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Congeneric bio-adhesive mussel foot proteins designed by modified prolines revealed a chiral bias in unnatural translation

Maud Larregola, Shannon Moore, Nediljko Budisa *

Department of Chemistry, Biocatalysis Group, Berlin Institute of Technology/TU Berlin, Franklinstraße 29, D-10587 Berlin, Germany

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ABSTRACT

Chiral bias in the unnatural translation and 'sticky' mussel proteins. The residue-specific *in vivo* incorporation of hydroxylated amino acids as well as other synthetic analogs, such as fluoroprolines, emerges as the method of choice for recombinant synthesis of Pro-rich mussel adhesive protein congeners. Chemical diversifications introduced in this way provide a general route towards bio-adhesive congeners endowed with properties not developed by natural evolution. Most importantly, we have found that the co-translational incorporation of (4*R*)-, and (4*S*)-hyroxylated and fluorinated analogs into mussel proteins presented a chiral bias: the expressed protein was only detectable in samples incubated with analogs with (4*R*)-substituents. Possible relationship of these stereochemical preferences for (4*R*)-stereoisomers in the translation to intracellular tRNA concentrations, ribosomal editing and proofreading or structural effects such as preorganization remains to be addressed in future studies. These studies will generally provide a mechanistic framework for the flexibility of the translational machinery and establish the boundaries of the unnatural translation.

1. Introduction

Temporary or permanent physical adhesion to biotic or abiotic surfaces is an essential mechanism used by a variety of organisms, and is particularly useful to a number of species living in aquatic environments [1]. Marine mussels for example are able to form flexible yet durable adhesion in environments characterized by high salinity, humidity and detaching forces such as water turbulence. Mussels form this attachment by the secretion of protein-based water resistant bio-adhesives and can adhere to a large variety of organic and inorganic surfaces such as wood, glass, plastic, stone, metal, concrete and Teflon [2]. The attachment works over solid supports by elastic byssal threads, each ending in an adhesive plaque with specific mussel "glue" protein cocktails. One of the best-characterized marine bio-adhesives is produced by the mussel *Mytilus galloprovincialis* and consists of five distinct proteins, referred to as foot proteins (Mgfps). Each foot protein plays a specific role such as matrix building (Mgfp-2 and Mgfp-4), protective coating (Mgfp-1) and adhesion (Mgfp-3 and Mgfp-5) [3].

Many adhesive proteins are scleroproteins containing repetitive sequence motifs and posttranslational modifications [2] such as hydroxylations of specific residues, which are most commonly tyrosine (Tyr), proline (Pro) and arginine (Arg) [1]. As these hydroxylated residues are responsible for the adhesive properties of mussel proteins, it is impossible to use bacterial expression with recombinant DNA technology to produce these substances in large amounts because bacteria lack the necessary modification machinery found in eukaryotic cells. Therefore, currently available adhesive protein products are extracted from homogenized raw mussels [4]. This technique however is both costly and time consuming and produces very small quantities of adhesive protein product with extremely limited possibilities of further re-design. Thus, an alternative approach is needed for research, medical and industrial applications.

Here, we describe a novel method for the synthesis of modified mussel adhesive proteins by exploiting the substrate tolerance of the cellular systems of uptake, metabolism, and translational apparatus to analogs of natural amino acids [5]. In addition to hydroxylated analogs, other modified residues such as fluoroprolines could be incorporated into target sequences in a residue-specific manner to give access to a class of biomaterials which have not been developed by natural evolution [6]. Congeners of mussel adhesive proteins are expected to be translated from the same genetic sequence, but will have most of their Pro-residues exchanged by hydroxylated or fluorinated counterparts. Despite the promise of mussel adhesive proteins as candidates for wet adhesives and as a biological bone glue [2], no report exists to date of the incorporation of synthetic amino acids into mussel adhesive proteins. In addition, whereas the role of Tyr-hydroxylation has been extensively studied [7,8], the hydroxylation of Pro to (2*S*,4*R*)-4-hydroxyproline ((4*R*)-OHPro), in spite of its presence as one of the most abundant residues in Mfp-1 [9], remains to be investigated. To address these gaps, we have developed experiments to determine the feasibility of

* Corresponding author. Fax: +49 30 314 28279.
E-mail address: budisa@biocat.tu-berlin.de (N. Budisa).

