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Investigations of albumin-insulin detemir complexes using molecular dynamics simulations and free energy calculations

Line A. Ryberg^{a*}, Pernille Sønderby^a, Jens T. Bukrinski^b, Pernille Harris^a, and Günther H. J. Peters^{a*}

^a Department of Chemistry, Technical University of Denmark, 2800 Kongens Lyngby, Denmark and ^b CMC assist ApS, 2500 Copenhagen, Denmark.

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ABSTRACT: Insulin detemir is a lipidated insulin analogue that obtains a half-life extension by oligomerization and reversible binding to human serum albumin. In the present study, the complex between a detemir hexamer and albumin is investigated by an integrative approach combining molecular dynamics (MD) simulations, molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) free energy calculations and dynamic light scattering (DLS) experiments. Recent reported small angle X-ray scattering data could not unambiguously resolve the exact binding site of detemir on albumin. We therefore applied MD simulations to deduce the binding site and key protein-protein interactions. MD simulations were started from initial complex structures based on the SAXS models and free energies of binding were estimated from the simulations by using the MM-PBSA approach for the different binding positions. The results suggest that the overlapping FA3-FA4 binding site (named FA4) is the most favorable site with a calculated free energy of binding of -28 ± 6 kcal/mol and a good fit to the reported SAXS data throughout the simulations. Multiple salt bridges, hydrogen bonds and favorable van der Waals interactions are observed in the binding interface that promote complexation. The binding to FA4 is further supported by DLS competition experiments with the prototypical FA4 ligand, ibuprofen, showing displacement of detemir by ibuprofen. This study provides information on albumin-detemir binding on a molecular level, which could be utilized in a rational design of future lipidated albumin-binding peptides.

INTRODUCTION

Human serum albumin is the most abundant protein in the circulatory system with a blood plasma concentration of 35-50 mg/mL and has a half-life of 19 days in the body due to recycling by the neonatal Fc receptor.¹ The biological function of albumin is manifold, and albumin plays an important role in the regulation of the colloidal osmotic pressure of the blood and in the transport of long-chain fatty acids, bilirubin², metal ions, and a great number of therapeutic drugs³⁻⁵. The pharmacokinetic properties of albumin makes it interesting in a pharmaceutical perspective as a platform for half-life extension of biopharmaceuticals.⁶ One of the major challenges for administering biopharmaceuticals and especially peptide-based drugs is their limited half-lives due to enzymatic degradation and clearance in the body by renal filtration.⁷ To overcome these shortcomings, one successful route is the covalent or non-covalent association to albumin, and hence, albumin has emerged as a versatile protein used in drug delivery of biopharmaceuticals. Lipidation is a successful strategy to obtain non-covalent albumin association that utilize albumin's natural affinity for fatty acids. Multiple products using this principle are available

on the market,^{8,9} for instance the insulin analogues, insulin detemir and insulin degludec, and the glucagon-like peptide 1 analogues, liraglutide and semaglutide. Apart from leading to albumin association, lipidation of the peptide-based drugs also leads to self-association, which additionally prolongs their half-lives. Although the self-association properties of the insulin analogues¹⁰⁻¹⁶ and liraglutide¹⁷ are well studied, their association to albumin has not been fully explored and hence, a molecular understanding of the complex formation has not been established. The focus of this study is on detemir's association to albumin.

While the albumin-detemir binding has been the topic of other studies, there is yet no consensus on the correct binding site. Kurtzhals *et al.*¹⁸ studied the binding between albumin and detemir and found that long chain fatty acids (>12C) displace detemir to a higher extent than octanoate, suggesting that detemir binds to a fatty acid binding site with preference for longer fatty acids. Kjeldsen *et al.*¹⁹ studied the interactions between detemir and the individual albumin domains I and III. The authors concluded that detemir binds to domain III and only weakly to domain I. Performing spectrofluorophotometric studies in combination with molecular docking, Fatima *et*

*al.*²⁰ proposed that detemir binds to albumin in-between domain I and III. The binding stoichiometry between albumin and detemir was studied by Havelund *et al.* in 2004²¹, and it was found that albumin binds to both dimeric and hexameric detemir.

Recently, small-angle X-ray scattering (SAXS) solution structures of albumin-detemir/degludec complexes in 1:6, 1:12, and 2:12 (albumin:insulin analogue) stoichiometries were presented²². Due to the limited resolution of SAXS and the symmetric shape of albumin, an exact binding site for the two insulin analogues could not be identified.

To close the gap and to gain a more detailed molecular understanding of the protein-protein interactions, we apply molecular dynamics (MD) simulations on complexes between albumin and a detemir hexamer. MD simulations and docking methodologies have been used previously to provide a molecular explanation of experimental results on albumin and insulin alone. Computational studies on insulin and insulin analogues have for instance investigated the dimerization pathway of the peptides,^{23,24} the effect of mutations on protein stability and potency,^{25,26,27} interactions with excipients at different physicochemical conditions,^{28,29} and the effect of water molecules and hydrogen bond network on peptide stability.^{30,31} The main focus of albumin studies has been albumin's capability to bind potential therapeutic, small organic molecules,^{32-33,34} chemical additives,³⁵ and natural products such as monosaccharides³⁶ and flavonoids³⁷ to determine binding poses of the ligands, binding free energy of the ligands and key interactions between albumin and the ligands. Albumin-ligand and albumin-ion interactions have also been studied in different physicochemical conditions.^{38,39} Possible conformational changes on binding of fatty acids to albumin have been observed through MD simulations,⁴⁰ high- and low-affinity fatty acid binding sites on albumin have been identified based on binding free energy calculations and the pathway for the binding process of fatty acids to the highest affinity binding site has been reported.⁴¹ Furthermore, studies have been performed to study the interactions of albumin with other biomolecules such as Interferon α -1b⁴² and polyamidoamine dendrimers⁴³ for drug delivery purposes and peptides to modulate protein-protein interactions.⁴⁴ Albumin flexibility has been studied using principal component analysis of the dynamics of subdomains revealing that conformational protein flexibility favorable precede ligand complexation and that ligand binding can induce allosteric effects to other binding sites.⁴⁵ These studies emphasize the important role of computational tools to complement experimental studies and to provide the necessary molecular understanding.

