

# Camelid nanobodies raised against an integral membrane enzyme, nitric oxide reductase

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**Abstract:** Nitric Oxide Reductase (NOR) is an integral membrane protein performing the reduction of NO to N<sub>2</sub>O. NOR is composed of two subunits: the large one (NorB) is a bundle of 12 transmembrane helices (TMH). It contains a *b* type heme and a binuclear iron site, which is believed to be the catalytic site, comprising a heme *b* and a non-hemic iron. The small subunit (NorC) harbors a cytochrome *c* and is attached to the membrane through a unique TMH. With the aim to perform structural and functional studies of NOR, we have immunized dromedaries with NOR and produced several antibody fragments of the heavy chain (VHHs, also known as nanobodies<sup>TM</sup>). These fragments have been used to develop a faster NOR purification procedure, to proceed to crystallization assays and to analyze the electron transfer of electron donors. BIAcore experiments have revealed that up to three VHHs can bind concomitantly to NOR with affinities in the nanomolar range. This is the first example of the use of VHHs with an integral membrane protein. Our results indicate that VHHs are able to recognize with high affinity distinct epitopes on this class of proteins, and can be used as versatile and valuable tool for purification, functional study and crystallization of integral membrane proteins.

**Keywords:** nitric oxide reductase; camelid antibodies; VHH domain; SPR; phage display

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*Abbreviations:* cAb, camel antibody; CDR, complementary determining regions; DDM, n-dodecyl-β-D-maltopyranoside; IPTG, isopropyl-(D-thiogalactopyranoside; NOR, Nitric Oxide Reductase consists in a complex of NorB and NorC; PBS, phosphate saline buffer; *Ps. nautica*, *Pseudomonas nautica*; SPR, Surface Plasmon Resonance; vH, variable heavy chain domain of antibody; VHH, variable heavy chain domain of camel heavy chain antibody.

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## Introduction

In the past 12 years, camelid VHH domains (or nanobodies)<sup>1,2</sup> have been widely used for basic research studies, as potential reagents<sup>3–7</sup> or drugs.<sup>5,8–11</sup> A wealth of crystal structures against protein antigens<sup>4,12–22</sup> or haptens<sup>23,24</sup> has revealed the amazing versatility of their binding modes. They can recognize enzyme clefts (lysozyme, amylase), use a unique CDR for providing interactions, interact with haptens laterally, or use part of their framework for proteins binding (amylase, etc). To date, however, all the complexes reported were from camelid Igs obtained through llama or dromedary immunization with soluble proteins.

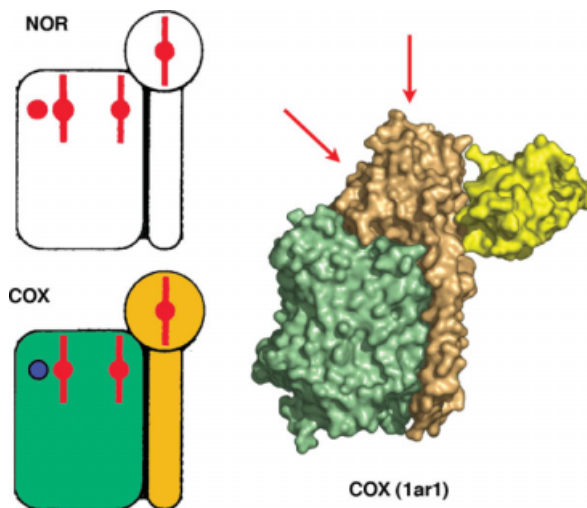
During our study of the enzymes from the denitrification cascade, catalyzing the reduction of  $\text{NO}_3^-$  to  $\text{N}_2$ ,<sup>25</sup> we have undertaken experiments aiming at a better understanding of Nitric Oxide Reductase (NOR), the last of the four enzymes whose structure still remains unknown.<sup>26–31</sup> NOR is composed by two subunits: NorB, a 12 transmembrane helix *b*-type cytochrome harboring two heme groups and a non-heme Fe center, and NorC, a membrane bound mono-hemic *c*-type cytochrome<sup>25</sup> (see Fig. 1). It catalyses the reduction of NO to  $\text{N}_2\text{O}$ :  $2\text{NO} + 2e^- + 2\text{H}^+ \rightarrow \text{N}_2\text{O} + \text{H}_2\text{O}$ . NOR resembles strikingly bacterial cytochrome *c* oxidase (COX), which possess a Cu ion instead of the Fe center<sup>32</sup> in the catalytic site (see Fig. 1). NOR and COX sequences are similar: *Ps. nautica* NOR (the enzyme studied here) large subunit shares 56% sequence identity (72% similarity) with the large subunit of *Paracoccus denitrificans* COX, of known structure.<sup>32</sup> On the basis of this similarity, it has been proposed that they might be evolutionary related.<sup>33</sup>

