Characterization of the Vitamin E-Binding Properties of Human Plasma Afamin†

Andreas F. Voegele,‡,|| Lidija Jerković,‡,|| Bernd Wellenzohn,§ Patricia Eller,|| Florian Kronenberg,|| Klaus R. Liedl,§ and Hans Dieplinger*,‡,||

Vitateq Biotechnology GmbH, Innsbruck, Austria, Department of Theoretical Chemistry, University of Innsbruck, Austria, and Institute of Medical Biology and Human Genetics, University of Innsbruck, Austria

Received July 25, 2002; Revised Manuscript Received September 8, 2002

ABSTRACT: Human plasma afamin, the fourth member of the albumin gene family, is shown to be a specific binding protein for vitamin E. A radio ligand-binding assay followed by Scatchard and Hill analysis are used to show that afamin has a binding affinity for both α-tocopherol and γ-tocopherol, two of the most important forms of vitamin E, in vitro. The binding-dissociation constant was determined to be 18 μM, indicating that afamin plays a role as vitamin E carrier in body fluids such as human plasma and follicular fluid under physiological conditions. Additionally, we demonstrate that afamin has multiple binding sites for both α- and γ-tocopherol. Due to the large binding capacity of afamin for vitamin E, it might take over the role of vitamin E transport in body fluids under conditions where the lipoprotein system is not sufficient for vitamin E transport. To confirm the experimental results, we performed homology modeling and docking calculations on the predicted tertiary structure, which showed coincidence between calculated and in vitro results.

The human albumin gene family is comprised of four members encoding the proteins human serum albumin (HSA)[1] (*1*), α-fetoprotein (AFP), vitamin D-binding protein (DBP), and the most recently discovered afamin (*1*−*7*). Many physiological properties of the former three have been known for quite a long time whereas no functional data are so far available for afamin. HSA is the most abundant plasma protein and is known to be responsible for the transport and delivery of a wide variety of metabolites, drugs, anionic ligands, and cations (*8*). The structure of HSA has been studied extensively, yielding a tertiary structure composed of three major domains subdivided into minor domains composed of three loops (*8*−*11*). These domains are dominated by a high α-helix content (∼67%) and a total of 17 disulfide bonds. Several binding sites have been characterized. The two most important ones are sites I and II (*12*−*16*). However, HSA is capable of binding more than two ligands. Therefore, a much higher number of potential binding sites exist, which was shown, e.g., for fatty acids (*10*).

From the evolutionary point of view the vitamin D-binding protein—also known as group-specific component (Gc)—is the oldest of the four proteins in the albumin gene family (*1*, *2*, *7*). DBP is the most important transport protein for vitamin D and vitamin D metabolites in human plasma (*17*, *18*). Similar to HSA, several studies on DBP report additional binding properties for other ligands such as actin or fatty acids (*19*−*21*).

AFP is expressed only during fetal life (*22*), whereas in adults AFP has been reported to increase significantly under certain pathological conditions (*23*−*25*). Like HSA, AFP is a transporter of several ligands, for instance ions, fatty acids, and bilirubin (*22*, *26*−*28*). AFP is one of the human plasma proteins most thoroughly investigated during the last three decades, and therefore, we refer to recent reviews (*22*, *28*).

The fourth and so far latest member of the albumin multigene family was discovered by Bélanger et al. (*4*) and Lichenstein et al. (*3*) and was named α-albumin for the rat and afamin for the human homologue. Araki et al. (*29*) showed that afamin is identical to α1T-glycoprotein, a protein described for the first time in 1964. In contrast to the other three family members, afamin is highly glycosylated which enlarges the molecular mass from 66 576 to 74 400 Da according to the amino acid sequence (determined by MALDI-TOF, *29*). The apparent molecular mass in SDS−PAGE is about 87 000 under reducing conditions (*3*). There are four potential glycosylation sites, and Araki et al. (*29*) indeed characterized four predominant fully sialylated carbohydrate chains. The only report on afamin and its pathophysiological role was suggested by Wu et al. (*30*), who found a significant down-regulation of afamin expression in hepatocellular carcinoma tumor tissue.

In a recent study, we demonstrated co-purification of afamin with vitamin E by multistep chromatography of human plasma and also showed that afamin is abundant in human follicular fluid, seminal fluid, and cerebrospinal fluid (CSF). Subsequently we saw that afamin is not only

† This work was supported by the Austrian Science Fund (FWF, P12358-MED), the Austrian National Bank (5363), and the Austrian Industrial Research Fund (FFF).

