Charles University
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Bc. Kateřina Jarosilová

Preparation and characterization of Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2)

Příprava a charakterizace Ca^{2+}/kalmodulin-dependentní proteinkinasy kinasy 2 (CaMKK2)

Diploma thesis

Supervisor:
Prof. RNDr. Tomáš Obšil, Ph.D.

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Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně pod vedením mého školitele prof. RNDr. Tomáše Obšila, Ph.D. a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

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Podpis ……

Kateřina Jarosilová

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ABSTRAKT

Kalmodulinová proteinkinasová kaskáda je signální dráha, která se podílí na buněčné odpovědi na zvýšenou koncentraci vápenatých iontů uvnitř buňky. Ca^{2+} patří mezi sekundární posly, které zajišťují odpověď na mnoho podnětů. Většina těchto odpovědí začíná navázáním vápenatých iontů na kalmodulin, receptorový protein uvnitř buňky. Vazbou se zvyšuje afinita kalmodulinu k dalším proteinům, které jsou součástí signální kaskády. Jedním z interakčních partnerů kalmodulinu je kalcium/kalmodulin-dependentní proteinkinasa kinasa 2 (CaMKK2).

CaMKK2 je serin/treoninová proteinkinasa, která se podílí zejména na regulaci genové exprese, dále také na regulaci energetické rovnováhy. CaMKK2 se skládá z několika funkčních oblastí, z nichž nejprozkoumanější je kinasová doména (její struktura již byla vyřešena díky krystalografickým studiím). Ta se nachází v prostřední části proteinu (přibližně 298 aminokyselinových zbytků) o velikosti 588 aminokyselin. U zbytku proteinu se předpokládá, že je převážně nestrukturovaný a částečně se strukturalizuje až po navázání ligandu.

Cílem této práce bylo připravit několik expresních konstruktů lidské CaMKK2. Tyto konstrukty jsou určeny pro strukturní studie a dále také pro studium interakcí a aktivity jednotlivých proteinů. Při navrhování a přípravě expresních konstruktů se vycházelo z předpokladu, že CaMKK2 je regulována místě-specifickou fosforylací.

Výsledky této práce prokázaly, že interakce CaMKK2 s proteinem 14-3-3 je závislá na fosforylaci motivů v sekvenci CaMKK2. V rámci této práce byly připraveny čtyři konstrukty CaMKK2 lišící se v počtu a umístění fosforylačních míst specifických pro protein kinasu A. Některá z těchto míst se zároveň shodovaly s motivy typickými pro vazbu proteinu 14-3-3. Všechny konstrukty byly úspěšně exprimovány a purifikovány. Enzymová aktivita připravených proteinů byla ověřena pomocí kinetického měření. Interakce fosforylované CaMKK2 s proteinem 14-3-3 byla ověřena pomocí nativní elektroforézy a analytické ultracentrifugace.

ABSTRACT

Calmodulin kinase cascade is a signaling pathway which is involved in the response to the increasing intracellular calcium levels. Ca^{2+} is a ubiquitous second messenger which promotes wide-range of cellular signaling events. Many of these signaling pathways start with the binding of Ca^{2+} to its primary intracellular receptor calmodulin. Calmodulin in turn binds to its downstream targets in the Ca^{2+}/calmodulin signaling cascade. One of the most important enzymes of this cascade is a Ca^{2+}/calmodulin-dependent protein kinase kinase 2 (CaMKK2).

CaMKK2 is a serine/threonine protein kinase which regulates for example gene transcription or energy homeostasis by phosphorylation of its downstream targets. Catalytic domain (which provides kinase activity) is located in the middle part of the protein and possesses structure typical for kinases. CaMKK2 consists of 588 amino acids but the secondary structure is known only for the region of the kinase domain (298 residues). The rest of the protein is assumed to be unstructured as long as CaMKK2 is not bound to any interaction partner.

The aim of this study was to prepare several constructs of human isoform of CaMKK2 for the further structural and activity studies. It is believed that CaMKK2 is regulated by site-specific phosphorylation. Phosphorylation of some particular residues should enhance the activity of the kinase whereas other phosphorylated residues promote inhibition. Putative regulation of CaMKK2 also involves interaction with the scaffolding protein 14-3-3.

Results of this thesis showed that 14-3-3 binding to CaMKK2 is a phosphorylation-dependent interaction. Four different constructs of human CaMKK2 (which differ in number or location of specific phosphorylation sites) were prepared. Some of these phosphorylation sites also correspond to the putative 14-3-3 binding sites. All prepared CaMKK2 constructs were successfully expressed and purified. The activity of prepared enzymes was confirmed by kinase assay. The interaction between phosphorylated CaMKK2 and 14-3-3 protein was verified by native electrophoresis and analytical ultracentrifugation.

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LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AID	autoinhibitory domain
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
APS	ammonium persulfate
ATP	adenosine triphosphate
AU	absorbance units
AUC	analytical ultracentrifugation
β-ME	β-mercaptoethanol
BAD	Bcl-2-associated death promoter protein
BL21(DE3)	strain of *E. Coli*
bp	base pairs
CaM	calmodulin
CaMK	calcium/calmodulin-dependent protein kinase
CaMKI	calcium/calmodulin-dependent protein kinase I
CaMKIV	calcium/calmodulin-dependent protein kinase IV
CaMKK	calcium/calmodulin-dependent protein kinase kinase
CaMKK1	calcium/calmodulin-dependent protein kinase kinase 1
CaMKK2	calcium/calmodulin-dependent protein kinase kinase 2
cAMP	cyclic adenosine monophosphate
CBD	calmodulin binding domain
CD	catalytic domain
CREB	cAMP response element-binding protein
DH5α	strain of *E. Coli*
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ds	double-stranded
DTT	dithiothreitol
ε	molar extinction coefficient
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum

GB1	B1 domain of G protein
GLS	gel loading solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPTG	isopropyl β-D-1-thiogalactopyranoside
LB medium	lysogeny broth medium
MCS	multiple cloning site
M_w	molecular weight
MWCO	molecular weight cut-off
NPY	neuropeptide Y
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PCRp	PCR products
PDB	Protein Data Bank
PKA	protein kinase A
PMSF	phenylmethylsulfonyl floride
RD	regulatory domain
RLU	relative luminescence units
RNA	ribonucleic acid
RP insert	Arg-Pro-rich insert
rpm	revolutions per minute
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
ss	single-stranded
SV-AUC	sedimentation velocity analytical ultracentrifugation
TB medium	terrific broth medium
TBE	Tris/Borate/EDTA
TEMED	tetramethylethylenediamine
TEV	Tobacco etch virus
UV	ultraviolet
WT	wild-type

1 INTRODUCTION

Overweight and obesity is a today's serious worldwide problem. According to the World Health Organization, more than 1.9 billion adults were overweight and over 600 million of them were obese in 2014. Obesity is associated with various life threatening diseases such as type 2 diabetes, cardiovascular disorders and many others. Despite the finding of several "obesity genes", it seems that environmental factors have the greatest influence on increasing adiposity.

It was found that calcium intake is a crucial factor inducing obesity. Lower calcium intakes lead to the higher levels of intracellular calcium which then trigger Ca^{2+} signaling pathways. Ca^{2+} is a common signaling element with many physiological roles. Ca^{2+} regulates a wide-range of cellular processes such as cell development, neurogenesis, learning and memory, but also saliva secretion or muscle contraction. More importantly, intracellular calcium levels affect lipogenesis and lipolysis.

Whole-body energy balance is regulated by Ca^{2+}-dependent signaling cascade. The energy homeostasis is determined by ATP to AMP ratio in the cell. Pathways consuming energy (such as proteosynthesis or gene transcription) are down-regulated after metabolic stress when ATP to AMP ratio is reduced. On the other hand, pathways producing ATP (such as fatty acid oxidation or glycolysis) are up-regulated in such cases. Another impact of signaling pathways is a stimulation of appetite by triggering specific hormones.

In the center of Ca^{2+} signaling is Ca^{2+}/calmodulin-dependent protein kinase cascade. This cascade involves multifunctional kinases which transfer the signal by phosphorylation of the downstream targets. New members of this signaling pathway were discovered over the years. And now, it is getting to be much clearer how great influence Ca^{2+} possesses in the cell.

2 LITERATURE REVIEW

2.1 Ca^{2+} signaling pathways

Calcium ions (Ca^{2+}) are one of the most ubiquitous second messenger which play crucial role in many signaling pathways (physiological as well as pathological), especially in neuronal tissues. Its concentration affects cell destiny. Ca^{2+} is essential for life as a signal molecule but its long-term high intracellular levels are lethal for the cell. There is no way to metabolically degrade Ca^{2+} therefore cell has to strictly regulate its intracellular concentration. Extracellular levels of Ca^{2+} range around 2 mM which is 20 000-fold higher than normal intracellular 100 nM concentration. This demand for low levels inside the cell is probably caused by the natural property of Ca^{2+}, precipitating phosphates. Maybe the involvement of Ca^{2+} in the signaling events is just a side effect of cellular effort to reduce Ca^{2+} cytosolic levels. Ca^{2+} binds to the proteins with specific helix-loop-helix binding motif (EF hand). After Ca^{2+} binding, proteins change conformation and can be involved in modulation of other members of signal transduction. Interestingly, Ca^{2+} ions are not usually able to diffuse further than 0.5 μm before they are captured by a binding protein [1]. After entering the cell, Ca^{2+} usually binds its primary receptor calmodulin.

2.2 Calmodulin

Calmodulin (calcium-modulated protein, CaM) is an intracellular Ca^{2+} receptor. Ca^{2+}/CaM complex is involved in many cellular signaling pathways where allosterically activates various proteins. Each calmodulin molecule is able to bind four Ca^{2+} ions (Figure 2.1) [2]. This binding induces conformational changes which result in exposition

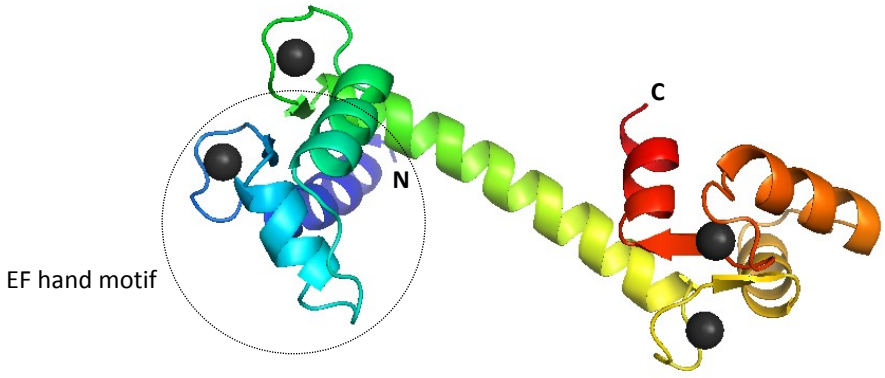


Figure 2.1 | Crystal structure of human calmodulin
Calmodulin is a typical Ca^{2+} binding protein with four EF hand motifs. Ca^{2+} ions are shown as black spheres. Secondary structure of the protein is colored from the blue N-terminus to the red C-terminus. Adapted from the structure deposited in the Protein Data Bank (PDB) [2].

of hydrophobic regions of calmodulin. These regions are then responsible for the interaction of calmodulin with its downstream targets [3]. Calmodulin cascade is involved in many biological processes such as long-term memory formation, synapse formation or energy balance [4]. CaM kinase cascade is not determined only by the Ca^{2+} signal. It is also negatively regulated on the level of CaMK (calcium/calmodulin-dependent protein kinase) kinases by cAMP-dependent protein kinase (PKA) [5].

2.3 Ca^{2+}/calmodulin signaling cascades

The Ca^{2+}/calmodulin-dependent protein kinase cascade involves three main kinases (Figure 2.2). CaMKK (calcium/calmodulin-dependent protein kinase kinase; with two

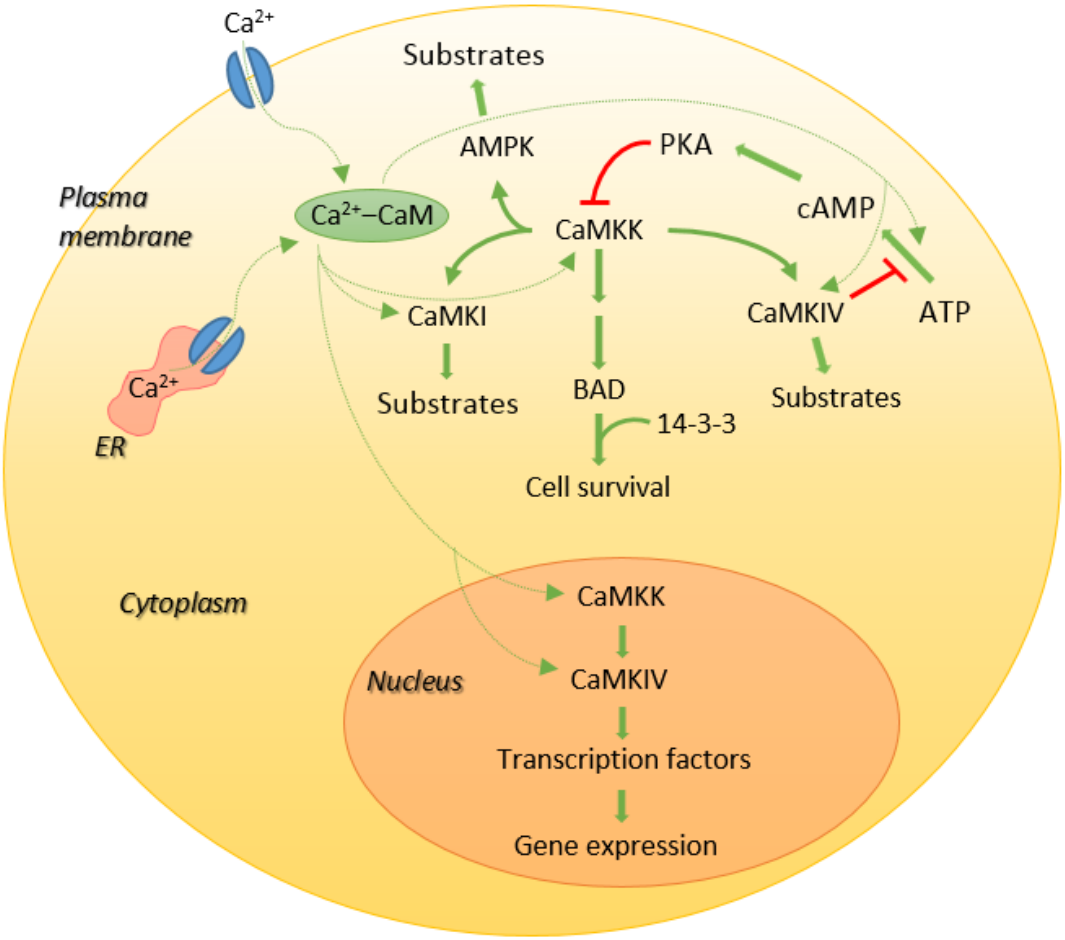


Figure 2.2 | CaM kinase cascade
Increased levels of intracellular Ca^{2+} lead to the formation of Ca^{2+}/calmodulin complex which triggers many cell responses. Individual pathways are described in the following text. ER – endoplasmic reticulum; CaM – calmodulin; AMPK – AMP-activated protein kinase; PKA – protein kinase A; cAMP – cyclic adenosine monophosphate; ATP – adenosine triphosphate; CaMKK – calcium/calmodulin-dependent protein kinase kinase; CaMKI – calcium/calmodulin protein kinase I; CaMKIV – calcium/calmodulin protein kinase IV; BAD – Bcl-2-associated death promoter protein. Adapted from [6].

isoforms known as α and β or 1 and 2, respectively) transiently phosphorylates (and thus activates) CaM kinases, CaMKI (calcium/calmodulin-dependent protein kinase I) and CaMKIV (calcium/calmodulin-dependent protein kinase IV). This cascade occurs mainly in brain and T lymphocytes but also in other key metabolic tissues where it is activated in response to the increase in the intracellular Ca^{2+} levels [6].

Creation of Ca^{2+}/CaM complex triggers higher affinity of calmodulin to its binding partners (such as various CaM kinases). Ca^{2+}/CaM binding itself is not sufficient for full activation of these downstream targets but causes conformational changes of the ATP-binding domain and also probably abolishes autoinhibitory interaction between catalytic and regulatory domain [7],[6]. Phosphorylation on the activation loop is usually required to achieve a full biological activity. In case of CaM kinases CaMKI and CaMKIV phosphorylation is provided by CaMKK2 [8]. It was shown that the same kinase is also the upstream kinase for AMPK (AMP-activated protein kinase) [9],[10]. CaMK cascade is also linked to apoptosis through the influence on transcription factors and through inactivating of proapoptotic protein BAD (Bcl-2-associated death promoter protein) [6], [11].

2.3.1 CaMKI

CaMKI (Figure 2.3) is expressed in many tissues and probably provides wide range of cellular roles, mainly connected with cell proliferation [12],[4],[13]. CaMKI is composed of catalytic domain which is followed by inhibitory and CaM-binding region [14]. Binding of Ca^{2+}/CaM complex to the CaMKI causes slight increase in activity and more importantly triggers autophosphorylation to strengthen the enzyme activity [15]. For its maximal kinase activity, CaMKI requires phosphorylation on threonine 177 which is located in kinase

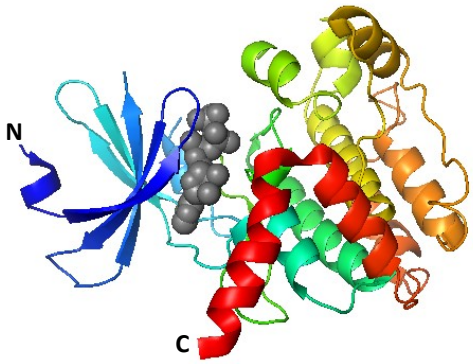


Figure 2.3 | Crystal structure of human CaMKI
CaMKI is a member of CaM kinase cascade. This is a structure of CaMKI (C-terminal region of 50 amino acids is flexible and thus missing) in complex with ATP. ATP is shown in gray. Secondary structure of the protein is colored from the blue N-terminus to the red C-terminus. Adapted from the structure deposited in the Protein Data Bank (PDB) [13].

activation loop [16]. Phosphorylation of this residue stabilizes and organizes the structure of the activation loop [14].

2.3.2 CaMKIV

From the cellular point of view, CaMKIV (Figure 2.4) is predominantly localized in the nucleus but is also present in the cytoplasm [17]. CaMKIV occurs in brain and some other tissues where mediates transcription regulated by Ca^{2+}. For the activation, transcription factors of specific genes have to be directly phosphorylated by CaMKIV [12]. CaMKIV itself has to be phosphorylated on specific residue (Thr196) on the activation loop to become active [18]. Moreover, it was described that CaMKIV is autophosphorylated after phosphorylation of Thr196 by CaMKK. This autophosphorylation occurs on the N-terminus which is rich in serines and threonines. CaMKIV then exhibits Ca^{2+}-independent activity which can prolong activation caused by increase in intracellular Ca^{2+} levels. Ca^{2+}/CaM binding is then prevented by autophosphorylation in the CaM-binding domain in this case [6].

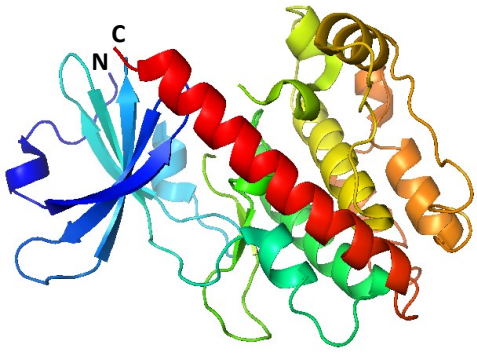


Figure 2.4 | Crystal structure of human CaMKIV
Structure of the catalytic domain of CaMKIV. Secondary structure of the protein is colored from the blue N-terminus to the red C-terminus. Adapted from the structure deposited in the Protein Data Bank (PDB), PDB ID: 2W4O.

2.4 Protein kinase A (PKA)

The cAMP-dependent protein kinase (protein kinase A, PKA) is a member of AGC group (subfamily of the kinome) which responds to the changing levels of second messenger (cyclic AMP) in the cell. It is a serine/threonine kinase which is involved in numerous signaling cascades [19]. PKA is a tetrameric enzyme which consists of two catalytic subunits which are maintained in an inactive state by two regulatory subunits [20]. Structure obtained from crystallization of PKA catalytic subunit (Cα) showed organization of catalytic domain which is characteristic for many different protein kinases. Kinase domain possess bi-lobed

structure with smaller N-terminal ATP binding site and larger C-terminal substrate binding site (Figure 2.5). C-terminus is relatively conserved through protein kinase family whereas the active site is the most variable region. ATP-binding site is not conserved so much which makes it an ideal target for selective inhibitors for each kinase [21].

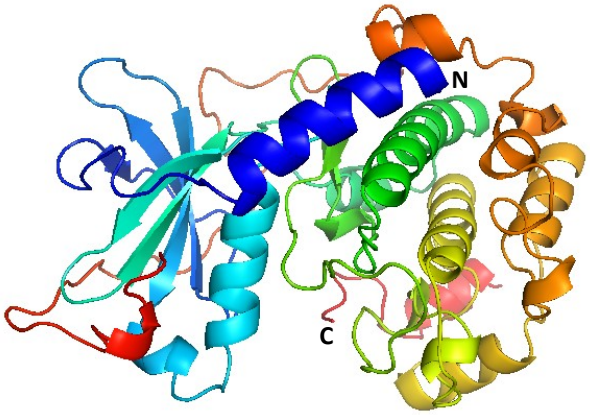


Figure 2.5 | Crystal structure of human PKA catalytic subunit
Catalytic subunit of PKA consists of two lobes (β-strands on the N-terminus, α-helixes on the C-terminus). Secondary structure of the protein is colored from the blue N-terminus to the red C-terminus. Adapted from the structure deposited in the Protein Data Bank (PDB) [21].

