Chimeric Avidin – NMR Structure and Dynamics of a 56 kDa Homotetrameric Thermostable Protein

Helena Tossavainen1, Sampo Kukkurainen2,4, Juha A. E. Máätä2,3,4, Niklas Kähkönen2,3, Tero Pihlajamaa1, Vesa P. Hytönen2,3,4, Markku S. Kolumaa2,3, Perttu Permi1*

1 Program in Structural Biology and Biophysics, Institute of Biotechnology, University of Helsinki, Helsinki, Finland, 2 Institute of Biomedical Technology, University of Tampere and Tampere University Hospital, Tampere, Finland, 3 BioMediTech, Tampere, Finland, 4 Fimlab Laboratories, Tampere, Finland

Abstract

Chimeric avidin (ChiAVD) is a product of rational protein engineering remarkably resistant to heat and harsh conditions. In quest of the fundamentals behind factors affecting stability we have elucidated the solution NMR spectroscopic structure of the biotin–bound form of ChiAVD and characterized the protein dynamics through 15N relaxation and hydrogen/deuterium (H/D) exchange of this and the biotin–free form. To surmount the challenges arising from the very large size of the protein for NMR spectroscopy, we took advantage of its high thermostability. Conventional triple resonance experiments for fully protonated proteins combined with methyl–detection optimized experiments acquired at 58°C were adequate for the structure determination of this 56 kDa protein. The model–free parameters derived from the 15N relaxation data reveal a remarkably rigid protein at 58°C in both the biotin–bound and the free forms. The H/D exchange experiments indicate a notable increase in hydrogen protection upon biotin binding.

Introduction

Chicken egg–white avidin and its bacterial analogue streptavidin from Streptomyces avidinii bind their natural ligand biotin with an extremely high affinity (dissociation constant \( K_d \approx 10^{-15} \) M). In addition, they are remarkably stable against heat and harsh conditions such as proteolysis, denaturants and extremes of pH. These exceptional properties are widely employed in streptavidin biotechnological applications which typically rely on bridging a biotinylated target molecule binder to (strept)avidin [1,2] often in solution conditions very unnatural to proteins. Chemical and genetical engineering of avidin and streptavidin have further extended the diversity of the techniques [3].

Despite low sequence similarity, proteins of the avidin family have a remarkably similar molecular structure composed of four identical subunits (of 128 residues in avidin, Figure 1). The monomeric unit consists of an antiparallel eight–stranded β barrel each of which accommodates one biotin molecule at one end of the barrel. The four avidin subunits are arranged in a dimer of dimers [4]. This quaternary structure results in three distinct interfaces: the 1–4 interface is characterized by hydrophobic and polar interactions of such extent that the dimer can be considered as a single structural unit whereas the 1–3 interface is the weakest, composed only of three residues in avidin. The 1–2 interface is important for the tetramer stability and biotin binding affinity. There, a crucial tryptophan residue interacts with biotin bound in the adjacent subunit [4,5]. Excitingly, the structural similarity encompasses also the biotin–free forms of the proteins. The binding site is preformed in the free form, and no significant tertiary or quaternary structure rearrangements are needed in order to achieve the tight protein–ligand interaction. The stability of the free form is, however, markedly lower than that of the bound form. This is reflected in lower unfolding and oligomer dissociation temperatures [6].

ChiAVD(I117Y), hereafter referred to as ChiAVD, is a product of rational protein engineering [7,8]. It is a hyperthermostable hybrid of avidin and avidin-related protein 4 (AVR4) [9–11] obtained by replacing a 23–residue segment in avidin with the corresponding segment found in AVR4 and additionally introducing an Ile to Tyr, \( \pi-\pi \), 1–3 interface–stabilizing point mutation. ChiAVD is remarkably resistant to heat with a transition midpoint temperature, \( T_m \), for thermal unfolding of 111.1°C in the free and \( \sim 130°C \) in the bound form. In the presence of SDS, it dissociates into monomers only at \( \sim 95°C \) and 110°C in the free and bound form, respectively. ChiAVD is the most thermostable avidin studied to date. It is also resistant to harsh conditions such as extremes of pH and various organic solvents, even at high temperature. The biotin–binding properties of ChiAVD are comparable to those of AVR4 [7]. Since ChiAVD has successfully been applied in novel approaches in biotechnology [12,13], the understanding of the molecular properties of the protein is of our
Figure 1. The molecular structure of avidin family of proteins. The structure, here represented with biotin-bound avidin (PDB identifier 2AVI), is a homotetramer composed of units of ~128 residues. Subunits are numbered according to [4]. Each subunit binds one biotin molecule, shown in stick representation. ChiAVD is a hybrid of avidin and AVR4, in which the segment highlighted in orange in avidin (residues 38–60, 23 residues) is replaced by the sequentially related segment found in AVR4 (residues 38–58, 21 residues). Also, Ile 117 of avidin, shown in red, is replaced by a tyrosine found in AVR4.

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Expression and purification of the 13C/15N–labelled ChiAVD

The coordinates of the final ensemble have been deposited to the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) with the accession code 2mif6. Structure figures were created with UCSF Chimera [21].

