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Application Note

Hydrodynamic diameter and thermal stability measurements of protein kinase A using switchSENSE®

Keywords: protein kinase A, hydrodynamic (Stokes) diameter, sizing, melting temperature

Background

Protein kinases are enzymes that play a key role in cellular regulation. One of the best understood kinases from a biochemical point of view is protein kinase A (PKA). PKA is a cAMP dependent seronine/threonine kinase, which is composed of two regulatory (PKA-R) and two catalytic (PKA-C) subunits. The enzyme undergoes multiple conformational changes and thus, information on size and shape of the subunits, or the tetrameric holoenzyme is of immense value. Another important parameter which is indicative of a correctly folded protein and if a protein's binding pocket for small molecule is occupied or vacant, is the protein's melting temperature T_M. Classical techniques to determine hydrodynamic size information include neutron scattering, circular dichroism or analytical gel chromatography. Melting temperatures are commonly measured employing thermal shift assays or differential scanning calorimetry. Here we show for the first time how both parameters, D_H and T_M, are measured simultaneously in the same assay setup.

Methods and Results

Analysis of complex formation by protein size measurements

The hydrodynamic (Stokes) diameters of PKA-C and PKA-R were investigated in a switchSENSE protein sizing experiment. For this purpose, a standard multi-purpose chip with 48 bp DNA tagged with a fluorophore at its 3'-end (MPC-48-2-Y1) was used. PKA-C and PKA-R were covalently conjugated to a complementary strand with a functional group at its 5'-end using the Dynamic Biosensors amine coupling kit 1 (CK-NH2-1-B48). To preserve enzymatic activity after conjugation, the active site of PKA-C was protected with 2 mM Mg^{2+} and 0.5 mM ATP in the coupling buffer.

For accurate size measurements, protein-DNA conjugates were purified from unreacted free DNA using an ÄKTA-system and then concentrated to a final concentration of 200 nM. Hybridization of conjugates to the biochip was followed in real-time by an increase of the fluorescence signal upon formation of double-stranded DNA. The hybridization was completed (i.e., app. 100% of the DNA nanolevers were functionalized with protein) after 15 minutes.

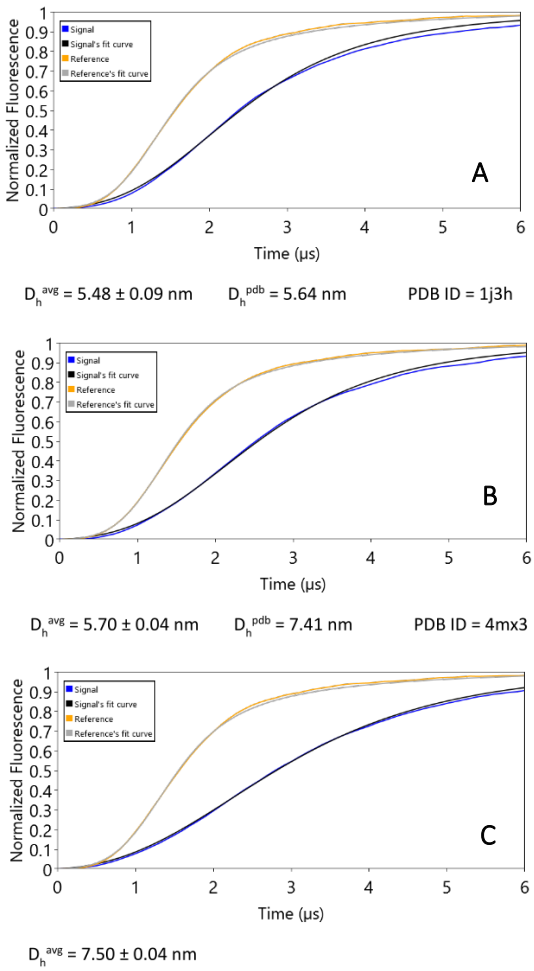

Figure 1 | Upward switching fluorescence response for bare DNA (reference, orange) and protein-DNA conjugates (blue), as well as derived hydrodynamic diameters (D_h^{avg}) and calculated diameters from the crystal structure (D_h^{pdb}):
A PKA-C, **B** PKA-R and **C** PKA-C-PKA-R complex (PKA-R was flown over immobilized PKA-C – DNA conjugate).

Switching speed measurements of double-stranded DNA molecules with and without protein were performed in a 10 mM Tris buffer (pH 7.4), containing 40 mM NaCl, 1.5 mM $MgCl_2$ and 0.5 mM TCEP. The switching dynamics of DNA-protein complexes gradually slow down with increasing protein size. By comparing the measured upward switching fluorescence response of bare DNA and DNA-protein conjugates to an analytical model that treats the protein as a sphere on top of a charged DNA cylinder, the effective hydrodynamic protein diameter can be determined with subnanometer accuracy [1] (Fig. 1).

The obtained diameter of PKA-C from switchSENSE is in excellent agreement with the value calculated with the switchANALYSIS software (WinHydroPro 10 algorithm) from the PDB file, as well as with results from analytical gel chromatography measurements [2] (c.f. Table 1).

The diameter of the regulatory subunit R was determined at 5.7 nm in the switchSENSE subunit analysis, which indicates that the regulatory subunit was conjugated as a monomer to the DNA nanolever on the chip surface (calculated diameter of a R monomer from the PDB file: 5.44 nm). This is noteworthy, because the regulatory subunit is known to form stable

Table 1 | Comparison of hydrodynamic diameter measurements.