PKA is an intracellular cAMP receptor which is activated by elevated cAMP levels in the cell. Two molecules of cyclic AMP bind to each regulatory subunit which then dissociate from catalytic subunits and thus activate them. Catalytic subunits phosphorylate serine or threonine residues of their downstream targets [22]. Phosphorylated residues of PKA targets are located in specific sequence (PKA phosphorylation motif) which is characterized by the presence of arginine residues prior to the phosphorylated residue (-R-R-X-pS/T-X-) [23], [24].

2.5 14-3-3 proteins

14-3-3 proteins are family of ubiquitous dimeric regulators which bind to a wide range of proteins. 14-3-3 are about 30 kDa acidic proteins which create typical cup-shaped dimer (Figure 2.6, page 17) [25],[26]. Seven isoforms of mammalian 14-3-3 protein were identified. 14-3-3 proteins participate in cell signaling through several different mechanisms. 14-3-3 proteins change activity of their binding partners, influence the interaction between their partners and other compounds and determine the localization of their partners in the cell. 14-3-3 binding can also induce conformational changes of the ligand which then also affect its biological activity [27].

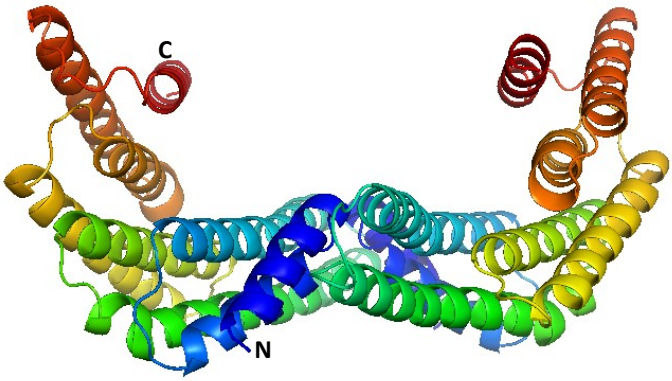


Figure 2.6| Crystal structure of human 14-3-3 dimer
14-3-3 protein creates dimer through the interaction between N-terminal helixes. Secondary structure of the protein is colored from the blue N-terminus to the red C-terminus. Adapted from the structure deposited in the Protein Data Bank (PDB) [26].

14-3-3 proteins bind to the phosphorylated serine which occurs in the specific sequence. It was shown that arginine in either the position -3 or -4 increases binding affinity. Serine in the -2 position was then identified as another plausibly important residue. The last residue critical for specific binding was proline in the +2 position [28]. These motifs are present in majority of the known 14-3-3 binding proteins [29]. The sequences of the binding motifs were later defined as R-[S/Φ]-[+]-pS-[L/E/A/M]-P and R-X-[Y/F]-[+]-pS-[L/E/A/M]-P where Φ is an aromatic residue, + is a basic residue, pS indicates phosphorylated serine and X means any residue [26]. Additionally, the third motif (C-terminal sequence, pS/pT-X-(X)-COOH) was defined [30]. Nevertheless, there is still a lot of 14-3-3 binding proteins with sequence which do not match with these optimal motifs.

14-3-3 ligands very often contain more than one 14-3-3 binding site. Phosphorylation of one of them is then usually necessary for the 14-3-3 binding whereas the second one provides conformational changes of the ligand which initiate its involvement in downstream metabolic or signaling pathways. The dominant 14-3-3 binding site (so called “gatekeeper”) is required for the initiation of the 14-3-3 binding. Without its involvement, the second binding site is not able to create stable interaction with 14-3-3 protein [27].

2.6 CaMK kinases

There are two variants of CaMK activators in the CaMK cascade, CaMKK1 and CaMKK2 (CaMKKα and CaMKKβ, respectively) [8]. The sequences of these distinct proteins share very high similarity (80%) and also identity (65%) [12]. Both kinases contain common catalytic kinase domain as was confirmed even for CaMKK2 (Figure 2.7) [31]. Originally, two regulatory domains of CaMKK1 were identified [32]. The same regions can be also found in the sequence of CaMKK2 (Figure S1, page 83) where the additional third regulatory region was observed [33]. Initial studies mention only CaMKK, the existence of two isoforms was shown later.

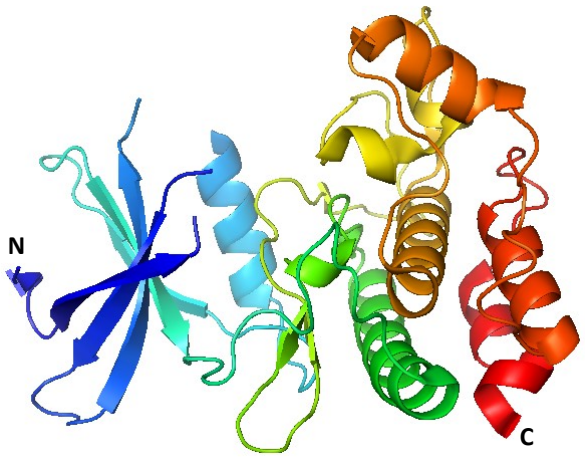


Figure 2.7 | Crystal structure of catalytic domain of human CaMKK2
Catalytic domain of CaMKK2 was crystalized with its specific inhibitor STO-609 (not shown). Solved structure confirmed that CaMK kinases possess catalytic domain organization typical for kinases. Secondary structure of the protein is colored from the blue N-terminus to the red C-terminus. Adapted from the structure deposited in the Protein Data Bank (PDB) [31].

2.6.1 CaMKK substrate recognition

CaMKKs activate their downstream kinases by phosphorylation of the specific residue located in the activation loop of the target. This specific residue (which functions as an activation site, usually serine or threonine) is surrounded by typical amino acids which provide easier recognition by upstream kinases. CaMKs share similar sequences (on the both sides from the activation site) with many other kinases with activation loop. Despite that, there is additional different mechanism of their recognition because other kinases are not phosphorylated by CaMKKs [34]. Moreover, it was shown that CaMKKs lack acidic residues which are conserved in the catalytic domain in other kinases and which serve as spots for substrate recognition [6].

The mammalian CaMKKs contain Arg-Pro-rich (RP) insert which does not occur in the sequences of the other protein kinases. This segment is involved in a selective recognition of CaMKI and CaMKIV kinases. But the insert is not necessary for autophosphorylation of CaMKKs or substrate phosphorylation of synthetic peptide (which corresponds with CaMKIV activation site) [34].

2.6.2 Regulation of CaMKKs

CaMKKs consist of an N-terminal catalytic kinase domain and regulatory region at the C-terminus. The regulatory region contains autoinhibitory domain and CaM-binding domain (Figure 2.8). This domain organization is shared by both CaMKK isoforms and their substrates CaMKI and CAMKIV.

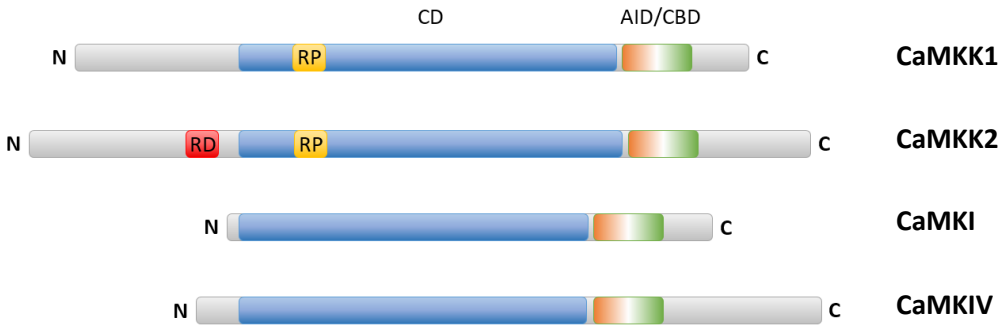


Figure 2.8 | Domain organization
CaM signaling cascade consists of several kinases which all possess similar domain organization. Catalytic kinase domain (CD) is shown in blue. Overlapping autoinhibitory domain (AID) and CaM-binding domain (CBD) are shown in orange and green. RP insert is shown in yellow. Regulatory domain (RD) is shown in red. Adopted from [34].

Protein kinases usually retain in the inactive state if there is no signal such as phosphorylation or messenger molecule. Inactive form of CaMKKs is maintained by autoinhibition. Substrate binding site and ATP-binding site of the catalytic domain are covered by autoinhibitory domain (AID) in the non-activated state. AID mimics a substrate and thus blocks substrate-binding site. ATP-binding site is not inhibited directly. AID causes conformational changes which do not allow binding of ATP. In the presence of Ca^{2+}/CaM complex, AID is dissociated from the catalytic domain because of the binding of the complex to its site which overlaps the AID [14]. CaMKKs are also able of intramolecular autophosphorylation in the presence of Ca^{2+}/CaM [5].

2.6.3 CaMKK1 and PKA

The phosphorylation of CaMKK1 is enhanced by the addition of PKA which is able to phosphorylate CaMKK1 in the absence of Ca^{2+}/CaM complex. CaMKK1 is then inhibited by PKA phosphorylation and thus is not able to phosphorylate its downstream substrates (such as CaMKIV). Moreover, PKA phosphorylation of CaMKK1 suppresses its binding to the Ca^{2+}/CaM complex due to phosphorylation of Ser458 in the CaM-binding domain (but also probably through some additional mechanism). On the other hand, binding of Ca^{2+}/CaM complex in the absence of PKA protects CaMKK1 against subsequent phosphorylation. Second regulatory PKA phosphorylation site (Thr108) was found just before the catalytic domain [5]. Three additional sites were identified as Ser52, Ser74 and Ser475 [35],[36]. Since Ca^{2+} and cAMP/PKA signaling pathways are closely linked together, these results which indicated PKA-dependent regulation of CaMKK1 were not surprising.

2.6.4 CaMKK1 and 14-3-3

Two primary PKA phosphorylation sites of CaMKK1 (Thr108 and Ser458) are connected with direct inhibition by PKA. The remaining sites were analyzed by a data base search. The analyses predicted (among others) regulatory 14-3-3 binding site [17]. Typical 14-3-3 binding site shares the same residue (arginine) at the -3 position from the phosphorylated serine as the one which can be found in the sequences phosphorylated by PKA [28],[23]. Sequence which surrounds Ser475 fulfills parameters for 14-3-3 binding site with -3 arginine and proline at position +2. But (according to study from 2004) this site is only poorly phosphorylated by PKA in the presence of Ca^{2+}/CaM complex thus it has no significant effect on the 14-3-3 binding [36], [17]. The experiments showed that "gatekeeper" 14-3-3 binding site is set in the N-terminus of CaMKK1 where two putative sites were considered (Ser52 and Ser74). Pull down assay later confirmed that Ser74 is responsible for the interaction with 14-3-3 protein. Despite the fact that sequence with Ser74 (containing glutamine in the +2 position) is not typical 14-3-3 binding site, one of the isoform (14-3-3γ) is able to interact with such substrate [17]. In a later study (from 2008), 14-3-3 isoforms η, β, γ and ε were described as CaMKK1 binding partners. It also disproved the hypothesis that only phosphorylated Ser74 is responsible for 14-3-3 binding. Both Ser74 and Ser475 are involved in the interaction with 14-3-3 isoforms [37].

14-3-3 protein assists to the PKA in the inhibition of the CaMKK1 activity. CaMKK1 alone can phosphorylate its downstream substrates. This activity is not affected

by the presence of 14-3-3 protein (in the absence of PKA). Suppressed CaMKK1 activity occurs only in the presence of PKA and inhibition is even stronger when 14-3-3 binds to the PKA phosphorylated CaMKK1. This effect may originate from binding of 14-3-3 protein which sterically blocks dephosphorylation of Thr108 but also from the direct allosteric inhibition of CaMKK1 activity [17].

Additionally to the CaMKK1 regulation by PKA/14-3-3, CaMKK2 as well was shown to interact with 14-3-3η after PKA phosphorylation [37].

2.7 CaMKK2

CaMKK2 regulates many important processes in the cell. Its increased activity is triggered by the elevation of intracellular Ca^{2+} levels. Kinase is then involved in signaling cascade as a central enzyme but also as a scaffolding protein. CaMKK2 participates in the creation of signaling complex which comprises kinase itself, calmodulin, CaMK target of CaMKK2, downstream substrate of CaMK and probably some additional components [4]. CaMKK2 was originally found in brain but it is also present in other tissues [8],[38],[39].

CaMKK2 is involved in two major signaling pathways. One of them is CaMK cascade involving the activation of CaMKI and CaMKIV while the other one involves the activation of AMP-activated protein kinase (AMPK). AMPK is then connected to the regulation of energy balance. CaMKI and CaMKIV regulate transcription. Expression of CaMKK2 in the tissues quite well correlates with expression of its downstream target CaMKIV [12]. CaMKI, CaMKIV and AMPK are three well-characterized substrates of CaMKK2.

2.7.1 Structure of CaMKK2

Human CaMKK2 is approximately 65 kDa protein relatively well conserved among different species. CaMKK2 consists of several domains. From the N-terminus of the protein, it is regulatory domain (residues 129-151), catalytic kinase domain (residues 165-446) and autoinhibitory region (residues 472-477) which overlaps CaM-binding domain (residues 475-500) [33],[ES1]. It was shown that catalytic domain is a relatively conserved region among CaM-kinase family (or in general among the catalytic domains of Ser/Thr protein kinases) [40]. Catalytic domain consists of two structural regions. N-terminus is created by β-strands (ATP-binding site) whereas bigger C-terminal region is composed mainly of α-helixes (substrate binding site) [41],[14]. Catalytic domain also contains unique RP insert (residues 200-230) which is involved in substrate recognition [31]. Autoinhibitory domain contains small differences in amino acid sequence and length compared to its closely related

kinases (such as CaMKI) [31]. CaM-binding domain fulfills characteristics of CaM-binding domain (hydrophobic and basic residues which could possibly form a basic amphipathic α-helix) but was not found by the homology search [40].

2.7.2 Regulation of CaMKK2

Protein kinases are usually regulated by the phosphorylation on the activation loop. The activation loop is localized inside the catalytic domain, on the interface of two structural regions [41]. Whole activation segment (residues 330-357), which lies between the DFG and APE motifs, is composed of activation loop and P+1 loop which is involved in substrate binding [31],[42]. The phosphorylation of the residue on the activation loop induces conformational changes into an active state which is characterized by "DFG-in" conformation. The sequence Asp-Phe-Gly is a highly conserved motif which is located at the N-terminus of the activation loop. Despite the fact that the sequence of activation loop of CaMKK2 includes several serine and threonine residues, they do not correspond to the phosphorylated residues of other kinases. Interestingly, CaMKK2 is able to achieve the active conformation independently on the activation loop phosphorylation [31].

Like other CaM kinases, CaMKK2 activity is suppressed by its autoinhibitary domain. Autoinhibition is relieved by creating a complex with Ca^{2+}/CaM. Autophosphorylation of specific residue does not allow the enzyme to form autoinhibited conformation even after Ca^{2+}/CaM dissociation [12]. Autonomous activity is probably provided by the regulatory domain which situated at the N-terminal part of the protein (specifically residues 129-151). Deletion of this region causes decrease in the autophosphorylation (which is independent on the Ca^{2+}/CaM binding) and also in overall kinase activity [33].

2.7.2.1 Regulation by Ca^{2+}/CaM

Activation of CaMKI and CaMKIV by CaMKK2 is a Ca^{2+}/CaM-dependent process. It was shown that binding of Ca^{2+}/CaM complex to the CaMK (I or IV) allows the access of CaMKK2 to the respective residues which can be then phosphorylated [12]. CaMKK2 and CaMKs possess similar CaM-binding region [40]. However, autonomous activity of CaMKK2 was demonstrated in the absence of Ca^{2+}/CaM complex [12]. This fact is supported by the x-ray structure of Ca^{2+}/CaM – CaMKK2 CBD complex which differs from other CaM-binding domains [31]. Activation status (more precisely binding of Ca^{2+}/CaM complex) of CaMKK2 has no effect on the association with its substrates. The only exception is

the interaction with AMPK where binding of Ca^{2+}/CaM complex to the CaMKK2 is required [43],[44].

2.7.2.2 Regulation by PKA

PKA phosphorylation of CaMKK1 results in inhibition of kinase activity [5]. However, the effect of PKA on the CaMKK2 is not fully explored and explained [45]. It was shown that PKA phosphorylates CaMKK2 much slower than CaMKK1. Also the autophosphorylation of CaMKK2 reaches higher levels than in case of CaMKK1 [36].

The alignment of amino acid sequences of human CaMKK1 and CaMKK2 (Figure S1, page 83) points out possible PKA phosphorylation sites in the CaMKK2 sequence. These sites correspond to the ones which were proven as PKA phosphorylation sites in CaMKK1. CaMKK2 thus contains four putative PKA phosphorylation sites (Ser100, Thr145, Ser495, Ser511). But their function has to be verified.

2.7.2.3 Interaction with 14-3-3 protein

Interaction between CaMKK2 and 14-3-3 protein was not fully established. But according to the high similarity of corresponding sites on both CaMKK isoforms, it is very likely that they both interacts with 14-3-3 in a similar way. One published study already confirmed that PKA phosphorylated CaMKK2 binds 14-3-3 protein (η isoform) [37].

2.7.3 Signaling cascades involving CaMKK2

Misregulation of the Ca^{2+} signaling pathways closely participates in health problems such as obesity or diabetes. Ca^{2+} binds calmodulin and this complex activates CaM kinase cascade. The central enzyme of this cascade is CaMKK2 as an upstream regulator. CaMKK2 has also great impact on processes in key metabolic tissues resulting in whole-body energy homeostasis [46],[47]. Besides that, CaMKK2 is involved in many other signaling pathways usually leading to the pathological effects.

2.7.3.1 Appetite

Appetite is regulated in hypothalamus by hormones ghrelin and leptin. These hormones are upstream signals which control AMPK which is also member of CaMKK2 signaling cascade. This CaMKK2-AMPK pathway (according to the present studies) regulates production of hormone NPY (neuropeptide Y, an appetite stimulant) which influences food intake. This hypothesis is also supported by fact that CaMKK2 is abundantly expressed

in brain. Suppression of CaMKK2 by its specific inhibitor STO-609 leads to the decrease in expression of NPY followed by body weight loss. Experiments with CaMKK2 null mice shown that mice without CaMKK2 have reduced refeeding after a fast in comparison to the wild-type mice. These CaMKK2 null mice were also resistant to a high-fat diet due to loss of appetite and their glucose tolerance retained at normal levels (whereas wild-type mice fed with high-fat diet were less tolerant to the glucose). The same results occurred with the test for insulin sensitivity. CaMKK2 null mice remained normally sensitive to the insulin after diet, whereas wild-type mice became more resistant. All these results lead to the conclusion that CaMKK2 null mice are protected against glucose intolerance and insulin resistance which are associated with obesity caused by high-fat diet [48].

2.7.3.2 Glucose tolerance

CaMKK2 also probably regulates hepatic glucose homeostasis. Several experiment with CaMKK2 null mice and mice with reduction of hepatic CaMKK2 expression were performed to prove that. CaMKK2 null mice were fed *ad libitum* (free access to food and water). Wild-type mice were divided into two groups. One of them was fed *ad libitum*, whereas the second one was pair-fed (they obtained the amount of food which corresponded to the CaMKK2 null mice intake). CaMKK2 involvement in glucose homeostasis was also shown by studies on mice with reduced levels of CaMKK2. After high-fat diet, these mice had reduced blood glucose. Also glucose tolerance was better while CaMKK2 was missing in liver. Additionally, it was shown that CaMKK2 null mice have reduced glucose production in hepatocytes and elevated fat metabolism. These experiments demonstrate that deletion of CaMKK2 causes not just the reduction of appetite but also directly affects and improves glucose metabolism [38].

2.7.3.3 Insulin resistance

Biosynthesis of a glucose takes place in liver and kidney in response to stress, exercise or fasting. Gluconeogenesis is suppressed by insulin (in the presence of glucose from food) resulting in reduction of blood glucose concentration. In type 2 diabetes, insulin resistance (together with other effects of this disease) causes hyperglycemia [38]. Insulin is secreted by β-cells in pancreas. In pancreatic β-cells, CaMKK2 regulates secretion of insulin. Whereas in peripheral tissues which are sensitive to this hormone (such as liver, skeletal muscle or plasma), CaMKK2 is involved in reduction of insulin sensitivity. The experiments were performed on CaMKK2 null mice compared with wild-type mice. High-fat diet-induced

insulin resistance occurred in wild-type mice, whereas knock-out mice were not affected. Another significant difference can be found in insulin production and secretion which is enhanced in CaMKK2-deficient β-cells and also in the presence of CaMKK2 selective inhibitor STO-609 [46].

The role of CaMKK2 in insulin-sensitive peripheral tissues was determined on mice fed with low-fat diet, normal diet and high-fat diet. The analysis of metabolites (which could be affected by loss of CaMKK2 and thus different circulating insulin levels) from tissues shown concentration changes between CaMKK2 null mice and wild-type mice. The biggest differences were visible in case of low-fat diet (relating to the diet) and skeletal muscle (relating to the tissue). Further experiments shown that CaMKK2 null mice have increased fatty acid catabolism in the liver. Interestingly, there was almost no difference in plasma metabolites between the absence and the presence of CaMKK2 [46].

