Final Report

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1.- Introduction

The project "Enzymatic functionalization of an artificial α/β barrel protein" is a very ambitious project with the final goal of creating the first 100% artificial enzyme. The project is divided in several steps and is fronted with two different designs named Octarellin V and Octarellin VI (Figure 1).

The first protein was designed in collaboration with S. Mayo (Caltech, USA) using the Orbit Software. The Octarellin V was produced, and characterized in a previous work published in 2003 (Offredi *et al*, 2003) by our laboratory. However this protein was not soluble in bacteria and very unstable, two features that make it impossible to obtain a large amount of the protein for structural determination by X-Ray or NMR techniques. To solve this, a directed evolution process over this protein was performed in the past, producing a soluble variant named Octarellin V.1, but this evolved variant was not characterized.

The second protein, Octarellin VI, was designed later in collaboration with J, Meiler and D. Baker (University of Washington, USA) using the Rosetta Software. The protein was expressed in bacteria and few characterizations were done. Also this protein showed insolubility and instability.

To achieve the final objective of generating an artificial enzyme, one the most important steps is to verify if our protocol to design proteins *in silico* works perfectly. Verification must be interpreted as the structure designed in the computer being the same as the protein expressed and purified from the bacteria. This is not a trivial step, because we need large amounts of protein to solve its structure, and finally, if the real experimental structure does not match the *in silico* design, it is necessary to re-create the design in an iterative process. Under this scenario, the main work for this project is to solve the inconvenience of insolubility and instability of Octarellin V and Octarellin VI which then would allow the determination of the real structure of these proteins.

In this report, we will indicate the strategies, results, and conclusions obtained after 18 months working on this project focused in solving the experimental structure of one Octarellin.

2.- Objectives

We have determined very specific objectives that are to be achieved:

1) Make a complete biophysical characterization of Octarellin V.1
2) Make a complete biophysical characterization of Octarellin VI
3) Improve Octarellin VI by directed evolution
4) Determine the best strategy to get the experimental structure of Octarellin V.1

3.- Results

The postdoctoral research was focused in the characterization of the proteins Octarellin V.1 and Octarellin VI. The Octarellin V.1 is the evolved version of the Octarellin V. This evolved version was created by Directed Evolution using the Error Prone PCR technique. The Octarellin VI was designed *in silico* using the software Rosetta and the design and its characterization have been published in 2013 in the PloS One journal (Figueroa *et al*, 2013). The detailed results are shown.

3.1 Characterization of the artificial protein Octarellin VI

The artificial protein Octarellin VI was expressed in bacteria and purified from the inclusion bodies (Figueroa *et al*, 2013). The renatured protein was used for biophysical characterization using Circular Dichroism spectroscopy (CD), Fluorescence spectroscopy and Nuclear Magnetic Resonance spectroscopy (NMR).

The Far-UV CD spectrum of Octarellin VI (Figure 2) shows a secondary structure content in accordance with the *in silico* design (Table 1). The Near-UV CD spectrum (Figure 2) shows positive bands, but with a weak signal at 280 nm, indicating a poor tertiary structure.

To test the tertiary structure through biophysical techniques, we used CD but also Fluorescence and NMR. The chemical denaturation of the protein followed by CD (ellipticity at 222 nm) and Fluorescene (tryptophan emission) shows a non-cooperative unfolding (Figure 3). This information plus the weak signal at 280 nm in the Near-UV CD spectrum suggests a non well structured protein, probably at a molten globule state. To verify this we decided to perform a NMR 2D spectrum, where it will be easiest to see whether the protein is well folded or not.

The NMR 2D spectrum showed a non-structured protein, with absence of tertiary structure (data not shown). This indicates that we have a protein with a secondary structure but not a tertiary structure.

3.2 Directed Evolution over Octarellin VI

Due to the poor solubility and lack of tertiary structure, we decided to perform a Directed Evolution process following the increment of solubility inside the bacteria. We used the Error Prone PCR technique and the Green Fluorescent Protein (GFP) as a solubility reporter (Waldo, 2003), creating a fusion protein between the Octarellin VI mutated variants and GFP. The principle of the technique is to add random mutations into Octarellin VI and then to select the soluble variants through the fluorescence observed in the bacterias; this fluorescence is in agreement with the idea that if the fusion protein is soluble, the GFP will be well folded and it will produce fluorescence. However, if it is not well folded, the fusion protein will be directed to inclusion bodies, where the GFP will be not fluorescent because lack of tertiary structure. We selected the most fluorescent variants and then we sequenced them to analyze the mutations. These new variants were used for a new cycle of Error Prone PCR. In total 8 cycles were performed and a phylogenetic tree of this process can be observed in the Figure 4.

The analysis of the mutations revealed a 92% of identity and 95% of similarity. Most of the mutations (8 of 17 mutations) appeared in the 3rd quarter of the primary structure (Figure 5) when we compare the “best variant”, due to its fluorescence level, with the sequence of the original Octarellin VI protein. At this moment, we are working in the standardization of a protocol to produce and purify the best variants coming from the 8 cycle of Error Prone PCR in order to then

characterize these proteins by biophysical techniques and subsequently, try to determine their real structure by X-Ray diffraction.

3.3 Biophysical characterization of the artificial protein Octarellin V.1

The artificial protein Octarellin V.1 was produced through a Directed Evolution process performed over the protein Octarellin V. The Octarellin V protein was previously designed and characterized (Offredi *et al*, 2003), but due to its insolubility in bacteria, not allowing production at large scale to determine its tertiary structure, it was necessary to perform the Directed Evolution process. This procedure was done before the arrival of Dr Figueroa.

The Octarellin V.1 was produced after 8 cycles or Error Prone PCR. It has 93% of identity with the parental protein Octarellin V, but it is produced almost exclusively in the soluble fraction of the bacteria (Figure 6).

