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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,

and 240 min after dosing. The glucose concentration in the samples was measured using a Lifescan OneTouch® Meter (Johnson & Johnson, Fremont, CA). The animal studies were conducted in accordance with the principles outlined in the Guide for the Care and Use of Laboratory Animals, National Research Council, and had received approval by the Institutional Animal Care and Use Committee (IACUC) at the local authorities.

3. Results and discussion

3.1. Formation of an insulin/lauryl sulfate complex through HIP

Insulin is a polypeptide composed of 51 amino acidic residues having six basic groups and six acidic groups (Garrett and Grisham, 1995). Since the isoelectric point of insulin is 5.5, we used acidified water (pH 2.5) to prepare the HIP complex. At this pH, all six acidic groups in insulin were un-ionized while all six basic groups became protonated. Therefore, the positively charged insulin could be bound with the negatively charged sulfate groups of SDS. Upon slow addition of SDS to the insulin solution, the solution became cloudy and precipitation occurred, thereby leading to a sharp decrease in the insulin aqueous solubility (Fig. 1). It was also observed that addition of a stoichiometric amount of SDS to the insulin solution (6:1, SDS/insulin molar ratio) resulted in the lowest aqueous solubility of insulin and a maximum yield of the precipitate. The results indicate that the positively charged insulin can be stoichiometrically ion-paired with the negatively charged SDS, and thus precipitates out of the solution due to the low aqueous solubility of the complex formed. The result observed in the study is in good agreement with the findings reported previously that SDS formed complex precipitates with proteins such as lysozyme, conalbumin, insulin, and ovalbumin (Hegg, 1979; Matsuura et al., 1993; Yoo et al., 2001).

It was also interesting to notice that after reaching the stoichiometric ratio (6:1, SDS/insulin molar ratio), an addition of an excessive amount of SDS into the insulin solution decreased the turbidity of the solution. This solution eventually turned clear with high insulin solubility (Fig. 1). This phenomenon was also observed using other proteins or peptides with different complexing agents such as fatty acids, surfactants, and phospholipids (Choi and Park, 2000; Yoo and Park, 2004). After adding a stoichiometric amount of SDS, all insulin in the solution was bound with SDS, and the additional SDS in the solution then formed a micelle. An insulin/lauryl sulfate complex was likely resolubilized into the micelles. This hypothesis was further confirmed by conducting another experiment in which the insulin solution was slowly added dropwise into the SDS solution instead. In this experiment, neither clouding nor precipitation was observed at the beginning, indicating that the insulin/lauryl sulfate complex formed initially was solubilized in the SDS micelles. However, precipitation occurred suddenly once the amount of the insulin exceeded its stoichiometric ratio to SDS.

The formation of the insulin/lauryl sulfate complex was also investigated using an isothermal titration calorimeter at 30 °C. Upon addition of the SDS to the insulin solution in a sample cell, the thermal response was recorded by the calorimeter. The enthalpy versus the SDS/insulin molar ratio was calculated after correction with a control group. As shown in Fig. 2, the SDS bound to the insulin in a stoichiometric ratio, which was consistent with the result shown in Fig. 1. In addition to the binding molar ratio, the ITC study also provided a quantitative description of the complex formation with the values for the apparent binding constant (K), which is usually a constant for the measurement of binding strength, and enthalpy (ΔH). In this study, K was 8.25E5 ± 2.2E5, and ΔH was −2753 ± 25.7 cal/mole. It should be pointed out, however, that these were the values for the combined events of the binding of SDS to the insulin and the complex precipitation because the binding and precipitation occurred almost simultaneously.

It was reported that SDS bound lysozyme, conalbumin, insulin, and ovalbumin in a stoichiometric manner (Hegg, 1979; Matsuura et al., 1993; Yoo et al., 2001). The stoichiometric ratio reported in the previous literature, however, was indirectly calculated based on the unbound proteins in a supernatant after separation of the precipitates and the total protein added. Direct measurements of the composition of a protein/SDS complex were not revealed by those studies. In our study, in addition to the indirect measurement of binding ratio of SDS to the