CaMKK2 null mice produce higher levels of insulin regardless to the diet (but it is a constant level within the physiological range). Mice are then protect against high-fat diet-induced insulin resistance and glucose intolerance. On the other hand, wild-type mice generate normal levels of insulin. Increased glucose intake induces oversecretion of insulin to compensate these changes. Chronic metabolic stress then leads to type 2 diabetes and insulin resistance associated with it [46].

2.7.3.4 Memory formation

Long-term memory formation is initiated by the Ca^{2+}-dependent gene transcription in hippocampus. CaMKK2 and activation of CREB (cAMP response element-binding protein, a transcription factor) participates in spatial memory formation through activation of splicing factors. Alternative splicing affects memory formation by regulation of neuronal plasticity [49].

2.7.3.5 Bone remodeling

Physiologically, bones are constantly degraded by osteoclasts and formed again by osteoblasts. CaMKK2 was identified as an inhibitor of osteoblast differentiation and thus factor causing remodeling imbalance resulting in osteoporosis [39].

3 AIMS OF DIPLOMA THESIS

- Preparation of three different expression constructs of human CaMKK2 from the original construct.
- Expression and purification of all four forms of CaMKK2.
- Optimization of phosphorylation protocol and verification of the result of phosphorylation reaction by mass spectrometry.
- Test of the interaction between prepared forms of CaMKK2 and 14-3-3 using native PAGE and analytical ultracentrifugation.
- Verification of the enzyme activity of prepared CaMKK2 using a kinase assay based on ADP-Glo^{TM} kit.

4 MATERIAL AND METHODS

4.1 Material

4.1.1 Chemicals

β-mercaptoethanol	Sigma-Aldrich, USA
acetic acid	PENTA – Ing. Petr Švec, Czech Republic
acrylamide	Carl Roth GmbH, Germany
agarose GTQ	Carl Roth GmbH, Germany
ammonium persulfate (APS)	Sigma-Aldrich, USA
ampicillin	Sigma-Aldrich, USA
bacto tryptone	Carl Roth GmbH, Germany
bacto yeast extract	Carl Roth GmbH, Germany
bis-acrylamide	Carl Roth GmbH, Germany
boric acid	LACH-NER, s.r.o., Czech Republic
bromophenol blue	Carl Roth GmbH, Germany
buffer for BamHI	Fermentas, Canada
buffer for PfuUltra DNA polymerase	Agilent Technologies, Inc., USA
Coomassie Brilliant Blue R 250	LKB Bromma, Sweden
deoxyribonucleotide triphosphate mix (dNTP)	Thermo Fisher Scientific, USA
disodium ethylenediaminetetraacetate dihydrate (Na_2-EDTA·$2H_2O$)	LACH-NER, s.r.o, Czech republic
ethanol	PENTA – Ing. Petr Švec, Czech Republic
glucose	PENTA – Ing. Petr Švec, Czech Republic
glycerol	PENTA – Ing. Petr Švec, Czech Republic
glycine	Carl Roth GmbH, Germany
hydrochloric acid	PENTA – Ing. Petr Švec, Czech Republic
isopropyl alcohol	Genomed, Germany
isopropyl β-D-1-thiogalactopyranoside (IPTG)	EMD Biosciences, Inc., Germany
kanamycin	Sigma-Aldrich, USA
LB agar	Carl Roth GmbH, Germany
LB medium	Carl Roth GmbH, Germany
magnesium chloride	PENTA-Ing. Petr Švec, Czech Republic
magnesium sulfate	PENTA-Ing. Petr Švec, Czech Republic
methanol	PENTA – Ing. Petr Švec, Czech Republic

N,N,N´,N´-tetramethylethylenediamine (TEMED)	Carl Roth GmbH, Germany
peptone (from casein)	Carl Roth GmbH, Germany
Rapid Ligation Buffer	Fermentas, Canada
sodium azide	Sigma-Aldrich, USA
sodium dodecyl sulfate (SDS)	Carl Roth GmbH, Germany
sodium chloride	Carl Roth GmbH, Germany
sodium hydroxide	PENTA – Ing. Petr Švec, Czech Republic
tris(hydroxymethyl)aminomethane (Tris)	Carl Roth GmbH, Germany
tryptone	Carl Roth GmbH, Germany
yeast extract	Carl Roth GmbH, Germany

4.1.2 Enzymes and other material

ADP-Glo^{TM} Kinase Assay	Promega, USA
BamHI	Fermentas, Canada
Blue Protein Ladder	BIOSYSTEMS, Czech Republic
cAMP-Dependent Protein Kinase, Catalytic Subunit	Promega, USA
centrifuge tubes	Carl Roth GmbH, Germany
chelating sepharose fast flow	GE Healthcare, UK
dialysis membrane (type 27/32)	Carl RothGmbH, Germany
DNA oligonucleotides	Generi Biotech s.r.o., Czech Republic
Escherichia coli, strain BL21(DE3)	Stratagene, USA
Escherichia coli, strain DH5α	Thermo Fisher Scientific, USA
Escherichia coli, strain Rosetta	EMD Millipore, USA
gel loading solution (GLS)	New England BioLabs, USA
GelRed Nucleic Acid Stain	New England BioLabs, USA
Gene Ruler 1 kb DNA ladder	Fermentas, Canada
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific, USA
laboratory glass	Simax, Czech Republic
MassRuler Low Range DNA ladder	Fermentas, Canada
microsyringe Hamilton	HAMILTON Bonaduz AG, Switzerland
microtubes	Eppendorf, Germany
NdeI	Fermentas, Canada
PCR Master Mix	Thermo Fisher Scientific, USA
peptide	Pepscan, Netherlands

PfuUltra DNA polymerase	Agilent Technologies, Inc., USA
plasmid pRSFDuet-1 + CaMKK2	obtained from the supervisor
Precision Plus Protein™ Standards (Dual-color, BIO-RAD)	Bio-Rad Laboratories, USA
QuikChange Multi Site-Directed Mutagenesis Kit	Agilent Technologies, Inc., USA
T4 DNA ligase	Fermentas, Canada
tips for automatic pipettes	Axygen, USA

4.1.3 Instruments

AmiconUltra concentrator (30 kDa)	EMD Millipore, USA
autoclave	Systec GmbH, Germany
automatic pipettes	Eppendorf AG, Germany
centrifuge 5415 D	Eppendorf AG, Germany
centrifuge 5804 R (rotor A-4-44)	Eppendorf AG, Germany
centrifuge HERMLE Z323K	Hermle, Germany
centrifuge SIGMA 8K (rotor 12510-H)	Sigma, Germany
column HiLoad 16/600 Superdex 200 pg	GE Healthcare, UK
column HiLoad 16/600 Superdex 75 pg	GE Healthcare, UK
column HiLoad 26/600 Superdex 75 pg	GE Healthcare, UK
electric cooker	Eta, Czech Republic
electrophoresis power supply	Sigma-Aldrich, USA
horizontal electrophoresis	Carl Roth GmbH, Germany
HPLC Watrex	Watrex, Czech Republic
Implen NanoPhotometer P300	Implen, Germany
incubator hood TH 15	Edmund Bühler GmbH, Germany
Infinite M200 Pro (multimode reader)	TECAN Group Ltd., Switzerland
laboratory scales	KERN & SOHN GmbH, Germany
magnetic stirrer Variomag Maxi, Komet	Thermo Fisher Scientific, USA
peristaltic pump	IDEX, Swizerland
pH meter JenWay 3505	Felsted, UK
ProteomLab XL-I Analytical Ultracentrifuge	Beckman Coulter, USA
sonicator 3000 Misonix	Cole-Parmer, USA
spectrophotometer Agilent 8453	Agilent, USA
thermoblock	Grant, UK
thermocykler Mastercycler Personal	Eppendorf, Germany

thermostat	Melag Brutschrank Incubat, Germany
transilluminator UVT-14M	Herolab GmbH, Germany
vertical electrophoresis	Bio-Rad Laboratories, USA
vortex Rx^3	VELP Scientifica, Italy
water bath	Memmert GmbH, Germany

4.2 Methods

4.2.1 Preparation of CaMKK2 constructs for the expression

Expression in bacteria is one of the options how to obtain protein from the living organisms. It requires a gene of a protein of the interest inserted in a suitable vector and a strain of bacteria which is competent for transformation.

4.2.1.1 Cultivation media for bacteria

There are two main possibilities how to cultivate bacteria (two types of growth media). The liquid growth medium is used in cases when it is not necessary to distinguish single colonies. This type of medium is utilized for the multiplication of bacteria. The second type of growth media, the solid one, is very useful for the selection of single colony (for example after mutagenesis). The solid growth medium is usually placed into Petri dishes.

There are several types of growth media which can be used, such as lysogeny broth (LB) medium, terrific broth (TB) medium or auto-induction medium ZYP-5052, which does not require induction of expression by IPTG (isopropyl β-D-1-thiogalactopyranoside). Conical flasks with 900 ml of medium are used for large-scale protein expression. Whereas 5 ml aliquots (called minipreps) take place during plasmid DNA (deoxyribonucleic acid) manipulations such as site-directed mutagenesis or introduction of cleavage site to the plasmid.

4.2.1.2 Preparation of LB medium

Minipreps were prepared for cultivation of bacteria in small volume. 1.25 g of LB medium (Table 4.1, page 32) was dissolved in 50 ml of deionized water. This solution was aliquoted into 5 ml aliquots in glass tubes using automatic pipette. The aliquots were sterilized by autoclaving (121 °C for 15 minutes, then cooled to 80 °C) and stored at 4 °C.

To prepare media for the large volume cultivation, 22.5 g of LB medium was dissolved in 900 ml of deionized water in conical flask. Flasks were covered with cotton plug and aluminum foil to prevent water evaporation during sterilization. Media were sterilized by autoclaving.

Table 4.1 | LB medium
LB medium is a nutritionally rich medium used for bacteria cultivation. Medium is composed of yeast extract which provides trace elements, various organic compounds and vitamins. Sodium chloride provides sodium ions which are important for osmotic balance and transport. Essential amino acids are provided by tryptone which is a mixture of peptides. Amounts shown in the table have to be dissolved in deionized water to the final volume of 100 ml.

Compound	Amount [g]
yeast extract	0.5
NaCl	1.0
tryptone	1.0

4.2.1.3 Preparation of ZYP-5052 medium

Auto-induction medium ZYP-5052 (Table 4.2) is developed for IPTG-inducible recombinant protein expression in *E. coli*. The basic principle of the medium is based on diauxic growth when in the first growth phase glucose (with limited concentration in medium) is metabolized preferentially. Bacterial culture achieves high cell density. Depletion of glucose in the second growth phase causes uptake of lactose which is converted to allolactose. Allolactose deactivates *lac* repressor which prevents expression of T7 RNA (ribonucleic acid) polymerase. Newly synthesized T7 RNA polymerase allows expression of recombinant proteins under control of T7 promoter. The whole mechanism of IPTG induction will be described in chapter 4.2.1.5. ZYP-5052 medium was prepared in conical flasks for large-scale protein expression.

Table 4.2 | ZYP-5052 medium
ZYP-5052 medium has a complex composition in comparison with LB medium. Na_2HPO_4 and KH_2PO_4 are sources of ions and function as a buffer of pH changes occurring due to metabolic processes in the cell. $(NH_4)_2SO_4$ provides a source of nitrogen and sulfur. Magnesium ions are provided by $MgSO_4$. Glycerol and glucose are presented in low amount so they are depleted before bacterial culture is overgrown. Metabolization of lactose induces recombinant protein expression.

Compound	Final concentration
tryptone	1%
yeast extract	0.5%
Na_2HPO_4	50 mM
KH_2PO_4	50 mM
$(NH_4)_2SO_4$	25 mM
glycerol	0.5%
glucose	0.05%
α-lactose	0.2%
$MgSO_4$	2 mM

4.2.1.4 Preparation of LB agar plates

8 g of LB agar (Table 4.3) was dissolved in 200 ml of deionized water in small conical flask closed by cotton plug (covered with aluminum foil) and sterilized by autoclaving (121 °C for 15 minutes, then cooled to 80 °C; stored at 4 °C). LB agar powder does not have to be completely dissolved in solution before sterilization, it dissolves during autoclaving.

Table 4.3 | LB agar
LB agar has the same composition as LB medium described above. LB agar additionally contains inert solidifying agent, polysaccharide agar. Amounts shown in the table has to be dissolved in deionized water to the final volume of 100 ml.

Compound	Amount [g]
yeast extract	0.5
NaCl	1.0
tryptone	1.0
agar	1.5

Solid sterilized agar was melted in the water bath and cooled to 50 °C before usage. 50 °C is a temperature when the agar is still liquid but the antibiotic is not degraded yet. The solution was gently swirled to mix after adding the desired amount of appropriate antibiotic (ampicillin – 100 μg/ml; kanamycin – 50 μg/ml; chloramphenicol – 25 μg/ml). The LB agar was split into 10 cm Petri dishes (approximately 25 ml per dish) and allowed to solidify. Inverted plates were stored at 4 °C. Bench area was sterilized by working near a flame for the whole time.

4.2.1.5 Expression bacterial strains

Protein expression in bacteria is a very popular and well-established biochemical procedure which has undeniable advantages (such as easy transformation with plasmid DNA, available growth media composed of inexpensive components or fast growth kinetics) [50].

Two *E. coli* strains which were used are BL21(DE3) and Rosetta cells. These strains are specially modified for recombinant protein expression. BL21(DE3) cells are B strain thus lack two proteases in cytoplasm and outer membrane. Rosetta cells are derived from BL21 cells but contain codons rarely used in *E. coli* to enhance the expression of eukaryotic proteins.

Genes of recombinant proteins are under control which does not allow the expression until the induction. Gene of the protein is placed after T7 promoter sequence in the plasmid which means that transcription is initiated only in the presence of T7 RNA polymerase (an RNA polymerase from the T7 bacteriophage) and this sequence is not recognized

by *E. coli* RNA polymerase. DE3 strains carry artificial gene for T7 RNA polymerase under control of *lac* promotor. *Lac* repressor is released from the *lac* operator in the presence of allolactose or its analog IPTG. This allows endogenous RNA polymerase to transcript gene for T7 RNA polymerase. T7 RNA polymerase in turn starts with transcription of gene under T7 promoter control.

BL21(DE3)pLys strains offer even more stringent control (useful for expression of toxic proteins). Strains with the "pLys" signification carry plasmid with information to produce T7 lysozyme, natural inhibitor of T7 RNA polymerase. T7 lysozyme is able to inactivate small amount of T7 RNA polymerase which occurs in the cell even in the absence of induction. T7 RNA polymerase is produced in huge amounts after induction so T7 lysozyme is not able to affect its activity.

4.2.1.6 Expression vector

An expression plasmid is a collection of multiple cloning site (MCS), selection markers (usually antibiotic resistance), fusion protein, but also replicon (containing the origin of replication and the control of copy number as well) and promoters (very often *lac* promoter) [50]. Plasmid which was used for expression of CaMKK2 was derived from pRSFDuet-1 (Figure 4.1). Plasmid has a size of almost 4 kbp and contains various restriction sites along the entire length, RSF origin of replication, lacI gene for *lac* repressor protein. Plasmid further contains kanamycin resistance gene and two MCSs under control of T7 promoter.

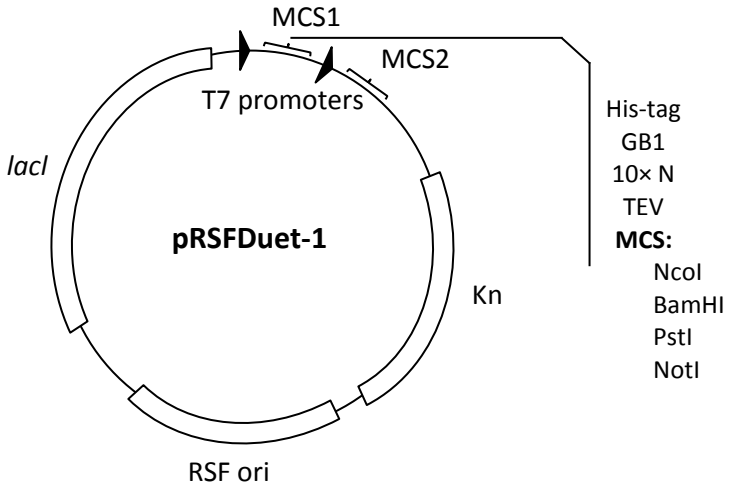


Figure 4.1 | Plasmid pRSFDuet-1
Plasmid map of edited pRSFDuet-1. RSF ori – RSF origin of replication, *lacI* – gene for *lac* repressor protein, Kn – kanamycin resistance gene, MCS – multiple cloning site. CaMKK2 nucleotide sequence was inserted between restriction sites BamHI and NotI. Adopted from [ES2].

Plasmid region which was translated into a protein consisted of N-terminal polyhistidine tag, GB1 protein (the protein G B1 domain), poly-N linker, TEV (Tobacco Etch Virus) protease cleavage site and CaMKK2 gene itself. Translated sequence started with His-tag which allowed easier purification of the protein by affinity chromatography. Sequence for GB1 protein was included in the plasmid prior to the sequence of CaMKK2. The domain was fused with studied protein to increase its solubility and stability in solution during expression and purification. Sequence of GB1 protein was followed by ten asparagine residues which flexibly connect GB1 and CaMKK2. TEV cleavage site was set almost just before the first nucleotide of CaMKK2 gene allowing cleavage of artificial part of the protein when it is necessary.

4.2.1.7 Amino acid sequence of CaMKK2

The full-length human CaMKK2 (isoform 1) is 588 amino acids long. Its structure is still unknown (except for kinase domain) but according to predictions of secondary structure CaMKK2 has unstructured both N- and C-terminus (Figure 4.2, page 36). Unstructured regions often cause problems with purification. For this reason we decided to work only with the middle part of the protein, specifically with sequence from His93 to Leu517. The original construct did not contain any point mutations.

4.2.1.8 Additional mutations

Four PKA (protein kinase A) phosphorylation sites were predicted in the sequence of CaMKK2, two of them were also expected 14-3-3 binding sites. To study the effect of the individual 14-3-3 binding sites two phosphorylation sites which are not involved in 14-3-3 binding (Thr145 and Ser495) were removed. Those amino acids were mutated to alanine because it preserves the secondary structure of mutated region and does not have any reactive groups. Two remaining phosphorylation sites were individually shut down by additional mutation to alanine. This mutations were performed to achieve higher homogeneity of the protein sample for further activity experiments where well defined sample is required.

Another mutation was inserted to the sequence by RNDr. Veronika Obšilová, Ph.D. to eliminate autophosphorylation of CaMKK2. Mutation of the residue in the activation segment of CaMKK2 (aspartic acid in the position 330 was mutated to alanine) provided so called “kinase dead” mutant which is ideal for structural studies because it lacks heterogeneity caused by nonspecific phosphorylation.

Figure 4.2 | Prediction of secondary structure
Secondary structure of CaMKK2 was predicted in PSIPRED [ES3]. Unstructured N- and C-terminal regions (sequence in grey) were not included in the original construct. The secondary structure prediction of the part of the protein which was used for further studies (sequence in black) shows multiple strands (yellow arrows) and helixes (purple cylinders). Coil is represented as a line.

4.2.1.9 Horizontal gene transfer

In nature, this mechanism serves for the spread of antibiotic resistance among bacteria. But in laboratory, horizontal gene transfer is a way how to artificially add DNA to bacterial cells (very often *E. coli*). *E. coli* is a bacterial strain with cell wall which has to be partially disintegrated when accepting foreign DNA from its surroundings. Heat shock is a widely used method of introducing the required gene into *E. coli* cells. Bacteria used in laboratories for transformation (so called competent cells) are specially adapted to accept inserted plasmids.

Competent *E. coli* cells DH5α are used for routine cloning applications such as plasmid DNA multiplication. Bacteria were transformed with plasmid pRSFDuet-1 which contained inserted sequence of CaMKK2 gene. Plasmids were stored at -20 °C.

DH5α cells were taken out of -80 °C and were thawed on ice. 1 µl of DNA was gently mixed with 45 µl of competent cells in microcentrifuge tube. The mixture was incubated on ice for 15 min. The tube was then placed into a 42 °C water bath for 45 sec. The tube was put back on ice for 2 min. 450 µl of LB medium (without antibiotics) prewarmed to 42 °C was then added to the mixture. Bacteria were grown in 37 °C shaking incubator for 45 min at 180 rpm (*revolutions per minute*). This step provides the bacteria time to take advantage of the information about antibiotic resistance encoded in the plasmid. The transformation mixture was then plated onto a LB agar plate (stored at 4 °C, prewarmed in 37 °C incubator) containing kanamycin. Bench area had to be sterilized by working near a flame to prevent sample contamination by bacteria naturally occurring in the air. Plates were incubated overnight (approximately 16 hours) at 37 °C.

Next day, 5 ml aliquots of sterilized LB medium with kanamycin (50 µg/ml) were prepared. Only a single bacterial colonies were picked from the plate and were placed into the glass tubes to inoculate LB medium. Bacteria in medium were then incubated overnight (approximately 16 hours) in 37 °C shaking incubator at 180 rpm.