In order to characterize biophysically this protein, we performed first a Far-UV CD spectrum, and in basis of its deconvolution an estimation was made of the secondary structure content. The Far-UV CD spectrum is shown in the Figure 7 and the secondary structure content was estimated as matching with the *in silico* design (table 2). Following this analysis, a 2D NMR spectrum was performed to verify the presence of a tertiary structure (Figure 8). The NMR analysis clearly showed a well-structured protein indicating the presence of a tertiary structure.

Then we determined whether the protein unfolded cooperatively or not. We used the CD technique at 222 nm and also the emission fluorescence of tryptohans. Both spectroscopic techniques showed us a cooperative unfolding (Figure 9), a feature of natural proteins. Also it allowed us to determine an unfolding ΔG of 46 KJ/mol, which is an improvement in comparison with value obtained for the parental protein Octarellin V (35 KJ/mol; Offredi *et al*, 2003). Indeed, this apparent improvement at stability level was studied by Differential Scanning Calorimetry (DSC), Dynamic Light Scattering (DLS) and Circular Dichroism (CD).

The DSC spectrum was performed under 2 different conditions: with and without Guanidine hydrochloride (GdnHCl, at 0.5 M). The reason for these 2 different conditions is based on an observation made by Offredi *et al*, where they described a stabilization of Octarellin V in presence of GdnHCl at concentrations below 1 M. We obtained similar results for our evolved protein, observing a Tm of 66 °C in absence of GdnHCl and an increment of the Tm in presence of this chaotropic agent, shifting to 83 °C (Figure 10). The Tm value without GndHCl is equivalent with this one reported for Octarellin V into the Offredi paper, meaning that the directed evolution process have not affected this parameter. However, the stability of the protein is not high. The protein showed a lifetime of just a few days. We repeated the DSC experiment only 3 days after the production and purification of the protein and the results are incredibly dramatic: the protein is unfolded and it precipitates into the cuvette after increasing the temperature (Figure 10). This loss of tertiary structure does not correlate with the secondary structure. The CD spectra before and after several days is the same (data not shown for after several days), indicating only lost of the tertiary structure. Moreover, following the CD value at 222 nm and increasing the temperature up to 95 °C, it is impossible to see any change (Figure 7), leading to a wrong interpretation of high thermostability. Complementary to these experiments, the DLS characterization showed that Octarellin V.1 is monodisperse, yet it depends on the protein concentration and the temperature. The protein remains monodisperse at very low concentrations (below 0.2 mg/ml) up to 90 °C, but it starts to precipitate if the concentration is higher (Figure 11).

All these experiments showed clearly that Octarellin V.1 is a soluble but unstable protein with a lifetime of few days, but showing native-like features as cooperative unfolding and with a secondary structure content matching with the *in silico* design.

It is clear that the instability of the protein is a flaw to be solved in order to determine the experimental structure of the Octarellin V.1. The best alternative is to use "binders" capable of stabilizing the protein without changing its structure.

3.4 "Binders" selection for Octarellin V.1 stabilization

Due to the protein instability showed by Octarellin V.1, we have decided to use "binders" that can recognize and stabilize the protein. Currently the use of some antibodies as binders is one of the most used techniques to achieve this desired result. Among the antibodies, the called "nanobodies" (Hamers-Casterman *et al*, 1993; Loris *et al*, 2003) are taking more importance because of their properties, such as their small size, high affinity and selectivity, conformational selectivity, and their easy production and purification from bacteria. A second group of "binders" is that one created artificially based on natural proteins. Inside this second group is highlighted the so called "αRep" (Urvoas *et al*, 2010), which are designed based on proteins with "heat" repeats and are capable of interacting with proteins through a large interaction surface (in comparison with nanobodies), and are very stable, conformational selectively and are easy to produce and purify from bacteria as well.

We decided to work with both systems in order to increase our chance of success, but also to probe and then determine whether the structure is or is not influenced by the selected binder. The nanobodies were obtained thanks to our collaboration with the Free University of Bruxelles (VUB), Belgium, and the αRep proteins were obtained thanks to another collaboration with the University of Paris Sud 11, France.

Seven different nanobodies were obtained after the immunization of the llamas using the Octarellin V.1. The genes which codify for the these seven nanobodies were subcloned into a procariotic expression vector system and then they were expressed and purified from bacteria *E. coli* BL21(DE3). To verify their interaction with high affinity for Octarellin V.1, the purified nanobodies were incubated in presence of purified Octarellin V.1 and then the complex Nanobody-Octarellin V.1 was isolated and purified by size exclusion chromatography (Figure 12). By this procedure we could purify the seven expected complexes. They were characterized by Isothermal Titration Calorimetry (ITC) showing a stoichiometry 1:1 and a Kd of 15 nM (Figure 13). All the complexes were suitable for crystallization screenings.

On the other hand, 3 different αRep were identified and in the same way as we did with the nanobodies, the proteins were expressed and purified from *E. coli* BL21(DE3). However, only one complex was able to be isolated and purified by size exclusion chromatography (Figure 14). This complex was also characterized by ITC showing a stoichiometry 1:1 and a Kd of 0.45 μM (Figure 15). The complex was suitable for crystallization screening.

3.5 Crystallization screening for the complexes Octarellin V.1-Binders

The 8 complexes (7 with nanobodies and 1 with αRep) were subjected to crystallization screening, testing around 200 different conditions for each complex using the vapor diffusion

technique. The nanobody complexes were concentrated up to 10 mg/mL and the αRep complex up to 20 mg/mL to perform the indicated screening.

From the 7 nanobody complexes, 2 of them produced crystals (Figure 16). The first complex produced “needle crystals” which were improved using a combination of streak seeding and adding 10% of ethanol. The second complex produced 3D crystals in the screening, but they weren't reproduced in the attempt to get bigger crystals to be diffracted.

