Atomic structure of a nanobody-trapped domain-swapped dimer of an amyloidogenic β2-microglobulin variant

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Edited by David S. Eisenberg, University of California, Los Angeles, CA, and approved December 6, 2010 (received for review June 17, 2010)

Atomic-level structural investigation of the key conformational intermediates of amyloidogenesis remains a challenge. Here we demonstrate the utility of nanobodies to trap and characterize intermediates of β2-microglobulin (β2m) amyloidogenesis by X-ray crystallography. For this purpose, we selected five single domain antibodies that block the fibrillogenesis of a proteolytic amyloidogenic fragment of β2m (ΔN6Δ2m). The crystal structure of ΔN6β2m in complex with one of these nanobodies (Nb24) identifies domain swapping as a plausible mechanism of self-association of this amyloidogenic protein. In the swapped dimer, two extended hinge loops—corresponding to the heptapeptide NHVTLSQ that forms amyloid in isolation—are unmasked and fold into a new two-stranded antiparallel β-sheet. The β-strands of this sheet are prone to self-associate and stack perpendicular to the direction of the strands to build large intermolecular β-sheets that run parallel to the axis of growing oligomers, providing an elongation mechanism by self-templated growth.

Results

Nanobodies Efficiently Block β2m Fibrillogenesis. The use of specific antibodies offers promising strategies for inhibiting and even reversing the fibril formation by amyloidogenic proteins (4, 14, 15). The aim of this study was to generate antibodies that stabilize early intermediates along the pathway of β2m fibrillogenesis and to use these antibodies for the structural investigation of such intermediates. For this purpose, camel and llamas were immunized with β2m and ΔN6β2m. According to standard protocols, we have selected 16 nanobody clones. Eight nanobodies representing unique sequence families were chosen for further analysis. Selected nanobodies with Kf’s in the nanomolar to micromolar range for β2m and ΔN6β2m variants were tested as inhibitors of ΔN6β2m fibrillogenesis (Fig. 1). Inhibition experiments were performed by incubating ΔN6β2m in the presence or absence of an equimolar amount of each nanobody. As a negative control, we also included a nanobody (Nb108) generated against another antigen. Fibrillogenesis was monitored by measuring the increase of the thioflavin T (ThT) fluorescence (16), by EM imaging, and by SDS-PAGE (Fig. 1 and Figs. S1 and S2). Considering that ΔN6β2m variant aggregates within hours, five nanobodies (Nb22, Nb23, Nb24, Nb30, and Nb272) were selected as aggregation inhibitors and tested as cocrystallization chaperones of prefibrillar intermediates.

Author contributions: K.D., E.P., M.S., L.W., V.B., and J.S. designed research; K.D., S.V., V.S., E.P., I.A.M., L.W., and J.S. performed research; K.D., V.S., E.P., I.A.M., L.W., and J.S. analyzed data; and K.D. and J.S. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The coordinates of the crystal structure have been deposited with the Protein Data Bank [www.pdb.org (PDB ID code 2X8B)].

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1008560108/-/DCSupplemental.
Chaperone-Assisted Crystallization of ΔN6β2m Amyloidogenic Protein. We reasoned that antibodies inhibit protein aggregation (1) by binding to and stabilizing native or native-like states of the protein (2), by kinetically trapping early intermediates, or (3) by sterically hindering the formation of large oligomers (15). Therefore, we tested five aggregation-inhibiting nanobodies as cocrystallization chaperones for prefibrillar intermediates of β2m amyloidosis. Nanobody-antigen complexes with a molecular weight of a 1:1 heterodimer were obtained by mixing the purified components followed by calibrated size exclusion chromatography in a 20 mM Tris buffer containing 100 mM NaCl at pH 7.5. Despite extensive screening, diffracting crystals were obtained only from ΔN6β2m (ΔN6) was incubated for one week at 37 °C in 50 mM NaAc, pH 5.0 in the absence or presence of excess amounts (42.5 μM of ΔN6β2m versus 50 μM of Nb) of nanobodies 20, 21, 22, 23, 24, 30, 31, or 272, or 108 (Nb108 is a β2m-unrelated nanobody, raised against another antigen). The kinetics of fibrillogenesis were monitored by measuring the increase in ThT fluorescence after 0, 24, and 124 h (A). To visualize remaining soluble protein, samples were centrifuged and the soluble fractions were analyzed by SDS-PAGE (B). To visualize the accumulation of protein aggregates, 124-h samples were fixed on carbon-coated grids and subjected to transmission electron microscopy (C). Fig. S1 shows the EM images in high resolution. The table (D) shows the affinities of nanobodies for β2m and ΔN6β2m as measured by surface plasmon resonance.