Materials and Methods

NMR spectroscopy, experiments and data analysis

Expression and purification of the 13C/15N–labelled ChiAVD as well as resonance assignment have been described previously [16]. Spectra for structure determination were acquired with a Varian INOVA 800 MHz spectrometer equipped with a cryogenic probehead, at 58°C. The spectra were processed with Vnmr 6.1C (Varian Inc.) and analysed with Sparky 3.110 (T. D. Goddard and D. G. Kneller, University of California, San Francisco).

Distance restraints were obtained from NOE peaks picked from 13C, 1H NOESY–HSQC, and 15N, 1H NOESY–HSQC spectra acquired from a sample dissolved in 92/8% H2O/D2O and 13C, 1H HSQC–NOESY acquired from a sample in 100% D2O, the latter being especially useful for NOEs arising from methyl groups. By inspection of available avidin crystal structures in the RCSB protein data bank (PDB), intersubunit (1–2 and 1–4) NOE peaks were identified and manually assigned. With Cyana [17] version 2.1, two hundred 1–4 dimer structures were calculated with automatic assignment carried out for the intrasubunit peaks, and manually assigned NOE peak lists for 1–4 intersubunit restraints. In addition to distance restraints, H–bond (from H/D exchange experiments), 0/ψ restraints from TALOS [18], χ2 restraints deduced from J(C–C') and J(C–N)–coupling spectra and 1H, 15N residual dipolar couplings (RDCs) from spectra acquired from a sample in dilute solution of bicelles at 40°C (see next subheading) were used. Twenty structures with the lowest target function were selected. An initial tetrameric structure was built by duplicating a dimer structure and positioning the two dimers at an approximately correct orientation. From each starting tetramer a set of 10 structures was calculated with XPLOR–NIH [19] version 2.29 using all the available restraints. Of the resulting 200 structures, twenty lowest–energy structures were minimized with Amber 8 [20] and selected to represent the ChiAVD structure in solution. The coordinates of the final ensemble have been deposited to the RCSB Protein Data Bank (http://www.rcsb.org/pdb/) with the accession code 2mif6. Structure figures were created with UCSF Chimera [21].
H/D exchange experiments were carried out by first lyophilising a sample in D$_2$O and then dissolving it to H$_2$O. Increase in cross peak intensity was followed by measuring $^1$H, $^{13}$N HSQC spectra at 58°C, 800 MHz. The first time point was at approximately 15 minutes after dissolution. The last time point for biotin-free ChiAVD sample was at 96 hours, and for the bound form weeks after dissolution. Peak intensities were fitted to a three–parameter equation of the form $I(t) = I(0)*[1+\exp(-k_m*t)].$ Residue specific protection factors [24,25] were derived from the H/D exchange rates $k_m$ using the spread sheet available from the Englander lab’s website, http://hx2.med.upenn.edu/download.html

RDC measurements

As ChiAVD is positively charged at the sample pH, we used bicelles, composed of 5% (w/V) DMPC/DHPC phospholipids at a molar ratio of 3:1, as the liquid crystal medium. Due to the instability of this liquid crystal medium at elevated temperatures we measured $^1$H–$^{13}$N RDCs at 40°C. As no deuterium labelling was utilized, we employed a modified version of the MQ–HINC–TROSY experiment that has been successfully used for measuring $^1$H–$^{15}$N RDCs in the 558–residue Filamin A 16–21 fragment [26].

RDCs were applied as constraints in all four subunits.

Molecular dynamics simulations

The X–ray crystallographic structure of chimeric avidin [8] (PDB identifier 3MMO) was completed for the missing residues in L6,7 of chains E, F, G, and H (1–3 residues each) and L3,4 of chain G (Asn43) using Modeller 9v5 [27]. Biotin was placed into the binding pockets of ChiAVD with the help of wild–type avidin [4], PDB identifier 2AVI. Coocrystallized water molecules from within a 5 Å radius were included, and waters clashing with biotin were removed. Hydrogens were added using PDB2PQR 1.3.0. [28,29]. GAFF parameters were assigned for the biotin using the antechamber module, and Amber_99SB parameters [30] were assigned for the protein in the tleap module in Amber 10 [20]. The tetrameric protein with or without biotin was placed in a 75 A x 86 A x 90 A box filled with TIP3P water molecules. 11 and 15 Cl$^-$ ions were added, resulting in a total of 52844 and 52973 atoms in the bound and ligand–free systems, respectively. Energy minimizations and molecular dynamics simulations were carried out in NAMD 2.6 [31]. Three 4000-step conjugate gradient minimizations were carried out for the ligand–bound complex: first with protein and ligand frozen, second with the ligand and CaX atoms frozen, and third without restraints. For the ligand–free system the second minimization was omitted. The systems were heated from 0 to 310 K in 31 ps and equilibrated for 2 ns in 310 K. The simulations were continued for 10 ns at three temperatures: 310, 333, and 523 K. The simulations were carried out in NPT conditions (1 atm) using the Berendsen thermostat and barostat. A 1–fs timestep was used in all simulations. The trajectory was superimposed by the CaX atoms using the RMSD Visualizer Tool plugin, and root mean square fluctuation (RMSF) was calculated using the “measure rmsf” command and a 1–ps step in VMD 1.9.1 [32].

