Three Camelid VHH Domains in Complex with Porcine Pancreatic α-Amylase: Inhibition and Versatility of Binding Topology

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ABSTRACT

Camelids produce functional antibodies devoid of light chains and CH1 domains. The antigen-binding fragment of such heavy-chain antibodies is therefore comprised in one single domain, the VHH. We report here on the structures of three dromadery VHH domains in complex with porcine pancreatic α-amylase. Two VHHs bind outside the catalytic site and do not inhibit or inhibit only partially the amylase activity. The third one, AMD9, interacts with the active site crevice and is a strong amylase inhibitor (Ki=10 nM). In contrast with complexes of other proteinaceous amylase inhibitors, amylase keeps its native structure. The VHHs water accessible surface areas covered by amylase range between 850 and 1150 Å^2, values similar or even larger to those observed in the complexes between proteins and classical antibodies. These values could certainly be reached because a surprisingly high extent of framework residues are involved in the interactions of VHHs with amylase. The framework residues that participate in the antigen recognition represents 25-40% of the buried surface. The inhibitory interaction of AMD9 involves mainly its CDR2 loop whereas the CDR3 loop is small and certainly not protruding as in the cAb-Lys3, a VHH inhibiting lysozyme. AMD9 inhibits amylase although it is outside direct reach of the catalytic residues, therefore it is to be expected that inhibiting VHH’s might also be elicited against proteases. These results illustrate the versatility and efficiency of VHH domains as protein binders and enzyme inhibitors and are arguments in favor of their use as drugs against diabetes.

Abbreviations: Ig, immunoglobulin; VH, camelid heavy-chain antibody VH; PPA, porcine pancreatic α-amylase; CDR, complementarity determining region; NCS, Non crystallographic symmetry; CNS, Crystallography and NMR System; RMSD, Root mean square deviation; vdW, van der Waals.
INTRODUCTION

The fundamental molecular recognition molecules of the humoral immune response are remarkably homogeneous throughout the vertebrate phylum. All immunoglobulins are multimers of heterodimeric chains, where each heavy chain (H) of 4 or 5 domains is linked by disulfide bridges to a light chain (L) of two domains (1). The antigen-binding part of the immunoglobulins is formed invariably by the N-terminal domains of both, the H and L chain. These domains display a large sequence variation however concentrated in three regions per domain, the CDRs.

Important deviations of this conserved immunoglobulin organization were however observed. In some immunoglobulin isotypes of camelids from the old world (camels, dromedaries) or from the new world (llamas, vicugna) the L chain is missing (2). Furthermore, their H-chain is devoid of the CH1 domain (3,4) due to an unconventional splicing event during the mRNA maturation. The antigen binding fragment of the heavy-chain antibodies is therefore comprised in one single domain, the unique N-terminal variable domain referred to as VHH that replaces a four-domain Fab fragment in the Ig structure (5). This VHH domain is obtained after a DNA recombination between dedicated VHH-germline gene segments with D and J minigenes. The dromedary VHH germline genes are quite diverse. They could be grouped into seven subfamilies (6), and contain additional hotspots for mutation that will add to the diversity of the antigen binding repertoire. Moreover, the domain often acquires a disulfide bond between its CDR3 and CDR1 or position 45 (5). A considerable interest in the humoral immune system of Camelidae comes from the observation that their heavy chain antibodies and the recombinant VHHs as well, contain a much higher proportion of molecules that interact directly with the active site cleft of enzymes (7). From a structural viewpoint, the three-dimensional structures of VHH complexes with lysozyme, RNase, carbonic anhydrase and two dye haptens, as well as an unbound VHH have been determined (8-14). All three dromedary anti-enzyme VHHs of known structure are derived from the same VHH germline subfamily (subfamily 2a), and only one of these is inhibiting the enzymatic activity of its antigen. This cAb-Lys3 inhibitor of chicken egg-white lysozyme has a remarkable paratope architecture where part of its long CDR3 protrudes from the remaining antigen-binding site and inserts into the active site of the enzyme, mimicking the lysozyme natural substrate (15).

