Potent enzyme inhibitors derived from dromedary heavy-chain antibodies

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Evidence is provided that dromedary heavy-chain antibodies, in vivo-matured in the absence of light chains, are a unique source of inhibitory antibodies. After immunization of a dromedary with bovine erythrocyte carbonic anhydrase and porcine pancreatic α-amylase, it was demonstrated that a considerable amount of heavy-chain antibodies, acting as true competitive inhibitors, circulate in the bloodstream. In contrast, the conventional antibodies apparently do not interact with the enzyme’s active site. Next we illustrated that peripheral blood lymphocytes are suitable for one-step cloning of the variable domain fragments in a phage-display vector. By bio-panning, several antigen-specific single-domain fragments are readily isolated for both enzymes. In addition we show that among those isolated fragments active site binders are well represented. When produced as recombinant protein in Escherichia coli, these active site binders appear to be potent enzyme inhibitors when tested in chromogenic assays. The low complexity of the antigen-binding site of these single-domain antibodies composed of only three loops could be valuable for designing smaller synthetic inhibitors.

Keywords: camel/inhibitors/panning/single-domain antibody fragment/VH

Introduction

Enzyme inhibitors, both low-molecular weight compounds and proteinaceous molecules, have emerged as important pharmaceutical agents. Recent advances in molecular biology and protein characterization, as well as the abundant information gathered from the genome sequencing projects, have led to the identification of a steadily growing number of new targets and created the need for the rapid development of specific inhibitors. Strategies to develop such inhibitors are often based on the synthesis of transition state analogues. In a number of cases molecules are identified after the screening of large numbers of chemical compounds or natural sources. The generation of antibody-based molecules forms an obvious alternative. It is widely recognized that the immune system is the preferred tool to generate specific binders or reporter molecules against virtually all agents (Paul, 1993). However, despite the omnipotence of the antibody repertoire, the number of conventional antibodies (i.e. heterotetramers of two light chains and two heavy chains) acting as competitive enzyme inhibitors remains disappointingly low. A satisfactory explanation for this scarce occurrence is given by the incompatible surface topography of the enzyme’s active site and the antigen-binding site of conventional antibodies. From a recent survey of enzyme structures it appears that the active site is found almost exclusively in the largest cleft on the protein surface (Laskowski et al., 1996). Likewise, the antigen-binding surface of conventional antibodies forms either a cavity, a groove or flat surface depending on whether an interaction with haptens, oligopeptides or proteins is observed (Webster et al., 1994). It is striking that convex antigen-binding surfaces are not found in conventional antibodies.

In this respect, we now demonstrate that functional heavy-chain antibodies from Camelidae behave quite differently in comparison with the conventional four-chain antibodies. More specifically, the heavy-chain antibodies acquired the potential to recognize protein cavities and as such the ability to inhibit enzymes. Camelidae produce an important fraction of their functional immunoglobulins as homodimers of only heavy chains, devoid of light chains (Hamers-Casterman et al., 1993). Specific heavy-chain antibodies can be raised in a dromedary or llama with a variety of protein antigens. The N-terminal variable region of these heavy-chain antibodies (referred to as VH) contains a minimum-sized antigen-binding domain (Sheriff and Constantine, 1996). The structure of a dromedary VH in complex with lysozyme revealed the unusual surface topography of the antigen-combining site of this single-domain antibody fragment (Desmyter et al., 1996) and the importance of the CDR3 loop for the binding interaction. The N-terminal part of the 24 amino acid-long CDR3 loop protrudes from the antigen-binding surface and penetrates deeply into the active site of lysozyme. However, this single observation does not allow the claim that the on average long CDR3 loop found in dromedary heavy-chain antibodies (Muyldermans et al., 1994) systematically prefers to interact with antigen clefts and automatically generates competitive inhibitors.

