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Interfacial adsorption of insulin Conformational changes and reversibility of adsorption

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ABSTRACT

The adsorption of human insulin to Teflon particles was studied with respect to conformational changes and the reversibility of adsorption was examined by total internal reflection fluorescence (TIRF).

Adsorption isotherms for the adsorption of human insulin indicated high affinity adsorption, even at electrostatic repulsive conditions. The plateau value for adsorption was in accordance with a protein layer consisting primarily of insulin monomers. Conformational changes of the insulin upon adsorption, was investigated by circular dicroism (CD) and fluorescence spectroscopy. The results suggested unfolding of adsorbed insulin, as observed by a decrease in α-helix and increase in random coil conformation. The changes in protein structure was not only related to the adsorbed species being monomeric, since CD and fluorescence results were different for adsorbed insulin compared to a monomeric analog of human insulin. Furthermore, the thermal stability in the adsorbed state was changed compared to insulin in solution.

On the basis of the TIRF studies with FITC-labelled insulin it was not possible to firmly conclude whether exchange between human insulin in the adsorbed state and in solution takes place, due to the limited time range investigated. However, the desorption mechanism appeared to be different with unlabelled insulin in the bulk solution compared to phosphate buffer.

1. Introduction

The loss of material due to adsorption to interfaces has frequently been reported in the development of highly potent protein pharmaceuticals. Since the adsorbed amount of most proteins reaches saturation of the interface over a large concentration range (Norde, 2000), protein adsorption must be taken into account for dilute solutions of proteins in particular (Wu and Chen, 1989; Petty, 1974). However, reduced physical stability and biological activity of proteins in solution, after

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being exchanged from the adsorbed state, should receive even more attention. Especially interfaces, which allow hydrophobic interactions between the protein and interface, e.g., hydrophobic materials or the air–water interface have been reported to cause permanent structural changes (Norde and Giacomelli, 2000; Tzannis et al., 1996) and to accelerate the aggregation of proteins in solution (Sluzky et al., 1992).

When proteins adsorb to interfaces they often undergo conformational changes favouring the interaction with the interface. The adsorbed protein is commonly tightly adsorbed to the interface with several points of interaction and may not easily be displaced upon dilution. However, adsorbed proteins have frequently been reported to undergo exchange with proteins in the bulk solution (Lok et al., 1983; Norde and Giacomelli, 2000). Proteins with altered structure which distribute back into solution are often considered the main precursor for aggregation in the presence of interfaces (Vermonden et al., 2001; Norde and Giacomelli, 2000).

As being one of the most successful recombinant protein pharmaceuticals, the aggregation of insulin has been widely investigated. Human insulin exhibits complicated self-association behaviour, organizing into dimers and hexamers in a concentration and pH dependent manner (Brange, 1994). The hexameric state of insulin is further stabilized by certain divalent ions and phenolic derivates, e.g., phenol and *m*-cresol.

Human insulin aggregates into large fibrous structures, the so-called fibrils (Nielsen et al., 2001a; Whittingham et al., 2002). The role of surface adsorption in insulin fibrillation has been explored because of the potential use of the protein in various drug delivery systems to obtain controlled release administration (Thurow and Geisen, 1984; Bringer et al., 1981; Kwon et al., 2001; Chawla et al., 1985). The major focus of insulin adsorption has been on interactions of insulin with solid/liquid interfaces occurring in pump devices (Lougheed et al., 1983). The presence of hydrophobic interfaces has been observed to accelerate the rate of insulin aggregation, possibly by facilitating unfolding of the protein (Sluzky et al., 1991; Brange et al., 1997; Kwon et al., 2001). Furthermore, solutions of human insulin at hexamer-inducing conditions (e.g., neutral pH and in the presence of zinc ions) are more prone to fibrillate at low concentration where the relative content of monomer is higher, and monomerisation of oligomeric insulin is thus considered to precede the fibrillation process. This is explained by increased occupation of interfacial sites by dimers and hexamers at higher concentration, reducing the surface area available to unfold the monomer (Sluzky et al., 1991).

The denaturation of insulin by guanidinium hydrochloride (GdnHCl) has identified at least two intermediates during unfolding of human insulin (Millican and Brems, 1994). In the first intermediate it is suggested, that the C-terminal segment of the B-chain is perturbed, whereas the native helical structure is retained. At higher concentrations of GdnHCl however, the first intermediate further denatures into an intermediate with major unfolding of the secondary and tertiary structure (Millican and Brems, 1994). The intermediates identified during denaturation are thought to be involved in the fibrillation of human insulin. Studies of the kinetics of insulin fibrillation using Thioflavin T (ThT) resulted in a model suggesting that unfolded intermediates of the insulin monomer, with an increased propensity to oligomerise, forms a nucleus for fibrillation (Nielsen et al., 2001b). Thus, it is highly likely that adsorption of insulin to hydrophobic interfaces induces the formation of partially unfolded intermediates. The aim of the present study is to characterize human insulin in the adsorbed state with regard to structural changes upon adsorption to Teflon. Furthermore, the reversibility of the adsorption is investigated, since the structurally perturbed protein may distribute back into solution again to induce insulin fibrillation.

2. Materials and methods

2.1. Materials

Freeze dried bulk preparations of recombinant human insulin containing two Zn^{2+} per hexamer and Asp^{B28} insulin and Trp^{B30} insulin analogs, were kindly donated by Novo Nordisk A/S (Bagsvaerd, Denmark) and used as received. The bulk preparations of the two insulin analogs did not contain zinc.

Fluorescein isothiocyanate (FITC, isomer I) was obtained from Molecular Probes, The Netherlands. Chlorodimethyl (3,3,3-trifluoropropyl)silane (Lancaster), used for silanization of surfaces, was obtained from Chemtronica, Sweden. All other chemicals were of analytical grade. Quartz slides for TIRF were obtained from BioElectrospec, U.S.

2.2. Sorbent materials

2.2.1. Teflon particles

A suspension of Teflon particles prepared by emulsion polymerization of tetrafluorethylene and perfluorovinylether was used as sorbent material. The Teflon suspension was kindly donated by Du Pont de Nemours (Du Pont de Nemours SA, Le Grand-Saconnex, Switzerland). The particles were free of non-ionic stabilizers and other contaminants. Colloidal stability of the suspension was obtained by charge repulsion due to the location of sulphate groups on the surface of the particles originating from the polymerization initiator potassium persulphate. A sessile drop of 10 mM phosphate buffer resulted in a contact angle of 96° for the pelletised particles. Thus, the charged groups on the surface only cover a minute fraction of the surface area of the particles. The average diameter of the particles was 215 nm and the specific surface area was 12.5 m^2/g as determined by the Brunauer–Emmett–Teller (BET) method. The refractive index of the Teflon particles is 1.35 which is about equal to the refractive index of water (1.33) and furthermore, the preparation does not contain UV-absorbing double bonds or aromatic groups. Hence, very low scattering of light is seen when the particles are used in optical techniques, which makes them suitable for studying protein adsorption.

2.2.2. TIRF surfaces

Hydrophobic quartz surfaces for TIRF were prepared by silanization. The preparation of polytetrafluoroethylene (PTFE)-like surfaces by silanization has previously been