As mentioned above, our computational study focusses on albumin-detemir interactions. We used the molecular mechanics Poisson-Boltzmann surface area (MM-PBSA) approach to rank different binding positions and in combination with SAXS data to identify the most structurally and energetic favorable binding site and key residues involved in protein-protein interactions. MM-

PBSA is an attractive method, since it is computationally less expensive than other more advanced types of free energy calculations such as alchemical transformations and potential of mean force⁴⁶. MM-PBSA has previously successfully been used to discriminate between high and low affinity fatty acid binding sites in albumin⁴⁷, as well as ranking of native and non-native poses of protein-protein complexes⁴⁸. Only a limited number of computational protein-insulin studies have been reported.^{20,49,50}

To the best of our knowledge, the present work is the first systematic molecular dynamics study on complexes between albumin and a long-acting insulin analogue. The findings in this study can provide an atomic-level description of detemir's binding to albumin and thereby its prolonged action. The knowledge on the albumin-detemir binding could be transferable to other lipidated peptides, and might enable a more rational design of lipidated albumin-binding peptides in the future.

MATERIALS AND METHODS

Molecular dynamics simulations

Construction of systems

Initial protein coordinates for detemir (PDB entry: 1XDA⁵¹, chain A-D) and for albumin (PDB entry: 1BJ5⁵²) were obtained from the Protein Data Bank (PDB). Albumin consists of three homologous domains that are asymmetrically assembled (Figure 1A). In the structure, seven common binding sites have been identified for medium- and long-chain fatty acids: FA1-FA7⁵³ (Figure 1B). Coordinates of the fatty acid chains in 1XDA (HETATM entries MYR) were combined with the coordinates of the acylated lysines (ATOM entries LYS) to common acylated lysine residues, hereafter referred to as Aly. PyMOL (version 1.8.2.3, Schrödinger, LLC) was used for building missing residues, and applying symmetry operations to form the detemir hexamer.

Four albumin-hexamer complexes with binding in respectively FA1, FA3-FA4, FA6, and FA7 were generated as described in the Results section, MD simulations. As the binding sites FA3 and FA4 are overlapping (Figure 1A), they were treated as one binding site and named FA4 throughout the article. The initial structures for the simulations are shown in Figure 1C-F. Prior to the simulations of the complexes, the hexamer was simulated alone. From that simulation, a hexamer conformation was extracted, in which one Aly fatty acid chain protrudes from the hexamer. This conformation was used for building the complexes. All ligands originally present in the crystal structures were kept in the simulations (Zn²⁺, phenol, and myristates), except for the myristates that would compete with detemir binding in the complexes. Thus, the FA1 bound myristate was deleted in the FA1 simulations, and the FA3 and FA4 bound myristates were deleted in the FA4 simulations. No myristates were bound to FA6 or FA7 in the albumin crystal structure used for the simulations.

The CHARMM36⁵⁴⁻⁵⁶ all-atom force field was used for the simulation (including Zn²⁺ parameters⁵⁷, and Na⁺ and Cl⁻ parameters⁵⁸). A topology entry for the acylated lysine

Figure 1. (A) Albumin shown in a cartoon representation colored by domain: turquoise (domain I), purple (domain II), and light blue (domain III). (B) Albumin shown in a grey cartoon representation with myristates bound to FA1-FA7 shown as vdW spheres. The myristates in the binding sites investigated by simulation are colored in magenta (FA1), orange (FA3 and FA4), green (FA6), and blue (FA7). Due to the overlap of FA3 and FA4, the two binding sites are considered as one and is for simplicity be named FA4 throughout the article. (C-F) Initial structures of albumin-hexamer complexes with binding in respectively FA1, FA4, FA6, and FA7. The hexamers are colored using the color coding of the myristates in (A). The albumin structure (PDB entry: 1E7G⁵³) was used in (A-B) to illustrate the seven fatty acid binding sites.

residue was built based on the CHARMM36 topology entry for an acetylated lysine and is provided in the supporting information. MolProbity^{59,60} was used for determining the protonation states of titratable residues and for flipping sidechains to optimize the hydrogen bond network. The structures were prepared for simulation by the VMD PSFgen Plugin and solvated in a box of TIP3P⁶¹ water molecules with paddings of 10–15 Å using the VMD Solvate Plugin version 1.6. Sodium ions were added to neutralize the systems (18 for detemir hexamer, 15 for albumin and one per myristate bound to albumin) and sodium and chloride ions were added to a concentration of 0.025 M, as complex formation between albumin and detemir was observed at this and higher NaCl concentrations.²² The ions were added using the VMD Autoionize Plugin, version 1.3.

Simulation setup

The simulations were performed with NAMD⁶² version 2.12. The integration time step was set to 2 fs. Initially, the systems were minimized using a conjugate gradient algorithm for 1500 steps for the complexes and 500 steps for the albumin and hexamer systems. The simulations were carried out in an *NPT* ensemble at 310 K and 1 atm. The Langevin method was applied for temperature control with a damping coefficient of 1 ps⁻¹, and the Langevin piston method⁶³ was applied for pressure control with a piston period of 100 fs and a piston decay of 50 fs. The neighbor

list was updated every 2 fs and set to a distance of 14 Å. The short-range van der Waals (vdW) interactions were cut off at 12 Å in combination with a switching function starting at 10 Å. The long-range electrostatics were calculated with the particle mesh Ewald method^{64,65} with a 1 Å grid spacing and full electrostatic evaluation every 4 fs. All hydrogen bonds were kept rigid using the SHAKE algorithm⁶⁶. The simulations of the complexes were run in duplicates and each of them for 200 ns. The duplicates were initiated from the same minimized structure but using different seeds for the generation of the initial velocities.