**Fig. 1.** The effect of nanobodies on ΔN6β2m fibrillogenesis. ΔN6β2m (ΔN6) was incubated for one week at 37 °C in 50 mM NaAc, pH 5.0 in the absence or presence of excess amounts (42.5 μM of ΔN6β2m versus 50 μM of Nb) of nanobodies 20, 21, 22, 23, 24, 30, 31, or 272, or 108 (Nb108 is a β2m-unrelated nanobody, raised against another antigen). The kinetics of fibrillogenesis were monitored by measuring the increase in ThT fluorescence after 0, 24, and 124 h (A). To visualize remaining soluble protein, samples were centrifuged and the soluble fractions were analyzed by SDS-PAGE (B). To visualize the accumulation of protein aggregates, 124-h samples were fixed on carbon-coated grids and subjected to transmission electron microscopy (C). Fig. S1 shows the EM images in high resolution. The table (D) shows the affinities of nanobodies for β2m and ΔN6β2m as measured by surface plasmon resonance.

**Chaperone-Assisted Crystallization of ΔN6β2m Amyloidogenic Protein.** We reasoned that antibodies inhibit protein aggregation (1) by binding to and stabilizing native or native-like states of the protein (2), by kinetically trapping early intermediates, or (3) by sterically hindering the formation of large oligomers (15). Therefore, we tested five aggregation-inhibiting nanobodies as cocrystallization chaperones for prefibrillar intermediates of β2m amyloidosis. Nanobody-antigen complexes with a molecular weight of a 1:1 heterodimer were obtained by mixing the purified components followed by calibrated size exclusion chromatography in a 20 mM Tris buffer containing 100 mM NaCl at pH 7.5. Despite extensive screening, diffracting crystals were obtained only from ΔN6β2m-Nb24 complex with the hanging drop vapor diffusion method using 0.2 M ammonium sulphate and 6% PEG 4000 as the precipitants in 0.1 M sodium acetate (pH 4.6). Free nanobody did not crystallize under the same conditions and purified ΔN6β2m aggregated within minutes under the crystallization conditions, indicating that the nanobody serves as an efficient crystallization chaperone for the intrinsically unstable ΔN6β2m variant. Low temperature X-ray diffraction data of the crystallized ΔN6β2m-Nb24 complex extended to 2.2 Å resolution (Table S1). The coordinates of full-length monomeric β2m (1BMG) were used as a search model to solve the crystal structure of ΔN6β2m by molecular replacement. Remarkably, the asymmetric unit of the crystal contains four molecules of ΔN6β2m, but only three nanobodies bound to three ΔN6β2m molecules (Fig. S3). As the nanobodies are fairly stable and the ΔN6β2m-Nb24 complex was purified as a 1:1 complex by analytical gel filtration, we exclude the possibility that one of the nanobodies was proteolytically removed during crystallization. We favor a second explanation and believe that the fourth Nb24 is present but highly mobile. There is enough space in the crystal lattice to accommodate it (Fig. S3), and we observe residual density in the area where we would expect the fourth nanobody to bind, especially in the area of ΔN6β2m-Nb interface. Partial occupancies of whole protein domains (17) or entire proteins (18) have previously been observed in crystal lattices of other protein-protein complexes.

**Nb24 Stabilizes a 3D Domain-Swapped Dimer of ΔN6β2m.** The crystal structure of ΔN6β2m-Nb24 complex reveals that ΔN6β2m exchanged identical subdomains between two monomers to form a 3D swapped dimer. Each domain is composed of six β-strands contributed by one subunit (A, B, C, D, and F) and one swapped C-terminal β-strand (strand G: residues 91–94, β2m numbering) contributed by the other (Fig. 2A). The short NHVTLSQ peptide (residues 83–89) serves as the hinge loop. In the monomer, the closed conformation of the hinge loop connects strands F and G. In the domain-swapped dimer the hinge adopts an extended conformation, lengthening the F strand by four amino acids (Fig. 2F). The extended hinge loops form a new long two-stranded antiparallel β-sheet, interrupted only by Pro90 (Fig. 2F). The main chain NH and CO groups of His84, Thr86, and Ser88 are hydrogen-bonded to the carbonyls and the amides of Ser88, Thr86, and His84 on the adjacent strand, respectively (Fig. 2D). The backbone donor and acceptor sites of Val85 and Leu87 are exposed to solvent (indicated by arrows on Fig. 2D), prone to stack with other β-strands in a parallel or antiparallel configuration.