Here we report the 3D structures of three complexes between porcine pancreatic α-amylase (PPA, 16) and camelid VHH fragments. These three binders originated from VHH germline genes of
three different subfamilies, two for which no structural information was yet available. Crystal structures of complexes between PPA and carbohydrate or proteinaceous inhibitors are known at atomic resolution (17-19). We investigate inhibitors that were raised by the humoral immune response in a few weeks time and that possess an enzyme inhibiting potency similar to the natural inhibitor that co-evolved with amylase over many millions of years.

MATERIALS and METHODS

VHH preparation and characterization

Periplasmic expression and immobilized metal affinity chromatography (IMAC) purification of the three PPA binder VHH proteins, in fusion with a C-terminal His6 tail, was performed according to Lauwereys et al. (7). The VHH proteins were further purified by gel filtration, mixed with PPA in a 2 to 1 molar ratio, and the complexes were separated from free antibody on Superdex 75 (Pharmacia) in 50 mM Tris (pH 7.5) 100 mM NaCl. The inhibition of the enzymatic activity of PPA by the various VHHs was tested on 2-chloro-4-nitrophenyl maltotrioside or on ‘blue-starch’ (Phadebas, Pharmacia-Upjohn) according to the protocols in ref. (7), or as recommended by the supplier, respectively.

Crystallographic procedures

All crystals were obtained using the hanging drop method of vapor diffusion, by mixing 1 µl of protein solution with 1 µl of reservoir solution. A single crystal of the PPA/AMB7 complex was obtained in 10-15 % PEG 20000, 0.1-0.2 M imidazole malate (pH 8.0). A unique monoclinic crystal was obtained, which could not be further reproduced (Table 1). Triclinic crystals of the PPA/AMD9 complex were obtained in 0.8 M phosphate buffer (NaH2PO4 and K2HPO4) at pH 7.0 (Table 1). Triclinic crystals of the PPA/AMD10 complex were produced in 32% PEG 4000, 0.1 M sodium-citrate and 0.2 M ammoniumacetate (pH 5.0) (Table 1).

Data were collected at ESRF (Grenoble, France) at beamline ID14-EH2 for AMB7 and AMD10 on a ADSC-Q4 detector. AMD9 was collected at beamline BM14, with a imaging plate detector. Data were integrated with DENZO (20) and reduced with SCALA (21). Collection statistics, presented in Table 1, indicate a good quality for the AMD9 and AMD10 complexes, whereas the data are incomplete for AMB7 due to spots overlap.
The three structures were solved by molecular replacement with AMoRe (22). The initial solution for the complex with AMB7 was obtained using native amylase (16,1FJH) and the anti-RR6 VHH fragment R2 (12,1QDO) as search models. The three amylases were positioned readily by AMoRE, yielding a correlation coefficient of 0.53 and an R-value of 37.6 at 4.0 Å resolution. The VHHs, however, were positioned manually by visual inspection of the difference maps using Turbo-Frodo (23). After rigid body and minimization refinement with CNS (24), R and Rfree dropped to 28.4 and 33.5%, respectively, at 2.0 Å resolution. For the AMD9 complex, the same search models were used. The amylase molecules were found by AMoRE, resulting in R and Rfree values of 36.8 and 39.0%, respectively, at 3.5 Å resolution. The packing consists of a dimer related by a pure 2-fold axis, and a translated dimer. The complete model of the 4 complexes was subjected to a rigid-body minimization and B-factors refinement, which lowered R and Rfree to values of 33.4 and 35.2%, respectively, at 1.8 Å resolution. For the PPA/AMD10 complex, the VHH search model was cAb-Lys3 (8, 1MEL). The two amylase molecules and the two VHH fragments were readily located by AMoRe, leading to a correlation coefficient of 0.56 and a R-value of 36.9% at 2.7 Å resolution.

The same refinement procedure was used with the three complexes, using CNS (24). Rounds of minimization/B-refinement were alternated with model rebuilding at the display with Turbo-Frodo. The complexes with AMD9 and AMD10 exhibit an excellent geometry and good R values (Table 1). In contrast, the complex with AMB7 suffers from data incompleteness and hence, exhibits a geometry quality closer to a model at medium resolution. The water accessible surfaces were calculated with DSSP (25) implemented in Turbo-Frodo (23). The radius of the water probe used was 1.5 Å. The coordinates have been deposited in the Protein Data Bank at RCSB (http://www.rcsb.org/pdb/) as entries 1KXT, 1KXQ and 1KXV.
RESULTS and DISCUSSION

