivo bioactivity of the protein complex.

The objective of this study was to characterize comprehensively the physiochemical and biological properties of the insulin/lauryl sulfate HIP complex in a solid state or in an aqueous medium, using octanol/water partition measurement, isothermal titration calorimetry (ITC), size-exclusion high-performance liquid chromatography (SEC-HPLC), ultraviolet-circular dichroism (UV-CD) and Fourier transform infrared spectroscopy (FTIR). In addition, the in vivo bioactivities of both the insulin HIP complex and free insulin were evaluated in rats.

## 2. Materials and methods

### 2.1. Materials

Human recombinant insulin and sodium dodecyl sulfate (SDS) were purchased from Sigma–Aldrich (St. Louis, MO). All other chemicals were from sources well known in the art.

### 2.2. Preparation of an insulin/lauryl sulfate complex

Ten milligrams of insulin and a corresponding amount of SDS (6:1, SDS/insulin molar ratio) were dissolved separately in 1 mL of acidified water (pH 2.5). The SDS solution was added slowly into the insulin solution. The cloudy solution was centrifuged at 14,000 rpm for 2 min at room temperature. The insulin concentration in the supernatant was analyzed using an HPLC method, and the unbound insulin fraction was calculated based on the initial amount of insulin added. The white precipitates recovered were rinsed with deionized water, lyophilized, and stored at −20 °C before further use.

### 2.3. Isothermal titration calorimetry

The binding between insulin and SDS was investigated using an isothermal titration calorimeter (VP-ITC, MicroCal, Northampton, MA) at 30 °C. The titration was carried out by a stepwise injection of 20 μL of 6.88 mM SDS solution into the sample cell filled with 0.172 mM insulin solution. The time interval between each injection was set to 2 min. The enthalpy change and binding molar ratio were calculated by the Origin 7 Software (OriginLab, Northampton, MA).

### 2.4. Insulin/lauryl sulfate complex composition

The insulin/lauryl sulfate complex was dissolved in phosphate-buffered saline (PBS) (pH 7.4) at 1 mg/mL, and the solution was then diluted with the mixture of acetonitrile–water (30:70, v/v, with 0.1% trifluoroacetic acid [TFA]) to reach the concentrations in the range of 2–100 μg/mL. Twenty-five microliters of the solution was injected into a Thermo BioBasic SEC-120 (5 μm) (Thermo Electron, Waltham, MA). The sample was eluted at 30 °C with the mixture of acetonitrile–water (30:70, v/v, with 0.1% TFA) at 1 mL/min, and the concentrations of the insulin and SDS were quantified using an evaporative light scattering detector SEDEX55 (SEDERE Inc., Lawrenceville, NJ).

### 2.5. Partitioning coefficient of an insulin/lauryl sulfate complex in a 1-octanol/water system

An insulin solution (1.72 mM) and SDS solutions with various concentrations were prepared separately in acidified water (pH 2.5). After slow addition of 0.6 mL of each SDS solution into 0.6 mL of the insulin solution, 1.2 mL of 1-octanol was added to the insulin/SDS mixture. After 4 h of agitation at room temperature, the mixture was spun for 10 min at 14,000 rpm. The insulin concentrations in both the octanol phase and the aqueous phase were measured at 280 nm by a UV spectrophotometer (UV160U, Shimadzu, Columbia, MD) using a 1 cm quartz cell.

### 2.6. FTIR

Fourier transform infrared spectroscopy was used to characterize the complex formation and the secondary structure of insulin in the complex. The insulin/lauryl sulfate complex in powder form was tested using a Perkin-Elmer Spectrum 2000 FTIR with an attenuated total reflectance (ATR) Accessory (Perkin-Elmer, Boston, MA). The resultant spectra were smoothed with a seven-point Savitsky–Golay smooth function to remove the noise. Second-derivative spectra were obtained with the derivative function of Omnic software (Nicolet, Waltham, MA). The inverted second-derivative spectra were obtained, and the curves were fitted with Gaussian band profiles as described previously (Dong et al., 1990, 1995). The fraction of a component in infrared second-derivative amide spectra was determined by computing the area of the component peak divided by the sum of areas of all the component peaks of the amide I band.

### 2.7. Ultraviolet-circular dichroism (UV-CD)

The insulin/lauryl sulfate (1:6 molar ratio) complex prepared as described previously was dissolved in PBS (pH 7.4) at the insulin-equivalent concentrations of 1 and 0.1 mg/mL, respectively, for near-UV CD and far UV-CD analysis. All CD measurements were conducted with an Aviv 62DS CD spectrophotometer (Aviv Associates, Lakewood, NJ) equipped with a temperature control device. The length for both near-UV CD and far-UV CD was 1 mm, and the sample temperature was maintained at 25 °C unless specified otherwise.

### 2.8. In vivo bioactivity

The in vivo bioactivity of insulin in the complex was evaluated in rats. The insulin/lauryl sulfate (1:6 molar ratio) complex was dissolved in PBS (pH 7.4) at the insulin-equivalent concentration of 1 mg/mL. Male and/or female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200–450 g were injected i.v. with (1) free insulin and (2) insulin complex at 2.2 IU/kg of an insulin-equivalent dose. The blood samples from the rats were taken at 0, 2, 15, 30, 60, 90, 120, 180,