The complex αRep-Octarellin V.1 gave us several hits in the crystallization screening (Figure 16), but one condition is distinguished because of the size of the crystals and the time needed to obtain them, which one it is only a couple of days.

All these crystals have been diffracted in a X-Ray generator, thanks to a collaboration with the Free University of Bruxelles. With this analysis we have determined which crystals can be then diffracted at a synchrotron to get a full data set and then solve the structure.

4.- Conclusions

The Octarellin project started more than 20 years ago. Several models have been created since the beginning using the current technologies and knowledge available each time. The current generation of Octarellins have been designed using a combinatorial design, which is composed by an *in silico* design using the best algorithms; and a second part made by directed evolution, where we use the natural selection to improve the solubility of our artificial proteins. Using this approach we have created the Octarellin V and VI.

The most critical step in the *de novo* design is to determine if the protocol used to design the artificial protein agrees with the expectations. That means to test that the protein designed in the computer will adopt the same structure as *in vivo*. This can generate an iterative cycle where if the real structure differs with the *in silico* design, we can use this information to improve the softwares. Clearly, until now, there is no evidence of successfully designing proteins of more than 150 amino acids, but there are very interesting successes with little proteins, such as Top7, created by Baker's laboratory (Kuhlman *et al*, 2003) where they could design a protein with a complete new fold, previously not present in nature. These kinds of results pushed us to continue working on our idea to create artificial proteins of more than 200 amino acids adopting the (β/α)8 fold, the most common in nature, but bringing now a new sequence unlike any other natural protein inside this family.

Now we are currently presenting the characterization of the artificial proteins Octarellin V.1 and Octarellin VI: our proteins coming from the last generation of Octarellins. Together with the characterization, we also showed the strategy to obtain the experimental structure of the Octarellin V.1.

The Octarellin VI was designed using the software Rosetta, the same used to design Top7. The protein was expressed in *E. coli* BL21(DE3) and purified from the inclusion bodies. The renatured protein showed some non-ideal features such as instability, non-cooperative unfolding and very poor solubility. The directed evolution technique was selected to improve the protein. Currently we are working on the standardization of a protocol to produce and purify these evolved variants to then try to determine their experimental structure.

The Octarellin V.1 was produced in the past by directed evolution over the Octarellin V,

improving its solubility inside the bacteria. This evolved protein showed native-like features such as cooperative unfolding and appearing well-structured by NMR 2D spectroscopy. However, its stability is not high, showing a lifetime of only a few days. In order to determine its experimental structure we decided to use “binders” which can interact with Octarellin V.1 and stabilize it, improving the lifetime by a complex formation. Two different kinds of binders were used: nanobodies and αRep proteins. Both systems allowed us to purify complexes and these complexes were used in crystallization screenings. Finally, we were able to determine crystallization conditions which allowed us to obtain crystals with good quality to be diffracted by synchrotron X-Ray radiation.

The experimental structure of Octarellin V.1 will be solved very soon, and this will be the biggest step in the history of Octarellins, not only because it will be the first Octarellin's structure solved, but will be the first structure of an artificial protein of more than 200 amino acids. Independent of the result, the information that we will get from the structure will be precious. It will allow us to design an artificial enzyme, and/or to improve the softwares used for protein design.

5.- References

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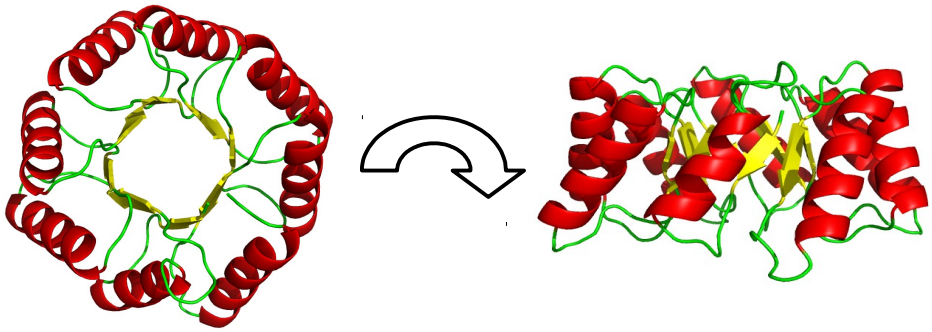
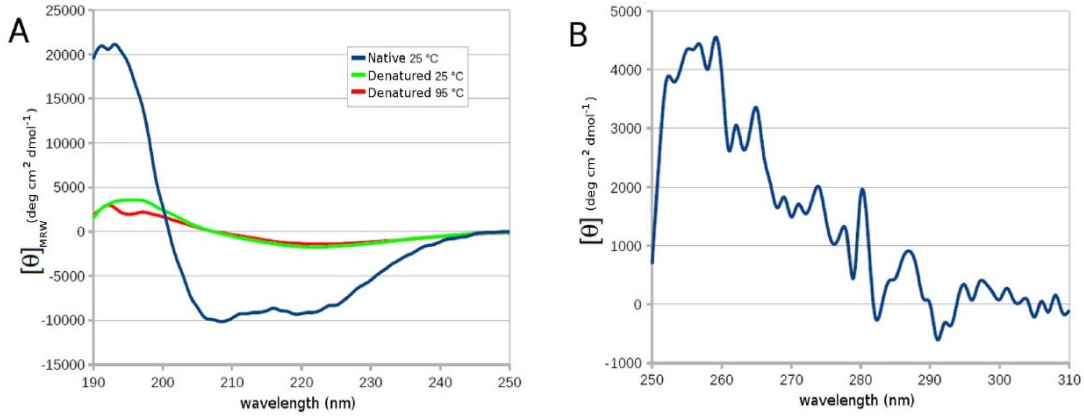
6.- Figures