**Nb24 Disrupts β2-Microglobulin Aggregates in Vitro but Does Not Disrupt the Fibrils.** We also investigated whether Nb24 can disrupt preformed ΔN6β2m aggregates or fibrils in vitro. Therefore, 45 μM of monomeric ΔN6β2m was incubated at pH 5.0 in the presence of ThT, and fibrillogenesis was followed by electron microscopy imaging and by measuring the ThT fluorescence increase (Fig. S4). After 5 h of incubation the ThT fluorescence was no longer increasing. EM imaging confirmed the presence of nonamorphous aggregates, but no amyloid fibrils were observed at this time point (Fig. S4A). Those nonamorphous protein ag-
gregates bind thT resulting in increased sample fluorescence (Fig. S4B). Under these conditions, the \( \Delta N6\beta 2m \) amyloid fibrils form only after two to four weeks of incubation (Fig. S2). Addition of Nb24 to preformed nonamorphous \( \Delta N6\beta 2m \) aggregates (obtained after 5 h of incubation at 37 °C and pH 5.0) caused a significant decrease of the ThT fluorescence (Fig. S4B) concomitant with an increase of resolubilized \( \Delta N6\beta 2m \) as shown by SDS-PAGE analysis of the soluble fraction (Fig. S4C). To investigate the stoichiometry of this reaction, \( \Delta N6\beta 2m \) aggregates were mixed with Nb24 at \( \Delta N6\beta 2m:Nb \) ratios of 1:1 and 4:1. Equimolar amounts of \( \Delta N6\beta 2m \) and nanobody were needed to completely disrupt the preformed \( \Delta N6\beta 2m \) fibrils, as indicated by the reduction of the ThT fluorescence to background levels. The \( \beta 2m \)-unrelated Nb108 did not interfere with \( \Delta N6\beta 2m \) aggregation (Fig. S4B). We have also grown amyloid fibrils of \( \beta 2m \) and \( \Delta N6\beta 2m \) and found that these fibrils in contrast to aggregates are stable for days in the presence of excess amounts of Nb24 (Fig. S5).

**Nb24 Does not Bind the MHC-I Complex.** The potency of Nb24 to recognize \( \beta 2m \) in the MHC-I was evaluated by FACS. A series of nanobodies raised against monomeric \( \beta 2m \) were conjugated with phycoerythrin and incubated with two human cell lines expressing MHC-I on their surface. Remarkably, most of the nanobodies, including Nb24, did not bind to MHC-I, exposed on the surface of these cells (Fig. S6).

**Discussion**

Many proteinaceous aggregates form through a nucleation mechanism followed by a self-templated growth where the ends of existing filaments recruit soluble molecules into aggregates (13). Consistent with this model, the assembly of \( \beta 2m \) into amyloid-like fibrils is characterized by an initial lag phase where little or no change in fibril concentration can be detected (19). This is followed by an elongation phase where a large mass percentage of the starting protein material is converted into fibrils. The lag phase can be shortened or ultimately abolished in vitro by adding fibrillar seeds or by using designed unstable mutants (13, 15, 20). The isolation and characterization of the oligomeric species that are present in solution prior to the appearance of fibrils remains a challenge. In this work, we have trapped and characterized the structure of an amyloidogenic \( \beta 2m \) variant that lacks six N-terminal amino acids. The crystal structure of \( \Delta N6\beta 2m \) in complex with Nb24 identifies a swapped dimer as a plausible structural nucleus that may serve as a mold for the self-templated growth of \( \beta 2m \) fibrils (Fig. 3).

