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Catalytic activity of mutants of yeast protein kinase CK2α

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Yeast CK2 is a highly conserved member of the protein kinase CGMC subfamily composed of two catalytic (α and α') and two regulatory (β and β') subunits. The amino-acid sequences of both catalytic subunits are only 60% homologous. Modelling of the tertiary structure of the CK2α displays additional α-helical structures not present in the CK2α' subunit, connecting the ATP-binding loop with the catalytic and activation loops. Deletion of this part causes drastic structural and enzymatic changes of the protein (CK2$\alpha^{\Delta 91-128}$) with characteristics similar to yeast CK2α' (low sensitivity to salt, heparin and spermine). Additionally, the deletion causes an over 5-fold decrease of the binding affinity for ATP and ATP-competitive inhibitors (TBBt and TBBz). The structural basis for TBBt and TBBz selectivity is provided by the hydrophobic pocket adjacent to the ATP/GTP binding site, which is smaller in CK2 than in the majority of other protein kinases. The importance of hydrophobic interactions in the binding of specific inhibitors was investigated here by mutational analysis of CK2α residues whose side chains contribute to reducing the size of the hydrophobic pocket. Site-directed mutagenesis was used to replace Val67 and Ile213 by Ala. The kinetic properties of the single mutants CK2$\alpha^{Val67Ala}$ and CK2$\alpha^{Ile213Ala}$, and the double mutant CK2$^{Val67Ala\ Ile213Ala}$ were studied with respect to ATP, and both inhibitors TBBt and TBBz. The K_m values for ATP did not change or were very close to those of the parental kinase. In contrast, all CK2α mutants analysed displayed higher K_i values towards the inhibitors (10 to 12-fold higher with TBBt and 3 to 6-fold with TBBt) comparing to recombinant wild-type CK2α.

Keywords: protein kinase CK2, mutagenesis, ATP binding, ATP-competitive inhibitors, heparin, spermine, protein phosphorylation, yeast, *Saccharomyces cerevisiae*

INTRODUCTION

Posttranslational phosphorylation of proteins is considered to be a major mechanism controlling cell functioning (Cohen, 2002; Manning *et al*., 2002). This covalent modification regulates a number of important cell processes including cell metabolism, response to external stimuli, cell cycle, transcription and translation, cell proliferation, differentiation and transformation (Krebs, 1994). Dysregulation of the activity of protein kinases is a major hallmark of human disease, particularly cancer (Blume-Jensen & Hunter, 2001). One of the kinases commonly disturbed in cancer is protein kinase CK2 (Faust *et al*., 1996).

CK2 is a highly conserved serine/threonine protein kinase known to occur as a tetrameric complex of catalytic α and/or α′, and a dimer of regulatory β subunits (reviewed in Pinna, 2002; Litchfield, 2003). CK2 is an enzyme with a broad substrate range able to phosphorylate over 300 proteins *in vitro* (Meggio & Pinna, 2003). Such pleiotropy may explain the unique property of CK2 of being constitutively active, both in the form of its possible heterotetrameric structures $\alpha\alpha'\beta_2$, $\alpha_2\beta_2$ and $\alpha'_2\beta_2$, and as monomeric catalytic subunits. Distinct isoenzymatic

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Abbreviations: CK2, casein kinase type 2; CK2α and CK2α′, α and α′ catalytic subunits of CK2; PMSF, phenylmethylsulfonyl fluoride; rP2B, recombinant yeast acidic ribosomal protein P2B; TBBt, 4,5,6,7-tetrabromo-1*H*-benzotriazole; TBBz, 4,5,6,7-tetrabromo-1*H*-benzimidazole; TCA, trichloroacetic acid.

forms of the catalytic subunit of CK2 have been identified in many organisms (Glover, 1998; Hilgard *et al*., 2002; Litchfield, 2003).