Figure 1: Idealized (β/α)8 proteins



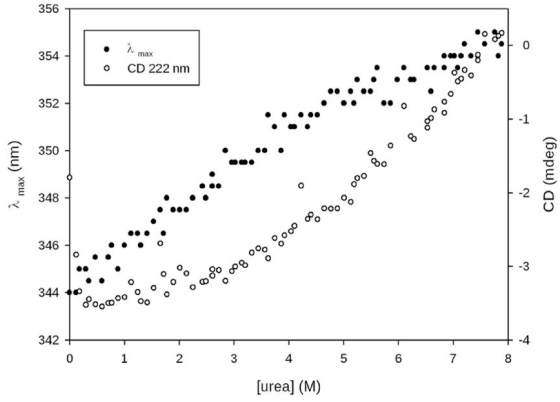
The artificial proteins Octarellin V and VI were designed to fold as the model displayed. The model is composed by 8 repetitions of the motif “beta strand - alpha helix”. The softwares used, Orbit and Rosetta, gave us 2 different amino acids sequences (identity less than 10%) and were completely different in comparison with any natural protein.

Figure 2: CD spectra for Octarellin VI



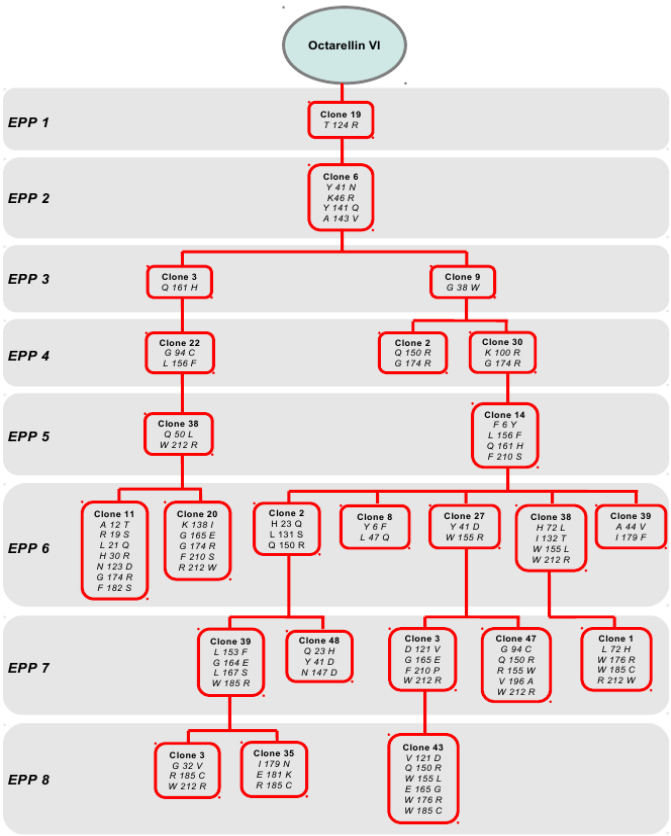
A) Far-UV CD spectrum of Octarellin VI (0.1 mg/mL) recorded at 25 °C, after denaturation at 95 °C, and after cooling from 95 to 25 °C. **B)** Near-UV spectrum of Octarellin VI (1 mg/mL) at 25 °C.

Figure 3: Urea unfolding of the artificial protein Octarellin VI



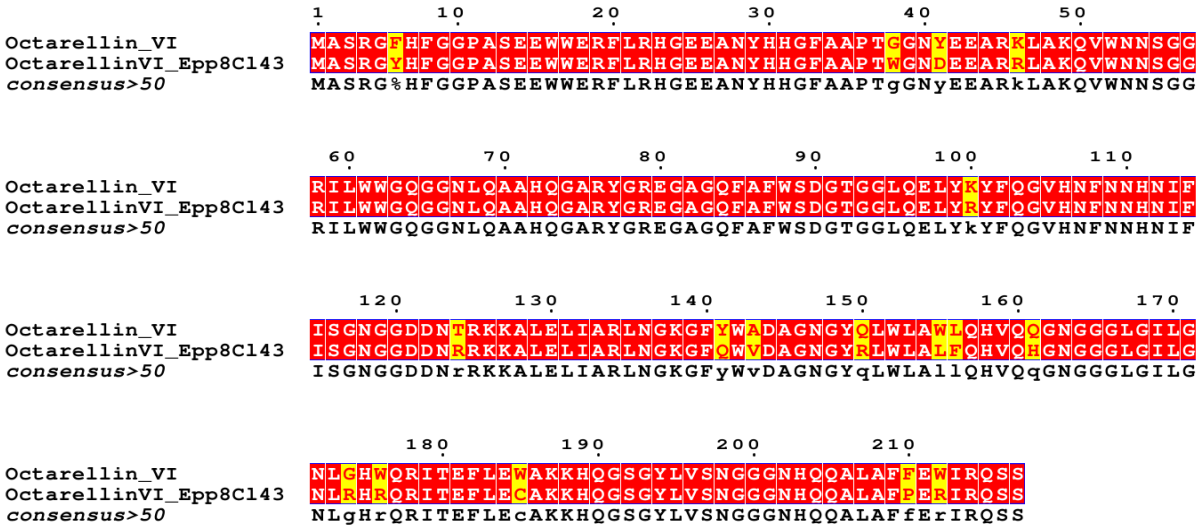
Change in the λmax of fluorescence emission (after exitation at 280 nm) and change on CD signal at 222 nm as a function of the urea concentration. The change in λmax of fluorescence emission shows a shift from 344 nm (folded) to 355 nm (unfolded) as the urea concentration increases, and the same behavior is observed for the CD signal at 222 nm.

