Direct Visualization of Protein Kinase A Activation on DNA Origami using Fast-Scan Atomic Force Microscopy

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ABSTRACT DNA origami nanostructures provide a unique platform for the direct observation of protein-protein interactions at the single-molecule level. Here, we used DNA origami in combination with fast-scan atomic force microscopy to observe the activation-induced dissociation of individual protein kinase A (PKA) holoenzymes. The PKA holoenzyme consists of two regulatory (R) and two catalytic (C) subunits. When cAMP binds to the R subunits it causes dissociation of the C subunits from the R subunit dimer and activation of the enzyme. Using a DNA origami platform, we were able to observe the activation of PKA in response to photolysis of caged cAMP. Furthermore, exploiting the potential of DNA origami for precise positioning of biomolecules, we were able to position the catalytic subunit of adenylyl cyclase in close proximity to PKA and to observe the activation of PKA in response to cAMP produced by adenylyl cyclase. We provide tools for the observation of signalling pathways at the single-molecule level and show that the C subunits of PKA dissociate from the holoenzyme but stay within ~10 nm of the R subunit dimer upon activation.

The great potential of DNA origami nanostructures for functionalization, and the variety of attachment mechanisms available¹⁻⁸, have given rise to a number of origami-based studies of single-molecule interactions *in vitro*^{7,9-11}. Here, we exploit the precise control of protein positioning on DNA origami tiles to observe the activation of protein kinase A (PKA) by cAMP at the single-molecule level. PKA is one of the simplest kinases, and so has served as a model for the study of other members of the protein kinase family¹². It is a key component in intracellular signalling and in the control of metabolism, and dysregulation of PKA is thought to play a role in various diseases¹³, and in ageing¹⁴.

The PKA holoenzyme consists of two regulatory (R) and two catalytic (C) subunits¹⁵. The R subunits serve two main roles: capture of the C subunits and consequent inhibition of their catalytic activity, and localization of the PKA holoenzyme through binding to AKAP scaffold proteins¹⁶⁻¹⁹. Each of the R subunits contains two cAMP binding sites. Once four cAMP molecules are bound to the R subunit dimer, the two C subunits become activated²⁰. Although PKA is a very well-studied protein, there are still unanswered questions about its mechanism of action, in particular regarding the dynamics of the interaction between the C subunits and the R subunit dimer during the activation/deactivation cycle. Two recent studies on the potential mechanisms underlying this process in the cellular context have generated conflicting

results^{21,22}. Specifically, Smith *et al.*²¹ reported that at physiological concentrations of cAMP (1-2 μ M) 70-80% of the C subunits remain associated with the AKAP/R subunit complex, and that substantial release of the C subunit was only seen at supraphysiological concentrations of cAMP (10-90 μ M). In contrast, Walker-Gray *et al.*²² found that the C subunits are rapidly released upon activation and then recaptured.

Here, we use single-molecule imaging by fast-scan atomic force microscopy (FS-AFM) to demonstrate that 2-D DNA nanostructures can be used as platforms on which to observe signalling at the single-molecule level. We use this technology to observe the behavior of the C subunits of PKA during activation, in near-real time.

We designed and synthesized a rectangular (100 x 70 nm) DNA origami 'tile' onto which we could place the catalytic domain of adenylyl cyclase and PKA at a distance of ~20 nm apart (Figure 1.A). The tile carries an 'orientation loop' on one side so that the attachment of proteins to the target region can be assessed (Figure 1.B). Tile assembly occurred with an efficiency of 92 $\pm$ 5% (n=5 experiments, 100 tiles each, Supplementary Figure 1). The two proteins of interest are attached onto their specific positions on the origami tile through streptavidin-biotin interactions for adenylyl cyclase and a SNAP tag-benzylguanine interaction for PKA (Figure 1.C).

Figure 1. DNA origami template and protein modification. **1.A:** Map of the DNA origami tile (100 x 70 nm) used in the present study. The tile carries biotin and benzylguanine (BG) modifications, ~20 nm apart, for the attachment of adenylyl cyclase and PKA, respectively. **1.B:** Representative AFM image of an origami tile showing the orientation loop. **1.C:** Schematic representation of the protein assembly used for this study. The biotinylated catalytic domain of adenylyl cyclase is attached to the origami tile through a biotin-streptavidin bridge while the PKA holoenzyme is held on the tile through a SNAP tag modification of the R subunit, which binds a benzylguanine-modified staple. Created with BioRender.com

We used a stepwise approach to protein attachment. Following incubation of the tiles with streptavidin, we found that $96 \pm 8\%$ (5 experiments, 250 tiles in total) of the tiles carried a streptavidin molecule, positioned at the known location of the biotinylated staple, on the edge of the tile, to the left of the orientation loop (Figure 2.A). The high efficiency of streptavidin binding was expected, given the high affinity of streptavidin for biotinylated targets^{23–25}. The peak volume of the bound molecules was $124 \pm 3 \text{ nm}^3$ ($n=80$), close to the calculated theoretical volume of a streptavidin tetramer (molecular mass, 55 kDa; theoretical volume, 105 nm^3)²⁶.