4.2.1.10 Isolation of plasmid DNA

GeneJET Plasmid Miniprep Kit is a commercial kit which was used for isolation of plasmid DNA. The bacterial cultures were harvested to the microcentrifuge tubes by centrifugation at room temperature (RT) at 13 000 rpm in a microcentrifuge for 1 min (centrifuge 5415 D). Supernatants were discarded. The pelleted cells were resuspended in 250 µl of the Resuspension Solution. The cell suspensions were additionally resuspended by vortexing. 250 µl of Lysis Solution was added to each tube and the phases were mixed by inverting the tubes several times. 350 µl of the Neutralization Solution was added to the viscous and transparent solution and the tubes were immediately mixed by inverting several times. Cell debris and chromosomal DNA was pelleted by centrifugation (RT, 13 000 rpm, 5 min). The supernatants were carefully transferred to the GeneJET spin columns by pipetting without disturbing the white precipitate. The columns were centrifuged for 1 min at RT at 13 000 rpm. The flow-throughs were discarded and the columns were placed back into the same collection tube. The columns were washed by adding 500 µl of the Wash Solution. They were centrifuged for 1 min at RT at 13 000 rpm and the flow-throughs were

discarded. The previous step with the Wash Solution was repeated one more time. The columns were additionally centrifuged for 1 min (RT, 13 000 rpm) to remove residual Wash Solution (especially ethanol). The columns were transferred into a fresh microcentrifuge tubes. 40 µl of the Elution Buffer was added to thecenter of column membrane and it was incubated at RT for 2 min. The plasmid DNA was eluted from the membrane by centrifugation for 2 min (RT, 13 000 rpm). The purified plasmid DNA was stored at -20 °C [ES4].

4.2.1.11 PCR

Polymerase chain reaction (PCR) is an *in vitro* method for a fast multiplication (so called amplification) of DNA segment. This technique allows generating up to millions of copies of a DNA from a single copy. PCR mixture consists of DNA template (sequence of DNA for replication), DNA polymerase (an enzyme which creates new DNA strands), DNA primers (short segments of DNA allowing DNA polymerase binding to the short double-stranded region of DNA on the template), mixture of deoxynucleotide triphosphates (dNTPs; building blocks for new DNA strand) and buffer (providing optimal chemical environment for DNA polymerase).

DNA polymerase is only able to add deoxynucleotide triphosphates to the 3' end of already created double-stranded DNA (*ds-DNA*) segment. Which means that two DNA primers has to be complementary to the 3' ends of each of the strands of DNA segment which is meant to be amplified. In this case PCR was used for introducing the point mutations to the original construct. It means that both primers were designed to be complementary to the particular segment of DNA template with a deviation in a single triplet.

PCR consists of a series of three main steps that are repeated many times. Every cycle begins with denaturation of the double-stranded DNA. This first temperature change is a heating of the reaction mixture to 95 °C for 30 sec. It is followed by lowering the temperature to allow the primers bind to the template (so called annealing). The temperature has to be high enough for specific hybridization of the primer to the appropriate sequence on the template, but low enough to create sufficient bond between primer and strand. The annealing temperature is dependent on a length and composition of the primer and usually ranges between 50 °C and 60 °C. The last step is elongation which means that the reaction temperature is raised to the optimum for appropriate DNA polymerase.

Designing PCR primers is a crucial step for successful amplification. These oligonucleotides are usually circa 30 bases long and their sequence has to be unique to target selected region on the template. It is also necessary to avoid a hairpin or loop

formation which is caused by unmindful design of a primer with nucleotide repeats or palindromes.

Four pairs of primers were designed for site-directed mutagenesis of the CaMKK2 sequence (Figure 4.3). Oligonucleotides were ordered from Generi Biotech. They were dissolved in deionized water according to the manufacturer's instructions to the final concentration 0.1 mM. Primers were then additionally 10× diluted before adding to the PCR mixture.

CaMKK2_S100A_forward
5′ cc ggt cgc aag ctg **gcg** ctg caa gag cgg tcc 3′

CaMKK2_S100A_reverse
5′ gga ccg ctc ttg cag **cgc** cag ctt gcg acc gg 3′

CaMKK2_T145A_forward
5′ ctg ccc cgg cgg ccg **gca** gtg gag tct cac 3′

CaMKK2_T145A_reverse
5′ gtg gtg aga ctc cac **tgc** cgg ccg ccg ggg cag 3′

CaMKK2_S495A_forward
5′ g ata cgt aaa cgc **gcc** ttt ggg aac cca ttc 3′

CaMKK2_S495A_reverse
5′ gaa tgg gtt ccc aaa **ggc** gcg ttt acg tat c 3′

CaMKK2_S511A_forward
5′ gag gaa cgc tca ctg **gca** gcg cct gga aac ttg 3′

CaMKK2_S511A_reverse
5′ caa gtt tcc agg cgc **tgc** cag tga gcg ttc ctc 3′

Figure 4.3 | PCR primers
Mutated triplets are highlighted in red in the sequences of designed primers.

The original construct contained four targets for mutation. So, in this case it was convenient to mutate more sites in a single step and for this reason QuikChange Multi Site-Directed Mutagenesis Kit was used. This kit allows mutating plasmid DNA at up to five different sites simultaneously and only one primer is required for each site. PCR mixture (Table 4.4) is then a bit different from the one which is commonly used and which is described above [ES5].

Table 4.4 | PCR mixture from QuikChange Multi Site-Directed Mutagenisis Kit
PCR reaction was performed in PCR microtubes in total volume of 25 µl. Three separate mixtures were set up. Two of them were designed for triple mutation and one of them (values in brackets) for double mutation.

Compound	Volume [µl]
10× QuikChange Multi reaction buffer	2.5
deionized water	17 (18)
ds-DNA template	0.5
primers	3× (2×) 1
dNTP mix	1
QuikChange Multi enzyme blend	1

QuikChange Multi enzyme blend includes *PfuTurbo* DNA polymerase which has high fidelity in DNA synthesis (6-fold higher than *Taq* DNA polymerase). *PfuTurbo* DNA polymerase has a temperature optimum about 65 °C and it requires 2 min to synthetize one kilobase of new DNA. *PfuTurbo* DNA polymerase extends primers to the length of the whole plasmid and thus generate ds-DNA molecule. This double-stranded molecule consists of a one new strand with mutations and one template strand without mutations. The primers were annealed to the same strand of the denatured DNA in the PCR reaction (Table 4.5).

Table 4.5| PCR reaction cycling parameters
The first step of ds-DNA dissociation was initial step and was not repeated during PCR cycling. Steps 2 to 4 were repeated thirty times. Step number 5 was additional step which was performed to allow DNA polymerase to finish synthesis. The temperature was maintained at 4 °C after completion of the reaction.

Step	Temperature	Time
1	95°C	2 min
2	95°C	1 min
3	55°C	1 min
4	65°C	10 min
5	65°C	10 min
6	4°C	continuous

The second step takes place after PCR reaction when each mixture of PCR products is treated with 1 µl of DpnI at 37 °C for 1 hour. The DpnI is a restriction endonuclease which is specific for methylated DNA. The newly created strand is purely unmethylated so this is a sufficient way how to selectively digest template DNA.

The third step (transformation of DNA to the cells and preparation of glycerol stocks) is described in chapters 4.2.1.13 and 4.2.1.14 and was performed by my dear colleague Mgr. Dana Kalábová.

4.2.1.12 Agarose gel electrophoresis

Electrophoresis was used for a verification of the PCR reaction, PCR products were expected to be approximately 5 kbp (base pair) long. Starting PCR mixture contains small primers (which are too small to be mistaken with PCR products) and DNA template (which was digested by DpnI after PCR reaction). It means no band on the gel in case of insufficient PCR reaction. Generally, gel electrophoresis is a separation method based on the different mobility of the molecules with various charge and size. DNA molecules are negatively charged due to their phosphate groups which means they move through the agarose matrix

towards the anode in an electric field. Agarose matrix is prepared by mixing agarose and appropriate buffer. Gel density is designed according to the size of the analyzed DNA fragments. It is necessary to realize that smaller fragments move faster.

0.8% agarose gel was prepared by mixing 0.56 g of agarose and 70 ml of 1× concentrated TBE (Tris/Borate/EDTA) buffer pH 8.0 (Table 4.6). The mixture was slowly heated up to the complete dissolution of the agarose. 1 µl of fluorescent nucleic dye (GelRed Nucleic Acid Stain) was gently added to the agarose solution and the mixture was poured into a cast. Wells in the gel were created by placing a comb in the cast. Gel was allowed to solidify at room temperature.

Table 4.6| 1× TBE buffer (pH 8)
TBE buffer should protect DNA against degradation. EDTA (ethylenediaminetetraacetic acid) is a chelating agent of magnesium cations so inactivates nucleases which can occur in the samples as contaminants. The pH of the buffer is slightly basic so the DNA is deprotonated and thus soluble in water. Amounts shown in the table has to be dissolved in deionized water to the final volume of 1000 ml.

Compound	Final concentration [mM]	Amount
Tris-HCl	100	10.8 g
boric acid	90	5.5 g
EDTA	1	4 ml

The agarose gel was then covered with 1× TBE buffer. The DNA samples were mixed with appropriate amount of 6× concentrated GLS (*gel loading solution*). GLS contains colored dyes for an easier monitoring of migration during electrophoresis and glycerol for raising the density of the sample (and easier loading of the sample to the well). A commercial mixture of DNA fragments with defined length (GeneRuler 1kb DNA ladder) was used as a standard of migration. Electrophoresis was carried out at 75V for 1 hour. DNA was visualized with a UV transilluminator.

4.2.1.13 Transformation of XL10-Gold ultracompetent cells

Mutated and amplified single-stranded (ss) plasmid DNA was transformed into XL10-Gold ultracompetent cells where the ds-DNA was created from the circle ss-DNA. XL10-Gold ultracompetent cells were included in the QuikChange Multi Site-Directed Mutagenisis Kit and had to be stored at -80 °C. Cells were thawed on ice. 2 µl of the β-mercaptoethanol (β-ME) was added to each 45 µl aliquot of cells. The tubes were gently mixed and cells were incubated on ice for 10 min. 1.5 µl of the DNA (treated with DpnI) from each mutagenesis reaction was transferred to a separate tube with competent cells.

The tubes were gently mixed and cells were incubated on ice for 30 min. The tubes were placed into a 42 °C water bath for 30 sec and then were incubated on ice for 2 min. 500 µl of NZY^+ broth (preheated to 42 °C; Table 4.7) was added to each tube and the cells were grown in 37 °C shaking incubator for 1 hour. Each transformation mixture was then plated onto a LB agar plate (with kanamycin). Plates were incubated overnight (approximately 16 hours) at 37 °C.

Table 4.7 | NZY^+ broth
NZY^+ medium has a similar basal composition as LB medium**.** Chemicals in the solid state which are mentioned in the table (peptone, yeast extract and NaCl) were dissolved in deionized water to the final volume of 100 ml. The pH was adjusted to the value of 7.5 using NaOH. Additional filter-sterilized solutions were added to the autoclaved medium.

Compound	Amount
peptone (from casein)	1 g
yeast extract	0.5 g
NaCl	0.5 g
1M $MgCl_2$	1.25 ml
1M $MgSO_4$	1.25 ml
2M glucose	1 ml

Next day aliquots of sterilized LB medium with kanamycin (50 µg/ml) were prepared. Only a single bacterial colonies were picked from the plates and were placed into the glass tubes to inoculate LB medium. Bacteria in medium were then incubated overnight (approximately 16 hours) in 37 °C shaking incubator at 180 rpm. Plasmid DNA was isolated as was described above (4.2.1.10) and samples of the isolated plasmids were sent to the Centre for DNA Sequencing (Institute of Microbiology of the Czech Academy of Sciences). All results were thoroughly checked (in Chromas) and plasmid with correct mutations were used in further experiments.

4.2.1.14 Glycerol stocks

Glycerol stocks are useful for long-term storage of bacteria containing plasmids. They can be stored stably at -80 °C for many years. Verified plasmids from the previous chapter (4.2.1.13) were transformed as was described above (4.2.1.9). Original construct was transformed to the Rosetta cells whereas constructs carrying mutation were transformed to the BL21(DE3) cells. It was necessary to use smaller volume of transformation mixture (about 100 µl) to avoid formation of bacterial plaque on plate in this case. Then it was easier to pick a single colony to inoculate 5 ml aliquot of LB medium with kanamycin (and chloramphenicol in case of Rosetta cells). Bacterial cultures in the glass tubes were then

homogenized by vortexing after overnight incubation. 700 µl of the culture was gently mixed with 300 µl of sterile glycerol in microcentrifuge tube. The mixtures were immediately placed into -80 °C.

4.2.2 Expression and purification of CaMKK2

Several different constructs were prepared for further experiments. Original construct contains four PKA phosphorylation sites which two of them are also predicted 14-3-3 binding sites. Constructs were designed to check the function of individual sites (Table 4.8).

Table 4.8| CaMKK2 constructs
Original construct was mutation free (wild-type, WT). The second construct contained N-terminal 14-3-3 binding site only. C-terminal 14-3-3 binding site was presented in the third construct. The fourth construct had both 14-3-3 binding sites in the sequence. 14-3-3 binding sites are written in italics.

Construct	PKA phosphorylation sites	Required mutation
CaMKK2$^{93\text{-}517}$ WT	*S^{100}*, T^{145}, S^{495}, *S^{511}*	none
CaMKK2$^{93\text{-}517}$ S^{100}	*S^{100}*	T^{145}A, S^{495}A, S^{511}A
CaMKK2$^{93\text{-}517}$ S^{511}	*S^{511}*	S^{100}A, T^{145}A, S^{495}A
CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}	*S^{100}*, *S^{511}*	T^{145}A, S^{495}A

4.2.2.1 Cultivation of bacteria for production of CaMKK2 variants

Optimization of cultivation is the first step when creating the new purification protocol. Bacteria containing original construct were cultivated in LB medium. It was necessary to perform several expression test to find out which conditions lead to the best yield of the protein.

Big cultivation flasks (preparation in chapter 4.2.1.2) were inoculated with 5 ml of bacteria culture. Each 900 ml of the LB medium contained kanamycin at the final concentration of 50 µg/ml. The flasks were incubated in 37 °C shaking incubator for about 5 hours until the optical density (OD) reached at least the value of 0.7. The optical density was measured at 600 nm which is typical wavelength for measuring of bacterial density. Recombinant protein expression in bacteria was induced by adding IPTG. Tested flasks were incubated for various time period in shaking incubator at 180 rpm at different temperatures.

Auto-induction medium ZYP 5052 was used for the mutant forms of CaMKK2. Protocol of purification was optimized also in this case. Optimization was carried in a similar way with a little difference, there is no need to add IPTG.

4.2.2.2 Cell harvesting and sonication

Cell harvesting is a first step in gaining proteins from the bacteria culture. Cells were pelleted by centrifugation at 4 °C at 3 400 rpm in a centrifuge for 30 min (centrifuge SIGMA 8K). Cell pellets were resuspended in 40-75 ml of the lysis buffer (Table 4.9) with or without lysozyme.

Table 4.9| Lysis buffer
Lysis buffer is designed to create the environment for a cell lysis. PBS buffer (phosphate-buffered saline, Table 4.10) is a solution of different salts. Lysozyme was added to the whole volume of lysis buffer before cell lysis in some cases. In other cases, lysozyme was added in small volume of lysis buffer to the thawed lysates. Amounts shown in the table has to be dissolved in deionized water to the final volume of 100 ml.

Compound	Final concentration	Amount
10× PBS	1×	10 ml
NaCl	1M	5.84 g
imidazole	2 mM	0.0136 g
β-ME	4 mM	27.8 µl
Optional: lysozyme		0.01 g

Table 4.10| 10× PBS buffer
PBS is buffer with composition which mimics isotonic environment inside the human body. pH value has to be adjusted to 7.4. Amounts shown in the table has to be dissolved in deionized water to the final volume of 100 ml.

Compound	Amount [g]
NaCl	8
KCl	0.2
KH_2PO_4	0.24
$Na_2HPO_4 \cdot 2H_2O$	1.44
NaN_3	0.04

Lysates were then immediately deep-frozen at -80 °C. Cells were thawed on ice and were prepared for sonication by incubation with serine protease inhibitor PMSF (phenylmethylsulfonyl floride) in cold room for 30 min. Sonication was then used to disrupt cell membrane of the bacteria so the protein of the interest was released to the solution. Sonication program was set to 3 ON/17 OFF with total time 8 min. It means that the samples were sonicated for 8 min in total, but every 3 seconds sonication was stopped for 17 seconds. The sonication was carried out on ice to prevent an overheating of the samples.

Sonicated cells were spun down in a centrifuge at 4 °C at 13 500 rpm for 45 min (centrifuge HERMLE Z323K). Samples were then ready for a further purification.

4.2.2.3 Nickel chelate affinity chromatography

Nickel chelate affinity chromatography is a separation technique used for the purification of recombinant proteins. Protein has to be tagged with a sequence of six histidines (which is very unique in naturally occurring proteins). These fusion proteins are then able to retain in a column thanks to specific coordinate covalent bond of imidazole group of histidine to nickel ions immobilized in a column. Bond created between histidine and metal is reversible and can be easily broken by using an excess of imidazole.

1 ml of slurry (chelating sepharose) was pipetted into the tube with filter to set up a new column. Slurry was washed with 40 ml of deionized water, incubated with 20 ml of 0.1 M $NiSO_4$ and washed again with 20 ml of deionized water and 20 ml of binding buffer (Table 4.11). Sonicated cells were slowly and carefully placed into the column to prevent creation of air bubbles. The column was kept at 4 °C during the purification of proteins. The slurry was washed with 150 ml of washing solution (elution buffer (Table 4.12) diluted in binding buffer in the ratio 1:9). This step was done to ensure that proteins weakly or non-specifically bounded to the slurry were washed away. The purified protein was eluted by pouring 8 ml of elution buffer to the column. Eluted solution was collected to the 15 ml conical tube with 10 ml of dialysis buffer (Table 4.13, page 46).

Table 4.11 | Binding buffer
Salt in the buffer reduces nonspecific hydrophobic interactions between proteins and slurry. Amounts shown in the table has to be dissolved in deionized water to the final volume of 100 ml.

Compound	Amount
10× PBS	10 ml
NaCl	5.84 g
imidazole	6.8 mg
β-ME	14.3 µl

Table 4.12 | Elution buffer
Higher concentration of imidazole allows the trapped protein to leave the column. Amounts shown in the table has to be dissolved in deionized water to the final volume of 100 ml.

Compound	Amount
10× PBS	10 ml
NaCl	5.84 g
imidazole	4.1 g
β-ME	14.3 µl

4.2.2.4 TEV protease cleavage of 6xHis-GB1 tagged fusion proteins

Purified proteins were fused with B1 domain of G protein to improve the solubility during the purification and also with histidine tag for easier purification. It is necessary to cleave the fusion tag before further biophysical characterizations of the proteins. Tobacco Etch Virus (TEV) protease is a widely used tool to remove artificial part of a protein. This protease allows high sequence specificity cleavage of fusion proteins.

The cleavage was performed during dialysis. Dialysis is a separation method designed to eliminate small contaminants from protein solution or to exchange buffer. The sample is placed in a large volume of dialysis buffer (separated by semi-permeable membrane). The membrane causes selective diffusion (only molecules smaller than the membrane pores can freely diffuse through the membrane, whereas macromolecules are captured inside the original solution). Concentration of small freely diffusing molecules (both from the sample and dialysis buffer) is aligned on the both sides of the membrane when the dialysis is done.

Appropriate amount of TEV protease was added to the protein solution after nickel chelate affinity chromatography. This mixture was separated from the dialysis buffer (Table 4.13) by a semi-permeable membrane with molecular weight cut-off (MWCO) of 14 kDa. Protein sample was dialyzed to 1 l of dialysis buffer at 4 °C for 16 hours.

Table 4.13 | Dialysis buffer
Composition of the buffer is designed to maximize the stability of a protein. For example, EDTA removes nickel ions which cause aggregation of His-tagged proteins. Amounts shown in the table has to be dissolved in deionized water to the final volume of 1000 ml.

Compound	Amount	Final concentration
Tris-HCl (pH 8)	50 ml	50 mM
NaCl	29.25 g	500 mM
EDTA (pH 8)	10 ml	5 mM
glycerol	100 g	10%
β-ME	143 µl	2 mM

Efficiency of TEV cleavage was verified by SDS-PAGE. Samples before and after addition of TEV proteases were compared.

4.2.2.5 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a widely-used method for separation of proteins in the electric field. The speed of individual proteins or peptides in the polyacrylamide gel is dependent on their electrophoretic mobility.

This mobility is a function of several properties of the molecule, such as conformation, charge or size. An anionic detergent sodium dodecyl sulfate (SDS) is added to the protein samples to remove native structure. Proteins are linearized and negatively charged proportional to their mass. Mobility is then mainly dependent on the length of the molecule. SDS denaturates higher-order structures. The denaturation of the proteins is usually supported by heating the samples prior to loading onto the gels.

The gel for polyacrylamide electrophoresis consists of separating gel (Table 4.14) and stacking gel (Table 4.15). Separating gel was poured between two glass plates and covered with a thin layer of isopropanol to create flat interface. Stacking gel was added on the solidified separating gel. Wells for the samples were created by a comb inserted on the top of the gel.

The concentration of the acrylamide in the gel can vary. Molecules with low molecular weight are better resolved on the gels with higher acrylamide concentration. 12% gels are sufficient to resolve CaMKK2 samples.

Table 4.14 | Separating gel (12%)
Gel consists of the buffer to adjust pH, acrylamide/bis-acrylamide mixture to form polymers with cross-links. Polymerization is initiated by adding ammonium persulfate (APS) as a source of free radicals and TEMED (tetramethylethylenediamine) as a stabilizer. Separating gel provides separation of the proteins based on their molecular weight.

Compound	Volume [µl]
deionized water	3000
1.5 M Tris-HCl (pH 8.8); SDS	2000
acrylamide 30%/bis-acrylamide 0,8%	2600
10% APS	75
TEMED	8

Table 4.15 | Stacking gel (12%)
Stacking gel differ a little bit from the separating gel in composition. Different pH value of the buffer allows concentrate the proteins to the one zone so they reach the gel interface at the same time.