With in mind to study *Ps. nautica* NOR structure and function, we have immunized a dromedary with the purified protein. We have obtained six VHHs against this enzyme with four of them displaying  $K_d$  values in the nM range. Using Surface Plasmon Resonance (SPR) and electron transfer assays, we could determine that several epitopes have been targeted. These nanobodies are presently used as tools towards *Ps. nautica* NOR crystallization. Finally, we report here, for the first time, that the complete and efficient procedure for immunization, VHHs selection and purification documented on soluble proteins can be adapted readily to integral membrane proteins.

## Results

### NOR purification

The NOR two-subunits complex has been successfully extracted and purified from DDM solubilized membranes of *Ps. nautica* cells (Timóteo CG, et al. in preparation). Calculated from the absorption measurement at 410 nm, this procedure yielded ~10 mg of purified protein per 200 L of *Ps. nautica* fermentor culture. Protein content, purity and homogeneity were estimated by SDS-PAGE and MALDI-TOF mass spectrom-



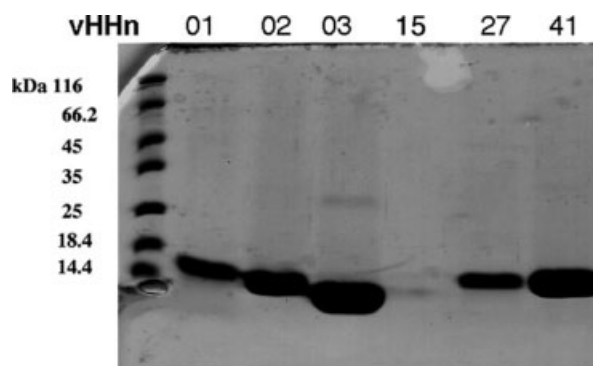
**Figure 1.** Schematic representation of nitric oxide reductase (NOR, upper left) and cytochrome *c* oxidase (COX, lower left). The heme groups and iron ions are colored red and the copper ion blue. Right side: compact model of *Paracoccus denitrificans* COX (1AR1).<sup>32</sup> The large subunit is green, the cytochrome *c* small subunit is orange, and the VH domain from the bound Fv is yellow. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

etry. NorB, which is an integral membrane protein with twelve predicted transmembrane helices, appears on gel as a band with an apparent mass of 38 kDa, smaller than the expected MW of 54.4 kDa, and it is not detectable by mass spectrometry, as often noticed with integral membrane proteins. NorC appears on gel as a band of the expected MW (17.6 kDa) and in mass spectrometry as a sharp symmetrical peak at the expected MW, indicating the absence of proteolysis (see Fig. 2). Purified protein in 100 mM K phosphate buffer at pH 7.0 with 0.02% DDM and 0.01% phenyl ethanol could be concentrated up to 30 mg/mL with no evidence of aggregation. The electron transfer activity of purified NOR has been tested both with cytochrome  $c_{552}$  and ascorbate as electron donors (see later).

### VHHs selection and production

Dromedary immunization was performed with purified NOR. Lymphocytes were isolated from blood samples, and a phage display library of the VHHs was generated using standard procedures (see Methods). Recombinant antibody fragments were selected from this phage display library by panning against NOR. Six VHHs binders (01, 02, 03, 15, 27, and 41) were obtained and sequenced (see Fig. 3). Binder sequences were aligned with the human POT vH<sup>35</sup> and the dromedary VHHly3, an inhibitor of hen egg-white lysozyme.<sup>12</sup> Several features confirm the heavy-chain antibody origin of the antigen-specific binders<sup>2</sup>: (1) they show substitutions Leu11Ser, Val37Phe/Tyr, Gly44Glu,





**Figure 4.** SDS-PAGE of purified VHHn01, 02, 03, 15, 27 and 41 of respectively 15.3, 14.3, 13.5, 14.5, 15.0, and 14.7 kDa (Lane1: MM marker).

detergent is  $\sim 100$  kDa (see Fig. 5), indicating that  $\sim 196$  molecules of DDM (510.6 Da) maintain NOR in solution. NOR-VHH complexes purified by either method showed identical kinetic behavior (see enzymatic activity).

