Caged Compounds

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Aptamer-Guided Caging for Selective Masking of Protein Domains**

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The exogenous control of biological phenomena is of critical importance to study macromolecular function in spatiotemporal resolution. The recent years have seen many approaches being developed and among them photochemical tools using photoswitches or phototriggers (so-called "cages") have gained emerging interest.[1] The caging strategy uses photolabile groups in strategic locations to temporarily block a function and liberate it upon need with light under exact control of location and time.

A particularly formidable problem is the control of protein activity with light. Epitopes to be regulated may be small catalytically active sites or extended areas on the surface interacting with other macromolecular complexes, for example, for the regulation of protein function. Most of the proteins have a size beyond the capabilities of today's solid-phase synthesis and translational incorporation of non-natural caged amino acids is the method of choice.[2] By far the most difficult problem is to control the interaction of proteins with other macromolecules, which happens by a multitude of weak interactions on often unknown surface areas. However, preparing a light-activatable protein with a multitude of serendipitously distributed caged amino acids is not strategically wise since it frequently leads to an unspecific simultaneous blocking of all protein functions.[3]

As a protein we chose the serine protease thrombin because of the intricate possibilities for the regulation of its function (Figure 1). In addition to its active site, which contains the catalytic center, thrombin coordinates its multiple interactions with different macromolecular substrates through its sub-domains termed "Exosite I" and "Exosite II". For example, Exosite I stabilizes the binding of fibrinogen, whereas Exosite II among other things mediates the binding of antithrombin through glycosaminoglycans.[4]

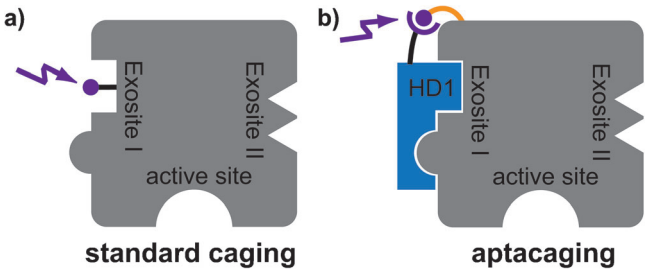

Figure 1. Standard caging (a) of a single amino acid residue with a limited radius of interaction inhibition and apta-caging (b) for masking of protein domains. Apta-cages are like plaster casts which are able to block regulatory protein surfaces with their aptamer domain and template the covalent cross-linking of the photolabile tether with the target protein. Upon irradiation, the covalent attachment of the aptamer to the protein is cleaved and enables the release of the epitope surface.

A useful technology to modulate individual sub-domain interactions of a protein is provided by aptamers.[5] Aptamers can be considered as plaster casts of their target molecule. Importantly, the development of aptamers does not involve structural knowledge but can be guided to individual protein sub-domains through appropriate selection schemes. Herein we use an aptamer to go beyond the local influence of individual cages. Instead, the aptamer HD1—together with a light-triggered release strategy—become effectively a supra-cage ("apta-cage") for reliable but temporary masking of protein regulatory surfaces. HD1 which has been shown to cover Exosite I and to block fibrinogen binding to thrombin,[6] has a calculated interaction surface of approximately 915 Å^2. In comparison, a classical nitrophenylethyl (NPE) cage covers a surface of about 110 Å^2, illustrating the superiority of apta-caging in reversible epitope inhibition.