ChiAVD mutants G42A and G42F

Glycine 42 mutations to alanine and phenylalanine were done with conventional PCR mutagenesis. Protein expression was performed using pET101/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) in BL21-AI E. coli (Invitrogen). Protein purification with 2-imino-biotin agarose (Affiland, Belgium) was performed as described in [16].

The dissociation rate constant ($k_{diss}$) of fluorescently labelled ArcDiaTM BF560 (ArcDia, Turku, Finland) biotin was determined by fluorescence spectrometry essentially as described in [33]. The assay was performed at 50°C using a QuantaMasterTM Spectrofluorometer (Photon Technology International, Inc., Lawrenceville, NJ, USA) equipped with circulating water bath thermostat. The fluorescence probe was excited at 560 nm and emission was measured at 570 nm.

Determination of hydrodynamic radius was performed by dynamic light scattering (DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd) in 50 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, 100 mM NaCl, pH 7 at 25°C. Six measurements were performed each consisting of 10 × 10 s measurement. Data was analysed using Zetasizer software v7.01 (Malvern Instruments Ltd) using “General purpose” model and volume distribution.

The transition midpoint ($T_{m}$) of the ChiAVD forms was measured by differential scanning calorimetry (DSC) using VP-capillary DSC (GE Healthcare, MicroCal) in 50 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, 100 mM NaCl, pH 7. The scanning was carried out from 20°C to 140°C at a rate of 120 C/h, using a 3 s filter period and a low feedback mode. Measurements were done using 13.8 μM protein in the presence or absence of 36 μM D-biotin (Fluka prod. no. 14400). Data analysis was made using the Origin 7 software (GE Healthcare, MicroCal). The $T_{m}$s were determined using a Non-2-state fitting model.

Results and Discussion

Structure of biotin–bound ChiAVD

We have solved the first solution structure of a member of the avidin–family of proteins. The structure of this 56 kDa protein was solved without resort to the laborious, yet relatively expensive method in which perdeuteration is combined with selective methyl protonation [34]. The deciding factor was the thermostability of the protein – a raise of the measurement temperature to 58°C reduces the protein’s overall rotational correlational time (~25 ns at room temperature) to half the value. Amide proton exchange rate with solvent significantly increases, however, along with temperature. To address this problem, a set of methyl proton detection experiments with high sensitivity and resolution [14] were employed in the assignment of methyl containing residues [16].

The homotetrameric symmetrical structure simplifies the NMR spectra of ChiAVD at the expense of losing potential NOE distance restraints at the very center of the protein structure (Arg114, Val115) where intra- and intersubunit correlations are indistinguishable. Intermolecular NOE cross peaks, here referring to those between ChiAVD subunits, were manually assigned from the methodology utilized earlier to produce monomeric avidin [8]. To overcome the symmetry issue it would have been possible to create a tetrameric ChiAVD with differentially labelled subunits by using the methodolodgy utilized earlier to produce monomeric avidin which tetramerizes upon addition of biotin [35]. Alternatively, dual chain avidin technology could have been applied [36].

Several NOE cross peaks from ChiAVD to biotin were observed in the NOE spectra of ChiAVD at the expense of losing potential NOE distance restraints at the very center of the protein structure (Arg114, Val115) where intra- and intersubunit correlations are indistinguishable. Intermolecular NOE cross peaks, here referring to those between ChiAVD subunits, were manually assigned from the methodology utilized earlier to produce monomeric avidin [8]. To overcome the symmetry issue it would have been possible to create a tetrameric ChiAVD with differentially labelled subunits by using the methodolodgy utilized earlier to produce monomeric avidin which tetramerizes upon addition of biotin [35]. Alternatively, dual chain avidin technology could have been applied [36].
for structure calculation were obtained from chemical shifts, J-coupling constants, H/D exchange experiments, and D2H RDCs. **RDC measurements.** To measure D2H RDCs in ChiAVD, the newly modified MQ-HNCO-TROSY scheme (MQ-HNCO-TROSY+, Figure 2) was devised. The pulse scheme reduces losses associated to exchange broadening due to solvent exchange or J couplings. This experiment enables the determination of H–15N RDCs by measuring 1J(NH) (in water) and 1(J NH) 2T component. This is especially pronounced in non-deuterated anti-TROSY component is always smaller than for the downfield component. Therefore, in the case of ChiAVD, we were able to measure 93 RDCs. In two experiments that select either the upfield or the downfield 15N–{1H} component. The former signal decays with a rate exp[−1/2(l–170–172+174)] and the latter with exp[−1/2l(l)R2A + R2T], where R2A and R2T are the transverse relaxation rates for the anti-TROSY and TROSY components. Therefore, in the MQ-HNCO-TROSY+ experiment, the linewidth for the upfield component is larger than for the downfield component. The narrower linewidth and the increased S/N for the upfield component are due to a differential relaxation effect between the two experiments that select either the upfield or the downfield 15N–{1H} components. The former signal decays with a rate exp[−1/2(l–170–172+174)] and the latter with exp[−1/2l(l)R2A + R2T], where R2A and R2T are the transverse relaxation rates for the anti-TROSY and TROSY components. Therefore, in the MQ-HNCO-TROSY+ experiment, the linewidth for the upfield component is always smaller than for the downfield component. This is especially pronounced in non-deuterated samples. The MQ-HNCO-TROSY+ spectrum of 15N, 13C–15N, 13C–1H RDCs in ChiAVD, the effective linewidth, with respect to the TROSY component, can be fine-tuned with two parameters K (0 < k < 1) and λ (λ > 0). In the case of ChiAVD, the 1J+D2H RDCs were measured by recording two MQ-HNCO-TROSY+ spectra in an interleaved manner with k = 0.5 and λ = 0.5 i.e. the apparent splitting measured in 15N dimension corresponds to 1J+D2H/2, hence doubling the random error. As highlighted in the figure, the upfield 15N–{1H} component generally exhibits a narrower linewidth with respect to the downfield 15N–{1H} component. In loops the free bound RMSD values range from 0.28 ± 0.04 and 1.34 ± 0.04 Å for the backbone and heavy atoms, respectively.