In this study we report the immunization of a dromedary with additional enzymes and demonstrate that a substantial proportion of the polyclonal heavy-chain antibodies binds into the active site of the enzymes. The antigen-specific VHVs, binding with nanomolar affinity are easily cloned from the peripheral lymphocytes. We consider that cloning and expression in Escherichia coli of recombinant dromedary VH antibody fragments is a general and powerful strategy to obtain a new type of potent and specific enzyme inhibitor in a short period of time.
Enzyme-inhibiting antibody fragments

Results

Two enzymes, porcine pancreatic $\alpha$-amylase and bovine erythrocyte carbonic anhydrase, were selected to test the generality of producing enzyme-inhibiting dromedary heavy-chain antibodies. Both enzymes are readily available, while inhibitors of small molecular weight and proteinaceous nature are known for each (Vértesy et al., 1984; Alkazzz et al., 1996; Wuebbens et al., 1997); in addition, simple enzymatic activity assays have been described (Pocker and Stone, 1968; Winn-Deen et al., 1988).

Specific antibodies in the heavy-chain IgG subclasses

Injection of a dromedary with 1 mg of both immunogens every 7 days during a 2-month period resulted in successful immunization as indicated by ELISA (Figure 1A). A sharp increase in titre was observed after three to four injections for both antigens. Compared with the response of porcine $\alpha$-amylase, a 1-week delay in response was observed for bovine carbonic anhydrase; maximal titre was obtained after 5 weeks and remained constant thereafter for at least an additional 3 weeks.

Four IgG subclasses can be purified from the dromedary serum by differential absorption on Protein A and Protein G. The IgG1 subclass contains the conventional heterotetrameric antibodies composed of two light and two heavy chains, whereas IgG2a, IgG2b and IgG3 are the homodimeric heavy-chain antibodies, devoid of light chains (Hamers-Casterman et al., 1993). Two experiments proved that all dromedary IgG subclasses recognized the antigens. In the first experiment we purified the individual IgG subclasses (IgG1, IgG2a, IgG2b and IgG3) from the sera and tested them individually in a solid-phase ELISA (Figure 1B and C). From the binding curves it was clear that antibodies with specificity for $\alpha$-amylase or carbonic anhydrase were present in all the IgG subclasses. The prevalence of specific heavy-chain antibodies was confirmed following a separate approach in which total serum was incubated with the native enzymes immobilized on Sepharose. The captured proteins were thereafter analysed on reducing and non-reducing SDS–PAGE (Figure 1D). In the serum collected at days 28 and 54, in addition to the conventional IgG1 antibodies of $M_r$ 160 000 Da, huge amounts of heavy-chain antibodies with $M_r$ of $\sim$95 000 Da were retained on the beads with immobilized $\alpha$-amylase (Figure 1D, lanes 2 and 3). As shown in lanes 4 and 5 of Figure 1D, the same samples contain a mixture of two bands ($M_r$ 45 000 and 42 000 Da) under reducing conditions. These are the monomeric heavy chains of the dromedary IgG2 and IgG3, respectively (Hamers-Casterman et al., 1993). The controls in which equivalent amounts of pre-immune serum (day 0) were used, revealed that only minor amounts of proteins were adsorbed onto the beads.
the beads (Figure 1D, lane 1). Similar results were obtained with carbonic anhydrase (data not shown).

**Presence of inhibitory antibodies in the heavy-chain subclasses**

Two experiments were performed to demonstrate that a substantial proportion of the polyclonal heavy-chain antibodies interact specifically with the active site of the enzymes. In a first set of experiments the individual isolated subclasses were tested in a competitive ELISA. It was demonstrated that an important fraction of the antibodies of IgG2a, IgG2b and IgG3 subclasses were prevented from binding to α-amylase in the presence of acarbose, a pseudohexasaccharide competitive inhibitor (Figure 2A). Apparently, ~50% of the heavy-chain antibodies are displaced upon addition of acarbose, which is known to bind into the active site of α-amylase (Gilles et al., 1996). In contrast, no significant difference in signal is observed with the conventional antibody IgG1 subclass. A similar observation was made for carbonic anhydrase with dorzolamide as competitive inhibitor. Here, the drop in signal is most pronounced in the IgG3 subclass (Figure 2B).

