Albumin binding of insulins acylated with fatty acids: characterization of the ligand–protein interaction and correlation between binding affinity and timing of the insulin effect *in vivo*

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Albumin is a multifunctional transport protein that binds a wide variety of endogenous substances and drugs. Insulins with affinity for albumin were engineered by acylation of the ε-amino group of Lys^{B29} with saturated fatty acids containing 10–16 carbon atoms. The association constants for binding of the fatty acid acylated insulins to human albumin are in the order of 10^4–10^5 M^{-1}. The binding apparently involves both non-polar and ionic interactions with the protein. The acylated insulins bind at the long-chain fatty acid binding sites, but the binding affinity is lower than that of the free fatty acids and depends to a relatively small degree on the number of carbon atoms in the fatty acid chain. Differences in affinity of the acylated insulins for albumin are reflected in the relative timing of the blood-glucose-lowering effect after subcutaneous injection into rabbits. The acylated insulins provide a breakthrough in the search for soluble, prolonged-action insulin preparations for basal delivery of the hormone to the diabetic patient. We conclude that the biochemical concept of albumin binding can be applied to protract the effect of insulin, and suggest that derivatization with albumin-binding ligands could be generally applicable to prolong the action profile of peptide drugs.

INTRODUCTION

The therapeutic applicability of a biologically active peptide depends on the possibility of delivering it at its site of action with a suitable time-profile. Peptide and protein drug delivery is associated with several problems [1]. For example, peptides must in general be administered by injection because they are susceptible to enzymic breakdown and penetrate poorly through mucosal membranes. Furthermore, most peptides have a short half-life within the circulation and must be gradually released into the bloodstream to have a sustained effect. Advanced controlled-delivery systems for peptides, such as pumps, liposomes and microspheres, have not proved successful [2].

Insulin is a peptide hormone that has been in clinical use for decades. To meet the requirement for a constant basal supply of the hormone, diabetic patients receive daily subcutaneous injections of long-acting insulin suspensions [3]. In recent years, much effort has been devoted to the development of soluble, long-acting insulin analogues with a more reproducible and a more prolonged effect than the insulin suspensions [4–6], but no analogues have shown improved clinical results.

Albumin is a multifunctional transport protein that binds reversibly a wide variety of endogenous substances and drugs [7–10]. Owing to the restricted passage of albumin–drug complexes across membranes, the pharmacokinetic parameters of many drugs can be altered by modification of their affinity for albumin [11,12]. To test whether albumin binding can be applied to protract the effect of peptide drugs, we have engineered insulin derivatives with affinity for albumin by acylation of the hormone with fatty acids (Figure 1). In the present study we investigate the interaction between the fatty acid acylated insulins and human serum albumin (HSA), using albumin immobilized on agarose. Differences in affinity of acylated insulins for albumin are correlated to the relative degree of protraction of the insulins in rabbits. The suitability of using albumin binding to protract insulin action is discussed.

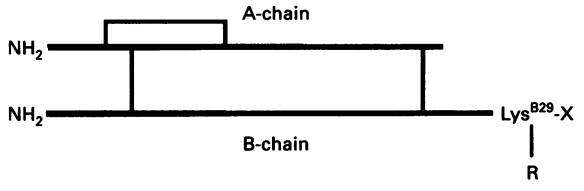

Figure 1 Schematic representation of the fatty acid acylated insulins

R denotes the fatty acid attached by an amide bond to the ε-amino group of Lys^{B29}. In the present study we have prepared derivatives of des-(B30) human insulin (X is deleted) in which R has from 10 to 16 carbon atoms, and analogues of human insulin (X is Thr) in which R has 10 or 14 carbon atoms.

EXPERIMENTAL

Materials

Fatty acid-free (< 0.005%) and globulin-free HSA purchased from Sigma were used throughout this study. Fatty acids of analytical grade were from Fluka AG (Switzerland) or Aldrich (Germany). Divinylsulphone-activated Sepharose 6B (Mini-Leak Low) was obtained from Kem-En-Tec A/S (Copenhagen, Denmark). Insulin Protaphane (NPH-insulin) and human insulin were supplied by Novo Nordisk A/S (Denmark). Des-(B30) human insulin was prepared as previously described [13]. [9,10-^{3}H]Myristic acid (33.5 Ci/mmol) was supplied as an ethanolic

Abbreviations used: Boc, t-butyloxycarbonyl; HSA, human serum albumin; NPH-insulin, Neutral Protamine Hagedorn insulin (Insulin Protaphane); Tris/TX-100, 0.1 M Tris/HCl, pH 7.4, containing 0.025% (v/v) Triton X-100; RA, relative receptor affinity.

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solution (1 mCi/ml) from DuPont NEN. All other chemicals used were of reagent grade or better.