We believe thrombin represents an ideal model case to test our ability to block and reactivate protein regulatory domains with light. We synthesized a modified version of HD1—termed fHD1—which bears a photo-cleavable tether at its 3′-end followed by a terminal amine group (Scheme 1 a). We have already shown that it is essential to modify the 3′-end of this aptamer and not its 5′-end because the 5′ case leads to a dramatic reduction of aptamer activity.[7] In the present case this modification has no influence on aptamer affinity towards thrombin (Supporting Information, Figure S1). Further functionalization with an ethylene glycol bis[succinimidylsuccinate] (EGS) which has two sulfo-NHS-groups yielded an aptamer-based reagent (Scheme 1 a) that upon recognition of thrombin guides the remaining sulfo-NHS-group towards

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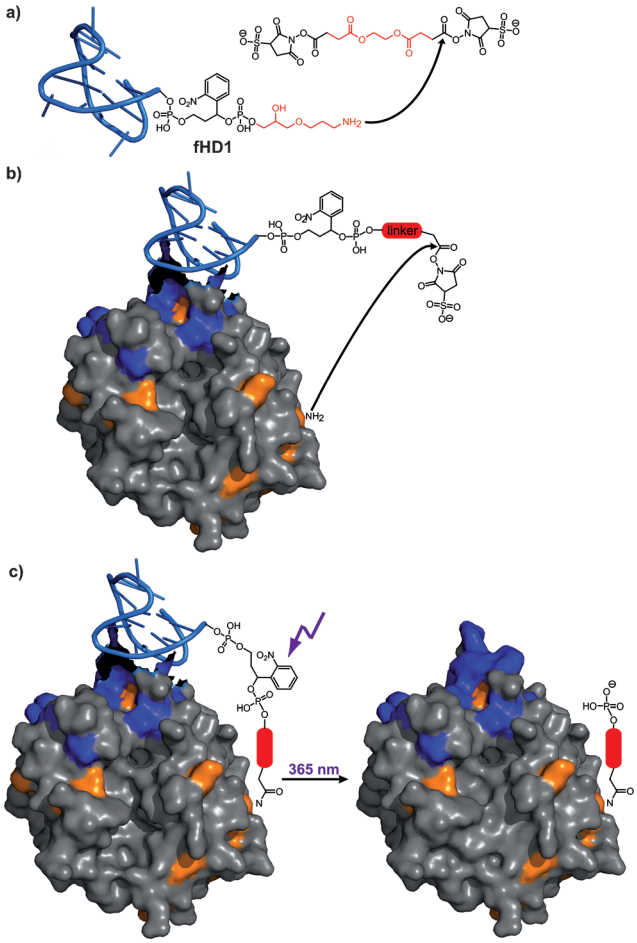

Scheme 1. a) Preparation of an aptamer-based reagent and b) templated reaction for apta-caging of thrombin. c) Upon irradiation the linker is cleaved and active thrombin liberated. protein data bank (PDB): 4DII (Exosite I blue, lysine residues orange).

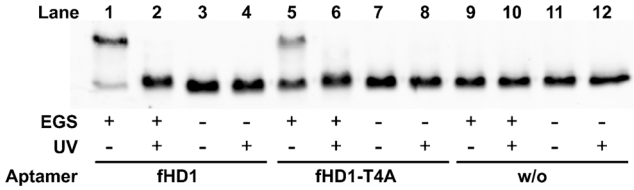

Figure 2. Western blot analysis of apta-caged thrombin. Aptamer fHD1 (lanes 1–4), the point mutant fHD1–T4A (lanes 5–8), or no aptamer (lanes 9–12) was employed for the aptamer-templated formation of apta-caged thrombin. The cross-linking reagent (EGS) and UV light ($\lambda = 365$ nm) were applied as indicated. For quantification see Figure S2.

covalent complex formation with one of the adjacent lysine residues (Scheme 1 b). This photolabile tether is essential since with a noncovalent complex of aptamer and thrombin-active protein would leak out of the complex if the local concentration gets close to or below the dissociation constant, which in the present case is approximately 100 nM (Figure S1).