We then incubated streptavidin-decorated tiles with a biotinylated adenylyl cyclase (bio-AC) that is derived from the catalytic domain of the CyaA toxin from *Bordetella pertussis*²⁷ (Figure 2.B). To ensure the activity of the catalytic domain of bio-AC, we incubated the protein with calmodulin (CaM), prior to incubation with streptavidin. The molecular mass of the recombinant calmodulin²⁷ is 19.5 kDa, and therefore the molecular mass of the bio-AC-CaM complex is 75 kDa in total. This corresponds to a theoretical volume of 142 nm^3 . The majority ($68 \pm 9\%$; $n=75$) of the structures showed an increase in volume after incubation with adenylyl cyclase. The average volume measured for the streptavidin-bio-AC-CaM complex (combined molecular mass, 130 kDa; theoretical volume, 247 nm^3) was $263 \pm 12 \text{ nm}^3$ ($n=50$). The extent of the increase indicates that on average one bio-AC-CaM complex bound to each streptavidin molecule; however, the increased width of the volume distribution for this sample suggests that some streptavidin molecules might carry more than one adenylyl cyclase molecule, consistent with the fact that bound streptavidin has three sites available for binding biotin. It should be noted that as the bio-AC was biotinylated *in vivo* (see Supplementary Information), it contains a single biotin tag per

enzyme molecule. All the proteins prepared and used in these experiments were characterized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Supplementary Figure 2).

Next, R subunits of PKA were attached to the tiles in the designated position, $\sim 20 \text{ nm}$ away from the binding site for the streptavidin/adenylyl cyclase complex, and immediately to the left of the orientation loop. The tiles were decorated with the RII α subunit with an efficiency of $63 \pm 16\%$ ($n=55$) (Figure 2.C), and the mean volume of the attached particles was $233 \pm 11 \text{ nm}^3$ ($n=50$). The theoretical volume of the R subunit of PKA is 123 nm^3 for a monomer and 246 nm^3 for a dimer, indicating that the majority of the attached particles are RII α dimers. When the tiles were incubated with the PKA holoenzyme, the volume distribution showed two peaks, one at $200 \pm 8 \text{ nm}^3$ ($n=69$) and another at $488 \pm 23 \text{ nm}^3$ ($n=68$), indicating that not all the R subunit dimers were associated with C subunits to form holoenzymes; hence, a mixed population of holoenzymes and R subunits was present (Figure 2.D). The attachment efficiency of the holoenzyme on the tiles was $60 \pm 9\%$ ($n=150$). The correct placement of the RII α subunits on the tiles was confirmed by measuring the distance of the bound particles from the ends and the sides of the tiles. The observed distances were as expected given the known position of the benzylguanine-modified staple (Supplementary Figure 3). In contrast, when the tiles were incubated with C subunits of PKA, which do not bear any modification, only a very small number of decorated tiles were seen ($5 \pm 2\%$ of all well-formed tiles; $n=55$). Furthermore, the observed particles were in random positions on the tiles (Supplementary Figure 3). The above result confirms that the binding of the R subunit of PKA to the DNA origami tiles is precise and controlled.

Figure 2. Attachment of proteins to the DNA origami tile. Insets show representative AFM images. Images are either 200 x 200 nm (**2.A-C**) or 100 x 100 nm square (**2.D**). Height range, 0-5 nm. Asterisks indicate the orientation loops. **2.A:** Volume distribution of streptavidin attached to the tile via the biotinylated staple. The peak volume corresponds to one streptavidin molecule. **2.B:** Volume distribution of attached streptavidin-bio-AC-CaM complexes. The volume increase corresponds to one bio-AC-CaM complex attached to each streptavidin molecule. **2.C:** Volume distribution of attached SNAP-tagged PKA R subunit. The peak volume corresponds to one R subunit. **2.D:** Volume distribution of attached particles after incubation of tiles with the PKA holoenzyme. The distribution shows two peaks, one corresponding to the R subunit dimer (as in **2.C**) and one to the holoenzyme.