Trajectory analysis

Root-mean-square deviation (RMSD) was calculated based on backbone atoms (C, O, N, CA) and were calculated relative to the minimized structures. The fits to experimental SAXS curves were calculated every 2 ns with CRY SOL⁶⁷ using 500 points in the theoretical curve and fitting up to $q_{max} = 0.4 \text{ Å}^{-1}$ with constant background subtraction and by fitting the effective atomic radius and the contrast of the solvation layer. Based on the RMSDs and CRY SOL fits throughout the trajectories, it was evaluated that the systems were in equilibrium after 100 ns, and the equilibrium analyses described in the following were thus carried out for the last 100 ns.

Binding free energy calculations

The MM-PBSA approach was used for estimating binding free energies (ΔG_{bind}) of the protein complexes in order to rank the different binding sites. ΔG_{bind} is calculated as the difference between the free energy of the complex ($G^{complex}$) and the free energies of the unbound proteins ($G^{albumin}$ and $G^{detemir}$):

$$\Delta G_{bind} = G^{complex} - G^{albumin} - G^{detemir} \quad (1)$$

In the MM-PBSA approach, the free energy is estimated as a sum of gas-phase and solvation energies:

$$G = E_{int} + E_{elec} + E_{vdW} + G_{pol} + G_{np} \quad (2)$$

E_{int} , E_{elec} and E_{vdW} are respectively internal, electrostatic and vdW energy contributions to the gas-phase energy. G_{pol} and G_{np} are polar and non-polar contributions to the solvation energy, respectively. The gas-phase energies were calculated by NAMD Energy Plugin, version 1.4 in VMD using the same force field parameters as for the MD simulations. G_{np} was calculated from the solvent accessible surface area (SASA) by the relation: $G_{np} = \gamma SASA + \beta$. The empirical constants γ and β were set to 0.00542 kcal/mol/Å² and 0.92 kcal/mol, respectively, as applied in previous studies^{68,69}. SASA was calculated using VMD and a probe radius of 1.4 Å. G_{pol} was calculated using the Adaptive Poisson-Boltzmann Solver⁷⁰ (APBS) for solving the linear Poisson-Boltzmann (PB) equation. The PDB2PQR⁷¹ software was used for generating input files for the APBS calculations. Sørensen et al. found that G_{pol} calculations using Poisson-Boltzmann solvers are sensitive to input variables, including grid spacing and internal dielectric constant of the protein (ϵ_{in}). Prior to the MM-PBSA calculations, the grid spacing sensitivity was tested and the optimal grid spacing for the system was found to be 0.4 Å (data not shown), which was used in the calculations. As proteins cannot be considered continuum dielectric media, ϵ_{in} is not a physical constant that can be measured experimentally. Values of ϵ_{in} ranging from 1 to 20 are commonly employed in MM-PBSA studies, and it has been suggested that the optimal value to use is system and method dependent.⁷²⁻⁷⁴ In an MM-PBSA study of protein complexes, Simões *et al.*⁶⁹ found better agreement with experimental data using values for ϵ_{in} ranging from 7 to 11. Based on their study, ϵ_{in} values were tested in this range. The ranking of the binding sites were found to be independent of the chosen ϵ_{in} . As the lowest standard deviations were found using $\epsilon_{in} = 7$, this value was applied in the calculations. The exterior dielectric constant is set to 80. The gas-phase and non-polar solvation energies were evaluated every 10 ps of the last 100 ns of simulations thus amounting to 10000 frames. The polar solvation energies were, however, only calculated every 100 ps as the calculations were very computationally demanding. The standard deviations of the energy contributions were estimated by block averaging⁷⁵.

The MM-PBSA calculations were set up using the single-trajectory approach, where the structures of the unbound proteins are extracted from the trajectory of the protein complex, which results in $E_{int} = 0$ in equation 2. The single-trajectory approach has been successfully employed in a benchmark study⁴⁸ in which protein-protein complexes were ranked using MM-PBSA. Entropy contributions to ΔG_{bind} were not considered in this study, as including entropy does not necessarily improve the ranking of relative binding affinities^{74,76} and it was assumed that the different binding sites would contribute equally.

Interface analysis

Interactions in the albumin-detemir interface were identified by counting albumin and detemir residues that were within 5 Å proximity of each other using an in-house script. The structures were clustered based on contacts between albumin and detemir using the Fraction of Common Contacts Algorithm⁷⁷ with a distance cut-off of 5 Å and a threshold of 0.75.

Dynamic light scattering

Dynamic light scattering (DLS) titration experiments were carried out for albumin-detemir mixtures in a 1:6 molar albumin:detemir ratio. Insulin detemir (detemir) was obtained from Levemir® (Novo Nordisk A/S) and dialyzed into a buffer containing 5 mM Na₂HPO₄, 15 mM phenol, 13 mM *m*-cresol, 173 mM glycerol, and 20 mM NaCl and adjusted with HCl to pH 7.4. Recombinant human serum albumin was obtained from Recombum® Elite (Albumedix Ltd) and dialyzed into a buffer containing 25 mM NaH₂PO₄ and 215 mM NaCl and adjusted with HCl to pH 6.5. The proteins were mixed in a 1:6 ratio to a total protein concentration of 3.8 mg/mL and diluted with detemir's dialysis buffer. The mixtures were equilibrated for two hours at room temperature without stirring. Ibuprofen sodium salt was thereafter added in stoichiometric amounts from 1-25, and the samples were further equilibrated for three hours. After ligand addition, the final protein concentration was 3.2 mg/mL and the final buffer compositions was 6 mM Na₂HPO₄, 14 mM *m*-cresol, 13 mM phenol, 166 mM glycerol, 28 mM NaCl at pH 7.4.