Protein concentrations were determined by UV absorbance, taking molar absorption coefficients of ϵ_{279} 35.7×10^3 $M^{-1} \cdot cm^{-1}$ for HSA [7] and ϵ_{276} 6.2×10^3 $M^{-1} \cdot cm^{-1}$ for the insulins.

Preparation of fatty acid acylated insulins

Human insulin and des-(B30) human insulin were treated with di-t-butyldicarbonate in DMSO/triethylamine (20:1, v/v), and the Gly^{A1}, Phe^{B1} di-Boc (where Boc represents t-butyloxycarbonyl) insulins were separated from the isomers by reversed-phase HPLC. The hydroxysuccinimide esters of fatty acids were prepared from fatty acids and *N*-hydroxysuccinimide by using dicyclohexylcarbodiimide in dimethylformamide for ester formation, and ethanol for recrystallization of the products. The ϵ-amino group of Lys^{B29} was selectively acylated by treatment of Gly^{A1}, Phe^{B1} di-Boc insulin with the fatty acid hydroxysuccinimide esters in dimethylformamide/DMSO (1:7, v/v) at 15 °C, using 20 equivalents of a tertiary amine such as 4-methylmorpholine. The protecting groups were removed with trifluoroacetic acid and the Lys^{B29}-acylated insulins were purified by reversed-phase HPLC. The identity of the fatty acid acylated insulins was confirmed by mass spectrometry, determining the molecular mass of the insulin derivative, of the B-chain after treatment with dithiothreitol, and of the B-chain fragment containing Lys^{B29} obtained by treatment with *Staphylococcus aureus* protease.

Mono ^{125}I-(Tyr^{A14})-labelled insulins were prepared as previously described [14].

Immobilization of HSA

HSA was coupled to the activated agarose matrix (Mini-Leak Low) according to the guidelines of the manufacturer. For each gram of gel was added 2 ml of 5% (w/v) HSA and 2 ml of 30% (w/v) PEG 20000 in 0.3 M $NaHCO_3$, pH 8.6. The suspension was gently agitated overnight at 23 °C. Excess active groups were blocked by treatment with 30 mM ethanolamine at pH 9.0 for 5 h at 23 °C. The molar content of HSA in the gel was 0.4–0.6 nmol per mg suction-dried gel as determined by titration with HSA in solution as described below. At pH 8.6, amino groups and thiol groups can be coupled to the activated matrix, whereas hydroxyl groups react at pH > 10 [15]. Because HSA contains 59 lysine residues and a single free cysteine [7], the coupling is expected to involve primarily ϵ-amino groups of lysine residues.

Equilibration of ligands with immobilized HSA

Immobilized HSA was washed with 2–3 volumes of 0.1 M Tris, pH 7.4, on a suction filter and drained until cracks were seen in the gel. A portion of the gel was weighed out and suspended in 0.1 M Tris, pH 7.4, containing 0.025% (v/v) Triton X-100 (Tris/TX-100). The concentration of immobilized HSA in the suspension was less than 40 mg/ml. The exclusion volume provided by the Mini-Leak matrix (less than 4% of the total volume) is considered to be insignificant. Portions of the suspension were pipetted into vials during stirring and combined with the radiolabelled ligand and Tris/TX-100 to give a final volume of 1.00 ml. When binding was examined in the presence of fatty acid or albumin in solution, these components were included in the mixture before Tris/TX-100 was added to the final volume of 1.00 ml. The concentration of radioactivity in the final mixture was 0.01–0.05 μCi/ml. The vials were rotated at 30 rev./min for 2 h at room temperature and centrifuged for 5 min at 1800 *g*. Incubation for 2 h was found to be sufficient to reach equilibrium.

When ^{125}I-labelled ligands were used, the total radioactivity in each vial (T) and the radioactivity in 500 μl of supernatant ($\frac{1}{2}F$) were counted on a Packard Cobra Auto-Gamma instrument (Packard, Meriden, U.S.A.). The bound radioactivity (B) was determined as $B = T - F$.

When ^{3}H-labelled myristic acid was used, 500 μl of supernatant was transferred to a scintillation counting vial, 10 ml Ultima Gold (Packard) was added, and the radioactivity ($\frac{1}{2}F$) was counted on a Packard Tri-Carb liquid scintillation analyser. In this case, the total radioactivity in each vial was estimated from a measurement of the radioactivity ($\frac{1}{2}T$) in 500 μl of a mixture treated as described above but in the absence of immobilized albumin.

No unspecific binding was seen when radiolabelled acylated insulins were incubated as described above with Mini-Leak Low blocked with ethanolamine. Triton X-100 was included in the buffer to prevent non-specific binding of the acylated insulins to vials and pipettes. By varying the concentration of the detergent between 0 and 0.025% (v/v) it was found that 0.025% (v/v) Triton X-100 prevented non-specific binding without affecting the binding to albumin.

