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Research Paper

Displacement of Adsorbed Insulin by Tween 80 Monitored Using Total Internal Reflection Fluorescence and Ellipsometry

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Purpose. This study was conducted to investigate the mechanism of action in the displacement of adsorbed insulin from a hydrophobic surface by Tween 80 and of the competitive adsorption of the two species.
Methods. Total internal reflection fluorescence (TIRF) and ellipsometry were used as *in situ* methods to examine the processes taking place at hydrophobic model surfaces in the presence of insulin and Tween 80.
Results. TIRF studies showed that the displacement of insulin by Tween 80 could be fitted to a sigmoidal function, indicating a nucleation-dependent process. Furthermore, a linear dependence between the apparent rate constant and the logarithm of the Tween 80 concentration was found. Competitive adsorption from solution mixtures of insulin and Tween 80 indicated that insulin was adsorbed first, but subsequently displaced by the surfactant. This displacement proved also to be dependent on the concentration of Tween 80 in the mixture.
Conclusions. The results indicate that Tween 80 at concentrations above critical micelle concentration can be used to protect insulin against surface adsorption. The presence of a lag phase in the displacement at low surfactant concentration indicates that the mechanism of action for Tween 80 to reduce adsorption of insulin may be by competing for sites at the surface.

KEY WORDS: FITC; insulin; protein adsorption; protein displacement; TIRF; Tween.

INTRODUCTION

Adsorption of proteins to interfaces is of major concern in the development of protein pharmaceuticals. Proteins are exposed to interfaces during purification, processing, and during storage and delivery of the therapeutic protein. Particularly in advanced drug delivery systems, the risk of surface-induced denaturation should not be ignored (1,2).

The stabilization of protein formulations by surfactants has been widely investigated (3–8). Among the surfactant types, the nonionic class has proven to be the most promising as they interact weakly with proteins and are effective in low concentrations. However, most of the studies carried out in the past were based on simple shaking or contact experiments, which do not allow any conclusions as regards the mechanisms of action of the applied surfactants (3–5). More detailed studies reported in recent years suggest that there may not be a single mechanism of action for a distinct group of surfactants as, for example, nonionic surfactants of the Tween type (6–8). Instead, this may be dependent on the protein applied. The following mechanisms of action for the stabilizing effect of nonionic surfactants on surface-induced protein denaturation have been suggested:

1. Competition of surfactant and protein at the interface. With the surfactant having a greater binding affinity for the interface, a reduction in the number of available binding sites for the protein is observed (6,7). It is generally thought that surfactant monomers are the competing species. Thus, an effective concentration close to the CMC value of the surfactant, where the concentration of monomers in the bulk solution is high, is expected (9).

2. Interaction of surfactant with the protein surface. The interaction is caused either by the surfactant acting to cover hydrophobic sites where aggregation and surface adsorption may potentially occur, or by acting as an artificial chaperonin that catalyzes refolding of the protein (8). Other possible mechanisms may be that the surfactant binds to the native state of the protein to a greater degree than the denatured state, which results in a decrease in the free energy of the native state relative to the denatured state (6), or that the surfactant

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ABBREVIATIONS: AFM, atomic force microscopy; BCA, bicinchoninic Acid; CD, circular dichroism; CMC, critical micelle concentration; FITC, fluorescein isothiocyanate; MALDI-TOF, matrix assisted laser desorption ionization-time of flight; TIRF, total internal reflection fluorescence.

destabilizes the partially unfolded protein. When protein–surfactant interactions are involved, the concentration dependence of the surfactant effect correlates with the molar ratio of surfactant to protein (9).

3. Preferential exclusion mechanism. A mechanism originally suggested by Timasheff (10) for the stabilizing effect of sugars on proteins in solution. As surfactants are generally operative at very low concentrations, volume exclusion effects can generally be neglected (9).

Displacement of already established protein layers may be related to the interactions proceeding in bulk solutions of protein/surfactant mixtures. The so-called orogenic displacement model was shown to be generally applicable to the displacement of protein layers by nonionic surfactants at various interfaces (11–13). The proposed model involves surfactant adsorption at localized defects in the protein film, due to packing limitations of the protein. This generates nucleation and subsequent growth of surfactant domains, which compresses the protein layer, leading to gradual and complex displacement of protein from the surface.

Whereas displacement of proteins from interfaces by surfactants has been widely investigated in food science, only a few studies have been carried out using pharmaceutically relevant proteins and nonionic surfactants directly at the interface. The lack of available surface-sensitive techniques allowing the study of interfacial phenomena *in situ* and in real-time could be one of the explanations for this.

The aim of the present study is to elucidate the mechanism of stabilization of insulin against surface adsorption by a nonionic surfactant of the Tween type. This is done by investigating the competition of human insulin and Tween 80 for a hydrophobic interface as well as the displacement of an already established protein layer by the same surfactant.

Ellipsometry and total internal reflection fluorescence (TIRF) are probed as analytical tools for this investigation. The use of surface-sensitive techniques on protein–surfactant systems may provide further insight into the interactions taking place at the interface during stabilization of proteins in solution against surface-induced denaturation.

MATERIALS AND METHODS

Materials

Freeze-dried bulk preparations of recombinant human insulin containing 2 Zn^{2+} per hexamer were kindly donated by Novo Nordisk (Bagsvaerd, Denmark) and used as received.

Fluorescein isothiocyanate (FITC, isomer I) was obtained from Molecular Probes (Leiden, The Netherlands). Tween 80, Ph. Eur. (polyoxyethylene sorbitan monooleate) was purchased from Merck-Schuchardt (Hohenbrunn, Germany). Chlorodimethyl(3,3,3-trifluoropropyl)silane (Lancaster), used for silanization of surfaces, was obtained from Chemtronica (Stockholm, Sweden). All other chemicals were of analytical grade. Quartz slides for TIRF were obtained from BioElectrospec (Harrisburg, PA, USA) and silica wafers with a thermally grown oxide layer of approximately 300 Å were kindly provided by Stefan Welin-Klintström, Linköping University.

Preparation of Human Insulin Solution

Stock solutions of insulin were prepared by dissolution of insulin at low pH by addition of 0.1 N HCl, whereupon phosphate buffer was added and the pH adjusted to 7.4 with 0.1 N HCl or NaOH. The solutions were stored at 4°C and used within a few days. All experiments were performed at room temperature at pH 7.4 using 10 mM phosphate buffer. For circular dichroism studies at pH 1.6, insulin solutions were prepared by dissolving human insulin directly in a solution of 25 mM HCl and 100 mM NaCl.

Preparation of Monolabeled FITC-Insulin

Human insulin was monolabeled with fluorescein isothiocyanate (FITC) on the N-terminal amino acid on the B chain (B1) following the procedure described by Hentz *et al.* (14) by reacting insulin and FITC in a molar ratio of 1:1. The labeling site was probed by *Staphylococcus aureus* V.8 protease digestion of the labeled insulin followed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) analysis. The results confirmed that B1 was the labeling site. The concentration of FITC-insulin was determined on a UV spectrophotometer using BCA protein assay. The prepared FITC-insulin was stored at −20°C until use. MALDI-TOF analysis indicated that the labeling ratio did not change significantly upon storage.

