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RESEARCH PAPER

Influence of Acylation on the Adsorption of Insulin to Hydrophobic Surfaces

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ABSTRACT

Purpose To study the effect of acylation on the adsorption of insulin to hydrophobic polystyrene beads.

Methods Adsorption isotherms for adsorption of insulin and acylated insulin to hydrophobic polystyrene beads were established, and the adsorption of the two proteins was compared further with isothermal titration calorimetry. In addition, the secondary structure and the association behavior of the two proteins were studied with circular dichroism.

Results Insulin and acylated insulin adsorbed with high affinity to the hydrophobic polystyrene beads. More acylated insulin molecules than insulin molecules adsorbed per unit surface area from solutions containing monomer-dimer mixtures of acylated insulin and insulin, respectively. In contrast, no difference was observed in the number of insulin and acylated insulin molecules adsorbing per unit surface area, when adsorption occurred from solutions containing monomer-dimer-hexamer mixtures.

Conclusion The influence of acylation on the adsorption behavior of insulin depends on the association degree of insulin, possibly due to a greater difference in hydrophobicity between monomeric insulin and acylated insulin than between the hexameric forms of these two proteins.

KEY WORDS acylated insulin · insulin · protein adsorption · ITC · CD

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INTRODUCTION

Acylation is the covalent attachment of a fatty acid chain to proteins or peptides (hereinafter referred to as proteins). It is a naturally occurring post-translational modification, but in the last three decades the use of protein-engineered acylation to facilitate the delivery of protein drugs by increasing their plasma half life has gained increased interest. The prolonged plasma half is obtained by binding of the fatty acid chain to serum albumin in plasma and through sustained release from the local administration site (1–3).

Acylated protein drugs are, like other protein drugs, exposed to a diversity of solid-liquid interfaces during purification, production, storage and use. Typical hydrophobic solid surfaces that protein solutions come into contact with during storage and use are closures, plungers, infusion bags, tubing and valves. Adsorption at interfaces during storage and use will result in protein loss from solution and may therefore lead to incomplete delivery to patients. Furthermore, adsorption may cause surface-induced structural changes in adsorbed and possibly desorbed protein molecules. Such structural changes may lead to aggregation and reduced biological activity and

potentially cause adverse immunological responses to the protein drug. Acylation will affect the protein characteristics and is therefore likely to affect the protein adsorption behavior at solid-liquid interfaces. Therefore, a thorough understanding of the affect of acylation on protein adsorption would be of great value in the future development of acylated protein drug products.

Insulin is a 5808 Da protein consisting of an A-chain and a B-chain linked by two disulfide bonds, with an additional intra-chain disulfide bond in the A-chain (4). In solution, insulin exhibits a complicated self-association behavior, where monomers exist in equilibrium with dimers and hexamers. The self-association of insulin is affected by a range of factors, including the insulin concentration, pH, ionic strength, divalent metal ions and various ligands (5). At concentrations below 0.1 μM, insulin exists as a monomer, whereas dimers are formed at higher concentrations (6). The hexamer form is promoted in the presence of Zn^{2+} ions. In the presence of Zn^{2+} ions and in the pH range 4–8, hexamers are formed at concentrations above 0.01 mM (6), but in the absence of Zn^{2+} ions, the hexamer is formed at concentrations above 2 mM (7). The monomer has hydrophobic amino acids exposed to the solution; therefore, it is thermodynamically favorable for two monomers to assemble into a dimer, where some of the hydrophobic amino acid residues are buried between the two insulin molecules (8). The formation of a heaxmer results in further burial of hydrophobic amino acid residues and the surface of a hexamer is almost entirely polar (8). When two monomers assemble, a shared anti-parallel β-sheet structure is formed (6), which makes the monomer-monomer interaction stronger than the interactions involved in the formation of hexamers (6,8).

Adsorption of insulin at solid-liquid interfaces has been studied with regard to varying hydrophobicity of the solid surface, association state of insulin, ionic strength and pH (9–11). Studies by Nilsson *et al.* (11) indicate that it is the monomeric form of insulin that adsorbs to a hydrophobic surface, while the adsorption plateau values observed by Mollmann *et al.* (9,10) are slightly higher than expected for a monolayer consisting of insulin monomers and correspond to monolayers consisting of a mixture of insulin monomers and dimers (9). To the best of our knowledge, the effect of acylation on the adsorption behavior of insulin has not been studied.

Today, an acylated insulin variant and an acylated GLP-1 variant are marketed, while acylation has been reported to increase the plasma half-life of leu-enkephalin (12), desmopressin (13,14), octreotide (15), salmon calciotonin (16) and interferon-alpha (17).

Lys^{B29} (N-tetradecanoyl) des-(B30) human insulin, which is marketed under the name Levemir®, is a long-acting acylated human insulin variant in which Thr^{B30} has been removed and a 14-carbon myristoyl fatty acid has been covalently bound to the side chain of Lys^{B29} (Fig. 1). This 5917 Da acylated insulin variant has a higher free energy of unfolding than insulin (18). The crystal structure of Lys^{B29} (N-tetradecanoyl) des-(B30) human insulin shows that the hydrophobic fatty acid group at the end of the chain has no significant effect on the conformation of the insulin monomer or its ability to form hexamers, but the fatty acid chains are involved in hydrophobic interaction between two adjacent hexamers (18).