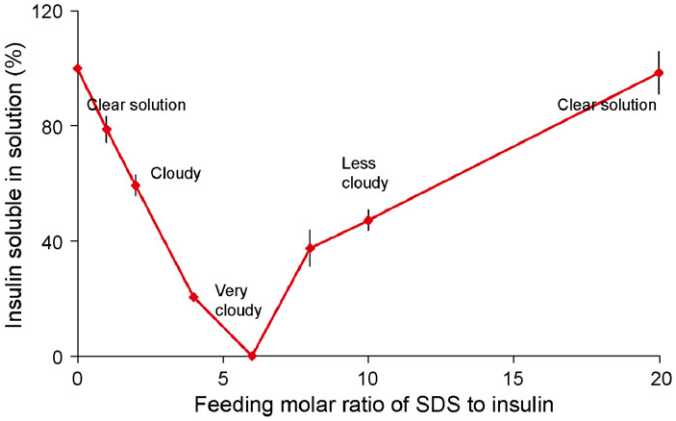


Fig. 1. Insulin aqueous solubility upon addition of SDS to insulin solution.

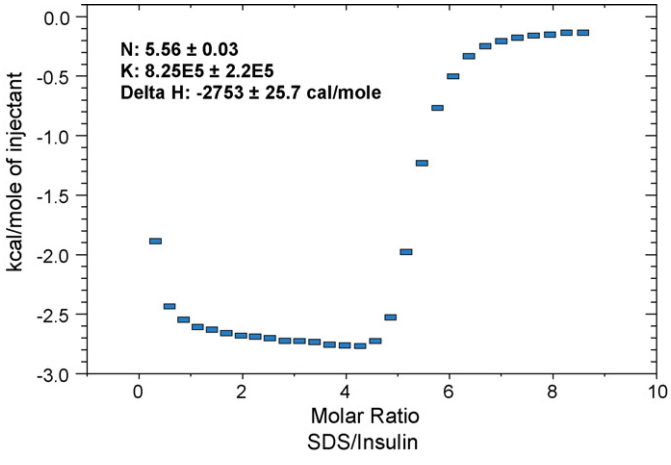


Fig. 2. Calorimetric titration of 6.88 mM SDS into 0.172 mM insulin at 30 °C. N: binding molar ratio; K: apparent binding constant; ΔH: enthalpy.

Table 1
Composition of insulin/lauryl sulfate complex by HPLC

Feeding molar ratio (SDS/insulin)	Measured insulin content (wt.%)	Measured lauryl sulfate content (wt.%)	Calculated molar ratio (lauryl sulfate/insulin)
4/1	74.0 ± 4.1	19.0 ± 1.1	5.2/1
6/1	69.4 ± 1.9	20.9 ± 0.5	6.1/1
10/1	67.8 ± 1.5	20.3 ± 0.3	6.0/1

insulin using the methods as reported and the ITC measurement result (Fig. 2), we also directly analyzed the composition of a lyophilized insulin/lauryl sulfate complex by an SEC-HPLC method (Table 1). The composition analysis of an insulin/lauryl sulfate demonstrated clearly that SDS bound to insulin in a stoichiometric ratio in the complex formation, which was again in good agreement with the results observed in Figs. 1 and 2.

In order to confirm the complex formation of the insulin through an ionic pairing, the precipitates obtained upon addition of SDS to the insulin solution were analyzed using FTIR. As depicted in Fig. 3, peaks of SO_2 stretch (asymmetric 1219 cm^{-1}, and symmetric 1083 cm^{-1}) in the insulin/SDS complex were shifted to the low field relative to the unbound SDS, which is attributed to the salt formation of sulfate groups. The FTIR results suggest that it is the sulfate group in SDS that binds to insulin in the complex formation. Due to the complexity of an FTIR spectrum of the basic groups of insulin, we were unable to identify the peak shift associated with these basic groups that interacted with the sulfate groups in SDS.

As shown in Fig. 1, we hypothesized that the insulin and SDS molecules formed a more lipophilic complex, and that the complex decreased the aqueous solubility, but could be resolubilized into the micelles due to the enhanced lipophilicity of the complexes. In order to confirm the lipophilicity of the complex, the solubility of insulin in 1-octanol and its partitioning coefficient in the 1-octanol/water system were measured (Fig. 4). The solubilities of insulin and the insulin complex in acidified water (pH 2.5) are >10 and 0.012 mg/mL, respectively. By complexing with SDS molecules, the insulin became very lipophilic. Insulin solubility in 1-octanol increased up to 5 from 0.02 mg/mL of the free insulin. The complex's partitioning into 1-octanol increased by five orders of magnitude compared to free insulin. The enhanced lipophilicity of the insulin was definitely due to the hydrophobic counterpart in the complex. This provided another clear indication for formation of the insulin/lauryl sulfate complex.