* To whom correspondence should be addressed: Phone: +43-512-507-3452. Fax: +43-512-507-2861. E-mail: Hans.Dieplinger@uibk.ac.at.

‡ Vitateq Biotechnology GmbH.

§ Department of Theoretical Chemistry, University of Innsbruck.

|| Institute of Medical Biology and Human Genetics, University of Innsbruck.

[1] Abbreviations: HSA, human serum albumin; AFP, α-fetoprotein; DBP, vitamin D-binding protein; IC_{50}, inhibitor concentration that displaces 50% of the labeled, bound ligand; K_D, binding-dissociation constant; SPR, surface plasmon resonance; BIA, biomolecular interaction analysis; EDC, *N*-ethyl-*N*′-(3-dimethyl aminopropyl)-carbodiimide hydrochloride; NHS, N-hydroxysuccinimide; HRPO, horseradish peroxidase; PDB, Protein Data Bank.

10.1021/bi026513v CCC: $22.00 © 2002 American Chemical Society
Published on Web 11/14/2002

associated with vitamin E in human plasma but also in human follicular fluid. We found clear evidence that afamin is a transport protein for vitamin E in follicular fluid and probably in other body fluids such as CSF (*31*).

Vitamin E is an important fat-soluble antioxidant and plays several protective roles in physiological processes (e.g., infertility and atherosclerosis) (*32*−*38*). In human plasma it is transported rather unspecifically by lipoproteins (*39*−*41*) and is transferred between lipoproteins by the phospholipid transfer protein (*42*). However, the lipoprotein pattern in follicular fluid, seminal fluid, or CSF is totally different from that of plasma. Therefore, the transport of vitamin E in such body fluids is still not understood. Of the eight naturally occurring forms of vitamin E, α-tocopherol and γ-tocopherol are the most abundant ones.

This study evaluates in detail the vitamin E-binding properties of afamin proposed in a recent study (*31*). Additionally, it determines the protein structure of afamin by homology modeling and confirms the in vitro binding properties of vitamin E by docking calculations on the proposed structure.

MATERIALS AND METHODS

Materials. All chemicals were analytical-grade and obtained from Sigma (Munich, Germany) or Merck (Darmstadt, Germany) if not otherwise stated. Human serum albumin (HSA) of approximately 99% purity was essentially fatty acid free and obtained from Sigma (Munich, Germany).

Purification of Afamin from Human Plasma. Afamin was purified from human plasma according to the procedure described in our previous study (*31*). In short, lipoproteins of human plasma were precipitated, and the supernatant was purified in three consecutive chromatography steps that included hydrophobic interaction chromatography, anion exchange chromatography, and hydroxyapatite chromatography. Purified afamin was stored at −20 °C.

SDS−PAGE, Silver Staining, Immunoblotting, ELISA. SDS−PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed with a standard 15% SDS−PA gel. Proteins in the SDS polyacryl amide gel were visualized with a Bio-Rad kit according to the protocol supplied, and an SDS−PAGE Broad Range Standard was used to determine the molecular mass of the proteins. Alternative to silver staining, the proteins in the gel were visualized by immunoblotting according to a standardized protocol described elsewhere (*31*) using a monoclonal mouse-antibody (IgG-N13) for afamin as primary and an anti-mouse IgG from goat (Calbiochem, San Diego, CA) as secondary antibody (*31*).

Afamin concentrations were measured with an enzyme-linked immunosorbent assay (ELISA) using a polyclonal, affinity-purified anti-afamin antibody for coating and the monoclonal HRPO-conjugated IgG-N13 for detection (*31*).