The samples were measured in triplicate on a DynaPro DLS plate reader (Wyatt Technology Corporation, Santa Barbara, CA). Wyatt Dynamics software was used for data collection and analysis. The samples were loaded onto 96 well non-binding Corning® microplates and centrifuged for 2 min at 2000 rpm at room temperature to remove air bubbles. The sample volume was 100 µL, the temperature was 298 K, and each sample was measured ten times for five seconds. Z-averages obtained by cumulants analysis are reported in the results section as hydrodynamic radius (R_h) and have been corrected for viscosities.

Figure 2. Rigid body modelling of the albumin-detemir hexamer complex gives models with binding at the domain interfaces, domain I/III, domain II/III, and domain I/II. The fits of the models to the experimental data (grey) are shown in respectively red, blue, and orange. The lower inset shows error-weighted residual plots for the model fits. The models are shown with albumin in grey, and the hexamers with same color-coding as in the plots.

Small-angle X-ray scattering

The SAXS data used for setting up the MD simulations and the *ab-initio* model shown in Figure 2 were previously presented.²² The experiments were performed at the I911-SAXS beamline⁷⁸ at the MAX IV Laboratory (Lund, Sweden). The experimental details can be found in Table 1 and Table 2 as well as the Materials and methods section in Ryberg *et al.* 2019.²² *SASREFCV*^{79,80} (*SASREF*) was used for rigid body modelling with standard settings for X-ray data and based on data up to $q = 0.43 \text{ \AA}^{-1}$. The input structures were the same albumin and detemir hexamer structures that were used for setting up the simulations.

The NaCl titration SAXS dataset presented in Supplementary Figure S19 was measured at the P12 BioSAXS beamline⁸¹ at PETRA III (DESY, Hamburg, Germany). Data collection parameters are given in Supplementary Table S1. Albumin and detemir were mixed in a 1:6 molar ratio, corresponding to 1.8 mg/mL albumin and 3.3 mg/mL detemir. The recombinant albumin was obtained from Recombum® Alpha (Albumedix Ltd). The samples were measured in a buffer consisting of 5 mM Na_2HPO_4 , 15 mM phenol, 13 mM *m*-cresol, 173 mM glycerol, and 20 mM NaCl that was adjusted with HCl to pH 7.4. The

SAXS sample preparation and data collection were carried out as described in Ryberg *et al.* 2019.²² The samples were titrated with 0–100 mM NaCl.

Data analysis and modelling were carried out using the *ATSAS* Program Package version 2.8.3⁸². Guinier analysis was performed on the baseline subtracted SAXS curves using *PRIMUS*⁸³. If inter-particle interactions were observed, the curves were truncated at low q -values. Volume fractions of the different species in the samples were investigated using *OLIGOMER*⁸³ with a form factor file that was produced by *FFMAKER*⁸³ using *CRY SOL* with standard settings and 256 points in the theoretical scattering curve. The molar 1:6 stoichiometry was used as a constraint in *FFMAKER*. The *OLIGOMER* analysis was carried out up to a maximum scattering vector $q_{\text{max}} = 0.4 \text{ \AA}^{-1}$ and with addition of a constant component. The structures used as input were fatty acid free albumin (PDB entry 1AO6⁸⁴), an insulin hexamer and di-hexamer (generated from PDB entry 1TRZ⁸⁵). Rigid body models of the insulin tri-hexamer, albumin-hexamer, albumin-di-hexamer and albumin-di-hexamer-albumin were taken from the small-angle scattering biological data bank

(SASBDB)⁸⁶ entries SASDEV5²², SASDEX5²², SASDE26²² and SASDEY5²², respectively.

RESULTS

SAXS modelling

The solution structure of a complex between albumin and a detemir hexamer was modelled using SAXS data in a previous study carried out in our group. The rigid body models of the complex were not conclusive with respect to detemir's binding site on albumin due to the low resolution of the SAXS data and the symmetric shape of albumin.²² In the current study, we utilize MD simulations in combination with MM-PBSA calculations to qualify which of the binding site is the most probable site.

Initially, we wanted to identify possible binding positions and carried out rigid body modelling using *SASREF*. The 22 generated models were clustered into three clusters based on the binding position of the hexamer on albumin. The best fitting model from each cluster and its fit to the data are presented in Figure 2. The models suggest that the hexamer binds at one of the domain interfaces. The fits of the models with binding near domain I/II ($\chi^2 = 1.31$) and near domain II/III ($\chi^2 = 1.96$) do not differ much considering that conformational changes of the proteins upon binding could be expected. The model with binding near domain I/III model has a slightly worse fit to the data ($\chi^2 = 3.32$). The ambiguity of the binding position on albumin can be explained by albumin's triangular shape. With SAXS data alone, it is not possible to distinguish the three sites. We therefore carried out MD simulations to investigate the binding sites on the atomic level.