3.2. Structure integrity of the insulin in the complex

It is always a big concern whether or not the protein retains its native structure in the HIP complex. The amide I band of protein/peptides in FTIR spectra (between 1600 and 1700 cm^{-1}) has been widely used as an indication for the secondary structure (backbone) conformation of protein/peptides (Haris and Chapman, 1994). The amide I band of a lyophilized insulin/lauryl sulfate complex and free insulin was examined and compared. As indicated in Fig. 5, amide I bands of the insulin in the complex and free insulin were similar, suggesting that the insulin in the complex has a similar secondary structure conformation to the native one. As a control, the insulin solution, which was heated at 50 °C for 1 h, did show a change in the amide I band in the spectra.

In order to quantitatively compare the secondary structural compositions of insulin/lauryl sulfate complex with those of insulin, we carried out the second-derivative and curve-fitting procedures to resolve the overlapping components under the amide I band according to the methods described previously

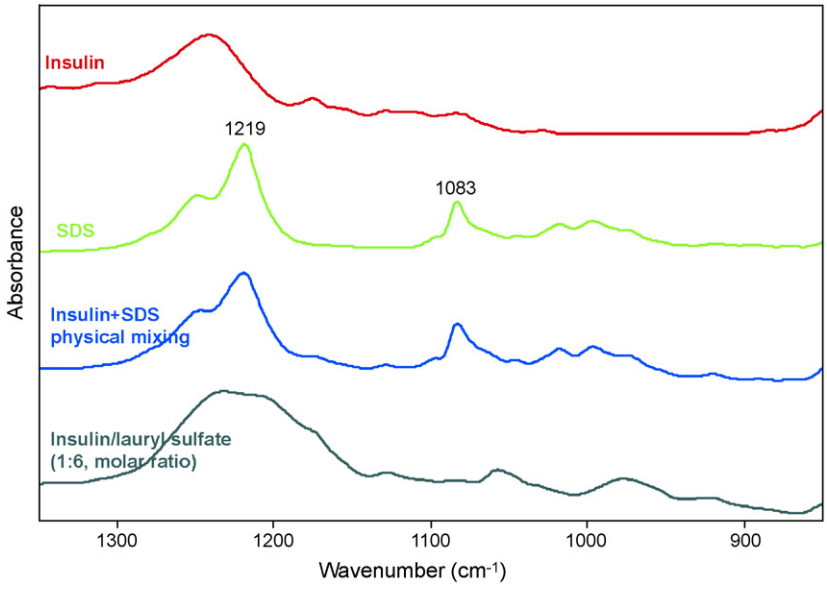


Fig. 3. FTIR spectra of insulin/lauryl sulfate complex and insulin.