In loops the free bound RMSD values range from 0.80 ± 0.04 Å for the for the backbone, H/D exchange experiments, and D2H RDCs. backbone atoms of the biotin–bound ChiAVD solution structure superimpose well with those of the biotin–free form crystal structure (8) (Figure 5A). For the monomer the average backbone RMSD (N, Cα, C, O atoms in β strands, 232 atom pairs) is 0.73 ± 0.04 Å and that for heavy atoms (N, Cα, O atoms in β strands, 468 atom pairs), 1.24 ± 0.04 Å. For the tetramer (960/1944 atom pairs) the RMSDs are slightly larger, 0.80 ± 0.04 and 1.34 ± 0.04 Å for the backbone and heavy atoms, respectively.

In loops the free bound RMSD values range from 0.80 ± 0.04 Å for the four–residue loop connecting β strands 1 and 2 (L1,2, residues 13–16 of the tetramer) to 1.60 ± 0.28 Å found for L4,5 (residues 54–62). The higher RMSDs in loops are the result of increased mobility as compared to the structured parts (see below the 15N relaxation analysis). Notably, this is true also for L3,4 (residues 35–46) in which the backbone atom RMSD to the free form is 1.29 ± 0.13 Å. In the biotin–free form residues Pro41– Gly42 of this loop fold to a helix-like conformation (φ/ψ angles on average −64°/−9° and −69°/−22°) whereas in the biotin–bound form these residues are found in multiple conformations. None of the conformations preclude the formation of the stabilising intramonomeric salt bridge between side chains of Asp39 and Arg114 from β8 observed in the structures of biotin–free ChiAVD and AVR4 (Asp39–Arg112, [8,9]). This salt bridge is present in half of the structures of the ensemble.

Although NOEs to biotin were excluded from the structure calculations, residues in the biotin binding site are well defined (Figure 5B). Some of the side chains of polar residues have, however, mutually different orientations. Besides the lack of biotin resonance assignments, here also the fact that no attempts were made to assign the side chain hydroxyl and amide group protons contributes to the differences observed.

### Biotin–free ChiAVD in solution

The biotin–free ChiAVD sample deteriorates substantially faster than that of the bound form at the high temperature needed for sufficient NMR experiment sensitivity. Cross peaks become wider and their shape gets distorted although remaining at same positions with no additional peaks appearing over time. We suspect that over time in the prevalent solution conditions the protein molecules transiently interact to form higher molecular weight states. Assignment of the backbone resonances was however successfully conducted at 70°C [16]. Methyl group chemical shifts of free ChiAVD are listed in Table S2.

The structural similarity of the bound and free forms is evident from chemical shift comparison. The Δδ Cα, Cβ, N, HN (free-bound) persuasively show that the chemical environments within monomers differ uniquely at residues located in the biotin binding site (see Figure S2 for Δδ Cβ). The largest shift differences, up to 3–5 ppm, are observed in L3,4 for residues Val37 and Ala38. The 1H, 13C chemical shifts of methyl groups located at the interfaces also match. A comparison of the 1H, 13C HSQC spectra of free (at 80°C) and bound (38°C) ChiAVD reveals that the methyl-containing residues at the 1–4 dimer interface exhibit comparable side chain chemical shifts (Δδ13C < 0.27 ppm, Δδ1H < 0.04 ppm) in the two forms. No significant methyl chemical shift changes are observed for Met96, Thr113, or Val115 at the 1–2 and 1–3 interfaces either. The N15–H15 pair of Trp110 bridging monomers 1 and 2 shifts notably. This is, however, caused by direct interaction with biotin.

Because of the different acquisition temperatures, the 1H line widths in the 1H, 13C HSQC spectra of free and bound ChiAVD cannot be directly compared. However, the measured line widths in both spectra can be divided in to three categories depending on their magnitude. We observe that each methyl resides in the same line width category in both protein states. We deduce that in the two forms the methyl groups at the interfaces have similar dynamical characteristics arising from similar chemical environments.

In all, the chemical shift and line width data indicate a close tertiary and quaternary structure similarity between the free and bound forms.
the bound form. It is thus just used to structure the form of the bound form in solution at 58°C in the analysis of the 15N relaxation data of both forms. When extracting diffusion tensor parameters from the relaxation data this structure also gave lower $\chi^2$ target function values as compared to the crystal structure of the free form. Details of the diffusion tensor parameters and their derivation are given in Figure S3.