It has been shown that perturbations in the expression or activity of CK2 are associated with human diseases. Abnormally high levels of CK2 have been observed in human cancers of the breast (Romieu-Mourez *et al*., 2002), prostate (Yenice *et al*., 1994), kidney (Stalter *et al*., 1994), lung (Scaglioni *et al*., 2006), head, and neck (Faust *et al*., 1996). Elevated CK2 activity has been associated with phosphorylation resulting in promotion of tumorigenesis by the prevention of the degradation of proto-oncogenic products (c-Myc, c-Myb, c-Jun) as well as transcription activators (NF-κB, Max, β-catenin), promotion of the degradation of tumor suppressor proteins (PML) or their inactivation (PTEN) (reviewed in Duncan & Litchfield, 2008). Consequently, protein kinase CK2 has emerged as a potential therapeutic target, and attempt to inhibit this enzyme have been ongoing in preclinical trials. A number of quite specific and selective ATP-competitive inhibitors of CK2 have been developed, among them halogenated derivatives of 1*H*-benzimidazole and 1*H*-benzotriazole (Meggio *et al*., 1990; Szyszka *et al*., 1995; Sarno *et al*., 2005a).

Yeast CK2 is composed of two catalytic (α and α′) and two regulatory (β and β′) subunits giving three forms, namely $\alpha_2\beta\beta'$, $\alpha'_2\beta\beta'$ and $\alpha\alpha'_2\beta\beta'$ of the holoenzyme, where both regulatory β and β′ subunits are required to form an active tetramer (Domańska *et al*., 2005; Kubiński *et al*., 2007). Sequence analysis of CK2 subunits from yeast to human reveals strong conservation in the structure and function. The amino-acid sequences of the CK2 catalytic subunits of all eukaryotic species contain 12 typical regions that are common to this superfamily of protein kinases (Hanks & Quinn, 1991; Hanks & Hunter, 1995). Human CK2α and CK2α′ exhibit approx. 90% identity in their catalytic domains (reviewed in Litchfield & Lüscher, 1993). In contrast to this high similarity, the amino-acid sequences of yeast CK2α and CK2α′ display only 59% identity. Additionally, the yeast catalytic subunit CK2α contains a unique 38-amino acid-long loop between αC and β4 region (position 91–128) (Fig. 1).

The present paper is a continuation of our studies on the structure and function of yeast protein kinase CK2 catalytic subunits. Here we describe expression and purification of four mutants of yeast CK2α, and their kinetic properties with ATP and ATP-competitive inhibitors. We also compare the structure and biochemical properties of both yeast catalytic CK2α and CK2α′ subunits with those of the $CK2\alpha^{\Delta 91-128}$ mutant.

A number of specific and potent ATP-competitive inhibitors of protein kinase CK2 are available (Sarno *et al*., 2002a; 2005a; 2005b). The structural basis for their selectivity is provided by a hydrophobic pocket adjacent to the ATP/GTP binding site, which in CK2 is smaller than in the majority of other protein kinases.

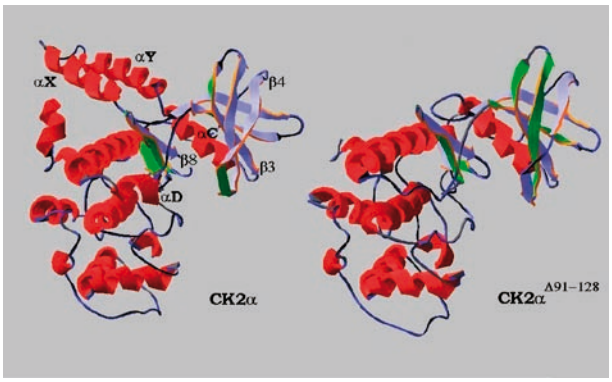

Figure 1. Model structures of yeast catalytic subunit CK2α and its mutant $CK2\alpha^{\Delta 91-128}$.
Models of catalytic subunits CK2α and $CK2\alpha^{\Delta 91-128}$ mutant were made with use of SWISS-MODEL Workspace for protein structure homology modeling (Kopp & Schwede, 2004; Arnold *et al*., 2006) and 1ds5D structure as template (Battistutta *et al*., 2000). Typical secondary structures of the N-terminus of CK2 catalytic subunit are marked.

We investigated the importance of hydrophobic interactions in the binding of specific inhibitors by mutational analysis of yeast CK2α residues whose side chains contribute to reducing the size of the mentioned hydrophobic pocket. Site-directed mutagenesis was used to replace Val67 and Ile213 (homologs of Val66 and Ile174 in human CK2) with Ala. The kinetic properties of the individual mutants $CK2\alpha^{Val67Ala}$ and $CK2\alpha^{Ile213Ala}$ and the double mutant $CK2^{Val67Ala\ Ile213Ala}$ were studied with respect to ATP and the competitive inhibitors TBBt and TBBz.