**Domain Swapping Generates a Plausible Nucleus for \( \beta 2m \) Fibrillogenesis.** Three-dimensional domain swapping has been proposed as a
general mechanism for the self-association of proteins (21, 22). The ΔN6β2m dimer we trapped with Nb24 meets all common properties of domain-swapped oligomers (23). First, only one small C-terminal segment of the protein (the rest retaining the native-like structure) participates in the oligomerization, without disrupting the core of the protein fold. Second, the single disulfide bond (Cys25-Cys80) does not need to be broken to swap the domains. Finally, all sites of local perturbation that have been related to β2m self-association colocalize with the hinge region at one end of the immunoglobulin fold opposite to the nanobody binding site (Fig. 4). Most remarkably, this swapped dimer meets many characteristics that have been attributed to prefibrillar intermediates of β2m fibrillogenesis. Phe30, His31, and Pro32—three residues particularly involved in amyloidogenesis—are located on the tip of the first loop that connects strands B and C. In the native β2m monomer Pro32 adopts the cis conformation and makes hydrophobic contacts with the hinge loop and the N-terminal segment. Using NMR and mutagenesis, Radford and co-workers (24, 25) identified a specific folding intermediate that contains a nonnative trans-Pro32 isomer as a direct precursor of dimeric species and oligomers that accumulate before the development of amyloid fibrils. Using Cu$^{2+}$ as an oligomerization trigger, Miranker and co-workers (26) also identified the cis to trans isomerization at Pro32 concomitant with a dramatic rotation of Phe30 from the hydrophobic core toward solvent as critical switches enabling aggregation. Consistent with these findings, Pro32 adopts the trans conformation and Phe30 takes a solvent exposed position in the swapped dimer (Fig. 4B). Cu$^{2+}$ coordination at His84 contained in the hinge loop itself also induces structural rearrangements of β2m, freeing its C terminus and allowing the formation of a domain-swapped dimer (27). In the native monomer, parts of all three connecting loops are shielded from solvent by the N-terminal peptide that is missing in ΔN6β2m variant, explaining why the truncated species is less stable—and unlike wild-type protein—has a higher tendency to self-associate and forms amyloid fibrils even at physiological pH (10, 28). The different sites of local perturbation that cause the onset of β2m fibrillogenesis define the local environment of the hinge loop. It thus appears that partial unfolding at one end of the rigid β-sandwich causes the formation of fibrils via a domain-swapped intermediate that forms upon refolding of the hinge loops. The Pro32 cis to trans switch and the dramatic rotation of Phe30 are key structural signatures of this transition.

Under physiological conditions, Nb24 forms a stable nanomolar 1:1 complex with ΔN6β2m. Because Nb24 was generated in vivo by immunization with the native monomer and cloned by library selection against the same protein, it is very likely that it binds one of the lowest energy states of β2m. Thermodynamically, antibodies pay a huge energetic penalty if they first bind to a low energy state and then distort the antigen’s structure into a more energetically unfavorable state (29).
high-energy conformation that does not appreciably exist in the absence of the bound antibody (29). Consistently, Nb24 does not distort the structure of β2m upon binding (Fig. S7). It thus appears that the self-association step follows a gain-of-interaction mechanism (30) where an extensive portion of the native structure of the monomer (including the Nb24 epitope) is maintained in the dimer.

The Swapped Dimer Is Predisposed to Elongation by a Mechanism of Self-Templated Growth. During the self-association of ΔN6β2m, two hinges that correspond to the heptapeptide NHVTLSQ, refold into extended β-strands, and stack into a unique two-stranded antiparallel β-sheet (Fig. 2). Interestingly Ivanova et al. (31) showed that the NHVTLSQ heptapeptide forms amyloids in isolation demonstrating that this peptide by itself has a high propensity to form amyloid structure upon exposure. In the newly formed two-stranded sheet, the backbone donor and acceptor sites of Val85 and Leu87 are exposed to solvent (Fig. 2D), prone to stack with other β-strands in a parallel or antiparallel configuration. Indeed, other strands may associate perpendicularly to build large intermolecular β-sheets that run parallel to the axis of the growing oligomers (Fig. 3). It thus appears that the swapped dimer can serve as a structural nucleus for the growth of the cross-β spine of elongating fibrils by templating the hydrogen-bonding network connecting the strands. In 3D-swapped ΔN6β2m (this study), the refolded adjacent β-strands expose a hydrophobic patch (Fig. 2E). This “dry surface” may provide the driving force for β-sheets of growing oligomers to associate and interdigitate. The remaining core domains may decorate the spine and protect it from solvent. In the growing oligomer, a transition from stacked swapped dimers to a runaway domain swap—where each monomer swaps a domain into the next monomer along the fibril—could generate more stable open ended protofibrils (Fig. 3). Remarkably, a llama nanobody (VHH-R9) missing the first seven amino acids was found to self-associate and stack following a similar mechanism (32). In the crystal structure of AN7VHH-R9, the last β-strand of the immunoglobulin fold associates with a symmetry-related molecule to form a domain-swapped dimer, its CDR3 loop refolds to generate a unique two-stranded β-sheet (Fig. 2C). In the packing of AN7VHH-R9 crystal, these two-stranded β-sheets stack with symmetry-related molecules to build a crystal-wide β-sheet structure. There is evidence that such a cross-β spine with a domain swap is also present in a designed nanobody to the core domain of β2m upon binding (Fig. S7). It thus appears that the selected nanobody efficiently blocks the fibrillation of ΔN6β2m, without interfering with the biological function of β2m suggesting that antibodies that stabilize particular oligomeric intermediates could be developed as therapeutic tools to prevent amyloid deposits in dialysis patients.

