Unique Thermal & Chemical Stability of the ChromoTek GFP-Binding Protein:GFP Complex

Abstract

The study of protein interactions by methods such as surface plasmon resonance requires the immobilization of a protein of interest on a biosensor. Ideally, this immobilization is unaffected by a large number of regeneration cycles under varying, often harsh chemical conditions. Here, we show that the ChromoTek GFP-binding protein (GBP, ChromoTek gt-250) in combination with the widely used GFP-tag specifically answers to the need of high chemical stability and is thus an ideal candidate for a biosensor capture molecule. Using nanoDSF technology (Prometheus NT.48, Nanotemper, http://www.nanotemper-technologies.com), we analyse the thermal and chemical stability of GBP and its complex with GFP. Strikingly, the melting temperature of this ChromoTek GBP:GFP complex is as high as 83 °C and the complex is almost insensitive to urea up to 8 M. This extraordinary stability of the ChromoTek GBP:GFP complex emphasizes why it is considered an excellent alternative to conventional immobilization techniques in biosensor-based technologies.

Summary

1. The combination of the ChromoTek GFP-binding protein (GBP) and GFP is ideal for applications that require a highly stable interaction between a capture molecule and a protein tag such as surface plasmon resonance analysis.
2. This GBP:GFP complex may be considered as an alternative to the streptavidin:biotin complex.
3. GFP-binding protein is stable and functional up to 68°C, whereas the GFP-binding protein-GFP complex is stable up to even 83°C.
4. The GFP-binding protein efficiently binds GFP even under denaturing conditions of 6-8 M urea or 1-2 M guanidinium hydrochloride (GdCl) after prolonged incubation for 16h. Short term exposure at even elevated denaturant concentrations may be tolerated.
5. The GFP-Binding protein:GFP complex is stable in 8 M urea with a melting point T_m of 51 °C.
6. The ChromoTek GFP-Trap comprises the GBP; its superior performance for immunoprecipitation of GFP fusion proteins is also a result of that extraordinarily stable complex formation.
7. Guanidinium hydrochloride at concentrations of 5-6 M may be used to elute GFP-fusion proteins bound to the GFP-Trap. This is an alternative for elution by boiling in Laemmli buffer.

Introduction

V_HH (also called nanobodies) are a remarkable class of single-domain antibodies. They constitute the heavy chain variable domain derived from camelid heavy chain-only antibodies and are the smallest, functional antibody fragments (13-15 kDa). Like conventional antibodies, V_HH bind to their cognate antigen with high affinity, i.e. with dissociation constants (K_D) in the nanomolar to low picomolar range. Unlike most conventional antibodies, however, V_HH tend to be extremely stable and remain functional at high temperatures and under harsh chemical conditions (van der Linden et al. 1999, Dumoulin et al. 2002, Muyldermans 2013).

One such highly stable V_HH is the ChromoTek GFP-binding protein (GBP). The ChromoTek GBP binds very tightly to green fluorescent protein (GFP) and many GFP derivatives with a K_D of 1 pM[1]. GBP is widely used for the analysis of GFP-fusion proteins by methods such as immunoprecipitation (ChromoTek GFP-Trap), immunofluorescence (ChromoTek GFP-Booster), and kinetics studies. Even denaturing conditions such as 8 M urea have been shown not to interfere with GFP-binding by GBP immobilized on a matrix such as agarose beads (ChromoTek GFP-Trap, see application note "Ubiquitination of GFP-tagged proteins"). This extreme resistance of GBP to urea has been successfully exploited in the analysis of covalent modifications of GFP-fusion proteins such as ubiquitination (see, for example, Min et al. 2013, Despras et al. 2016).

The analysis of protein interactions has also benefitted from the high stability of GBP and its complex with GFP. Methods to determine binding kinetics such as surface plasmon resonance (SPR), biolayer interferometry (BLI), surface acoustic wave SAW), or SwitchSense all rely on the immobilization of a protein on the surface of a biosensor chip or tip. Furthermore, these methods require the immobilized protein to remain on the biosensor for prolonged times and under varying chemical conditions owing to consecutive cycles of analyte binding and regeneration. GBP and its complex with GFP meet these needs through a very slow dissociation rate constant ($k_{off} = 3 \cdot 10^{-5}$ s^{-1}) and tolerance to even harsh chemical conditions.