Labeling of proteins involves the risk of altering the affinity of interfaces. However, the FITC-labeled insulin was shown to have similar adsorption behavior as the native human insulin (Mollmann *et al.*, unpublished data). A mixture of FITC-insulin and nonlabeled human insulin in a molar ratio of 1:4 and total concentration of 10^{-1} mg/mL was used for the TIRF experiments to eliminate the risk of self-quenching of the highly fluorescent FITC-insulin, when located at the surface.

Preparation of Hydrophobic Surfaces

Hydrophobic surfaces were prepared by silanization. Silane-modified quartz slides were used for TIRF, whereas silica wafers were used in ellipsometry measurements. Modified surfaces are widely used in both techniques applied. This allows measurements on surfaces with comparable characteristics in regard to hydrophobicity and surface potential as the pharmaceutically relevant material of interest (15). Quartz slides and silica wafers were cleaned using the procedure described by Landgren and Jönsson (16) in a mixture of 25% NH_3, 30% H_2O_2, and H_2O (1:1:5) at 80°C for 5 min, followed by cleaning in a mixture of 30% HCl, 30% H_2O_2, and H_2O (1:1:5) at 80°C for 5 min. The surfaces were subsequently rinsed three times in H_2O and ethanol, and finally stored in ethanol. This cleaning procedure leaves the quartz and silica surfaces essentially hydrophilic. Prior to treatment with chlorodimethyl(3,3,3-trifluoropropyl)silane, the surfaces were dried using a steam of nitrogen gas and placed on the bottom plate in a desiccator. A small volume (approximately 0.5 mL) of chlorodimethyl(3,3,3-trifluoropropyl)silane was added to a beaker at the bottom of the desiccator. The silane was allowed to evaporate and deposit on the wafers overnight in a nitrogen atmosphere. Vapor

deposition minimizes the risk of polymerization and aggregation of the silane at the surface (17). After treatment, the surfaces were rinsed and stored in ethanol. Prior to use, the surfaces were rinsed in ethanol and dried using a steam of nitrogen gas. Silica surfaces prepared in the same manner have previously been characterized and proved to be homogenous and highly hydrophobic with a contact angle of 85–90° (18).

Circular Dichroism

Circular dichroism (CD) spectra were obtained at ambient temperature on a Jasco J-810 Circular Dichroism spectrophotometer. In the far-UV region, spectra were obtained using a human insulin concentration of 0.5 mg/mL, and a 0.1-mm cell was applied. A concentration of 1.5 mg/mL and a 2-mm cell were used for near-UV spectra. Three consecutive scans were averaged for each sample. The spectra were recorded using a scanning speed of 20 nm/min and a bandwidth of 1 nm. CD spectra of the appropriate reference solutions were collected and subtracted from the protein spectra, whereupon data were converted to molar ellipticity.

Total Internal Reflection Fluorescence

The principles of total internal reflection fluorescence (TIRF) have been described in detail elsewhere (19–21). TIRF allows the detection of emitted light from fluorophores localized at or close to a surface. The fluorophores are excited by an exponentially decaying evanescent wave generated at the interface due to total internal reflection of an incident light beam. The instrumental setup in this study was a custom-built flow cell manufactured by BioElectrospec mounted into a Spex-Fluorolog-3 fluorescence spectrometer. The flow cell consisted of a 73° dove tail prism coupled to the hydrophobic quartz slide using glycerol as refractive index matching liquid. A Teflon gasket separated the slide from the back support, creating a flow cell having dimensions of 16 mm × 24 mm × 70 μm and securing laminar flow. Slits of 5 nm were used, and excitation and emission wavelengths for FITC-insulin were 490 and 520 nm, respectively. The penetration depth of the evanescent wave is a function of the angle of incidence θ, the wavelength λ, and refractive indices of the solid and liquid materials. The setup used in the present experiments results in a penetration depth of approximately 137 nm and thus only proteins with a distance no larger than the depth of penetration to the surface are excited. Contributors to the fluorescence detected can be divided into three categories: fluorescence from the adsorbed species, bulk fluorescence, and scatter (19). As the electrical field of the evanescent wave decays exponentially into the bulk solution, proteins adsorbed to the surface are detected with much higher sensitivity than proteins in the bulk solution. It should be noted that bulk contributions can generally be minimized by using low concentrations of the fluorescent species.

Experimental Procedure

Experiments were carried out at ambient temperature by passing phosphate buffer (10 mM, pH 7.4) through the flow cell until a stable baseline was established. A flow rate of 1 mL/min was used throughout the experiments. All adsorption experiments were carried out at least twice. The fluorescence signal varied less than 10% on individual measurements. This is in accordance with the reproducibility rate found by other authors using the same experimental setup (22).

Displacement Experiments

The FITC-insulin/human insulin mixture was introduced into the TIRF flow cell and adsorption was allowed to take place for 5 min monitored with a data interval of 30 s, or for 30 min with a data interval of 100 s. After the initial adsorption, a solution of Tween 80 of the given concentration was added and desorption of FITC-insulin was monitored. The fluorescence signal during this sequence was followed with a data interval of 30 or 100 s for the first short period of time, whereupon the data interval was increased to 500 s to minimize the risk of photo bleaching. The flow was kept constant at 1 mL/min throughout the experiment unless otherwise indicated.

Competition Experiments

The FITC-insulin/human insulin mixture was mixed with Tween 80 solution of the given concentration and allowed to equilibrate for 1 h. The mixture was introduced into the TIRF flow system and adsorption was monitored for 30 min with a flow of 1 mL/min. Data collection was set to an interval of 30 s for the first 5 min, and subsequently the interval was decreased to 500 s to minimize the risk of photobleaching. After monitoring adsorption for 30 min, phosphate buffer was introduced and the rinsing phase was monitored for 30 min.