Compound	Volume [µl]
deionized water	500
1.5 M Tris-HCl (pH 6.8); SDS	1000
acrylamide 30%/bis-acrylamide 0,8%	2250
10% APS	75
TEMED	8

5× concentrated sample buffer (Table 4.16, page 48) with SDS was added to the protein samples before electrophoresis. Samples were heated to finish denaturation.

Table 4.16 | SDS sample buffer
Glycerol increases the sample density for easier loading into the wells. SDS denatures proteins and charge them negatively. Bromophenol blue is a tracking dye with high electrophoretic mobility which allows to observe migration. β-mercaptoethanol breaks disulphide bonds.

Compound	Volume [ml]
50% glycerol	5
10% SDS	2
10% bromophenol blue	1
deionized water	0.9
1 M Tris-HCl	0.6
β-ME	0.5

Gel was set into the gel tank filled with 1× SDS running buffer (50 mM Tris-HCl, pH 8.3; 385 mM glycine; 0.1% SDS). A commercial mixture of proteins with defined size Precision Plus Protein Standards was loaded into the first well as a standard. Electrophoresis was carried out at 190 V for 1 hour.

Results of the SDS-PAGE analysis were visualized with protein stain Coomassie Brilliant Blue R-250. Gel was incubated in a staining solution (Table 4.17) for 1 hour.

Table 4.17 | Staining solution
Anionic dye is non-polar and thus is dissolved in methanol. Acetic acid fixes proteins in the gel.

Compound	Amount
methanol	450 ml
glacial acetic acid	100 ml
Coomassie Brilliant Blue R 250	1 g
deionized water	450 ml

Unbound dye which was incorporated into the gel was washed with destaining solution (Table 4.18).

Table 4.18 | Destaining solution
Incubation in destaining solution takes a few hours and has to be repeated several times to achieve a clear background with blue bands of proteins.

Compound	Volume [ml]
methanol	100
glacial acetic acid	100
deionized water	800

SDS-PAGE was used to clarify the success of the individual steps in protein purification process.

4.2.2.6 Protein sample concentration

Protein sample can become over diluted during the purification process. Required concentration of protein in the solution is then achieved by reduction of sample volume. Slow volume reduction leads to the increasing concentration of protein solution. It brings with it a risk of precipitation of the protein. Precipitation is caused by interaction between misfolded proteins molecules or their hydrophobic patches which get too close together. It is necessary to carefully handle it because protein can aggregate in any moment of concentration process.

Concentration of CaMKK2 samples was done in a concentrators with a semipermeable membrane (cut-off 30 kDa). Samples were carefully centrifuged at 2300 rpm at 4 °C (centrifuge 5804 R). Samples were gently stirred during the concentration process after each two minutes of centrifugation. This procedure continued until the desired concentration was reached or the protein started to precipitate.

4.2.2.7 Size exclusion chromatography

Size exclusion chromatography (SEC) is a technique used in the final step of protein purification. Columns for size exclusion chromatography differ in pore size of packing material. Molecules are separated in the column according to their size (more precisely according to their hydrodynamic volume). Small molecules penetrate into the pores so their way through the column is slower than in case of bigger molecules which do not enter the pores and their way is then much faster. Smaller molecules are eluted from the column later then bigger ones.

HiLoad 16/600 Superdex 200 pg column and HiLoad 26/600 Superdex 75 pg column were used for a final purification of prepared proteins. Chromatographic buffer (Table 4.19, page 50) similar to the dialysis buffer was filtered through the membrane with 0.45 μm pores to eliminate any bigger pieces of impurities in the solution. The column was equilibrated with the filtered buffer prior to run.

Table 4.19 | Buffer for size exclusion chromatography
Buffer has similar composition as dialysis buffer. Only β-mercaptoethanol was replaced by dithiothreitol (DTT). Amounts shown in the table has to be dissolved in deionized water to the final volume of 1000 ml.

Compound	Amount	Final concentration
Tris-HCl (pH 8)	50 ml	50 mM
NaCl	29.25 g	500 mM
EDTA (pH 8)	2 ml	1 mM
glycerol	100 g	10%
DTT	0.771 g	5 mM

Mixture of proteins after TEV cleavage was concentrated to the volume of 2 ml and filtrated through the membrane with 0.45 μm pores and then injected into the column. Fractions (which corresponded to the studied protein) were collected and verified by SDS-PAGE.

4.2.3 Phosphorylation

Protein kinase A (PKA) is a family of kinases which phosphorylate specific sites in the sequence of the other proteins. Its catalytic domain is commonly used for *in vitro* phosphorylation of natural downstream substrates or artificially created PKA phosphorylation sites.

Mixture for phosphorylation contained substrate, ATP, PKA and magnesium acetate (the optimized composition of the reaction mixture is listed on page 64). Protein of the interest was concentrated to the 1 mg/ml in the buffer with 50 mM Tris (pH 8). Mixture of a protein and compounds for phosphorylation was incubated in 30 °C water bath for 2 hours and at 4 °C overnight.

Protein was purified after phosphorylation by size exclusion chromatography on HiLoad 16/600 Superdex 75 pg column (chapter 4.2.2.7).

4.2.4 Native gel electrophoresis

Proteins prepared and analyzed in non-denaturing conditions are used in case of studying complex formation. SDS-PAGE is based on the denaturation and charging of the proteins. Secondary structure and native charge of the protein are maintained in the native gels. Molecules are then separated according to their molecular mass, volume of the packed molecule or complex and intrinsic charge.

Native gel (Table 4.20) was prepared in a similar way as was described above in the chapter about SDS-PAGE (chapter 4.2.2.5). Native gel consists of only one type of gel. Electrophoresis was run in the 1× TBE buffer at 4 °C for 4.5 hours at a constant voltage of 140 V.

Table 4.20 | Native gel (12%)
The gel composition is designed to keep proteins in non-denaturing conditions.

Compound	Volume [µl]
deionized water	3000
10× TBE buffer	600
acrylamide 30%/bis-acrylamide 0,8%	2400
10% APS	75
TEMED	8

4.2.5 Analytical ultracentrifugation (AUC)

Properties of macromolecules (such as proteins) in solution can be analyzed by analytical ultracentrifugation. The advantage is that proteins are in the native state and can be used for further experiments after AUC because the technique is nondestructive. Samples are spun at very high speeds which causes sedimentation of molecules according to their hydrodynamic and thermodynamic characteristics. This method provides observation of sedimentation by optical detection system in a real time. There are two types of analysis which could be done in analytical ultracentrifuge.

Sedimentation velocity is a technique which determines the size, shape and interactions of macromolecules (hydrodynamic characteristics). The experiment is based on sedimentation of molecules in a centrifugal field generated in a centrifuge. The meniscus is a region of the sample which is nearest to the center of the rotor in the sample cell. Proteins are very quickly centrifuged from the meniscus area and form a boundary which divides sample into area without protein (closer to the meniscus) and the area with a constant concentration of the protein (closer to the bottom of the cell). The rate at which the boundary moves is dependent on the shape and molecular weight of the analyzed proteins. The width of the sedimentation boundary correlates with number of species in the sample with similar sedimentation coefficients. Sedimentation coefficient (S) of the protein is defined as the ratio of the particle's velocity to the centrifugal field.

Sedimentation equilibrium is a method which observes molar mass and interactions of macromolecules (including association constants and stoichiometry of the complexes)

but is completely insensitive to the shape of molecule (thermodynamic characteristics). Samples are spun at a lower speed than in case of sedimentation velocity and produces a protein concentration gradient across the sample cell. The goal of the experiment is to achieve the balance between sedimentation and diffusion (which is opposite to the concentration gradient) of the molecules. The device measures the equilibrium concentration distribution across the centrifuge cell [51].

The samples of CaMKK2 alone and the mixture of 14-3-3 protein and CaMKK2 were analyzed by sedimentation velocity experiment. Proteins were dialyzed to the buffer (Table 4.21) prior to the analysis. The experiment was carried out at 42 000 rpm (ProteomLab XL-I Analytical Ultracentrifuge). The sedimentation was observed with an absorbance detector (standard double-beam spectrophotometer) set to the wavelength of 280 nm. The actual experiment and data processing were performed by my dear colleague Mgr. Olívia Petrvalská.

Table 4.21 | Dialysis buffer for AUC
The buffer for AUC contains only indispensable compounds to be as simple as possible.

Compound	Amount	Final concentration
Tris-HCl (pH 8)	50 ml	50 mM
NaCl	29.25 g	500 mM
β-ME	143 µl	2 mM

4.2.6 Kinetics

Kinases regulate cellular signaling pathways so they are ideal drug target candidates. The way of studying kinases and their effect on substrates is a monitoring of their kinase activity. ADP-Glo™ Kinase Assay was used for these purposes. The bioluminescent kinase assay monitor ADP production during a kinase reaction when studied kinase phosphorylates its substrate and converts ATP to ADP. Two-step assay was performed in a multiwell plate. Components of the kinase reaction were mixed and the reaction was allowed to proceed. An equal volume of ADP-Glo™ Reagent was added to the reaction to terminate the phosphorylation of substrate. The ADP-Glo™ Reagent also depleted remaining ATP after kinase reaction. The Kinase Detection Reagent then converted ADP to ATP which was measured by a luciferase/luciferin reaction using a luminometer. Luminescence was then proportional to the ADP production which was proportional to the kinase activity.

ADP-Glo™ Kinase Assay was performed in the 96-well plate. 25 µl of kinase reaction was run at room temperature. 25 µl of ADP-Glo™ Reagent was added to stop kinase reaction and deplete the remaining ATP. Mixture was incubated at RT for 40 min. 50 µl of Kinase

Detection Reagent was added to convert ADP to ATP and start luciferase/luciferin reaction. Mixture was incubated at RT for 30-40 min. The luminescence was measured by a plate-reading luminometer (Infinite M200 Pro - multimode reader) [52],[ES6].

5 RESULTS

5.1 Preparation of CaMKK2

Four constructs of CaMKK2 which differ in number of PKA phosphorylation sites were prepared. Original construct containing the catalytic kinase domain flanked by putative 14-3-3 binding motifs (at Ser100 and Ser511) was modified by PCR site-directed mutagenesis to obtain more variants of the protein. All forms were enzymatically active proteins with one, two or four PKA phosphorylation sites (Table 5.1). First, it was necessary to optimize the protocol of expression and purification prior to the further experiment.

Table 5.1| CaMKK2 constructs
The constructs differ in number of PKA phosphorylation sites but also in number of putative 14-3-3 binding motifs (written in italics).

Construct	PKA phosphorylation sites	Required mutation
CaMKK2$^{93\text{-}517}$ WT	*S^{100}*, T^{145}, S^{495}, *S^{511}*	none
CaMKK2$^{93\text{-}517}$ S^{100}	*S^{100}*	T^{145}A, S^{495}A, S^{511}A
CaMKK2$^{93\text{-}517}$ S^{511}	*S^{511}*	S^{100}A, T^{145}A, S^{495}A
CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}	*S^{100}*, *S^{511}*	T^{145}A, S^{495}A

5.1.1 Site directed mutagenesis

Three new constructs were prepared by mutation of four sites which were identified as putative PKA phosphorylation sites. Two of them were also predicted as 14-3-3 binding sites.

PKA phosphorylation site is a specific motif containing two basic residues (Arg or Lys) prior to the phosphorylated residue (Ser or Thr). Four PKA phosphorylation sites were found in the CaMKK2 sequence based on alignment with CaMKK1. These sites were selectively removed by mutating target residue to alanine.

^{97}RKL[S→A]L^{101} ^{142}RRP[T→A]V^{146} ^{492}RKR[S→A]F^{496} ^{508}RSL[S→A]A^{512}

Besides of the original construct possessing the sequence of wild-type CaMKK2 from His93 to Leu517 (CaMKK2$^{93\text{-}517}$ WT), three new constructs were obtained (CaMKK2$^{93\text{-}517}$ S^{100}; CaMKK293$^{\text{-}517}$ S^{100}, S^{511}; CaMKK2$^{93\text{-}517}$ S^{511}). The construct CaMKK2$^{93\text{-}517}$ S^{100}, S^{511} was prepared by mutating Thr145 and Ser495 thus removing two PKA phosphorylation sites and keeping both putative 14-3-3 binding sites intact. The last two constructs (CaMKK2$^{93\text{-}517}$ S^{100}; CaMKK2$^{93\text{-}517}$ S^{511}) contained only one PKA phosphorylation site/14-3-3 binding motif either at the N- or C-terminus (Ser100 and Ser511, respectively).

5.1.2 Optimization of expression of CaMKK2

It is always necessary to optimize the expression of new construct because every recombinant protein behaves differently. The first step is to choose optimal conditions for bacterial growth. Original construct was initially expressed in 900 ml of LB medium to test optimal conditions. Large-scale expression was then performed in 8× 900 ml of medium.

5.1.2.1 Original construct (CaMKK2$^{93-517}$ WT)

Construct CaMKK2$^{93-517}$ WT was transformed to the Rosetta cells and bacteria were cultivated in LB medium. Rosetta cells are derived from BL21 competent cells but they are specially modified for the expression of eukaryotic proteins. This strain contains plasmid which provides tRNAs for six additional codons (those which are rarely used in *E. coli*). The plasmid is coupled with chloramphenicol resistant. Therefore, it is necessary to use two antibiotics during the cultivation. One antibiotic is for elimination of bacteria which do not contain plasmid encoding the expressed protein. The second antibiotic is chloramphenicol to ensure the presence of additional codons.

5 µl of a glycerol stock of CaMKK2$^{93-517}$ WT in *E. coli* Rosetta cells was added to 5 ml of LB medium with appropriate amount of antibiotics kanamycin and chloramphenicol to start the cultivation. Bacteria were incubated for 16 hours at 37 °C in shaking incubator at 180 rpm. These bacterial suspensions were then used to inoculate 900 ml of LB medium. Cultures were incubated in shaking incubator at 37 °C until the OD reached the value of 0.7. Then IPTG was added (1 ml of 0.5 M IPTG per 900 ml of medium) to initiate the expression of the recombinant protein. Expression was performed at different temperatures for various time periods (Table 5.2).

Table 5.2 | Optimization of CaMKK2 expression in LB medium
The expression protocol was optimized to achieve the highest possible yield. Before induction by IPTG, all experiments were carried out under the same conditions. After that, temperature was changed as described in the table. For example, in experiment A the temperature was decreased to 25 °C. The temperature was then decreased again after 2.5 hours (to 15 °C for 16 hours).

Experiment	Temperature [°C]	Time [h]
A	25	2.5
	15	16
B	25	16
C	30	2
D	37	2

Cells were pelleted by centrifugation and resuspended in the lysis buffer. Cellular content was released by sonication.

Protein was preliminary purified by nickel chelate affinity chromatography. CaMKK2 was tagged with polyhistidine tag and thus retain in the column (reversible bound to the nickel ions immobilized in the column), whereas untagged proteins are not trapped within the column. CaMKK2 was eluted by the excess of imidazole which competitively binds to the nickel ions. Fractions were collected to the 1.5 ml microcentrifuge tubes placed on ice.

Expression of CaMKK2 was verified by SDS-PAGE (Figure 5.1). Samples were taken before sonication, after sonication and centrifugation (from the pellet and from the supernatant), and after nickel chelate affinity chromatography from each fraction.

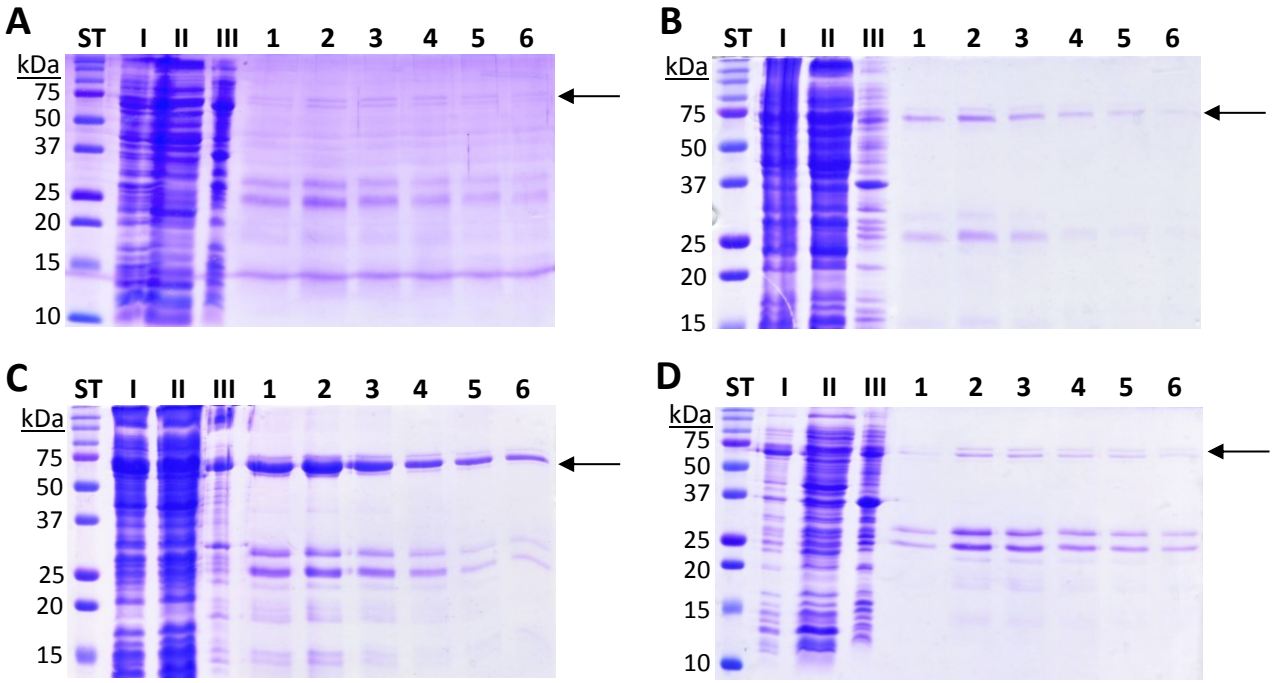


Figure 5.1 | Optimization of CaMKK2 expression in LB medium – 15% SDS-PAGE gels
Bands of CaMKK2 are marked with arrows. 5 µl of Precision Plus Protein Standards (ST) were loaded to the first well. 5 µl of lysate was mixed with 5 µl of sample buffer (well I). After sonication and centrifugation, 10 µl of supernatant was mixed with 5 µl of sample buffer (well II). Sample of the pellet (III) was prepared by dissolving amount of the pellet on the top of the tip in 1 ml of deionized water. 10 µl of the solution was mixed with 5 µl of sample buffer. 15 µl sample was taken from each fraction and mixed with 5 µl of sample buffer (wells 1-6). Electrophoresis was carried out at 200 V for 55 min.

The samples were only preliminary purified by nickel chelate affinity chromatography and thus the SDS-PAGE shows a lot of impurities. The extra bands correspond to the proteins which are present in *E. coli* and are trapped in the column.

The best result was obtained from experiment C (2 hours at 30 °C after induction). These conditions (with expression time extended up to 4 hours) were used for further expression of original construct of CaMKK2 (CaMKK2$^{93\text{-}517}$ WT).

5.1.2.2 Expression of CaMKK2 mutants

Other constructs (CaMKK2$^{93\text{-}517}$ S^{100}; CaMKK293$^{\text{-}517}$ S^{100}, S^{511}; CaMKK2$^{93\text{-}517}$ S^{511}) were transformed to the Rosetta cells and to the BL21(DE3) to compare expression in these two strains. However, experiments performed using the same procedure as described in chapter 5.1.2.1 revealed no significant differences (data not shown). Therefore, an easier option of BL21(DE3) strain was chosen. Only one antibiotic was then required to maintain the selectivity.

Auto-induction medium ZYP-5052 was another innovation in the development of expression protocol of mutant versions of CaMKK2. Auto-induction medium can be used for IPTG-inducible expression. The cultivation was optimized for the maximum yield. 10 µl of glycerol stock of CaMKK2$^{93\text{-}517}$ mutant form in *E. coli* BL21(DE3) cells was added to 5 ml aliquot of LB medium with required amount of antibiotic kanamycin. Bacteria were incubated for 16 hours at 37 °C in shaking incubator at 180 rpm. 900 ml of ZYP-5052 medium was inoculated by 2.5 ml of bacterial culture grown in LB medium. Large volume cultures were incubated at 37 °C in shaking incubator at 180 rpm for 5 hours. Temperature was then decreased to 20 °C for 15 hours.

5.1.3 Purification of CaMKK2 constructs

Purification was composed of several steps. Bacterial cultures were harvested by centrifugation and sonicated to gain cellular content. Expressed fusion protein was then preliminarily purified by nickel chelate affinity chromatography. TEV cleavage was performed to cleave the artificial part of protein. Final step of purification was size exclusion chromatography.

5.1.3.1 Nickel chelate affinity chromatography

Proteins were encoded in plasmid pRSFDuet-1 which contains GB1 fusion with N-terminal His-tag. Fusion part was later separated from CaMKK2 gene by TEV cleavage site. Originally, purified protein were trapped in the column at temperature 4 °C and eluted to the microcentrifuge tubes kept at 4 °C. But this procedure brought big losses through precipitation of protein in the tubes and during dialysis. It was probably caused by higher

concentration of the protein after it came off the column. Proteins were thus eluted to 10 ml of dialysis buffer to dilute them. It also worked against precipitation because of the presence of EDTA. EDTA was able to remove nickel ions which cause aggregation of his-tagged proteins via their tags.