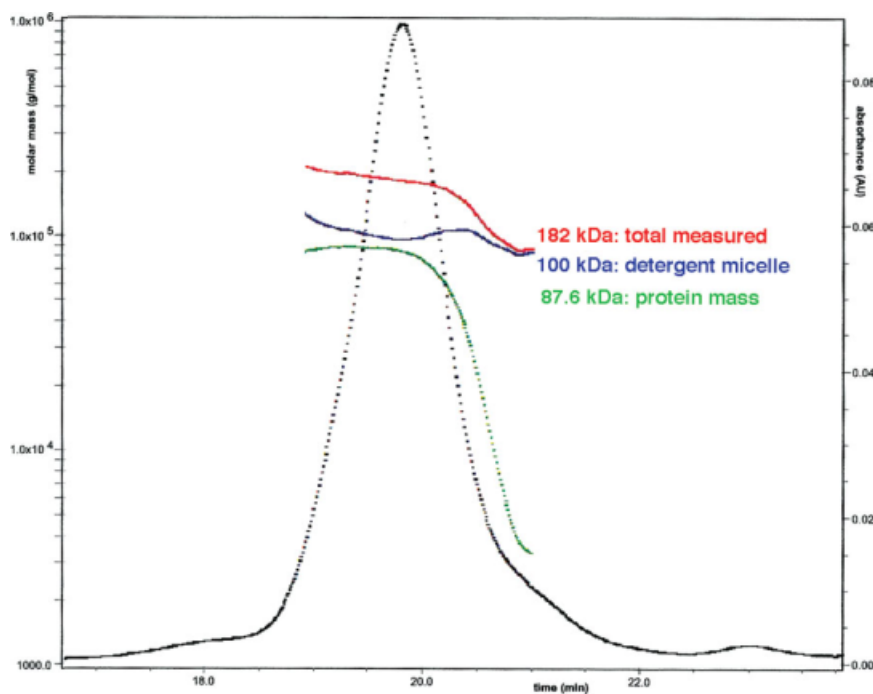
#### Affinity and epitope mapping of VHHs

We have determined the dissociation constants of the VHH binders for NOR by Surface Plasmon Resonance (SPR, BIAcore) experiments. Different VHHs were covalently linked to a BIAcore CM5 chip by amine coupling. We chose to attach the VHHs to the chip as they are more stable than NOR, making, thus, easy the regeneration procedure with ethylenglycol and/or SDS. Moreover,

the larger molecular mass of NOR makes it a more favorable candidate to be used as analyte. In a first step, the equilibrium dissociation constants of five individual VHH binders (01, 02, 03, 27, and 41) for NOR were determined and found to be in the nanomolar range (1–50 nM) (Table I). In a second step, epitope mapping of the five VHHs versus VHHn03 was performed, using as analyte the preformed binary complex of NOR with VHHn03. The binary complex was able to bind only to VHHn41 and VHHn27, with  $K_d$  values close to those determined for NOR alone (7 and 5.8 nM for VHHn41 and 11 and 45 nM for VHHn27). These results clearly show that VHHn03, VHHn41, and VHHn27 interact with NOR at independent sites. As a positive control, the preformed ternary complex NOR-VHHn03-VHHn41 was passed as analyte over VHHn27 covalently bound. The quaternary complex is formed; the affinity of VHHn27 for the ternary complex is two times higher than that for the binary complex (5.9 and 11 nM) and one order of magnitude higher than that for NOR alone (5.9 and 45 nM). On the basis of the results of these binding experiments, we decided to use the binary, ternary, and quaternary complexes in further crystallization trials.

#### Crystallization and X-ray diffraction

Conditions giving rise to diffracting crystals were not found with NOR alone, at least in our hands. On the basis of the experience of other laboratories and confident that complex formation with antibody fragments



**Figure 5.** HPLC-Wyatt analysis of NOR-VHHn03 complex. The *abscissa* indicates the time scale of the HPLC injection, the left *ordinate* indicates the molar mass in g/mol (Da) and the right *ordinate* the UV absorbance at 280 nm. Absorption peak in black and the curves indicating the molar mass for the complex in detergent, the protein alone and the detergent are in red, green, and blue, respectively. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

**Table I.** Kinetic Rate Constants and Equilibrium Dissociation Constant of NOR Antibody Fragments as Determined by BIAcore. Purified NOR or NOR in Complex with VHHn Were Injected over the Sensor Chip Coated with the Corresponding VHHn Fragments

	$k_{on} 10^5 M^{-1}s^{-1}$	$k_{off} 10^{-4}s^{-1}$	$K_{diss} 10^{-9} M$
NOR+			
VHHn01	0.7 ± 0.2	6.2 ± 1.2	9.2 ± 1.1
VHHn02	1.4 ± 0.3	6.7 ± 1.6	5.2 ± 1.7
VHHn03	1.5 ± 0.3	1.8 ± 0.2	1.3 ± 0.25
VHHn41	1.1 ± 0.35	5.6 ± 2.0	5.8 ± 1.6
VHHn27	0.3 ± 0.1	15 ± 6	45 ± 5
NOR-VHHn03+			
VHHn01	nc	nc	nc
VHHn02	nc	nc	nc
VHHn03	nc	nc	nc
VHHn41	1.2 ± 0.4	8.3 ± 2.0	7.0 ± 1.5
VHHn27	2.2 ± 0.1	22 ± 1.5	11 ± 1.4
NOR-VHHn03-VHHn41+			
VHHn27	7 ± 2	39 ± 0.5	5.9 ± 1.3

nc: not calculable.