MATERIALS AND METHODS

Reagents, plasmids and strains. Restriction enzymes, T4 DNA ligase and PCR reagents were obtained from Fermentas. Yeast extract, trypton and agar were purchased from Biocorp (Poland). Qiagen supplied the QIAquick PCR Purification Kit. Oligonucleotides for DNA amplification and mutagenesis were from the Oligonucleotide Synthesis Laboratory, Iinstitute of Biochemistry and Biophysics PAS (Warszawa, Poland). pUC19 and pYES2/CT vectors were obtained from Fermentas and Invitrogen, respectively. Hartmann Analytic GmbH was the supplier of [γ-^{32}P]ATP. Ni-NTA agarose and other chemicals were purchased from Sigma. *Escherichia coli* strain XL1Blue, used for cloning and mutagenesis, and *Saccharomyces cerevisiae* INVSc1, used for recombinant protein expression, were obtained from Stratagene and Invitrogen, respectively.

Construction of CK2α mutants. The gene of yeast *CKA1* was cloned into pUC19 vector in the *Eco*RI and *Hin*dIII sites according to standard procedures to create pUC19::*cka1*. This was modified by site-directed PCR mutagenesis according to the procedure described before (Allemandou *et al*., 2003). Two simultaneous PCR reactions generated two non-overlapping products A and B, using pUC19::*cka*1 as a template. Fragment A was amplified using a mutagenic phosphorylated primer M (complementary to *cka1*, containing desired mutation) and selection primer S (complementary to pUC19, carrying a *Sal*I restriction site). Fragment B was amplified using primer M′ (complementary to *CKA1*) coupled to the second selection primer S′ (containing the same restriction site as primer S). The primers: M1/M1′, M2/M2′ and M0/M0′ were used to generate the following constructs: CK2α^{V67A}, CK2α^{I213A} and CK2α$^{\Delta 91-128}$, respectively. The double mutant CK2α$^{V67A/I213A}$ was made using CK2α^{V67A} mutant DNA as a template and incorporating the second mutation.

The PCR reaction mixture (100 μl) contained 100 ng of pUC19::*cka1*, 10 pmol of each primer, 10 nM of each dNTP and 2 U of *Pfu* polymerase in 1×*Pfu* buffer. The cycling parameters were: 95°C for 30 s, followed by 11 cycles at 95°C for 30 s, 50°C for 30 s, and 72°C for 4 min with a final elongation step of 72°C for 10 min. The PCR products A and B were column-copurified, digested for 2 h with *Sal*I, again copurified and eluted with 20 μl of H_2O. An aliquot of 10 μl was incubated with T4 DNA ligase overnight at room temp. Digestion of template DNA was performed for 2 h using *Dpn*I. Reaction mixtures (10 μl) were transformed into *E. coli* XL1 Blue following standard method.

The pUC19::*cka1* mutants were purified, digested with *Eco*RI and *Hin*dIII and subcloned into *Eco*RI–*Hin*dIII-digested pYES2/CT. All constructs were verified by sequencing and introduced into *S. cerevisiae* INVSc1 using lithium acetate method.

Expression and purification of proteins. CK2α: The CK2α gene *CKA1* was amplified by PCR from *S. cerevisiae* W303 genomic DNA with primers: 5′ CGAAGCTTCGAACTATGAAATGCAGGGT 3′ and 5′ CGCGAATTCCGATTTTTCAATTTGTTCCCT 3′. The *Hin*dIII–*Eco*RI restriction fragment of CK2α was cloned into the respective restriction sites of the pYES2/CT vector (Invitrogen).

Recombinant CK2α was expressed from plasmid pYES2/CT::*cka1::v5::6his* as described earlier (Kubiński *et al*., 2007). Transformation of yeast was carried out using the lithium acetate method. Recombinant his$_{6v5}$-tagged CK2α was generated in yeast strain Y01837 (Δ*CKA2*), obtained from the Euroscarf Collection Centre. The CK2α expression strain was grown overnight at 30°C in a selective medium without uracil to OD_{600nm} of 2–2.5. Yeast cells were harvested by centrifugation, resuspended in ice-cold buffer A (50 mM Tris/HCl, pH 8.0, 0.5 mM EDTA, 6 mM β-mercaptoethanol, 0.5 mM PMSF) and disintegrated using glass beads (ϕ 0.5 mm). Cell extracts were ultracentrifuged (100 000×*g*, 1.5 h, +4°C) to obtain ribosome-free fraction (S-100). Recombinant CK2α was purified on Ni-NTA agarose (Sigma) according to the standard protocol with slight modifications. The S-100 fraction (20 mg of protein) containing recombinant CK2α was loaded twice on a Ni-NTA agarose column (0.5 ml) equilibrated with buffer A. The column was washed with buffer A containing 10 mM and 20 mM imidazole. Active fractions of protein kinase were eluted from the column with buffer A containing 250 mM imidazole and dialysed prior to use in buffer A.