In the second approach we tested the enzyme-inhibiting capacity of the heavy-chain antibodies. To avoid immunoprecipitation, we first prepared pure VHHs starting from IgG3 heavy-chain antibodies. IgG3 was chosen as it constitutes the most abundant heavy-chain subclass in dromedaries and llamas (Hamers-Casterman et al., 1993). These VHHs are readily obtained by the limited proteolytic digestion of IgG3 with endo-Glu V8 protease. This enzyme apparently cleaves in the short hinge region between the VHH and the CH2 domain (R.Hamers, in preparation). Undigested IgG3 and the Fc-part were retained on a Protein A column. The flow-through, containing the VHHs, was dialysed and subsequently tested in enzymatic assays. No inhibition could be demonstrated for carbonic anhydrase due to the insensitivity of the colorimetric assay (p-nitrophenylacetate hydrolysis). On the other hand, the cleavage reaction of 2-chloro 4-nitrophenyl maltotriose by porcine α-amylase is a much more sensitive assay. Here, preincubation of the enzyme with the purified polyclonal VHH fraction resulted in a substantial drop of the initial cleavage rate (Figure 2C). A VHH pool prepared from IgG3 of a non-immunized dromedary did not interfere with the enzyme activity. Thus, the observed inhibition is not due to trace amounts of co-purified contaminants or residual protease.

In summary, the first experiment revealed the presence of heavy-chain antibodies interacting with the active site of the enzymes, while the second experiment showed that the VHHs of the IgG3 subclass contained enzyme-inhibiting binders. Taken together, these observations strongly suggest that at least part of the VHHs of the IgG3 population are competitive enzyme inhibitors.

**Isolation of individual VHH binders**

The VHHs of 10⁷ peripheral blood lymphocytes were cloned in the pHEN4 vector after RT–PCR amplification (Ghahroudi et al., 1997). A VHH library of 5×10⁶ individual clones was obtained and panned for the presence of carbonic anhydrase or α-amylase binders. After the third round of panning, individual colonies were randomly

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**Fig. 2.** Competitive ELISA with small molecular weight inhibitors and enzyme inhibition of α-amylase by dromedary VHH fragments. (A, B) ELISA signals obtained after binding of IgG1, IgG2a, IgG2b and IgG3, isolated from serum collected at day 54, and at two different protein concentrations (0.6 and 0.3 μg/ml), to amylase (A) and carbonic anhydrase (B) in the absence (filled bars) or presence (open bars) of inhibitor. Detection of bound IgG was determined as described in the legend of Figure 1. (C) Inhibition of α-amylase by VHH fragments isolated from serum (collected at day 54). VHH fragments were prepared by limited proteolysis of the IgG3 fraction. Varying amounts of this pool of antibody fragments (range 0–300 μg/ml) were preincubated for 2 h with α-amylase at a concentration of 1.5 μg/ml in a final volume of 50 μl. The residual enzymatic activity (increase OD₄₀₅/min) was measured after addition of 950 μl 0.2 mM 2-chloro-4-nitrophenyl maltotrioside.
Enzyme-inhibiting antibody fragments

selected and the VHHs produced in their phage-attached form or as soluble protein in a TG1 E.coli strain.

For carbonic anhydrase, the detection of the binders in an ELISA with the anti-M13 or with the anti-decapeptide monoclonal antibody revealed that 23 out of the 24 clones were positive. The binding characteristics of these clones, produced as soluble periplasmic protein in TG1 cells, were examined in further detail in a competitive ELISA. In this assay the immobilized enzyme was preincubated with the inhibitor, dorzolamide, before adding the soluble VHH. From the optical reading, it appeared that VHH binding to carbonic anhydrase was inhibited by the presence of this low-molecular weight inhibitor in 14 of the 24 clones tested.

For α-amylase, 20 individual clones randomly selected after three rounds of panning with biotinylated α-amylase, were tested in solid-phase ELISA either in their phage-attached form or as soluble protein. The ELISA indicated 17 positive clones out of 20 tested. In order to identify putative active site binders a direct enzyme inhibition assay was used. In this assay an equal volume of a 10-fold diluted periplasmic fraction was preincubated with the enzyme for 30 min. Upon adding the chromogenic substrate, the residual enzymatic activity was determined spectrophotometrically. This allowed us to identify seven out of 20 clones as α-amylase inhibitors.

