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The dynamics of multimer formation of the amphiphilic hydrophobin protein HFBII

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ABSTRACT

Hydrophobins are surface-active proteins produced by filamentous fungi. They have amphiphilic structures and form multimers in aqueous solution to shield their hydrophobic regions. The proteins rearrange at interfaces and self-assemble into films that can show a very high degree of structural order. Little is known on dynamics of multimer interactions in solution and how this is affected by other components. In this work we examine the multimer dynamics by stopped-flow fluorescence measurements and Förster Resonance Energy Transfer (FRET) using the class II hydrophobin HFBII. The half-life of exchange in the multimer state was 0.9 s at 22 °C with an activation energy of 92 kJ/mol. The multimer exchange process of HFBII was shown to be significantly affected by the closely related HFBI hydrophobin, lowering both activation energy and half-life for exchange. Lower molecular weight surfactants interacted in very selective ways, but other surface active proteins did not influence the rates of exchange. The results indicate that the multimer formation is driven by specific molecular interactions that distinguish different hydrophobins from each other.

1. Introduction

Hydrophobins are amphiphilic proteins produced by filamentous fungi. Structural analysis by x-ray crystallography shows that the proteins are globular and have one distinct patch on their surface where only aliphatic side chains are exposed to solvent [1]. The presence of this hydrophobic patch in the otherwise hydrophilic surface of the proteins suggests how hydrophobins function as amphiphiles (Fig. 1). The discovery of these unusual amphiphilic structures presents the question of how these differ from more commonly encountered surfactants such as phospholipids or bile acids [2]. We find that hydrophobins have several unique functions such as their role in attachment or as coatings on fungal structures [3,4]. Hydrophobins are found in all filamentous fungi and sequence comparisons show that they can be divided into two families, class I and II [5]. Although clearly homologous, the two families have some properties that are distinct, and the discussion here uses class II as a starting point, and does not address whether the conclusions are applicable to class I or not.

Biophysical characterization reveals another distinct property; the surface elasticity of interfacial films of some hydrophobins is very high, even exceeding 1 N/m, being an order of magnitude higher than that of any other known type of surfactant. It is noteworthy that the build-up of these interfacial layers can take a very long time, up to hours to form [6,7]. We also understand that the properties at the air-water interface stem from a very precise molecular arrangement of interacting hydrophobin molecules [8]. While having these remarkable properties at interfaces many hydrophobins are very soluble in aqueous solution, easily exceeding 100 mg/ml. Experiments show that the high solubility is due to the formation of multimers in solution, predominantly tetramers that lead to shielding the hydrophobic patches and therefore high solubility (Fig. 1B).

Here we study the class II hydrophobin HFBII from *Trichoderma reesei* to address the question of the dynamics of multimers in solution. So far, we have a very limited understanding of the dynamics of interactions between HFBII molecules in solution. We may pose questions such as: What is the timescale of assembly of solution multimers? Can multimers be stabilized or destabilized? What types of molecules interact with multimers? Is there interplay between different types of hydrophobins in solution or does molecular recognition distinguish between different structures? Answers to these questions will help understand both the assembly pro-

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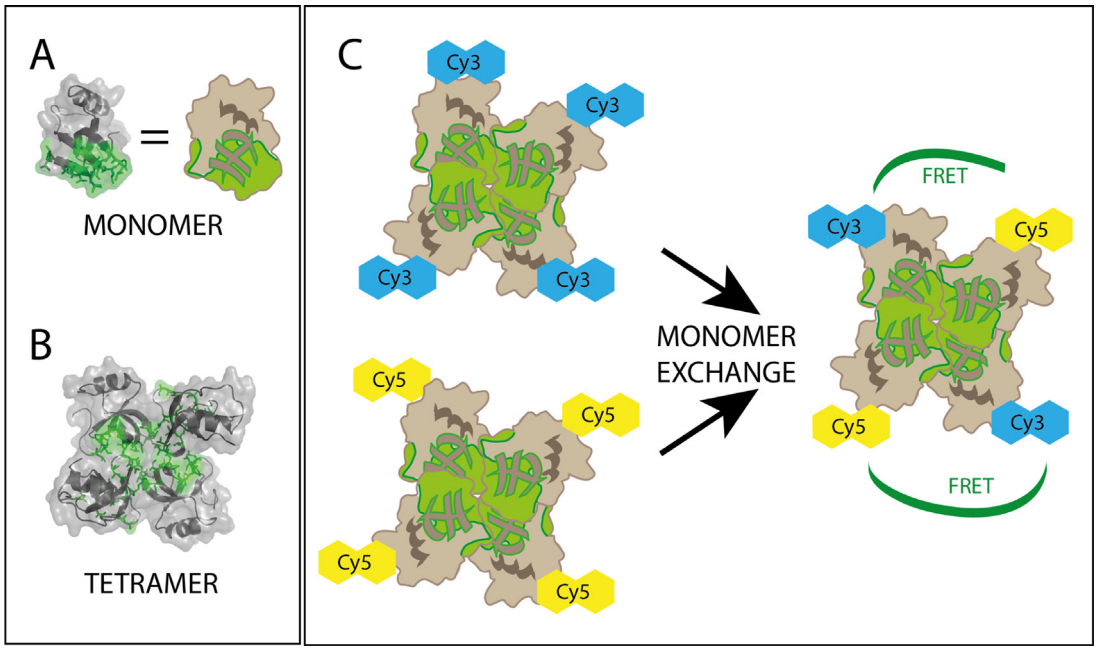