Titration of immobilized HSA for binding of fatty acids

A weighed amount (about 25 mg/ml) of immobilized HSA (HSA_{imm}) was equilibrated with 1.5 nM [9,10-^{3}H]myristic acid and varying concentrations (1–40 μM) of HSA in solution (HSA_{free}). As HSA_{free} is present in large molar excess over [9,10-^{3}H]myristic acid, a plot of F/B against $[HSA_{free}]$ is linear. Assuming that the binding constants to HSA_{imm} and HSA_{free} are equal, the slope of the line is $1/[HSA_{imm}]$. The molar content of HSA per mg of suction-dried gel is calculated from the amount of gel in the suspension (about 25 mg/ml) and $[HSA_{imm}]$. In one case, the albumin content of a hydrolysed sample of Mini-Leak HSA was determined by amino acid analysis. The amino acid analysis showed a 20% higher albumin content than found by titration, indicating that the binding properties of HSA are not completely unaffected by immobilization. It has previously been shown that a similar coupling of albumin to agarose does not alter the primary binding sites for most ligands, including fatty acids [16].

Receptor affinity

Relative affinities of ^{125}I-(Tyr^{A14})-labelled insulins for the soluble insulin receptor were determined by a modification of a previously described assay [17]. In brief, the soluble insulin receptor was immobilized on Mini-Leak to a concentration of 1 μM in the gel. Various concentrations (0–20 nM) of immobilized receptor were equilibrated with 10 pM of the radiolabelled insulin in a binding buffer (pH 7.8) containing 0.1 M Hepes, 0.1 M NaCl, 0.01 M $MgCl_2$ and 0.025% Triton X-100 for 2 h at 23 °C. Bound tracer was isolated by centrifugation and the relative receptor affinities (RA) were calculated from a plot of bound insulin against receptor concentration as described elsewhere [17]. Human insulin represents the RA of 100%.

Animal experiments

Studies in rabbits were performed on non-diabetic, fasted, male New Zealand White rabbits, 0.5–3 years of age and weighing 2.5–3.5 kg, receiving subcutaneous injections of either an acylated insulin or NPH-insulin. At least 1 h before dosing, the rabbits were fixed in pillories. Acylated insulins were given as aqueous solutions containing 600 nM (100 units/ml) of insulin. The dose

was 12 nmol of insulin per animal. Blood samples were drawn before and 1, 2, 4 and 6 h after injection. Glucose analysis was performed by the hexokinase method [18].

Euglycaemic glucose clamps were carried out in non-diabetic, conscious, female pigs, cross-bred from Danish Landrace, Yorkshire and Durok, 4–5 months of age and weighing 70–95 kg. Before the experiments the pigs were fasted overnight, for 18 h. Two catheters were inserted in the jugular veins, one for glucose infusion and one for blood sampling. The pigs were free to move in their pens during the clamp period. Five pigs received NPH-insulin and Lys^{B29}-tetradecanoyl, des-(B30) insulin in random order with an interval of 10 days. The dose was 216 nmol of insulin. The pigs were kept euglycaemic at their individual fasting glucose levels by infusion of a glucose solution (270 g/l) at a variable rate. Depending on changes in plasma glucose concentration obtained during frequent plasma glucose monitoring, the necessary adjustments of the glucose infusion were made empirically. Blood samples were collected in heparinized glass tubes every 15 min, plasma was separated, and glucose was determined within 1.5 min of blood sampling with a YSI (Yellow Springs Instruments) glucose analyser (glucose oxidase method).

RESULTS

Binding of acylated insulins to immobilized HSA

The interaction between albumin and insulins acylated with fatty acids at the ε-amino group of Lys^{B29} was studied using albumin immobilized on agarose. This method was chosen because the size of the ligands precludes performing binding studies by dialysis. Typical plots for binding of the insulins to immobilized HSA are shown in Figure 2. Scatchard plots are not linear, as exemplified in the inset to Figure 2, indicating that binding occurs at more than one class of sites. The initial part of the Scatchard plot is consistent with binding of at least 1 mol of Lys^{B29}-tetradecanoyl, des-(B30) insulin per mol of HSA with high affinity. The capacity of albumin for binding the insulins apparently exceeds 5 mol/mol, without any indication of saturation within the tested concentration range. However, the interpretation of binding data obtained at insulin concentrations above 10 μM is difficult owing to extensive insulin self-association and to the relatively low solubility of the acylated insulin analogues in the applied buffer.

