

Enhanced functional isolation of cAMP-binders using the C2-cAMP caproKit™ and the C8-cAMP caproKit™

Capture Compound Mass Spectrometry (CCMS) is an innovative technology to reduce the complexity of biological samples through selective isolation of targeted protein or enzyme families. Small synthetic molecules (Capture Compounds™) interrogate native proteins, even lipophilic membrane proteins. CCMS technology enables an efficient complexity reduction of the proteome and allows discovering, isolating and profiling members of functional protein families (e.g. cAMP binding proteins) within a variety of biological samples.

Introduction

Cyclic adenosine monophosphate is an important second messenger in many biological systems, ranging from unicellular organisms to metazoans (1). The formation or degradation of cAMP is triggered by extracellular signals that are transduced to adenylyl cyclases or phosphodiesterases. Among the key effectors are the cAMP-dependent protein kinases (PKAs), but also several ion channels as well as a range of other proteins (2-4).

In most known cases, cAMP acts on effector proteins through binding to cyclic nucleotide binding domains (CNBDs). However, cAMP can also bind to GAF domains in some eukaryotic phosphodiesterases and in a range of prokaryotic proteins (5, 6). Existing crystal structures (PDB: 2ZMF) suggest that the binding mode of cAMP may differ between GAF domains and CNBDs.

Recently, caprotec introduced a C8-cAMP Capture Compound attached to the Capture Compound (CC) scaffold via the 8-position of the adenine (C8-cAMP-CC). It was demonstrated that this CC effectively captures regulatory subunits of the Protein Kinase A

(PKA) and cAMP-regulated hyperpolarisation and cyclic nucleotide-gated (HCN) ion channels (7). However, phosphodiesterases were not among the proteins captured by this CC. This application note presents data obtained from experiments using a C2-cAMP-CC with a different attachment position of the CC scaffold, via the 2-position of the adenine (Figure 1). In this application note a comparison is presented of the capture profiles of the C8-cAMP-CC and the new C2-cAMP-CC when applied to a protein sample from rat brain synaptosomes. Indeed, the C2-cAMP-CC, unlike the C8-cAMP-CC, is capable of selectively capturing several phosphodiesterases, likely via interaction with the GAF domains of these phosphodiesterases. The data demonstrate the utility of the C2-cAMP-CC for capturing purified regulatory subunit 1A of cAMP-dependent protein kinase (KAPO, also termed PKA RI). With the C2-cAMP-CC and the C8-cAMP-CC caprotec now offers complementary cAMP caproKits for the comprehensive profiling of cAMP binding proteins from complex biological mixtures.

Materials

The C8-cAMP caproKit™ (Article No. 1-1030-050) contains the C8-cAMP Capture Compound at a stock concentration of 100 μ M, free C8-cAMP as the competitor solution at a stock concentration of 4 mM, streptavidin magnetic beads, 5x concentrated capture buffer, and 5x concentrated wash buffer as well as a preparation of purified regulatory subunit 1 of cAMP-dependent protein kinase (KAPO, also termed PKA RI, at a stock concentration of 1 mg/ml). The C2-cAMP caproKit™ contains the C2-cAMP Capture Compound at a stock concentration of 100 μ M and cAMP (20 mM) as the competitor. The other components correspond to the components of the C8-cAMP caproKit™. The structures of the different Capture Compounds are depicted in Figure 1.

with the caproBox™, and the beads were isolated using the caproMag™. As the protein sample, two rat brains were homogenized by 12 strokes at 900 rpm in a motor-driven glass-teflon homogenizer in a homogenisation buffer containing 0.32 M sucrose, 5 mM HEPES/NaOH pH 7.4, supplemented with protease inhibitors. 10 volumes of homogenisation buffer per gram of tissue wet weight were used. The homogenate was filtered through a nylon gauze to remove debris, and the filtrate was centrifuged for 10 min at 1000 x g to remove nuclei. The supernatant was centrifuged for 15 min at 4 °C at 12,000 x g to pellet organelles. The crude organelle pellet was resuspended in homogenisation buffer, re-homogenised with 6 strokes at 900 rpm in a motor-driven glass-teflon homogenizer, and centrifuged for 20 min at 4 °C at 12,000 x g. The organelle pellet was then resuspended 1.5 ml/gram of tissue wet weight