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incorporating Pro analogs into a Pro-rich fusion protein, Fp151. The recombinant Fp151 sequence is composed of Mgfp-1 repeats conjoined with an Mgfp-5 sequence (Fig. 1) [4]. Our aim was not only the incorporation of (4*R*)-OHPro – to afford a naturally hydroxylated protein – but more specifically to test the translational capacity of fluoroproline diastereoisomers (2*S*,4*R*)-4-fluoroproline ((4*R*)-FPro) and (2*S*,4*S*)-4-fluoroproline ((4*S*)-FPro) in order to generate and analytically explore the first synthetic congeners of mussel foot proteins.

Apart from these great possibilities for practical applications, we describe here an interesting phenomenon of stereochemical preferences for particular stereoisomers during the unnatural translation. Unfortunately, the studies about the mechanisms and features of unnatural translation and especially the understanding of how the incorporation of different synthetic noncanonical amino acids affects translation machinery and co-translational folding are largely missing. Therefore, our initial results by using Pro-rich protein Fp151 should provide a solid basis to systematically study the mechanisms and features of unnatural translation.

The choice of fluorination is motivated by the spectacular findings reported in the last decades [10]. For example, the most abundant protein in nature – hydroxylated Pro-rich triple polyproline II (PPII) helix of collagen – exhibited a dramatic increase in stability upon replacement of the (4*R*)-OHPro residues with (4*R*)-FPro [11]. Similarly, classical fluorinated polymers are generally known to show enhanced thermodynamic and hydrolytic stability as well as resistance to organic solvents [12]. Thus, we speculate that the co-translational incorporation of fluorinated Pro residues into a mussel protein would represent a general route towards novel bio-adhesives with properties that are not present in currently available biomaterials produced by standard procedures.

2. Materials and methods

All chemicals were purchased from Sigma (Sternheim, Germany) or Merck KGaA (Darmstadt, Germany) unless otherwise indicated. Fluoroproline analogs were obtained from Bachem (Weil am Rhein, Germany).

2.1. Fermentation, analog incorporation and protein purification

Protein expression was performed using the Pro auxotrophic *Escherichia coli* strain JM83. Cells were transformed with a pQE80L vector (Qiagen, Hilden, Germany) – carrying the fp151 gene (Supplementary Information) with an *N*-terminal His-tag – and, in the case of prolyl-tRNAsynthetase (ProRS) co-expression, with a pWK1 plasmid (compatible p15A replicon) carrying the ProRS expression cassette under the control of the $P_L$tet promoter. Transformants were grown in new minimal medium [13] containing 40 μM Pro as natural substrate until depletion of Pro and an optical density at 600 nm between 0.6 and 0.8. Subsequently, the proline analogs were added to the medium (final concentration 1 mM), and target protein expression was induced by addition of 1 mM isopropyl *β*-D-1-thiogalactopyranoside. Protein expression was performed for 4 h at 30 °C with vigorous shaking. After cell harvest, cells were lysed by sonication in lysis buffer (sodium dihydrogen phosphate 100 mM, Tris 10 mM, pH 7) and the lysate was centrifuged at a high speed (15 000 rpm, 4 °C, 30 min). For the parent Fp151 protein, the pellet was washed with lysis buffer and solubilized in 8 M urea before centrifugation. Target protein was then purified at room temperature using a Ni–NTA column (GE Healthcare, Munich, Germany) with an imidazole gradient (0–500 mM) in buffer (sodium dihydrogen phosphate buffer (50 mM, pH 8.0) containing 8 M urea and 500 mM NaCl). Purified protein fractions were dialyzed against 5% acetic acid at room temperature overnight. For the Fp151[(4*R*)-FPro] protein, the pellet was washed with lysis buffer and solubilized in a buffer (sodium dihydrogen phosphate buffer (50 mM, pH 8.0) containing 8 M urea, 500 mM NaCl, 5 mM betaine, 5 mM DTT and 26 mM imidazole) before centrifugation. The target protein was then purified using an Ni–NTA column (GE Healthcare, Munich, Germany). Protein was eluted with an imidazole gradient (26–880 mM) and protein fractions were dialyzed against 5% acetic acid containing 5 mM betaine and 5 mM DTT at room temperature overnight. For the Fp151[(4*R*)-OHPro] protein, the pellet was washed with lysis buffer and solubilized in buffer (sodium dihydrogen phosphate buffer (50 mM, pH 8.0) containing 8 M urea, 500 mM NaCl, 5 mM betaine, 5 mM DTT) before centrifugation. Target protein was then purified using a self-made 500μL Ni–NTA agarose (QIAGEN, Hilden, Germany) column. Protein was eluted with imidazole steps (25 mM and 100 mM) and the second elution fraction was submitted to dialysis against 5% acetic acid containing 5 mM betaine and 5 mM DTT at room temperature overnight.