Figure 4: Phylogenetic tree obtained after 8 cycles of Error Prone PCR for Octarellin VI



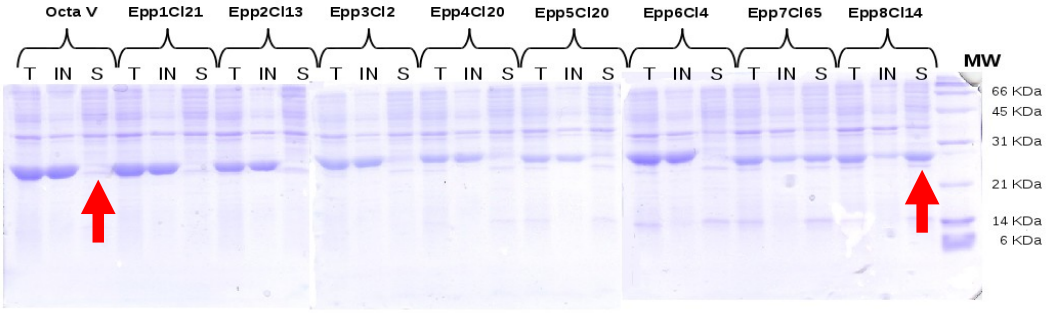
Using the GFP as solubility reporter, the best clones each of the cycles are displayed showing the mutations. All the selected clones from one cycle were used for the next cycle.

Figure 5: Sequence alignment between Octarellin VI and its variant Epp8Cl43



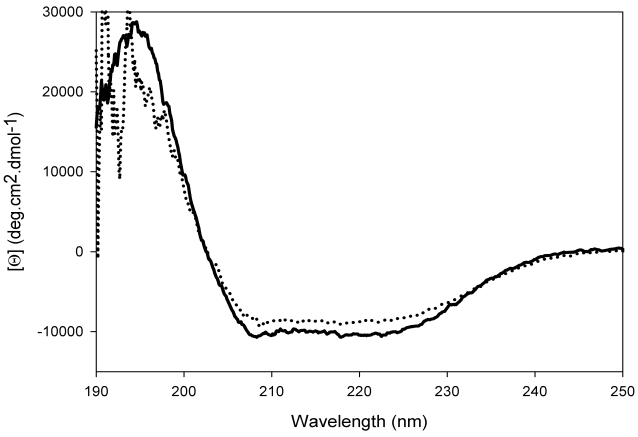
The alignment shows a high conservation for the variant 43 coming from the 8 cycles of Error Prone PCR (Epp8Cl43). The mutations are highlighted in yellow.

Figure 6: SDS-PAGE analysis for the expression of Octarellin V and its evolved variants



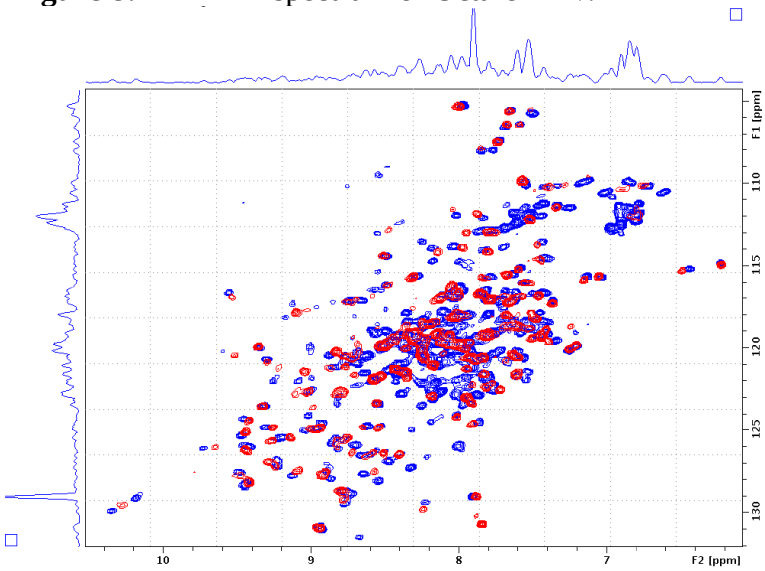
The expression was induced by IPTG 1 mM for 12 hours at 37 °C, and the total (T), insoluble (IN), and soluble (S) fractions were analyzed in SDS-PAGE stained with Coomassie Blue. After 8 cycles of Error Prone PCR, we can see how the protein shift from the insoluble fraction (Octa V lanes) to the soluble fraction (Epp8Cl14 lanes), as is indicated by the arrows.

Figure 7: Far-UV CD spectrum of Octarellin V.1



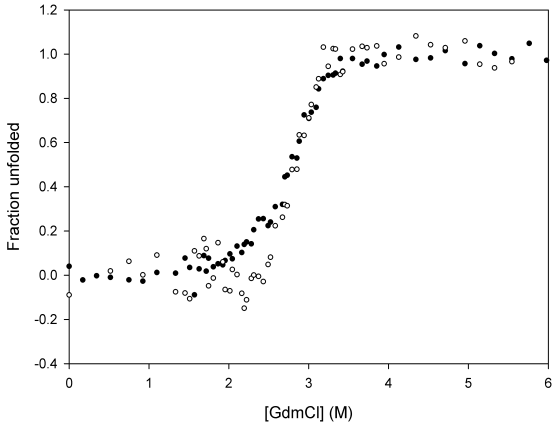
The Far-UV CD spectrum was obtained at 25 °C (solid line) and 90 °C (dotted line) using a protein concentration of 0.1 mg/mL. Both spectra are equals showing clearly a conservation of the secondary structure at 90 °C.