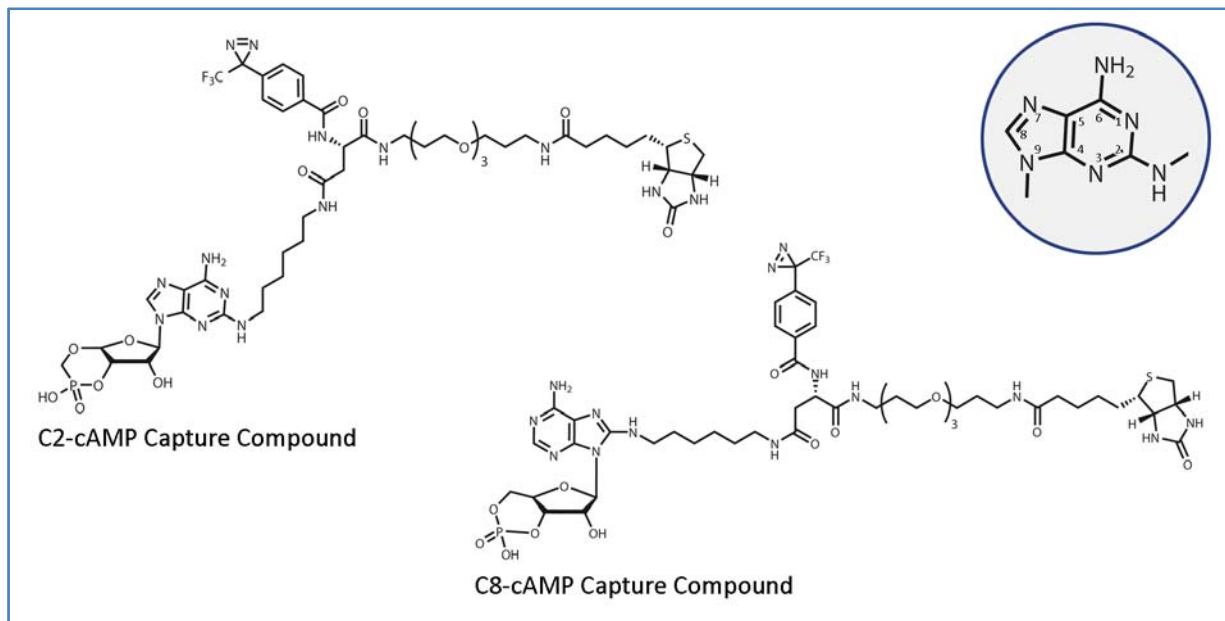


Figure 1 Structure of C2-cAMP and C8-cAMP Capture Compound™.

We recommend PCR Tube strips for volumes up to 200 μ l (Thermo Fisher, cat. No. AB-1114, component of the caproKits™) as reaction vessels to prepare the samples, conduct the capture experiments and to wash and isolate the magnetic beads. Note that at some stages of the experiment centrifugation of the Tube strips in a simple tabletop centrifuge with an appropriate butterfly rotor is desirable. Irradiation of the samples for photo cross-linking was performed

buffer B (0.32 M sucrose, 5 mM Tris/HCl pH 8.1) with a pastette and layered on top of a sucrose step gradient consisting of equal volumes of sucrose solution layers of 0.85 M sucrose, 1 M sucrose, and 1.2 M sucrose in an ultracentrifugation tube. Ultracentrifugation was carried out for 2 hours at 85,000 x g at 4 °C. The synaptosome fractions was recovered from the phase border between 1.0 M and 1.2 M sucrose, diluted with ≥ 4 volumes of PBS, and pelleted by centrifugation for 30 min at 40,000 x g. The synaptosome pellet was then

resuspended in homogenisation buffer, aliquoted, snap-frozen, and stored at -80 °C until further use.