Relevance to DRA? Is a domain-swapped dimer of ΔN6β2m physiologically relevant, or is it just a crystallographic artifact? In general, domain-swapped oligomers are obtained at high protein concentrations or at low pH. Other domain-swapped proteins are fragments of their complete molecules (34). Strikingly, the deposition of β2m amyloid in humans has been correlated to high protein concentrations, lower pH, and proteolysis. First, the concentration of β2m increases up to 60-fold in the body fluids of patients suffering from DRA as an inevitable consequence of long-term hemodialysis (7). Second, the deposits of β2m are mainly localized at inflammatory sites in the muscle skeletal system. The pH of the extracellular fluids in these inflammatory loci is known to be acidic. The induction of chronic inflammation only is sufficient to trigger β2m-amyloidosis (35, 36). Third, 25% or more of the β2m in these deposits is of ΔN6-truncated form (10). Finally, it has been shown that the addition of tiny amounts of ΔN6 to β2m rapidly leads to the formation of large aggregates, suggesting that this species can serve as seeds for β2m fibrillation (28). All this points to a domain-swapped ΔN6β2m dimer as a building block of the structural nucleus of amyloid formation in DRA. High protein concentrations and a low pH may be the triggers for its formation. However, it remains to be proven if the swapped dimer is kinetically and mechanistically constructive in the process.

Stabilization of Conformational Intermediates as a Therapeutic Strategy. Different explanations may account for the anti-amyloidogenic properties of Nb24. Most probably, binding of the nanobody to the core domain of β2m sterically hinders the self-template growth of the swapped intermediate (Fig. 3), thus preventing elongation. This is consistent with our observation that Nb24 can reverse the elongation phase of β2m nuclei (Fig. S3). If the recruitment of soluble molecules at the ends of existing oligomers is reversible, nanobodies that bind the interacting interface will decompose growing fibrils by mass action. Theoretically, it cannot be excluded that the elongation of the fibrils involves structural changes in the core domain of β2m, which may be prohibited by the binding of particular nanobodies.

Using FACS, we found that Nb24 does not bind MHC-I on the cell surface (Fig. S6). It thus appears that the selected nanobody efficiently blocks the fibrillation of ΔN6β2m, without interfering with the biological function of β2m suggesting that antibodies that stabilize particular oligomeric intermediates could be developed as therapeutic tools to prevent amyloid deposits in dialysis patients.

Materials and Methods

Generation and Selection of Nanobodies. One camel (Camelus dromedarius) and one llama (Lama glama) were immunized with recombinant full-length β2m, and another llama was immunized with recombinant ΔN6β2m. From each animal, an independent phage display library was constructed. Nb20, Nb21, Nb22, and Nb24 are nanobodies derived from camel and selected against β2m. Nb23, Nb30, and Nb31 derive from ΔN6β2m-immunized llama and Nb272 originate from the llama immunized with β2m. All selected nanobodies were recloned to the pHP6 (37) vector for expression in Escherichia coli as C-terminal His6-tagged proteins. Nanobodies were purified to homogeneity by immobilized-metal affinity chromatography and gel filtration (38).

Crystallization and Data Collection. Nanobody–antigen complexes were obtained by mixing the purified components followed by calibrated size exclusion chromatography in a 20 mM Tris buffer containing 100 mM NaCl at pH 7.5. Crystals were grown at 10 °C by mixing equal volumes of protein with a reservoir solution containing 0.5 M sodium citrate buffer, 0.2 M ammonium sulfate and 6% PEG 8000 in 0.1 M Na acetate pH 4.6. The selenium–methylionine labeled Nb24 produced isomorphous crystals in complex with ΔN6β2m. All X-ray diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) beamlines ID29 and BM16. Crystal diffraction to 2.16 Å and a complete dataset was collected. The selenium–methylionine labeled protein crystals diffracted not beyond 3.5 Å. All data were indexed, integrated, and scaled using Denzo and Scalepack (39). Subsequent data analysis was performed using the CCP4 suite of programs (40).