Sequence alignment

Sequence analysis of the 23 clones with specificity for carbonic anhydrase revealed that four different VHH fragments were selected, the deduced amino acid sequences of which are compiled in Figure 3. The clones encoding the enzyme-inhibiting camel single-domain antibody (cAb) fragments cAb-CA04, cAb-CA06 and the non-inhibiting cAb-CA05, cAb-CA10 occurred 12, four, four and three times respectively among the 23 selected clones.

For the α-amylase binders, the sequence results revealed that only two different inhibitory clones were present. These were referred to as cAb-AMD7 and cAb-AMD9 and occurred four and three times, respectively. Among the non-inhibitory VHH fragments, four different clones were present.

The amino acid sequences of the four different carbonic anhydrase binders (two enzyme inhibitors and two non-inhibitors) and the six α-amylase binders (two inhibitors and four non-inhibitors) are aligned along with the human POT VH (Fan et al., 1992) and the dromedary VHH inhibitors (Desmyter et al., 1996; Ghahroudi et al., 1997). It is clear that all isolated single-domain binders are derived from heavy-chain antibodies: they are VHs and not VHs. Indeed, the substitution of Leu11, Val37, Gly44, Leu45 and Trp47 by Ser11, Phe or Tyr37, Glu44, Arg45 (two clones with Cys45) and mostly Gly47 supports this statement (Muyldermans et al., 1994).

It is anticipated that these VHHs are generated by a recombination mechanism from a VHH germline gene (Nguyen et al., 1998). The divergence of the CDR1 and CDR2 region indicates that all binders are derived from different VH germline genes. This underlines the extended repertoire present within the dromedary heavy-chain antibodies.

The average length of the CDR3 of these new binders is 15.1 amino acids. With the notable exception of cAb-AMD9, all the binders contain an additional pair of cysteines (one in the CDR3 and one in either the CDR1 or at position 45).

We could not yet allocate the D germline minigenes. Neither did sequence comparison of the dromedary VHH inhibitors with naturally occurring proteinaceous carbonic anhydrase inhibitors or tendamistat reveal any homology. Nor could we detect any specific feature (sequence, CDR length, position of Cys in CDR3) to discriminate the inhibitors from the non-inhibitors.

VHH purification and affinity measurements

The four carbonic anhydrase and four α-amylase binders (cAb-AMB7, cAb-AMB10, cAb-AMD7 and cAb-AMD9) were selected for detailed characterization. These single-domain binders were expressed in the periplasm of E.coli WK6 as a fusion protein with a C-terminal (His6) purification tag. The proteins were purified to >95% homogeneity, as judged by SDS–PAGE, by two successive chromatographic steps. All purified proteins remained soluble and were present only in their monomeric form. For all VHH
fragments, 1–3 mg pure protein was obtained from a 3 l shakeflask culture.

Real-time binding was monitored with an IAsys Biosensor instrument. As the α-amylase lost all of its enzymatic activity upon immobilization to the cuvette, we carried out all binding experiments by immobilizing the purified antibody fragments to the dextran layer. The equilibrium dissociation constant \( K_D \) for the individual binders was derived from the ratio of the kinetic rate constants (Table I). As shown, the \( k_{\text{off}} \) rates range from \( 2.5 \times 10^4 \) up to \( 2.36 \times 10^5 \) M\(^{-1}\)s\(^{-1}\), whereas dissociation rates as low as 0.0008 s\(^{-1}\) for cAb-AM-D9 were obtained. Combined, this resulted in equilibrium dissociation constants of between 3.5 and 70 nM for all the binders with the exception of cAb-AMD7.