The use of GBP in SPR was extensively characterized by Della Pia & Martinez 2015. They showed that GBP outperforms conventional antibodies as a capture molecule in SPR. In addition, they reported that GBP is tolerant to heating and is functional over a wide pH range and how that benefits an SPR experiment.

Although GBP and its complex are thus well known to be of extraordinary stability, this stability has not been formally characterized owing to a dearth of practical methods applicable to the characterization of V_HH. Here, we employed a recently developed technology called nanoDSF (differential scanning fluorimetry). Using the Prometheus NT.48 (Nanotemper, http://www.nanotemper-technologies.com), we explored in detail the thermal and chemical stability of GBP and also the complex of GBP and GFP.

Results

Thermal stability of the GFP-binding protein and its complex with GFP

First, we analyzed the thermal stability of GBP and the complex GBP:GFP by monitoring protein autofluorescence at 350 nm and 330 nm during heating from 20 to 95 °C (Figure 1). The melting curve for GBP displays one sharp single transition, as would be expected for a single-domain protein. The melting temperature T_m of 68 °C indicates that GBP is indeed a highly stable protein.

[1] Kinetic parameters of the GFP-Trap have been determined using Dynamic Biosensors' switchSENSE® technology

In comparison, the melting curve of the complex GBP:GFP is characterized by a single transition with a T_m of 83 °C. In principle, melting curves of a protein complex may display several transitions if the melting of the single components is preceded by complex dissociation or a single transition if dissociation of the complex and melting of its subunits are concomitant. Thus, in the case of the complex GBP:GFP, the single transition indicates that the observed T_m is characteristic of the complex as a whole.

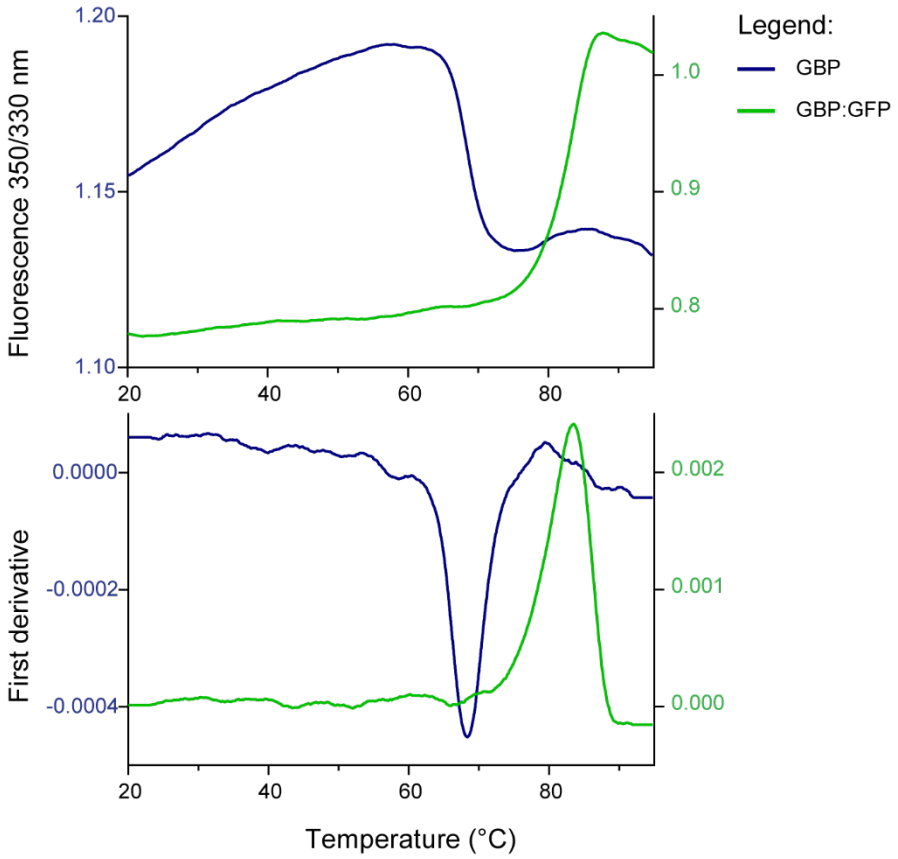

Figure 1 Melting curves for GBP and the complex GBP:GFP. *Shown is the ratio of the autofluorescence at 350 and 330 nm (top) and its first derivative (bottom).*