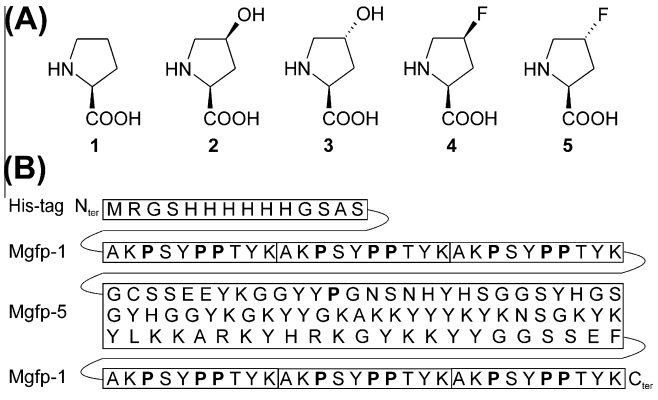

Fig. 1. Structures of Pro, its analogs and Fp151 protein (A) Natural amino acid Pro (**1**) and related analogs, (2*S*,4*S*)-4-hydroxyproline ((4*S*)-OHPro) (**2**), (4*R*)-OHPro (**3**), (4*S*)-FPro (**4**), and (4*R*)-FPro (**5**). (B) Pro-rich fusion protein Fp151 comprises an N-terminal His-tag and two arrays of triple Mgfp-1 decapeptide repeats fused with an Mgfp-5 sequence. The protein sequence is derived from a fusion protein described by Hwang et al. [4].

2.2. Mass analysis

For liquid chromatography-electrospray ionization–mass spectrometry (LC–ESI–MS), 20 μl aliquots of the purified proteins were pre-separated on a Discovery BioWide Pore C5 column (3.5 μm particle size, 100 × 2.1 mm, Supelco, Bellefonte, PA, USA) by eluting with a gradient from 10% to 90% B within 15 min and 90–95% B in 2 min (for Fp151 and Fp151[(4*R*)-FPro]: eluent A: 0.05% (v/v) TFA in water, eluent B: 0.05% (v/v) TFA in acetonitrile; for Fp151[(4*R*)-OHPro]: eluent A: 0.1% (v/v) HCOOH in water, eluent B: 0.1% (v/v) HCOOH in acetonitrile). A flow rate of 250 μl min^{-1} was used. The masses of the eluted fractions were analyzed on a MicroTOF ESI–MS (BrukerDaltonics) or an Exactive (Thermo Scientific). The absorbance was detected at 210 nm.