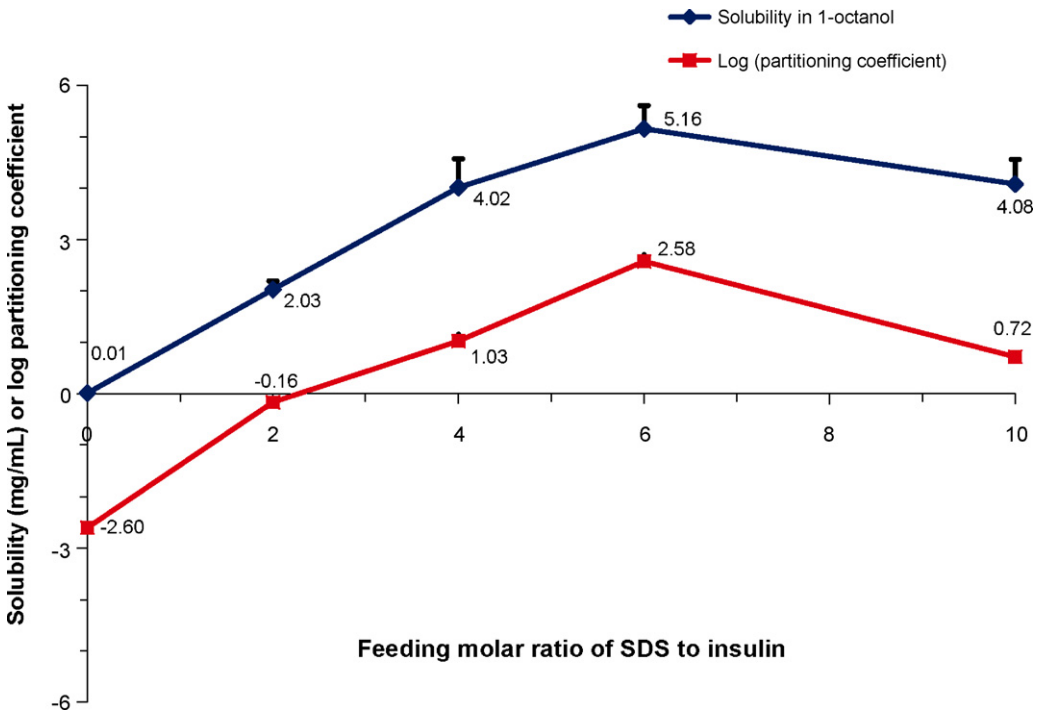


Fig. 4. Partitioning coefficients of insulin/lauryl sulfate complex and insulin in 1-octanol/water.

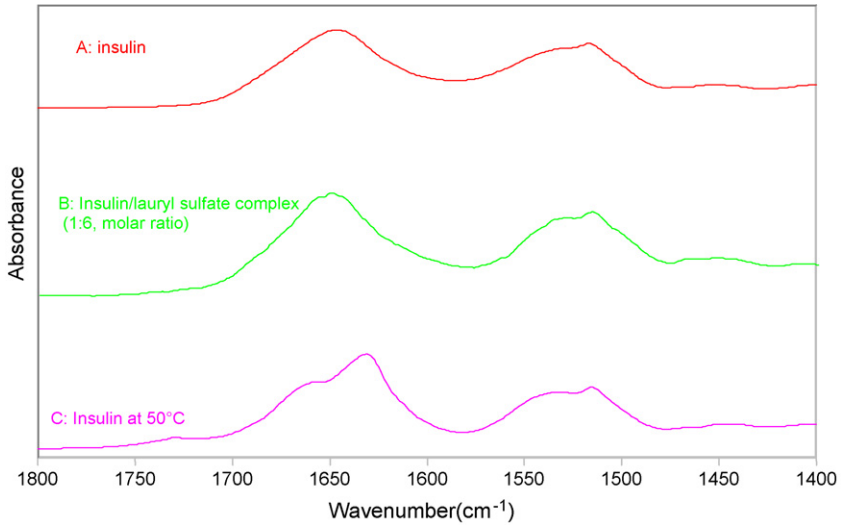


Fig. 5. FTIR spectra in the amide I region of insulin samples: (A) insulin at 25 °C; (B) insulin/sulfate complex at 25 °C; (C) insulin at 50 °C for 1 h.

Table 2
Comparison of the curve-fitted infrared second-derivative amide I spectra of insulin and insulin/lauryl sulfate complex

Insulin[a] (25 °C)		Insulin/lauryl sulfate complex[b] (25 °C)		Insulin[c] (50 °C for 1 h)		Assignment
Position (cm^{-1})	Area (%)	Position (cm^{-1})	Area (%)	Position (cm^{-1})	Area (%)	
1681.9	17.1	1678.5	18.7	1680.5	17.6	Beta-turn
1666.6	5.7	1662.3	3.5	1667.2	6.2	Beta-sheet
1647.5	51.3	1647.2	53.3	1647.7	41.6	Alpha-helix
1620.4	25.9	1620.0	24.5	1621.4	34.6	Low wavenumber beta-sheet

[a] Curve fitting $R^2 = 1.0000$.
[b] Curve fitting $R^2 = 1.0000$.
[c] Curve fitting $R^2 = 0.9998$.