Characterization of the VHH binders

A dromedary was immunized with PPA, and the antigen-binding repertoire of the heavy-chain antibodies was cloned in a phage display vector. After three rounds of panning with the antigen we identified several binders (7), of which three (AMB7, AMD9, and AMD10 VHH) were selected for structural investigation. The sequences revealed that the three binders are derived from germline genes of different subfamilies (6, Figure 1a). The VHH germline gene used to generate the AMB7 VHH binder is of subfamily 4b member since it has a 16 amino acid long CDR2, and a Cys (at position 45). Its CDR3 is 19 amino acids long and contains a Cys that could form a disulfide bridge with Cys45. The AMD9 VHH binder contains 17 amino acids in its CDR2, meaning that the VHH germline used is either of subfamily 2a or 5a. The presence of Phe37, Gly47, Ala49 and Val78 instead of Tyr, Leu, Ser and Leu respectively suggests that is most likely derived from 2a, the most frequently used subfamily in dromedary. This binder is special in the sense that the CDR1 is shortened by 3 amino acids possibly due to a deletion around the palindromic nucleotide sequence (codons 29-33 in clone cvhhp11). Since the deletion in the CDR1 has removed the Cys and since no Cys occurred in the CDR3 region of 14 amino acids in size, this structure is not stabilized by an interloop disulfide bridge. The VHH germline used for AMD10 binder is of subfamily 3b because it has the characteristic 16 amino acid long CDR2 and a Cys30, Tyr37, and Phe 47. Its CDR3 is short for a VHH (13 amino acids), and contains a Cys.

The affinity of the three VHHs for PPA ranged from 3.5 nM (AMD9) to 235 nM (AMB7), AMD10 having an intermediate affinity of 25nM as measured on an IAsys biosensor (7, Table 2). The gel filtration of a stoichiometric mixture of the binders with PPA proved that binding occurred at a 1:1 molar ratio. These binders were also chosen because they inhibit the PPA to different extents. Only AMD9 VHH has the capacity to inhibit the hydrolysis of the small organic 2-chloro-4-nitrophenyl maltohexaoside substrate (7, Table 2). The hydrolysis of ‘blue-starch’, a large water insoluble cross-linked starch polymer carrying a blue dye, by α-amylase to form water soluble blue fragments is almost completely blocked by AMD9 VHH, whereas the AMB7 VHH shows a largely retarded solubilization of the chromophore and AMD10 VHH exhibits only a weak effect (Table 2). This
suggests that the three VHHs interact at three different epitopes of the PPA and/or employ different enzyme inhibition modes.

The VHH structures

The VHH polypeptidic chains are not complete and are visible in density from residues 2 to 25 and 28 to 111 for AMB7 VHH (123 residues), from 1 to 111 for AMD9 VHH (118 residues) and from 2 to 112 for AMD10 VHH (119 residues) (Kabat numbering, 29). The three VHH structures adopt the classical immunoglobulin fold (Figure 1b) and do not present important deviations in their frameworks: the RMSD calculated with the 88 framework Cα-atoms range from 0.61 Å (AMB7/AMD9) to 0.84 Å (AMB7/AMD10). Indeed, most deviations are observed at the CDRs level, but some significant divergences occur also elsewhere: in the loop adjacent to the CDRs (amino acids 71-78), in the loop at the bottom of the VHHs (amino acids 39-44), and in the segment 102-106, just after the CDR3 in the PPA/AMD10 complex.

Besides the classical disulfide bridge formed between Cys 22 and Cys 92, dromedary VHHs possess frequently a second pair of cysteines, one being always located into the CDR3 (5) and the other one into the CDR1 (5), forming a second disulfide bridge (8,10). Two VHHs studied here, AMB7 and AMD10, possess four cysteines in their sequence (Figure 1a). A second disulfide bridge is observed in AMB7 VHH between the CDR3 cysteine residue 100h and, for the first time, a framework residue (Cys 45). In the case of AMD10 VHH, the second disulfide bridge tethers CDR3 Cys 100c and the CDR1 Cys 30. In the latter case, the relatively short CDR3 and the presence of this disulfide bridge may explain the deviation in the framework conformation observed between residues 102-106; in addition, the presence of two prolines (residues 100a and 100d) may also contribute to this effect.