may facilitate the crystallization of membrane proteins,<sup>32,36–38</sup> we systematically used NOR-VHH binary, ternary, and quaternary complexes for crystallization trials. For each complex, excepted for the ternary NOR-VHHn03 + 41, we have obtained crystals, in general after 2 weeks. Average resolution of the crystals was between 12 and 6 Å. A preliminary data set was collected at 7 Å resolution with NOR-VHHn02 crystals at the Swiss Light Source Synchrotron Radiation Facility at the Paul Scherrer Institut (SLS, Switzerland) beam line X10SA (PXII). Crystals belong to space group P2<sub>1</sub> with cell dimensions  $a = 85.0$  Å,  $b = 135.3$  Å,  $c = 137.2$  Å, and  $\beta = 95.6^\circ$ . The  $V_m$  values and water percentages calculated for the asymmetric unit are of 4.6 Å<sup>2</sup>/Da = 73%, 3.1 Å<sup>2</sup>/Da = 60%, and 2.3 Å<sup>2</sup>/Da = 47%, for 2, 3, and 4 molecules, respectively.

### Enzymatic activity

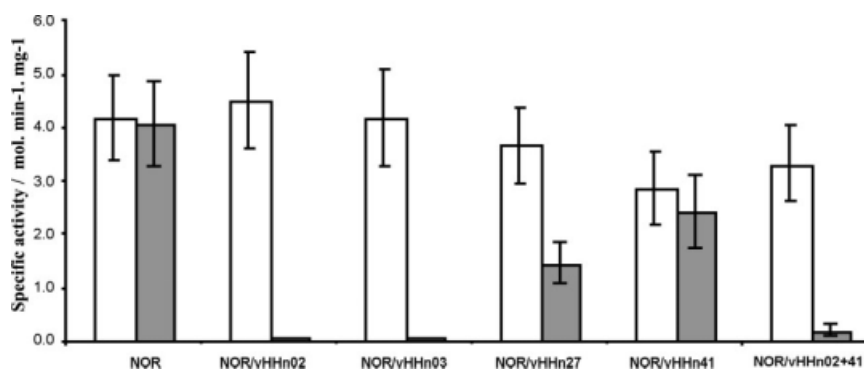
Reduced *Ps. nautica* cytochrome  $c_{552}$  or ascorbate/PMS are efficient electron donors for NOR (Timóteo CG, et al., in preparation), and were used to evaluate the activity of NOR in complex with VHHs in compari-

son to the activity of NOR alone. All complexes are active with ascorbate/PMS, although at different level (see Fig. 6); NOR complexed with VHHn02 and VHHn03 is inactive with reduced cytochrome  $c_{552}$  but active with ascorbate/PMS. The binding of VHHn27 partially affects the kinetic behavior of NOR with  $c_{552}$  as electron donor, while VHHn41 scarcely modifies activity in the presence of either electron donor system.

### Discussion

We report for the first time camelid immunization and VHHs selection against an integral membrane protein. The VHHs obtained by this means can be used, as Swiss knives, in a variety of situations: difficult protein purification, functional studies, epitope mapping, crystallization.

Using VHHs, we could purify NOR readily in two chromatography steps and reduce the purification time to two days. A similar approach was used for the first time to purify COX with antibody fragments.<sup>39</sup> In our previous protocol, three chromatography steps were necessary and the overall process lasted for 5 days. As membrane proteins are often fragile and detergents expensive, a shorter and faster purification may be crucial for obtaining a stable preparation. The idea of using VHHs for protein purification was also proposed recently in the case of soluble proteins.<sup>7</sup> As often observed with the VHHs domains, five of the six antigen-specific binders selected by panning could be expressed in *E. coli* readily and in quantities sufficient for BIAcore binding experiments, crystallization and enzymatic assays. Four of these VHHs have excellent  $K_d$  values, ranging from 1.3 to 9.2 nM, while a fifth has a  $K_d$  value of 45 nM. The affinity values class these VHHs among the best raised against soluble proteins.<sup>4,12–22</sup> The best binder, VHHn03 forms a strong complex with NOR (1.3 nM). When the complex NOR-VHHn03 was passed over the four other VHHs attached to the BIAcore chip, two of them (VHHn01 and 02) did not interact with the complex, indicating that they share the same epitope as VHHn03. In contrast, the two remaining VHHs (VHHn027 and 41) interact with the complex, demonstrating that these