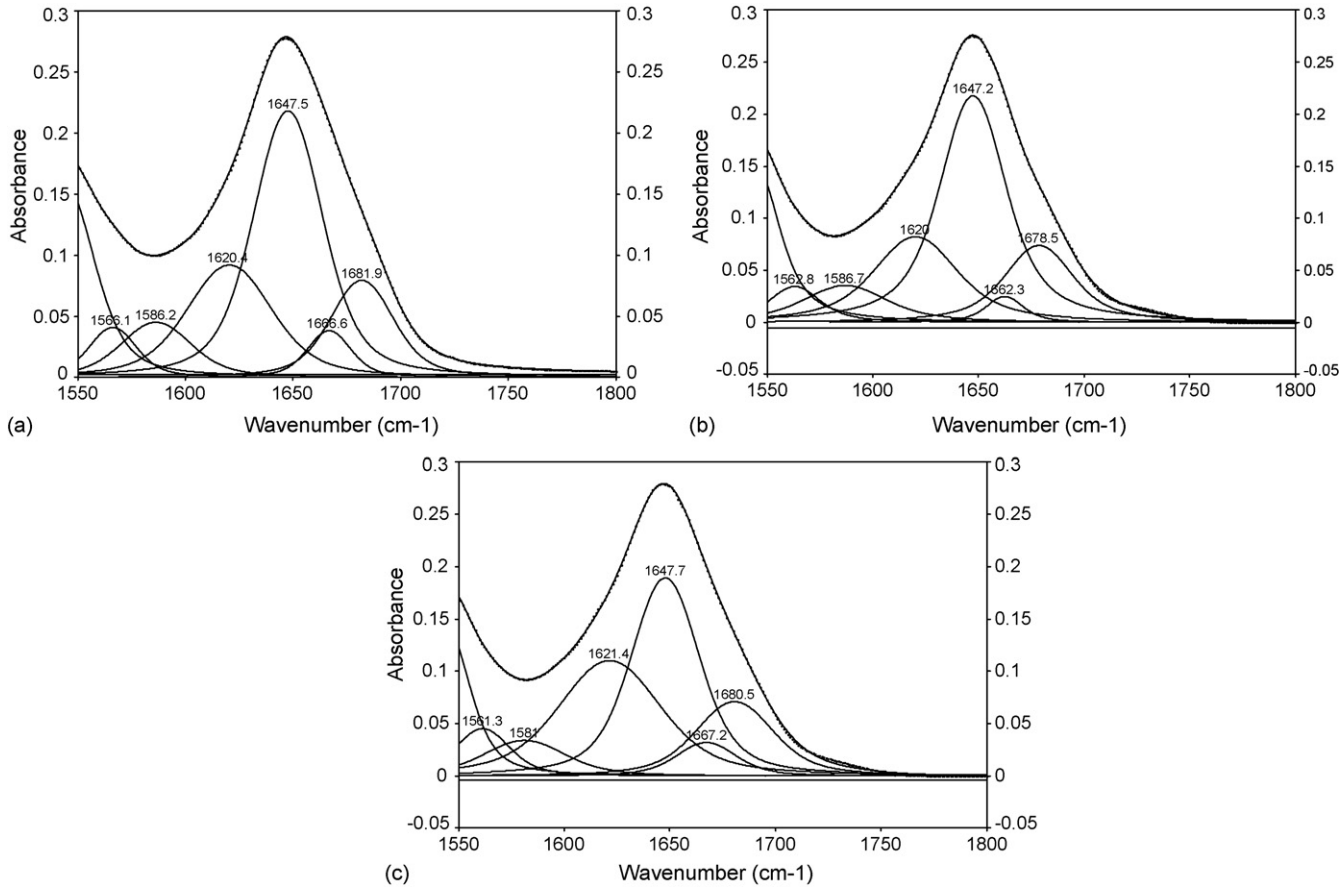


Fig. 6. Curve-fitted inverted second-derivative amide I spectra: (a) insulin at 25 °C; (b) insulin/sulfate complex at 25 °C; (c) insulin at 50 °C for 1 h. The curve-fitting procedure was carried out as described under Section 2.

(Dong et al., 1990, 1995). Fig. 6 shows the curve-fitted, inverted second-derivative spectra of insulin and the insulin/lauryl sulfate complex at 25 °C, and insulin that was heated at 50 °C for 1 h. The assignment of the FTIR peaks to the secondary structures of proteins has been studied extensively based on the spectra of a number of proteins and poly-amino acids with known secondary structures (Byler et al., 1986; Susi and Byler, 1986; Cabiaux et al., 1989; Dong et al., 1990, 1995, 2000). For the insulin samples tested here, band positions were assigned as follows: 1682 cm^{-1} to beta-turn, 1667 cm^{-1} to beta-sheet, 1648 cm^{-1} to alpha-helix, and 1620 cm^{-1} to low wavenumber beta-sheet. A quantitative comparison of the secondary structure components from the relative areas of the individual components of the deconvoluted amide I spectra is listed in Table 2.