5.1.3.2 TEV protease cleavage

TEV protease was prepared in our lab with specific activity of 250 units per 1 mg of purified protein (one unit is defined as an amount of TEV protease which cleaves >85% of 3 μg of substrate in one hour at pH 8.0 at 30 °C). Therefore, 0.125 μl of prepared TEV protease corresponded to the 1 unit. Concentration of CaMKK2 was measured only roughly because of the imidazole content in the solution. Protein was quantified by measuring absorption at 280 nm. UV light of this wavelength is absorbed by aromatic residues. It is possible to calculate a molar extinction coefficient (ε) from the protein sequence and then use the Lambert-Beer equation (A = εcl), where A is an absorbance (which is measured), ε is a molar extinction coefficient ($dm^3 \cdot mol^{-1} \cdot cm^{-1}$), c is a molar concentration ($mol \cdot dm^{-3}$) and l is a pathlength (cm), to calculate protein concentration. Elution buffer mixed with appropriate volume of dialysis buffer was used as a blank.

Fusion proteins were cleaved by incubation with TEV protease (250 units of TEV per mg of fusion protein) during overnight dialysis. The efficiency of the cleavage reaction was verified by SDS-PAGE (Figure 5.2, page 59).

5.1.3.3 Size exclusion chromatography

Cleaved proteins were slowly concentrated to the 2 ml volume before the final purification step. This volume was injected into the column. HiLoad 16/600 Superdex 200 pg column (bed volume 120 ml, flow rate 2.5 ml/min) was used to purify CaMKK2$^{93\text{-}517}$ WT. 1.5 ml fractions were collected to the microcentrifuge tubes. Proteins in eluted fractions were detected by continuous measuring absorbance at a wavelength of 280 nm (Figure 5.3, page 60). Fractions selected according to the chromatogram were analyzed by SDS-PAGE (Figure 5.4, page 60). SDS-PAGE electrophoresis was carried out in 12% gel at 190 V for 1 hour.

HiLoad 26/600 Superdex 75 pg column (bed volume 320 ml, flow rate 1.5 ml/min) was used to purify mutant forms of CaMKK2 (CaMKK2$^{93\text{-}517}$ S^{100}; CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}; CaMKK2$^{93\text{-}517}$ S^{511}). 1.5 ml fractions were collected to the microcentrifuge tubes. Proteins in eluted fractions were detected by continuous measuring absorbance at a wavelength

of 280 nm (Figure 5.5, 5.7 and 5.9; pages 61, 62 and 63). Fractions selected according to the chromatogram were analyzed by SDS-PAGE (Figure 5.6, 5.8 and 5.10; pages 61, 62 and 63).

SDS-PAGE electrophoresis was carried out in 12% gel (unless noted otherwise) at 190 V for 1 hour.

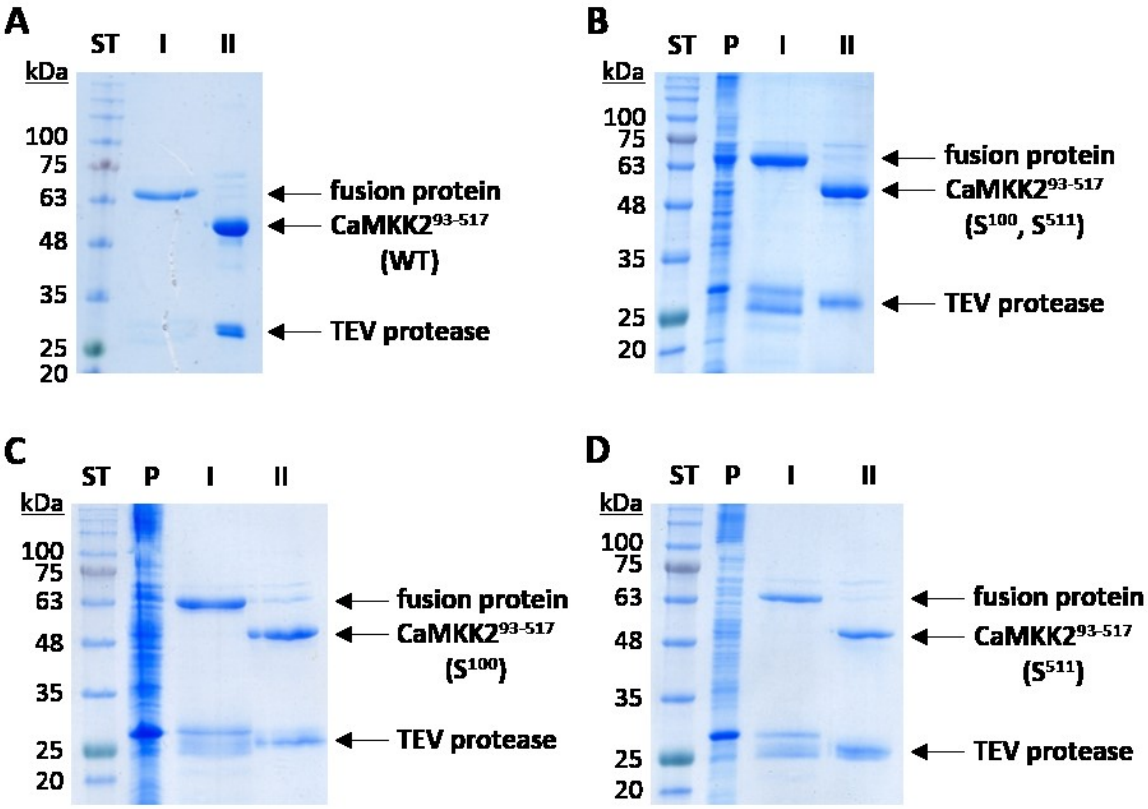


Figure 5.2 | TEV protease cleavage – 12% SDS-PAGE gels
5 µl of Blue Protein Ladder (BIOSYSTEMS) was used as a standard (ST). Sample of pellet (P) was prepared as described above (Figure 5.1, page 56). All samples were mixed with 5 µl of sample buffer. 20 µl of protein sample was taken before cleavage (I) and 20 µl sample was taken after cleavage (II). Electrophoresis was carried out at 190 V for 1 hour. Molecular weight of all fusion proteins is about 57 kDa, molecular weight of CaMKK2 after TEV cleavage is about 48 kDa. Molecular weight of TEV protease is 27.5 kDa. **A**, CaMKK2 without mutations. **B**, CaMKK2 with two PKA phosphorylation sites (Ser100 and Ser511). **C**, CaMKK2 with N-terminal PKA phosphorylation site (Ser100). **D**, CaMKK2 with C-terminal PKA phosphorylation site (S^{511}).

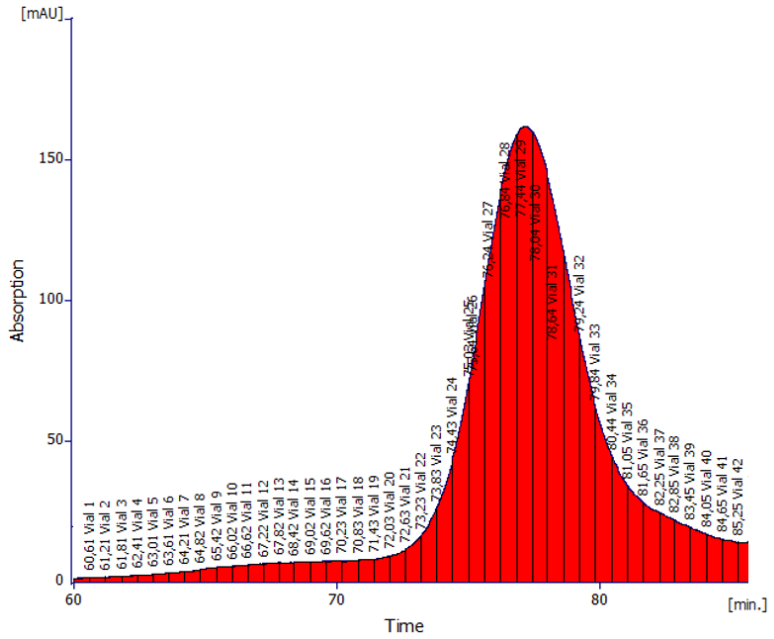


Figure 5.3 | Size exclusion chromatography – chromatogram of CaMKK2$^{93-517}$ WT
Chromatogram shows elution profile of CaMKK2 without mutations. The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red.

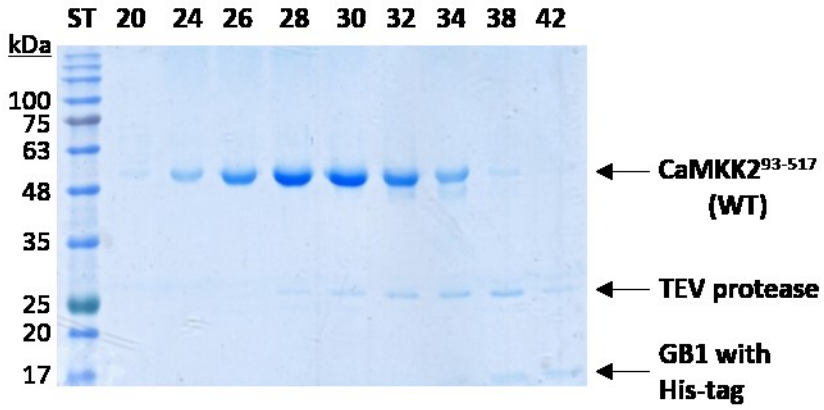


Figure 5.4 | Size exclusion chromatography – SDS-PAGE of eluted fractions with CaMKK2$^{93-517}$ WT
Fractions from the peak area were analyzed by SDS-PAGE to evaluate the purity of protein. 15 µl samples from selected fractions (numbers 20-42) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa, molecular weight of TEV protease is about 27.5 kDa and molecular weight of GB1–His-tag is about 10 kDa.

SDS-PAGE revealed overlapping peaks of CaMKK2 and TEV protease which were not visible in the chromatogram. Thus only fractions 24-30 from SEC were merged together for further experiments. CaMKK2 without mutations was purified with final yield 0.26 mg of pure protein from 900 ml of LB medium.

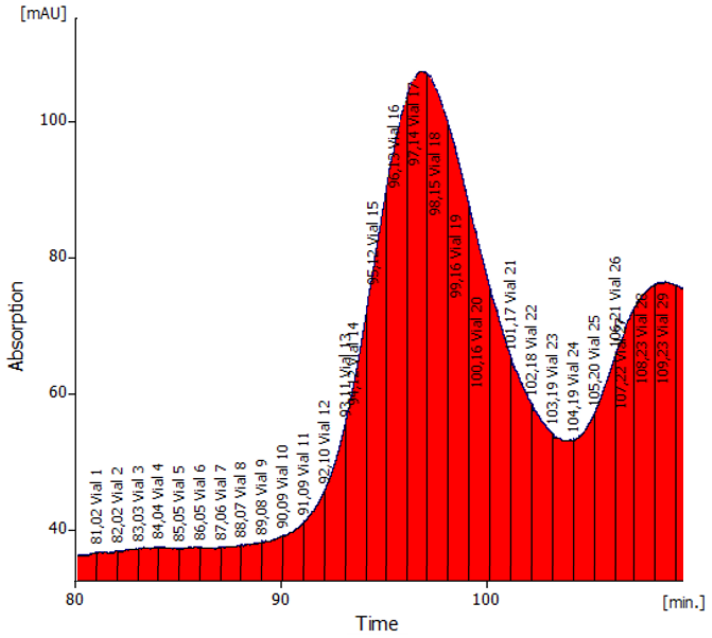


Figure 5.5 | Size exclusion chromatography – chromatogram of CaMKK2$^{93-517}$ S^{100}, S^{511}
Chromatogram shows elution profile of CaMKK2 with two PKA phosphorylation sites (Ser100 and Ser511). The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red.

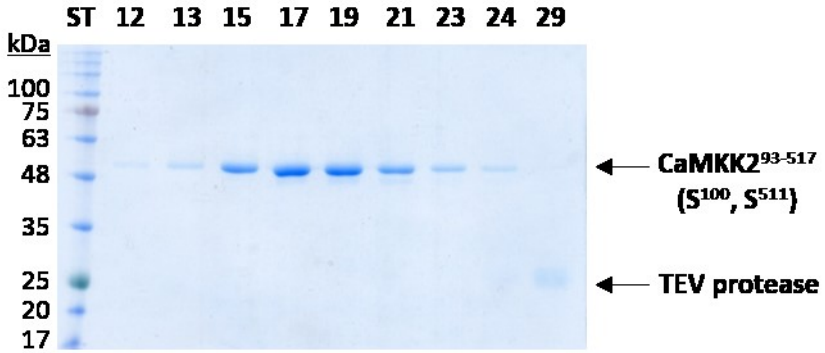


Figure 5.6 | Size exclusion chromatography – SDS-PAGE of eluted fractions with CaMKK2$^{93-517}$ S^{100},S^{511}
Fractions from the peak area were analyzed by SDS-PAGE to evaluate the purity of protein. 15 µl samples from selected fractions (numbers 12-29) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa, molecular weight of TEV protease is about 27.5 kDa.

HiLoad 26/600 Superdex 75 pg column provided better separation of CaMKK2 from contaminants compared to the HiLoad 16/600 Superdex 200 pg column, as documented by SDS-PAGE which revealed nice separation of proteins. Fractions 12-23 from SEC were merged together for further experiments. CaMKK2 with two PKA phosphorylation sites was purified with final yield 0.29 mg of pure protein from 900 ml of ZYP-5052 medium.

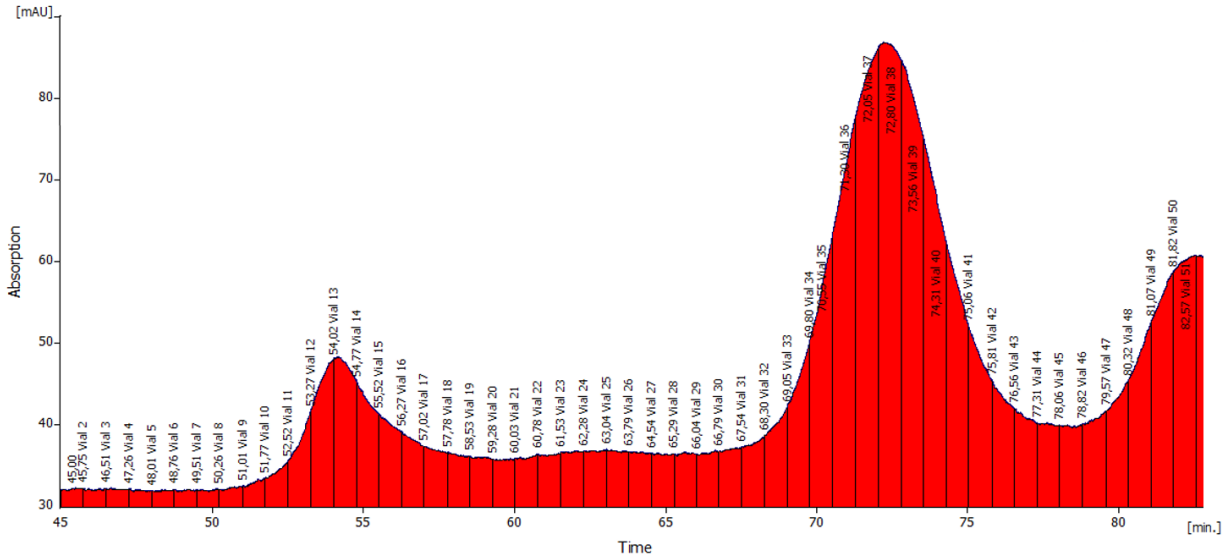


Figure 5.7 | Size exclusion chromatography – chromatogram of CaMKK2$^{93-517}$ S^{100}
Chromatogram shows elution profile of CaMKK2 with one PKA phosphorylation sites (Ser100). The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red.

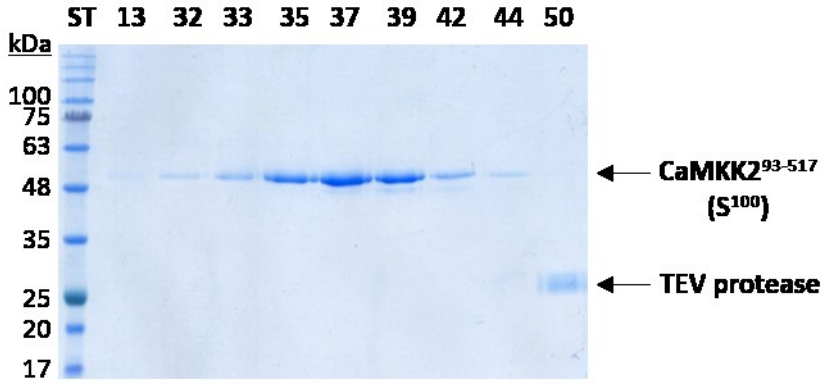


Figure 5.8 | Size exclusion chromatography – SDS-PAGE of eluted fractions with CaMKK2$^{93-517}$ S^{100}
Fractions from the peak area were analyzed by SDS-PAGE to evaluate the purity of protein. 15 µl samples from selected fractions (numbers 13-50) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa, molecular weight of TEV protease is about 27.5 kDa.

SDS-PAGE proved nice separation of proteins. Fraction 13 corresponds to the peak of CaMKK2 aggregates. Fractions 33-42 from SEC were merged together for further experiments. CaMKK2 with one PKA phosphorylation (Ser100) sites was purified with final yield 0.15 mg of pure protein from 900 ml of ZYP-5052 medium.

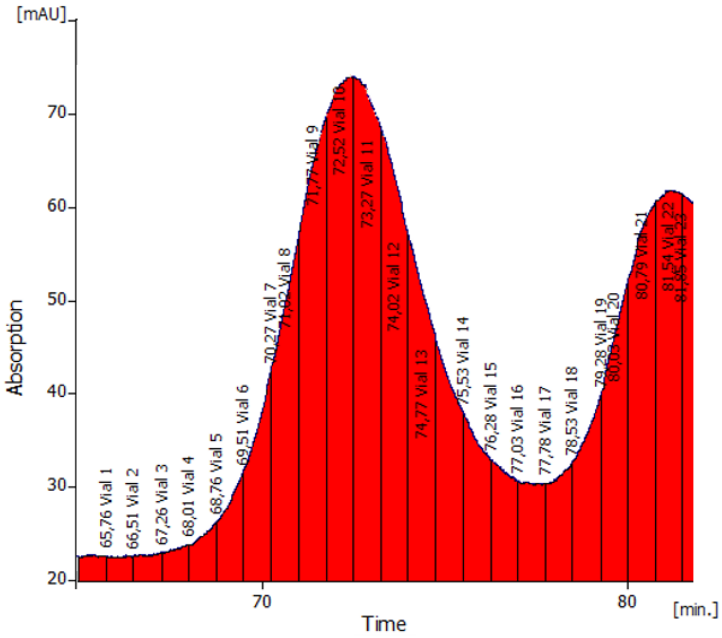


Figure 5.9| Size exclusion chromatography – chromatogram of CaMKK2$^{93-517}$ S^{511}
Chromatogram shows elution profile of CaMKK2 with one PKA phosphorylation sites (S^{511}). The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red.

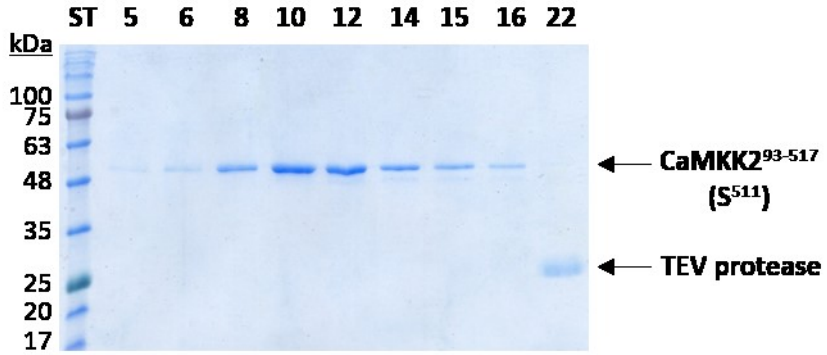


Figure 5.10| Size exclusion chromatography – SDS-PAGE of eluted fractions with CaMKK2$^{93-517}$ S^{511}
Fractions from the peak area were analyzed by SDS-PAGE to evaluate the purity of protein. 15 µl samples from selected fractions (numbers 5-22) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa, molecular weight of TEV protease is about 27.5 kDa.

SDS-PAGE proved nice separation of proteins. Fractions 5-16 from SEC were merged together for further experiments. CaMKK2 with one PKA phosphorylation (S^{511}) sites was purified with final yield 0.08 mg of pure protein from 900 ml of ZYP-5052 medium.

5.1.4 Phosphorylation

All purified proteins were phosphorylated by PKA for further studies. Standard phosphorylation protocol requires the presence of 12 mM magnesium acetate, 750 µM ATP and appropriate amount of PKA (1.5 U per nmol of PKA phosphorylation site; one unit is defined as amount of PKA which is required to incorporate 1 pmol of phosphate into the substrate in one minute at 30 °C; activity 102.8U/µl). Protein was incubated at 30 °C for 2 hours with this mixture with additional incubation overnight at 4 °C. In some cases, fraction of the protein was autophosphorylated. Mixture for autophosphorylation possesses the same composition as is required for phosphorylation with one difference, PKA is not present.

CaMKK2$^{93\text{-}517}$ WT contains four PKA phosphorylation sites. Standard protocol mentioned above was used for phosphorylation and autophosphorylation. Remaining ATP was removed by multiple dialysis. The result of phosphorylation was checked by mass spectrometry.

