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The dynamics of multimer formation of the amphiphilic hydrophobin protein HFBII



COLLOIDS AND SURFACES B

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1. Introduction

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 $22 \degree \text{C}$ 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.

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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.

worthy 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*

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 note-

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.

2. Experimental

2.1. Materials

The class II hydrophobin proteins HFBI and HFBII were purified from *T. reesei* mycelium (HFBI) or culture supernatant (HFBII)



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).

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



Fig. 3. A) Data from a stopped-flow FRET experiment at 17.5° C with the fitted single exponential curve (in red). B) Residuals show an even distribution throughout the range of data points. C) Residuals show a normal distribution around the exponential model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were done as described earlier [18]. HFBII-CysC was conjugated with either cyanine dye 3 donor (Cy3-monomaleimide), or cyanine dye 5 acceptor (Cy5- monomaleimide, both from GE-Healthcare) in order to form a FRET pair.

2.2. Fluorescence spectroscopy and FRET

The fluorescence spectra of the samples were measured with a fluorescence spectrophotometer, Cary Eclipse (Varian) with a thermostat set at 22 °C. The samples were excited at 516 nm (donor excitation), and the emission spectra from both the donor and acceptor were recorded. To cover the range of total protein from 1 to 225 μ g/ml, the FRET experiments were performed using the labelled protein without addition of non-labelled protein (1:1), and with unlabelled added to ratios 1:4.5 and 1:9. The high fluorescence signal of the labelled HFBII allowed measuring low concentrations of HFBII, and the addition of unlabelled HFBII was required for high protein concentrations, as otherwise the fluorescence would be very high and saturate the photomultiplier detector in the spectrophotometer. Calculations of the efficiency of energy transfer, E, were performed precisely as described previously [18].

2.3. Stopped-flow FRET measurements

Sample concentration and volumes: Stopped-flow FRET was measured between fluorescently labelled hydrophobin proteins using a Chirascan SF.3 spectrometer (Applied Photophysics) with a fluorescence detector and a CS/SF stopped-flow unit. Cy3- and Cy5-labeled HFBII-CysC at equal concentrations were mixed in the stopped-flow cell and excited with donor wavelength (516 nm). The FRET signal arises when labelled proteins are brought into close proximity, and a transfer of energy will occur from donor molecules to acceptor molecules. Growth of the acceptor emission (or decay of the donor emission) can be measured on a time scale suitable for following molecular processes. The samples were prepared with a 1:10 ratio of labelled HFBII-CysC to unlabelled HFBII. Each syringe was loaded with $10 \,\mu g/ml$ labelled HFBII and 90 µg/ml wild-type HFBII, with the Cy3 in syringe 1 and Cy5 label in syringe 2 resulting in a total HFBII concentration in each syringe of $100 \,\mu g/ml$. The $100 \,\mu g/ml$ total HFBII was set as reference. The addition of wild-type was made in order to reduce the very high fluorescence signal. Measurements of the FRET signal as a function of time were performed at acceptor emission of 665 nm and

Table 1

Ea, t_{1/2} diss for selected proteins including HFBII.*

Protein	t _{1/2} diss.	Ea (kJ/mole)	Temp (°C)
HFBII multimer	0.88 s	92	22
Insulin monomer-dimer [20]	6.08*10 ⁻⁹ s ^a	10.5 ass. 30,9 diss.	23
Phosphorylase b, two dimers to tetramer [21]	1.5 min ^a	12.3 ass. ^b 32,3 diss. ^b	25
recombinant humanized antibody (rhuMAb) VEGF self-association [22]	1 h ^a	45.2	30
Spectrin dimer – tetramer [23]	10 h ^a	250 ass. 460 diss.	29,5
Intermediate state of Cytochrom C [24]	0,04 s ^a	50	20
Bence-Jones protein Au variable fragment dimerization [25]	0,005 s ^a	N/A	20
TATA binding protein (TBP) dimer diss. [26]	7,4 min	N/A	25

* Data has been converted to the appropriate units when necessary.