The aim of this work was to examine how acylation of insulin influences the adsorption of insulin to hydrophobic polystyrene beads, using human insulin and Lys^{B29} (N-tetradecanoyl) des-(B30) human insulin as model proteins. Throughout the study, two different buffer systems were used, namely 10 mM NaH_2PO_4+1 mM EDTA pH 7.4, in which insulin and acylated insulin exist as a monomeric-dimeric equilibrium, and 10 mM NaH_2PO_4 pH 7.4, in which the two proteins exist as a monomeric-dimeric-hexameric equilibrium. Adsorption isotherms for the adsorption of the two proteins to polystyrene beads were compared, and isothermal titration calorimetry was used to further compare their adsorption. Near-UV and far-UV circular dichroism were used to compare the secondary structure and association behavior of the two proteins.

MATERIALS AND METHODS

Materials

Freeze-dried bulk preparations of recombinant human insulin (insulin) and Lys^{B29}-dodecanoyl des-(B30) insulin (acylated insulin) were kindly donated by Novo Nordisk A/S, Denmark. Both insulin and acylated insulin contained 2.3 Zn^{2+} ions per hexamer. In addition, the acylated insulin bulk contained one phenol molecule per protein molecule. Phenol can bind in the hydrophobic cavities between insulin dimers and thereby shift the hexamer configuration from the T-state to the more stable R-state (19), and this is also the case for acylated insulin (18). However, Zn needs to be present for insulin to form hexamers at the concentrations used in our experiments. Thus, the differences observed between insulin and acylated insulin at monomeric/dimeric conditions are unlikely to be a result of the presence of phenol in the acylated insulin solution. The T to R conformation change is accompanied by a large change in the 250–260 nm range of the near-UV spectrum (20). The near-UV signals measured for insulin and acylated insulin were all between 1.3 and 1.6. These values are of the same range as the values reported by Olsen and Kaarsholm (18) when no phenol is present. Moreover, they show that when only one phenol molecule per acylated insulin molecule is present, the fraction of

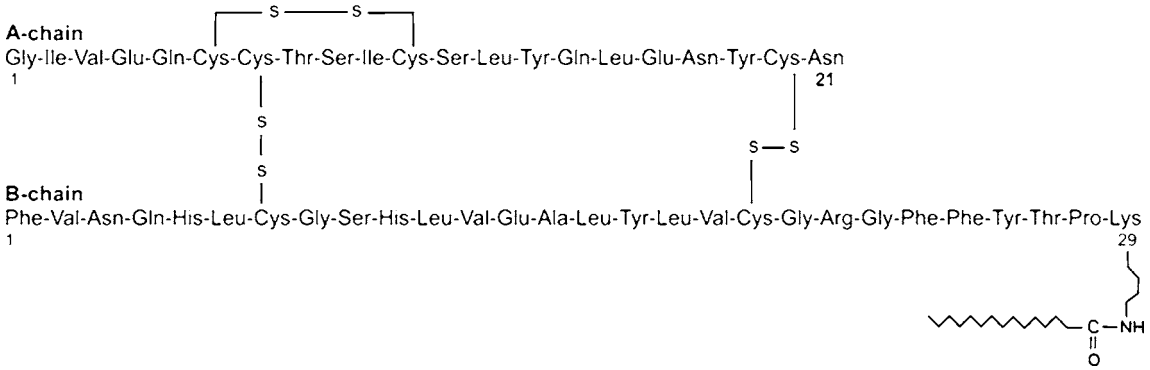

Fig. 1 Primary structure of acylated human insulin variant. Thr^{B30} has been removed, and a 14-carbon myristoyl fatty acid has been covalently bound to the side chain of Lys^{B29}. Modified from (3).

acylated insulin in the R-state is small (app. 2%). Therefore, the phenol present in our acylated insulin samples is unlikely to affect the adsorption of acylated insulin. The isoelectric point of the insulin monomer has been determined to 5.4 (21,22) and is expected to be even lower for the acylated insulin due to blocking of the positive charge at Lys^{B30}. Therefore, insulin and the acylated insulin will both be negatively charged at pH 7.4. All water used was Milli-Q grade, and all other chemicals were of analytical grade and were obtained from commercial sources.

Sample Preparation

Insulin was dispersed in water, and 0.1N HCl was added to dissolve the insulin, after which buffer was added and pH adjusted to 7.4 with 0.1N NaOH. Acylated insulin was dissolved in water, buffer was added and pH was adjusted to 7.4 with 0.1N HCl. The concentrations of the samples were determined from the absorbance at 276 nm, using an extinction coefficient of 6200 M^{-1} cm^{-1} (23). The buffer was either 10 mM NaH_2PO_4 pH 7.4 or 10 mM NaH_2PO_4+ 1 mM EDTA pH 7.4. EDTA was added to bind Zn^{2+} ions and thereby prevent hexamer formation of insulin and acylated insulin, which can form in the presence of free Zn^{2+} ions. Addition of 1 mM EDTA to a 300 μM insulin solution will result in a free Zn^{2+} concentration of 8.67×10^{-16}. (The concentration of free Zn^{2+} ions was calculated using MaxChelator (http://maxchelator.stanford.edu).)

Polystyrene Beads

An 8 w/v % suspension of polystyrene beads with sulphate functional groups on the surface suspended in distilled de-ionized water was obtained from Interfacial Dynamics Corporation (Eugene, OR, USA). Due to the sulphate groups, the surfaces of the polystyrene beads were partly negatively charged. The ionic strengths of the NaH_2PO_4 and NaH_2PO_4 + EDTA buffer solutions used for preparation of polystyrene beads solutions were adjusted to make sure that the final suspensions of polystyrene beads had were in 10 mM NaH_2PO_4 pH 7.4 or 10 mM NaH_2PO_4+1 mM EDTA pH 7.4.