Visualization of the formation of the covalent complex (fHD1–thrombin) and its light-induced cleavage was achieved by SDS-polyacrylamide gel electrophoresis followed by western blot analysis (Figure 2). As control experiments, we included reactions using an aptamer point mutant (fHD1–T4A) with reduced affinity to thrombin, reactions excluding the EGS linker and reactions in the absence of any aptamer. We purified the complexes by gel filtration and analyzed the resulting samples using a thrombin-specific antibody. Thrombin's light (6 kDa) and heavy chain (31.5 kDa), connected by a disulfide bond are cleaved under the reductive and denaturing conditions of the SDS-PAGE gel, resulting in a band of approximately 31.5 kDa. The covalent cross-link between aptamer (5.5 kDa) and thrombin's heavy chain results in a band that migrates as a 37 kDa complex. As shown in Figure 2, the reaction yielded an aptamer–thrombin complex (lane 1), which can be cleaved by irradiation with UV light ($\lambda = 365$ nm; lane 2). Using the point mutant fHD1–T4A the formation of a covalent complex was also observed (lane 5), albeit to a much lesser extent (for quantification see Figure S2). This complex was also cleaved by irradiation (lane 6). Omission of either the aptamer or the cross-linking reagent never produced shifted bands (lanes 3, 4 and 7–12). In addition to Exosite I recognition, recent studies report that HD1 also exhibits weak interactions with thrombin's polybasic Exosite II.[8] Our western blot studies also shed light on this finding. Complex formation of fHD1 and thrombin resulted in one dominant band, corresponding to the 1:1 complex and one additional slower migrating band that is most likely from the ternary complex built from two fHD1 molecules bound to one thrombin molecule (Figure S3 a). Both bands disappear upon irradiation with light. Neither the addition of equimolar concentration of non-modified HD1 nor the addition of HD1–T4A was able to block the formation of the ternary complex utilizing Exosites I and II. However, the addition of HD22, an aptamer that specifically recognizes Exosite II[9] clearly diminished the formation of the ternary complex (Figure S3 a). Similar results were observed when using fHD1–T4A for complex formation (Figure S3 b).

As shown in Figure 2 formation of the covalent complex is not quantitative and a residual amount of free thrombin remains detectable (Figure 2 lane 1). Further depletion of free thrombin was examined with a bead purification approach using HD1-coupled magnetic beads that should interact with free thrombin in the solution. These beads were incubated with the reaction products. While this assay works very well with thrombin solutions, further depletion of free thrombin from cross-linking reactions was not possible (Figure S4) indicating that the residual thrombin is not capable of interacting with HD1 and will be inactive in coagulation assays.

To assess the successful masking of Exosite I in the covalent complex we used an amidolytic assay with a fluorogenic peptide in the presence and absence of hirudin (Figure S5). Hirudin simultaneously recognizes Exosite I and the active site of thrombin with very high affinity (K_d value of 0.2 pM) and thereby blocks its amidolytic activity.[10] The results of the amidolytic assay are shown in Figure 3 and Figures S6–S8. Essentially, the covalent complex shows retained cleavage of the peptide in the presence of hirudin,

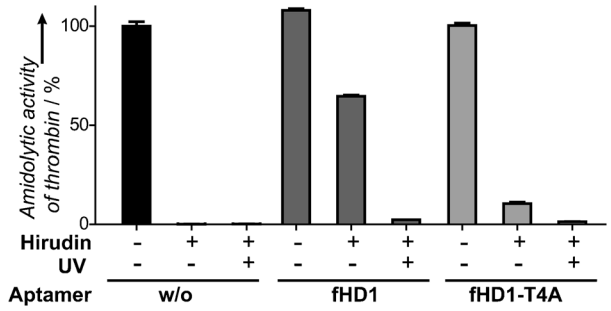

Figure 3. Amidolytic activity of thrombin measured by cleavage of a fluorogenic peptide substrate. Apta-caged thrombin [7 nM] was incubated with hirudin [15 nM] and the fluorogenic peptide substrate before and after irradiation with a UV LED ($\lambda = 365$ nm). The increase of fluorescence is measured over time and normalized to the sample in the presence or absence of hirudin and with or without UV-irradiation (as indicated). Formation of apta-caged thrombin was performed either without aptamer (w/o), with fHD1, or with fHD1–T4A, as indicated. One representative data set is shown. Error bars represent intra-experimental variance (see also Figure S7).