2.3. Amino acid analysis

The procedure for total hydrolysis, derivatization and gas chromatography-positive chemical ionization-mass spectrometry (GC–PCI–MS) analysis was adapted from the recently published paper of Pesic et al. [14]. Total hydrolyses of dry Fp151[(4*R*)-FPro] ($1.1x10^{-8}$ mol) were performed at 110 °C in aqueous 6 N hydrochloric acid solution (200 μl) under vacuum for 24 h in glass ampoules. After 24 h, the hydrolysates were transferred into ReactiVials and lyophilized. For GC–PCI–MS analyses, hydrolysates were transformed into their *N*-trifluoroacetyl/ethyl ester derivatives. To the dry hydrolysates a total volume of 200 μl of 2 M ethanolic

HCl–solution, generated from acetyl chloride in absolute ethanol (1:4, v:v) was added. The samples were heated for 30 min at 110 °C and excess of derivatization solution was removed at 110 °C in a gentle stream of nitrogen. Derivatization was completed through acetylation by adding 100 μL dichloromethane and 100 μL trifluoroacetic anhydride to the samples. The mixtures were heated again for 10 min at 110 °C. Excess of reagent was removed at ambient temperature in a gentle stream of nitrogen to avoid evaporation of amino acid derivatives. Resulting residues were dissolved in 100 μL anhydrous toluene and portions of 1 μL were subjected to GC/MS (5975C, Agilent Technologies, Waldbronn, Germany) (Supplementary Information). Amino acids were identified by prominent mass spectral fragments and their corresponding protonated masses $[M+H]^+$ in positive chemical ionization-(PCI)-mode. Full-Scan data for qualification and single ion monitoring (SIM) data for quantification were acquired by switching between both modes.

All expected amino acids could be identified except Arg and His, which are not detectable with this derivatization method, and Cys and Met, which were decomposed and oxidized, respectively, during acid hydrolysis. Finally, Asn is deaminated to Asp during this hydrolysis process. For quantification of proline (Pro) and (4*R*)-FPro, the integrated peak areas of the SIM spectra of internal standards Nle and (4*S*)-FPro were used, respectively (Supplementary Information). Both internal standards were added before total hydrolysis to the protein sample. This quantification was repeated to test the reproducibility.

3. Results and discussion

In our previous studies, we have reported the sensitivity of the translation machinery for chemical and chiral isosteric (isostructural) proline analogs that are incorporated into various proteins during unnatural translation [6]. In particular, we have demonstrated that stereochemical bias for protein folding with fluoroprolines is directly related to proline puckering conformation and different substituents are capable to induce preorganization effect in the structure of the target polypeptide [15]. In addition, we and others have found that some other subtle modifications in proline side chains (e.g. simple exchange CH2 → S → Se) impair chain elongation with unknown mechanism(s) [16]. These are important determinants for the incorporation efficiency, since the kinetic and proofreading of protein translation must be directly influenced by the chirality of the Pro analogs.

Most recently, we have also shown that both (4*R*)- and (4*S*)-OHPro or FPro can be incorporated into globular recombinant proteins [15,17]. In Pro-rich Fp151, however, the co-translational incorporation presented a chiral bias: the expressed protein was only detectable in samples incubated with (4*R*)-substituents (Fig. 2). Both (4*R*)-FPro and (4*R*)-OHPro were effectively incorporated into Fp151 using our supplementation incorporation method [5] assisted with the constitutive co-expression of the endogenous prolyl-tRNAsynthetase (ProRS) in *E. coli* (Fig. 2B) [18]. This is a crucial feature for translation enhancement, since overexpression of *E. coli* ProRS markedly enhances the intracellular level of the enzyme, which compensates for the lower affinities of ProRS towards Pro analogs [19]. This is best exemplified by the expression of Fp151[(4*R*)-OHPro], which was detectable only under the conditions of translation enhancement (Fig. 2A and B).

The stereoselective preference for (4*R*)-stereoisomers during Fp151 translation is most probably due to a preorganization effect in the structure of the target protein [20], although other factors such as ribosomal translational editing and proofreading cannot be excluded. However, the preorganization effect is favored by previous observations with model peptides: the repeat decapeptide sequence Ala-Lys-Pro-Ser-Tyr-(4*R*)-OHPro-(4*R*)-OHPro-Thr-Tyr-Lys present in Mgfp-1 was described to adopt a PPII helix in water [21]. Indeed, previous studies on collagen indicate that replacement of Pro by (4*R*)-OHPro or (4*R*)-FPro increases the protein stability [11,20]. The inductive effects of the hydroxyl group or fluorine atom dictate a predominant Cγ-exo pucker upon the pyrrolidine ring of (4*R*)-OHPro and (4*R*)-FPro residues – an effect especially prominent for fluorine – whereas (4*S*)-substituted analogs present a Cγ-endo pucker [22]. In fact, the absence of any traces of Fp151 expression with (4*S*)-substituted analogs revealed how 'wrong' chirality biased *in vivo* protein translation by possible protein misfolding and subsequent degradation in the cells, or by premature termination of the chain elongation at the ribosome.