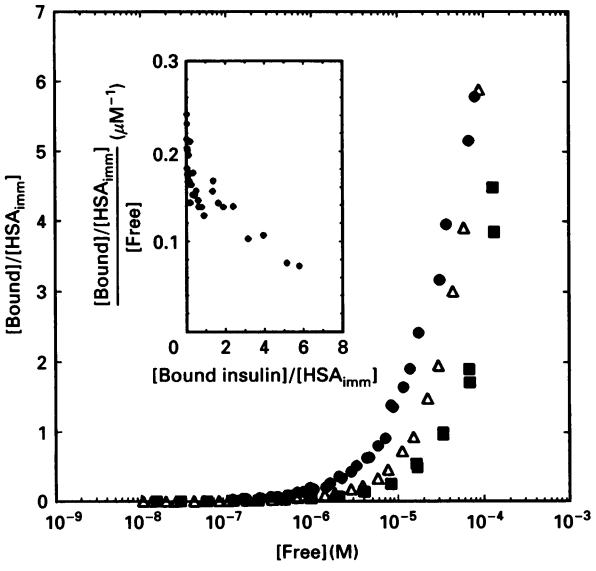

Figure 2 Binding of fatty acid acylated insulins to immobilized HSA

Various concentrations (0–150 μM) of ^{125}I-(Tyr^{A14})-labelled insulins were equilibrated with 5 μM of immobilized HSA at 23 °C. ■, Lys^{B29}-decanoyl, des-(B30) insulin; △, Lys^{B29}-dodecanoyl, des-(B30) insulin; ●, Lys^{B29}-tetradecanoyl, des-(B30) insulin. Inset: Scatchard plot of the data for Lys^{B29}-tetradecanoyl, des-(B30) insulin.

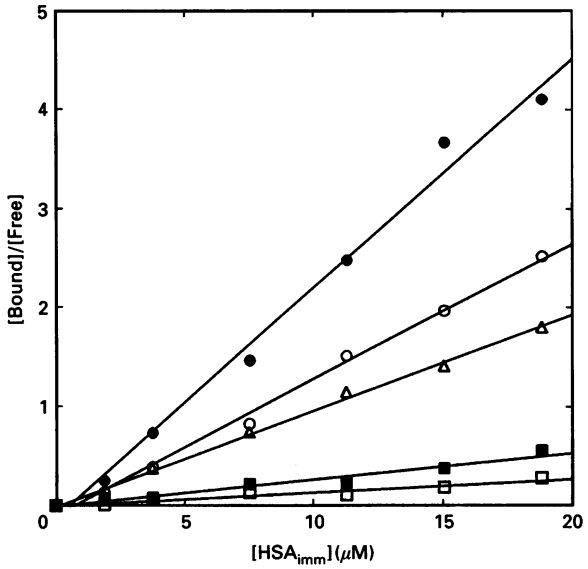

Figure 3 Plots of bound/free insulin concentration ratios against HSA_{imm} concentration

^{125}I-(Tyr^{A14})-labelled insulins (2.5 nM) were equilibrated with 1–20 μM immobilized HSA at 23 °C. □, Lys^{B29}-decanoyl insulin; ■, Lys^{B29}-decanoyl, des-(B30) insulin; △, Lys^{B29}-dodecanoyl, des-(B30) insulin; ○, Lys^{B29}-tetradecanoyl insulin; ●, Lys^{B29}-tetradecanoyl, des-(B30) insulin.

To quantify and compare the affinities of the fatty acid acylated insulins for HSA, ^{125}I-labelled insulins were equilibrated at 2.5 nM with various concentrations (1–20 μM) of immobilized HSA. At these experimental conditions less than 0.25% of the albumin is occupied by an insulin molecule, and the first association constant, K_a, for binding of insulin to albumin can be estimated by:

$$K_a = \frac{B}{F}\frac{1}{[HSA_{imm}]}$$

where B/F is the ratio between bound and free ^{125}I-labelled insulin and $[HSA_{imm}]$ is the total concentration of immobilized albumin [19]. Plots of B/F against $[HSA_{imm}]$ are linear with slopes that estimate K_a. Representative plots are shown in Figure 3. The K_a for binding Lys^{B29}-tetradecanoyl, des-(B30) insulin to HSA is determined as 2.4×10^5 M^{-1} by this approach. This value seems to be in good agreement with the estimate of K_a obtained by extrapolation of the Scatchard graph (Figure 2, inset) to the intercept with the ordinate axis [20,21]. Lys^{B29}-acylated des-(B30) insulins have higher affinities for albumin than their full B-chain counterparts. The increase in $-\Delta G$ for binding obtained by deletion of Thr^{B30} is about 0.35 kcal/mol for the decanoyl and tetradecanoyl derivatives (Figure 4). The location of the C-terminal carboxylate group closer to the lipophilic side chain may favour formation of an ionic bond to a basic residue at the binding site. K_a for binding the Lys^{B29} acylated des-(B30)-insulins to HSA rises from 0.28×10^5 M^{-1} to 2.4×10^5 M^{-1} when the