Figure 8: 2D NMR spectrum of Octarellin V.1



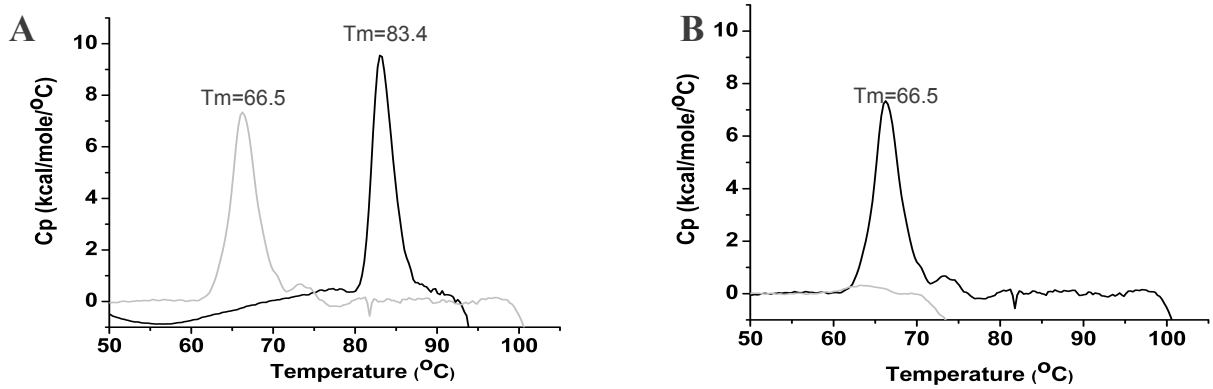
The 2D NMR spectrum was determined at two different pH (7=blue, 8.5=red) using a protein concentration of 0.8 mg/mL. It is possible to observe differences at the signal position but the frequency distribution is similar in both cases, which indicates that the protein is structured and stable in these conditions.

Figure 9: Chemical unfolding of Octarellin V.1



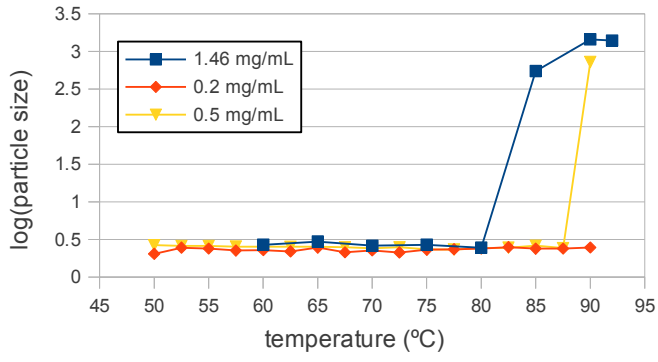
The chemical unfolding induced by guanidine hydrochloride (GdnCl) was followed by shift in λmax (open circles) and CD at 222 nm (black circles). The chemical unfolding is cooperative and using a 2 state model we determined a ΔG unfolding of 46 KJ/mol.

Figure 10: DSC spectrum of Octarellin V.1



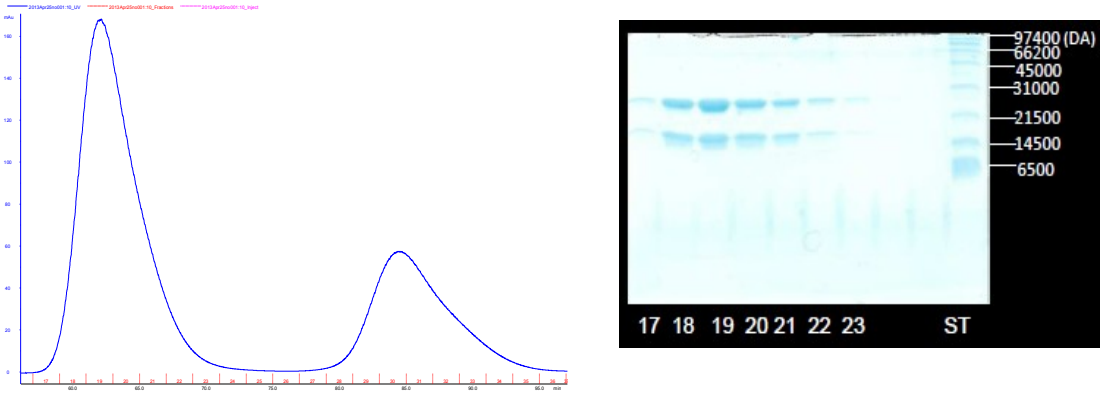
A) The DSC spectrum of fresh protein was determined in presence of 0.5 M of GdnHCl (black line) or in absence of GdnHCl (gray line). The native protein showed a Tm = 66.5 °C but it has an increment of the Tm in presence of GdnHCl (Tm=83.4 °C). **B)** The same experiment was repeated with the same batch of protein 3 days later. However, the protein didn't show a transition and it precipitated after 70 °C (gray line). The spectrum of the fresh protein is shown for comparison (black line).

Figure 11: DLS analysis for Octarellin V.1



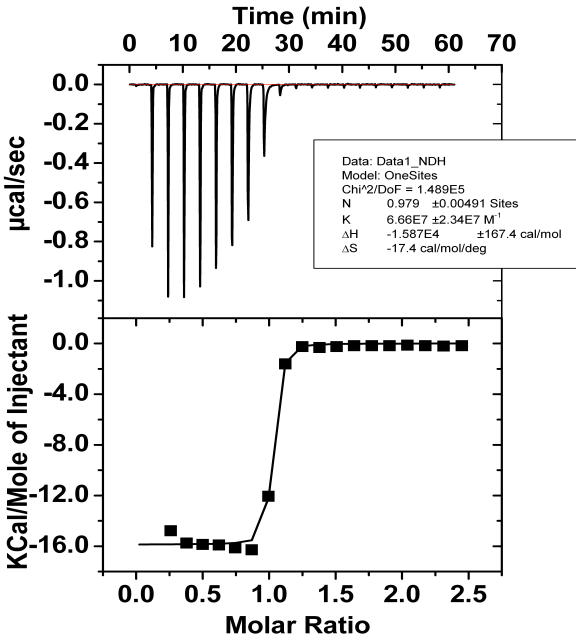
The DLS spectrum as function of the temperature was determined using 3 different protein concentrations. The protein remained soluble and without change in the size at low concentration (0.2 mg/mL). It precipitated under the same conditions but increasing the protein concentration.