Circular Dichroism (CD)

Far-UV and near-UV CD spectra were obtained on a Jasco-J-810 circular dichroism spectrophotometer (Jasco, Tokyo, Japan). One-hundred-μM insulin and acylated insulin samples were scanned in a 0.05 mm cell from 250 to 180 nm, and 300 μM samples were scanned in a 5 mm cell from 350 to 250 nm to obtain far-UV and near-UV spectra, respectively. A bandwidth of 1 nm, a response time of 4 s, a data pitch of 0.2 nm and a scanning speed of 50 nm/min were used. Each spectrum is an accumulation of 10 scans. Spectra of the appropriate reference solutions were recorded and subtracted from each protein spectrum. The molar CD signal $\Delta\varepsilon$ was expressed in M^{-1} cm^{-1} with far-UV and near-UV data normalized to the molar concentration of protein and peptide bond, respectively.

Adsorption Isotherms

Adsorption isotherms were established for insulin and acylated insulin adsorption to polystyrene beads. For each protein, solutions of varying concentration were added to 3.2 ml Beckman centrifuge polycarbonate tubes containing beads with at total surface area of 0.1 m^2. A total volume of 3 ml was added to each tube. At high concentration and in the presence of Zn^{2+} ions, insulin and acylated insulin can form hexamers and di-hexamers, respectively. A 10 mM NaH_2PO_4 buffer with pH 7.4 containing 1 mM EDTA was used in order to bind the Zn^{2+} ions and thereby prevent formation of hexamers. The samples were left to adsorb at room temperature for 1 h, after which they were centrifuged with 100,000 rpm (550,000 g) for 30 min at 25°C in a Beckman Coulter Optima Max-E Ultra Centrifuge using a

TLA110 rotor (Beckman Coulter, Fullerton, CA, USA). Subsequently, the supernatant was removed and the protein concentration determined by UV spectroscopy using ε_{276}= 6200 M^{-1} cm^{-1} (1,23), and the adsorbed amount was then determined by mass balance. In order to study whether desorption of insulin and acylated insulin occurred, the beads were re-suspended in 3 ml buffer and left overnight. The next day, the samples were centrifuged, and the concentration in the supernatant was determined.

Isothermal Titration Calorimetry Experiments

Isothermal titration calorimetry measurements were performed with a Nano ITC^{2G} from TA Instruments (Newcastle, DE, USA). In a typical experiment, the sample cell was filled with a suspension of polystyrene beads yielding a surface area of 0.11 m^2. The suspension of polystyrene beads was titrated with 5 μL aliquots of 300 μM insulin or acylated insulin. The temperature was 25°C, and the reference cell was filled with water. All solutions were degassed by stirring under vacuum before use. Four different experiments were performed: titration of insulin and acylated insulin into beads in 10 mM NaH_2PO_4+1 mM EDTA pH 7.4 and titration of insulin and acylated insulin into beads in 10 mM NaH_2PO_4 pH 7.4. The titration of insulin into beads suspended in 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4 was performed three times to examine the reproducibility. Since the reproducibility was high, the other experiments were performed only once or twice. Heat of dilution was subtracted for all heat signals before they were integrated using the NanoAnalyze software from TA instruments.

RESULTS

Circular Dichroism

The far-UV spectra for insulin and acylated insulin in 10 mM NaH_2PO_4+1 mM EDTA pH 7.4 showed structures dominated by α-helix with a strong negative CD signals at 208 and 222 nm and a strong positive CD signal around 195 nm (Fig. 2a). The negative CD signal at 208 nm and 222 nm as well as the positive CD signal around 195 nm were smaller for acylated insulin than for insulin, while the positive CD signal from 190–180 nm was higher for acylated insulin than for insulin. Furthermore, the crossover point at the x-axis and the positive band at 195 nm were blue-shifted for acylated insulin. In 10 mM NaH_2PO_4 pH 7.4 (Fig. 2b), far-UV spectra similar to those in 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4 were observed. Compared to insulin, the spectra for acylated insulin had a slightly larger negative CD signal at 208 nm and a slightly smaller negative CD signal at 222 nm.