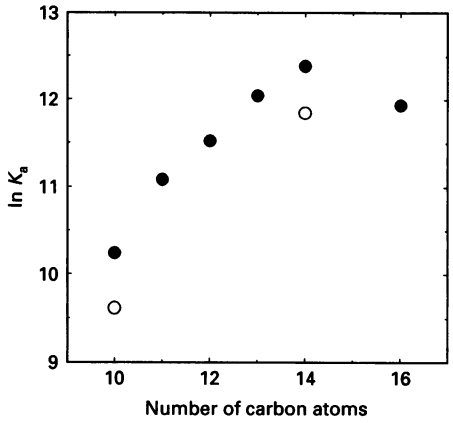

Figure 4 Correlation between K_a for binding of fatty acid acylated insulins to immobilized HSA and the number of carbon atoms in the fatty acid side chain

K_a was estimated as the slope of linear plots of B/F against [HSA_{imm}] as shown in Figure 3. ●, Lys^{B29}-acylated, des-(B30) insulins; ○, Lys^{B29}-acylated insulins with full B-chain. The points shown are the means of at least two determinations.

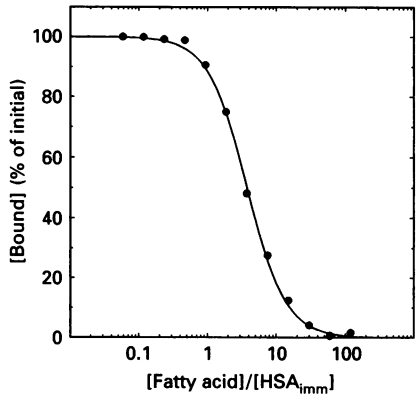

Figure 5 Displacement of Lys^{B29}-tetradecanoyl, des-(B30) insulin from immobilized HSA with lauric acid

^{125}I-(Tyr^{A14})-labelled Lys^{B29}-tetradecanoyl, des-(B30) insulin (2.5 nM) was equilibrated with 10.1 μM Mini-Leak HSA in the presence of 0.6 μM to 2.5 mM lauric acid at 23 °C.

number of carbon atoms in the acyl chain is increased from 10 to 14, reflecting that non-polar interactions contribute to the binding. The gain in $-\Delta G$ for binding obtained by extension of the fatty acid chain with one methylene group is 0.2–0.5 kcal/mol (Figure 4), which is in the region of one-half that reported for the long-chain free fatty acids [22]. Extension of the chain length to 16 carbon atoms does not increase the binding affinity any further. We note that human insulin *per se* does not bind with detectable affinity to immobilized HSA ($K_a < 10^3$ M^{-1}).

Competition with fatty acids

The influence of fatty acids on the binding of Lys^{B29}-tetradecanoyl, des-(B30) insulin to HSA is shown in Figure 5. A significant displacement of the insulin occurs in the presence of more than 1 mole of lauric acid per mol of HSA. Thus the trace amount of radiolabelled Lys^{B29}-tetradecanoyl, des-(B30) insulin binds with lower affinity when the first binding site for fatty acid

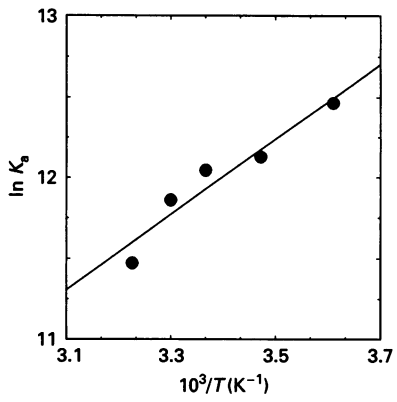

Figure 6 Van't Hoff plot for the binding of Lys^{B29}-tetradecanoyl, des-(B30) insulin to immobilized HSA

^{125}I-(Tyr^{A14})-labelled Lys^{B29}-tetradecanoyl, des-(B30) insulin (2.5 nM) was equilibrated with 1–10 μM immobilized HSA at various temperatures (4–37 °C). The buffer was 5 mM Tris, pH 7.4, containing 100 mM NaCl and 0.025% (v/v) Triton X-100. K_a was estimated as the slope of linear plots of B/F against [HSA_{imm}] as shown in Figure 3. The points shown are the means of four determinations.

is occupied. A 50% displacement is obtained at a fatty acid to HSA_{imm} concentration ratio of about 4, and the insulin derivative is quantitatively displaced as this ratio approaches 100. The results suggest that the fatty acid acylated insulin competes with long-chain fatty acids for binding at common sites. Alternatively the displacement of the acylated insulins by laurate might be due to a conformational change.