Model-free analysis

$R_1$, $R_2$ and heteronuclear Overhauser (hetNOE) 15N relaxation data were recorded on 600 and 800 MHz spectrometers at 58°C for free and bound ChiAVD. For the bound form also relaxation data at 40°C on 800 MHz were recorded. These data as well as the average values are presented in Figure S4 and Table S3. The relaxation data were interpreted using the Model-free approach [40,41] and are presented for the 800 MHz data. Similar results were obtained from the analysis of the 600 MHz data.

Assuming isotropic diffusion, the overall rotational correlation times ($\tau_c$) are 13.0 ± 0.4 ns for the biotin-bound ChiAVD, and 13.2 ± 0.4 for the free form at 58°C. At 40°C, a $\tau_c$ of 18.0 ± 0.6 ns is found for the bound form. By assuming a linear correlation between the ratio of solvent viscosity and temperature ($\eta/T$) and $\tau_c$, at 25°C ChiAVD has an overall rotational correlation time of 25.4 ns. It is interesting to note, that this $\eta$ differs markedly from that estimated by the empirical formula, $\tau_c = 0.5998 \times MW + 0.1674$, giving 33.8 ns. The Stokes-Einstein relation for the reorientation of a hard sphere gives an estimate of 21.0 ns for $\tau_c$ assuming a hydration radius of 3.2 Å. The observed rigidity of ChiAVD (see below) might lower the rotational correlation time towards the value predicted by the latter relation. This is consistent with the notably low $\chi_s$ found for the highly rigid β-lactamases TEM-1 [42] and PSE-4 [43].

The Model-free parameters $S^2$, $\tau_c$ and $R_{ex}$ are presented in Figure 6. The squared order parameter, $S^2$, provides information...
on the amplitude of the N–H bond vector motion. It varies between 0 and 1, with 1 corresponding to completely restricted bond vector motions and 0 to completely unrestricted motions. \( \tau_e \) gives the effective time scale of the bond vector motion, typically ranging from 0 to 100 ps but in loops and termini up to a few ns. \( R_{ex} \) reports on the presence of ms-ms time scale exchange.

**Comparison of free and bound ChiAVD.** On average the two forms are dynamically very similar but subtle differences are observed in loop regions. Average \( S^2 \), excluding the termini, are 0.86±0.03 for the bound and 0.85±0.02 for the free form. Secondary structure regions have an average \( S^2 \) of 0.89±0.02 in the bound and 0.87±0.02 in the free form. Altogether both forms are remarkably stable taking into account the high measurement temperature.

Corresponding data are available for 66 residues. Large reduction (\( \Delta S^2 > 0.1 \)) in the amplitude of motion upon biotin binding is observed for residues Thr19 (B2), Gly31 (B3), Ala38, Asn40 and Ile44 (L3,4), Gln53 (B4) and Arg100 (B7). The most impressive change is observed in L3,4 with \( \Delta S^2 \) values of 0.13–0.19. Mobility in L3,4 is however still present in the biotin–bound form as Asn40 and Ile44 have \( S^2 \) values of ~0.7 and Gly42 as low as 0.2. Two residues, Ser73 and Phe120, show significant increase in mobility upon biotin binding.

**Figure 3. Excerpt from the MQ-HNCO-TROSY+ spectrum.** The MQ-HNCO-TROSY+ spectrum of \( ^{15}N, ^{13}C \) labeled chimeric avidin was measured at 40 °C on 800 MHz in a dilute liquid crystal medium composed of 3:1 mixture of DMPC:DHPC phospholipids (bicelles). Panel (A) displays overlaid upfield (red contours) and downfield (black contours) components of \( ^{15}N-^{1}H \) doublets. Vertical dotted lines indicate the position of the corresponding \( ^{15}N \) traces shown in panel (B) for T14, N27 and D87. As the scaling parameters were set to \( \kappa = 0.5 \) and \( \lambda = 0.5 \), the measured couplings are scaled down by the factor of 2. The measured apparent \((J+D)/2\) couplings are shown next to each splitting for T14, D87 and N27.

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Slightly larger values (2.0 in the bound form, and seven residues in the free form, with relaxation are observed for residues in L3,4 and L5,6, with the more mobile when temperature is raised. Largest decrease in $S^2$ analysis shows that it is the biotin–binding region that becomes found for the two temperatures. Interestingly, a per residue the protein is structured from residues Lys3 to Leu123. Ensemble of fifteen structures of least restraint violations. ($u$ sets with an experimental temperature below 34°C entries including order parameters deposited in the BioMagResBank [53]. In both proteins slow exchange are located in secondary structure regions. Three of these are located in the lid–making loop L3,4. At the Ser73 and Arg114, all at the biotin–binding end of the –barrel. are in contact with the 1–2–related monomer via Trp110.

In both forms, it is relaxation active for several residues in loops and in a few loop–flanking residues. Largest contributions of $R_{ex}$ to relaxation are observed for residues in L3,4 and L5,6, with the bound form values outweighing those of the free form.