**Figure 6.** Specific activity plot of different NOR-VHHn complexes using two electron donating systems (white—ascorbate/PMS; grey—reduced *Ps. nautica* cytochrome  $c_{552}$ ).

three binders have distinct epitopes. It should be noticed that VHHn41  $K_d$  value does not change significantly when binding to NOR alone or to NOR-VHHn03 complex, indicating that VHHn41 should bind to an epitope quite distinct from that of VHHn03. In contrast, when VHHn27 binds to the NOR-VHHn03 complex, the  $K_d$  value decreases four-fold as compared to the binding to NOR alone. We propose, therefore, that VHHn27 binds to an epitope distinct but close enough to that of VHHn03, and that the association of VHHn03 to NOR may induce a conformational change favorable to the further binding of VHHn27. The ternary complex NOR-VHHn03-VHHn41 is still able to bind to the VHHn27, demonstrating that VHHn41 and VHHn27 have also distinct epitopes. The  $K_d$  of VHHn27 with NOR-VHHn03-VHHn41 is slightly lower (1.7-fold) than with the binary complex, suggesting some limited effect (conformational or steric) of VHHn41 on the subsequent binding by VHHn27.

It is interesting to know the location of the four VHH binders epitopes on NOR. To this end, we measured the electron transfer activity of NOR with ascorbate/PMS or cytochrome  $c_{552}$  as electron donors in presence of four VHHs. When the bulky dimeric cytochrome  $c_{552}$ <sup>40</sup> is used as electron donor, electron transfer is abolished by VHHn02 and VHHn03. This means that the epitope common to both VHHs coincides with the reduced cytochrome  $c_{552}$  binding site on NorC subunit, and that these VHHs prevent cytochrome  $c_{552}$  to transfer electrons to NOR. In contrast, when the small molecules of ascorbate and PMS are used in the experiments, electron transfer is not altered by any of the VHHs, meaning that they can sneak into the cytochrome domain close to the heme iron. Alternatively, ascorbate and PMS may bypass the cytochrome  $c_{552}$  electron delivery pathway and deliver electrons to the active site through a different mechanism that is not blocked by the VHHs. Torndyck et al.<sup>41</sup> also consider this possibility based on the information that ascorbate/PMS can also effectively deliver electrons to other classes of NORs which lack the *c* subunit.<sup>42</sup>

In contrast to VHHn02 and VHHn03, VHHn27 alters partially (by ~60%) the electron transfer, which suggests that they interact partially with reduced cytochrome  $c_{552}$ . BIAcore experiments have confirmed that VHHn27 binding site is different to that common to VHHn02 and VHHn03, the presence of which abolish completely cytochrome  $c_{552}$  binding. Therefore VHHn27 should not interact at all with the VHHn02 or VHHn03, but should interact partially with the large dimeric cytochrome  $c_{552}$  that binds at the same place as VHHn02 or VHHn03.

In conclusion, neither of the small VHHn02 nor VHHn03 molecules interfere with the VHHn27 binding site, while the large dimeric cytochrome  $c_{552}$  does. The binding of VHHn41 does not alter significantly the

electron transfer by reduced cytochrome  $c_{552}$ . Considering the respective sizes of VHHs and cytochrome  $c_{552}$  and that of the NorC subunit (as estimated from COX in Fig. 1), it is very likely that two or even three VHHs, such as VHHn02, VHHn27, and VHHn41, that we found able to co-crystallize with NOR, can bind simultaneously on NorC subunit (Fig. 1, right). The VHHn41, however, might also bind to a completely different epitope, on the cytochrome *c* small subunit side, or even internally.

It is well-established that binders favor protein crystallization, particularly that of integral membrane proteins.<sup>32,38</sup> In this later case, they may help by providing nondetergent covered protein surfaces, favorable to tight packing, or by stabilizing the flexible membrane protein in a given conformation.<sup>38</sup> This has been illustrated with COX, a topological relative of NOR,<sup>32</sup> with the  $\beta_2$ -adrenergic receptor<sup>38</sup> and in several other cases. Here we have shown that all our complexes of VHHs with NOR crystallize readily, while NOR alone does not, despite several years of intensive efforts. However, most crystals diffract to 6 Å at best. We expect that further optimizations will make it possible to get crystals diffracting at higher resolution.