CK2α′: Recombinant CK2α′ was expressed from plasmid YEplac181::*cka2*::*6his*::cMyc (a generous gift from Dr. H. Riezman, University of Geneva (Schaerer-Brodbeck & Riezman, 2003)). Transformation of yeast was carried out using the lithium acetate method. The recombinant his$_6$-cMyc-tagged CK2α′ was expressed in yeast strain Y01837.

CK2α mutants: CK2α^{V67A}, CK2α^{I213A}, CK2α$^{V67A/I213A}$ and CK2α$^{\Delta 91-128}$ were expressed in *S. cerevisiae* INVSc1 as N-terminal hexahistidine and V5 epitope fusion proteins. Cells were grown overnight with intensive shaking at 30°C to initial OD_{600nm} of 0.5 in one-liter uracil-free selective medium containing 2% galactose. Harvested cells were disintegrated in buffer B (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5 mM PMSF, pH 8) by vortexing with glass beads (ϕ 0.5 mm). Lysates were cleared and supernatants were agitated with Ni-NTA agarose for 1 h at 4°C. The wash and elution steps of purification were carried out on a column according to manufacturer's instructions.

Elf1 protein: was overexpressed and purified to homogeneity according to a procedure described before (Kubiński *et al*., 2006).

rP2B: recombinant acidic ribosomal proteins P2B were purified according to a previously described method (Tchórzewski *et al*., 1999).

Enzyme kinetics. The standard protein kinase assay mixture contained in a final volume of 50 μl: 20 mM Tris/HCl (pH 7.5), 15 mM $MgCl_2$, 6 mM β-mercaptoethanol, 10 μM [γ-^{32}P]ATP (spec. activity 500–1000 c.p.m./pmol), 2 μg of a protein substrate (Elf1 or P2B proteins) and 5–20 pmol of wild type or mutant CK2α subunits. Reactions were carried out for 5 min at 30°C and terminated by addition of 100 μl of 10% TCA. Samples were filtrated through GF/C filter (Whatman), washed with 20 ml of 10% TCA, dried, and the radioactivity was measured in a scintillation counter. Enzyme preparations used in the assays were purified to almost homogeneity. The

duration of incubation was chosen to represent the linear part of the kinetic curve.

Michaelis constants K_m were determined for CK2 nucleotide substrate (ATP) and Elf1 or P2B protein with the use of the Lineweaver-Burk plot method. The same method was used for determination of V_{max} values for wild type and mutated CK2 forms. K_m for ATP was determined by varying its concentration from 0 to 100 µM.

Inhibition constants K_i were determined for inhibitors (TBBt, TBBz) *vs.* CK2 forms and Elf1 protein with the use of Dixon plots. K_i values were determined at 0, 4.8 and 9.6 µM concentration of TBBt and TBBz and varying ATP concentration from 0 to 100 µM. Apparent K_m and K_i values were calculated using the computer program GraphPad Prism4. The kinetic data represents average results obtained from 3–5 individual experiments.

CK2α modelling. Modeling of CK2 catalytic subunits from *S. cerevisiae* was done using SWISS-MODEL Workspace and a web-based environment for protein structure modelling (Arnold *et al.*, 2006; Kopp & Schwede, 2004).