**Comparison of bound ChiAVD at two different temperatures.** The rigidity of the protein is almost completely retained when increasing the temperature from 40 to 58°C. The average $S^2$ of 0.86±0.03 observed at 58°C for all residues excluding the flexible residues at the termini has only slightly decreased from the 0.88±0.03 observed at 40°C. If only residues in secondary structures are considered the same average $S^2$ value is found for the two temperatures. Interestingly, a per residue analysis shows that it is the biotin–binding region that becomes more mobile when temperature is raised. Largest decrease in $S^2$ are observed for residues Gly15, Ala36, Gly42, Ile44, Thr45, Ser73 and Arg114, all at the biotin–binding end of the –barrel. Three of these are located in the lid–making loop L3,4. At the lower temperature the number of residues with a $R_{ex}$ contribution is larger. Unexpectedly, the majority of the residues exhibiting slow exchange are located in secondary structure regions.

The Model–free parameters derived from the relaxation data imply that ChiAVD is a remarkably rigid protein. A search of entries including order parameters deposited in the BioMagResBank (http://www.bmrb.wisc.edu/) reveals that in the thirty data sets with an experimental temperature below 34°C the calculated average $S^2$ (omitting possible flexible terminal residues) ranges from 0.71 to 0.92. Only a few deposited data sets with an experimental temperature above 40°C are available. In these the average $S^2$ are 0.90 at 45°C for Trp repressor (MRB entry 17041), 0.86 at 50°C for C1BM11 [1839], 0.82–0.84 at 47–75°C for calmodulin–peptide complex (4970), and 0.81 at 44°C for azurin (6243). From the literature we find, in addition, average $S^2$ of 0.54 (50°C) for the B1 domain of Streptococcal protein G [44], 0.62 (45°C) for cardiac troponin C [43], 0.81–0.76 at 15–47°C for ubiquitin [46], and 0.88 (β strands only, 45°C) for OspA [47]. Considering that for most proteins studied to date the temperature $S^2$ dependency of $S^2$ is negative, ChiAVD with an average $S^2$ of 0.88 (40°C) and 0.86 (58°C) ranks among the most rigid proteins studied. The current understanding that among factors potentially increasing chemical and thermal stability is the reduction of conformational flexibility [48] is nicely in line with the fact that ChiAVD is extremely stable towards harsh conditions. It is, however important to note that the $^{1}H$–$^{1}H$ vector motions represent only a subset of the backbone dynamics, and acquisition of motional data of the $^{13}C$–$^{15}N$ vector would result in a more comprehensive perception of the overall backbone motions [49].

**Conformational entropy**

The contribution of conformational entropy changes to binding free energy can be derived from the $S^2$ values [50,51]. For the 66 residues considered the net loss in conformational entropy is $\Delta S = -122.9 \text{ J mol}^{-1} \text{ K}^{-1}$. This figure includes only the fast ps–ns time–scale motion of amide bond vectors of a subset of residues of the protein. It is however close to the experimentally determined AS of $-115.7 \text{ J mol}^{-1} \text{ K}^{-1}$ found for the structurally related protein AVR4/5(G122S) [7] meaning that this type of motion makes a significant contribution to the entropic term of the Gibbs free energy of binding biotin to ChiAVD.

**H/D exchange**

H/D exchange studies were performed at 58°C for the free and biotin–bound ChiAVD. Data (partly qualitative) were obtained for 89 (free) and 104 (bound) residues (see Figure 7 and Figure S5 showing the curve fitting to the data). Twenty–five (free) and thirty–seven (bound) backbone amide hydrogens as well as Trp10 (free) and Trp10, 70, 97, and 110 (bound) side chain $H^e$ hydrogens are very efficiently buried and/or hydrogen bonded. Their $^{1}H$, $^{1}N$ HSQC cross peak remain unperturbed for days after solvent exchange. Most of these are located at subunit interfaces: residues in B–β7 at the 1–4 interface, residue 114 and residues 115–116 in β8 at the 1–2 and 1–3 interfaces, respectively. Trp70, 97 and 110 are essential in providing hydrophobic interactions to biotin. Trp10 is located at the opposite end of the β-barrel and forms, in both free and bound ChiAVD, a hydrogen–bond with its $H^{b}$ to Leu6 carbonyl oxygen.

An estimate for the lower limit of the highest rate constant was calculated from peak intensities in the first spectrum after solvent addition [52]. Residues which had exchanged before the first time point have a rate constant of $>2.9 \times 10^{-3} \text{ s}^{-1}$ (represented with a protection factor $P$, expressed as log $P$, of 2.5 in Figure 7). Forty and thirty–nine residues belong to this group in the free and bound ChiAVD, respectively. For residues which had exchanged before the second time point the approximate rate constant is $1.7 \times 10^{-3}<k_{ex}<2.9 \times 10^{-3} \text{ s}^{-1}$ (log $P$ 2.8). Six (free) and one (bound) residues belong to this group. Backbone amide protons of fifteen (free) and twenty–five (bound) residues exchanged within the experimental time. On average, the rates for free ChiAVD are $0.28 \times 10^{-3} \text{ s}^{-1}$ faster than for the bound form.