Figure 12: Purification of the complex Nanobody-Octarellin V.1



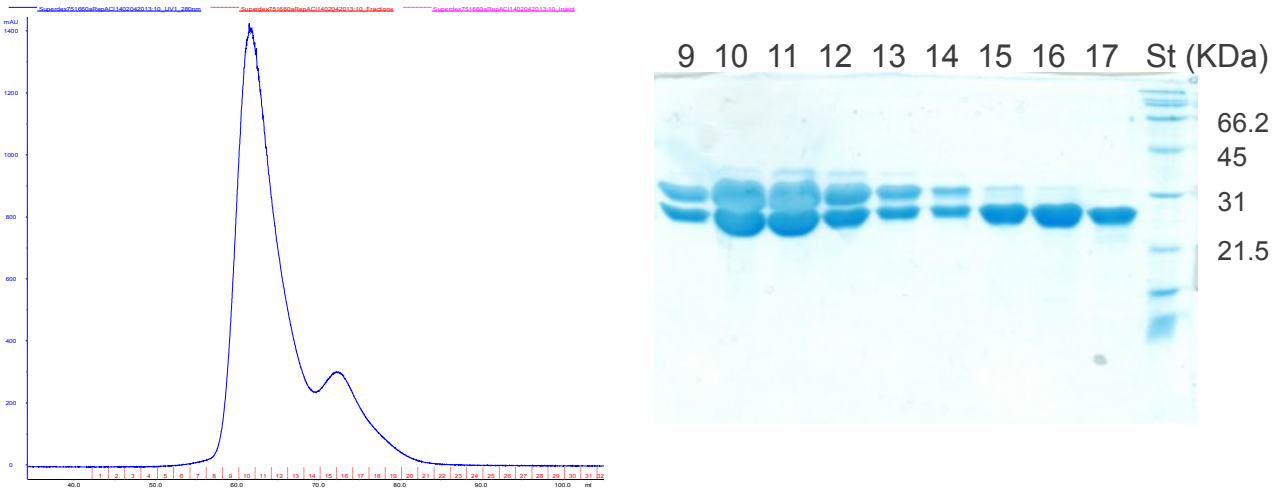
The complex Nanobody-Octarrellin V.1 was purified by size exclusion chromatography (SEC) and the chromatogram is displayed. To perform the purification, the purified proteins were incubated in a ratio of 1:2 (Octarellin:Nanobody) in order to guarantee all the Octarellin would participate in the complex and in this way isolate more easily from the free protein (complex size: 39 KDa, Octarellin size: 24 KDa, Nanobody size: 15 KDa). The SDS-PAGE analysis of the fractions corresponding with the complex peak is also shown.

Figure 13: ITC characterization of the interaction between the Nanobody and Octarellin V.1



Octarellin V.1 (30 µM) was titrated with a nanobody (280 µM). The model of two states was used to determine the stoichiometry (N=0.979) and the Kd (15 nM).

Figure 14: Purification of the complex αRep-Octarellin V.1



The complex αRep-Octarrellin V.1 was purified by size exclusion chromatography (SEC) and the chromatogram is displayed. To perform the purification, the purified proteins were incubated in a ratio of 1:2 (αRep:Octarellin) in order to guarantee all the αRep will participate in the complex and in this way isolate more easy from the free protein (complex size: 54 KDa, Octarellin size: 24 KDa, αRep size: 30 KDa). The SDS-PAGE analysis of the fractions corresponding with the two peaks is also shown. The fractions 9-13 were collected for further studies.

Figure 15: ITC characterization of the interaction between the αRep and Octarellin V.1

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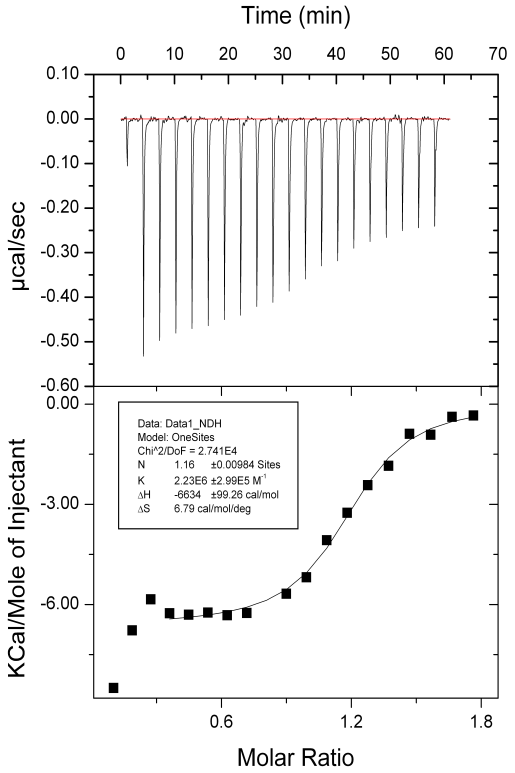
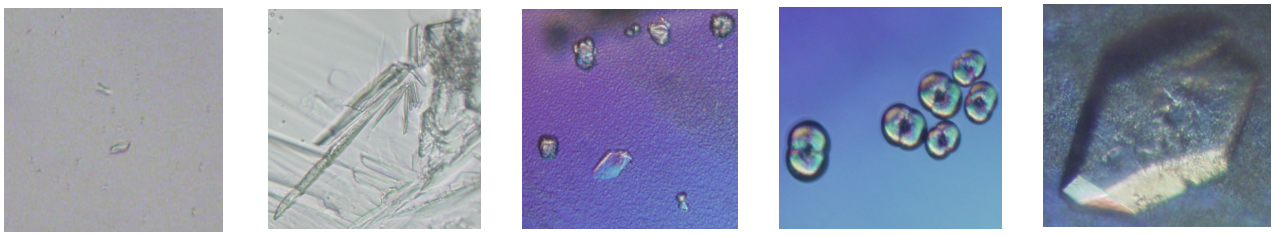