Initially, we demonstrated the successful incorporation of (4*R*)-OHPro and (4*R*)-FPro into Fp151 analytically by in-gel trypsin digestion and mass spectrometry MS/MS analysis of the tryptic peptides (Supplementary Information). This was followed by performing the protein purifications of parent Fp151 protein (yield: 0.3 mg L^{-1}; determined from UV profile) and its congeners Fp151[(4*R*)-OHPro] (0.03 mg L^{-1}) and Fp151[(4*R*)-FPro] (2 mg L^{-1}). As expected, ESI–MS revealed that it is possible to replace all Pro residues in Fp151 as the major peaks of the Fp151[(4*R*)-OHPro] and Fp151[(4*R*)-FPro] spectra correspond to the replacement of all 19 Pro residues by the respective analog (Fig. 2C). In this way, we succeeded in replacing up to 12% of the Fp151 protein sequence with (4*R*)-OHPro or (4*R*)-FPro. The heterogeneity of the congener ESI-MS profile can be interpreted as a mixture of species with different degrees of substitution, but this does not quantitatively reflect the level of analog incorporation. Thus, since the expression level of Fp151[(4*R*)-OHPro] was significantly lower and requires further optimization, we proceeded further with amino acid analysis of Fp151[(4*R*)-FPro]. We performed quantitative GC–PCI–MS analyses of Pro → (4*R*)-FPro substitutions in Fp151[(4*R*)-FPro] (Fig. 2 D and E) after total protein hydrolysis with 6 N HCl at 110 °C for 24 h under vacuum followed by *N*-trifluoroacetyl/ethylester derivatization in both the absence and presence of internal standards, (4*S*)-FPro and norleucine (Nle). As expected, these analyses revealed that 93% of Pro are replaced by (4*R*)-FPro in Fp151[(4*R*)-FPro].

4. Conclusions

In the present manuscript we have reported new yet unobserved properties of the unnatural translation. This could be both an exciting new tool and a new conceptual approach to understand the basic mechanisms of protein translation. On the other hand, our results could also be used as a solid basis for the design of components of the translational machinery in order to create an efficient expression of biomaterials with high biomedical relevance such as marine mussel-based 'biological glues' for bone-surgery and bone-regeneration.

First, we have tackled a fundamental problem of unnatural translation, that is, how alternative readings of particular gene sequence by various synthetic amino acids influenced cellular expression capacity. In our previous studies, we have discovered the stereochemical bias for the protein folding with fluoroprolines which is directly related to Pro puckering conformation. We are currently performing detailed examinations (analog-tRNA discrimination by EFTu, influence on peptide bond formation or translocation, etc.) in order to elucidate a mechanistic basis for the plasticity of the ribosome for unnatural substrates.

Second, by using the best known biological model for wet adhesion – mussel proteins from genus *Mytilus*– we are able to demonstrate that a simple recombinant expression and purification in native and modified forms is indeed feasible. Therefore, we anticipate that our approach will allow for the development