Ellipsometry

Ellipsometry measures changes in the polarization state of light upon reflection from a flat surface (23). Adsorption of proteins on the surface will influence the optical properties and hence the polarization of the reflected light will be different for a surface with adsorbed protein when compared to the bare surface. Changes in optical properties are reflected in the ellipsometric angles Ψ and Δ, which are determined with a time resolution of a few seconds. All measurements were performed on a Rudolph thin film ellipsometer (type 43603-200E; Rudolph Research, Flanders, NJ, USA). A xenon lamp, with a 4,015-Å wavelength was used as the light source. A detailed description of the experimental setup for null-ellipsometry is given by Landgren and Jönsson (16). Prior to each adsorption experiment, the optical properties of the bare surface were determined in air and buffer by four-zone null ellipsometry (24). The risk of entrapment of air at the interface during measurement was minimized by filling the cuvette with ethanol and then replacing ethanol with phosphate buffer from the bottom of the cuvette. After establishing a baseline for the substrate, the sample was added from a stock solution, giving a final volume of 5 mL in the cuvette and diluting the sample to the concentration preferred. The solution was thermostated to

25°C and stirred at approximately 50 rpm with a magnetic stirrer. Polarization angles of the polarizer and analyzer were continuously monitored in one zone during the entire experiment. Adsorption from individual human insulin and Tween 80 solutions as well as from mixtures of human insulin and Tween 80 were allowed to take place for 60 min, whereupon the cuvette was flushed for 5 min with phosphate buffer using a flow rate of 12 mL/min. The polarizer and analyzer angles were monitored for 30 min during desorption of the adsorbed layer. The thickness (δ) and refractive index (n) of the adsorbed layer were calculated from the experimental data by the McCrackin algorithm (25). These data were used to obtain the adsorbed amount according to the method described by Cuypers *et al.* (26). The ratio of the molar weight to molar refractivity of human insulin was set to the previously determined value of 4.1 g/mL (27). The value used for the partial specific volume was 0.75 mL/g (28). The adsorbed amount can be determined much more accurately than δ and n individually (26), and thus, this value is presented here. The thickness of an adsorbed layer of Tween 20 monomers was reported to be barely detectable by ellipsometry (29). Thus, adsorbed layers of protein/surfactant mixtures were treated in the same manner as adsorbed layers of the protein alone, similar to previous displacement studies involving other proteins and surfactants (30).

Data Evaluation of Kinetics of Displacement

Displacement of insulin by Tween 80 after initial desorption of loosely attached insulin can be described by a sigmoidal curve defined by a lag phase, where no or only minor displacement occurs, a subsequent displacement phase and a final equilibrium phase. Sigmoidal curves are commonly used to describe nucleation-dependent processes (31). Fluorescence intensity measured in TIRF experiments were plotted against time and fitted in SigmaPlot® by a sigmoidal curve to derive the rate constant for displacement. The sigmoidal curve can be described by the following equation:

$$Y = \frac{a}{1 + e^{-[(x-x_0)/\tau]}} \quad (1)$$

where Y is the fluorescence intensity, a is the maximum fluorescence intensity during the lag phase, x is the time, and x_0 is the time to 50% of maximum fluorescence intensity. The apparent rate constant for displacement is given by $1/\tau$ and the lag time is described by $x_0 - 2\tau$.

RESULTS

Displacement Experiments—Rinsing with Phosphate Buffer

A representative adsorption curve for FITC-insulin is shown in Fig. 1a. FITC-insulin adsorbs rapidly to the hydrophobic surface and reaches a steady level. After 5 min of adsorption, phosphate buffer is introduced into the cell. After a rapid decrease in fluorescence intensity, the fluorescence reaches a plateau of approximately 80% of the initial fluorescence. FITC-insulin did not adsorb to unmodified quartz

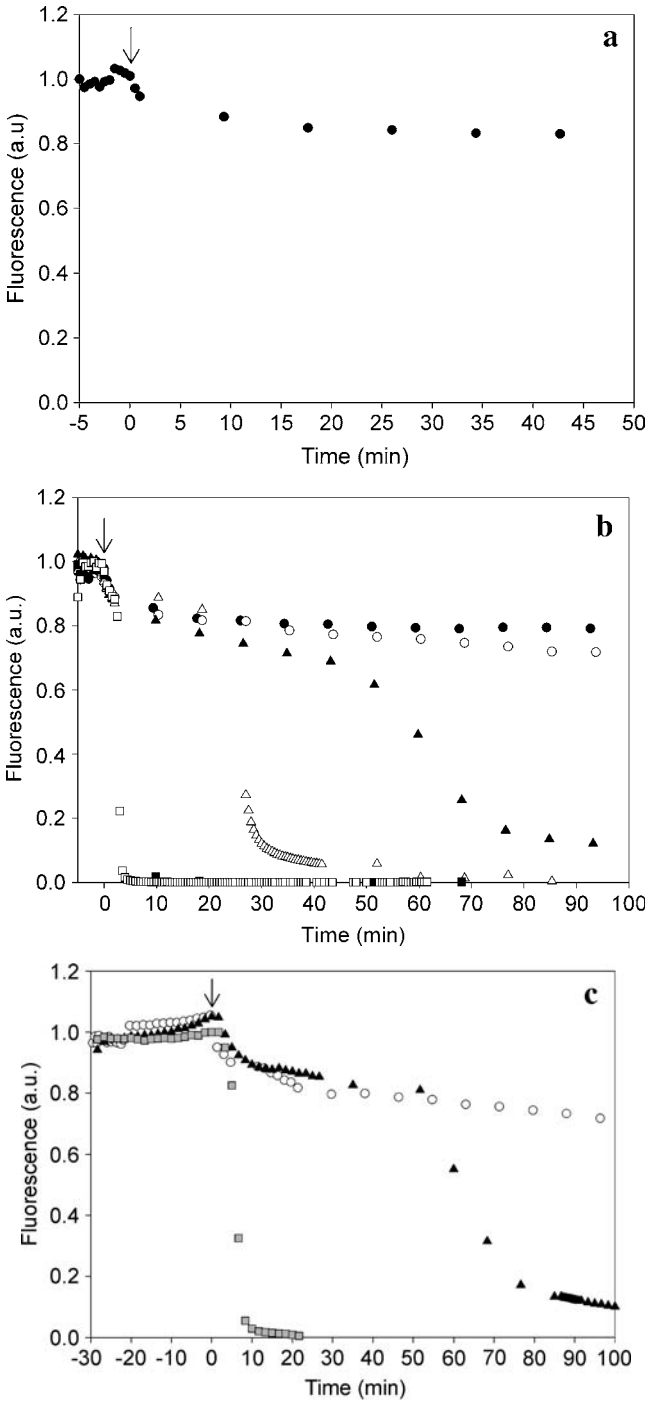

Fig. 1. (a) Typical TIRF experiment. At $t = -5$, a mixture of FITC-labeled and unlabeled human insulin is injected into the flow. The arrow indicates the onset of rinsing with phosphate buffer. (b) Displacement of FITC-insulin by Tween 80. At $t = -5$, a mixture of FITC-labeled and unlabeled human insulin is injected into the flow. At $t = 0$, phosphate buffer or a Tween 80 solution is introduced into the flow cell. Filled circles: 10 mM phosphate buffer. Open circles: 10^{-4} mg/mL Tween 80. Filled triangles: 2×10^{-4} mg/mL Tween 80. Open triangles: 10^{-3} mg/mL Tween 80. Filled squares: 10^{-2} mg/mL Tween 80. Open squares: 10^{-1} mg/mL. (c) Investigation of the effect of adsorption time on displacement. At $t = -30$, a mixture of FITC-labeled and unlabeled human insulin is injected into the flow. At $t = 0$, the protein is displaced by 10^{-4} mg/mL Tween 80 (open circles), 10^{-3} mg/mL (black triangles), or 10^{-2} mg/mL (gray squares).

surfaces, as only a slight increase of approximately 3% of the initial fluorescence signal at hydrophobic surfaces was observed when FITC-insulin was introduced to the flow cell (data not shown).