αRep (25 µM) was titrated with the Octarellin V.1 (216 µM). The model of two states was used to determine the stoichiometry (N=1.16) and the Kd (0.45 µM).

Figure 16: Crystallization screening

A) αRep/Octarellin V.1 crystals



0.1 Sodium malonate pH 7.0
0.1 M HEPES pH 7.0
0.5% v/v Jeffamine ED-200

0.2 M Ammonium acetate
0.1 M BIS-TRIS pH 5.5
25% w/v PEG 3500

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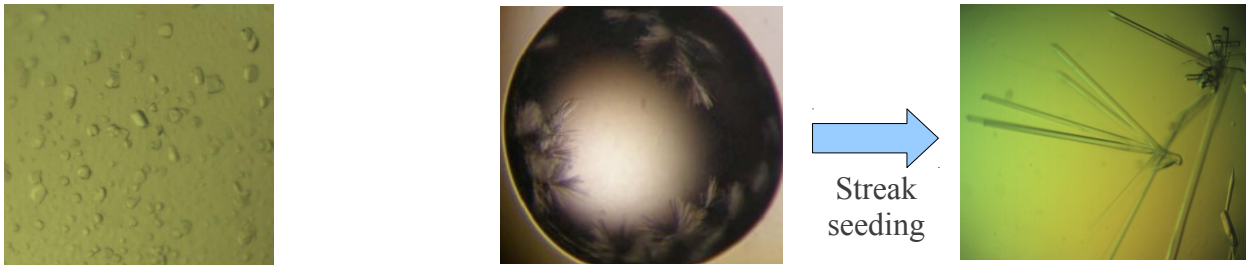
0.1 M BIS-TRIS pH 5.5
25% w/v PEG 3500

B) Nanobody/Octarellin V.1 crystals



0.1 M Bicine pH 8.5
20% w/v PEG 6000

0.1 M Tris pH 8.5
25% w/v PEG 3350

0.1 M Tris pH 8.5
22.3% w/v PEG 3350
10% v/v ethanol

Crystals obtained by crystallization screening using Crystal Screen I, II and INDEX from Hampton Research. The Nanobody-Octarellin V.1 needle crystal was improved by streak seeding and using ethanol 10% as an additive.

Table 1

	Helix (%)	**Strand (%)**	**Turn (%)**	**Unordered (%)**
Octarellin VI	34 ± 3 (45.8)	18 ± 2 (15.3)	19 ± 1 (7.9)	29 ± 2 (31.0)
Octarellin V.1	32 ± 3 (48.0)	22 ± 4 (11.2)	19 ± 1 (12.0)	26 ± 5 (28.7)

Values in parentheses were obtained from the 3D coordinate file (computational design) with the DSSP software.

7.- Contributions

a) Publication

Figueroa M, Oliveira N, Lejeune A, Kaufmann KW, Dorr BM, Matagne A, Martial JA, Meiler J, Van de Weerdt C (2013) **"Octarellin VI: using Rosetta to design a putative artificial (beta/alpha)8 protein"**
PloS One 8(8): e71858

b) Conferences

1. **"The artificial protein Octarellin V.1 is soluble, thermostable and monomeric but apparently differs with the *in silico* design"**
   Figueroa M, Jacquin O, Matagne A, Damblon C, Goormaghtigh E, Valerio-Lepiniec M, Minard P, Martial JA, Van de Weerdt C.
   GIGA day (Liege, Belgium, 2013)

2. **"*In silico* and *in vivo* combinatorial design of Octarellin VI, an artificial protein modeled on the (beta/alpha)8 fold"**
   Figueroa M, Taralla S, Buscetta M, Sanjuan W, Matagne A, Lejeune A, Martial JA, Van de Weerdt C.
   PEGS Europe (Vienna, Austria, November 2012)

3. **"Improving solubility and stability of Octarellin V, a TIM-like artificial protein, by directed evolution"**
   Figueroa M, Jacquin O, Vendevenne M, Parvizi G, Atoout S, Evrard C, Matagne A, Goormaghtigh E, Martial JA, Van de Weerdt C.
   PEGS Europe (Vienna, Austria, November 2012)

4. **"*In silico* and in vivo combinatoral design of Octarellin VI, an artificial protein modeled on the (β/α)8 fold"**
   Figueroa M, Taralla S, Buscetta, Sanjuan W, Matagne, Lejeune A, Martial JA, Van de Weerdt C.
   GIGA day (Liege, Belgium 2012)

5. **"*De novo* design of artificial α/β barrel proteins: step forwards to determine the structure"**
   Figueroa M, Taralla S, Irrthum A, Evrard C, Lejeune A, Martial JA, Van de Weerdt C.
   Encuentros Conference (Berkeley, USA, 2011)

6. **"Evolutionary optimization of an artificial protein for improved solubility: bioinformatics analysis"**
   Figueroa M, Irrthum A, Martial JA, Van de Weerdt C.
   GIGA day (Liege, Belgium, 2011)