Temperature dependence and thermodynamic parameters

The temperature dependence of the association constant for binding of Lys^{B29}-tetradecanoyl, des-(B30) insulin to HSA is shown in Figure 6. K_a decreases with increasing temperature in the range 4–37 °C, in agreement with previous results for the interaction between HSA and fatty acids [21]. The K_a for binding of this insulin analogue to HSA at 37 °C is 1×10^5 M^{-1}. Hence at an HSA concentration of 0.6 mM, corresponding to the albumin level in human plasma, the bound fraction is 98.4%. The almost linear correlation between $1/T$ and $\ln K_a$ allows us to estimate the enthalpy change that accompanies binding from the van't Hoff equation, $\ln K_a = \Delta H°/RT + \text{constant}$ [23]. The change in free energy and entropy at 298 K can be obtained from the equations $\Delta G° = -RT\ln K_a$ and $\Delta G° = \Delta H° - T\Delta S°$. The estimated values for $\Delta G°$, $\Delta H°$ and $T\Delta S°$ are −29.5, −19.4 and 10.1 kJ/mol, respectively, indicating that the association is driven by favourable changes in both enthalpy and entropy.

Effect profiles *in vivo*

The effect profiles of the fatty acid acylated insulins after subcutaneous injection into rabbits are shown in Figure 7(a). NPH-insulin, which is the most generally used long-acting insulin suspension in the treatment of diabetes, was given as a reference. The fall in blood glucose within the first hour after injection shows that the acylated insulins have a significantly slower onset of action than NPH-insulin. Furthermore, the time until maximal effect is generally longer for the acylated insulins than for NPH-insulin. However, this time cannot be determined for all insulins, because the blood glucose was not followed beyond 6 h. As shown in Figure 7b, the initial fall in blood glucose depends on

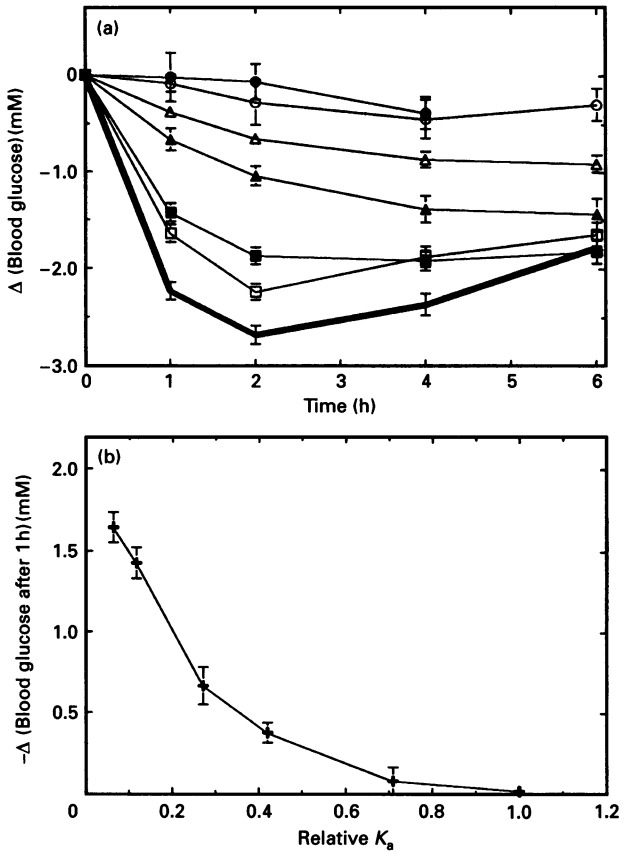

Figure 7 Blood-glucose-lowering effect of fatty acid acylated insulins after subcutaneous injection into rabbits

(**a**) □, Lys^{B29}-decanoyl insulin ($n = 24$); ■, Lys^{B29}-decanoyl, des-(B30) insulin ($n = 36$); ▲, Lys^{B29}-undecanoyl, des-(B30) insulin ($n = 12$); △, Lys^{B29}-dodecanoyl, des-(B30) insulin ($n = 18$); ○, Lys^{B29}-tridecanoyl, des-(B30) insulin ($n = 4$); ●, Lys^{B29}-tetradecanoyl, des-(B30) insulin ($n = 6$). The heavy line shows the effect of NPH-insulin ($n = 34$). Changes in blood glucose are given as means ± S.E.M. (**b**) Correlation between the relative affinities of the acylated insulins for HSA and the decrease in blood glucose 1 h after injection. Lys^{B29}-tetradecanoyl, des-(B30) insulin represents a relative affinity of 1.0.