whereas irradiation led to a full inhibition of peptide cleavage. This result clearly indicates that in the complex Exosite I is masked by the aptamer. Control experiments with fHD1–T4A or without aptamer show light-independent inhibition of thrombin's amidolytic activity, strictly depending on the hirudin concentration. Increased concentrations of hirudin were shown to reduce the complex's amidolytic activity (Figure S6). This situation might be due to outcompeting fHD1 of the complex by hirudin (which has a 500000-fold higher affinity compared to HD1) or residual interaction of hirudin with the active site even when the Exosite I is sequestered.

We next examined whether the apta-caged thrombin can be used in a human blood-plasma-based assay for the light-dependent induction of clot formation. To achieve this we employed a sensitive spectrometry-based assay that makes use of an increased absorption of UV light upon the onset of coagulation at low thrombin concentrations (the weak probing UV light for this assay is not sufficient to perform any uncaging).[11] As the fibrinogen-binding site is masked in the covalent fHD1–thrombin complex, coagulation of plasma is not induced upon its addition (Figure 4a). However, irradiation ($\lambda = 365$ nm) induces release of thrombin, which led to clot formation (Figure 4b), indicated by an increase in absorption. In contrast, when using fHD1–T4A, no significant effect on the clotting activity before and after photocleavage is observed (Figure 4c,d), the same applies to control reactions without any oligonucleotide and cross-linker (Figure 4e,f). In accordance with the western blot analysis data, clot formation is also detected in samples prepared without linker or aptamer (Figure S9 and S10). These data clearly show that the covalent complex between thrombin and fHD1 masks Exosite I and that this site is essentially reactivated upon irradiation.

In a final experiment we sought to visualize light-triggered thrombin release and clot formation. Therefore we incubated fibrinogen with the covalent complex in two glass capillaries and irradiated one over a small area with UV light ($\lambda =$ 365 nm), whereas the other remained untreated. We recorded clot formation by light microscopy (Supporting Information, Movie 1) and a representative picture prior (Figure 5a) and after irradiation (Figure 5b) is depicted in Figure 5.

In conclusion, we have introduced a novel caging-method enabling the selective masking of protein domains. Essentially, we described the synthesis of an aptamer-based reagent that upon interaction with its specific protein target forms a stable covalent complex. Irradiation with light subsequently releases the active protein. This apta-caging approach can be generally applied for any target protein for which aptamers are available or can be generated. Aptamer-guided chemistry