Displacement Experiments—Rinsing with Tween 80

The removal of FITC-insulin by rinsing with Tween 80 at various concentrations is displayed in Fig. 1b. When 10^{-4} mg/mL Tween 80 used in the rinsing step, fluorescence drops to a level corresponding to the removal of FITC-insulin by phosphate buffer, whereupon a slow and continuous decrease in fluorescence is seen. After rinsing for 90 min, the fluorescence level was not significantly different from the fluorescence of surface adsorbed species after rinsing with phosphate buffer.

At 10^{-3} and 2×10^{-4} mg/mL Tween 80 a different pattern for displacement takes place. A rapid decrease in fluorescence intensity to approximately 80% of the initial intensity is observed, followed by a plateau phase, where fluorescence decreases only slightly. The length of this phase is different at the two concentrations applied and is approximately 60 and 25 min for 2×10^{-4} and 10^{-3} mg/mL, respectively. After the plateau region, the adsorption curves decreases rapidly to approximately 10–20% of the initial fluorescence intensity. Subsequently, a slow decrease approaching baseline levels occurs. The time, when the rapid decline in fluorescence is observed, is quite reproducible as the onset varied less than 5 min on three independent measurements with Tween concentrations of 2×10^{-4} mg/mL.

At concentrations of 10^{-2} mg/mL Tween 80 or higher, a rapid decrease to baseline fluorescence level is observed, indicating complete displacement of FITC-insulin.

Reasonable fits were obtained when the displacement of insulin was fitted to a sigmoidal curve as described by Eq. (1) (Fig. 2a) at concentrations $< 10^{-1}$ mg/mL. The rate constant for displacement of insulin exhibits a linear dependence on the logarithm of the Tween 80 concentration (Fig. 2b) and furthermore, there is an inverse linear relationship between the logarithm of the lag time and the logarithm of the Tween 80 concentration (Fig. 2c).

As proteins may optimize their interaction with the surface over time, the displacement of FITC-insulin by Tween 80 after 30 min of adsorption was also examined (Fig. 1c). During the adsorption phase, the fluorescence intensity of the adsorbed layer increases slightly with time. In general, however, the displacement by Tween 80 proceeds after the same pattern as when FITC-insulin is adsorbed for 5 min.

It was tested whether the characteristic displacement by 10^{-3} and 2×10^{-4} mg/mL Tween 80 could also be induced by the flow of solvent itself. To this purpose, the Tween 80 solution (10^{-3} mg/mL) was exchanged with phosphate buffer after approximately 10 min desorption/displacement before the rapid decline in fluorescence was observed. This procedure did not cause a decrease in fluorescence, and thus, the removal of adsorbed FITC-insulin is not flow-induced.

The effect of the rate of transport was examined using various flow rates. By increasing the solution flow rate, it was examined whether the lag period was dependent on the rate of transport to the surface. The displacement of FITC-insulin with 10^{-3} mg/mL Tween 80 at a flow rate of 0.25–2 mL/min is shown in Fig. 3a, where a marked reduction in the length of

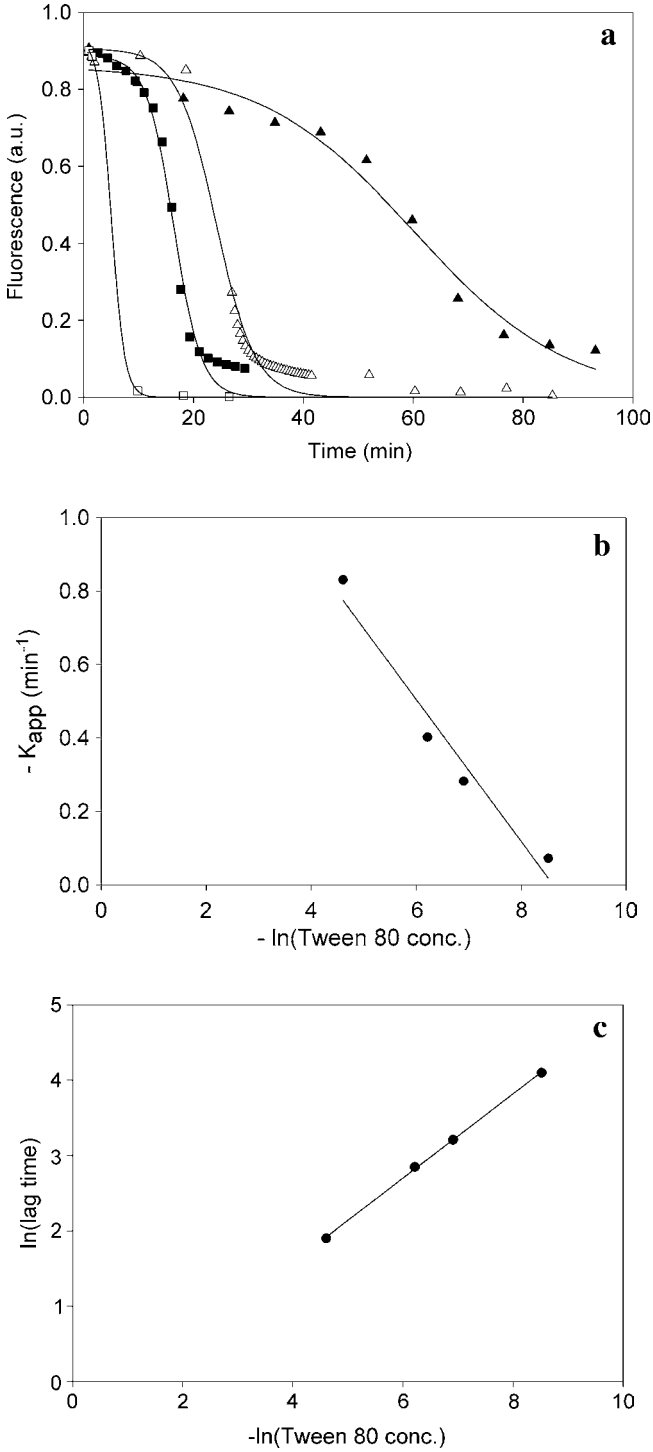

Fig. 2. (a) Fitting of displacement curves to a sigmoidal function. Filled triangles: 2×10^{-4} mg/mL Tween 80. Open triangles: 10^{-3} mg/mL Tween 80. Filled squares: 2×10^{-3} mg/mL Tween 80. Open squares: 10^{-2} mg/mL. (b) Relation between the concentration of Tween 80 and the apparent rate constant for displacement. (c) Relation between the concentration of Tween 80 and lag time before displacement.