As shown in Fig. 6, the second-derivative and deconvoluted spectrum of the insulin/lauryl sulfate complex is similar to that of free insulin. A quantitative analysis (Table 2) revealed that the complex contained 53.3% of alpha-helix, 24.5% of low wavenumber beta-sheet, and 18.7% of beta-turn; which is comparable to 53.3% of alpha-helix, 25.9% of low wavenumber beta-sheet, and 17.1% of beta-turn in free insulin. The alpha-helix content in the insulin/lauryl sulfate complex is consistent with the values determined by other FTIR studies (40–53%) (Wei et al., 1991; Vecchio et al., 1996; Pikal and Rigsbee, 1997), CD analysis (∼57%) (Melberg and Johnson, 1990; Van Stokkum et al., 1990), and X-ray crystallographic measurement (53%) (Baker et al., 1988). In addition, the contents of beta sheet and unordered structure observed in the insulin/lauryl sulfate complex are in agreement with those reported previously in the insulin studies (Wei et al., 1991; Vecchio et al., 1996).

The results of second-derivative and curve-fitting FTIR spectra suggested that the insulin/lauryl sulfate complex retained similar secondary structural compositions to the free insulin. As a negative control, we heated a solution of free insulin at 50 °C for 1 h. As we expected, the area of the alpha-helix band for

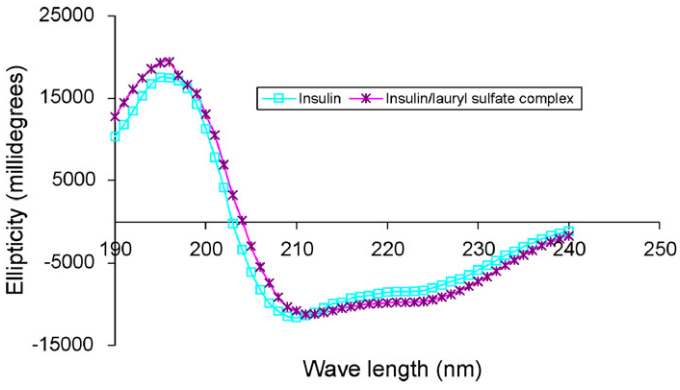


Fig. 7. Far-UV CD spectra of insulin/lauryl sulfate complex and insulin.

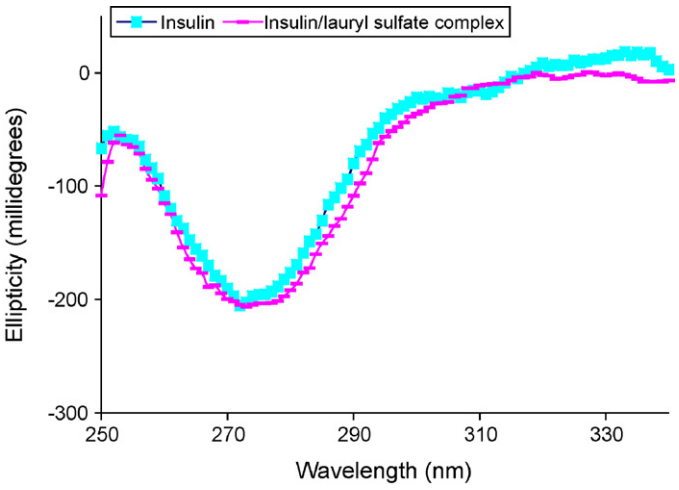


Fig. 8. Near-UV CD spectra of insulin/lauryl sulfate complex and insulin.

the heated insulin decreased dramatically from 51.3% to 41.6% (Fig. 6), indicating a thermally induced conformational change (Pikal and Rigsbee, 1997; Dong et al., 2000).

To further assess the secondary structure conformation of the insulin in the complex, a far-UV CD spectrum of the complex in an aqueous medium was measured and compared with that of the free insulin solution (Fig. 7). Native insulin has two negative maximal bands at 222 and 209 nm in the far-UV region. The 222 nm band is assigned in large part to the beta structure, while the 209 nm band is proposed to be mainly associated with the alpha-helical structure. In the aqueous medium in the studies, the insulin/lauryl sulfate complex showed the far-UV CD spectrum to be similar to that of free insulin, indicating that the insulin secondary structure conformation was not perturbed in the complex. This finding is consistent with our FTIR result (Figs. 5 and 6) and studies of the protein HIP complex in the organic solvents previously reported (Matsuura et al., 1993; Meyer et al., 1995, 1996).