A detailed description of the methods can be found in SI Materials and Methods.

ACKNOWLEDGMENTS. We acknowledge the work of Maja Debulpaep, who performed EM imaging and the use of the beamlines at the ESRF. This work was supported by grants from the Interuniversity Attraction Poles (project P6/19), the Ministero dell’Istruzione, dell’Università e della Ricerca (Fondo per gli Investimenti della Ricerca di Base e Programmi di Ricerca di Interesse Nazionale), the European Union Framework 6 EURAMY Amyloidosis in Europe (project LSHM-CT-2005-037525) and Fondazione Cariplo and Regione Lombardia. K.D. and S.V. received doctoral fellowships of the Fonds Wetenschappelijk Onderzoek and the Innovatie door Wetenschapen Technologie, respectively.


Supporting Information

Domanska et al. 10.1073/pnas.1008560108

SI Materials and Methods.

Generation and Selection of Nanobodies. In order to obtain β2-microglobulin (β2m) specific nanobodies, one camel (Camelus dromedarius) and one llama (Llama glama) were immunized with recombinant full-length β2m. Another llama was immunized with recombinant ΔN6β2m. Animals were injected at days 0, 7, 14, 21, 28, and 35 with 200 μg of the antigen, using GERBU LQ (GER-BU biotechnick) as the adjuvant. Peripheral blood lymphocytes were isolated from blood samples that were collected at day 39. From these lymphocytes, total RNA was extracted according to the method of Chomczynski and Sacchi (1). Fifty micrograms of total RNA was used to prepare cDNA using SuperScript II Reverse Transcriptase (Invitrogen) and a N6 primer according to the manufacturer's instructions. DNA fragments encoding heavy chain variable (VHH) domains (nanobodies) were amplified by nested PCRs and phage display libraries (>5×10^6 independent clones per library) have been constructed according to Conrath et al. (2). β2m-specific phages were enriched in vitro on Maxisorp (Nunc) 96-well plates coated with 5 μg β2m or with 5 μg ΔN6β2m in sodium bicarbonate buffer pH 8.2. Antibody bound phages were recovered from antigen-coated wells with 100 mM thriethylamine pH 11.0 and neutralized with Tris-HCl pH 7.0. After two and three rounds of selection, ELISAs were performed on periplas- matic extracts of individual colonies to screen for β2m and ΔN6β2m-specific nanobodies, respectively. Nb20, Nb21, Nb22, and Nb24 are nanobodies derived from camel and selected against β2m. Nb23, Nb30, and Nb31 derive from the ΔN6β2m-immunized llama and Nb272 originates from the llama immunized with β2m. All selected nanobodies were recloned to the pHEN6 (3) vector for expression in His6-tagged proteins. Nanobodies were purified to homogeneity by immobilized metal affinity chromatography and gel filtration. Selenomethionine-labeled nanobodies were expressed in minimal media (M9) containing seleno-L-methionine and purified using standard protocols.

ΔN6β2m Fibrillogenesis Inhibition Experiments. Fibrillogenesis inhibition experiments were performed by incubating 42.5 μM of ΔN6β2m at 37 °C and pH 5.0 in the absence (positive control) or presence of an equimolar amount of each nanobody. As a control, we also included a not related Nb (Nb108) that recognizes another antigen. Fibrillogenesis was monitored on the basis of the thioflavin T fluorescence (ThT) (5). In parallel, samples were subjected to ultracentrifugation, and the supernatant was analyzed on SDS-PAGE to visualise soluble protein.

Preparation of Fibrils. The β2m fibrils were prepared by incubating 100 μM of the purified protein at 37 °C in 50 mM phosphate buffer and 100 mM NaCl, pH 7.4, in the presence of 20% (vol/vol) TFE and 20 μg/mL fibrillar seeds. Fibrils were collected after 3 wk by centrifugation and washed with deionized water. Fibrils of ΔN6β2m were prepared by incubating 100 μM of the purified protein at 37 °C in 50 mM acetate buffer, pH 5.0 and 100 mM NaCl and collected after 4 wk. The quality of the fibrils was analyzed by ThT fluorescence measurements and using electron microscopy imaging. Protein samples (5 μL) were applied drop- wise on carbon-coated grids (Formvar/carbon on 400 Mesh Copper). The grids were dried with filter paper and negatively stained with 1% (wt/vol) uranyl acetate. Micrographs were recorded on a Jeol Jem-2100 electron microscope at 200 kV.