Besides, an interesting observation is the different autofluorescence 350/330 ratio of GBP and the complex GBP:GFP. This ratio is correlated with the transition of tryptophan residues from (usually) a hydrophobic environment within the protein core to solvent exposure after protein unfolding. In the case of GBP, the autofluorescence 350/330 ratio starts high and drops with protein denaturation, whereas the ratio for the complex GBP:GFP shows the opposite behavior. This difference can be explained by the presence of two tryptophan residues on the surface of GBP (in addition to one tryptophan each within the hydrophobic cores of GBP and GFP). These two tryptophan residues are already exposed to the solvent in folded apo-GBP, but reside within the paratope and are thus partly buried in the interface of the GBP:GFP complex. Thus, the different behavior of the autofluorescence ratio is in agreement with the notion that the unusually high T_m of 83 °C may be attributed to the GBP:GFP complex.

The shift in T_m from 68 to 83 °C indicates that GBP, which is already fairly heat-resistant by itself, is further stabilized by GFP. Thermal stabilization of a protein by a cognate ligand is a well-known effect (e.g. see Vedadi et al. 2006), and the complex of GBP:GFP may thus be another example.

Chemical stability of the GFP-binding protein and its complex with GFP

Next, we characterized the chemical stability of GBP and the complex GBP:GFP in two commonly used denaturants, guanidinium hydrochloride (GdCl) and urea. The proteins were incubated with 0-6 M GdCl or 0-8 M urea at room temperature for 16 h and their autofluorescence analysed using the Prometheus NT.48 (Figure 2).

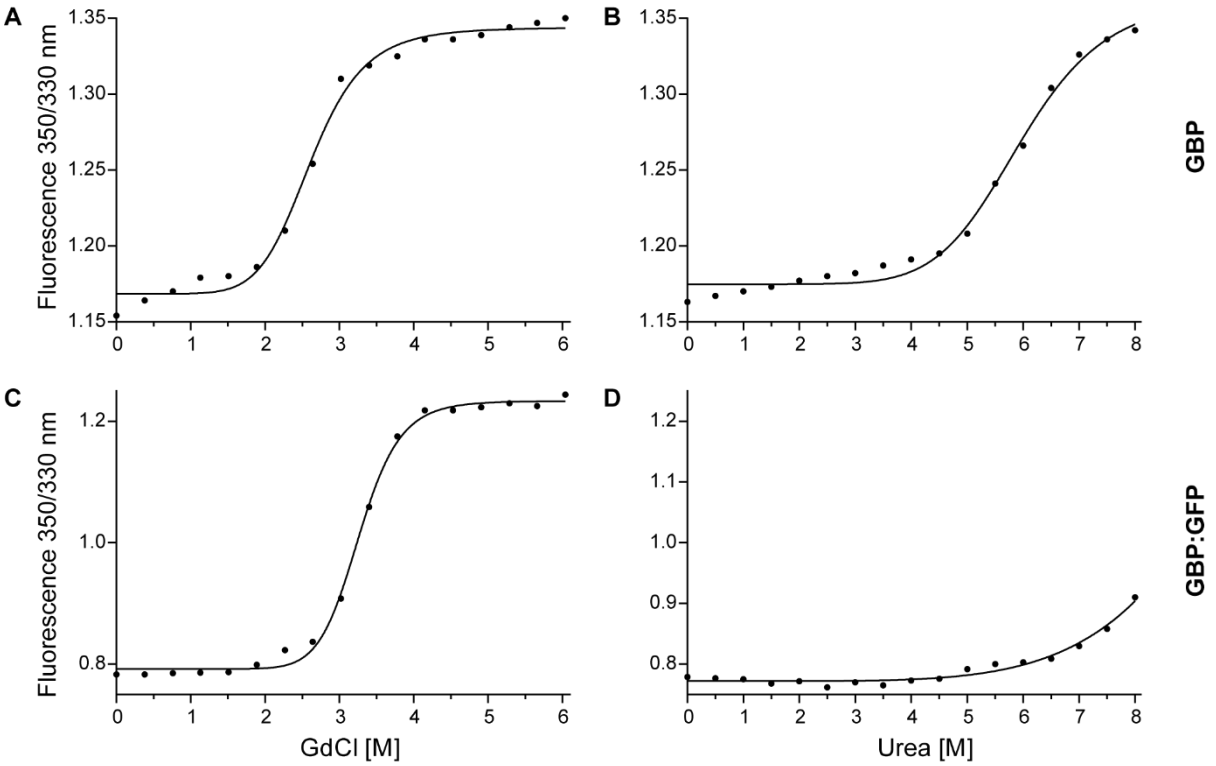

***Figure 2 Chemical denaturation of GBP and the complex GBP:GFP**. GBP (A, B) and the complex GBP:GFP (C, D) were incubated with 0-6 M guanidinium hydrochloride (GdCl; A, C) or 0-8 M urea (B, D). After overnight equilibration, fluorescence was recorded at 350 and 330 nm.*