Capture experiment

The assays were performed according to the caproKit™ OnBead Guideline (see www.caprotec.com/support/downloads) and prepared as follows: for preparation of the caproBeads™, per assay 50 µl streptavidin magnetic beads were mixed with 25 µl of the C8-cAMP-CC or C2-cAMP-CC solution, respectively and incubated for 2 min at room temperature under vigorous shaking. Afterwards, the tube strip was fitted in the caproMag™, the beads collected in the tube lids, and washed twice with wash buffer and ultimately collected in the tube lid according to the caproMag™ Guideline (www.caprotec.com/support/downloads). In the meantime, the assays were prepared in the following order: Water, 5x capture buffer, and lysate were mixed by vortexing to achieve a final reaction volume of 100 µl with a final protein concentration of 3.5 mg/ml. Note: for the competition sample add free competitor prior to the addition of the lysate. The caproBeads™ were thoroughly resuspended in the sample solutions and allowed to incubate for 3 hours at 4 °C under rotation. The samples were very briefly centrifuged in a tabletop centrifuge with a butterfly rotor, in order to remove any liquid from the tube lids, but short enough to avoid pelleting of the caproBeads™. Afterwards, in 12 intervals of 2.5 min the samples were irradiated in the caproBox™ at 2-4 °C and finally washed six times with wash buffer WB1. The capture samples were then washed twice with 200 µl 80 % acetonitrile and once with 200 µl MS-grade water, and subjected to OnBead tryptic digestion as described in the respective caproKit™ guideline MassSpec (<http://www.caprotec.com/support/downloads>).

Mass spectrometry and database research

LC-MS/MS experiments were performed using a nanoflow HPLC system (Proxeon) coupled on-line to an ESI-LTQ-Orbitrap system. From the LC column, peptides were eluted during an 90-min linear gradient from 5 % ACN/0.1 % FA to 40 % ACN/0.1 % FA followed by additional 2 min to 100 % ACN/0.1 % FA and remaining at 100 % for another 8 min with a controlled flow rate of 300 nl/min. MS/MS fragmentation was performed in a data-dependent mode using one survey MS scan followed by four MS/MS scans per second. Proteins were identified by automated database searching against the human UniProtKB/Swiss-Prot database using SEQUEST and Tandem X! as implemented in the software scaffold 2.0. Specific search parameters were 5 ppm precursor tolerance, 1 amu fragment ions tolerance, and full trypsin specificity allowing for up to two missed cleavages. Phosphorylation at serine, threonine, and tyrosine, oxidation of methionines, deamination at asparagines and glutamine, acetylation at lysine and serine, formylation at lysine, and methylation at arginine, lysine, serine, threonine and asparagine were allowed as variable modifications. No fixed modifications were used in database search.

Results

Distinct capture profiles were observed from capture experiments with C8-cAMP-CC versus C2-cAMP-CC. Both Capture Compounds performed equally well on the regulatory subunits of PKA (KAPs, see also Table 1 for the nomenclature).

Table 1: Overview of the nomenclature for human and rat regulatory subunits of cAMP-dependent protein kinases.

Acronym	Gene name	UniProtKB acc	Protein name	Organism
KAP0_Human	PRKAR1A	P10644	cAMP-dependent protein kinase type I-alpha regulatory subunit	Human
KAP1_Human	PRKAR1B	P31321	cAMP-dependent protein kinase type I-beta regulatory subunit	Human
KAP2_Human	PRKAR2A	P13861	cAMP-dependent protein kinase type II-alpha regulatory subunit	Human
KAP3_Human	PRKAR2B	P31323	cAMP-dependent protein kinase type II-beta regulatory subunit	Human
KAP0_Rat	Prkar1a	P09456	cAMP-dependent protein kinase type I-alpha regulatory subunit	Rat
KAP1_Rat	Prkar1b	P81377	cAMP-dependent protein kinase type I-beta regulatory subunit	Rat
KAP2_Rat	Prkar2a	P12368	cAMP-dependent protein kinase type II-alpha regulatory subunit	Rat
KAP3_Rat	Prkar2b	P12369	cAMP-dependent protein kinase type II-beta regulatory subunit	Rat

Equal efficiency among the different cAMP-CCs was also observed for cGMP-dependent protein kinase 2 (KGP2, UniProtKB accession no. [Q64595](#)). In this case, both attachment variants are compatible with successful capturing due to some promiscuity between cAMP and cGMP binding (Figure 2).