It is evident from these data that the bound form is considerably more stable in terms of $H^b$ protection arising from hydrogen bonding and/or burial. The free and bound ChiAVD differ in $H^b$ protection at the side facing solvent (residues in B–β3) as well as in L7,8 and the following 310 helix (Figure 7B). Similar results have been obtained for streptavidin by H/D exchange and mass spectrometric studies [53]. In both proteins β1–β3 at the solvent–exposed face of the structures, as well as the 310 helix at the 1–2
interface show a reduction in exchange upon biotin binding. At the 1–4 interface a larger number of amide hydrogens are labile in the biotin-free form of streptavidin as compared to that of ChiAVD: In addition to residues in β5–β6 and β8 protected in both proteins, in ChiAVD solvent protection covers also residues in β7. H/D exchange and infrared spectroscopic studies with avidin [54] also indicated a reduction in the proportion of exchangeable hydrogens and reduction in the fast exchange kinetic constant upon biotin binding. ChiAVD has a biotin binding affinity similar to that of AVR4 [7], which is higher than that observed for streptavidin [55]. This mutual difference might partially be accounted for the differences observed in hydrogen protection. In ChiAVD the more extensive hydrogen bond network in the free form would imply a smaller loss in entropy upon biotin binding which would have a favourable effect on the thermodynamics of the reaction and thus increase affinity. A further entropic benefit for ChiAVD might result from L3,4 retaining at least partially its mobility (again a smaller loss in entropy) as opposed to streptavidin in which a reduction of exchange is observed for the entire loop.

MD analysis

Molecular dynamics simulations were carried out at three temperatures for 10 ns. Movement of the protein chain is visualized by plotting the root mean square fluctuation over time (RMSF; Figure 8). A clear correlation between secondary structure and RMSF is observed: all eight β strands are found to show much lower fluctuation as compared to the loops connecting them. L3,4 and L6,7 are the most mobile. Biotin stabilizes most loops in all the temperatures tested, but the stabilizing effect is rather modest. Using an average of five residues to calculate the D_RMSF, the highest degree of stabilization is observed in sequence stretches Thr35–Asp39 for 310 K (D_RMSF: 2.32%) and 333 K (D_RMSF: 2.32%) and Trp70–Phe74 for 523 K (D_RMSF: 2.31%). RMSF is

Table 1. Structural Statistics of Biotin–bound ChiAVD.

| Structural restraints per ChiAVD monomer | Distance restraints | Angle restraints |
|----------------------------------------|--------------------|-----------------|
|                                       |                       |                 |
| Distance restraints                     | intramolecular       |                 |
|                                        | 2002                |                 |
| short-range |i–j| ≤ 1               | 917             |                 |
| medium-range 1<|i–j| < 5               | 188             |                 |
| long-range |i–j| ≥ 5                | 897             |                 |
| hydrogen bond restraints                | 27                  |                 |
| intermolecular, 1–2 interface           | 289                 |                 |
| intermolecular, 1–3 interface           | 99                  |                 |
| Angle restraints                        | φ/ψ                 |                 |
|                                        | 68/67               |                 |
|                                        | χ                   |                 |
|                                        | 11                  |                 |
| N^1–H^2 RDC restraints                  | 58                  |                 |
| Agreement with experimental data        |                      |                 |
| Average RMS deviations from restraints  |                      |                 |
| distance restraints (Å)                 | 0.019 ± 0.001       |                 |
| dihedral restraints (°)                 | 0.23 ± 0.15         |                 |
| RDC restraints (Hz)                     | 0.55 ± 0.02         |                 |
| Average number of violations            |                      |                 |
| distance restraints >0.5 Å              | 0                   |                 |
| dihedral restraints >5°                 | 0.4 (max. 8.4°)     |                 |
| RDC restraints >1 Hz                    | 3.7 (max. 2.3 Hz)   |                 |
| Average RMS deviations from ideal covalent geometry |          |                 |
| bonds (Å)                              | 0.011 ± 0.002       |                 |
| angles (°)                             | 2.31 ± 0.06         |                 |
| Average RMS deviation from mean structure in ChiAVD tetramer (Å)* |                      |                 |
| backbone atoms                         | 0.23 ± 0.04/0.32 ± 0.05 |               |
| heavy atoms                            | 0.59 ± 0.05/0.71 ± 0.04 |             |
| Ramachandran plot regions (%) in ChiAVD tetramerb |                      |                 |
| most favored regions                   | 83.4/87.4           |                 |
| additionally allowed regions            | 13.6/11.5           |                 |
| generously allowed regions              | 2.1/0.8             |                 |
| disallowed regions                     | 0.9/0.3             |                 |

*Residues in β strands/Five residues excluded from the termini.

bAll residues/Five residues excluded from the termini.

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NMR Structure and Dynamics of Chimeric Avidin

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found to correlate with the temperature used. Movement of Ala22 in L2,3 increases in the presence of biotin. The results of the molecular dynamics simulation agree remarkably well with the order parameter data, with the exception of L6,7. The simulation nicely exposes the small differences observed in the experimental data between the free and the bound protein form, namely the larger number of mobile residues in L3,4 of the free form and the loop’s overall higher amplitude of motion. Also the lower stability of the 310 helix in the free form is evident. However, the order parameters show no indication of high mobility for L6,7. In fact the simulation data indicates higher mobility for twelve residues, encompassing half of the β strands flanking the three–residue L6,7.