Crystalization and Data Collection. Nanobody–antigen complexes with a molecular weight of a 1:1 heterodimer were obtained by mixing the purified components followed by calibrated size exclusion chromatography in a 20 mM Tris buffer containing 100 mM NaCl at pH 7.5. Despite extensive screening at the High Throughput Crystalization Laboratory of the European Molecular Biology Laboratory (EMBL) Grenoble Outstation (https://htxlab.embl.fr), diffracting crystals were obtained only from the ΔN6β2m-Nb24 complex. Crystals were grown at 10 °C by mixing equal volumes of protein with a reservoir solution containing 0.2 M ammonium sulfate and 6% PEG 4000 in 0.1 M Na acetate pH 4.6. Free Nb did not crystallize under the same conditions, whereas purified ΔN6β2m aggregated within minutes under the crystallization conditions, indicating that the nanobody serves as a crystallization chaperone for the intrinsically unstable ΔN6β2m variant. The selenium–methionine labeled Nb24 produced isomorphous crystals in complex with ΔN6β2m. All X-ray diffraction data were collected at the European Synchrotron Radiation Facility (ESRF) beamlines ID29 and BM16. Prior to data collection, crystals were flash frozen in liquid nitrogen. Crystal diffracted to 2.16 Å and a complete dataset was collected. The selenium–methionine labeled protein crystals were small, diffracted not beyond 3.5 Å, and were also subjected to radiation damage. A redundant dataset was collected from one single crystal that diffracted to 4.5 Å. All data were indexed, integrated, and scaled using Denzo and Scalepack (6). Subsequent data analysis was performed using the CCP4 suite of programs (7).

Structure Determination and Refinement. The crystal structure of ΔN6β2m-Nb24 complex was determined using a combination of molecular replacement (MR) and single wavelength anomalous diffraction techniques. Initially, the structure solution was attempted using molecular replacement with β2m (1BMG) and Nb (1HCV) molecules as models. The program PHASER (8) was used for the MR descalculations and an initial electron density map was computed. A partial model was manually built and could be refined using Refmac (9) to values of R = 37% and R_free = 42%. Several heavy atom derivatives of the complex were prepared to improve the phases without much success. Therefore, a Se-Met labeling of the nanobodies was made, and a highly redundant dataset was collected at the peak wavelength of the selenium to a resolution of 4.5 Å. The Se positions were calculated using SHELX (10), phases computed and combined with the phases from the partial model using SIGMAA (7, 11). The electron density map improved after density modification using density modification package but the automatic model building was not successful either with ARP/wARP (12) or Buccaneer (13). A manual building of the model was done using Crystallographic Object-Oriented Toolkit (COOT) (11) and refinement was done with Refmac (9). This process was iterated several times and the electron density map improved. Four ΔN6β2m and three Nb molecules were identified in the asymmetric unit. The phases at this point were input to Buccaneer (13) for automatic model building, and seven molecules could be located. The electron density for one of the Nb molecules is very weak and could not be traced into the model. Further manual building of the loops and few residues was done with COOT (11). The water molecules...
were added using ARP/wARP (12) and the model refined to values of $R = 24.1\%$ and $R_{\text{free}} = 28.9\%$. A further Translation/Libration/Screw refinement of the model was done with Refmac that brought the values down to $R = 23.8\%$ and $R_{\text{free}} = 26.7\%$.


