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Structural diversity of a collagen-binding matrix protein from the byssus of blue mussels upon refolding

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ABSTRACT

Blue mussels firmly adhere to a variety of different substrates by the byssus, an extracorporal structure consisting of several protein threads. These threads are mainly composed of fibrillar collagens called preCols which are embedded in a proteinaceous matrix. One of the two so far identified matrix proteins is the Proximal Thread Matrix Protein 1 (PTMP1). PTMP1 comprises two von Willebrand factor type A-like domains (A1 and A2) in a special arrangement. Here, we describe the refolding of recombinant PTMP1 from inclusion bodies. PTMP1 refolded into two distinct monomeric isoforms. Both isomers exhibited alternative intramolecular disulfide bonds. One of these isomers is thermodynamically favored and presumably represents the native form of PTMP1, while the other isoform is kinetically favored but is likely non-native. Oligomerization during refolding was influenced by, but not strictly dependent on disulfide formation. The conformational stability of PTMP1 indicates an influence of intramolecular disulfides on the native state, but not on unfolding intermediates. Monomeric PTMP1 exhibited a high thermal stability, dependent on the pH of the surrounding environment. Especially under acidic conditions the disulfide bonds were critically involved in thermal stability.

1. Introduction

Sessile marine mussels adhere to several kinds of substrates by a unique holdfast, the mussel byssus. In case of the blue mussel (*Mytilus galloprovincialis* Lamarck, 1819) the byssus is composed of several dozens of threads which are attached to a byssus stem arising from the mussel foot. Each byssal thread can be morphologically divided into three different sections, a proximal portion with a wavy surface shape, a distal portion with a rather smooth (but granular) surface and an adhesive plaque (Brown, 1952). The latter represents an oval disc-like structure and mediates firm attachment of a thread to the substratum. Apart from their differences in appearance, the proximal and distal thread sections also exhibit distinct mechanical properties. Whereas the distal portion shows a high stiffness and yielding behavior, the proximal portion is elastic and much more extensible (Bell and Gosline, 1996). Thereby, the mussel avoids weak spots caused by sharp transitions between the soft mussel tissue and hard substrates (Waite et al., 2004). Overall, byssal threads show a high mechanical toughness necessary to function as shock-absorbing tethers upon waves and currents (Waite et al., 2002).

Byssal threads are to a large extent composed of proteins, with special fibrillar collagens, called preCols (Qin and Waite, 1995), representing the main load-bearing components (Hagenau et al., 2011), which are surrounded by matrix proteins, characterizing the byssus as a biological composite (Sun et al., 2001). The content

Abbreviations: CD, circular dichroism; ESI, electrospray ionization; FTIR, Fourier transform infrared spectroscopy; GdmCl, guanidinium hydrochloride; IAEDANS, 5-[2-[(2-Iodo-1-oxoethyl)amino]ethylamino]-1-naphthalenesulfonic acid; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PTMP1, Proximal Thread Matrix Protein 1; SH, sulfhydryl; tet, tetracycline; TCEP, Tris(2-carboxyethyl)phosphine; TMP, thread matrix protein; TOF, time-of-flight; VWA, von Willebrand factor type A-like.

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of matrix proteins differs significantly in the respective proximal and distal thread sections (Bairati and Vitellaro-Zuccarello, 1976; Hagenau et al., 2009). While matrix proteins represent only a minor portion in the distal section (~4%), in the proximal section they represent more than one third of the dry weight (Waite et al., 2002). In respect to their abundance, especially in the proximal thread section, the biological role of the byssal matrix proteins was assumed to function as lubricant (Sagert and Waite, 2009), fibril connector (Waite et al., 2002), as well as spacer and mediator of integrity (Suhre et al., 2014b). To date, two different types of byssal matrix proteins were identified: the thread matrix proteins (TMPs) (Sagert and Waite, 2009; Sagert et al., 2006), and the Proximal Thread Matrix Protein 1 (PTMP1) (Sun et al., 2002). The latter was detected in proximal thread sections via mass spectrometry by direct laser ablation of thread sections, and the respective protein could be purified from the byssus (Sun et al., 2002). The molecular weight of PTMP1 appeared to be 50 kDa and the protein was observed to be moderately post-translationally modified by glycans. Immunohistochemical analysis revealed that the protein was exclusively associated with the proximal thread section. The cDNA sequence of PTMP1 was deduced from a cDNA-library by PCR employing degenerate primers towards the DNA sequences encoding the experimentally determined amino- and carboxyterminal protein sequences of PTMP1. The sequence revealed that the protein mainly consists of two tandem-repeated sequence stretches belonging to the group of von Willebrand factor type A-like (VWA) domains, commonly found in extracellular proteins (Whittaker and Hynes, 2002) and often associated with collagen binding (Brondijk et al., 2012; Emsley et al., 2000; Fresquet et al., 2007; Klatt et al., 2011). Prominent examples of such proteins with VWA domains are collagen type VI (Beecher et al., 2011), integrins (here, these domains are called I-domains) (Lee et al., 1995; Qu and Leahy, 1995), von Willebrand factor (Emsley et al., 1998; Huizinga et al., 1997), and the matrilins (Wagener et al., 2005). All VWA-domains share a common Rossmann-fold (Rossmann and Argos, 1981) exhibiting a central β-sheet surrounded by α-helices. Often, the edges of these domains are clamped by a stabilizing intramolecular disulfide bond (Cruz et al., 1995; Huizinga et al., 1997; Lacy et al., 2004).