In GdCl, GBP and the complex GBP:GFP unfolded cooperatively in a single transition with the midpoint C_m at 2.6 and 3.3 M GdCl, respectively. As in the case of thermal stability, the complex GBP:GFP is thus more stable than GBP alone. The observed C_m for GdCl compare favorably with data for other V_HH (Dumoulin et al. 2002).

In the case of our urea titration, we were unable to record complete unfolding curves for GBP and the complex GBP:GFP (Figure 2). We tested a concentration range of 0-8 M urea, which was not sufficient for complete denaturation of GBP alone and had almost no impact at all on the complex GBP:GFP. For GBP, the C_m is estimated to be in the range of 6-7 M urea, whereas it seems to be well above 8 M for the complex GBP:GFP. Thus, GBP is highly resistant to urea and extremely so in complex with GFP.

Using the Prometheus NT.48, we also performed a combined analysis of chemical and thermal stability of GBP and the complex GBP:GFP. To this end, we titrated proteins with GdCl and urea as above and subjected these reactions to heating to 95 °C (Figure 3, Figure 4). First, the presence of either denaturant reverses the behavior of the ratio of autofluorescence at 350 and 330 nm for GBP, as is already evident from the chemical denaturation experiment described in Figure 2. Second, increasing the concentration of either denaturant lowers T_m of GBP or the complex GBP:GFP proportionally. Strikingly, however, T_m of the complex GBP:GFP is still at 51 °C in 8 M urea (Figure 4). A T_m of 51 °C would be considered normal for a protein under physiological conditions (see e.g. Vedadi et al. 2006), but the same T_m in 8 M urea underscores the extraordinary stability of the GBP:GFP complex.