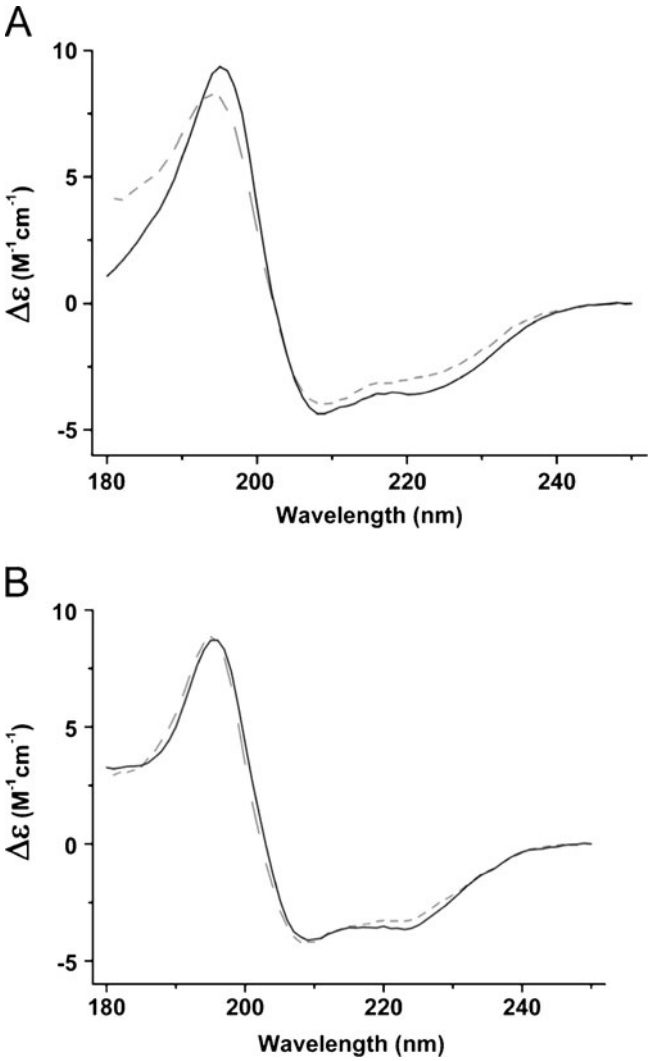

Fig. 2 Far-UV CD spectra of insulin (*black line*) and acylated insulin (*gray dashed line*) obtained on 100 μM solutions in 0.5 mm cells. (**A**) In 10 mM NaH_2PO_4+1 mM EDTA pH 7.4. (**B**) In 10 mM NaH_2PO_4 pH 7.4.

Furthermore, the crossover point at the x-axis and the positive band at 195 nm were blue-shifted for acylated insulin compared to insulin. Although differences in the far-UV CD spectra for insulin and acylated insulin were observed, the spectra for both proteins were dominated by α-helical structure in both 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4 and 10 mM NaH_2PO_4 pH 7.4.

Near-UV CD spectra of insulin and acylated insulin, respectively, in both buffers are shown in Fig. 3. All four spectra are dominated by a tyrosyl signal with a minimum at 276 nm. Insulin dimers and hexamers exhibit a characteristic tyrosyl CD signal at approximately 276 nm. The two main contributors to this signal are interactions in the monomer-monomer interface of the dimers and in the dimer-dimer interfaces of the hexamers (24,25). The near-UV CD spectra for both insulin variants showed a larger negative CD signal around 276 nm in 10 mM NaH_2PO_4

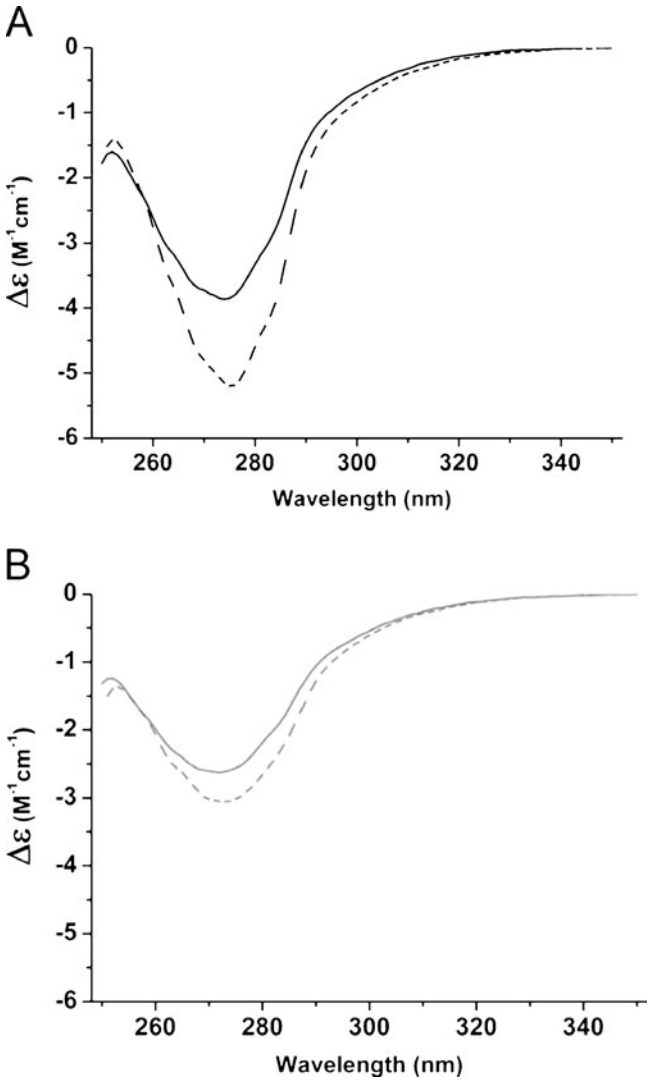

Fig. 3 Near-UV CD spectra of insulin and acylated insulin obtained on 300 μM solutions in 0.5 mm cells. (**A**) Insulin in 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4 (*black line*) and in 10 mM NaH_2PO_4 pH 7.4 (*black dashed line*). (**B**) Acylated insulin in 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4 (*gray line*) and in 10 mM NaH_2PO_4 pH 7.4 (*gray dashed line*).

pH 7.4 than in 10 mM NaH_2PO_4+1 mM EDTA pH 7.4, indicating that the monomer-dimer-hexamer equilibrium was shifted further toward the hexamer form when the solution contained Zn^{2+} ions. The observed difference in the intensity of the CD signal for insulin and acylated insulin is in accordance with previous near-UV CD measurements on Zn-free human insulin and Zn-free acylated insulin (18).