The recently resolved crystal structure of full-length PTMP1 revealed two VWA domains arranged and interconnected in a unique fashion by a highly stable β-sheet linker (Suhre et al., 2014b). This special arrangement is critical for the high structural stability of the entire protein and is also a prerequisite for its capability to bind collagens with high affinities. Upon binding to collagens, PTMP1 affects fibril assembly and arrangement. On the one hand, it might be involved in spacing the collagen fibrils, on the other hand in preventing their slippage out of the matrix and, therefore, preventing micro-scale failure of the thread upon stretching.

Here, we show that denatured full-length PTMP1 refolds into two distinct stable monomeric isoforms being in equilibrium. Both isomers are fully, but differently disulfide bridged, and the disulfide bonds of PTMP1 are critically involved in structural stabilization of the native state of the protein.

2. Materials and methods

2.1. Cloning and expression

PTMP1 and its variants were cloned and recombinantly produced as SUMO fusion constructs as described earlier (Suhre et al., 2014a,b). Additionally, cysteines were replaced by serines in the full length sequence using site directed mutagenesis creating the variants C244S/C440S (C244S, C440S) and NoCys (C49S, C241S, C244S, C248S, C437S, C440S).

2.2. Protein purification and refolding

PTMP1 and its variants were purified from bacterial inclusion bodies as described previously (Suhre et al., 2014a,b). Briefly, inclusion bodies were isolated from bacterial pellets by detergent and ultrasonic treatment. Afterwards, inclusion bodies were denatured in 8 M urea, reduced with 20 mM Dithiothreitol (DTT) and purified by affinity chromatography (Ni-loaded IMAC) under reducing conditions (5 mM DTT). The SUMO-tag was removed by ULP1-treatment in presence of 1 M urea, 1 mM DTT and a subsequent second IMAC step. Afterwards, proteins were refolded upon extensive dialysis against buffer A (20 mM sodium phosphate pH 8, 150 mM sodium chloride) at protein concentrations below 1 mg/mL. To separate refolding and disulfide bond formation, the first dialysis step was performed in the presence of 1 mM DTT. Proteins were dialyzed against 10 mM ammonium bi-carbonate and lyophilized. Resolubilized and refolded proteins were separated by size exclusion chromatography (SEC, HiLoad 26/60 Superdex 200 pg, GE Healthcare) in buffer A at a flow rate of 3 mL/min. Fractions of 3 mL were collected. To store proteins, they were dialyzed against 10 mM ammonium bi-carbonate and lyophilized. For analysis, proteins were resolubilized in buffers, and insoluble protein was removed by ultracentrifugation (186,000*g* for 30 min). Protein concentrations were determined by UV spectroscopy using the following calculated extinction coefficients (ProtParam, www.expasy.org): PTMP1 (15,275 M^{-1} cm^{-1}), C244S/C440S (15,150 M^{-1} cm^{-1}), NoCys (14,900 M^{-1} cm^{-1}), A1 and A2 (4595 M^{-1} cm^{-1}).

2.3. Size exclusion chromatography coupled with multi-angle-light-scattering (SEC–MALS)

SEC–MALS experiments were performed using a Superdex 200 10/300 GL column (GE Healthcare) at an 1100 series HPLC device (Agilent). Protein solutions (200 μL) were injected in phosphate buffer at a flow rate of 0.8 mL/min. Chromatograms were recorded by measuring the absorbance at 280 nm, as well as the refraction index (Shodex RI-71, Showa Denko). MALS was recorded using a DAWN EOS (Wyatt Technology) light scattering detector. Molecular weights were calculated employing the Astra 6.0.1 software (Wyatt Technology) using the Zimm-model (Zimm, 1948).

2.4. Determination of free sulfhydryl groups (Ellman's test)

Free sulfhydryl (SH^-) groups were determined by cleavage of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) (Ellman, 1959). 2 μM of the respective protein were incubated in 100 mM Tris/HCl pH 8.0, 0.3 mM DTNB, 0.03 mM EDTA in water for 20 min, and the absorbance was recorded and background corrected at 412 nm. To detect SH-groups buried inside the structure, all experiments were additionally performed in presence of 6 M guanidinium hydrochloride (GdmCl) leading to similar results. The concentration of free SH-groups was calculated employing molar absorption coefficients of 13,700 M^{-1} cm^{-1} (GdmCl) or 14,150 M^{-1} cm^{-1} (water) (Riddles et al., 1983).

2.5. Circular dichroism (CD) spectroscopy

CD spectra were recorded in 1 mm cuvettes using a Jasco J-815 spectropolarimeter (Jasco) at a scanning speed of 50 nm/min, with a response time of 2 s and accumulating 3–5 single spectra. All spectra were buffer-corrected. Thermal stability of the proteins was evaluated by recording the CD signal of 0.2 mg/mL protein solutions in 10 mM sodium phosphate (pH 8) or 10 mM sodium acetate (pH 4) in the absence and presence of 1 mM Tris(2-carboxyethyl)phosphine (TCEP) at a wavelength of 222 nm with a heating and cooling rate of 60 K h^{-1}. Stability against denaturation