Tocopherol-Binding Assay. We performed an assay according to Mallon et al. (*17*) using dextran-coated charcoal with several modifications for our system. It was necessary to use a different approach for the α-tocopherol binding assay than used by Dutta-Roy and co-workers (*40*, *43*) and Dahlberg et al. (*44*) because we had to use higher concentrations of α-tocopherol due to a lower affinity of afamin. This required the addition of a small amount of detergent, which turned out to be incompatible with the Lipidex-1000 methods. The dextran coated charcoal suspension was prepared using a 1:1 mixture of a 10% (w/v) charcoal suspension and a 5% (w/v) dextran (Dextran 32, pharm. pyrogen-free, Serva, Heidelberg, Germany) solution both in 10 mM Tris, 0.9% NaCl (pH 8.2) at 4 °C. A 250 μL sample of a 2.0 μM afamin solution in 10 mM Tris-HCl, 0.9% NaCl at pH 8.2 in 12 × 75 mm disposable glass tubes, was used for all experiments. A small amount of Tween 80 (0.0025%, w/v) was added to avoid adherence of α-tocopherol to the surface wall. The following steps were carried out at 0 °C in a cold room under ice cooling. The afamin solution was incubated with 15−250 μM D-α-(5-methyl-^{14}C)tocopherol (specific activity 12.4 Ci mol^{-1}, Henkel Düsseldorf, Germany). Displacement experiments were used to determine the amount of specifically and nonspecifically bound D-α-(5-methyl-^{14}C)tocopherol. For this purpose a 1−120-fold excess of D-α-tocopherol (>99%, Fluka, Buchs, Switzerland) or D-γ-tocopherol was added to the solution. Tocopherols were added as ethanolic solutions in a total volume of 30 μL, which turned out to be an amount that did not influence the binding behavior significantly. Incubation was started by removing the ice cooling and lasted for 2 h at 25 °C. This period was sufficient to ensure equilibrium of the reaction. After incubation, the assay vials were put on ice for 10 min to stop and "freeze" the reaction. One aliquot of 50 μL was taken, pipetted into well plates containing 1 mL of scintillation fluid (OptiPhase Super Mix, Wallac, Turku, Finland), and finally the radioactivity was measured in a scintillation counter (1450 MicroBeta Trilux, Wallac, Turku, Finland). The assay mixtures were kept at 0 °C, and 50 μL of ice-cooled dextran coated charcoal mixture was added and constantly mixed with. It turned out that 40 min was sufficient to remove unbound α-tocopherol, after which the dextran-coated charcoal was removed by centrifugation at 2700*g* at 4 °C for 10 min (GS-15R centrifuge, Beckman, Palo Alto, CA). Again, 1 aliq of 50 μL was taken and the radioactivity measured. Data were analyzed as described in the literature (*45*−*48*). Specific binding was calculated as the difference between total and nonspecific binding. Nonspecific binding was determined in the presence of a 100-fold excess of nonlabeled ligand. Binding experiments were also performed with afamin that was treated with charcoal before the binding assay in order to remove bound hydrophobic ligands. However, the binding behavior of afamin was not influenced by this treatment.

Association and Dissociation Kinetics. Association and dissociation kinetics were measured with surface plasmon resonance (SPR) using biomolecular interaction analysis (BIA). For details we refer to recent reviews (*49*−*51*). SPR studies were carried out using a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden) accoring to Reiffert et al. (*52*). Afamin was immobilized on a carboxymethyl dextran surface chip (CM5) by activating the surface using EDC and NHS, followed by injection of afamin in 10 mM NaOAc, pH 5.0, running buffer 20 mM MOPS, pH 7.0, 150 mM NaCl. D-α-Tocopherol was injected dissolved in 100% ethanol at a concentration of 100 mM. Immobilized afamin was regenerated using 0.01% Tween 20 in running buffer, followed by 50 mM glycine, pH 9.5 solution, to remove bound D-α-tocopherol. Additionally, we performed the same kind of experiments with human plasma albumin to test

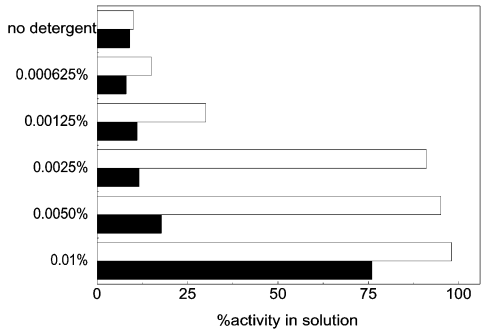

FIGURE 1: Dependence of the solubility of D-α-(5-methyl-^{14}C)-tocopherol as a function of the Tween 80 concentration (white bars). The black bars show the efficiency of dextran-coated charcoal as a function of the Tween 80 concentration in separating off free ligand (fraction remaining in solution). A Tween 80 concentration of 0.0025% (w/v) turned out to be the best compromise between solubilizing vitamin E and being able to remove vitamin E again afterward.

whether HSA is also a binding protein for D-α-tocopherol. The used HSA was purchased from Sigma as approximately 99% fatty acid free lyophilized powder and then redissolved in assay buffer.