^a Calculated from k_{diss} assuming first order kinetics $(t_{1/2} = \ln(2)/k_{diss})$.

^b Approximated using two-point Arrhenius.

Table 2

Effect of different solution additives on half-life, energy of activation (Ea), and frequency factor (A). All additions were made to the 100 µg/ml HFBII sample, and comparison in per cent (%) change are made to this sample. Values for half-life are given for 20 °C.

Sample	Half-life [s]	% change relative to 100 μg/ml HFBII	Ea [kJ/mol]	A [1/s]
HFBII (100 µg/ml)	0.88 ± 0.08		92.51	1.95E+16
+100 μg/ml HFBII	0.79 ± 0.08	-10	88.17	3.51E+15
+100 µg/ml HFBI	0.60 ± 0.06	-32	41.38	2.26E+07
+1 mg/ml βCas	0.90 ± 0.08	2	69.44	1.46E+12
+1 mg/ml βLG	0.90 ± 0.08	2	86.83	1.88E+15
+5% ethanol	0.26 ± 0.04	-71	83.38	1.60E+15
+0.1% Tween	0.40 ± 0.04	-55	91.81	3.31E+16
+0.01% SDS	0.92 ± 0.08	5	151.19	5.27E+26
+10 mM NaCl	0.92 ± 0.08	5	92.42	1.77E+16
+50 mM NaCl	1.02 ± 0.09	16	99.33	2.70E+17
+100 mM NaCl	1.31 ± 0.09	49	84.88	5.72E+14
+200 mM NaCl	1.57 ± 0.09	78	92.7	1.17E+16

the drive volume was set to 140 µl. As the protein concentration did not change during the course of each measurement the observed change in FRET signal corresponds to the rate of monomer exchange in the multimers. Activation energies were determined by measuring the exchange rate at three different temperatures (21.5 °C, 17.5 °C and 12.5 °C). Furthermore, the effect of additional proteins: β -lactoglobulin (β LG) and β -casein (β Cas) (1 mg/ml), (Sigma-Aldrich), surfactants Tween-20 (0.1%), Sodium dodecylsulfate (SDS) (0.01%) (Sigma-Aldrich, USA) as well as ethanol (5%) and salt (NaCl 10, 50, 100 and 200 mM) on HFBII dynamics were examined and compared to the reference of 100 µg/ml total HFBII. In all cases the concentrations and ratios of all components were identical in both syringes, except for the structure of the fluorophore (i.e. Cy3 or Cy5).

2.4. Data collection and analysis

A single exponential curve was fitted to the FRET fluorescence data using Microcal origin (Microcal) or Pro-data viewer (Applied Photophysics, UK) software and the Marquardt-Levenberg algorithm. From the equation for the exponential decay (Eq. (1)):

$$c(t) = ae^{-kt} + c_0 \tag{1}$$

where c(t) is the FRET signal at time t and k is the rate constant. The half-life was calculated by Eq. (2):

$$t_{1/2} = \frac{\ln(2)}{k}$$
 (2)

2.5. Arrhenius equation and activation energy

Three temperatures were chosen, $21.5 \,^{\circ}$ C, $17.5 \,^{\circ}$ C and $12.5 \,^{\circ}$ C in order to examine the temperature dependency of the multimer

exchange of HFBII by stopped-flow FRET. The Arrhenius equation, Eq. (3):

$$k = Ae^{-E_{q}/RT}$$
(3)

gives activation energy E_a and frequency factor A representing the frequency of collisions between monomers by plotting ln(k) vs. 1/T, where k is the reaction rate constant from fitting and T temperature.