Since protein tertiary structure is vital to protein bioactivity, the near-UV CD spectrum of the insulin/lauryl sulfate complex, which reflects any conformational changes in the tertiary or quaternary structures of the insulin, was measured. Native insulin in the aqueous solution exhibits a comparatively large negative extremum at 274 nm (Ettinger and Timasheff, 1971). This negative extremum is assigned to the contribution of the aromatic residues in the insulin. Any attenuation of this band, therefore, may be correlated with aggregation and denaturation.

As depicted in Fig. 8, negligible changes in both a near-UV CD spectrum and band intensity at 274 nm were found between the insulin complex and native insulin. This suggests that the insulin in the complex retained its native-like conformational tertiary structures, and that no appreciable amount of aggregates were found in the insulin complex compared with native insulin.

All UV-CD studies were conducted in PBS (pH 7.4). Insulin could exist in either free ionized form or a soluble HIP complex (Lengsfeld et al., 2002). We are unable to quantitate the ratio of free ionized insulin to the soluble insulin HIP complex. Therefore, UV-CD studies measured the conformational structures of both ionized insulin and the soluble insulin HIP complex in PBS.

To further confirm this, SEC-HPLC was also used to detect the existence of any possible aggregates. As shown in Fig. 9, no significant amount of aggregates (<0.4%, w/w) was observed in the insulin/SDS complex. These data indicate that the insulin

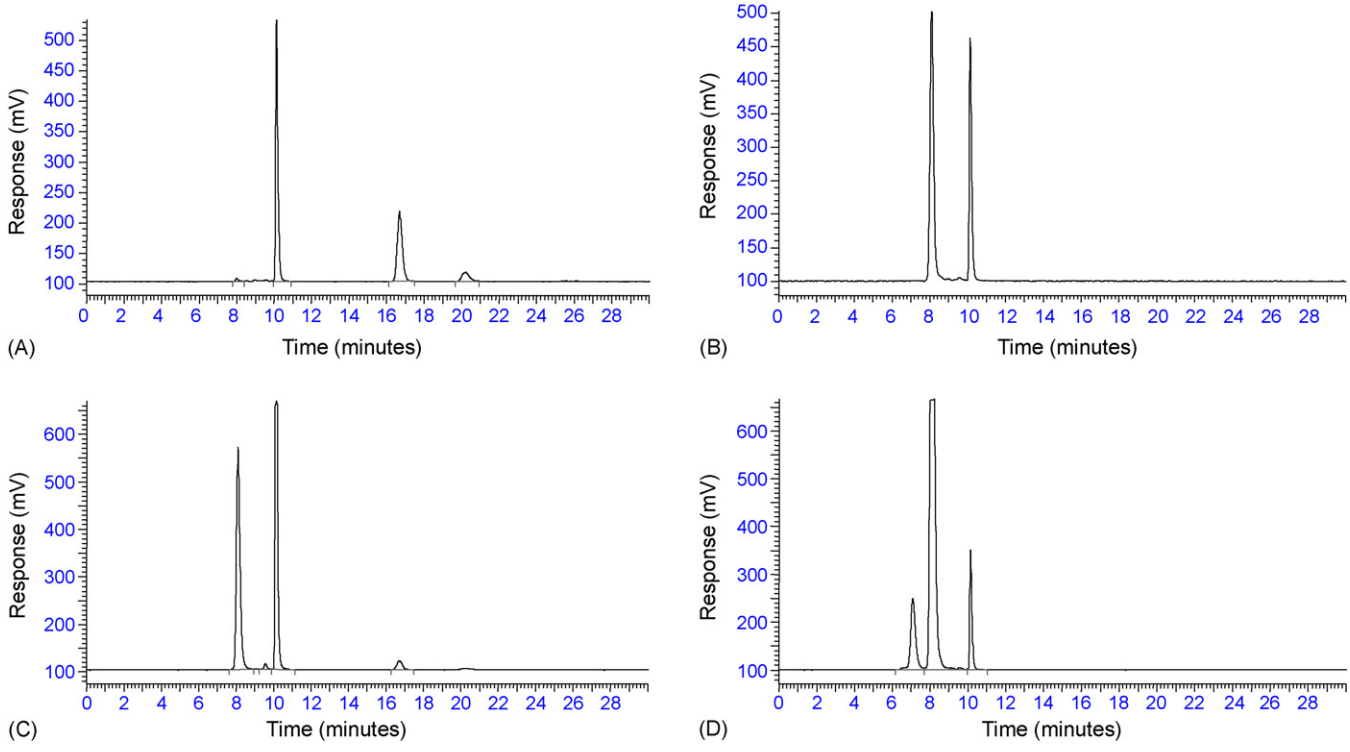
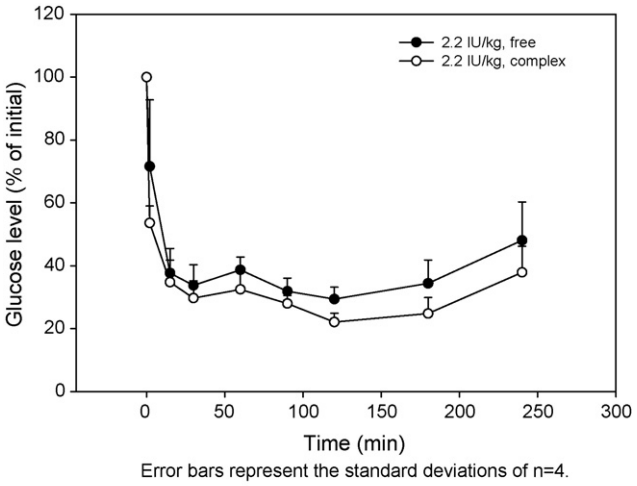