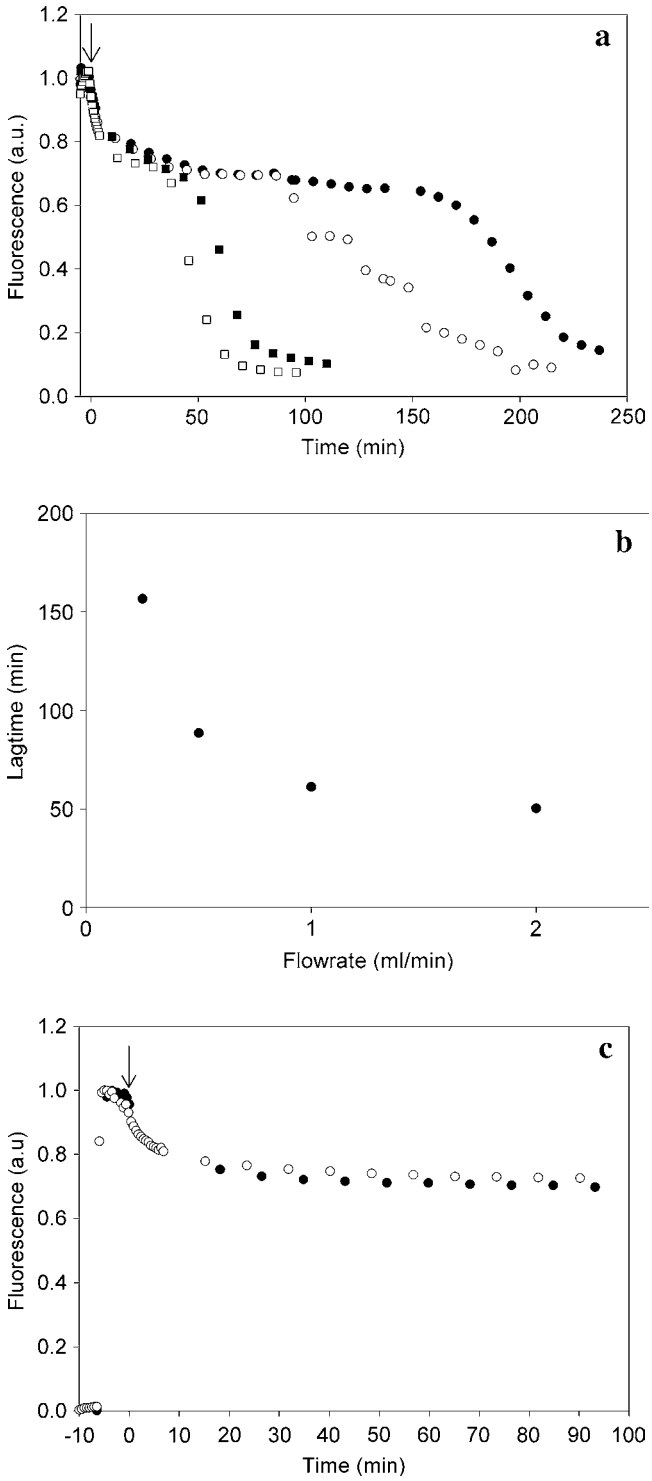

Fig. 3. Investigation of the effect of flow rate on displacement. (a) Displacement of FITC-labeled insulin by 2×10^{-4} mg/mL Tween 80 at different flow rates. Filled circles: 0.25 mL/min. Open circles: 0.5 mL/min. Filled squares: 1.0 mL/min. Open squares: 2.0 mL/min. The arrow indicates the onset of rinsing. (b) Lagtime before displacement as a function of flow rate. (c) Desorption in the presence of 10 mM phosphate buffer at different flow rates. Filled circles: 1 mL/min. Open circles: 2 mL/min. The arrow indicates the onset of rinsing.

the lag phase is observed with increasing flow rate (Fig. 3b). It should be noted that a higher flow rate also results in increased shear in the flow cell. However, no changes in desorption of FITC-insulin by phosphate buffer was observed upon changing the flow rate from 1 to 2 mL/min (Fig. 3c).

Insulin–Tween 80 Interactions in Solution

Nonionic surfactants have been reported to interact directly with proteins, especially in the partly denatured state (32). Circular dichroism spectra of protein/surfactant mixture may indicate whether interactions take place in solution. Far- and near-UV spectra of native human insulin at pH 1.6 and 7.4 are shown in Fig. 4a and b, respectively. The far-UV CD spectra display the characteristic negative minima at 208 and 222 nm as expected for α-helical proteins. At pH 1.6, human insulin is primarily monomeric in solution (33). However, this results only in slight changes in the far-UV CD spectra. The characteristics of the near-UV spectra of human insulin at pH 1.6 and 7.4 are in accordance with the ones reported

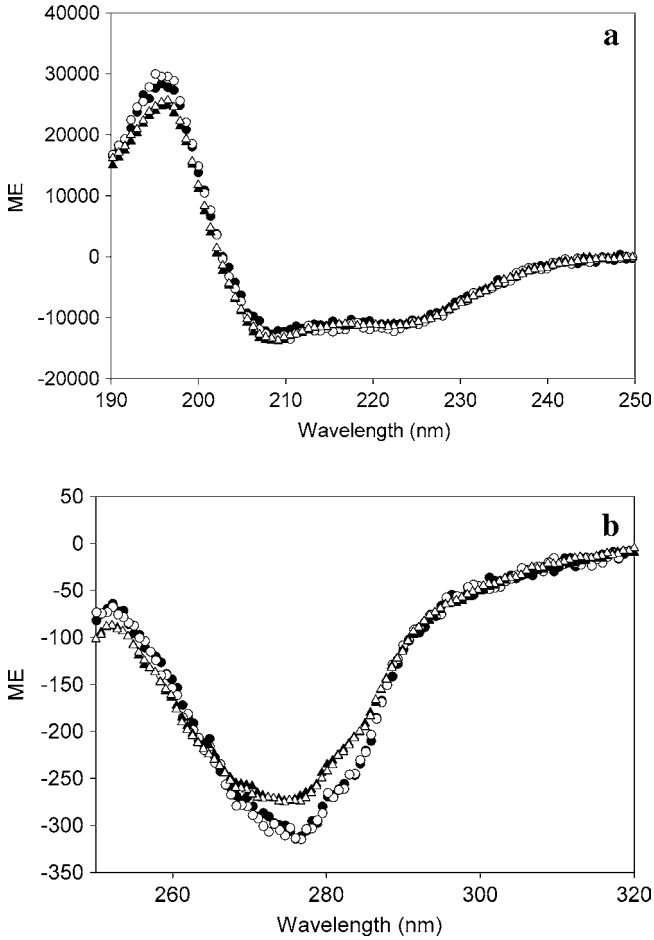

Fig. 4. Insulin structure in the presence and absence of Tween 80. (a) Far-UV circular dichroism spectra of 0.5 mg/mL human insulin. Filled circles: in phosphate buffer. Open circles: in 10^{-1} mg/mL Tween 80. Filled triangles: in phosphate buffer, pH 1.6. Open triangles: in 10^{-1} mg/mL Tween 80, pH 1.6. (b) Near-UV circular dichroism spectra of 1.5 mg/mL human insulin. Filled triangles: in phosphate buffer. Open triangles: in 10^{-1} mg/mL Tween 80. Filled triangles: in phosphate buffer, pH 1.6. Open triangles: in 10^{-1} mg/mL Tween 80, pH 1.6.

elsewhere (34). The presence of Tween 80 at a concentration of 10^{-1} mg/mL was not observed to affect the far- and near-UV CD spectra of insulin at either pH value. Thus, the results indicate that Tween 80 does not induce changes in the secondary and tertiary structures of native human insulin in solution in the concentration range applied in this study.