3. Results and discussion

Initially we established that a FRET signal was formed in a concentration dependent manner when Cy3 and Cy5 labelled HFBII were mixed together, confirming that HFBII forms multimers in solution with higher concentrations driving molecules towards interactions (Fig. 2). The results were consistent with an earlier study of HFBI and show that the FRET signal increases at increasing concentrations of protein [18]. This means that multimers are favoured at high HFBII concentrations, and since multimers provide a mechanism for shielding the hydrophobic part of the protein, this observation offers an explanation for the high solubility of HFBII. The sigmoidal shape of the curve shows that multimer formation occurs to a lesser extent at low concentrations and at high protein concentrations an increasing fraction of proteins are found in the multimers. Towards high concentrations (over 250 µg/ml) there is a trend towards saturation, i.e. that HFBI molecules are predominantly in the tetramer state.

The good overlap of the curves using different ratios of labelled to non-labelled HFBII (Fig. 2) showed that there was no adverse effect on the protein function caused by labelling. Similarly, changing the ratio of labelled to non-labelled protein by doubling or reducing it to a half did not affect stopped-flow FRET results as described below, showing that labelled protein functioned identically to the wild-type.

We next turned to the question of the dynamics of the HFBII multimers in solution. We hypothesized that multimers are not static,



Fig. 4. The half-life of HFBII complexes at different temperatures with different additives. A) with HFBI, β -lactoglobulin (β LG), and β -casein (β Cas). B) With ethanol, Tween 20, and SDS. D) With different concentrations of NaCl.



Fig. 5. Data for representative cases showing the linear relation between ln(k) and 1/T as predicted by the Arrhenius equation. Results for HFBII (squares), HFBII + HFBI (circles), and HFBII + β LG (triangles) are shown.

but that an exchange of molecules occurs continuously between them. The dynamics of exchange of monomers between the multimer complexes are likely to affect properties such as assembly at interfaces. To study the dynamics of the multimers we used a stopped-flow apparatus to mix two solutions of HFBII, one containing HFBII labelled with Cy3 (HFBIICy3) the other containing HFBII labelled with Cy5 (HFBIICy5). HFBIICy5 was loaded in one syringe and HFBIICy3 was loaded in another. This was done so that the concentration of HFBIICy3 was the same as HFBIICy5. After mixing the samples in the stopped-flow apparatus, we measured the rate at which the FRET signal was formed. Since the overall concentration of HFBII remained constant throughout each stopped-flow experiment, there was no net formation of multimers, only an equilibrium exchange of protein molecules between multimers. The FRET signal therefore formed as Cy3 and Cy5 labelled HFBII molecules exchanged between multimers, resulting in mixed Cy3 - Cy5 multimers.

Since the experiment was set up to measure the rate at which individual protein molecules exchange between multimers at equilibrium, we expected the kinetics to follow an exponential decay model. Indeed, a first order exponential model showed excellent agreement with the measured data (Fig. 3) giving a half-life ($t_{1/2}$) for the complex. Residuals are evenly spread around the fitted curve over the entire range of data. The $t_{1/2}$ –values show a clear dependency on temperate (Fig. 4). At 20 °C the $t_{1/2}$ for HFBII was 1.2 s at a protein concentration of 100 µg/ml.

Comparing the $t_{1/2}$ of the HFBII complex to other measured rates of protein interactions we find that the exchange is slow compared to several dimerization interactions (Table 1), which have significantly smaller $t_{1/2}$ –values. On the other hand another group of interactions can have $t_{1/2}$ –values up to hours. These slow exchange rates are, however, typically found in ligand interactions that fulfil a defined regulatory or immunosensing functions. Searching the literature we could not find other examples of protein complexes with lifetimes ($t_{1/2}$) in the range of seconds.

Measuring the temperature dependency of rate (Fig. 4) allowed us to calculate the activation energy and the frequency factor of the exchange of monomers between multimers by applying the Arrhenius equation to the data as shown in Fig. 5. The activation energy (Ea) for the HFBII multimer exchange at 100 μ g/ml was 92 kJ/mol and the Frequency factor (A) was 1.95E+16.