The CDRs 1-3 in the VL and 1-2 in the VH domains of immunoglobulins have been shown to adopt a restricted set of conformations, depending on their length and amino acid sequence (26). In the VHHs of camelids, the set of conformations of CDR1 and CDR2 has been found to extend beyond that observed in classical VHs (26). The CDRs in the three VHHs are well defined in density, except for 2 residues which are not visible in the CDR1 of AMB7 VHH. The conformation of this CDR1, however, resembles that of canonical type 1. For the two other VHHs (AMD9 and AMD10) the CDR1s do not fit with any CDR1 of known canonical type, or with any VHH known structures. In contrast,
CDR2 canonical types are readily identified as type 1 for AMB7 and AMD10 VHHs, and as type 2 for AMD9 VHH.

The CDR3 loops do not obey to canonical types, even if some conformational preferences and classes have been identified (28). Their length of 19, 14 and 13 residues, respectively, in the three VHHs can be considered as average for dromedary VHHs (Figure 1a). Amazingly, when looking at the VHH side bearing the CDR3 (Figure 1b), one CDR3 is going to the left (AMB7), another to the right (AMD9) and the last one upwards (AMD10). Clearly, the disulfide bridges may play a role in this conformational dispersion. In the AMB7 VHH, the disulfide bridge pulls the CDR3 in a zone never observed in any of the other camelid VHHs. Proline 96 twists the CDR3 chain towards solvent. It adopts an anti-parallel 2 stranded β-sheet structure (β-hairpin) between residues 99 and its end at residue 100k (Figure 1b). A proline at this position redirects the chain close to the protein core with a classical framework conformation. The AMD9 CDR3 covers the VH/VL interface as shown in other VHH studies and does not present any special features. In the AMD10 VHH, the disulfide bridge between CDR1 and the short CDR3 maintains the loops close together and makes it possible for the CDR3 to protrude as observed in the anti-lysozyme VHH (8).

Structure of the complexes

Overall structures

In the three complexes, the complete PPA polypeptidic chain is visible in the electron density map. The structures of the PPA complexes with AMB7, AMD9 and AMD10 VHHs contain 3, 4 and 2 PPA/VHH complexes in their asymmetric unit, respectively. When the PPA/VHH complexes contained in the asymmetric unit from the same crystal form are superimposed, very low RMSD on the Cα atoms are observed, below 0.3 Å in all cases. Superimposing the PPAs belonging to the three different complexes yields surprisingly low RMSD values, below 0.5 Å for all atoms.

The PPA/VHH associations

Superposition of the three complexes using the PPA coordinates indicates clearly the different location of the VHHs at the PPA surface (Figure 2a). The AMD9 VHH interacts directly with the PPA’s active-site region and blocks the entrance of the V-shaped crevice. AMB7 VHH binds PPA at one end of the elongated active site crevice but far from the catalytic residues (Figure 2A). In contrast, AMD10 VHH binds PPA far from the catalytic crevice (Figure 2a).
Calculations of water accessible surfaces have been used to evaluate the contacts of each VHH with its PPA partner. In Table 3, the surface areas of the VHH shielded from the solvent by bound PPA have been reported. AMD9 VHH has the largest contact area with a total of 1151 Å² (Table 3). This belongs to the highest values currently observed in complexes between classical antibodies (Fabs or Fvs) and proteins. The two other fragments, AMB7 and AMD10 VHHs, interact with PPA with large contact surface areas of 854 and 882 Å², respectively (Table 3).

In all three complexes, several parts of the VHHs interact with PPA. Dissecting these interactions indicates a surprisingly large contribution of the framework residues to the VHH/PPA contacts with 28, 25 and 40% of the total contact surface in the three VHHs, respectively (Table 3). Some contribution of framework residues to the interaction with the antigen have already been reported for classical immunoglobulins. However, the extent of these interactions is much smaller than for the present VHHs, the amount of buried surfaces covered by framework residues being comprised between 1 and 9% of the total interaction (29). Such interactions have also been observed to be important in VHHs/haptens interactions (13). The main interactions, however, are provided by the three CDRs, CDR3 being the main contributor in the complexes of AMB7 and AMD10 VHHs, and the least for AMD9 VHH. In this latter case, CDR2 dominates the interaction and provides the inhibitory contact (Table 3). In all cases, the CDR1 is very little involved in the interactions.