### Enzyme inhibition

All carbonic anhydrase-specific VHH fragments were tested for their inhibitory potency in an esterolytic assay. Dorzolamide was developed as an inhibitor for human carbonic anhydrase. We have evidence that the bovine carbonic anhydrase is also inhibited by dorzolamide when tested under identical conditions (IC\(_{50} = 3 \) μM). From our observation that this drug prevented the binding of two VHH fragments (cAb-CA04 and cAb-CA06) in an ELISA assay, we anticipated that both VHH fragments would inhibit the enzymatic activity of carbonic anhydrase.

As shown in Figure 4A, cAb-CA04 and cAb-CA06 clearly inhibit the esterolytic activity of bovine erythrocyte carbonic anhydrase. The lower IC\(_{50} \) for cAb-CA06 (1.5 μM) versus cAb-CA04 (2 μM) is in agreement with its higher affinity as determined by IAsys. No inhibition of p-nitrophenylacetate hydrolysis was observed for cAb-CA05 or cAb-CA10, even when tested at concentrations as high as 10 μM.

The crude periplasmic proteins from two out of four selected α-amylase-specific clones inhibited the enzymatic activity of the porcine pancreatic α-amylase. This observation was confirmed with the purified proteins (Figure 4B). Both cAb-AMD9 and cAb-AMD7 appeared to be potent inhibitors (IC\(_{50} \) of 10 and 25 nM, respectively). The order of potency is in agreement with the \( K_D \) values determined by IAsys biosensor measurements. In contrast, neither cAb-AMB7 nor cAb-AMB10, when tested at micromolar concentration, had any effect on the catalytic efficiency of the enzyme. This proves that the observed inhibition was due to the VHH and not as a result of a co-purified contaminant. For comparison, the IC\(_{50} \) for the α-amylase with the non-antibody inhibitor acarbose was found to be in the order of 5 μM when measured under similar conditions.

### Discussion

In conventional antibodies the antigen-binding site is formed by combining the variable regions of light and heavy chains. Residues present in all six hypervariable regions (three in each domain) may be actively involved in the molecular interaction with the antigen (Padlan, 1994, 1996). They create a sufficiently large surface area which is essentially flat for protein antigens (Webster et al., 1994; Padlan, 1996). These would be seen frequently at the antigen-binding surface (Wu et al., 1993; Padlan, 1996). These would be seen frequently at the antigen-binding surface (Wu et al., 1993; Padlan, 1996).

The catalytic amino acids are mostly buried inside a
of choosing the optimal amino acid sequence to derive small molecular recognition units (Sheriff and Constantine, 1996).

The generality of a protruding CDR3 loop in camelid heavy-chain antibodies might be questioned upon examination of the structure of a llama VHH fragment (Spinelli et al., 1996). This antibody fragment has a moderate affinity ($K_D = 300 \text{nM}$) for the $\alpha$-subunit of human chorionic gonadotrophin hormone. Here, the first hyper-variable loop protrudes due to the presence of amino acid substitutions at key positions for the loop configuration. Hence, the CDR1 loops of VHVs might adopt many as yet unknown exposed conformations (Muyldermans et al., 1994; Spinelli et al., 1996). The CDR3 of this llama VHH is only six amino acids long. A recent comparison of dromedary and llama VHH amino acid sequences indicates that such a short CDR3 loop is rather uncommon as most VHVs have CDR3 loops of, on average, 15–16 amino acids (Vu et al., 1997).

All these observations directed us to the working hypothesis that the long CDR3 loops (or the exposed CDR1) protrude from the remaining antigen-binding surface in camelid VHVs and that these antibody regions should bind preferentially in clefts or cavities present on the antigen surface. As the active site of a vast majority of enzymes is located in the major cleft (Laskowski et al., 1996), obtaining several inhibitory antibodies from camelid heavy-chain antibodies should indirectly support the above hypothesis.

Immunizing a dromedary with small quantities of enzymes generates heavy-chain antibodies that react with the enzymatic active site. The solid-phase ELISA showed that addition of a competitive enzyme inhibitor displaced a measurable quantity of heavy-chain antibodies. In contrast, binding of the conventional IgG1 subclass was not affected. This corroborated the notion that the antigen-binding loops of conventional antibodies are largely unable to penetrate the cleft of the enzymatic active site. In addition, by using purified VHVs from the heavy-chain IgG3 subclass, we could demonstrate unambiguously that these polyclonal VHVs contain sufficient competitive enzyme inhibitors to obtain a concentration-dependent inhibition of $\alpha$-amylase.