Fig. S1. The effect of nanobodies on $\Delta N_6\beta_2m$ fibrillogenesis. $\Delta N_6\beta_2m$ was incubated for one week at 37 °C in 50 mM NaAc, pH 5.0 in the absence (control) or presence of excess amounts (50 μM of Nb versus 42.5 μM of $\Delta N_6\beta_2m$) of nanobodies 20, 21, 22, 23, 24, 30, 31, 272, or 108 (Nb108 is a $\beta_2m$-unrelated nanobody that was raised against another antigen). (a) Representative EM images of these samples are shown. Protein aggregates were observed in the control sample ($\Delta N_6\beta_2m$) and in the samples of $\Delta N_6\beta_2m$ coincubated with nanobodies Nb20, 21, 31 or the $\beta_2m$-unrelated nanobody Nb108. Consistent with the ThT data (Fig. 1), no aggregates or fibrils could be found in the samples of $\Delta N_6\beta_2m$, coincubated with nanobodies Nb22, 23, 24, 30, 31, 272, or 108. (b) Obvious differences in turbidity among samples from this experiment: inhibitor corresponds to Nb24 and noninhibitor to Nb31.
Fig. S2. Fibrillogenesis of ΔN6β2m followed by electron microscopy. ΔN6β2m was incubated for 28 days at 37 °C and pH 5.0 (50 mM acetate buffer) in the absence of nanobody. Samples were taken after 5, 14, and 28 d and subjected to electron microscopy imaging. After 5 d, only nonamorphous protein aggregates were observed in the sample. After 2 wk of incubation, the first amyloid fibrils started to grow out of these aggregates. After 4 wk, all aggregates were converted into amyloid fibrils.

Fig. S3. Crystal structure of ΔN6β2m in complex with Nb24. View of the unit cell with four ΔN6βm (gray and purple) and three Nb24 molecules (blue). For clarity complementarity determining regions (CDRs) have been highlighted on one of the nanobody molecules: CDR1 in red, CDR2 in yellow and CDR3 in green. The putative position of the nonvisible fourth nanobody is indicated with a dashed circle.

Fig. S4. Disruption of preformed ΔN6β2m aggregates by Nb24. ΔN6β2m was incubated at 37 °C in 50 mM NaAc, pH 5.0 to form aggregates. (a) Electron micrograph of ΔN6β2m aggregates after 5 h of incubation in an acetate buffer pH 5.0. (b) After 5 h (dashed line in b), these preformed aggregates were mixed with fresh monomeric ΔN6β2m (O), Nb24 in a 1:1 Nb-ΔN6β2m ratio (□), Nb24 in a 1:4 Nb-ΔN6β2m ratio (▵) or an irrelevant Nb (◆), respectively. The assembly/disassembly of ΔN6β2m aggregates was monitored by ThT fluorescence for 24 h. (c) The disassembly of ΔN6β2m aggregates by Nb24 was confirmed by SDS-PAGE analysis of the soluble fraction after centrifugation.
**Fig. S5.** Nb24 does not disassemble β2m nor ΔN6β2m fibrils. Fibrils of β2m (a) and ΔN6β2m (b) were prepared as described in Materials and Methods. Excess of Nb24 was added to these fibrils and the fibrils stability was followed by ThT fluorescence measurement.

![Thioflavin T fluorescence](image1)

**Fig. S6.** Binding of nanobodies on the Major Histocompatibility Complex class I of living cells. (a) A series of nanobodies raised against monomeric β2m were conjugated with phycoerythrin (PE) and incubated with human cells expressing MHC-I on their surface and analyzed by FACS. (b) A representative FACS analysis of a β2m binder (Nb30) that cross-reacts with MHC-I, expressed on the surface of BT474 cells. Addition of the Nb30-PE conjugate causes a dramatic increase in the fluorescence of the BT474 cells (green line) as compared to the autofluorescence of untreated cells (red line). No such shift was observed with the Nb24-PE conjugate (c), indicating that Nb24 does not cross-react with MHC-I, expressed on the surface of a human cells.

**Table:**

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<th>Cell line</th>
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<th>Nb23</th>
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<th>Nb31</th>
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![FACS analysis](image2)
Fig. S7. Crystal structure of Nb24 bound to ΔN6j2m. (a) Nb24 is given in its surface representation. The Nb24 paratope contains residues from CDR2 (yellow) and CDR3 (green). The epitope region of ΔN6j2m is represented in sticks. The ΔN6j2m epitope consists of residues from the loop connecting β-strands C and D (Lys41—Arg45, shown in light pink) and the loop connecting β-strands E and F (Lys75—Asp77, shown in purple). (b) Comparison of the overall conformation of the epitope in the binary complex (light pink and violet) and free j2m (1LDS, cyan). Binding of the nanobody causes the reorientation of solvent exposed residues but has virtually no effect on the conformation of the main chain (c).

Table S1. The X-ray data collection and structure refinement statistics

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<th>Dataset</th>
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<td>α = 90, β = 105.115, γ = 90</td>
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*R_sym = Σ(|I - 〈I〉|)/Σ(〈I〉), where I is the intensity measurement for a given reflection and 〈I〉 is the average intensity for multiple measurements of this reflection.

†With respect to Engh and Huber parameters.