## Material and Methods

### Materials

Detergents (Anatrace), S-sec-phenyl-ethyl-alcohol (Sigma), DEAE-Biogel A gel ion exchange (Bio-rad), Macroprep Ceramic hydroxyapatite type I and Bio-Scale CHT20-I column (Ceramic hydroxyapatite type I) (BioRad), Ni-NTA column, 6xHis ladder and penta-His-HRP conjugate (Qiagen), ECL chemiluminescent detection system, Superdex 75 column, His-trap crude FF column (GE Healthcare), PVDF membranes (Roche), bicinchoninic acid (BCA) protein assay kit (Pierce), Amicon-Ultra cutoff 10–50 kDa concentrators (Millipore).

### *Ps. nautica* NOR (NORn) production

*Ps. nautica* cells were grown as described by Prudêncio et al.<sup>43</sup> *Ps. nautica* medium culture contained: 11.7 g/L NaCl, 12.3 g/L  $MgSO_4 \cdot 7H_2O$ , 0.75 g/L KCl, 6.05 g/L Tris, 3 g/L  $NH_4Cl$ , 2 mL sodium lactate 60%, 1 g/L Yeast-Extract, 1 g/L  $KNO_3$ , 2 mg/L  $FeSO_4 \cdot 7H_2O$ , 74.4 mg/L  $K_2HPO_4$ , 1.47 g/L  $CaCl_2 \cdot 2H_2O$ , 1 ml/L Starkey oligo-elements stock solution pH7.5. 1000 $\times$  oligo-elements stock solution: 263 mM MgO, 20 mM  $CaCO_3$ , 22.3 mM  $FeSO_4 \cdot 7H_2O$ , 5 mM  $ZnSO_4 \cdot 7H_2O$ , 3.6 mM  $MnSO_4 \cdot H_2O$ , 0.1 mM  $CuSO_4 \cdot 5H_2O$ , 3.2 mM  $CoSO_4 \cdot 7H_2O$ , 0.97 mM  $BO_3H_3$ , 0.8 mM  $Mo_7(NH_4)_6(O_2)_4H_2O$ , 0.14 mM  $Ni(NO_3)_2 \cdot 6H_2O$ , 0.12 mM  $Na_2SeO_3$ . A volume of 200 ml of medium, inoculated with a 20 mL *Ps. nautica* overnight culture, were first grown in bottle under aerobic conditions at 30°C. The 200 ml culture was used to inoculate 2 L of medium

and let grown 24 hours under anaerobic conditions to reach an OD<sub>600</sub> of 0.8. This 2 L culture was used to inoculate 18 L of medium and let grown 5 hours under aerobic conditions (air supply of 0.2 volume per volume per minute) and 19 hours under anaerobic conditions to reach an OD<sub>600</sub> of 0.8. Volume of 200 L of medium in a 300 L working volume fermentor (CHEMAP) was finally inoculated with this 18 L culture and grown again at 30°C 5 hours under aerobic conditions (air supply of 0.2 v/v per minute) and 19 hours under anaerobic conditions. Cells were harvested by centrifugation at 14000g at 4°C under N<sub>2</sub> atmosphere (100 L/h). Resulted ~250 g wet pellet was resuspended in ~1 L 10 mM Tris pH 7.2, disintegrated (1.8 Kbars) and stored aliquoted at -20°C.

### ***Ps. nautica* NOR purification**

NOR was purified as described by Timóteo C. G. et al. (manuscript in preparation): 250 mL of defrost *Ps nautica* fermentor pellet (corresponding to 50 L of culture) were diluted two times in 50 mM Tris pH8.0 at 4°C and ultracentrifuged 1H30 at 40,000 rpm, 4°C. Pellets were resuspended in ~500 mL 50 mM Tris pH 8.0, 100 mM KCl with a Potter-S homogenizer (Braun). Protein concentration was quantified following the BCA method using bovine serum albumin as a standard and brought to a concentration of 10 mg/mL. Resuspended pellets were sonicated, washed two times in 50 mM Tris pH 8.0 by ultracentrifugation 1H30 at 40,000 rpm, 4°C, aliquoted in 3 × 250 mL at ~10 mg/mL and stored at -80°C. Solubilization of membranes from 250 mL defrost sample was done by addition of 0.6% (w/v) *n*-dodecyl-β-D-maltopyranoside (DDM) and 0.01% (v/v) *S*-sec-phenyl-ethyl alcohol (phenyl ethanol) 15 min at 4°C under agitation and membrane proteins were recovered in supernatant after ultracentrifugation 1H30 at 40,000 rpm, 4°C. Sample was applied on DEAE column equilibrated in 50 mM Tris pH 8.0, 0.02% DDM, 0.01% phenyl ethanol. After washing the column, elution of NOR protein by a linear gradient from 0 to 500 mM NaCl was followed spectrophotometrically at 410 and 278 nm absorbance. Eluate was buffer exchanged and concentrated in 10 mM K phosphate pH 7.0, 400 mM NaCl, 0.02% DDM, 0.01% phenyl ethanol (buffer A), aliquoted in 3 × 20 mL and stored at -80°C. A 20 mL defrost sample was brought to 0.1% DDM and centrifuged 15 min at 7000g before being applied on a Macrorep hydroxyapatite column equilibrated in buffer A. A gradient from 0 to 100% of buffer B (1.5M K phosphate pH 7.0, 0.02% DDM, 0.01% phenyl ethanol) was applied to the column and eluted fractions were pooled, buffer exchanged and concentrated in buffer A. Pool was brought again to 0.1% DDM and centrifuged 15 min at 7000g before being applied on a Bio-Scale hydroxyapatite column equilibrated in buffer A. A gradient from 0 to 100% of buffer B was applied to the column. Eluted fractions were pooled, buffer