Progress in molecular biology has generated straightforward tools for isolation and characterization of antibody fragments (Winter et al., 1994). The identification of individual binders from the cloned antigen-binding repertoire is greatly simplified when starting from the peripheral blood lymphocytes of an immunized dromedary. The VHVs of the heavy-chain antibodies of these animals belong to one single family (Muyldermans et al., 1994) and consequently only one set of PCR primers is required to amplify the entire repertoire (Ghahroudi et al., 1997). Note that no scrambling of VL and VH pairs occurs and that VL cloning is not required. Hence, small libraries of $\sim 10^7$ individual colonies are adequate for most purposes.

From the cloned VHH repertoire we isolated four and six different binders to carbonic anhydrase and $\alpha$-amylase, respectively, that originated from different heavy-chain antibodies. For both target enzymes the two inhibitors use totally different CDR sequences. This proves that different germline VHH genes were used in the recombination event to produce the parental antibody. It also implies that

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**Fig. 5.** Crystallographic model of mouse D1.3 and dromedary cAb-Lys3 antibodies with lysozyme. Ribbon representation of crystal structures of lysozyme (blue) in complex with the mouse D1.3 VH-VL fragments (grey, left of lysozyme) and the dromedary single-domain cAb-Lys3 (grey, below lysozyme). The CDR1, CDR2 and CDR3 of D1.3 VH and VL, shown in red, orange and yellow, respectively form the flat antigen-binding surface characteristic for most protein binders. The lower complexity of the cAb-Lys3 compared with the mouse D1.3 is evident, as well as the large protruding CDR3 loop which penetrates into the active site of lysozyme. The catalytic residue Asp35 of lysozyme is shown in ball-and-stick representation for reference. The pdb file 1VFB of D1.3 (Bhat et al., 1994) and pdb file 1MEL of cAb-Lys3 (Desmyter et al., 1996) in complex with lysozyme were used to generate this figure. The lysozyme of both complexes were superimposed on each other. Both antibodies bind to their original epitope as determined by crystallography.
the dromedary finds different solutions to interact with the same epitope. In addition, no evident sequence homology was found with tanexamistat or known proteinaceous carbonic anhydrase inhibitors (Vertesy et al., 1984; Wuebbens et al., 1997). Therefore, we believe that immunization of a dromedary and subsequent characterization of individual binders is one of the alternatives to generate specific enzyme inhibitors. We consider our dromedary single-domain binders as de novo-generated or ‘man-made’ enzyme inhibitors. It is worth mentioning that these single-domain binders were obtained in a time span of only a few months.

The binders interact with their target with affinities of up to a few nM. The absence of better binders might be inherent in the single-domain nature of the binders or due to the method of panning. Approximately 40% of the selected clones scored as inhibitors—a number which agrees nicely with our expectations based on the competitive ELISA result with heavy-chain antibodies and which argues for unbiased cloning and panning. More importantly, the high yield of binders with enzyme-inhibiting potential supports the statement that dromedary heavy-chain antibodies bind preferentially to clefts or cavities. The fact that cAb-AMD9 has a short CDR3 loop lacking a cystine bond indicates that other inhibition mechanisms might be present among the dromedary VHHs besides those using a large protruding loop as found in cab-Lys3. We also isolated and characterized several non-inhibitory antibody fragments. The CDR3 loop of these binders was comparable in length. Whether these non-inhibitory antibody fragments interact with other clefts present on the protein surface is not clear at present.

It is tempting to correlate the presence of enzyme-inhibiting heavy-chain antibodies in dromedaries with the reported low incidence or low susceptibility to viral and bacterial ‘Artiodactyl’ pathogens (Wernery and Kaaden, 1995). However, at this stage the correlation requires formal proof.