MD simulations

The MD simulations were set up based on the rigid body models presented in Figure 2. There is strong evidence⁸⁷ and a general consensus in literature^{6,18,19,88} that detemir binding occurs at a fatty acid binding site as C8-C16 fatty acids and small molecule ligands that bind in albumin's fatty acid binding sites can displace detemir's binding to albumin.^{18,87} Thermodynamic experiments suggest that the albumin-detemir binding is a result of both ionic and hydrophobic interactions between albumin and detemir's acylated lysine residue (Aly) that is also the C-terminal of detemir's B chain. More specifically, the C-terminal carboxylate is predicted to form an ionic bond to a basic residue near one of albumin's binding sites, and the Aly fatty acid chain is predicted to form non-polar interactions with the residues in the binding site.⁸⁷ This information was utilized for setting up the initial structures for the simulations. First of all, by studying the proximity of the binding positions in Figure 2 and the seven common fatty acid binding sites in Figure 1B, it was evaluated that detemir could bind to FA1, FA4, FA6 or FA7. Secondly, the hexamers in the rigid body models presented in Figure 2 were rotated and translated to position the Aly fatty acid chain at the entry of the fatty acid binding sites while ensuring that the C-terminal carboxylate in proximity to a basic residue. The initial simulation structures are shown

in Figure 1C-F. More detailed views of the binding interfaces are shown in Supplementary Figures S1-S4.

Trajectory analysis

The RMSD evolutions of the simulations are presented in Figure 3. For all simulations, there is a steep initial increase in RMSD, which is largely due to conformational changes in the albumin and detemir structures to accommodate the binding (Supplementary Figure S5). Apart from the FA6a simulation (Figure 3C), the RMSDs stabilize and fluctuate around a constant value after 100 ns indicating that the simulations have converged. In addition to RMSDs, the fits to the experimental SAXS data throughout the trajectories are calculated and presented in Figure 4. The FA4a, FA4b, and FA7a simulations have the best fits to the experimental data with average χ^2 values based on the last 100 ns of simulations of 2.8 ± 0.2 , 2.0 ± 0.3 , and 2.0 ± 0.5 , respectively. The FA6 simulations have slightly worse fits to the data and larger fluctuations (average χ^2 values of 3 ± 1). The FA1a, FA1b, and FA7b do not fit the data with average χ^2 values of 9 ± 4 , 6 ± 1 , and 13 ± 3 , respectively. Based on the average χ^2 values of the duplicates, the FA4 and FA6 simulations fit the data best.

Figure 3. RMSD of backbone atom positions calculated relative to the minimized structures for the (A) FA1, (B) FA4, (C) FA6, and (D) FA7 trajectories.

Figure 4. SAXS fits of the complexes calculated by CRY SOL throughout the trajectory.

Binding free energy calculations

The MM-PBSA approach was used to assess the energetics of the simulations in order to rank the complexes. For all simulations, the electrostatic and vdW energies fluctuate around constant values after 100 ns of simulations (Supplementary Figures S8 and S9) supporting the conclusion that the simulations have converged. The MM-PBSA calculations were thus carried out for the last 100 ns. The results are presented in Table 1. The FA4 binding site has the most favorable binding energy, $\Delta\bar{G}_{bind} = -28\pm6$ kcal/mol, followed by the FA1 binding site with $\Delta G_{bind} = -16\pm2$ kcal/mol. The FA6 and FA7 binding sites have the least favorable binding energies with $\Delta\bar{G}_{bind} = 14\pm31$ kcal/mol and $\Delta\bar{G}_{bind} = 10\pm13$ kcal/mol, respectively.

Table 1. Binding free energy components of the complexes. The standard deviations are given in parentheses.

		ΔE_{elec}	ΔE_{vdW}	ΔG_{pol}	ΔG_{np}	$\Delta E_{elec} + \Delta G_{pol}$	ΔG_{bind}^a	$\Delta\bar{G}_{bind}^b$
		kcal/mol	kcal/mol	kcal/mol	kcal/mol	kcal/mol	kcal/mol	kcal/mol
FA1	a	-69 (1)	-37.2 (0.4)	98 (2)	-6.0 (0.1)	29 (2)	-15 (2)	-16 (2)
	b	-82 (1)	-74.5 (0.7)	150.6 (0.9)	-11.1 (0.1)	68 (1)	-17 (1)	
FA4	a	-125.0 (0.8)	-68.1 (0.6)	172 (2)	-11.0 (0.1)	47 (2)	-32 (2)	-28 (6)
	b	-132.9 (0.8)	-68.8 (0.6)	190 (2)	-11.77 (0.04)	57 (2)	-24 (2)	
FA6	a	48 (1)	-46.8 (0.4)	41 (2)	-7.1 (0.1)	89 (2)	36 (2)	14 (31)
	b	29 (1)	-58.5 (0.6)	29 (1)	-7.4 (0.1)	58 (1)	-8 (1)	
FA7	a	17.7 (0.9)	-41.3 (0.3)	48 (2)	-5.7 (0.1)	66 (2)	19 (2)	10 (13)
	b	-8.0 (0.9)	-46.3 (0.5)	61 (1)	-6.7 (0.1)	53 (2)	0 (2)	

^a $\Delta G_{bind} = \Delta E_{elec} + \Delta E_{vdW} + \Delta G_{pol} + \Delta G_{np}$

^b $\Delta\bar{G}_{bind}$ is the average ΔG_{bind} for duplicate simulations with standard deviations given in parentheses.

For the FA1 and FA4 complexes, the protein-protein electrostatic energies favor binding. Considering the overall negative charges of albumin and detemir, this indicates that the binding interfaces are complementary. The favorable ΔE_{elec} are opposed by unfavorable protein-solvent ΔG_{pol} contributions. In agreement with these observations, ΔE_{elec} and ΔG_{pol} are often found to partly cancel out^{68,72}, as more charged residues in the protein-protein interface are buried. The desolvation of charged residues consequently leads to less favorable protein-solvent interactions. However, the total electrostatic contribution to binding ($\Delta E_{elec} + \Delta G_{pol}$) is sensitive to the definition of the boundary between protein and solvent and the internal dielectric constant, which can lead to an overestimation of the solvation penalty.^{89,90} For all the complexes, the vdW energies favor binding with the most favorable energies for the FA4 complexes accounting for $\Delta\bar{E}_{vdW} = -68.5\pm0.5$ kcal/mol.