To gain a better understanding of the nature of the mechanisms of multimer stabilization, we studied how changing solvent conditions or adding possibly interacting compounds to the solution affected HFBII multimers. For these different conditions we determined the t_{y_2} as well as Arrhenius parameters (Table 2). First, doubling the HFBII concentration to 200 µg/ml, did not significantly affect t_{y_2} . However, if HFBI was added at a final concentration of 100 µg/ml there was a clear decrease of t_{y_2} by over 30%. To interpret this result we can consider the following reasoning. If no change had occurred there could have been two alternative conclusions: either the added HFBI would behave identically to HFBII (similarly as the equal addition of HFBII did not affect t_{y_2}) or that HFBI and HFBII multimerization would be completely independent (one not affecting the other in any way). We conclude that the decrease in t_{y_2} observed here therefore shows that there is a significant interaction between HFBI and HFBII multimers in solution, leading to some sort of destabilization of the multimer formation of HFBII.

Notably, the destabilization of HFBII multimers by HFBI was seen in the temperature dependency of this interaction, resulting in an activation energy (Ea) that was about half of that with only HFBII. No other conditions (as described below) resulted in such a lowering of activation energies. Adding HFBII to an even higher concentration (200 μ g/ml) gave only a small increase in exchange rate.

Next we screened different categories of molecules, other proteins, solvents, surfactants, and ionic strength (Table 2). Addition of the surface-active proteins β -casein and β -lactoglubulin (also Fig. 4) did not affect the t_{1/2} of HFBII indicating that an interaction with other proteins, even surface active-ones, is not a general property of HFBII. This understanding can be useful as many potential uses of hydrophobins are to stabilize interfaces in complex mixtures, for example involving milk proteins, as some suggested applications of hydrophobins are in milk-based foods [7,14]. The addition of 5% ethanol gave a very significant reduction in t_{1/2}. This finding is consistent with earlier observations that solvents, especially ethanol, can break up assemblies of class II hydrophobins [27]. It is likely that ethanol molecules interact favourably with the side chains of the hydrophobic patch of HFBII, thereby increasing solubility of the monomeric HFBII.

The effects of the surfactants SDS and Tween 20 were very different. While Tween 20 resulted in a similar effect as ethanol resulting in a significantly faster exchange, there was practically no effect by adding SDS. This result was unexpected since it previously has been concluded that SDS is efficient in breaking up complexes of Class II hydrophobins, and suggests that SDS does not work on the level of solution multimers, but rather on higher-level assemblies. The similarities between ethanol and Tween 20 in lowering t_{y_2} results suggest that the non-ionic Tween 20 would act by stabilizing the monomeric form of HFBII. Addition of increasing concentrations of NaCl was used to study the hydrophobic nature of multimerization interactions. There is a clear increase in the t_{y_2} with increasing salt concentrations. This is in line with a strengthening of hydrophobic interactions as can be expected.

Measuring the time scale for multimer dynamics of hydrophobins, broadens our insight in the behaviour of hydrophobins as amphiphiles and verifies the currently accepted model for function where solution multimerization results in a high solubility of hydrophobin. Hydrophobin multimers in solution are dynamic and continuously disassemble and reassemble. This study shows the overall dynamics of monomer exchange between multimers, but we cannot determine from these data if the mechanism goes through monomers or dimers or even how many intermediates there are, or what the rate-limiting step is. We can, however, say that it is a relatively slow process, happening only at the scale of seconds, but the process is still not as slow as to be rate-limiting for interfacial assembly which can take substantially longer, up to hours [6]. The interference by HFBI on the multimerization of HFBII indicates that the molecular recognition between hydrophobins is not so strict as to discriminate between different types, and that HFBI and HFBII can affect the functions of each

other, indicating that molecular recognition is an important part of multimer formation. The exact nature of this possible mechanism of molecular recognition is unclear. A future challenge will be to understand how the different details of hydrophobin function are related to their biological functions and the mechanism in which multimers in solution assemble into interfacial membranes.

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