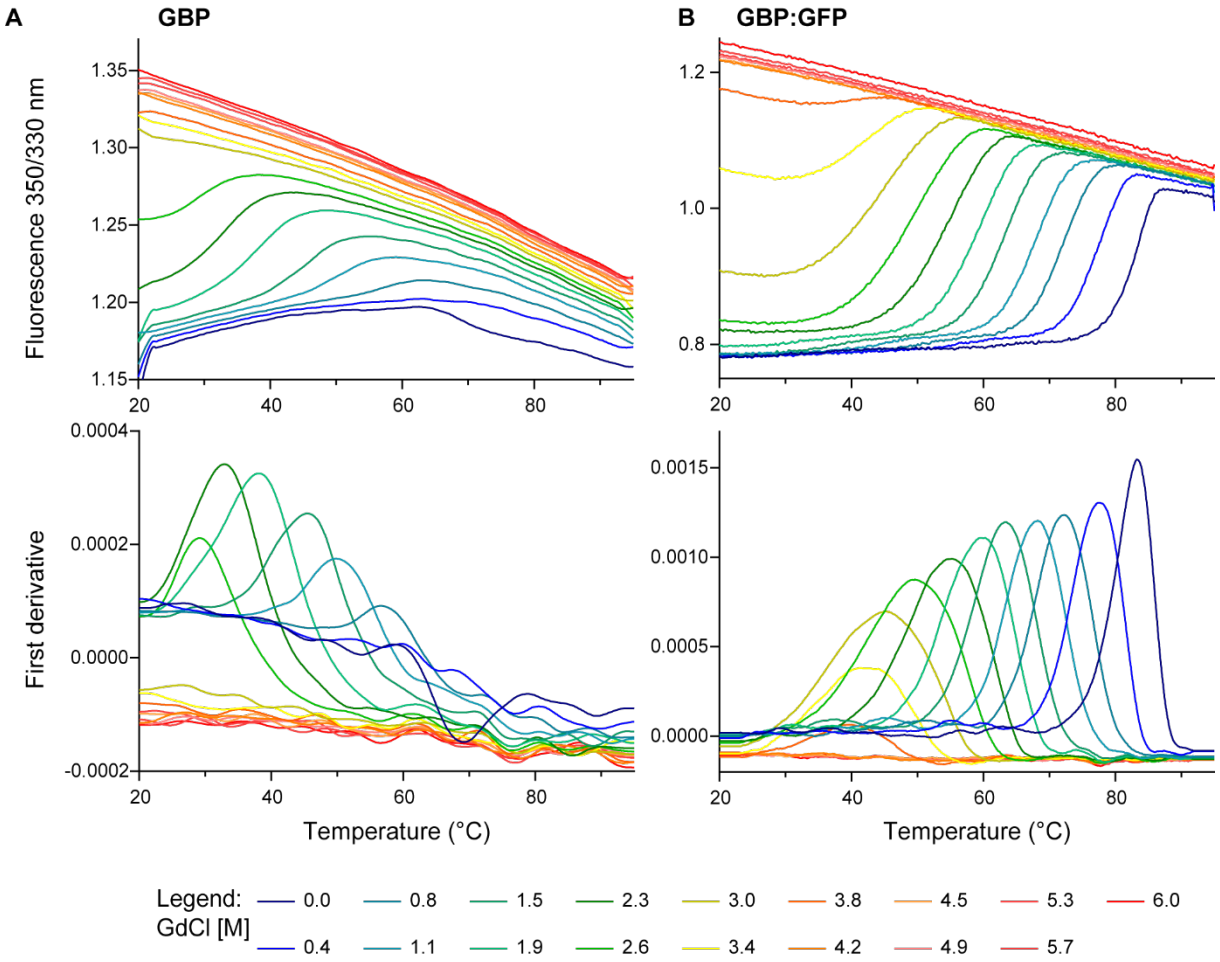

Figure 3 Melting curves for GBP and the complex GBP:GFP in increasing concentrations of guanidinium hydrochloride (GdCl). *Shown is the ratio of the fluorescence at 350 and 330 nm (top) and its first derivative (bottom) for GBP (A) and GBP:GFP (B).*

Conclusion

Our analysis quantitates the well-known stability of the ChromoTek GFP-binding protein (GBP) and its complex with GFP. To our knowledge, this is the first in-depth study of the chemical and thermal stability of GBP, despite its wide-spread use in research. This absence of prior data is partly due to the cumbersome nature of the experiments that have traditionally been used to probe the thermal or chemical stability of V_HH (e.g. van der Linden et al. 1999, Dumoulin et al. 2002). The use of the Prometheus NT.48 has now allowed us to characterize the stability of V_HH routinely as part of ChromoTek's research and development program, as well as in quality control.

In summary, GBP is stable and functional at temperatures up to 68 °C (melting point T_m), whereas the complex GBP:GFP is stable up to even 83 °C. The complex GBP:GFP is also almost insensitive to urea at concentrations up to 8 M. Even at a urea concentration of 8 M, which is denaturing for most known proteins, the complex has still a T_m of 51 °C. This unusual stability explains the suitability of ChromoTek GBP for applications such as immunoprecipitation under denaturing conditions or the use in protein interaction analysis such as surface plasmon resonance (SPR).