Adsorption Isotherms

Adsorption and desorption isotherms were obtained for adsorption of insulin and acylated insulin to polystyrene beads in 10 mM NaH_2PO_4+1 mM EDTA, pH 7.4 (Fig. 4). The initial part of the adsorption isotherms merges with the y-axis, indicating that both proteins adsorb with high affinity

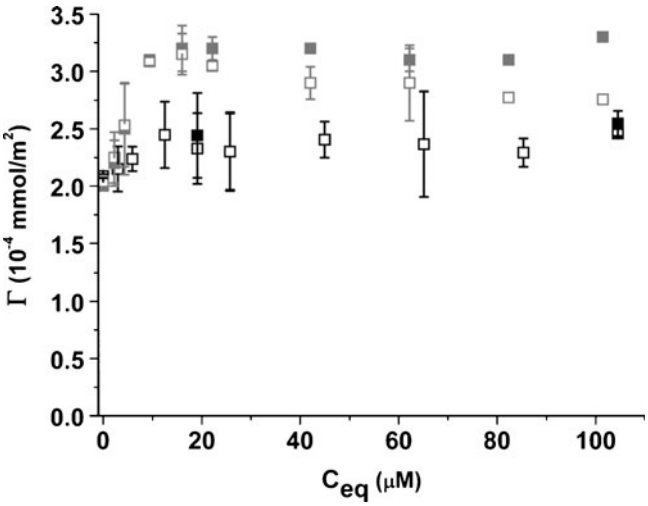

Fig. 4 Adsorption isotherms for absorption of insulin (■) and acylated insulin (■) to polystyrene particles in 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4. Insulin (□) and acylated insulin (□) after desorption. If no desorption was detected, the adsorbed amounts per unit surface area is shown only with an open square.

to the polystyrene beads. For insulin and acylated insulin, adsorption saturation was reached above approximately 12 μM with plateau values of $2.4\pm0.3\times10^{-4}$ mol/m^2 and $3.2\pm0.1\ 10^{-4}$ mol/m^2, respectively. For insulin, the desorption isotherm could generally not be distinguished from the adsorption isotherm. For two points on the isotherm the average values for the adsorption and desorption differed, but the observed differences were within the standard deviation and therefore no indication of desorption. Slight desorption was observed for acylated insulin yielding a plateau value of $3.0\pm0.2\ 10^{-4}$ mol/m^2 for the desorption isotherm. Even though a slight desorption was observed for acylated insulin, the plateau value after desorption was still higher for acylated insulin than for insulin.

Isothermal Titration Calorimetry

Representative raw data for titration of insulin and acylated insulin into 10 mM NaH_2PO_4+1 mM EDTA pH 7.4 and into a suspension of polystyrene beads in the same buffer are shown in Fig. 5. Blank titrations of insulin into buffer revealed small exothermic heat flows that gradually decreased with the number of injections, and towards the end a small endothermic heat flow was observed after each injection. The size of the heat flow signals for the blank titration of insulin into buffer indicates that the contribution from dissociation of insulin dimers will have minute influence on the experiments with insulin. For titration of acylated insulin into buffer, a steady small endothermic heat flow was observed for each injection, indicating that heat flow from dissociation for acylated insulin will not affect the results for titrations of acylated insulin into a suspension of polystyrene