exchanged and concentrated in 100 mM K phosphate pH 7.0, 0.02% DDM, 0.01% phenyl ethanol. Purified NOR concentration was determined spectrophotometrically from absorbance at 410 nm.

### ***Dromedary immunization, VHH library construction, and binders selection***

A dromedary was injected subcutaneously on days 0, 7, 14, 21, 28, 35 with about 500 µg (per injection) of purified NOR in Gerbu adjuvant. On day 39, anticoagulated blood was collected from the vein for the preparation of lymphocytes.

The lymphocytes from the 'immune' blood were purified on Lymphoprep. The total RNA from the peripheral blood lymphocytes was extracted and used as template for first strand cDNA synthesis with oligo (dT) primer. Using this cDNA, the VHH encoding sequences were amplified by PCR according to Saerens et al.,<sup>44</sup> digested with PstI and NotI, and cloned into the PstI and NotI sites of the phage-display phagemid vector pHEN4.<sup>45</sup> A VHH library of about 4 × 10<sup>7</sup> independent transformants was obtained. About 82% of these transformants harbored the vector with an insert corresponding to the expected VHH gene size. Three consecutive rounds of phage display and panning (10<sup>11</sup> phages per well of microtiter plate) were performed on solid-phase coated NOR (100 µg/mL, 10 µg/well). The enrichment for antigen-specific phages within the pools at each round of panning was assessed by polyclonal phage ELISA. A clear enrichment was seen after the second and third round of panning. Totally 96 individual colonies (48 from 2nd round and 48 from third round) were randomly selected and analyzed by ELISA for the presence of antigen specific VHHs in their periplasmic extracts. The VHH sequences from the ELISA-positive colonies were subject to RFLP analysis using HinfI restriction enzyme and nucleotide sequencing. Seven different nanobodies were identified.

### ***VHHs expression and purification***

VHH genes of the binders were sequenced and subcloned in pHEN6 vector in fusion with pelB sequence on N-ter and six histidine tag on C-ter.<sup>46</sup> *Escherichia coli* WK6 cells transformed with the appropriate vector were grown at 37°C in Terrific Broth medium containing 100 µg/mL ampicillin and 0.1% glucose until optical density reached 0.8. Expression was induced by the addition of 1 mM IPTG and growth was continued for an additional 16 hours at 28°C. Periplasmic fraction containing the VHH fragments was prepared.<sup>47</sup> The His-tail-containing fusion protein was purified by immobilized metal affinity chromatography on a 5 mL Ni-NTA column in 50 mM Na/K phosphate pH 8.0, 300 mM NaCl, 10% glycerol. Eluted fractions in 250 mM imidazole were concentrated on a Amicon-Ultra 10 kDa cutoff concentrator prior to be loaded on a HiLoad 10/30 Superdex75 PG gel filtration column in

PBS. Protein concentration of the VHHs was determined by UV spectrometry from absorbance at 278 nm, using their calculated extinction coefficient.

### **NOR-VHHs complexes purification**

NOR-VHH complexes were prepared following two different procedures.