PKA subunit /complex	MW (kDa)	D_H (nm) switchSENSE	D_H (nm) calculation from PDB file	D_H (nm) from [2] analytical gel chromatography
C	41	5.48 ± 0.09	5.64 [1J3H]	5.42 ± 0.04
R	43	5.70 ± 0.04	5.44 [4MX3] monomer	
R_2	86		7.41 [4MX3] dimer	8.64 ± 0.06
C-R_2	127	7.50 ± 0.04	n.a.	n.a.
C_2-R_2	168	n.a.	9.51 [3TNP] heterotetramer	9.20 ± 0.16

dimers in solution (R_2) [2]. Hence a possible product of the coupling reaction of the regulatory subunit to the DNA could have been DNA-R_2, but if the regulatory subunit was conjugated as a dimer, the measured diameter would be significantly larger: for comparison, the calculated diameter of the R_2 dimer is 7.4 nm and the diameter that has been determined experimentally with a complementary technique (analytical gel chromatography) was 8.6 nm [2].

When a solution of the regulatory subunit R was flown over the immobilized catalytic subunit C, the diameter of the C-R complex was measured at 7.5 nm in the presence of Mg^{2+} and ATP. A straightforward interpretation of this result is that a C_1R_1 complex has formed on the surface, because the increase in diameter from 5.5 to 7.5 nm is roughly what be expected when combining two proteins of 40-50 kDa each. However, this result is surprising since the regulatory subunit is expected to form dimers in solution. An alternative explanation is that C_1R_2 complexes were formed, but that the diameter is being underestimated.

Thermal stability

Directly after measuring the hydrodynamic diameter, the thermal stabilities of PKA-C and PKA-R were investigated. For this purpose, the biochip was heated to 70°C while the change in absolute fluorescence was monitored as the temperature increased gradually. Upon unfolding, the hydrophobic interior of the proteins is exposed, which can be observed in a stronger quenching of the fluorescence of the probing dye adjacent to the protein. This effect can be used to investigate the influence of cofactors (small molecules, other proteins) on the melting temperature of a protein (Fig. 2).

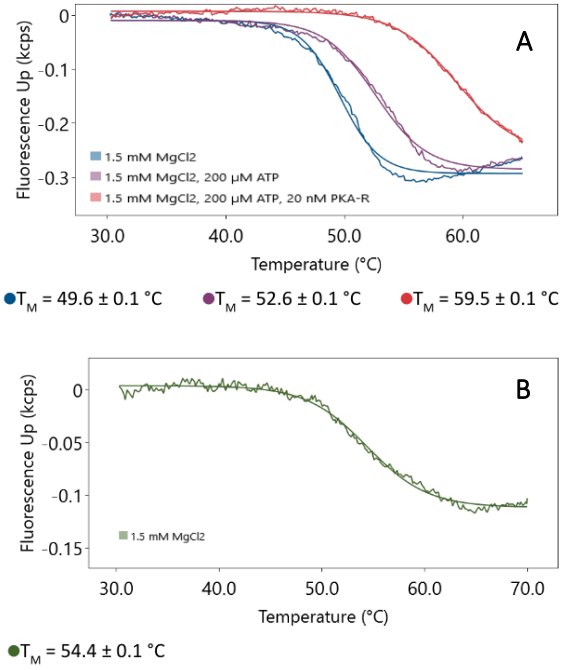

Figure 2 I Melting curves. **A** PKA-C without and with ATP and PKA-R. **B** PKA-R.

While PKA-C shows a relatively low melting temperature of less than 50°C, incubation with Mg^{2+}, ATP and PKA-R led to a strong stabilization which could be observed in the shift of the melting temperature of 3°C, respectively 10°C (c.f. Table 2).

Table 2 | Melting temperatures of PKA-C and PKA-R and shifts ΔT_M upon incubation with different analytes.

Subunit/complex	Melting buffer	T_M (°C)	ΔT_M (°C)
C	1.5 mM $MgCl_2$	49.6 ± 0.1	-
C	1.5 mM $MgCl_2$, 200 µM ATP	52.6 ± 0.1	3.0 ± 0.2
C	1.5 mM $MgCl_2$, 200 µM ATP, 20 nM PKA-R	59.5 ± 0.1	9.9 ± 0.2
R	1.5 mM $MgCl_2$	54.4 ± 0.1	-

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Conclusions

This study demonstrates that the hydrodynamic diameter as well as thermal stability of the catalytic and regulatory PKA subunits could be investigated in the same switchSENSE assay. The switchSENSE sizing measurements provided valuable information on the type of complex (stoichiometry) formed on the surface, which is an important pre-requisite for the interpretation of binding kinetics experiments.

Changes in the size and shape of proteins could be monitored with two complementary switchSENSE measurement modes (signals): the Dynamic Response measurement mode was used to measure the protein diameter. The Fluorescence Proximity Sensing mode was used for melting experiments; here, the dye fluorescence is quenched when the protein's interior hydrophobic core becomes exposed as a result of a thermally induced unfolding process. The assay is set up in a few minutes and the screening of molecules which elicit conformational changes or shifts in thermal stability is straight-forward.

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