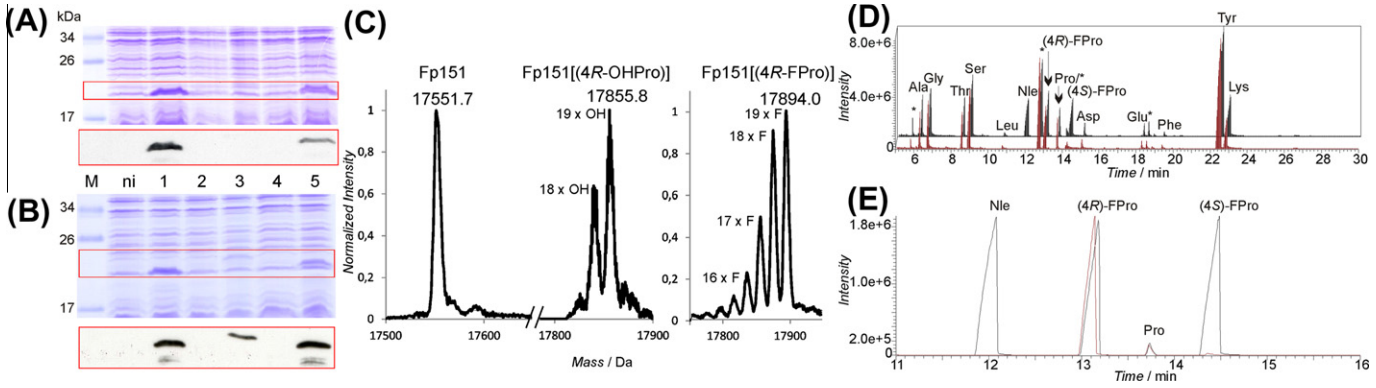

Fig. 2. Expression profiles and analyses of parent and congeneric Fp151 proteins (A) Top: Coomassie staining of cell lysates after expression of Fp151 with Pro (1), (4*S*)-OHPro (2) (4*R*)-OHPro (3), (4*S*)-FPro (4) and (4*R*)-FPro (5) using Pro-auxotrophic *E. coli* K-12 strain JM83. Bottom: Western blot generated by anti His-Tag antibodies. M, marker; ni, non-induced sample. Note that only parent and (4*R*)-FPro-protein were expressed. (B) Expression of Fp151 and its congeners in the same strain with co-expressed *E. coli* ProRS. Note stronger expression of 5 and appearance of 3. (C) ESI-MS analyses of Fp151 (Mw[calculated]: 17552,5 Da), Fp151[(4*R*)-OHPro] and Fp151[(4*R*)-FPro]. Beside dominant fully substituted protein (19 (4*R*)-OHPro residues: Mw[calculated] = 17856,5 Da and 19 (4*R*)-FPro residues: Mw[calculated] = 17894,5 Da), other mass species containing 18, 17 or 16 proline analogs are present. This correlates well with MS/MS results (Supplementary information). (D and E) GC–MS quantitative analyses of Pro → (4*R*)-FPro substitutions in Fp151[(4*R*)-FPro]: GC-PCI-MS chromatograms of the Fp151[(4*R*)-FPro] hydrolysate alone (red) and in presence of internal standards Nle and (4*S*)-FPro (black). Peaks marked with an asterisk (*) are unidentified byproducts. (D) Whole scan spectra. (E) Single ion monitoring (SIM) spectra used for quantification (SIM and Scan spectra were acquired at the same time). To overcome the different ion abundance between Pro and (4*R*)-FPro, we chose (4*S*)-FPro as an internal reference in order to precisely quantify (4*R*)-FPro. This calculation revealed a high level of substitution, (93%), indicating an expected 7% of residual Pro. However, by using Nle as a usual internal standard for amino acids in proteins [23], we calculated 3.5% of residual Pro. This small discrepancy is most probably due to the differences in ionization between Nle and Pro.

of bio-adhesive congeners with unique chemical properties by using an extended set of synthetic amino acids. Currently, we are investigating adhesive and other mechanical properties of our fluorinated Fp151, as it is reasonable to expect that fluorination will endow this protein with novel properties not exhibited by natural mussel proteins.

Generally speaking, it should be possible to use popular site-specific incorporation methods [24] for bio-adhesive recombinant production. However, these methods are less suitable as the efficiency of translation significantly decreases with increasing numbers of stop/non-triplet codons [25]. Furthermore, like many other biological phenomena [17], adhesive properties of mussel proteins are based on the collective effects of amino acid modifications at multiple positions in the polypeptide sequence, rather than at a single position [2]. Therefore, residue-specific replacement is unrivaled for this purpose as it allows one or more types of modified amino acids to be co-translationally incorporated into large portions of target protein sequences [26–28].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.031.

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