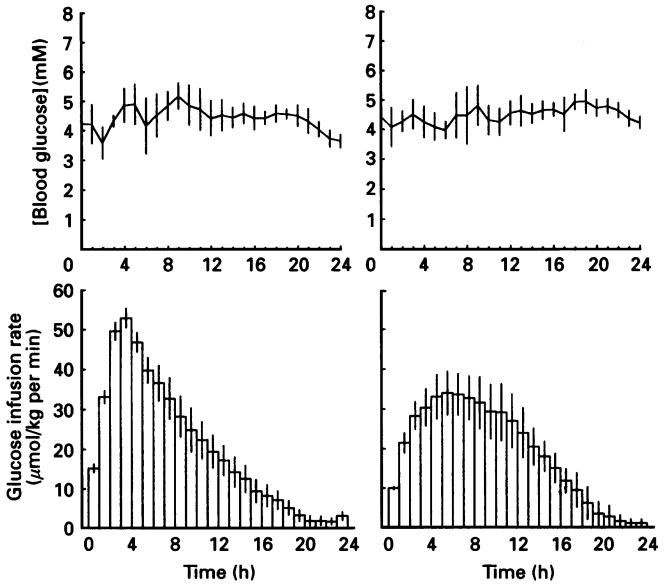

Figure 8 Euglycaemic glucose clamp after subcutaneous injection into pigs

Blood glucose levels (means ± S.D.; top) and glucose infusion rates (means ± S.E.M., $n = 5$; bottom) during euglycaemic glucose clamp after subcutaneous injection of 216 nmol NPH-insulin (left) and Lys^{B29}-tetradecanoyl, des-(B30) insulin (right) into pigs.

the affinity of the acylated insulin for albumin, suggesting that the protracted effect of the acylated insulins is due to albumin binding.

The receptor affinity relative to human insulin for Lys^{B29}-decanoyl insulin; Lys^{B29}-decanoyl, des-(B30) insulin; Lys^{B29}-dodecanoyl, des-(B30) insulin; and Lys^{B29}-tetradecanoyl, des-(B30) insulin was found to be 76%, 76%, 54% and 46% respectively; the RAs for Lys^{B29}-undecanoyl, des-(B30) insulin and Lys^{B29}-tridecanoyl, des-(B30) insulin were not determined. It has previously been shown that insulin analogues with 20–300% receptor affinity have the same biological potency *in vivo* [24]. To preclude the possibility that the diminished action on blood glucose is related to a decreased bioefficacy of the acylated insulins and to study the effect profile over a longer time course, we performed a 24 h euglycaemic glucose clamp after subcutaneous injection of 216 nmol Lys^{B29}-tetradecanoyl, des-(B30) insulin and NPH-insulin into pigs. The resulting profiles are shown in Figure 8. The cumulative glucose infusion (0–24 h) was the same after injection of Lys^{B29}-tetradecanoyl, des-(B30) insulin (2.5 ± 1.3 mol) and NPH-insulin (2.6 ± 0.6 mol), indicating that the two insulin preparations are equipotent *in vivo*. Both preparations resulted in a significant glucose consumption for at least 18 h. However, Lys^{B29}-tetradecanoyl, des-(B30) insulin showed a more protracted glucose utilization profile than NPH-insulin, the times to peak effect being 6.4 ± 2.2 h and 3.4 ± 0.2 h, respectively.

DISCUSSION

Derivatization of the insulin molecule with albumin-binding ligands provides a new approach to protracting the blood-glucose-lowering effect after subcutaneous injection of the hormone. Albumin is the most abundant protein in the extracellular fluid. The concentration of HSA in human plasma is about 0.6 mM [25], whereas the level in subcutaneous interstitial fluid is about 60% of that in plasma [12]. The protein binds a wide variety of endogenous substances and drugs with binding constants that are typically in the order of 10^4–10^6 M^{-1} for organic anions, and about 10^8 M^{-1} for long-chain fatty acids [7–10]. We have obtained insulins with affinities of 10^4–10^5 M^{-1} for HSA by acylation of the ε-amino group of Lys^{B29} with saturated fatty acids containing 10 to 16 carbon atoms.

Binding mechanism

The binding of aromatic anions and fatty acids to albumin occurs by a combination of hydrophobic and ionic interactions [26–30]. In accord with this general mechanism, the C-terminal carboxylate group and the acyl side chain of the fatty acid acylated insulins seem to take part in the interaction with HSA. Thus the interaction between albumin and fatty acid acylated des-(B30) insulins is believed to involve the fatty acid carbon chain and the side chain (1 nitrogen plus 4 carbon atoms), the α-carbon and the carboxylate group of Lys^{B29}. The binding site apparently cannot adapt sufficiently for further non-polar interactions with the ligand when the number of carbon atoms in the

fatty acid at Lys^{B29} increases beyond 14. Alternatively, accommodation of the C_{16} chain in the binding cavity might impede the favourable ionic interaction at the binding site. The K_a for binding of fatty acids to HSA similarly tends towards an upper limit, as an increase in carbon chain length from 16 to 18 carbon atoms results in a relatively small increase in K_a [22]. Studies with fatty acids containing a higher number of carbon atoms were not reported. The thermodynamic data for binding of Lys^{B29}-tetradecanoyl, des-(B30) insulin to HSA are compatible with the involvement of both ionic and hydrophobic interactions in binding of the ligand [21]. However, conformational changes in the protein can be expected to accompany ligand binding [10], and the interpretation of entropy and enthalpy changes is therefore speculative.