Computational Details. The three-dimensional structure of afamin was generated by subscribing the sequence to the Swissprot database using the Swiss-Model tool (*53*,*54*). The Swiss-Model predicts the structure of proteins using methods of comparative modeling (*55*). The first step in comparative modeling is to identify homologue proteins with already known 3D structures. Generally it is accepted that a sequence identity of about 35% and more results in models that deviate only 1.0−1.5 Å from their experimental counterpart (*55*, *56*). (In our case the sequence identity to albumin was 36.52%.) HSA was used as template for comparative modeling of afamin. In detail the structures with the PDB codes E7IA, E7HA, E7GA, E7FA, E7EA, E7CA, E7BA, E7BB, E7AB, E7AA (*57*), BKE (*10*), BM0B, BM0A, BJ5, AO6A (*58*), AO6B, and UOR were used as templates. To overcome the problem of the molecular environment, we performed a 30 ps simulated annealing procedure using explicit water and counterions, starting from the comparative model structure. During this simulation the temperature was raised to 700 K in the first 4 ps and then slowly cooled to 0 K. All calculations were performed using the AMBER (*59*) suite of programs. The final structure served as receptor for the docking calculation of α-tocopherol with the Autodock3 program (*60*). As the docking algorithm, we chose the Lamarckian genetic algorithm and used random ligand positioning. The atomic charges for α-tocopherol were elucidated with the RESP (*61*) charge-fitting procedure.

RESULTS

Tocopherol-Binding Experiments. Verification of the Method. Dextran-coated charcoal was used as adsorption material to separate free from bound ligand. After testing several materials for the reaction vials, it was decided to use disposable glass tubes. The amount of α-tocopherol loss due to adsorption on the vessel wall was lowest with glass tubes and was improved by addition of 0.0025% Tween 80 (Figure 1). Lower Tween 80 concentrations did not sufficiently mediate solubilization of α-tocopherol, whereas with higher Tween 80 concentrations dextran-coated charcoal did not remove enough of the free concentration of α-tocopherol.

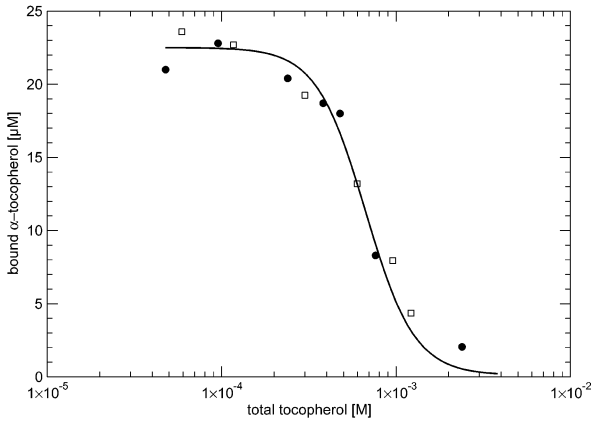

FIGURE 2: Displacement experiment for afamin with bound D-α-(5-methyl-^{14}C)tocopherol and D-α-tocopherol (solid circles) and D-γ-tocopherol (open squares) as "inhibitors". Both inhibitors perform equally well in displacing the bound ligand. The IC_{50} constants are 0.81 ± 0.10 mM (n = 3) and 0.75 ± 0.12 mM (n = 3) for α- and γ-tocopherol, respectively. (The Figure shows data determined in one representative experiment. The concentrations used were 2.0 μM afamin, 48 μM D-α-(5-methyl-^{14}C)tocopherol, and 48 μM to 25 mM D-α-tocopherol and D-γ-tocopherol. Incubation time was 2 h, which turned out to be sufficient to reach equilibrium (data not shown).)

Binding Experiments. Displaceable or specific binding of α-tocopherol to afamin was determined in a displacement experiment. In this experiment bound D-α-(5-methyl-^{14}C)-tocopherol was displaced by an excess of D-α-tocopherol (see Figure 2). Nonspecific binding was 21% under the chosen conditions and was subtracted from total binding (not shown). The IC_{50} (inhibitor concentration that displaces 50% of the labeled, bound ligand) was determined to be 0.81 ± 0.10 mM in three experiments for D-α-tocopherol.