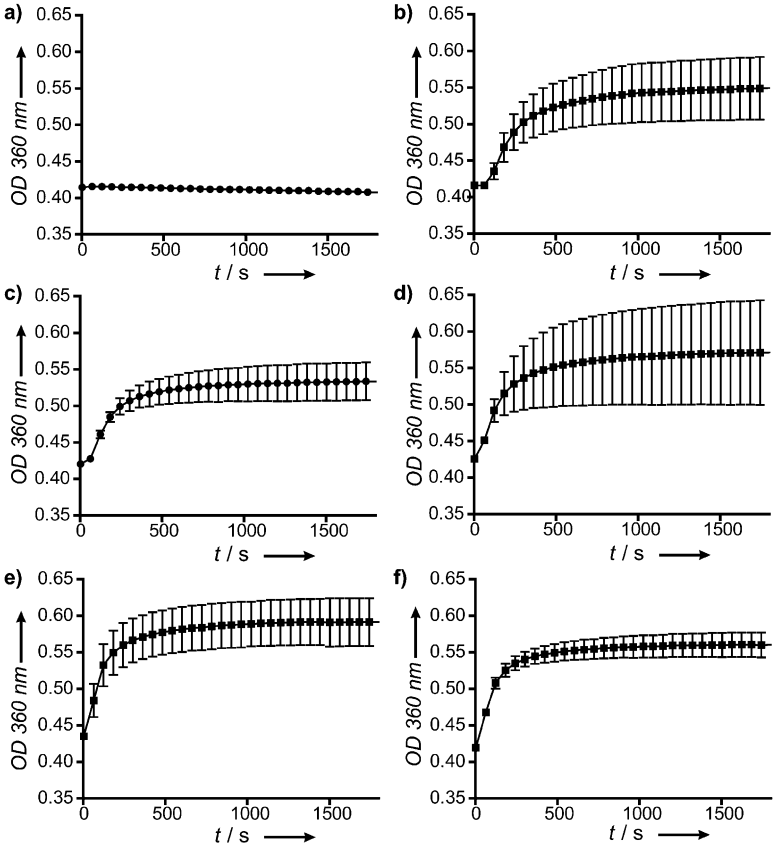

Figure 4. Plasma coagulation assays. Absorption at 360 nm is measured in the presence of apta-caged thrombin in dependence of UV-LED ($\lambda = 365$ nm) irradiation. Measurement was monitored for 30 min in human pool plasma. The formation of a plasma clot leads to an increase in signal. a) thrombin apta-caged with fHD1 before UV irradiation; b) thrombin apta-caged with fHD1 after irradiation; c) thrombin apta-caged with fHD1–T4A before irradiation; d) thrombin apta-caged with fHD1–T4A after irradiation e) thrombin in reaction mixture without aptamer and cross-linker before irradiation f) thrombin in reaction mixture without aptamer and cross-linker after irradiation. One representative data set is displayed. Error bars represent intra-experimental variance (see also Supporting Information data).

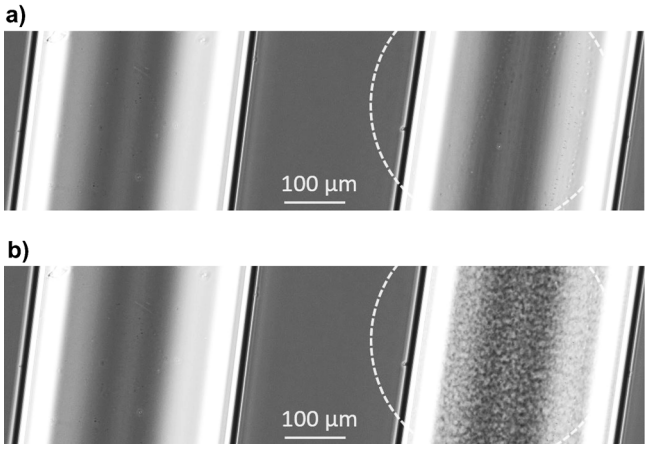

Figure 5. Microscope pictures of clot-formation in glass capillaries before (a) and after (b) UV irradiation ($\lambda = 365$ nm). Both capillaries are filled with 8 mg mL^{-1} fibrinogen and 0.1 µg mL^{-1} apta-caged thrombin. The white dashed circles indicate the irradiation area. b) Image recorded 28 min after irradiation with a UV-Hg lamp. Irradiation time: 1 min.

has recently been shown to be suitable for masking specific reactive sites of aminoglycosides enabling the specific modification of certain residues.[12] Herein we followed a different approach, employing supramolecular chemistry to generate a covalent complex, which can be dissociated with spatiotemporal resolution. Using thrombin has not only a model-system character but also opens various biomedical applications. Locally induced blood clotting could be a powerful tool to restrict tumor blood supply or to stop uncontrolled bleeding in emergency situations. The use of an inducible thrombin would be a preferred way of doing this since it avoids the use of hemostatic agents, such as recombinant activated factor VII, thereby minimizing the risk of unwanted side effects, such as life-threatening thromboembolic complications. Future experiments with different photolabile groups suitable for in vivo activation will shed light on whether this approach can be translated into patient-relevant clinical settings. Furthermore, the approach described will also have an impact on synthetic and systemic biology, using masking and liberation of protein domains to control cellular networks and circuits.

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