Davies and Riechmann (1995) generated a ‘synthetic’ single-domain antibody library. For this purpose, a human VH scaffold was first ‘camelized’ to mimic the dromedary VH domain sequence. This construct was subsequently topped with randomized codons at the CDR3 position. Interestingly, Martin et al. (1997) extracted a hepatitis C virus protease inhibitor from this library. It indicates that a large ‘synthetic’ or a ‘naïve’ dromedary VH library might substitute a dedicated (immunized) library. However, the fact that the isolated proteins had a relatively low affinity and dimerized upon antigen interaction questions their potential and the single-domain nature of these binders. Moreover, other work of Riechmann’s group (Davies and Riechmann, 1996) stressed the importance of all three CDR loops. Consequently, it cannot be excluded that a synthetic VHH library of randomized CDR3 might only yield enzyme inhibitors for a limited number of antigens.

If binding to cavities is an acquired property of Camelidae heavy-chain antibodies, this may have important implications for the wider use of these antibody fragments (anti-idiotypes, receptor–ligand, cell surface molecules). In addition to their superior properties such as simple isolation, high solubility and stability, these antibody fragments can provide a valuable source of structural templates for designing molecular weight lead compounds based on their CDRs. However, antibodies or antibody fragments are in general much more specific for their target compared with small organic inhibitors which frequently fail to discriminate between the target enzyme or cell surface marker and related enzymes or homologous cell surface molecules.

Finally, we would like to point out the possible advantages of the dromedary VHHs for intracellular immunizations or intrabodies (Biocca and Cattaneo, 1995). The VHH form the minimal-sized intact antigen-binding site and their single-domain nature avoids the introduction of essential linkers used in scFv constructs which might lead to aggregation or susceptibility to proteolysis (Whitlow et al., 1993; Alibhan et al., 1995) followed by dissociation of the VH-VL domains. Moreover, it can be argued that the feasibility of intracellular immunization will in many instances be dependent on the enzyme-neutralizing capacity of the binders. We have proved here that, in this respect, the dromedary VHHs are also likely to be superior to conventional scFvs.

Materials and methods

Dromedary immunization

One adult male dromedary (Camelus dromedarius) was injected at days 0, 7, 14, 21, 28, 35, 42, 49 and 54 with 1 mg bovine erythrocyte carbonic anhydrase (EC 4.2.1.1; Sigma) and porcine pancreatic α-amylase (EC 3.2.1.1 Type IA; Sigma). Serum was collected prior to each injection to follow the immune response against the immunogens. At days 31 and 57, anticoagulated blood was collected for lymphocyte isolation. Peripheral blood lymphocytes were prepared with Unisep (WAK Chemie, Germany). Cells were counted and pelleted aliquots of 5 × 10⁶ cells stored at −80°C until further use.

Separation of the IgG subclasses

Serum was diluted 2-fold in phosphate-buffered saline (PBS) and IgG subclasses were obtained by successive affinity chromatography on 1 ml HiTrap Protein G and Protein A columns (Pharmacia). IgG3 and IgG1 were eluted from the Protein G column at pH 3.5 and 2.7, respectively. The flow-through was loaded on the Protein A column to recover two more fractions of heavy-chain antibodies. IgG2a and IgG2b were recovered at pH 4.5 and 3.5, respectively. The IgG protein concentrations were determined spectrophotometrically, assuming an ε₅₉₅ of 13.5 at 278 nm for all subclasses.

Capping enzyme-specific antibodies

The carbonic anhydrase and α-amylase, dissolved in 0.1 M NaHCO₃ pH 8.3, were coupled to CNBr-activated Sepharose (Pharmacia) at room temperature at a density of 3 mg protein/ml resin. Beads were washed several times with PBS before adding an equal volume of serum from days 0, 28 or 54 (200 μl). After washing five times with 800 μl PBS to remove unabsorbed proteins, the pelleted beads were resuspended in a solution containing essentially 1% SDS, boiled, and applied to a SDS-polyacrylamide gel to analyse the captured proteins.