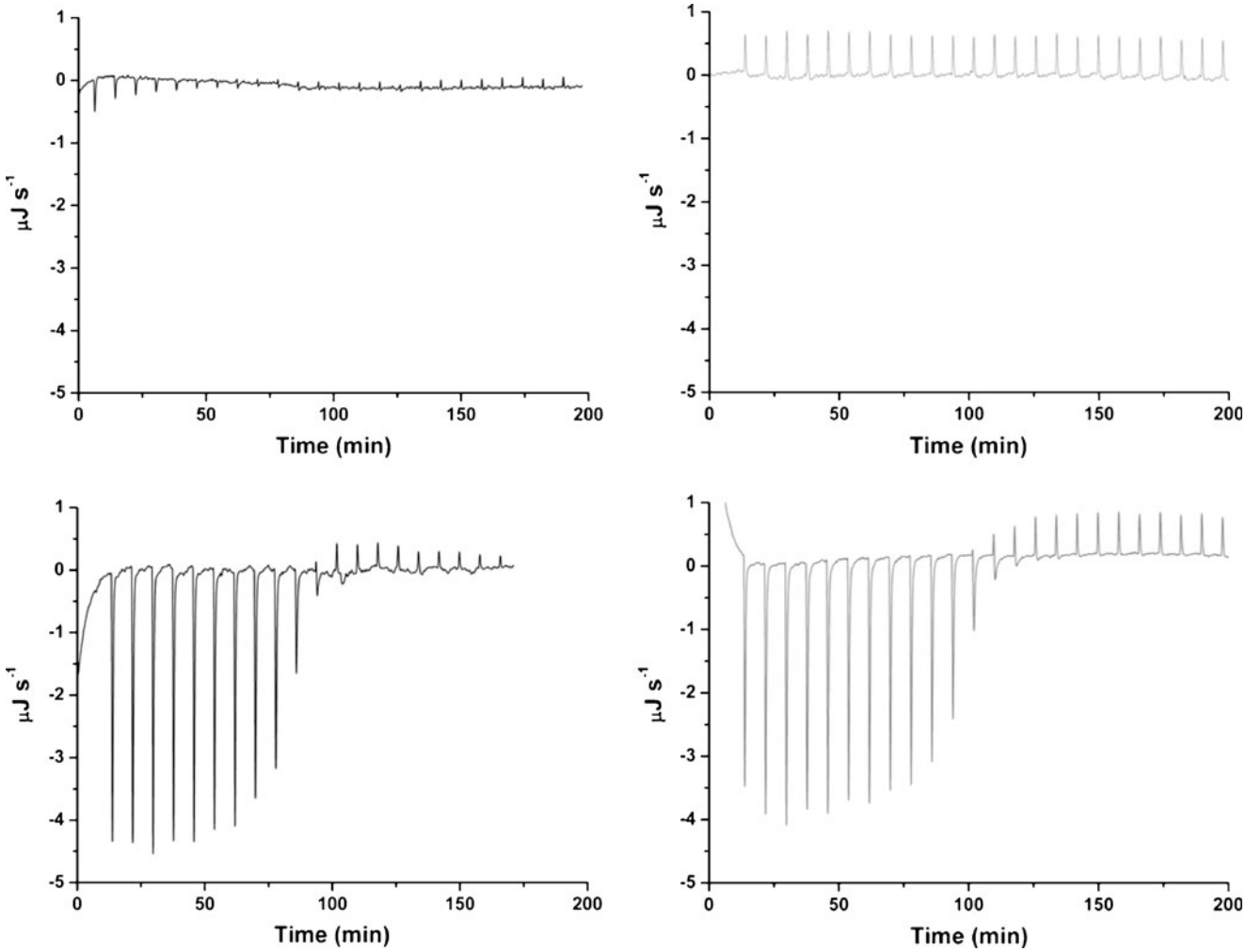

Fig. 5 Raw heat signals from isothermal titration calorimetric experiments obtained at 25°C. Top: titration of 5 μL aliquots of 300 μM insulin (-) and 300 μM acylated insulin (-) into 10 mM NaH_2PO_4 + 1 mM EDTA, pH 7.4. Bottom: titration of 5 μL aliquots of 300 μM insulin (-) and 300 μM acylated insulin (-) into a suspension of polystyrene beads with a total surface area of 0.11 m^2 in the cell.

beads. Titration of insulin and acylated insulin into the suspension of polystyrene beads was accompanied by exothermic heat flows, which after a few injections decreased gradually until a small endothermic heat flow was observed following an injection. A steady heat flow was obtained after 13 injections for insulin, whereas it was obtained after 16 injections for acylated insulin. Both the enthalpogram for insulin and acylated insulin were s-shaped (Fig. 6).

In 10 mM NaH_2PO_4 pH 7.4, s-shaped enthalpograms were also observed for insulin and acylated insulin titrations into polystyrene beads (Fig. 7). The enthalpogram for insulin was similar to the enthalpogram obtained in 10 mM NaH_2PO_4+1 mM EDTA pH 7.4. The enthalpogram for titration of acylated insulin into the suspension of polystyrene beads in 10 mM NaH_2PO_4 + pH 7.4 was accompanied by a lower exothermic heat flow than upon titration into the suspension of polystyrene beads in 10 mM NaH_2PO_4+ 1 mM EDTA pH 7.4. The heat flow signals for blank titration of insulin and acylated insulin, respectively, into 10 mM NaH_2PO_4 pH 7.4 were similar to the heat flows observed for titrations into 10 mM NaH_2PO_4+1 mM EDTA (data not shown).

DISCUSSION

Due to the self-association behavior of insulin and acylated insulin, their adsorption behaviors were compared in two buffer systems. First, in a 10 mM NaH_2PO_4+1 mM EDTA pH 7.4 buffer, EDTA binds the Zn^{2+} ions and thereby prevents hexamer formation, resulting in a solution containing only monomers and dimers. Using the dimer association constant 7.5×10^5 M^{-1} (26) for human insulin at pH 7.0, the monomer-dimer distribution at 300 μM and 8 μM is calculated to 10:90 and 50:50, respectively. Below 1 μM, the solution will be dominated by monomers. Near-

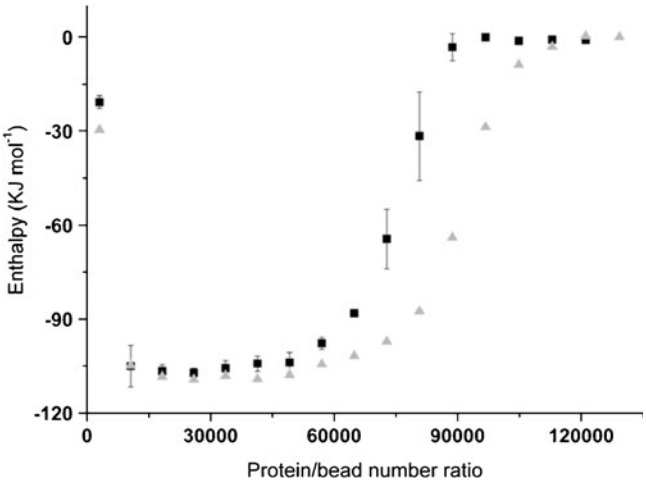

Fig. 6 Isothermal titration calorimetric data for the titration of 300 μM insulin (■) and 300 μM acylated insulin (▲) into a suspension of polystyrene beads in 10 mM NaH_2PO_4 + 1 mM EDTA, pH 7.4 at 25°C. The raw heat signal peaks have been integrated to obtain the enthalpy change per mole of protein injected. The total surface area of the polystyrene beads in the cell was 0.11 m^2.