The same experiment was performed again with afamin and D-α-(5-methyl-^{14}C)tocopherol as the labeled ligand but with D-γ-tocopherol as inhibitor. The results are also shown in Figure 2, and the IC_{50} was determined to be 0.75 ± 0.12 mM.

For full characterization of the binding properties, a saturation experiment was carried out to obtain a binding isotherm and perform both a Scatchard and a Hill analysis. Figure 3 shows specific binding of D-α-tocopherol with afamin. The point of half-saturation (50% saturation of the binding sites)—representing the overall binding-dissociation constant (K_D)—was reached at a free D-α-(5-methyl-^{14}C)-tocopherol concentration of 18 ± 7.1 μM. The highest saturation was achieved at 35 μM bound D-α-(5-methyl-^{14}C)-tocopherol. Saturation was extrapolated to 36 ± 11 μM, showing a maximum number of binding sites (B_{max}) of 18 molecules D-α-tocopherol per molecule afamin.

From the data obtained, a Scatchard plot was created that is shown in Figure 4. The character of the Scatchard plot under these experimental conditions was clearly concave downward. Thus, it had the appearance of positive cooperativity. To clarify whether this curvature of the Scatchard plot was caused by nonequilibrium conditions, we repeated measurements at incubation times of up to 5 h but found the same appearance.

The maximum binding capacity (B_{max}) was determined as the point of intersection of the graph with the abscissa and

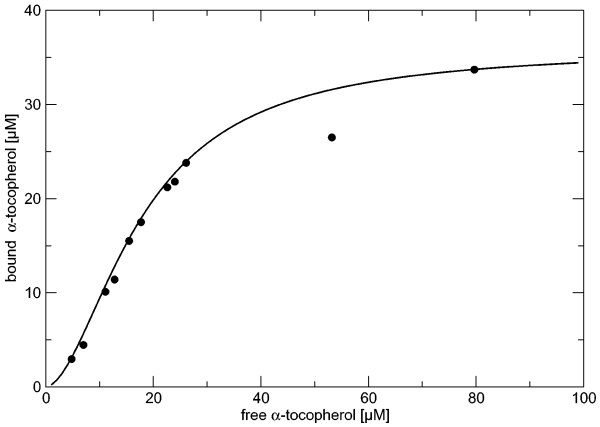

FIGURE 3: Binding isotherm for afamin with D-α-(5-methyl-^{14}C)-tocopherol. The shown points represent specific binding calculated as the difference between total and nonspecific binding. The graph shows one representative experiment out of three. The fitted curve was calculated with an assumed K_D of 18 μM ±7.1 and a Hill coefficient h of 1.8 (*47*).

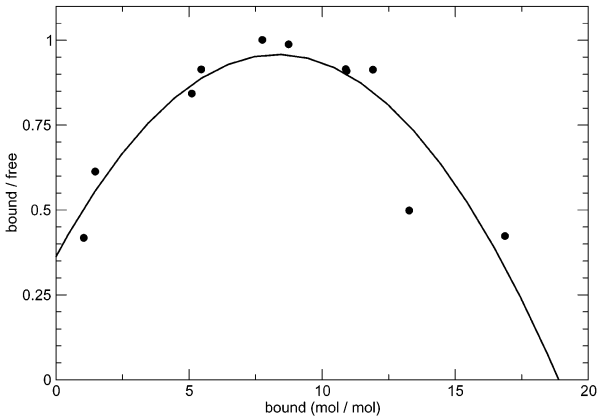

FIGURE 4: Scatchard plot for D-α-tocopherol and afamin binding. The shape of the plot is concave downward, indicating positive cooperativity in binding. The graph shows one representative experiment out of three.

compares well to the above result with 18 molecules per molecule afamin. For interpretation of the strength of binding, we performed a Hill analysis (data not shown) that gave us details about the type of cooperativity in binding. The Hill coefficient (h) was determined to be 1.8, indicating that the binding occurs with positive cooperativity. Additionally, we will test the binding affinity with docking calculations in the forthcoming section.