RESULTS

Comparison and modelling of yeast CK2 catalytic subunits

As can be seen in Table 1 and 3D models presented in Figs. 1 and 2, the amino-acid sequence and structure of yeast protein kinase CK2α differ from those at CK2α′ and other eucaryotic CK2α subunits by containing a 38-amino acid-long loop consisting of two α-helical structures (marked αX, and αY in Table 1 and in Fig. 1) between αC and β4. This part of the enzyme is situated in direct neighbourhood of the β3 and β8 as well as αC structures engaged in ATP/GTP and protein substrate binding, respectively (Niefind *et al.*, 2001; Sarno *et al.*, 1997). Using the SWISS-MODEL Workspace for protein structure homology modelling (Arnold *et al.*, 2006; Kopp & Schwede, 2004) and 1ds5D structure as a template (Battistutta *et al.*, 2000) we compared the structural features of both catalytic subunits CK2α and CK2α′ with that of CK2α$^{Δ91–128}$ (Fig. 2). The results obtained from this modeling show that after removing both α-helical motifs (Fig. 2B) the CK2α subunit assumes a structure which is more similar to that of CK2α′ than to the original structure of intact CK2α (Fig. 2C and 2A, respectively). These data of modelling raised a question: how does the deletion of the 38-amino acid loop influence the properties of the CK2α subunit. To address it, site-directed PCR mutagenesis was used to delete 114 nucleotides coding

Table 1. Alignment of amino-acid sequences of N-terminal parts of CK2α subunits*

				N-terminal segment			ATP-binding loop		↓
	1	11	21	31	41	51	61	71	81
H. sapiens CK2α	----------	---------M	SGPVPSRARV	YTDVNTHRPR	EYWDYESHV-	VEWG-NQDDY	QLVRKLGRGK	YSEVFEAINI	TNNEKVVVKI
Z. mays CK2α	----------	----------	----MSKARV	YADVNVLRPK	EYWDYEAL-T	VQWG-EQDDY	EVVRKVGRGK	YSEVFEGINV	NNNEKCIIKI
S. cerev. CK2α′	---------M	PLPPSTLNQK	SNRVYSVARV	YKNACEERPQ	EYWDYEQGVT	IDWG-KISNY	EIINKIGRGK	YSEVFSGRCI	VNNQKCVIKV
S. cerev. CK2α	----------	---------M	KCRVWSEARV	YTNINKQRTE	EYWDYENTV-	IDWSTNTKDY	EIENKVGRGK	YSEVFQGVKL	DSKVKI**V**IKM
secondary structure				<-αA->			<----β1---->	<---β2--->	<---β3-
	basic cluster								
	91	101	111	121	131	141	151	161	171
H. sapiens CK2α	LKPVKKKKIK	REIKILENLR	GG--------	----------	----------	----------	PNIITLADIV	KDPVSRTPAL	VFEHVNNTDF
Z. mays CK2α	LKPVKKKKIK	REIKILQNLC	GG--------	----------	----------	----------	PNIVKLLDIV	RDQHSKTPSL	IFEYVNNTDF
S. cerev. CK2α′	LKPVKMKKIY	RELKILTNLT	GG--------	----------	↓----------	----------	PNVVGLYDIV	QDADSKIPAL	IFEEIKNVDF
S. cerev. CK2α	LKPVKKKKIK	REIKILTDLS	NE**KVPPTTLP**	**FQKDQYYTNQ**	**KEDVLKFIRP**	**YIFDQPHNGH**	ANIIHLFDII	KDPISKTPAL	VFEYVDNVDF
secondary structure	-> <------αC----->		<-----αX----->		<-----αY----->		<---β4-->	<--β5-->	<-
				catalytic loop		activation loop			
	181	191	201	211	221	↓ 231	241	251	261
H. sapiens CK2α	KQLYQTLTDY	DIRFYMYEIL	KALDYCHSMG	IMHRDVKPHN	VMIDHEHRKL	RLIDWGLAEF	YHPGQEYNVR	VASRYFKGPE	LLVDYQMYDY
Z. mays CK2α	KVLYPTLTDY	DIRYYIYELL	KALDYCHSQG	IMHRDVKPHN	VMIDHELRKL	RLIDWGLAEF	YHPGKEYNVR	VASRYFKGPE	LLVDLQDYDY
S. cerev. CK2α′	RTLYPTFKLP	DIQYYFTQLL	IALDYCHSMG	IMHRDVKPQN	VMIDPTERKL	RLIDWGLAEF	YHPGVDYNVR	VASRYHKGPE	LLVNLNQYDY
S. cerev. CK2α	RILYPKLTDL	EIRFYMFELL	KALDYCHSMG	IMHRDVKPHN	VMIDHKNKKL	RL**I**DWGLAEF	YHVNMEYNVR	VASRFFKGPE	LLVDYRMYDY
secondary structure	-αD->	<---------αE---------	-><β6>	<--β7-->	<--β8->	<β9>		<αF>	

*Shown are *Homo sapiens* CK2α (P68400), *Zea mays* CK2α (P28523), *Saccharomyces cerevisiae* CK2α′ (P19454) and CK2α (P15790). Residues mutated or removed in this work are marked by black arrows and in bold lettering.