The CDR3 plays a minor role in PPA inhibition when considering the AMD9/PPA complex. It is not protruding from the paratope and not penetrating into the active site in the way that was reported for the lysozyme/cAb-Lys3 complex (8). This protruding geometry can certainly be established upon formation of a disulfide bridge between the CDR1 and the CDR3. This feature is however absent in AMD9 VHH. In contrast, it is observed in the AMD10 VHH, in which the CDR3 protrudes at the top of the VHH body. Despite this favorable conformation, AMD10 VHH does not bind to the catalytic crevice either. The AMD10/PPA interactions involve mainly framework residues on a side of the VHH, the CDR3 and other framework residues located close to it. In AMB7 VHH, the long hairpin CDR3 and some framework residues around it provide most of the interactions.

**Interaction of AMD9 VHH at the catalytic site**

The AMD9 VHH covers a large surface of PPA in the complex (1108 Å²) within the active site crevice. This VHH, however, does not occupy a central position in the active site, as compared to
other proteinaceous inhibitors of PPA (18,19). The CDR2 loop 50-55 contacts the upper side of the V-shaped extended active-site crevice, the side chains of Tyr 52 and Arg 52a filling the amylose path at its non-reducing end. Tyr 52 accessible surface is reduced from 32 to 9 Å² upon complexation, and that of Arg 52a from 179 to 46 Å², making this residue the largest contributor in the AMD9 complex formation. The Arg 52a guanidinium group is located 4.8 Å away from the catalytic residue Asp300 and establishes a strong stacking contact (d=3.6 Å) with PPA’s Trp59 indole ring, which has been shown to interact with the hydrophobic face of saccharides. This stacking was also observed in other structures of PPA in complex with proteinaceous inhibitors such as tendamistat and alpha-AI (18,19).

Comparison of the PPA structures in the VHH complexes with native amylase

The three current PPA structures remain very close to the native PPA structure. Native PPA has been shown to exhibit striking differences when inhibited by a small molecule or some proteinaceous inhibitor such as alpha-AI (but not by tendamistat) (18,19). The core A domain is very similar in all PPA structures, as well as in the VHH/PPA complexes, with the exception of some loops near the active site (see below). In the VHH complexes, the C domain of PPA adopts slightly different orientations compared with the native enzyme. The B domain stretch 125-155 is distorted, specially in the case of AMD9 VHH, while the effect is less pronounced with AMB7 VHH and inexistent in AMD10 VHH. Three loops deviate as compared to native PPA: loops 220-225, 237-241 and 348-354. The largest deviation with regards to the native enzyme involves the 348-354 loop. The effect is larger in AMD9 VHH (3Å displacement) and weaker in AMB7 and AMD10 VHHs. Two loop deviations are common between the complexes of PPA with VHH and with proteinaceous inhibitors. In this latter case, deviations are observed at PPA’s 137-153, 237-241, 350-359 and at the active site.

Correlates of binding topology and inhibition data

Among the three VHHs binders of PPA, AMD9 VHH is the only one to interact directly in the V-shaped active site crevice, close to the catalytic residues. Its binding affinity (Kd =3.5 nM, Table 2) correlates well with its very large interaction surface area with PPA, the largest found amongst VHH/protein interactions. The affinity value is of the same order of magnitude as the Ki value obtained upon competitive inhibition by AMD9 VHH of the PPA activity on a small pseudo-substrate 2-chloro-4-
nitrophenyl maltotrioside. This inhibition is also a clear indicator of the proximity of AMD9 VHH to the catalytic residues. In contrast, the two other binders are unable to inhibit the hydrolysis of the small substrate by the PPA, their epitope being located outside of the catalytic area. The Kd value of AMD10 VHH is 25 nM, while, that of AMB7 VHH is one order of magnitude larger, despite the same interacting surface area. The action of PPA on large substrates, mimicked by the ‘blue-starch’ Phadebas amylase test, is indeed inhibited by AMD9 VHH (Table 2). AMB7 VHH, unable to inhibit the activity of PPA on a small substrate, exhibits however a marked inhibitory effect (70%) of the PPA activity on starch. This can be explained by the localization of AMB7 VHH in the starch binding crevice ca 15Å away of the catalytic residues, at the reducing end, opposite to the AMD9 VHH location. AMD10 VHH is unable to inhibit PPA activity on small substrates and presents a very reduced effect with large substrates, which can be rationalized by its location far from the catalytic residues.