Competition Experiments

The competitive adsorption of mixtures of insulin and Tween 80 as monitored by TIRF is shown in Fig. 5. Two different scenarios were observed, depending on the concentration of Tween 80. At 10^{-1} mg/mL Tween 80, the fluorescence intensity initially increases to a level corresponding to the adsorption of FITC-insulin in the displacement studies. As opposed to the displacement studies, however, the fluorescence subsequently decreases rapidly again, approaching baseline levels. At 10^{-2} mg/mL Tween 80, the initial fluorescence intensity does not decline. Rather, as the adsorption proceeds, fluorescence intensity increases further to almost double the intensity values. Furthermore, no significant decrease in fluorescence intensity is observed upon rinsing with phosphate buffer. Emission scans of adsorbed FITC-insulin remained unchanged throughout the adsorption period (data not shown), indicating that the increase in fluorescence intensity was not due to changes in the emission maximum. It has been reported that FITC emission is sensitive to pH variation and changes in electrostatics (35). Thus, it was examined how ionic strength influenced the emission characteristics of FITC-insulin in solution. In this work, it was observed that the quantum yield of FITC-insulin increased with increasing ionic strength, whereas a similar increase in quantum yield was not observed for FITC-insulin in the presence of Tween 80 in increasing concentrations (data not shown).

Ellipsometry measurements monitoring the adsorption of mixed solutions of human insulin and Tween 80 are shown in Fig. 6a. The figure shows that the highest adsorbed amount before rinse of approximately 1.9 mg/m^2 is obtained from adsorption of a human insulin solution in the absence of Tween 80, whereas the pure Tween 80 solution results in lower adsorbed amount. Upon rinsing, a part of the adsorbed fraction is removed for all solutions tested. When Tween 80 is present in the insulin solution in a concentration of 2.8×10^{-3} mg/mL, an unexpected development in the adsorption is observed (see Fig. 6b). Within the first 10 min the adsorbed amount increases to a level reaching beyond the adsorption of pure Tween 80. Then, the adsorbed amount suddenly decreases again to a much lower level with about 0.8 mg/m^2 adsorbed, indicating the presence of a less dense layer of surfactant. When the concentration of Tween 80 was increased by a factor of 10 in the mixture, there also seems to be an adsorbed amount that is higher than pure Tween 80 in the early stages of the adsorption. However, the adsorbed amount decreases more rapidly to a level comparable to the adsorption of pure surfactant.

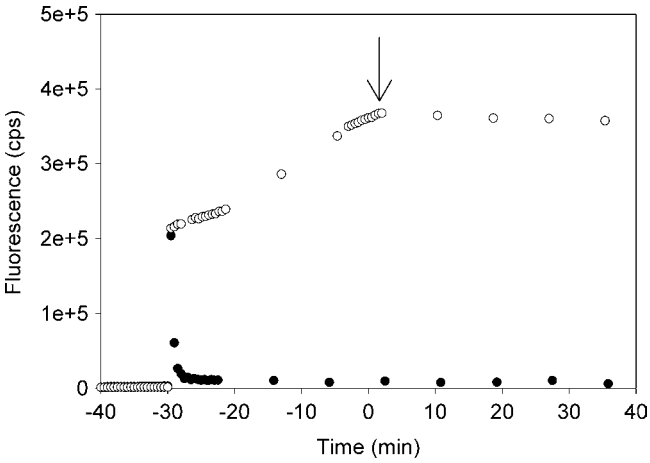

Fig. 5. Competitive adsorption of FITC-insulin and Tween 80. At $t = -30$, mixtures of insulin and Tween are injected. The arrow indicates the onset of rinsing with phosphate buffer. Open circles: 10^{-2} mg/mL Tween 80. Filled circles: 10^{-1} mg/mL Tween 80.

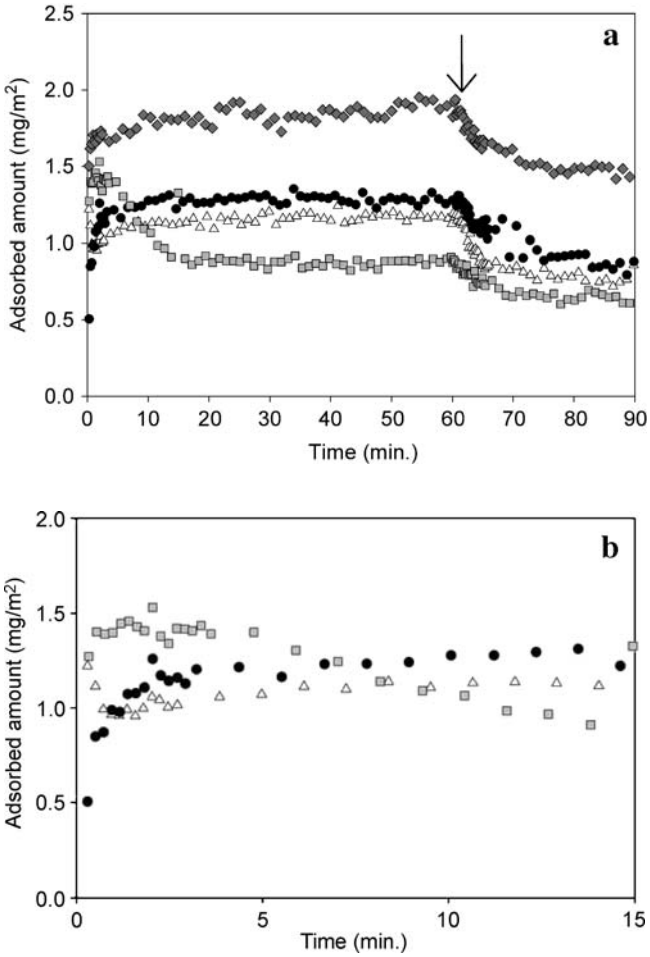

Fig. 6. Competitive adsorption investigated by ellipsometry. At $t = 0$, mixtures are added to the cuvette. The arrow indicates the onset of rinsing. (a) Dark gray diamonds: 10^{-1} mg/mL human insulin. Black circles: 2.8×10^{-2} mg/mL Tween 80. Open triangles: 10^{-1} mg/mL human insulin and 2.8×10^{-2} mg/mL Tween 80. Gray squares: 10^{-1} mg/mL human insulin and 2.8×10^{-3} mg/mL Tween 80. (b) Initial adsorption process. Black circles: 2.8×10^{-2} mg/mL Tween 80. Open triangles: 10^{-1} mg/mL human insulin and 2.8×10^{-2} mg/mL Tween 80. Gray squares: 10^{-1} mg/mL human insulin and 2.8×10^{-3} mg/mL Tween 80.