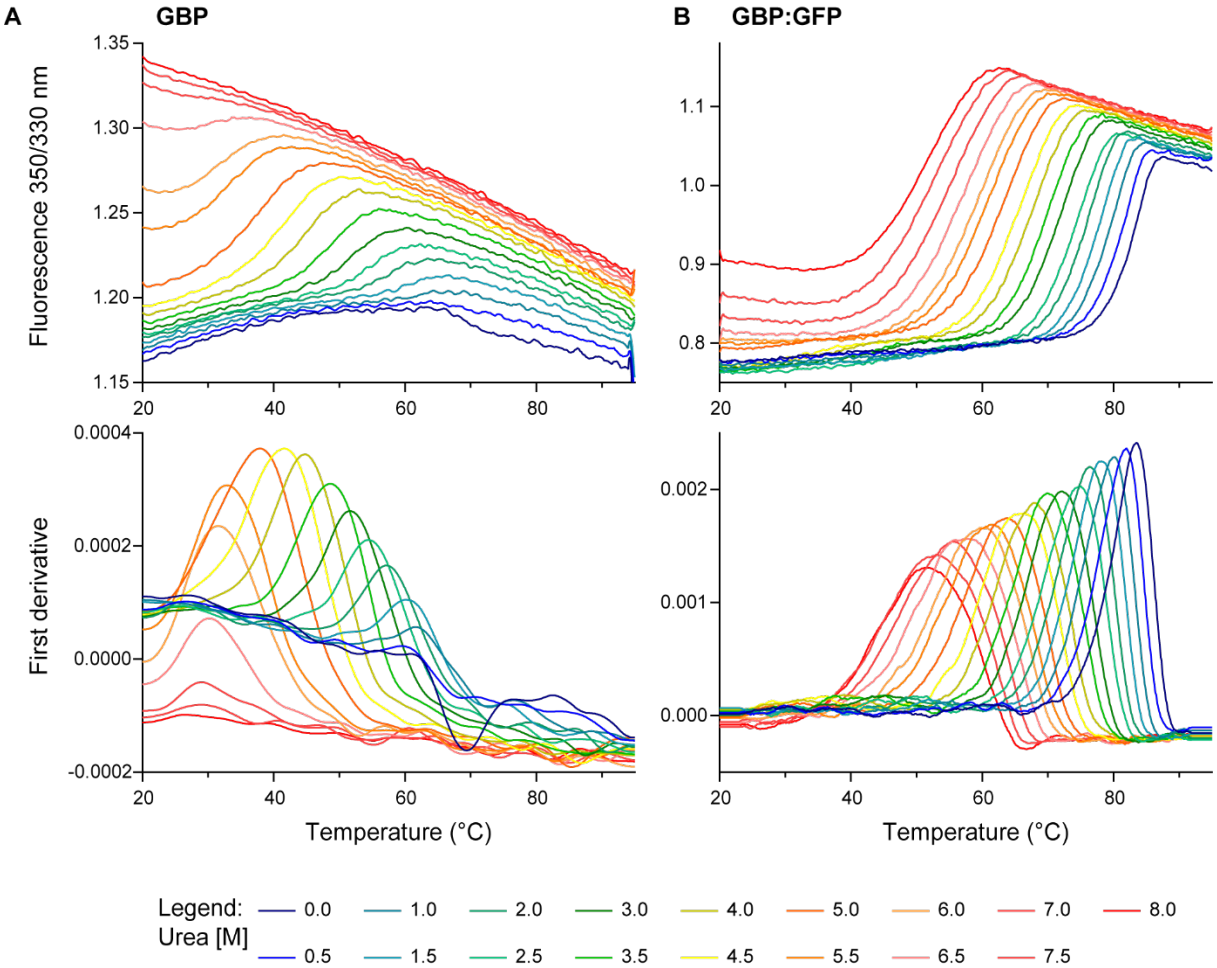

Figure 4 Melting curves for GBP and the complex GBP:GFP in increasing concentrations of urea. *Shown is the ratio of the fluorescence at 350 and 330 nm (top) and its first derivative (bottom) for GBP (A) and GBP:GFP (B).*

These stability data for GBP and its complex with GFP may thus yield helpful information for the application of GBP to following techniques (although we would like to point out that all experiments were performed with unfused GFP, and results may thus vary for different GFP-fusion proteins):

- **Immunoprecipitation:** The data fully support the use of the ChromoTek GFP-Trap (GBP immobilized on a matrix such as agarose beads) under denaturing conditions, e.g. in 6-8 M urea or 1-2 M GdCl. We would like to point out that all chemical denaturation data presented here were recorded after prolonged incubation (16 h) of GBP or its GFP-complex with the respective denaturants. In consequence, short exposure to even higher denaturant concentrations may also be tolerated by the GFP-Trap. Conversely, high concentrations of GdCl (5-6 M) may be used to elute a GFP-fusion protein bound to the ChromoTek GFP-Trap. This may be relevant if a user intends to elute his protein of interest without boiling it in Laemmli buffer.
- **Protein interaction analysis**: The study of protein interactions by methods such as surface plasmon resonance requires the immobilization of a protein of interest on a biosensor. Ideally, this immobilization is unaffected by a large number of regeneration cycles under varying, often harsh chemical conditions. Here, we show that the ChromoTek GFP-binding protein (GBP) is an ideal capture molecule for the binding analysis of GFP-fusion proteins using methods such as SPR (e.g. Biacore, GE Healthcare), biolayer interferometry (BLI, fortéBio), SwitchSense (Dynamic Biosensors) or surface acoustic wave (SAW, e.g. Nanotemper). The use of GBP in BLI was successfully tested in our own laboratory. In the case of SwitchSense, ChromoTek's GBP is already available pre-linked

to the necessary DNA-strand[2]. For the use of GBP in SPR, please refer to the Biacore procedure 28-9855-93 AB and the thorough study by Della Pia and Martinez 2015. All of these methods benefit from the high affinity of the GBP:GFP complex and its extreme chemical tolerance, which allows many cycles of binding analysis and biosensor regeneration.