CaMKK2$^{93\text{-}517}$ S^{100} contains only one PKA phosphorylation site. Standard protocol was also used in this case. The excess of ATP was removed by size exclusion chromatography using HiLoad 16/600 Superdex 75 pg column (bed volume 120 ml, flow rate 1 ml/min). Purity of protein fractions was verified by SDS-PAGE (Figure 5.11).

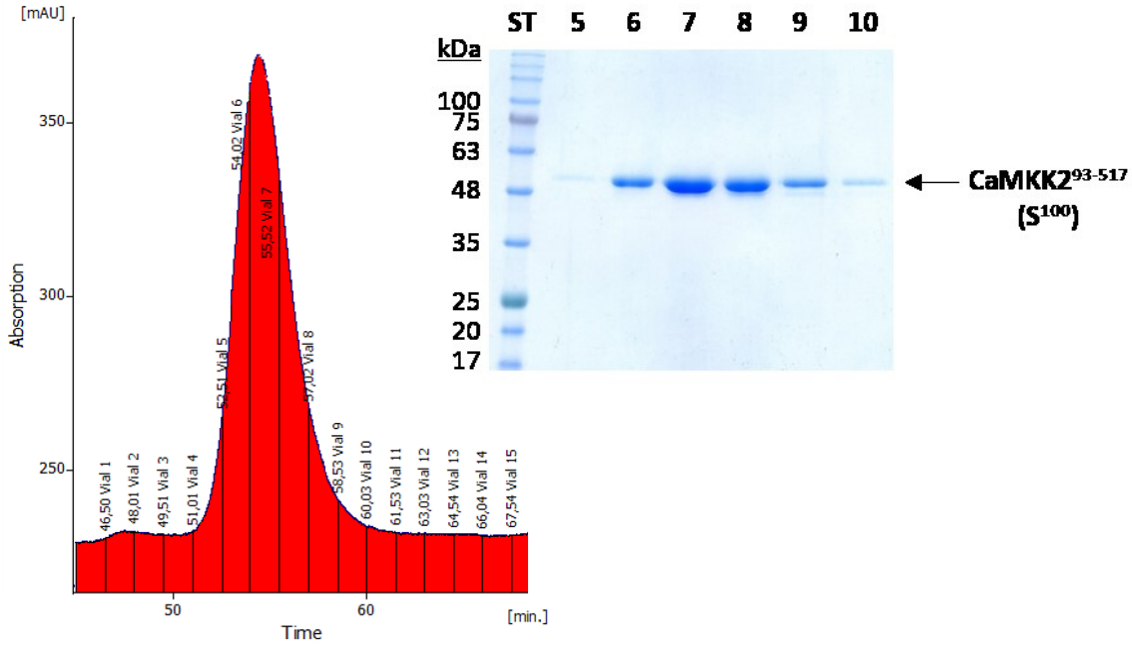


Figure 5.11 | Size exclusion chromatography – chromatogram and SDS-PAGE of CaMKK2$^{93\text{-}517}$ S^{100}
Chromatogram shows elution profile of phosphorylated CaMKK2 with one PKA phosphorylation sites (Ser100). The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red. Fractions from the peak area were analyzed by SDS-PAGE to evaluate the purity of protein. 15 µl samples from selected fractions (numbers 5-10) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa.

Fractions 5-9 from SEC were merged together. CaMKK2 with phosphorylated Ser100 was purified with final yield 0.08 mg of pure phosphorylated protein from 900 ml of ZYP-5052 medium. The result of phosphorylation was checked by mass spectrometry.

CaMKK2$^{93\text{-}517}$ S^{511} contains also only one PKA phosphorylation site. Standard protocol was used also in this case. The excess of ATP was removed by size exclusion chromatography using HiLoad 16/600 Superdex 75 pg column (bed volume 120 ml, flow rate 1 ml/min). Purity of protein fractions was verified by SDS-PAGE with 15% gel (Figure 5.12).

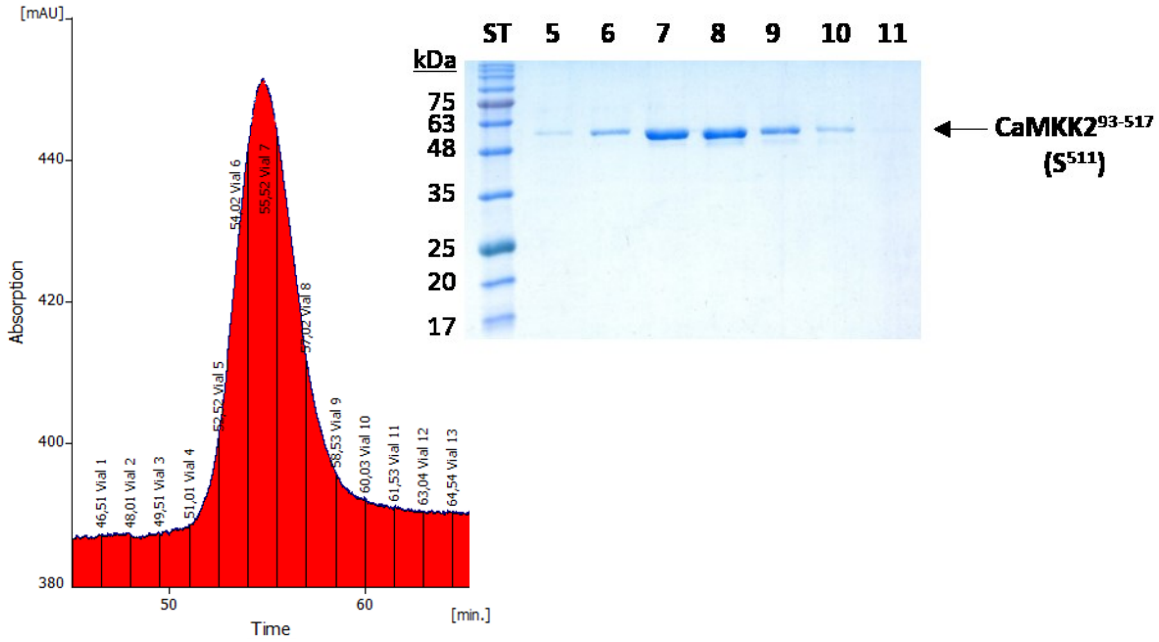


Figure 5.12 | Size exclusion chromatography – chromatogram and SDS-PAGE of CaMKK2$^{93\text{-}517}$ S^{511}
Chromatogram shows elution profile of phosphorylated CaMKK2 with one PKA phosphorylation sites (S^{511}). The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red. Fractions from the peak area were analyzed by SDS-PAGE to evaluate the purity of protein. 15 µl samples from selected fractions (numbers 5-11) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa.

Fractions 5-11 from SEC were merged together. CaMKK2 with phosphorylated S^{511} was purified with final yield 0.03 mg of pure phosphorylated protein from 900 ml of ZYP-5052 medium. The result of phosphorylation was checked by mass spectrometry.

Since the results of mass spectrometry measurements revealed uncomplete phosphorylation of Ser511, the phosphorylation protocol for CaMKK2$^{93\text{-}517}$ S^{100}, S^{511} was changed. The changed protocol involves higher amount of PKA (8 U per nmol of PKA phosphorylation site). In addition, the incubation time was also prolonged to 3 hours at 30 °C followed by overnight incubation at 4 °C. The excess of ATP was removed by size exclusion chromatography using HiLoad 16/600 Superdex 75 pg column (bed volume 120 ml, flow rate 1 ml/min). Purity of protein fractions was verified by SDS-PAGE.

In the case of autophosphorylated CaMKK2$^{93\text{-}517}$ S^{100}, S^{511} (Figure 5.13), fractions 5-10 from SEC were merged together. CaMKK2 with autophosphorylated Ser100 and Ser511 was purified with the final yield of 0.13 mg from 900 ml of ZYP-5052 medium. The result of autophosphorylation was checked by mass spectrometry.

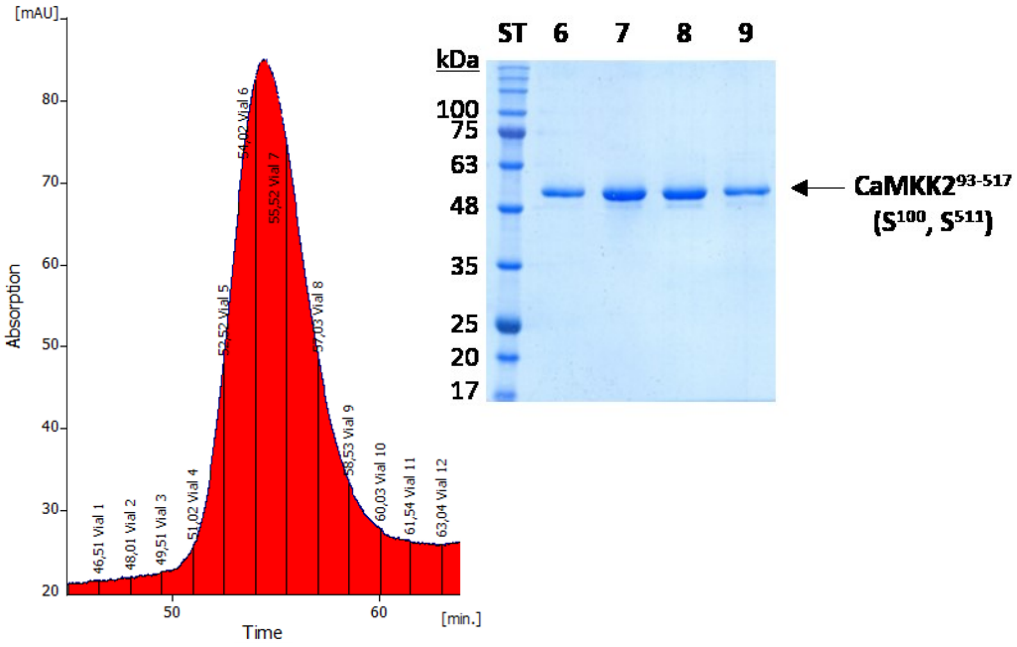


Figure 5.13 | Size exclusion chromatography – chromatogram and SDS-PAGE of PKA phosphorylated CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}

Chromatogram shows elution profile of autophosphorylated CaMKK2 with two PKA phosphorylation sites (Ser100 and Ser511). The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red. Fractions from the peak area were analyzed by SDS-PAGE to evaluate the purity of protein. 15 µl samples from selected fractions (numbers 6-9) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa.

In the case of phosphorylated CaMKK2$^{93\text{-}517}$ S^{100}, S^{511} (Figure 5.14), fractions 6-11 from SEC were merged together. CaMKK2 with phosphorylated Ser100 and Ser511 was purified with final yield of 0.16 mg from 900 ml of ZYP-5052 medium. The result of phosphorylation was checked by mass spectrometry.

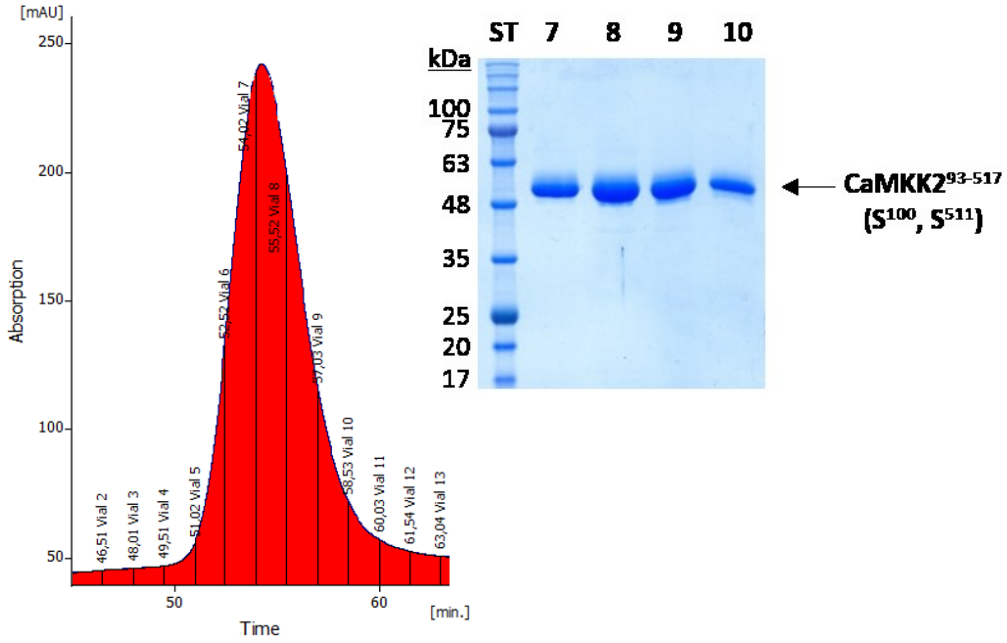
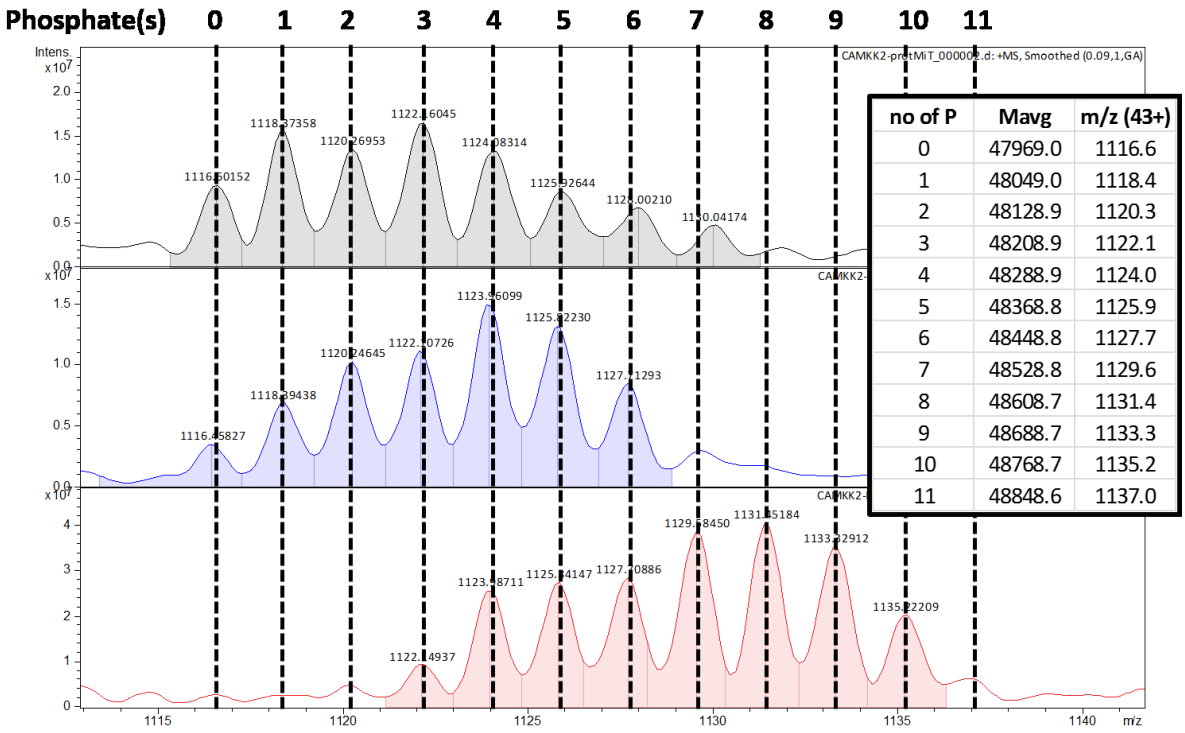


Figure 5.14 | Size exclusion chromatography – chromatogram and SDS-PAGE of autophosphorylated CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}
Chromatogram shows elution profile of autophosphorylated CaMKK2 with two PKA phosphorylation sites (Ser100 and Ser511). The absorbance in mAU (milli-absorbance units) on the y axis is plotted against elution time in minutes. Collected fractions are colored in red. Fractions from the peak area were analyzed by SDS-PAGE to roughly evaluate the purity of protein. 15 µl samples from selected fractions (numbers 6-9) were mixed with 5 µl of sample buffer. 5 µl of Blue Protein Ladder was used as a standard (ST). Molecular weight of CaMKK2 is about 48 kDa.

5.1.4.1 Results of mass spectrometry

All mass spectrometry measurements were performed by RNDr. Petr Man, Ph.D. and RNDr. Petr Pompach, Ph.D. in the laboratory of Protein Structure Characterization by Advanced Mass Spectrometry of Biocev, Czech Academy of Sciences. Analysis of CaMKK2$^{93\text{-}517}$ WT showed interesting fact - CaMKK2 is already heavily phosphorylated during expression in bacteria. The level of this phosphorylation is even higher in the case of autophosphorylated protein which was additionally incubated with ATP/Mg^{2+}. Up to 11 phosphorylated sites were identified in CaMKK2$^{93\text{-}517}$ WT phosphorylated by PKA. However, PKA phosphorylated only four sites, the rest was phosphorylated and/or autophosphorylated during expression and/or incubation with ATP/Mg^{2+} (Figure 5.15, page 68).



no of P	Mavg	m/z (43+)
0	47969.0	1116.6
1	48049.0	1118.4
2	48128.9	1120.3
3	48208.9	1122.1
4	48288.9	1124.0
5	48368.8	1125.9
6	48448.8	1127.7
7	48528.8	1129.6
8	48608.7	1131.4
9	48688.7	1133.3
10	48768.7	1135.2
11	48848.6	1137.0

Figure 5.15 | Mass spectrometry analysis of CaMKK2$^{93\text{-}517}$ WT
Comparison of in vitro nonphosphorylated (top panel), in vitro autophosphorylated (middle panel) and in vitro phosphorylated (bottom panel) sample. The 43+ charge state is shown. The vertical black dashed lines denote different phosphostates. Molecular weights (M_{avg}) of detected phosphostates are listed in the table shown in inset. It can be noticed that protein purified from *E. coli* cells (thus without any *in vitro* phosphorylation and/or incubation with ATP/Mg^{2+}) is already phosphorylated (top panel). PKA phosphorylates four sites within the sequence of CaMKK2 (as documented by the shift of bottom MS spectrum by mass corresponding to four phosphate groups).

Mass spectrometry analysis of purified and phosphorylated mutated proteins (CaMKK2$^{93\text{-}517}$ S^{100}; CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}; CaMKK2$^{93\text{-}517}$ S^{511}) revealed that Ser100 was phosphorylated from 93% or better (using standard phosphorylation protocol). Surprisingly, Ser511 was phosphorylated only from 60% under the same conditions. The changed phosphorylation protocol (higher PKA concentration) enabled higher phosphorylation of Ser511 as 99.7% phosphorylation of Ser100 and 85% phosphorylation of Ser511 were detected.

5.2 Native gel electrophoresis

Interaction between CaMKK2 and 14-3-3 protein was studied by native gel electrophoresis. CaMKK2$^{93\text{-}517}$ WT was used for this experiment. 14-3-3 (human zeta isoform) was mixed with PKA phosphorylated (or autophosphorylated) CaMKK2 in precisely defined molar ratio (Table 5.3, page 69) and the mixture was incubated at 4 °C for 30 min followed by analysis on native PAGE.

Table 5.3| Composition of samples for native gel electrophoresis
2 µl of sample buffer was added to 8 µl of each mixture. Autophosphorylated CaMKK2 is in grey, phosphorylated CaMKK2 in black in the table.

Well	CaMKK2 (autoP or P)	14-3-3ζ	Ratio CaMKK2/14-3-3 ζ
1	8.8 µM	-	-
2	8.8 µM	-	-
3	-	7.5 µM	-
4	7.7 µM	7.5 µM	1:1
5	7.7 µM	7.5 µM	1:1
6	7.7 µM	15 µM	1:2
7	7.7 µM	15 µM	1:2
8	7.7 µM	3.75 µM	2:1

10 µl of sample was loaded into the wells of 12% native gel (Figure 5.16). Electrophoresis was carried out at 140 V at 4 °C for 4.5 hours.

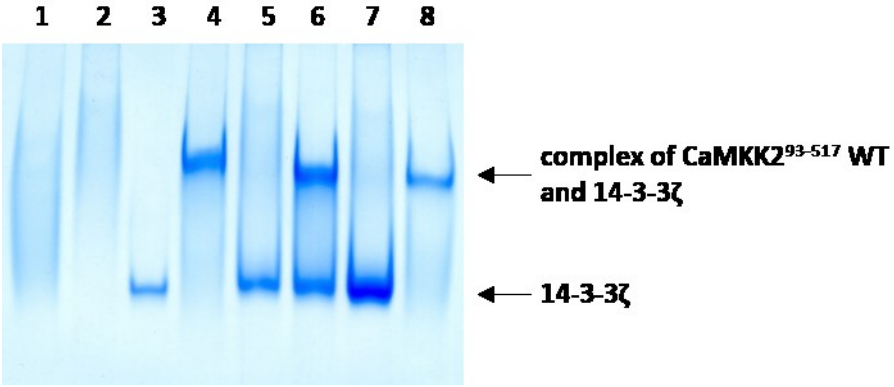


Figure 5.16| Native gel electrophoresis
1 – phosphorylated CaMKK2 alone; 2 – autophosphorylated CaMKK2 alone; 3 – 14-3-3ζ alone; 4,6,8 – mixture of phosphorylated CaMKK2 and 14-3-3ζ; 5,7 – mixture of autophosphorylated CaMKK2 and 14-3-3ζ. Complex formation is visible (as formation of a new band) in lanes number 4, 6 and 8.