Due to the relatively small binding-dissociation constant, we wanted to test whether a difference in binding occurs between native and denatured protein. If the denatured protein would show the same binding behavior, then only the primary structure of afamin would possess the binding properties. Thus, α-tocopherol binding would be rather unspecific. We performed binding experiments on (1) lyophilized and redissolved afamin, (2) afamin heated to 50 °C for 20 min, and (3) afamin heated to 100 °C for 10 min (*62*). Compared to the control experiment, the binding was reduced by 11% for 1, by 24% for 2, and by 95% for 3.

Association and Dissociation Kinetics. Rates of D-α-tocopherol association to afamin, and the following dissociation were measured by surface plasmon resonance. The rate of association of D-α-tocopherol to immobilized afamin was determined as $k_{ass} = 3.3 \times 10^1 \pm 1.6 \times 10^1$ M^{-1} s^{-1}. After

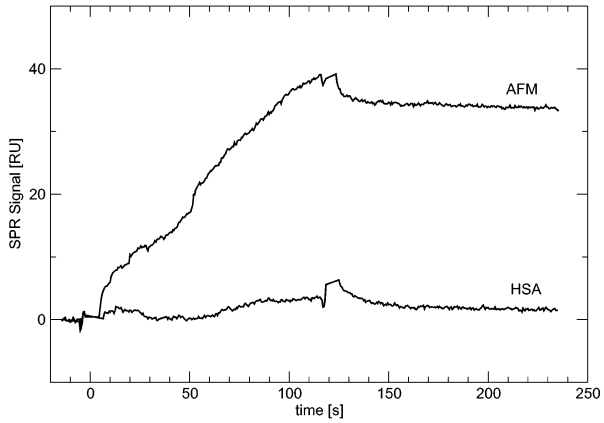

FIGURE 5: Kinetics of association and dissociation between D-α-tocopherol and afamin and human plasma albumin, respectively. Association was initiated at time = 0 s by injection of the α-tocopherol solution which was stopped after 120 s. Dissociation was monitored afterward in running buffer. HSA showed no signal after two minutes of association, whereas a clear signal for afamin was observed.

injection of 200 μM D-α-tocopherol, which is the highest applicable concentration under the chosen conditions, the molar ratio between immobilized afamin and bound D-α-tocopherol was 1:5. The apparent rate of dissociation of bound D-α-tocopherol was found as $k_{diss} = 1.12 \times 10^{-4} \pm 0.37 \times 10^{-4}$ s^{-1}. We evaluated the binding dissociation constant from the equilibrium rate constants according to the relationship $K_D = k_{diss}/k_{ass}$ and found a value of 3.4×10^{-6} M^{-1} (= 3.4 μM).

The SPR method was also used for HSA to test whether afamin is the only specific binding protein for D-α-tocopherol in the albumin gene family. Immobilized HSA showed no signal after passing D-α-tocopherol over it for several minutes (see Figure 5).

Structure of Afamin and Docking Results. The protein structure was determined by homology modeling using the Swiss-Model tool. Evaluation of the protein model is an important part of protein structure prediction. Beside the high sequence identity (36.52%, Smith Watermann score 1430), which normally results in excellent structures, a Ramachandran plot of the structure was calculated using Procheck (*63*). About 90% of all residues occupy the most favored region in this Ramachandran plot, which again indicates the high quality of our model. With the exception of the first 40 amino acids, which include the sequence of the signal peptide—explaining that a higher sequence identity was not observed—no large gaps exist in the pairwise alignment of afamin with its homologues. It is well-known that afamin consists of three domains and exhibits 17 Cys−Cys disulfide bridges (*6*). In our comparative model all respective pairs of Cys residues, which are involved in a disulfide bridge, are structurally close together. In our opinion, this again shows in an impressive way that the results of the comparative modeling can be trusted.

Comparison of both the comparative modeling and the simulated annealing structures by visual inspection shows that the principal fold does not change during this simulated annealing minimization procedure. A more detailed view of the structure and dynamics of afamin and the influence of the solvent elucidated by molecular mechanics calculations will be the topic of future research.