Fig. 1. A) The structure of HFBII shows that its hydrophobic patch comprises 12% of its surface area (shown in green). B) HFBII becomes highly soluble in water by shielding the hydrophobic patches in multimers. C) By labelling HFBII with Cy3 and Cy5 fluorescent labels, forming a FRET pair, the dynamics of the exchange of protein molecules between multimers could be measured. The exchange of monomers probably involves multiple intermediate steps. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cess of hydrophobins *in vivo* as well as industrial applications of hydrophobins [9–13]. Such applications are for example the use of HFBII as an emulsifier or for the production of extremely stable foams for the food industry [2,14]. In pharmaceutical applications hydrophobins are used for dispersion of drugs to modify uptake and distribution in organs [15]. Other applications are found in protein purification or immobilization [16]. Understanding the assembly process of hydrophobins is also important for understanding the physiology of fungi. Hydrophobins have roles in surface hydrophobicity and properties of for example fruiting bodies and spores, they can play a role in attachment to surfaces, and they affect the way in which fungi grow and interact with their environment [17].

Our current understanding is that multimer formation in solution makes hydrophobins highly soluble by shielding their hydrophobic patches. The behaviour of HFBI in solution has been shown to be dependent on hydrophobin concentration [18]. By increasing hydrophobin concentration, an increasing fraction of proteins were in the multimer form. The assemblies that hydrophobins form at interfaces have a distinctly different geometry than in solution, indicating that a reformation of assemblies is triggered by the interface [8].

To study the dynamics of multimer exchange we used fluorescent labels that formed a Förster Resonance Energy Transfer (FRET)-pair (Fig. 1C). By having two otherwise identical solutions of HFBII at equal concentration, but each labelled with one of the fluorophores forming the FRET pair, and mixing these in a stopped-flow apparatus we could obtain the half-time of exchange of HFBII in multimer complexes. This exchange is not driven by differences in concentration, but reflects exchange of protein molecules between multimers at equilibrium.

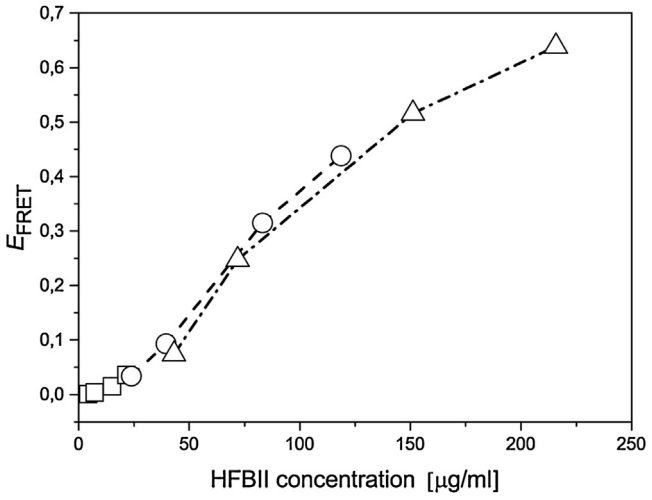

Fig. 2. Equilibrium experiment showing FRET after mixing equal amounts of Cy3 and Cy5 labeled HFBII. Increasing the concentration of HFBII gives an increased FRET efficiency. This shows that multimers are formed in a concentration-dependent manner. Experimental series with different ratio of labelled to unlabelled proteins overlap each other (squares 1:1, circles 1:4.5, and triangles 1:9).

2. Experimental

2.1. Materials

The class II hydrophobin proteins HFBI and HFBII were purified from *T. reesei* mycelium (HFBI) or culture supernatant (HFBII) using two-phase extraction and reversed phase chromatography as described earlier [18,19]. In order to conjugate HFBII at specific sites we engineered a variant termed HFBII-CysC that has an additional Cys residue at the C-terminus. The *T. reesei* hfb2 gene in plasmid pTNS3117 was mutated using a site-directed mutagenesis kit (QuikChange, Stratagene, USA) and oligonucleotides 5′-GCCATCGGCACCTTCTGCACCTAAGGATCCCCCGGG-3′ (sense) and 5′-CCCGGGGGATCCTTAGGTGCAGAAGGTGCCGATGGC-3′ (antisense) (Sigma-Genosys Ltd) in order to insert residues Cys-Thr before the stop codon. The resulting plasmid, termed pGZ14, contained the HFBII-CysC encoding sequence and was controlled by cbh1 promoter and terminator. Transformation into *T. reesei* (resulting in strain VTT-D-061177), expression and purification