UV CD and NMR on insulin and acylated insulin has shown that the acyl side-chain interferes with protein-protein recognition in the dimer interface, thereby increasing the dimer dissociation constant of acylated insulin of one order of magnitude or more compared to insulin (18). In the second buffer, 10 mM NaH_2PO_4 pH 7.4, both proteins will be present as monomer-dimer-hexamer equilibriums. In a solution of bovine insulin containing 2 Zn^{2+} ions per insulin hexamer and at pH 7.0, more than 75% of the total insulin has been shown to be hexameric at a concentration above 17 μM (27).

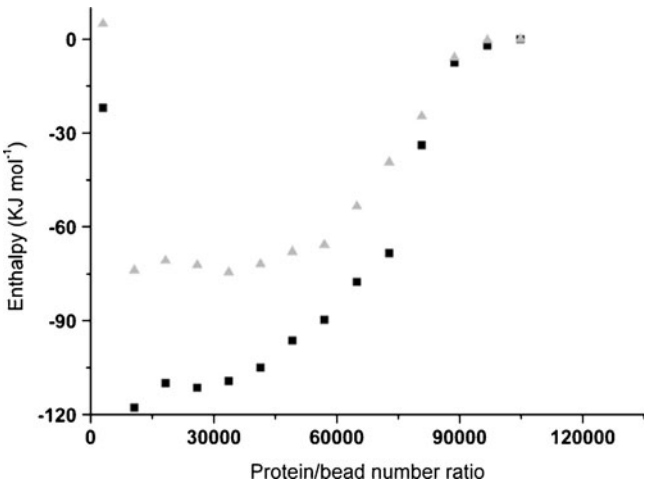

Fig. 7 Isothermal titration calorimetric data for the titration of 300 μM insulin (■) and 300 μM acylated insulin (▲) into a suspension of polystyrene beads in 10 mM NaH_2PO_4, pH 7.4 at 25°C. The raw heat signal peaks have been integrated to obtain the enthalpy change per mole of protein injected. The total surface area of the polystyrene beads in the cell was 0.11 m^2.

Adsorption from Solutions Containing Monomers and Dimers

Driving Forces and Reversibility

After attachment to a surface, protein molecules may form numerous contacts with a sorbent surface (28), likely leading to high-affinity adsorption. Both insulin and acylated insulin reached adsorption saturation at concentrations above approximately 12 μM (Fig. 4) under conditions of electrostatic repulsion. This indicates that both proteins have a high affinity for the polystyrene beads and that hydrophobic interactions are the dominating driving force for adsorption, which is in accordance with previous findings for adsorption of insulin to hydrophobic Teflon beads, a hydrophobic PTFE-like surface and a hydrophobic methylated silica surface (9–11). This observation is further supported by the slight negative slope observed for ΔH *versus* temperature (ΔC_p), which points to the hydrophobic effect as a driving force for the absorption (data not shown) (29).

For most proteins adsorbing to a surface with high affinity, a portion of the protein molecules adsorb irreversibly toward dilution (30), while another fraction of the protein molecules are only loosely adsorbed above monolayer coverage. Such loosely adsorbed protein molecules are expected to be only partly in contact with the surface or other adsorbed protein molecules, and they may be removed by dilution with buffer (31,32). The slight desorption of acylated insulin molecules revealed by the desorption isotherm indicates that only loosely adsorbed acylated insulin molecules were removed by dilution with 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4, thereby indicating that acylated insulin adsorbs irreversibly to the hydrophobic polystyrene particles. No desorption of insulin could be measured upon dilution with 10 mM NaH_2PO_4 + 1 mM EDTA pH 7.4, showing that insulin adsorbs irreversibly to the hydrophobic polystyrene particles. This is in accordance with previous findings for adsorption of insulin to hydrophobic Teflon beads and hydrophobic PTFE-like surfaces (9,10). It is possible that more acylated insulin molecules than insulin molecules adsorb loosely, due to interactions between the fatty acid chains of adjacent molecules.

Adsorbed Amounts

The plateau values for the adsorption isotherms after desorption were $2.4 \pm 0.3 \times 10^{-4}$ mol/m^2 (1.4 mg/m^2) and $3.0 \pm 0.2\ 10^{-4}$ mol/m^2 (1.8 mg/m^2) for insulin and acylated insulin, respectively (Fig 4). The amount of insulin molecules adsorbing per unit surface area to different hydrophobic surfaces has been reported to be between 0.8 and 1.7 mg/m^2 (9–11,31). It has been proposed that at higher protein concentration in solution, a surface becomes

covered more quickly, allowing less time for the molecules to relax at the surface (33). This may affect the orientation and the conformation of the adsorbed protein molecules and thereby the amount of molecules adsorbing per unit surface area (34). Therefore, the estimated amount of protein molecules adsorbing per unit surface area tends to depend on the experimental setup. In addition, the adsorption plateau value of a protein will depend on the hydrophobicity of the surface (35). Therefore, the adsorption plateau value for insulin of 1.4 mg/m^2, obtained from our adsorption isotherm (Fig. 4), corresponds well with the previous reported values of 0.8–1.7 mg/m^2.