- 1 Purified NOR in 100 mM KPB (potassium phosphate buffer) pH 7.0, 0.02% DDM, 0.01% phenyl ethanol (PE) was incubated with pure VHH protein in PBS pH 7.2, for 1 h on ice, in a 1:1.2 molar ratio. NOR-VHH complex was purified and separated from free antibody excess by gel filtration chromatography on a Superdex75 column in 100 mM KPB pH 7.0, 0.005% (w/v) DDM, 0.01% (v/v) PE.
- 2 Solubilized protein from membrane fraction (see NOR purification (Timóteo CG, et al. in preparation)) was dialyzed against 100 mM KPB pH 7.0, 0.02% DDM, 0.01% PE and incubated with pure VHH (in the same buffer), in 1:1.2 ratio for 1 h. The mixture was applied onto a His-trap FF crude column (GE Healthcare) equilibrated in the same buffer. The column was washed with 60 mM imidazole and eluted with 250 mM imidazole in the same buffer. Eluted fractions were pooled and concentrated. When necessary the complex was mixed with one or two additional distinct VHH. NOR-VHH complex was separated from free antibody excess by gel filtration chromatography as described in 1. NOR-VHH concentration was determined using NOR extinction coefficients ( $\epsilon_{411 \text{ nm}} = 295 \text{ mM}^{-1} \text{ cm}^{-1}$ ) (Timóteo CG, et al. in preparation) and purity was evaluated by SDS-PAGE.

### **NOR-VHHs complexes characterization**

**Western blot.** Proteins were transferred from a 15% SDS-PAGE gel to a Hybond PVDF membrane and blocked with 5% nonfat dried milk in 20 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Tween 20 at 4°C. The membrane was then treated with anti His-HRP primary antibodies. Visualization of VHH-specific bands was done by ECL chemiluminescence.

**Mass spectrometry.** Mass spectrometry was performed on a matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometer (Bruker Autoflex, Bruker Daltonics, Bremen, Germany) according to standard procedures, slightly modified for membrane proteins.

**MALS/UV/RI (Wyatt).** Size exclusion chromatography was performed on an Alliance 2695 HPLC system (Waters) using a Silica Gel KW804 column (Shodex) eluted with 50 mM Tris-HCl pH 8, 300 mM NaCl, 0.05% DDM at a flow rate of 0.5 mL/min.

30  $\mu\text{L}$  sample between 0.5 and 1 mg/mL was routinely applied on the column. Detection was performed using a triple-angle light scattering detector (Mini-DAWN<sup>TM</sup> TREOS, Wyatt technology, Santa Barbara, USA). Molecular weight determination was performed by ASTRA V software (Wyatt technology).

### **SPR affinity measurements**

Kinetic analysis of NOR-VHHs interactions were performed on BIAcore 1000 using CM5 (carboxymethylated dextran) sensor chip coated with 500–900 resonance units of individual purified VHH fragments immobilized by amine coupling. VHHs non-specific for NOR were used as control. For the kinetic constants determination, diluted solutions of NOR or NOR in complex with VHHs (0.3–40 nM in 10 mM Hepes pH 7.2, 150 mM NaCl, 2 mM EDTA, 0.005% surfactant P20) were passed over the flow-cell with the specific VHHs and on the control flow-cell. Binding traces were recorded for at least five concentrations of analyte, in triplicate. Each binding-regeneration cycle was performed at room temperature with a constant flow rate of 40  $\mu\text{L}/\text{min}$ . In each cycle, buffer (50  $\mu\text{L}$ ) was injected first to stabilize the baseline, the analyte (80  $\mu\text{L}$ ) was then injected and the spontaneous dissociation was followed for 6 min; regeneration of the binding surface was achieved by injection of 5  $\mu\text{L}$  of ethylenglycol and/or 0.5% SDS and further washing with the running buffer for 6 min. Curves obtained after subtraction of the reference and the buffer signals were fitted to a (1:1) Langmuir binding model and a (1:1) binding with mass transfer with BIAevaluation (BIAcore). Better results, in terms of  $\chi^2$  and standard error of the calculated values, were obtained with the first model.

### **Crystallization and X-ray diffraction**

Initial crystallization screening of NOR-VHHs complexes were performed with commercial kits MemStart, MemSys, MemGold I and II, MemPlus (Molecular Dimensions Ltd), MB I and II (QIAGE). The nanodrops experiments were carried out with a Cartesian HoneyBee X8 robot, using sitting-drops in Greiner plates (100–200–300) nL of protein mixed with 100 nL of well solution). In the first round of crystallization trials, the concentration of binary (NOR-VHHn02, NOR-VHHn03, NOR-VHHn27 and NOR-VHHn41), ternary (NOR-VHHn03 + 27 and NOR-VHHn03 + 41) and quaternary (NOR-VHHn03 + 27 + 41) complex varied between 3 and 12 mg/mL in the purification buffer 100 mM potassium phosphate pH 7.0, 0.01% phenyl ethanol, 0.005% DDM.

### **Kinetic activity assays**

NO reductase activity measurements were performed as described by Timóteo CG, et al. (manuscript in preparation). Purified NOR was incubated for 30 min on ice with each or mixtures of VHHs in equimolar



ratio (1:1). After incubation, the complexes were assayed for NO reductase activity with ascorbate/PMS or reduced *Ps. nautica* cytochrome  $c_{552}$  as electron donors.

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