Fig. 9. SEC-HPLC chromatograms: (A) SDS; (B) insulin at 25 °C; (C) insulin/lauryl sulfate complex at 25 °C; (D) insulin at 50 °C for 1 h.



	Pharmacodynamic Activity (%)	CV%
Free	60.4 (4.7)	7.8
Complex	65.3 (3.2)	4.9

P-value is 0.1358.

Fig. 10. Plasma glucose concentration after i.v. injection of the insulin/lauryl sulfate complex and free insulin.

in the complex retained not only the secondary structure conformation (Figs. 5 and 6), but also the tertiary structure of the protein.

3.3. In vivo bioactivity of the complex

All characterizations of the insulin complex structures so far indicated that the insulin in the complex retained its native secondary and tertiary structure. In addition, we wanted to evaluate directly its bioactivity in vivo and compare the bioactivity with that for native insulin. A pharmacodynamic study using the complex was conducted in rats. Fig. 10 shows the plasma glucose concentration profile for both free insulin and the complex. The pharmacodynamic activities of the complex and free insulin were calculated according to the following expression:

$$100 - \frac{\mathrm{AUC}_{0-t}}{t}$$

where AUC_{0-t} is the area under curve of the glucose level (percent of initial level) versus time, and t is the last time point at which all the rats were still alive.

The bioactivities (percentage of AUC decrease) of free insulin and the complex were 60.4% and 65.3%, respectively, but no statistical significance was found ($p > 0.1$). In this study, 2.2 IU/kg of insulin or insulin-equivalent dose (in the complex) was used. This insulin dose is within the appropriate dose range (1–5 IU/kg) for a noticeable glucose response (Kim et al., 1999; Chung et al., 2002; Varshosaz et al., 2004, 2006). Therefore, any changes in the AUC of the glucose level versus time in the pharmacodynamic study are indications of changes of bioactivities of insulin. This in vivo result clearly indicates that the insulin in the complex also retained its bioactivity in vivo, and was consistent with our in vitro characterization that the insulin retained its native-like structure integrity in the complex.

The in vitro enzyme activity of the HIP protein complex has been reported, mainly in organic solvents (Paradkar and Dordick, 1994a; Meyer et al., 1995; Sergeeva et al., 1997). The bioactivity level of the protein complex in organic solvents is two to three orders of magnitude greater than that of the corresponding free proteins suspended in the organic solvents, but still much less than that for the free proteins in aqueous solution. In addition, those studies are mainly concerned with the in vitro enzymatic activity of the complex in the organic solvent, not the direct evaluation of its in vivo bioactivity. Our in vivo study has clearly demonstrated that the HIP insulin/lauryl sulfate complex is as bioactive as the free insulin.

4. Conclusions

Insulin complexed with sodium lauryl sulfate at an approximately stoichiometric ratio, and it significantly ($p < 0.001$) increased its solubility in 1-octanol and partitioning into 1-octanol in an octanol/water system. In addition, insulin in the complex retained its native secondary and tertiary structures, and also showed in vivo bioactivity comparable to free insulin in the rats.

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