We used FS-AFM to track the activation of PKA, attached alone to the tiles. Tiles bearing PKA holoenzymes were visualized at a scanning rate of 4 frames per minute in the presence of caged cAMP (1 mM). A rapid (<20 s) dissociation of the two C subunits occurred upon UV photolysis of the cage (Figure 3.A,B). In contrast, in the presence of the PKA inhibitor (R_p) -cAMPS^{28,29} no dissociation of the PKA holoenzyme was seen (Figure 3.B,C). After photolysis of the caged cAMP, the volume of the largest attached particle (arrows, Figure 3.A) fell by $50\pm7\%$ ($n=6$), consistent with the release of the two C subunits. Two smaller particles can be observed next to the large particle, consistent with the two catalytic subunits being released but staying in the close proximity of the regulatory dimer. In contrast, no change in the appearance or volume of the measured particles was observed in the presence of (R_p) -cAMPS.

We next visualized the activation of PKA by cAMP produced from ATP by adenylyl cyclase. ATP was generated by UV photolysis of caged ATP (1 mM). It was not possible in these experiments to measure the concentration of cAMP to which the bound PKA was exposed, but it is likely that it is substantially lower than that achieved by UV photolysis of caged cAMP (above). A rapid dissociation of the PKA holoenzyme was again observed (Figure 4.A,B), and

the volume of the largest attached PKA particle (arrows, Figure 4.A) fell by $49\pm12\%$ ($n=6$). The dissociation can be seen in sections taken through the attached particles (Figure 4.C). Again, no dissociation of the PKA holoenzyme was seen in the presence of (R_p) -cAMPS (Figure 4.B,D). The distance between the central $R_{II}\alpha$ dimer and the C subunit particles in close proximity was 9.9 ± 1.4 nm ($n=13$; Supplementary Figure S4), agreeing with previous studies on the dissociation of the PKA holoenzyme²¹

Figure 3. Activation of PKA by UV photolysis of caged cAMP. **3.A:** Tiles bearing a PKA holoenzyme, imaged in the presence of caged cAMP. Upon UV photolysis of the caged cAMP, indicated by the flash, the holoenzyme underwent dissociation. **3.B:** Effect of UV photolysis of caged cAMP on the volumes of PKA holoenzymes. **3.C:** Images from a control experiment in the presence of the PKA inhibitor (R_p)-cAMPS.

Figure 4. Activation of PKA using cAMP produced from caged ATP by adenylyl cyclase. **4.A:** Tiles bearing a PKA holoenzyme and the catalytic subunit of adenylyl cyclase, imaged in the presence of caged ATP. Upon UV photolysis of caged ATP, adenylyl cyclase converts ATP to cAMP and the PKA holoenzyme undergoes dissociation. **4.B:** Effect of UV photolysis of caged ATP on the volumes of PKA holoenzymes. **4.C:** Cross-sections of the holoenzyme, taken at the positions indicated by the lines in 4.a, before and after activation. **4.D:** Images from a control experiment in the presence of the PKA inhibitor (R_p)-cAMPS.

The R and C subunits of PKA used in this study bear His₆-tags at the C termini of the proteins. It has been shown previously that the tag does not interfere with the activity of the subunits²⁸. In addition to the His₆ tag, the R subunit was modified to bear an additional SNAP tag at the N terminus, which contains the dimerization/docking domain of the protein³⁰. Nevertheless, dimerization of the RII α subunit did not seem to be inhibited in the present study, as suggested by the volume measurements of the RII α subunit. cAMP-induced activation of PKA has been studied before, for example using fluorescence polarization assays³¹ and hydrogen-deuterium exchange studies³². Further, the PKA holoenzyme has been visualized by negative-stain electron microscopy²¹. However, the effect of cAMP on PKA structure has never previously been observed at the single-molecule level. In our study, we provide a platform on which to study protein-protein interactions and parts of signalling pathways at the single-molecule level. Using this platform, we saw a

clear dissociation of the PKA holoenzyme, both when PKA was activated by caged cAMP, and when activated by cAMP generated by adenylyl cyclase from ATP. The two dissociated C subunits remained on the origami tile, within ~10 nm of the R subunit dimer. Note that, as mentioned above, individual C subunits do not bind efficiently to the tiles, so their retention likely involves a continuing interaction with the R subunit dimer, as proposed by Smith *et al.*²¹.

Overall, we show that the use of fast-scan AFM to observe the behavior of proteins anchored to DNA origami tiles offers a powerful new method with which to interrogate protein function and protein-protein interactions and highlight the potential of DNA nanotechnology as a tool in the observation of signalling pathways at the single-molecule level.

ASSOCIATED CONTENT

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Supporting Information (PDF)

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Author Contributions

IM designed and performed the experiments, analyzed the data and wrote the manuscript. DL, HS and ME provided resources and contributed to the experimental design and data interpretation. JME and RMH conceived the project idea, designed experiments and revised the manuscript. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

PKA, FS-AFM, SDS-PAGE

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