DISCUSSION

Displacement Experiments

Irreversible adsorption of proteins to different hydrophobic surfaces has been reported frequently (36,37). In this study, FITC-insulin was observed to adsorb rapidly onto the hydrophobic model surfaces. It was previously shown that human insulin adsorbs with high affinity to hydrophobized silica surfaces (18). After 5 min of adsorption interactions with the surface were strong, because approximately 80% of the initial fluorescence was not removed upon rinsing with phosphate buffer for 90 min. This is in accordance with the data obtained by ellipsometry (18). The decrease in fluorescence observed upon introduction of phosphate buffer may be explained by the removal of loosely attached protein and bulk protein from the flow cell (20). The bulk contribution can be estimated in a flow cell containing an unmodified quartz surface, because FITC-insulin was not observed to adsorb at the surface. The approximate increase in the fluorescence after the introduction of FITC-insulin was 3%, which is due the fluorescence from the bulk solution. Thus, there seem to be two populations of adsorbed protein: a weakly bound fraction that readily desorbs and a fraction of FITC-insulin that adsorbs with high affinity and can not be removed by phosphate buffer in the time scale investigated.

The fraction of adsorbed insulin that was not removed by phosphate buffer can be removed by other surface active molecules. Tween 80 in concentrations $>10^{-2}$ mg/mL easily displaces FITC-insulin from the surface, as shown in Fig. 1b. The critical micelle concentration (CMC) of Tween 80 was reported to be 1.4×10^{-2} mg/mL in water (38). FITC-insulin is readily removed from the hydrophobic surface by Tween 80 at concentrations about or above CMC. At concentrations of Tween 80 $<10^{-2}$ mg/mL, displacement proceeds in four phases. The first phase is due to the removal of loosely attached FITC-insulin and fluorescent molecules from the bulk solution, with kinetics similar to the decrease in fluorescence observed by rinsing with phosphate buffer. During the second phase, only a slight decrease in fluorescence is observed, indicating the presence of a lag phase in the displacement. The slight decrease could be a result of photobleaching of FITC-insulin in the adsorbed layer. The length of the lag phase depends on the concentration of Tween 80 and the flow rate of the solution (as shown in Fig. 3b). Thus, the net mass transport of Tween 80 to the surface is an important factor in the displacement process. In the next phase, a rapid decrease in fluorescence intensity is observed, which indicates that a major part of the labeled insulin is removed from the surface. The kinetics of the removal of FITC-insulin from the surface is considerably faster for the higher Tween 80 concentrations, which supports the observation that accumulation of a suitable amount of surfactant molecules is essential for the complete displacement of the protein. As accumulation at the surface occurs, the concentration of Tween 80 at the surface is possibly higher than in the bulk solution, and a turnover concentration where displacement takes place can thus not be directly related to the bulk concentration of the surfactant. The last phase of the displacement curve is characterized by a slow and continuous decrease in fluorescence that is approaching baseline fluorescence levels. The course of the displacement curve is in accordance with the orogenic displacement model reported by Mackie *et al.* (11,12) for air–water as well as oil–water interfaces. Using AFM, the authors observed that Tween surfactants are able to displace proteins from surfaces due to the higher surface activity. Tween was initially deposited at defects in the protein layer, whereupon the protein layer was compressed as the surfactant accumulated in large continuous domains followed by the final displacement of the protein (12).

The processes described by Gunning *et al.* (39) suggests that displacement is initiated by attachment of surfactant at certain points in the protein layer and may thus be characterized as a nucleation-dependent process. Single Tween molecules are not able to replace insulin by themselves; however, as the Tween 80 dominated domains grow, the protein layer is compressed and increases in density. The initial deposition of Tween 80 and the compression of the protein layer would not result in major changes in the fluorescence detected at the surface. The lag phase observed at Tween 80 concentrations of 10^{-2} mg/mL or below may therefore reflect these stages of the displacement. As Tween concentration increases, sufficiently high surface pressures are reached to induce failure of the protein network and release of protein in large clusters from the surface. In the TIRF experiments, this stage is observed by a sudden decrease in fluorescence, indicating displacement of a large amount of protein from the surface.

Nucleation-dependent processes such as protein fibril formation are often described by sigmoidal curves, as in Eq. (1) (31). Fitting of the displacement curves to this equation resulted in reasonable fits (Fig. 2a), which supports the idea that the mechanism of the displacement may be described by the formation of nucleation sites in the protein layer. At Tween 80 concentrations $> 10^{-2}$ mg/mL, no satisfactory fit could be obtained, as very rapid displacement with no detectable nucleation phase occurred. The apparent rate constant for displacement as derived from the curve fits indicates a linear dependence on the logarithm of Tween 80 concentration (Fig. 2b). Furthermore, an inverse linear relationship between the logarithm of the lag time and the logarithm of the Tween 80 concentration is observed (Fig. 2c). As the bulk concentration increases, the amount of Tween 80 molecules participating in surfactant domains at the surfaces also increases. The domains expand in two dimensions and compress the insulin layer until final collapse. Thus, as the rate for expansion of the surfactant domains increases due to increased supply of Tween 80, the lag time before release from the surface decreases and the rate of displacement increases.

Adsorption time has often been reported to be of importance for the reversibility of protein adsorption (40). However, a change in the length of the adsorption period for FITC-insulin from 5 to 30 min was not observed to have any effect on the displacement of protein by Tween 80 (Fig. 1c). A decreased desorption of protein after longer times of surface contact has been explained by the possibility of optimization of the attachment to the surface over time, and may involve the unfolding of the protein (35,41,42). However, the unfolding of proteins at surfaces is usually related to the surface coverage, where a low surface coverage allows

the protein to expand and optimize interactions in all dimensions (41,42). In the present study, a closely packed layer of FITC-insulin and human insulin molecules is expected, because the total concentration applied (10^{-1} mg/mL) lies within the plateau region of the adsorption isotherm of human insulin (18). This may prevent the insulin molecules to unfold further after adsorption. Thus the protein/surface interactions in the adsorbed layer may not change significantly over time, which is in accordance with the observations reported. Another possible explanation is that the unfolding and optimization has already taken place during the first 5 min of adsorption or that this process is slower than the time range studied.