CONCLUSIONS
The crystal structures of the three VHHs in complex with PPA reveal water accessible surface areas similar or even larger to those observed in the complexes between proteins and classical antibodies (32). For the first time, a strong involvement of framework residues in opportunistic interactions with a proteinaceous antigen is observed. Such interactions have recently been documented in the case of a complex of a VHH with a hapten (12). Indeed, the involvement of framework residues could compensate in some cases for the lack of VL and hence provide interaction surfaces of the same magnitude as for classical immunoglobulins. In brief, for a smaller footprint of VHHs compared to Fvs, involvement of framework residues makes it possible for VHHs to reach an interaction surface area as large, or even larger, than that observed with Fvs. Furthermore, it makes it possible for the VHH to interact with its protein antigen with different topologies of orientations: front wise, side-wise, penetrating, flat-wise. The participation of the framework residues in the antigen recognition is expected to occur more frequent for VHHs than for VHs, because we noticed an overall increased accumulation of mutations (i.e. variability) throughout the entire sequence relative to that of dromedary VHs (6). Hence, these mutants introduced by an active somatic hypermutation mechanism can be selected by during the affinity maturation.

This is confirmed by the anti-PPA VHH fragments AMD9 and AMD7 which exhibit excellent Kd and Ki values, around 10 nM. These values are much better that those obtained with small saccharidic
inhibitors such as acarbose (Ki=1 \mu M), a molecule used against diabetes. Indeed, anti-PPA natural proteinaceous inhibitors such as tendamistat and alpha-AI, that co-evolved with the amylase over millions of years, exhibit smaller Ki values (0.01 nM and 0.1 nM, respectively). These values are not out of reach with VHHs provided that directed evolution techniques are used.

**Acknowledgements:** The ESRF is greatly acknowledged for beam time allocation. We would like to thank Eric Blanc for his technical assistance with graphics. This study was supported in part by the EU BIOTECH Structural Biology project (BIO4-98-048).
REFERENCES


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**Legends to figures:**

**Figure 1.** Sequences and 3D structures of the three VHVs. The three CDRs (1 to 3) are colored red, green and blue, respectively and the cysteines are purple.  **a)** anti-PPA AMB7, AMD9 and AMD10 VHVs amino-acid sequences. The numbering is according to Kabat (30). **b)** From left to right, views of the AMB7, AMD9 and AMD10 VHVs in the same orientation, with the CDR3 oriented in front (View made with SPOCK (33)).

**Figure 2.** Views of the three VHVs superimposed on PPA with the acarbose molecule bound (pink). PPA surface is in gray and its visible catalytic residue Asp300 is in red. The VHVs Cα trace is orange and the three CDRs are colored red, green and blue, respectively; and each VH surface is transparent. **Inset:** Close-up of the PPA active site with the inhibitory AMD9 VH bound. The saccharidic inhibitor, acarbose, has been positioned in the active site according to the X-ray structure as a probe of the saccharide position. Two residues of the VH, Tyr52 and Arg52a (CPK yellow and orange), clash with the modeled acarbose. (Views made with SPOCK (33)).
Table 1: Data collection, structure determination and refinement summary.

<table>
<thead>
<tr>
<th>Data Collection</th>
<th>AMB7</th>
<th>AMD9</th>
<th>AMD10</th>
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<tbody>
<tr>
<td>Space group</td>
<td>P2₁</td>
<td>P1</td>
<td>P1</td>
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<tr>
<td>cell dimensions: a, b, c (Å)</td>
<td>52.8x286.8x66.0</td>
<td>65.2x100.9x103.7</td>
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<tr>
<td>α, β, γ (°)</td>
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<td>81.2, 79.1, 78.9</td>
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<td>4 / 2.39</td>
<td>2 / 2.19</td>
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<td>3531130 / 383526</td>
<td>716803 / 175069</td>
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<td>Completeness (overall /last shell)a</td>
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<td>99.8 / 99.5</td>
<td>99.8 / 99.5</td>
</tr>
<tr>
<td>% I / σI (overall / last shell)a</td>
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<td>5.0 / 1.7</td>
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<td>7.0 / 13.7</td>
<td>6.4 / 32.2</td>
<td>7.4 / 13.7</td>
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</tbody>
</table>