The MM-PBSA analysis shows that the FA4 complex is the most favorable binding site, which is in accordance with its good fit to the SAXS data.

Interface analysis

In the following, we examine the protein-protein interfaces of the complexes. As natural insulin does not bind to albumin²¹, it is expected that the Aly residue promotes binding of detemir to albumin in a manner similar to fatty acid binding.¹⁹

The frequencies of interfacial residue pairs and representative structures of the complexes are shown in Figure 5 (for the FA4b complex) and Supplementary Figures S10-S16 (for the remaining complexes). From the heatmaps, it is seen that the Aly residues have more interactions with albumin in the FA4 complexes compared to the other complexes. This is summarized in

Table 2. The FA4 complexes form in average 36 Aly-albumin contacts, which can be linked to their favorable vdW energies (Table 1). In comparison, the FA1a and FA7b complexes have the fewest Aly-albumin contacts, which is

also reflected in their relatively larger radii of gyration (R_g s) and center-of-mass (COM) distances (Supplementary Figures S6 and S7, respectively). This supports further the notion that FA4 is the most favorable binding site.

In the FA4 complexes, the Aly residues in chain D and H (Figure 5C; Aly^{D29} and Aly^{H29}, respectively) account for the majority of the Aly-albumin proximities (Table 2). The specific Aly-albumin residue pairs are highlighted in the heat maps in Figure 5A and Supplementary Figure S12A.

A zoom in on the Aly^{D29} and Aly^{H29} binding sites are shown in Figure 6 for the FA4b simulation and in Supplementary Figure S17 for the FA4a simulation. In both simulations, Aly^{D29} protrudes into the hydrophobic cleft of the FA4 binding site and Aly^{H29} is positioned in the albumin-detemir interface. Salt bridges are observed between Glu^{C4}-Lys⁴¹⁴ (notation: detemir residue-albumin residue), Glu^{C4}-Arg⁴¹⁰, Aly^{D29}-Arg⁴¹⁰, Aly^{H29}-Lys³⁷² and hydrogen bonds are observed between Phe^{D1}-Glu³⁹³ and Gln^{D4}-Gln³⁹⁰, indicating that these are important

interactions. Additionally, Gln^{D4}-Glu³⁹³ and Cys^{C7}-Asn³⁸⁶ interactions are observed in the FA4a complex, and Glu^{B21}-Lys⁵⁴¹, Gly^{C1}-Glu⁴⁹² and Glu^{G4}-Lys³⁷⁸ are present in the FA4b complex. Based on the heatmaps (Figure 5A and Supplementary Figure S12A), Aly^{D29} forms favorable vdW interactions with the hydrophobic/aromatic residues in the FA4 binding site: Leu³⁸⁷, Leu³⁹⁴, Phe⁴⁰³, Leu⁴⁰⁷, Tyr⁴¹¹, Leu⁴³⁰, Leu⁴⁵³, Phe⁴⁸⁸. In conclusion, Aly^{D29} in the FA4 complexes bind to an albumin fatty acid binding site by a combination of electrostatic and hydrophobic interactions, as expected from literature.⁸⁷

Apart from the FA4 complexes, only in the FA6b complex the Aly residue protrudes into the FA6 binding site. However, the binding energy is noticeably less compared to the one observed for the FA4 complexes indicating that this is not a primary binding site (Table 1).

Figure 5. Analysis of interactions in the FA4b simulation. (A) Interaction heatmap of the albumin-detemir interface with albumin residues on the y-axis, and detemir residues on the x-axis. The color scale goes from blue indicating that the residues are not within 5 Å of each other throughout the simulation to yellow that indicates that the residues are within 5 Å of each other in all simulation frames. The arrows in light pink and orange mark the fatty acid residues present in the binding interface, Aly^{D29} and Aly^{H29}, respectively. (B) The representative structure of the FA4b complex shown as cartoon with Aly residues shown as vdW spheres. The residues are colored according to their maximum interaction values from the heatmap in (A). The Alys located at the interface are marked with rectangles. (C) Schematic overview over chain IDs in a detemir hexamer. Each monomer consists of two chains: chain A shown in grey and chain B shown in light blue.

Figure 6. Analysis of interface in the FA4b simulation. (A) and (B) show the interacting residues around Aly^{D29} (light pink) and Aly^{H29} (orange), respectively. The detemir hexamer is colored green, and albumin is colored grey.

Table 2. Overview over the number Aly-albumin proximities present in more than 30 % of the last 100 ns of simulation for Aly^{D29}, Aly^{H29}, and Aly^{L29}.

	Aly ^{D29}	Aly ^{H29}	Aly ^{L29}	Total
FA1a	7	0	0	7
FA1b	11	6	8	25
FA4a	17	12	0	39
FA4b	22	8	2	32
FA6a	10	5	0	15
FA6b	21	0	1	22
FA7a	11	8	0	19
FA7b	0	8	0	8

presented in Figure 7. With the addition of one equivalent of ibuprofen, we observe a decrease in R_h corresponding to dissociation of the albumin-hexamer complex. From one to 25 equivalents, R_h steadily decreases with a less steep slope compared to the slope when only one equivalent of ibuprofen is added.