Competition Experiments

The competitive adsorption of FITC-insulin/Tween 80 mixtures may indicate whether the surfactant is able to stabilize liquid insulin formulation against surface adsorption. It has already been established that Tween 80 in a concentration of 1% vol/vol increases the stability of porcine insulin when subjected to continuous rotation (3). Also, other nonionic surfactants have been proven to improve the stability of insulin against aggregation (3,5).

Competitive adsorption measurements were studied by TIRF and ellipsometry. TIRF measurements on mixtures of insulin and Tween 80 proved to be complex and thus difficult to interpret. In the present study, the concentration of Tween 80 seems to be of importance for preventing the adsorption of FITC-insulin to the hydrophobic surface, as shown in Fig. 5. The initial increase in fluorescence intensity observed for mixtures containing 10^{-1} mg/mL Tween 80 was comparable to the fluorescence intensity observed for an adsorbed layer of insulin in the absence of Tween 80. This may be explained either by a faster adsorption of insulin to the surface compared to Tween 80 or by a slower exchange or desorption of adsorbed insulin molecules. Subsequently, however, Tween 80 molecules displace insulin at the surface. This may be due to a higher affinity to the surface or to the excess in the number of Tween 80 molecules in the solution. The TIRF data are in accordance with ellipsometry measurements on the mixtures of insulin and Tween 80, as shown in Fig. 6b, where the initially adsorbed amount decreases within the first 15 min of the measurement.

At a concentration of 10^{-2} mg/mL of Tween 80, FITC-insulin is not displaced by the surfactant in the TIRF experiments shown in Fig. 5. Estimations of the total surface area of the flow cell and tubing system indicated that the lack of displacement could not be attributed to the depletion of Tween 80 molecules in the sample volume. The increase in fluorescence intensity during the adsorption step and the lack of decrease in fluorescence upon rinsing indicates that changes in the adsorbed layer take place over time. The increase was only observed for mixtures of FITC-insulin and Tween 80, and thus the observed increase may be ascribed to the presence of Tween 80 in the adsorbing solution. A similar effect was also observed when Tween 80 was adsorbed first and FITC-insulin was present in the rinsing solution and at lower concentrations of Tween 80 (data not shown). Because the emission maximum of FITC-insulin was unchanged during the adsorption step, the increase in fluorescence intensity can be explained by an increase in quantum yield of the fluorophore. Another explanation could be an increased adsorption of FITC-insulin over time, as, for example, by the exchange of human insulin by FITC-insulin. However, this seems unlikely, because a significant exchange between the two species was not observed in the absence of Tween 80, as shown in Fig. 1c, where the fluorescence only increased slightly during the adsorption step.

Fluorescence emission spectra as well as CD spectra of insulin/Tween 80 mixtures did not indicate any interaction between insulin and Tween 80 in solution. The association states of insulin in solution and at the surface are probably different. The hexameric state of human insulin is predominant in solution under the conditions applied, whereas the protein is believed to adsorb in the monomeric form (18,43,44). Furthermore, the exterior of the insulin hexamer is mainly hydrophilic (45), whereas the monomer has hydrophobic patches exposed on the surface. Thus it was investigated whether interaction with Tween 80 would be observed for both species using different pH values. As indicated in Fig. 3a and b, no changes in secondary and tertiary structures were observed in the presence of Tween 80 for either association state. Nevertheless, weak interactions that do not alter the protein conformation or the interaction with a small fraction of the insulin species in the bulk solution can not be ruled out. Furthermore, the restricted motion and the smaller distance between the molecules in the adsorbed state may induce local changes that can not be observed in bulk solution. However, the above results indicate that the change in quantum yield of FITC-insulin is rather attributable to interaction of the FITC label itself with Tween 80 than to interactions between the protein and the surfactant.

The TIRF results indicate that Tween 80 should be effective in stabilizing insulin against adsorption to hydrophobic surfaces at concentrations above the CMC. It was observed for bovine serum albumin that adsorption to hydrophobic surfaces decreases sharply at Tween 20 concentrations near the CMC value of the surfactant (29). Because the experiments indicate competition for surface binding sites as the primary mechanism of displacement of insulin from the surface, an effective concentration in the same range would be expected for Tween 80.

However, the concentration of Tween 80 where effective displacement of insulin occurs was not comparable for the TIRF and ellipsometry data. The ellipsometry data in Fig. 6a indicate that insulin is displaced from the surface at all Tween 80 concentrations tested, as the amount adsorbed from the mixtures of insulin and Tween 80 indicated the formation of low density layers of Tween 80. The concentrations tested in the TIRF experiments are not similar to the Tween 80 concentrations used in the ellipsometry measurements. Thus, it is likely that displacement by Tween 80 can be observed in the TIRF cell at a concentration of 2.8×10^{-2} mg/mL. Nevertheless, at 10^{-2} mg/mL no displacement is observed, despite the fact that displacement can be registered by ellipsometry at even lower concentrations (2.8×10^{-3} mg/mL).

Inconsistency in the results may be explained by the experimental setup, as the adsorbing solution in the TIRF

experiment is run through tubing before entering the flow cell, allowing interactions of Tween 80 or insulin with other surfaces, whereas the ellipsometry measurements were carried out in a cuvette. Another option is that the increase in quantum yield observed in TIRF experiments is due to interaction between the FITC group and Tween 80 at the surface, and thus represents an artifact effect. As a consequence, the results obtained by ellipsometry are more likely to represent the processes taking place at the surface during competitive adsorption.

CONCLUSION

The displacement of surface-adsorbed human insulin by Tween 80 as well as the competitive adsorption of the two species was investigated by TIRF and ellipsometry.

FITC-insulin rapidly adsorbed to the hydrophobized quartz and silica surfaces and the major part of the adsorbed protein could not be removed by phosphate buffer. The displacement studies showed that Tween 80 is able to remove the adsorbed insulin in a concentration-dependent manner, as the apparent rate constant for displacement was linearly dependent on the logarithm of the Tween 80 concentration. The displacement data could be fitted to a sigmoidal curve function, which is in accordance with the assumption that displacement takes place through the formation of nucleation sites at the defects in the protein layer. Thus, the results indicate that Tween 80 reduces the adsorption of insulin by competing for sites at the surface. This is further supported by CD data, which did not indicate any general structural changes of insulin monomers or hexamers in the presence of Tween 80, suggesting that no or limited interaction between the two species occurs in solution.

Competitive adsorption from mixtures of insulin and Tween 80, as monitored by TIRF and ellipsometry, indicated that insulin initially adsorbs to the surface, but is replaced by Tween 80 in a later stage.

Displacement studies by TIRF may indicate the mechanism of action of the surfactant, whereas competitive adsorption studies can be used to determine the effective concentration range of the excipient. Thus, the use of surface-sensitive techniques in preformulation and excipient screening may provide valuable information about surface-induced denaturation and how excipients may improve solution stability.

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