ITC data have by several authors been fitted to reversible isotherm equations. However, other authors have pointed out that the fitting of experimental data on equilibrium amounts of adsorbed protein to reversible isotherm equations is questionable for an irreversible adsorbed amount of protein (34,36). As it is evident from our adsorption isotherms that the adsorption of both insulin and acylated insulin is irreversible, the ITC data have not been fitted. From the ITC experiments, it was observed that 16 injections of acylated insulin were needed to reach saturation in the ITC cell, whereas only 13 injections of insulin were needed (Fig. 6). This indicates that acylation increases the number of insulin molecules adsorbing per unit surface area and is in accordance with the findings from the adsorption isotherms. Several parameters can influence the number of protein molecules adsorbing per unit surface area, including the size, charge and hydrophobicity of the protein. The molar mass of acylated insulin is 5917 Da compared to the molar mass of 5808 Da for insulin. Assuming that the higher molar mass of acylated insulin is synonymous with a bigger size, acylation of insulin would be expected to decrease the number of insulin molecules adsorbing per unit surfaces area. A structurally stable protein is less prone to undergo structural changes upon adsorption than a more structurally labile protein and is therefore likely to cover a smaller surface area (37). Acylation has been shown to increase the Gibbs free energy of unfolding for acylated insulin (18) and may therefore lead to a closer packing of acylated insulin. It is also possible that it is the fatty acid chain of acylated insulin that interacts with the hydrophobic polystyrene beads and that an acylated insulin molecule adsorbed in this manner will take up less surface area than an insulin molecule.

Acylated insulin is likely to have a higher net negative charge than insulin, because the fatty acid chain is attached to the Lys^{B30} side chain, which is positively charged in insulin at pH 7.4. The higher negative charge will decrease the affinity of acylated insulin towards the hydrophobic and partly negatively charged polystyrene beads. However, acylation increases the hydrophobicity of insulin and is therefore likely to increase the affinity of insulin for the hydrophobic polystyrene beads. Since the major driving force for the adsorption is hydrophobic interactions, the contribution from the increased hydrophobicity should dominate over the increased electrostatic repulsion due to difference in charge.

Adsorption from Solutions Containing Monomers, Dimers and Hexamers

The enthalpogram obtained for titration of insulin into a suspension of hydrophobic polystyrene beads in 10 mM NaH_2PO_4+1 mM EDTA pH 7.4 was similar to the enthalpogram obtained for titration of insulin into a suspension of hydrophobic polystyrene particles in 10 mM NaH_2PO_4 pH 7.4 (Figs. 6 and 7). This indicates that the presence of 2 Zn^{2+} ions per hexamer did not affect the number of insulin molecules adsorbing per unit surface area, which is in accordance with a previous study where the adsorbed amounts of Zn-free human insulin and human insulin containing 2 Zn^{2+} ions per hexamer were compared by ellipsometry (11). In contrast, fever injections of acylated insulin were needed to obtain saturation in the ITC experiment in 10 mM NaH_2PO_4 pH 7.4 as compared to the ITC experiment in 10 mM NaH_2PO_4+1 mM EDTA pH 7.4 (Figs. 6 and 7). This indicates that the adsorption of acylated insulin to hydrophobic beads is influenced by the presence of free Zn^{2+} ions and thereby influenced by the association behavior of acylated insulin. For the ITC experiments in 10 mM NaH_2PO_4 pH 7.4, both insulin and acylated insulin reached saturation after 13 injections, indicating that the same amount of insulin and acylated insulin molecules adsorb to the hydrophobic polystyrene beads per unit surface area. The same amount of insulin and acylated insulin molecules has also been found to adsorb to hydrophobic gold surface in 10 mM NaH_2PO_4 pH 7.4 (see Supplementary Material I).

Due to the hydrophobic character of the fatty acid chain, acylation will increase the hydrophobicity of insulin. It is mainly the nonpolar residues of the insulin monomer that are involved in the association into dimers and hexamers, and the surface of the hexamer is almost entirely polar (8). In solutions containing acylated insulin hexamers, two acylated insulin hexamers can form di-hexamer, in which three fatty acid chains from each of two adjacent hexamers interact (38) and thereby shield the fatty acid chains from the water. Therefore, the differences in hydrophobicity of insulin and acylated insulin will be smaller in a solution containing monomers, dimers and hexamers than in a solution containing only monomers and dimers. We suggest that the decreased amount of acylated insulin molecules adsorbing per unit surface area in the presence of Zn^{2+} ions is a result of the decreased hydrophobicity of the acylated

insulin due to the hexamer and di-hexamer formation. It cannot be ruled out that the decreased hydrophobicity of insulin due to hexamer formation also affects the adsorbed amount of insulin. However, this could not be measured with the ITC setup used.

CONCLUSION

Insulin and acylated insulin were observed to adsorb with high affinity to hydrophobic polystyrene beads, and hydrophobic interactions seemed to be the major driving forces for the adsorption. The effect of acylation on the amount of insulin molecules adsorbing per unit surface area to hydrophobic polystyrene beads was dependent on the association degree of insulin. Upon adsorption from solutions containing monomeric and dimeric insulin and acylated insulin, respectively, acylation increased the number of insulin molecules adsorbing per unit surface area. In contrast, no differences in the adsorbed amounts of insulin and acylated insulin per unit surface area were detected upon adsorption from solutions of the two proteins containing monomers, dimers and hexamers. We suggest that this difference in the effect of acylation on the amount of insulin molecules adsorbing per unit surface area is due to a greater difference in hydrophobicity between monomeric insulin and acylated insulin than between the hexameric forms of these two proteins.

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