In principle, conventional antibodies may also be used for for immunoprecipitation and protein interaction analysis and often are. However, GBP and other V_HH easily outcompete conventional antibodies under challenging conditions such as elevated temperatures, varying pH or high denaturant concentrations. Indeed, the finding that the complex of GBP and GFP is still quite stable in 8 M urea with a T_m of 51 °C is setting a new limit in protein complex stability, virtually unrivalled by any other pair of capture molecule and protein tag.

Material and Methods

Denaturation

For denaturation assays, 200 µl reactions were set up in a 96-well microtiter plate. Each reaction contained 76 µM GFP-binding protein (GBP, ChromoTek gt-250) or equimolar concentrations of GBP and GFP (21 µM each). In addition, reactions were composed of PBS and 0 to 6 M guanidinium hydrochloride or 0 to 8 M urea. After incubation overnight at room temperature, reactions were loaded into nanoDSF Grade Standard Capillaries (Nanotemper PR-COO2).

Thermal unfolding experiments

Using a Prometheus NT.48 (Nanotemper) instrument, the denaturation reactions were subjected to a thermal gradient from 20 to 95 °C at a heating rate of 1 °C/min. Tryptophan fluorescence at 350 and 330 nm was recorded at a rate of 20 datapoints per minute. The melting temperature T_m was determined using the first derivative of the ratio of fluorescence at 350 and 330 nm. For denaturation analysis, the ratio of 350/330 at 20 °C was plotted against denaturant concentration.

References

ChromoTek 2014. Ubiquitination of GFP-tagged proteins. Application note (http://www.chromotek.com/fileadmin/user_upload/pdfs/Application_notes/Applicaton_note_Ubiquitination_140211.pdf)

Della Pia EA, Martinez KL 2015. Single domain antibodies as a powerful tool for high quality surface plasmon resonance studies. PLoS ONE 10(3): e0124303. doi:10.1371/journal.pone.0124303

Despras E, Sittewelle M, Pouvelle C, Delrieu N, Cordonnier AM, Kannouche PL 2016. Rad18-dependent SUMOylation of human specialized DNA polymerase eta is required to prevent under-replicated DNA. *Nat. Commun.* 7:13326

Dumoulin M, Conrath K, Van Meirhaeghe A, Meersman F, Heremans K, et al. 2002. Single-domain antibody fragments with high conformational stability. *Protein Sci.* 11:500–15

GE Healthcare Life Sciences 2011. GFP-Trap® capture surface for Biacore assays. Procedure 28-9855-93 AB (http://www.chromotek.com/fileadmin/user_upload/pdfs/Application_notes/LabGuide-GFPTrapCaptureSurface.pdf)

[2] GFP binding protein kit for 48 mer (CK-GFP-1-B48), GFP binding protein kit for 96 mer (CK-GFP-1-B96), see http://www.dynamic-biosensors.com/consumables/ for more information.

van der Linden RHJ, Frenken LGJ, de Geus B, Harmsen MM, Ruuls RC, et al. 1999. Comparison of physical chemical properties of llama VHH antibody fragments and mouse monoclonal antibodies. *Biochim. Biophys. Acta* 1431:37–46

Min M, Mayor U, Lindon C 2013. Ubiquitination site preferences in APC/C substrates. *Open Biol*. 3:130097

Muyldermans, S. 2013. Nanobodies: Natural single-domain antibodies. *Annu. Rev. Biochem.* 82:775-97

Vedadi M, Niesen FH, Allali-Hassani A, Fedorov OY, Finerty PJ, et al. 2006. Chemical screening methods to identify ligands that promote protein stability, protein crystallization, and structure determination. *Proc. Natl. Acad. Sci. U.S.A.* 103:15835-40