CaMKK2 creates blurry bands (it is caused by higher heterogeneity of the sample due to unspecific phosphorylation and unstructured regions), whereas band of 14-3-3ζ is nicely focused at the bottom of the gel. Another focused band (in the upper part of the gel) occurs when complex of CaMKK2 and 14-3-3ζ is formed.

5.3 Analytical ultracentrifugation

Interaction between CaMKK2 and 14-3-3ζ protein suggested by native gel electrophoresis (Figure 5.16, page 69) was next studied by sedimentation velocity analytical ultracentrifugation experiment (SV-AUC).

The first experiment was performed to find an optimal concentration of CaMKK2. The normalized continuous sedimentation coefficient distributions, c(s), obtained from these SV-AUC experiments are shown in Figure 5.17. As can be noticed, CaMKK2 forms dimers at higher protein concentrations (> 10 μM). Therefore CaMKK2 at concentration 10 μM was used in further experiments.

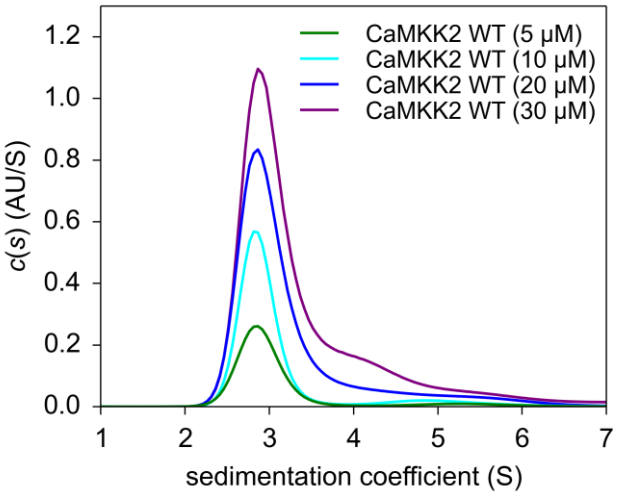


Figure 5.17 | AUC – sedimentation velocity of wild-type CaMKK2$^{93\text{-}517}$
The normalized continuous sedimentation coefficient distributions, c(s), obtained from SV-AUC experiments with CaMKK2 at different concentrations are shown. The curves for the protein concentration of 5 μM and 10 μM (green and cyan curve, respectively) show single peak. It means that only one type of particle is present in the sample. Higher concentrations (20 μM and 30 μM; blue and purple curve, respectively) possess other types of particle which broaden the distributions of sedimentation coefficients to higher values. It is a typical evidence of protein oligomerization.

The further experiments were done to study the interaction between CaMKK2 and 14-3-3 protein (isoform ζ). The first experiment (Figure 5.18, part A, page 71) confirmed that 14-3-3 protein does not bind to the nonphosphorylated protein (CaMKK2 which was not incubated either with ATP/Mg^{2+} or PKA). Distribution obtained for the mixture of nonphosphorylated CaMKK2 and 14-3-3ζ (blue) showed single peak with a sedimentation coefficient, *s*, value of 3.3 S and no peak at higher *s* values was observed. On the other hand, distribution obtained for the mixture of phosphorylated CaMKK2 and 14-3-3ζ mixed in 1:1

molar ratio (cyan) showed two peaks at *s* values of 3.3 and 4.9 S. This strongly suggests formation of the CaMKK2/14-3-3ζ complex (peak with the higher *s* value).

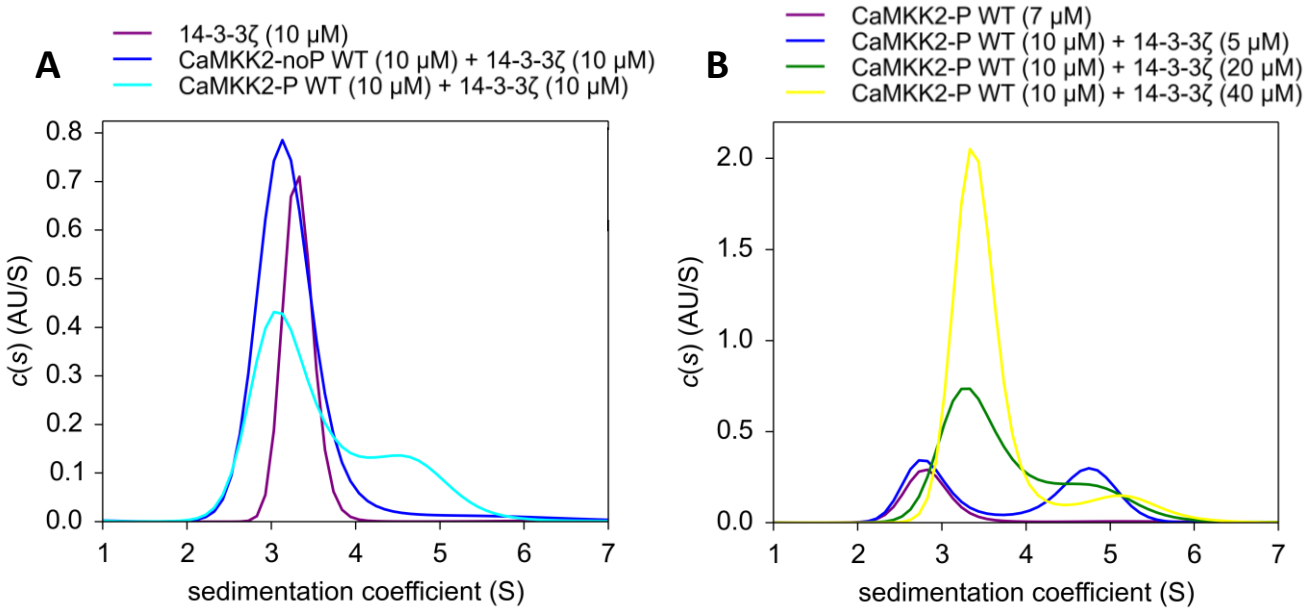


Figure 5.18 | AUC – sedimentation velocity of wild-type CaMKK2$^{93-517}$ in complex with 14-3-3ζ
The normalized continuous sedimentation coefficient distributions c(s) of mixtures of CaMKK2 and 14-3-3ζ at various concentrations are shown. The concentration of 14-3-3 protein is given for the monomer. **A**, comparison of the samples with 14-3-3 protein alone (purple curve), nonphosphorylated CaMKK2 in the mixture with 14-3-3 protein (blue curve) and phosphorylated CaMKK2 mixed with 14-3-3 protein (cyan curve). **B**, phosphorylated CaMKK2 mixed with 14-3-3 protein in different ratios.

The next experiment (Figure 5.18, part B) was aimed to elucidate the binding stoichiometry of the CaMKK2/14-3-3ζ complex. Sample of CaMKK2 alone was compared with two samples containing both phosphorylated CaMKK2 and 14-3-3ζ mixed at three different molar ratios (2:1, 1:2 and 1:4; blue, green and yellow distributions, respectively). As noticed, both blue, green and yellow distributions contain peak at *s* values between 4.5-5.2 S as a result of titration of CaMKK2 with increasing concentration of 14-3-3 protein. These values of *s* correspond to molecular mass of approx. 80-90 kDa, thus indicating 2:1 stoichiometry with a dimer of 14-3-3ζ binds one molecule of phosphorylated CaMKK2.

5.4 Kinetics

Kinase assay was performed using a procedure described previously [31]. In this assay, the AMPK peptide possessing the sequence surrounding the CaMKK2 phosphorylation site (^{167}GEFLRTSCGSP^{177}) was used as a substrate. The reaction mixture was composed of 500 µM peptide, 50 mM HEPES (pH 7.5; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 300 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 10% glycerol, 5 µM calmodulin and 1 mM $CaCl_2$. The 200 nM enzyme (CaMKK2 phosphorylated only on Ser100 or on both Ser100 and Ser511) and 400 µM ATP were added to the mixture to create final volume of 25 µl and start the reaction [31].

Kinase activity of CaMKK2 was monitored by ADP-Glo™ Kinase Assay. Kit calibration was done prior to the kinetic measurements. The calibration involves measuring of different ADP to ATP ratio to create a standard curve for conversion of ATP to ADP (Figure 5.19).

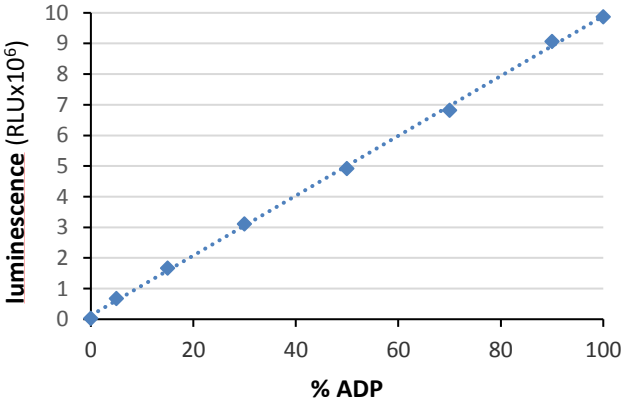


Figure 5.19| ATP-to-ADP conversion curve
The amount of ADP produced in kinase reaction can be estimated from a standard curve of conversion. % of ADP in the mixture is plotted against luminescence in relative luminescence units (RLU).

Time dependence was measured to verify the activity of prepared proteins (Figure 5.20, page 73). Similar results were obtained for two mutant forms (CaMKK2$^{93\text{-}517}$ S^{100} and CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}). The activity of the CaMKK2$^{93\text{-}517}$ S^{511} construct was not measured due to the low phosphorylation of Ser511 even in the presence of high PKA concentration. This suggests that Ser511 is most likely only partially (if at all) phosphorylated *in vivo*. Since the 14-3-3 binding is phosphorylation-dependent interaction, CaMKK2$^{93\text{-}517}$ S^{511} was excluded from further experiments.

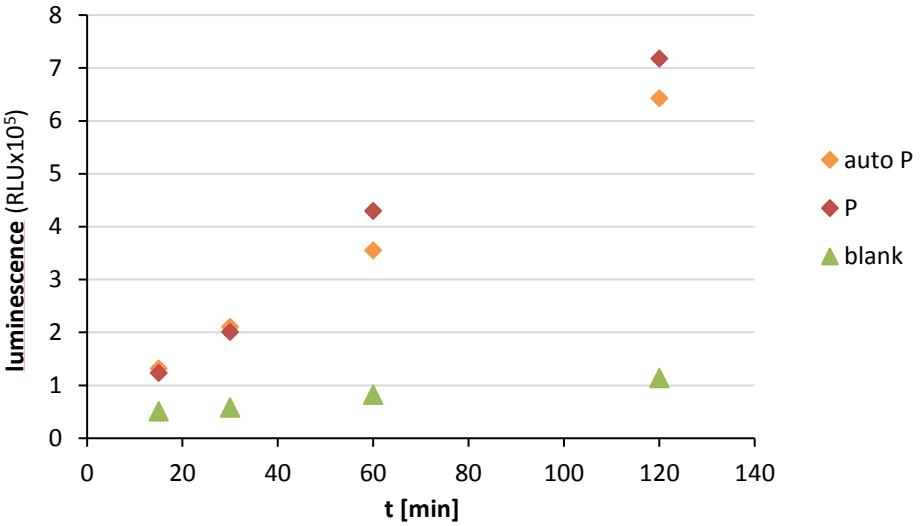


Figure 5.20| Dependence of the ADP production on the reaction time (CaMKK2$^{93\text{-}517}$ S^{100},S^{511})
The production of ADP is proportional to the time of kinase reaction. Phosphorylated CaMKK2 (red rhombuses) reaches higher luminescence than autophosphorylated CaMKK2 (yellow rhombuses). The blank mixture (green triangles) was identical with reaction mixture except for the peptide which was missing.

It was also necessary to optimize the time of reaction for further experiments, so the conversion does not exceed 5% and Michaelis-Menten kinetics can be used.

6 DISCUSSION

The prokaryotic systems are frequently used to prepare various eukaryotic proteins. Prokaryotic organisms (such as bacteria) lack some types of post-translational modifications (for example glycosylation), thus expressed proteins might be more suitable for further biophysical characterization thanks to the higher homogeneity of the sample. Bacterial expression is also much cheaper and easier than other (eukaryotic) expression systems.

Despite the fact that the decades of experience with bacterial expression provided the general approach for this technique, it is always necessary to optimize the expression of specific protein. Each protein is different. They vary in solubility, arrangement of the secondary structure, cellular toxicity and many other attributes. Sometimes it is even desirable to edit coding sequence for the protein prior to the transformation as it was done for CaMKK2 in this work. In this case, it was helpful to remove both N- and C-terminal regions to avoid difficulties with low solubility of recombinant protein. These regions of CaMKK2 probably lack any fixed secondary structure (Figure 4.2, page 36) and thus could cause aggregation and/or proteolytic instability of the protein during the purification. Different approaches of expression and purification were also tried to obtain maximal yield of the recombinant protein. These experiments involved screening for the optimal growth medium, optimal conditions of expression and effective purification (chapter 4.2.2). The most effective expression in LB medium and ZYP-5052 autoinduction medium was established (chapter 5.1.2). Purification protocol is described in chapters 4.2.2 and 5.1.3.

Some studied interactions are phosphorylation-dependent. This brings the requirement for the clearly defined phosphorylated sample with minimal deviations. The main problem with proteins expressed in a living organism is that they usually come modified from the host cell and kinases can also undergo autophosphorylation. To minimize these effects in this work, site-directed mutagenesis was used to delete unwanted phosphorylation sites (chapter 4.2.1.11). The rest of them were specifically phosphorylated by protein kinase A (PKA).

CaMKK2 is meant to be used in two types of experiments. First, structural studies will be used to reveal the structure of CaMKK2 regions beyond the borders of already known kinase domain. They could also provide understanding of the interactions between CaMKK2 and other proteins (such as 14-3-3 proteins, calmodulin or CaMKK2 downstream targets). CaMKK2 with silenced activity (so called kinase dead mutant) was prepared for these purposes. Based on homology with CaMKK1, it has been speculated that the 14-3-3 protein

binding affects the kinase activity of CaMKK2 [17],[37]. Experiments that would target this regulation will require the preparation of the fully active enzyme. Therefore, major part of this thesis was focused on preparation of such CaMKK2 variants.

The possible hypothesis of regulation of CaMKK2 was suggested after literature research and careful consideration. It was proposed that CaMKK2 activity is enhanced by the binding of Ca^{2+}/calmodulin complex. On the other hand, phosphorylation of CaMKK2 by PKA should partly inhibit CaMKK2 kinase activity. Full inactivation of CaMKK2 is then provided by binding of 14-3-3 dimer. 14-3-3 proteins bind only to the phosphoserine which occurs in specific sequence. This sequence possesses small analogy with PKA phosphorylation sites. Indeed, four PKA phosphorylation sites were predicted in the sequence of original construct of human CaMKK2$^{93\text{-}517}$ where two of them were also suggested as 14-3-3 binding sites.

Prediction of four PKA phosphorylation sites was confirmed by the results of mass spectrometry analysis. The analysis revealed a shift in phosphorylation level between autophosphorylated sample and sample which was exposed to the PKA phosphorylation. The shift exactly matched with four extra phosphorylation sites in the sequence of CaMKK2 (Figure 5.15, page 68). Three new mutant forms were thus prepared from the original construct of human CaMKK2$^{93\text{-}517}$. The first goal was to remove spare PKA phosphorylation sites which are not involved in 14-3-3 binding. This construct (called CaMKK2$^{93\text{-}517}$ S^{100}, S^{511}) possesses two PKA phosphorylation sites (Ser100 and Ser511), which are also putative 14-3-3 motifs. The other two mutants were prepared to confirm so called "gatekeeper" hypothesis. It assumes that 14-3-3 dimer binds to its ligand through one binding interaction on each monomer. It means that ligand possesses two motifs providing 14-3-3 binding where one of the 14-3-3 binding site on the ligand is crucial for the binding. The association between the 14-3-3 protein and its ligand cannot happen in the absence of the "gatekeeper" binding site because the second binding site is not able to sufficiently mediate strong and stable interaction on its own. Constructs with only one 14-3-3 binding motif (called CaMKK2$^{93\text{-}517}$ S^{100} and CaMKK2 S^{511}) were prepared to reveal the molecular basis of 14-3-3 binding and to check if one of these motifs plays a role of the "gatekeeper" binding site.

Creation of the complex between CaMKK2$^{93\text{-}517}$ WT and 14-3-3ζ was proven by the native gel electrophoresis (Figure 5.16, page 69). It was shown that the interaction is strictly phosphorylation-dependent process and autophosphorylation is not sufficient for the complex formation. The same result was also obtained from the analytical ultracentrifugation analysis (Figure 5.17, page 70). SV-AUC experiments also suggeted that

the molar stoichiometry of the CaMKK2/14-3-3ζ complex is 1:2 (dimer 14-3-3 binds one molecule of CaMKK2). Kinase activity of prepared proteins was verified by kinase assay where the peptide matching with CaMKK2 phosphorylation site in AMPK sequence was used as a substrate.

7 CONCLUSION

- Three different constructs of human CaMKK2 were prepared (with different number of PKA phosphorylation sites) from the original construct.
- All four forms (WT and three mutants) were successfully expressed and purified.
- Phosphorylation protocol was optimized and the result of phosphorylation reaction was verified by mass spectrometry.
- The phosphorylation-dependent interaction between CaMKK2 and 14-3-3ζ was confirmed using native PAGE and analytical ultracentrifugation. SV-AUC suggested that the molar stoichiometry of the CaMKK2/14-3-3ζ complex is 1:2 (dimer of 14-3-3 binds one molecule of CaMKK2).
- Enzyme activity of prepared proteins was verified by the kinase assay based on ADP-Glo^{TM} kit.

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SUPPLEMENTS

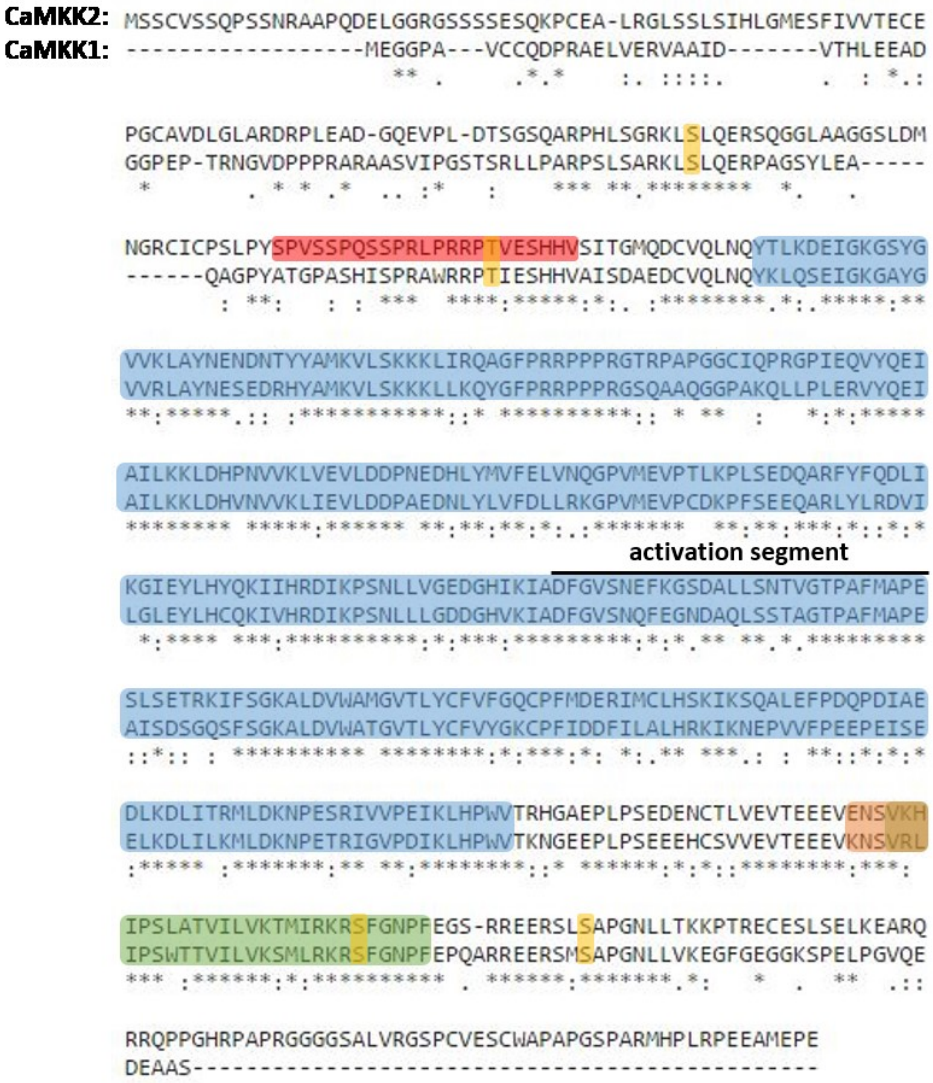

Figure S1 | Sequence alignment of CaMKK2 and CaMKK1
Both CaMKK isoforms possess similar sequences. The alignment shows that most of the protein is identical (* indicates fully conserved residue, : means conservation between residues with very similar properties, . indicates conservation between residues with less similar properties). It means that also domain organization is shared by both isoforms. Catalytic domain (CD) is highlighted in blue, autoinhibitory domain (AID) is highlighted in orange, calmodulin binding domain (CBD) is highlighted in green. Regulatory domain (RD) which occurs only in CaMKK2 is colored in red. Putative PKA phosphorylation sites in CaMKK2 (derived from phosphorylation sites in CaMKK1 which were proven) are colored in yellow. Activation segment between DFG and APE motifs is also labeled. Adapted from [ES1] and [ES7].