ChiAVD mutants G42A and G42F

Apart from the termini, three regions stand out from the average motional regime in ChiAVD: L3,4, L4,5 and L5,6. According to the $S^2$ and $\tau_e$ values residue Gly42 in L3,4 is the most mobile residue. In quest of further stability, we mutated Gly42 to alanine and phenylalanine. The Gly $\rightarrow$ Ala mutation represents the simplest attempt to create rigidity with a bulkier side chain whereas the objective of the Gly $\rightarrow$ Phe mutation was to create a stabilizing π–π aromatic interaction with Phe72 in the structurally opposite L5,6. (Strept)avidins have extensively been modified by mutagenesis [56], but to our knowledge the outcome of mutating Gly42 has not yet been described. Intriguingly, Chivers et al. [57] applied point mutations S52G and R53D to ChiAVD Gly42 in sequence) and obtained streptavidin with both decreased dissociation and association rates, but also with increased thermal stability. The mutants expressed efficiently (data not shown), folded properly to tetramers and had physical properties very similar to ChiAVD. A hydrodynamic radius of 3.04 $\pm$ 0.75 nm was measured by DLS for ChiAVD–G42A, 3.09 $\pm$ 0.70 nm for ChiAVD–G42F, and 3.18 $\pm$ 0.69 nm for ChiAVD. The main peak consisted of 99.7–100% of the volume–adjusted intensity. The obtained hydrodynamic size corresponds to globular protein with molecular weights of 45.4 kDa (ChiAVD–G42A), 47.2 kDa (ChiAVD–G42F) and 50.4 kDa (ChiAVD), which is very close to the theoretical size of the tetramer.

Mutation of Gly42 had no notable influence on the thermostability. However, we observed a slightly lower biotin dissociation rate for the mutants. At 50°C ChiAVD–G42A showed a slightly lower fluorescently labelled biotin dissociation rate than ChiAVD–G42F or ChiAVD: (4.64 $\pm$ 0.33) $\times 10^{-5}$ s$^{-1}$ as compared to (5.80 $\pm$ 0.40) or (6.39 $\pm$ 0.88) $\times 10^{-5}$ s$^{-1}$, indicating that both of the mutations were slightly beneficial for the binding of the conjugated biotin. The $T_m$ values obtained from DSC experiments were 110.5 $\pm$ 0.2/129.6 $\pm$ 1.0°C for biotin–free/bound ChiAVD, 110.1 $\pm$ 0.0/128.8 $\pm$ 0.0°C for ChiAVD–G42A, and 109.9 $\pm$ 0.0/128.2 $\pm$ 0.1°C for ChiAVD–G42F. We hypothesise that the introduced bulkier sidechains do not form the pursued, thermostability enhancing, stable interactions to their structural neighbors. Instead, the occasional interactions hinder the loop dynamics. A resulting smaller gain in entropy upon biotin release for the mutants would reduce the dissociation rate.

Conclusions

We have shown that at 58°C biotin-bound ChiAVD maintains its compact, well-defined structure. With an average order
Figure 7. Per residue protection factors of biotin-free and bound ChiAVD. (A) The protection factors of the free form are shown with red bars. Residues exhibiting fast exchange have been assigned the value 2.5, residues in the second fastest group (see text) the value 2.8, and residues with slow exchange the value 7.0. Residues with no data available have been assigned the value 20.1. Trp side chain data are marked with asterisks.

(B) Differences in protection factors shown in the structure of ChiAVD. In dark blue are shown residues with enhanced protection to exchange in both forms and in light blue residues for which the free form is more prone to exchange. Residues shown in red are susceptible to fast exchange in both forms. Residues colored in white have no comparable data.

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Figure S4 $^{15}$N R$_1$, R$_2$ and hetNOE data of biotin–bound and free ChiAVD. The 800 MHz data are represented with red (58°C) and blue (40°C) dots, and those of 600 MHz (58°C) with black dots. Trp N$^\beta$ data at 800 MHz, 58°C are shown with green dots. Secondary structure regions are highlighted with β strands in blue and 3_10 helices in red. The depicted secondary structure of the free form is that present in the crystal structure [1]. Average errors are 0.04 (800 MHz, 58°C), 0.03 (800 MHz, 40°C), and 0.03 (600 MHz, 58°C) s$^{-1}$ for R$_1$, 0.40/0.70/0.31 s$^{-1}$ for R$_2$ and 0.04/0.04/0.06 for hetNOE in the bound form and 0.03/0.03 (800/600 MHz, R$_1$), 0.31/0.48 (R$_2$) and 0.05/0.04 (hetNOE) for the free form. The amino acid sequence has a gap (highlighted in grey); His$^54$ is followed by Lys$^57$. The first three residues Gln(−3), Thr(−2) and Val(−1) do not belong to the original mature protein form, but result from the signal peptide construction used in the bacterial expression. (TIF)

Figure S5 Representative curves of H/D exchange experiments. Free ChiAVD is represented with empty circles and the bound form with filled circles. (TIF)

Table S1 ChiAVD experimental RDCs measured at 40°C, 800 MHz. (TIF)

Table S2 Methyl group chemical shifts of free ChiAVD at 80°C. (TIF)

Table S3 Average R$_1$, R$_2$ and hetNOE values and standard deviations for biotin–bound and free ChiAVD. (TIF)

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Author Contributions

Conceived and designed the experiments: HT SK JAEM NK TP VPH PP. Performed the experiments: HT SK NK TP VPH PP. Analyzed the data: HT SK NK VPH PP. Contributed to the writing of the manuscript: HT SK NK TP VPH PP.

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