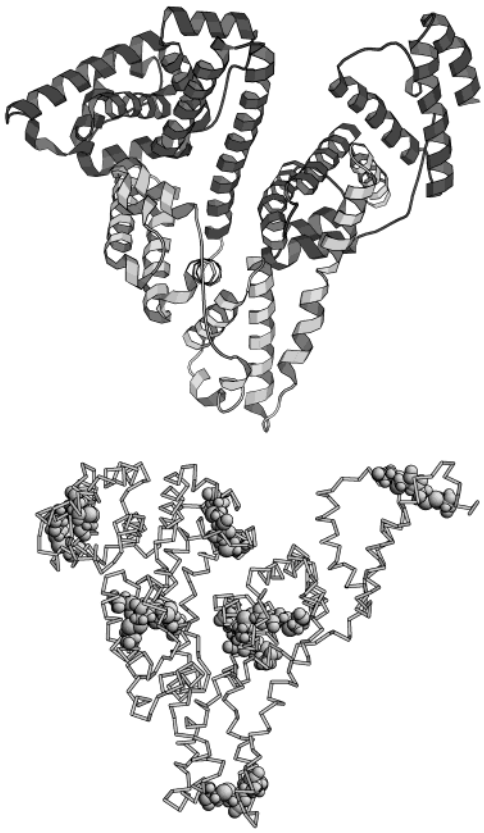

FIGURE 6: Predicted structure of afamin (top) based on a comparative modeling approach using serum albumin as template. The three domains are marked by different gray scales. The bottom structure shows the afamin model after the simulated annealing procedure. The cysteins building a disulfide bridge are indicated as spacefill.

The amount of α-helix was estimated to be about 75%, and although 12% of all backbone torsions (data are from the Ramachandran plot) are in the range of *β*-sheets, no such secondary structure element was found in the structure. Furthermore, the structure (Figure 6) shows that the N-glycosylation sites are located near the surface of the protein, allowing high accessibility.

The binding sites and thermodynamics of α-tocopherol were investigated using the Docking program Autodock3, starting from the simulated annealing structure as receptor. Summarizing these results, a great variety of different binding sites are found with the best results in the range of a free-energy change (ΔG) of 7.0 kcal mol^{-1} for binding (at 298.15 K). This was explained by the large flexibility of the alkyl chain and is in agreement with our experimental results. However, due to the lack of statistics these results should be interpreted with caution.

DISCUSSION

We recently showed that afamin has the ability to bind α-tocopherol (*31*). In this study we performed a detailed analysis of the binding properties of afamin. Experimentally, it was seen that afamin has an apparent binding-dissociation constant (K_D) of 18 ± 7.1 μM. The inhibitory constant that displaces 50% of bound ligand (IC_{50}) was determined to be 0.81 ± 0.10 mM, which is relatively high, even though it should be in the same order of magnitude with the K_D. Presumably, this can be partially explained by the high number of binding sites ($B_{max} = 18 \pm 5.5$). Even though the IC_{50} for D-*γ*-tocopherol (0.75 ± 0.12 mM) appears to be slightly lower, indicating a higher binding affinity, when considering the uncertainties in the experimental setup, it is much more likely that both D-α-tocopherol and D-*γ*-tocopherol are almost equally good inhibitors for bound D-α-(5-methyl-^{14}C)tocopherol.

To describe the binding properties of afamin for D-α-tocopherol in more detail, a Scatchard analysis was performed that did not yield a satisfactory explanation for the observed binding pattern. Presumably, the binding sites are filled step-by-step, and each binding site differs from the previous one since afamin does not possess 18 domains with identical structure. According to Dahlquist (*46*), determination of each binding-dissociation constant would require at least three points for each binding site, yielding an overall need of at least 54 different experimental values. This number is clearly out of the range and out of sensitivity of the employed method. Alternatively, we performed a Hill analysis and obtained a Hill coefficient of 1.8, indicating a slight positive cooperativity. The positive cooperativity can be best explained by the fact that incoming, hydrophobic α-tocopherol molecules increase the hydrophobicity of the protein−ligand complex and thus make this complex more accessible to forthcoming ligands. From the shape of the saturation and the Scatchard plot and from the value of the Hill coefficient, we can assume that all binding-dissociation constants are within the same range. Therefore, we rely on the previously determined value for K_D of $1.8 \times 10^{-5} \pm 0.71 \times 10^{-5}$ M, which represents the overall, apparent binding-dissociation constant, and on the maximum number of 18 binding sites. A different approach to investigate the binding properties of afamin was carried out with the surface plasmon resonance method. With this approach we determined a binding-dissociation constant of $3.4 \times 10^{-6} \pm 2.3 \times 10^{-6}$ M. This value is within 1 order of magnitude with the binding constant determined by the dextran coated charcoal assay. However, the immobilization of afamin might cause some of the afamin molecules to lose their activity. Additionally, immobilization will prevent D-α-tocopherol from accessing all possible binding sites, and therefore, we cannot determine the total binding capacity. Due to the hydrophobicity of α-tocopherol and due to the large number of binding sites, it is likely that in the course of dissociation rebinding of the dissociated ligand occurs at another binding site. Therefore, only an apparent rate of dissociation could be measured and the values must be used with care. The rate constants of association and dissociation are $3.3 \times 10^{1} \pm 1.6 \times 10^{1}$ M^{-1} s^{-1} and $1.1 \times 10^{-4} \pm 0.37 \times 10^{-4}$ s^{-1}, respectively.