Preparation of monomeric VHH by limited proteolytic digestion

IgG3 isolated from the serum of day 54 was dialysed against 0.1 M NH₄HCO₃, pH 8.0, before the Staphylococcus aureus V8 Endo-proteinase (Boehringer) was added at a 1/50 (w/w) ratio. After incubation for 2 h and dialysis against PBS, monomeric polyclonal VHH was recovered in the flow-through of the HiTrap Protein A column. The VHH concentration was determined spectrophotometrically as above.

Solid-phase binding ELISA

The carbonic anhydrase and α-amylase, at a concentration of 3 μg/ml in PBS were coated overnight at 4°C on Maxisorb 96-well plates (Nunc). Plates were blocked with 1% (w/v) casein in PBS for 2 h at room temperature. After incubation with either the diluted total serum or purified IgG subclasses, bound dromedary IgG was detected with a
rabbit anti-dromedary IgG antiserum. This antiserum recognized all dromedary IgG subclasses equally well, when coated on the polystyrene support. A goat anti-rabbit alkaline-phosphatase conjugate (Sigma) was used as secondary reagent.

Virion binding was revealed using the HRP-anti M13 conjugate (Pharmacia). Mouse anti-haemagglutinin decapetide tag (clone 16B12; BABCO, CA, USA) or anti-HIS tag (Dianova) were used as primary reagents in combination with an anti-mouse IgG-alkaline phosphatase conjugate (Sigma) for the detection of E.coli-produced VHH proteins. In the competition ELISA 1 μM dorzolamide (Trusopt®; MSD) or 1 μM acarbose (Miles) were present as carbonic anhydrase or α-amylase inhibitors.

**Biotinylation of porcine pancreatic α-amylase**

Crystalline porcine pancreatic α-amylase was biotinylated in the presence of 1 mM acarbose in 0.1 M NaHCO3 pH 8.0 with a 5-fold molar excess of biotin-X-sulfo-NHS (Calbiochem) for 2 h on ice. When tested with the chromogenic substrate, no loss of enzymatic activity was observed upon modification. The biotinylated enzyme was stored at 4°C until further use.

**VHH library construction and selection of binders**

The VHHS of 107 lymphocytes were cloned after RT–PCR amplification in the pHEN4 vector according to Ghahroudi et al. (1997). The VHHS were expressed on phage after infection with M13K07. The library was panned for the presence of binders respectively on solid-phase coated carbonic anhydrase (10 μg/well) in wells of microtitre plates or in solution with 100 nM biotinylated α-amylase in combination with streptavidin-coated magnetic beads (Dynal). Bound phage were eluted with 50 mM diethylamine.

**VHH expression and purification**

The VHH gene of the binders were sequenced and subcloned in a vector in which the haemagglutinin tag and the geneII between Nov and EcoRI sites were replaced by a fragment encoding six consecutive histidine codons followed by a termination codon. An overnight culture of WKK6 cells freshly transformed with the appropriate plasmid was used to inoculate 3 l of Terrific Broth medium containing 100 μg/ml ampicillin and 0.1% glucose. After growth at 37°C, until the optical density reached 0.75–1.0, expression was induced by the addition of 1 mM IPTG. Growth was continued for an additional 16 h at 28°C. After harvesting the cells by centrifugation, the periplasmatic fraction containing the VHHS was prepared (Skerra and Plückthun, 1988). The His tail-containing fusion protein was purified by chromatography on Ni-NTA (Qiagen) and Superdex-75 (Pharmacia). The protein concentration of the VHHS was determined spectrophotometrically using their calculated extinction coefficient and Mr (PC Gene, IntelliGenetics).

**Affinity measurements**

Kinetic analysis of the interactions were determined with IAsys Biosensor Instrument (Fisons). The individual VHHS fragments were immobilized in 10 mM sodium acetate, pH 5.0, on the carboxymethylated dextran layer using ECD/NHS chemistry. A coupling efficiency of 900–1200 in 10 mM sodium acetate, pH 5.0, on the carboxymethylated dextran fragment from camel heavy-chain antibodies. FEBS Lett. 238, 561–569.


Enzyme-inhibiting antibody fragments

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