Refinement

| number of protein / solvent /other atoms | 14439 / 1730 / 6 | 19296 / 2807 / 8 | 9571 / 1380 / 4 |
| number of reflections (all) / completeness (%) | 65698 / 50.1 | 317086 / 96.8 | 168160 / 95.9 |
| R- / R-free value (%)b | 20.3 / 23.6 | 19.7 / 21.9 | 20.5 / 22.9 |
| Residues in the 4 PROCHECK (31) areas (%) | 84.8 /14.4 / 0.5 / 0 | 88.9 /10.9 / 0.2 / 0 | 89.8 / 9.9 / 0.3 / 0 |

a last shell : 2.13-2.0 Å ; 1.75-1.65 Å ; 1.8-1.7 Å
b reflections in the test set (nr/ %): 1655/2.5; 9426/3; 3337/1.9.
Table 2  Summary of VHHs affinities and inhibitory effects on PPA.

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<th>Kd (nM)</th>
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<td>PPA/AMD9</td>
<td>3.5</td>
<td>10 nM</td>
<td>7 %</td>
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<tr>
<td>PPA/AMD10</td>
<td>25</td>
<td>&gt; mM</td>
<td>80 %</td>
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</table>

a IAsys biosensor assay (7).
b 2-Chloro-4-nitrophenyl maltotrioside (7).
c 'blue-starch' Phadebas amylase test (this work).

Table 3  Water accessible surfaces (in Å²) of the VHHs covered by PPA and of PPA covered by the VHHs upon complexation. The CDR definition is according to Kabat (30).

<table>
<thead>
<tr>
<th></th>
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<th>AMD 9</th>
<th>AMD10</th>
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<tr>
<td>Sum of CDRS</td>
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<td>864</td>
<td>528</td>
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<tr>
<td>Framework</td>
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<td>287</td>
<td>354</td>
</tr>
<tr>
<td>Sum of VHH</td>
<td>854</td>
<td>1151</td>
<td>882</td>
</tr>
<tr>
<td>% of total surface area of VHH</td>
<td>13.1</td>
<td>17.9</td>
<td>14.4</td>
</tr>
<tr>
<td>PPA</td>
<td>871</td>
<td>1108</td>
<td>849</td>
</tr>
<tr>
<td>Sum VHH+PPA</td>
<td>1725</td>
<td>2259</td>
<td>1731</td>
</tr>
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</table>
Desmyter et al., Figure 1

A

cAb-LYS3.DVQLQASGGSVQAGGLRSQCAGSYTGIPYCMGWRQAPGKEREGVAAINM-----GGGITYYADS
AMYL-B7..QVQLVESGGGLVQAGGLRSQCAGSYTFSSYPMGWRQAPGKEELVSRIPESE----SDGSANYAGS
AMYL-D9..QVQLVESGGGLVQAGGLRSACGNTYSSTMSKAAAYTID-----VGRWFAPGKEREGVAAAY----RNGIYSSADS
AMYL-D10..DVQLQASGGGLVQAGGLRSQCAGSYTIGPYCMGRQAPGKEREGVAAINM-----GGGITYVYDS

.................70!........80!...abc...90!......100!abcdefghijklmnop......110!...
cAb-Lys3.VKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTTYAYSEQQHLYSOGYDSWGQGTVVS
AMYL-B7..VKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTTYAYSEQQHLYSOGYDSWGQGTVVS
AMYL-D9..VKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTTYAYSEQQHLYSOGYDSWGQGTVVS
AMYL-D10..VKGRFTISQDNAKNTVYLLMNSLEPEDTAIYYCAADSTTYAYSEQQHLYSOGYDSWGQGTVVS

B

[Graphical representation of protein structures]

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Aline DESMYTER, Silvia SPINELLI, Françoise PAYAN, Marc LAUWEREYS, Lode WYNS, Serge MUYLDERMANS and Christian CAMBILLAU

J. Biol. Chem. published online April 17, 2002

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