To shed light on the complex binding behavior of afamin, we determined the structure of afamin by homology modeling and performed docking calculations with D-α-tocopherol on the structure. We found several binding sites with a ΔG of 7.0 kcal mol^{-1}. This value is equivalent to a K_D of 7.3×10^{-6} M, which is just between the two experimentally determined ones (*48*). Investigation of the ligand-afamin complex showed that due to the high flexibility of the hydrophobic alkyl tail of D-α-tocopherol the ligand can enter the binding pockets in several ways and a broader spectrum of equally strong binding sites could be determined. However, several binding sites with similar affinity are in very good agreement with the experimental results. Yet the

experimental as well as the theoretical approach to determine the binding-dissociation constant bear the potential of slight errors. First, due to the experimental setup in the dextran coated charcoal assay, there is an error potential of at least 1 order of magnitude, and the presented data can be seen as a worst-case interpretation. α-Tocopherol could not be completely dissolved in the assay vials; nor could the free ligand be totally removed afterward. Second, in the SPR approach, not all binding sites are accessible for the ligand, so several binding sites have to be neglected in this assay. Third, the theoretical considerations are based on a structure evaluated by homology modeling. Even though the homology of the protein (36.5%) is higher than the homology required (35%) for the predicted model to be considered almost equivalent to an experiment, still we cannot be absolutely sure that the structure and therefore the docking results represent the real structures of protein−ligand interactions.

However, taking all results into account we find that afamin is a specific binding protein for D-α-tocopherol and D-γ-tocopherol. Surface plasmon resonance experiments on HSA showed no binding for D-α-tocopherol. Binding experiments with afamin treated under denaturing conditions confirmed specific binding for α-tocopherol. Weakly denaturing conditions affected the binding behavior only slightly, whereas strongly denaturing conditions decreased the specific binding behavior of afamin for α-tocopherol tremendously.

The value of 1.8×10^{-5} M for the K_D of afamin for D-α-tocopherol appears to be physiologically relevant (*31*). The high number of 18 binding sites is remarkable and makes afamin a high-capacity binding protein for vitamin E. However, such a high capacity is not unique among the proteins of the albumin gene family. HSA, for instance, has been reported to possess several binding sites for fatty acids (*10*) and up to 29 binding sites for other ligands (*64*). The mean human plasma concentration of afamin is 61.4 μg mL^{-1}, which yields a total concentration in binding sites of 17.0 μM, assuming that each molecule of afamin binds 18 molecules of vitamin E (*31*). With a mean α- and γ-tocopherol concentration of 29.9 μM, it is very likely that at least a fraction of α- and γ-tocopherol is bound to afamin—even though the majority will be bound to lipoproteins (*31*). The concentrations of afamin and α- and γ-tocopherol in human follicular fluid are only slightly lower than in human plasma (*31*). Since the lipoprotein concentration in follicular fluid is very small, afamin might be important as transport protein for α- and γ-tocopherol there. Considering that the experimentally determined K_D might be even smaller—like the ones determined theoretically and by SPR—the importance of afamin for D-α-tocopherol transport in extravascular fluids is evident.

ACKNOWLEDGMENT

We are grateful to J. Striessnig for helpful discussions in the initial stage of this study and in final preparation of the manuscript. SPR measurements were performed by BIAFFIN GmbH & Co KG, D-44801 Bochum, Germany.

NOTE ADDED AFTER ASAP POSTING

This paper first appeared on the Web on November 14, 2002, without all revisions. The correct version was published on November 18, 2002.

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BI026513V