Binding site

The binding of a large number of aromatic anions to HSA seems to take place in two distinct binding pockets located in subdomains IIA and IIIA of the protein, respectively [26]. The binding sites for fatty acids are less well defined, but it seems that there are three primary binding sites for long-chain fatty acids, possibly located in subdomains IB, IIIA and IIIB [10,31], respectively, whereas a large number of fatty acid anions are bound with lower affinity [22,32]. The displacement of Lys^{B29}-tetradecanoyl, des-(B30) insulin from albumin after addition of one or more lauric acid equivalents to HSA provides strong evidence that the high-affinity binding of fatty acid acylated insulins occurs at a primary long-chain fatty acid binding site. Alternatively, binding of the acylated insulins might occur at a secondary fatty acid site, in which case the influence on insulin-binding of the first lauric acid equivalents could be ascribed to a fatty acid-induced conformational change in these binding sites. However, the first two long-chain fatty acids are generally believed to have little effect on binding at other sites of the albumin molecule [8,27,33,34].

The association constants for binding of the acylated insulins at the fatty acid binding sites of HSA are 1–3 orders of magnitude smaller than the first binding constants for the attached fatty acids themselves [22]. Furthermore, the K_a for binding of the fatty acid acylated insulins depends to a relatively small degree on the number of carbon atoms in the fatty acid side chain. Thus the changes in ligand structure caused by attachment of the free fatty acids to insulin have a significant influence on the binding properties. First, the binding of acylated insulins to albumin may be sterically hindered. Secondly, the presence of an amide function within the ligand may be unfavourable for binding owing to the polarity and diverging geometry of the amide bond relative to a carbon–carbon bond.

Protraction of insulin action by albumin binding

Insulin is secreted into the blood from the pancreatic beta-cells at a low basal rate in the fasting state and at a higher rate in response to the postprandial increase in the blood glucose level. To mimic the normal pattern of insulin release, a combination of rapid-acting and long-acting insulin preparations is used in the most intensive treatment of diabetes [3]. At present, a neutral solution of human insulin provides the rapid-acting component, whereas a prolonged action is obtained by injection of insulin suspensions of crystals with protamine or zinc. It has recently been convincingly shown that the onset of diabetic late complications can be delayed by intensive treatment with insulin [35]. To permit a tighter control of the blood glucose, it is desirable to develop rapid-acting insulins with a briefer effect than human insulin [36] and long-acting insulins with a smoother and more reproducible effect profile than the insulin suspensions [37]. The acylated insulins provide a breakthrough in the search for soluble, prolonged-action insulins for basal delivery of the hormone to diabetic patients. The mechanism of protraction is probably binding to albumin in the subcutaneous tissue, resulting in a lower absorption rate of the acylated insulins than of human insulin. Binding to albumin in plasma may increase the plasma half-life of the acylated insulins relative to that of human insulin and contribute to prolong the action profiles. The selection of a specific insulin derivative for drug development based on studies in a pig model will be discussed elsewhere.

As the acylated insulins compete with fatty acids for binding to human albumin, the plasma level of free fatty acids may influence the effect profile of the acylated insulins. In normal subjects, the molar ratio of free fatty acids to albumin varies between 0.5 and 1.0, depending on the nutritional state [38]. Provided that albumin is present in large excess over insulin, which is the case *in vivo*, the binding of acylated insulins is only affected to a minor degree by fluctuations in the free fatty acid level within this range. A transient high level of free fatty acids is expected to have a minor effect on the rate by which insulin is released into the bloodstream, because the absorption of unbound insulin occurs relatively slowly, i.e. with a half-time of 1–2 h [36].

Derivatization with albumin-binding ligands as described here may provide a general approach to prolonging the effect of peptide drugs. Owing to the linkage between binding affinity and degree of protraction, the action profile can be engineered by modification of the ligand structure. The knowledge of the crystal structure of human serum albumin and the possibility of performing X-ray analysis of albumin bound with ligands such as fatty acids [10,26] are likely to prove useful in the future design of peptide derivatives with an affinity for specific albumin-binding sites.

We thank Yvonne B. Madsen, Lene G. Andersen, Birgit D. Spon, Kate Müggler, Lene Drube, Lone Sørensen, Mette Dall and Erna Willert for excellent technical assistance; Dr. Per F. Nielsen for mass spectrometric analysis; and Dr. Asser S. Andersen and Dr. Thomas Kjeldsen for comments on the manuscript.

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Received 24 May 1995/2 August 1995; accepted 11 August 1995