In Figure 8, the binding of ibuprofen to FA4 is compared with detemir's binding in the FA4b simulation in order to explain the DLS results on a molecular level. Figure 8B and D show the important interactions between ibuprofen's carboxylic acid group and albumin: a salt bridge with Arg⁴¹⁰ and hydrogen bonds to Tyr⁴¹⁰ and Ser⁴⁸⁹. Figure 8A and C show that Aly^{D29} competes for the same binding site as ibuprofen. While Aly does not form specific interactions with Ser⁴⁸⁹, the salt bridge to Arg⁴¹⁰ is a key interaction for the binding as also illustrated in Figure 6A. Furthermore, hydrophobic interactions of the fatty acid chain are observed with Phe⁴⁸⁸ and Tyr⁴¹¹. The displacement of detemir with ibuprofen can thus provide further evidence that FA4 is the most favorable binding site.

DISCUSSION

MD simulations of albumin-detemir complexes with binding in FA1, FA4, FA6, and FA7 were performed to deduce which is the most favorable binding site. The FA1 and FA7 simulations show greater variabilities within the duplicates in the time evolution of RMSD, R_g and COM distance (Figure 3, Supplementary Figures S6 and S7, respectively), whereas the progress of the FA4 simulations is similar for the duplicate simulations. The large fluctuations observed for the FA1a simulation can be explained on the molecular level by the hexamer loosing and regaining contact with albumin's domain III. Similarly, the hexamer in the FA7b complex loses contact with albumin's domain I in the beginning of the simulation. When the hexamer is not in contact with the respective

DLS

From the previous results, FA4 seems to be the most favorable binding site. To test this experimentally, DLS competition experiments were set up. Albumin and detemir were mixed in a 1:6 ratio and titrated with the prototypical FA4 ligand ibuprofen.^{91–93} The results are

domains, the complex becomes too large to fit the SAXS data (Figure 4), which is also reflected in the RMSD, R_g and COM plots (Figure 3, Supplementary Figures S6 and S7, respectively). In contrast, the FA4 and FA6 complexes have the best fits to the SAXS data, which could indicate that these are the correct binding sites, if only the SAXS fits were considered. When also taking the MM-PBSA energetics of the complexes into account, it is clear that the FA6 binding site ($\Delta\bar{G}_{bind} = 14 \pm 31$ kcal/mol) is less favorable than the FA4 site ($\Delta\bar{G}_{bind} = -28 \pm 6$ kcal/mol). According to the MM-PBSA ranking, FA4 is the most favorable binding site followed by FA1, and the equally unfavorable binding sites, FA6 and FA7. The ranking suggests that FA1 could be a secondary

Figure 7. DLS competition experiment where the albumin-detemir complex is titrated with ibuprofen. With the addition of one ibuprofen equivalent (per albumin-hexamer complex), a significant decrease in R_h is observed indicating that the complex dissociates to a mixture of albumin and detemir.

Figure 8. The binding poses of Aly D29 (orange) from the FA4b simulation (A) and (C) and ibuprofen (PDB entry: 2BXG5, light blue) (B) and (D) are compared. (C) and (D) are rotated 60° relative to (A) and (B). The structures have been aligned

on the five alpha helices surrounding ibuprofen. The residues responsible for ibuprofen binding are shown as sticks.

detemir binding site. Intuitively, this seems to be contradicted by the SAXS fits of the FA1 complexes that indicate that FA1 binding is not observed in the experiments. This discrepancy can be explained by the 1:6 molar albumin:detemir ratio used in the SAXS experiments. At this ratio, the detemir hexamer solely saturates the primary binding site, and we would therefore not expect that a complex with binding at a secondary site fits the SAXS data. Our study can thus not exclude that FA1 could be a secondary binding site, but the main focus of the study is to investigate detemir's primary binding site. Trajectory analysis, binding free energy calculations and interface analysis suggest that FA4 is the primary binding site.

To experimentally test whether detemir does indeed bind at FA4, DLS competition experiments were set up, where the albumin-hexamer complex was titrated with ibuprofen. While ibuprofen is proposed to have multiple binding sites on albumin, there is a consensus that FA4 is the primary ibuprofen binding site with a ~10-100 times higher affinity than for the secondary ibuprofen binding site FA6.⁹⁴ The primary association constants of ibuprofen and detemir have been determined under the same experimental conditions to be $2.7 \cdot 10^6 \text{ M}^{-1}$ and $2.4 \cdot 10^5 \text{ M}^{-1}$, respectively.¹⁸ The addition of one ibuprofen equivalent can thus be expected to compete with detemir binding. With respect to possible allosteric effects, crystal structures of albumin with and without ibuprofen are compared. The backbone RMSD of the structures (PDB entries: 1AO6⁸⁴ and 2BXG⁵) is 0.7 Å, indicating that ibuprofen binding to FA4 does not lead to conformational changes in albumin. This is in agreement with previous studies showing ibuprofen binding to FA4 may not be allosterically linked to the FA1 binding site.^{95,96} In the competition experiment, the addition of one ibuprofen equivalent leads to a decrease in R_h indicating complex dissociation. For the above-mentioned reasons, the decrease in R_h can be considered a competition effect of ibuprofen binding to FA4, which confirms FA4 as the correct binding site. This result is in agreement with the findings of Kjeldsen *et al.*¹⁹ that detemir binds to albumin's domain III in which FA4 is embedded.

Another competition study of the albumin-detemir complex formation was carried out by Kurtzhals *et al.*¹⁸ The authors found that detemir could be displaced from albumin when more than one ibuprofen equivalent was added and that another FA4 ligand, diazepam, could not displace detemir. Based on this, the authors suggested that FA4 may not be the correct binding site. Their observation that diazepam cannot displace detemir can be explained by a crystal structure of albumin with diazepam bound (PDB entry: 2BXF⁵) that was published after the study of Kurtzhals *et al.* The crystal structure reveals that diazepam binds more deeply into the FA4 binding site than

ibuprofen does, and therefore does not compete with detemir Aly binding (Supplementary Figure S18).