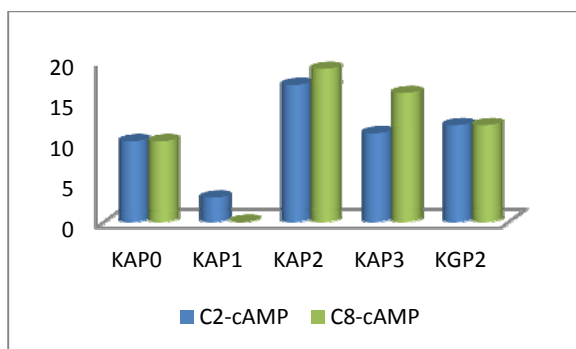


Figure 2 Capturing of KAPs and the cGMP-dependent protein kinase KGP2 by the two different C8-cAMP-CC and C2-cAMP-CC. Given are numbers of unique peptides from duplicate experiments.

In the case of other C8-cAMP-CC target proteins, the capture profiles are distinct reflecting different structural properties of the binding sites. On the one hand C8-cAMP-CC outperforms the C2-cAMP-CC on HCN ion channel proteins and the cAMP-dependent guanine nucleotide exchange factor Epac4 (Figure 3).

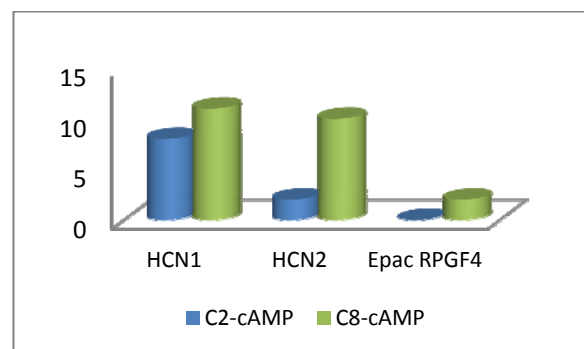


Figure 3 Capturing of HCN ion channels and the cAMP-dependent guanine nucleotide exchange factor Epac 4 by the two different cAMP-CCs. Given are numbers of unique peptides from duplicate experiments. The C8-cAMP-CC outperforms the C2-cAMP-CC with respect to these target proteins.

On the other hand the C2-cAMP-CC clearly outperforms the C8-cAMP-CC on some phosphodiesterases (Figure 4).

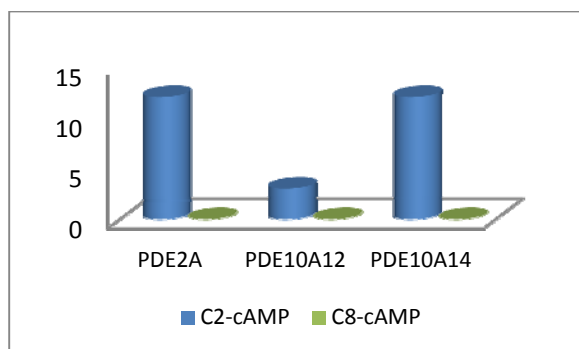


Figure 4 Capturing phosphodiesterases with the two different cAMP-CCs. Given are numbers of unique peptides from duplicate experiments. While the C8-cAMP-CC fails to capture these target proteins, the C2-cAMP-CC is very effective.

The latter observation cannot be explained on the basis of current knowledge about the orientation of cAMP binding to the active centres of phosphodiesterases. However, it is consistent with the observed orientation of cAMP binding to GAF domains that are present in some phosphodiesterases.

In summary, the capture profiles obtained from the two Capture Compounds show that they are complementary in the coverage of cAMP-binding target proteins. Furthermore, the two Capture Compounds reveal distinct structure-activity relationships for cAMP to target proteins and thus qualify the cAMP Capture Compounds as valuable tools for structural biology applications.

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www.caprotec.com/support/downloads

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Ordering information:

Item Nr.	Description
1-1030-050	C8-cAMP caproKit™ 50 rxns
1-1030-010	C8-cAMP caproKit™ 10 rxns
1-1031-050	C2-cAMP caproKit™ 50 rxns
1-1031-010	C2-cAMP caproKit™ 10 rxns

The caproKit includes the respective Capture Compound, all buffers, protein controls, competitor, and Streptavidin magnetic beads.

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