Detemir's binding site on albumin has previously been investigated using *in-silico* approaches by Fatima *et al.*²⁰ who based on docking proposed that detemir's chain B binds near FA1. The result is not directly comparable with ours, as the binding of a hexamer can be expected to differ significantly from the binding of only chain B of a detemir monomer. In particular, we observe that not only hydrophobic Aly (chain B) interactions are present in the albumin-detemir interface but also salt bridges and hydrogen bonds formed by residues in both chain A and B of detemir (Figure 6).

The binding free energy of the albumin-detemir complex has been determined experimentally to -7.1 kcal/mol by Kurtzhals *et al.*⁸⁷ and -3.8 kcal/mol by Fatima *et al.*²⁰. The deviation between the binding energies could reflect the different experimental conditions. In comparison, we calculated a lower binding energy of -28 ± 6 kcal/mol for the most favorable FA4 complexes. Such an overestimation of binding energies is commonly observed for the MM-PBSA approach.^{68,72,73,97} The strength of the method is not to estimate absolute binding energies but to rank protein-protein and protein-peptide complexes based on their relative energies,^{48,97,98} which puts confidence into the ranking of the albumin-detemir complexes.

In addition to ranking, the MM-PBSA results can give an indication of which energy terms contribute to the stability of the albumin-detemir complex. For the FA4 binding site, especially the electrostatic and vdW gas-phase energies are stabilizing the complexes. This result fits well with the findings of Kurtzhals *et al.*⁸⁷ who found that both ionic and non-polar interactions contribute to the complex stability. For the ionic interactions, the authors observed that adding an extra residue to detemir's B chain (Thr^{B30}) resulted in a weaker albumin binding suggesting that positioning the C-terminal on position 29 is favorable. The authors propose that this is due to an ionic interaction between the C-terminal carboxylate and a basic albumin residue. Indeed, in both FA4a and FA4b, strong interactions are observed between the Aly^{D29} C-terminal carboxylate and Arg⁴¹⁰ (Figure 6A) and the Aly^{H29} C-terminal carboxylate and Lys³⁷² (Figure 6B). For the non-polar interactions, Kurtzhals *et al.*⁸⁷ found that increasing the length of the Aly fatty acid chain up to 14 carbons led to a stronger binding and concluded that the Aly acyl chain takes part in the interaction with albumin. The many Aly-albumin interactions in the FA4 heatmaps (Figure 5A and Supplementary Figure S12A) clearly show that this is the case for the FA4 binding site. The agreement between our study and the predictions by Kurtzhals *et al.*⁸⁷ puts further confidence in FA4 as the correct binding site.

While Kurtzhals *et al.*⁸⁷ find that both electrostatic and hydrophobic interactions are important, Fatima *et al.*²⁰ find that the complex formation is dominated by hydrophobic interaction. These findings are not contradictory, as hydrophobic interactions are generally found to be the driving interactions in protein-protein

complexes, whereas the long-range electrostatic interactions are steering in the complex formation and contribute to interaction specificity.^{90,99-101} In agreement with the results of Fatima *et al.*²⁰, we have found that addition of NaCl promotes complex formation (Supplementary Figure S19), which indicates that hydrophobic interactions stabilize the albumin-detemir complex.

CONCLUSION

In this work, we have studied a complex between a detemir hexamer and albumin using MD simulations in combination with MM-PBSA free energy calculations and DLS competition studies. The simulations were set up based on SAXS results²² suggesting that detemir can bind to albumin close to the albumin fatty acid binding sites, FA1, FA6, FA7, or the overlapping binding sites FA3-FA4 (named FA4). The simulations were performed in duplicates for 200 ns.

The FA4 simulations had the best fits to the experimental SAXS data with an average χ^2 of 2.4 ± 0.4 , and the lowest binding free energies with an average of -28 ± 6 kcal/mol.

Several electrostatic interactions were identified between detemir and albumin: Glu^{C4}-Lys⁴¹⁴ (notation: detemir residue-albumin residue), Glu^{C4}-Arg⁴¹⁰, Aly^{D29}-Arg⁴¹⁰, Aly^{H29}-Lys³⁷², Phe^{D1}-Glu³⁹³ and Gln^{D4}-Gln³⁹⁰. Besides the specific interactions, we also monitored hydrophobic interactions between the fatty acid residue (Aly) and albumin. The hydrophobic interactions stemming from the Aly residues were found to be an important contribution to the free energy of binding as quantified by MM-PBSA analysis. The binding of detemir to FA4 was confirmed by a DLS competition experiment in which albumin-detemir complexes were titrated with the FA4 ligand, ibuprofen. Upon addition of one ibuprofen equivalent, a decrease in R_h was observed indicating that detemir was displaced by direct competition.

Thus based on structural and energetic analyses of MD simulations, competition experiments and comparison with the detailed studies by Kurtzhals *et al.*⁸⁷, we propose that FA4 is the most probable binding site for detemir. The results give a molecular insight into the binding of a lipidated peptide to albumin that could be exploited in the design of future drugs.

ASSOCIATED CONTENT

Supporting information:

Additional RMSD plots based on albumin and the hexamer structures; time evolutions of R_g , center-of-mass, electrostatic and vdW binding energies; Interaction heat maps; Clustered structures of the simulated complexes; Figure of the FA4a binding interface; Comparison of the FA4b detemir binding pose with the diazepam binding pose; SAXS data and analysis for titration with NaCl; CHARMM36 topology entry for the Aly residue.

This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

* linear@kemi.dtu.dk
* ghp@kemi.dtu.dk

Author Contributions

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ABBREVIATIONS

Aly, acylated lysine; DLS, dynamic light scattering; MD, molecular dynamics; MM-PBSA, molecular mechanics Poisson-Boltzmann surface area; RMSD, root-mean-square deviation; SAXS, small-angle X-ray scattering.

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