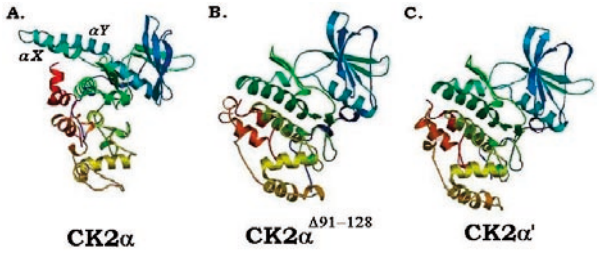

Figure 2. Comparison of model structures of yeast catalytic subunits CK2α and CK2α′ with CK2α$^{\Delta 91–128}$.

for the unique loop from the yeast CK2α subunit to obtain the M0 (CK2α$^{\Delta 91–128}$) mutant. The biochemical properties of CK2α′, CK2α and obtained M0 mutant were compared.

Biochemical properties of CK2 catalytic subunits and CK2α$^{\Delta 91–128}$

Wild type CK2α and its deletion mutant M0 were overexpressed in yeast, purified (Fig. 3) and biochemically characterized. Both yeast CK2 catalytic subunits together with the CK2α mutant M0 (Table 2) were tested for their kinetics using ATP as a substrate. The analyses were performed using either recombinant ribosomal acidic proteins rP2B or elongation factor Elf1 as substrates.

The K_m values for CK2α, M0 and CK2α′ for ATP were 7.2, 44.6 and 3.6 μM, respectively, using the Elf1 factor as a substrate (Table 3). The k_{cat}/K_m ratio of wild-type catalytic subunits CK2α and CK2α′ was almost identical, 0.55 and 0.58 $s^{-1} \times \mu M^{-1}$, respectively, while for the M0 mutant it was 0.05 $s^{-1} \times \mu M^{-1}$. This means that the deletion of the 38 amino acids from CK2α decreases its catalytic efficiency over ten fold. A comparable effect of losing the ATP-binding capacity and enzyme efficiency was observed when rP2B proteins were used as a substrate.

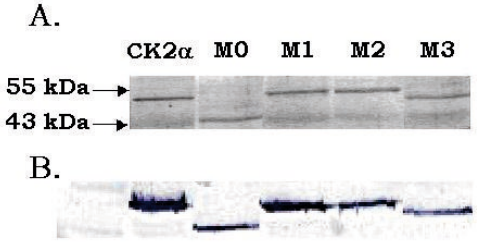

Figure 3. SDS/PAGE and Western blot analysis of CK2α and its mutants M0, M1, M2 and M3.
Recombinant CK2α and its mutants were purified on Ni-NTA agarose column, electrophoresed on 10% SDS/PAGE and Coomassie stained (A) or analysed by Western bloting (B). CK2 subunits were detected with anti-V5 monoclonal antibodies. Arrows on the right indicate positions of protein markers 55 and 43 kDa.

Table 2. Yeast CK2α mutants used in this work

Mutant	Plasmid	Protein
M0	pYES2/CT::*cka1*$^{A271–384}$	CK2α$^{\Delta 91–128}$
M1	pYES2/CT::*cka1*^{T200C}	CK2α^{V67A}
M2	pYES2/CT::*cka1*^{A637GT638C}	CK2α^{I213A}
M3	pYES2/CT::*cka1*^{T200CA637GT638C}	CK2α^{V67AI213A}

Influence of NaCl, spermine and heparin on CK2 catalytic subunits and CK2αΔ$^{91–128}$

Salt concentration, polyamines and heparin are known as natural modulators affecting activity of many enzymes. To compare the optimum of monovalent salt concentrations for wild-type subunits (α and α′) and the M0 mutant, the protein kinase activity was determined at various NaCl concentrations (0.02–250 mM). Both wild-type subunits showed different sensitivity to salt (Fig. 4A). Activity of CK2α′ was stable at concentrations between 0 and 150 mM and decreased to 70% at 250 mM salt. CK2α was much more sensitive to salt. The activity of this subunit at low (50 mM) NaCl concentration was 80%. However, in the presence of 100 mM NaCl a second drastic over 60% drop in activity was observed. In the case of the CK2α$^{\Delta 91–128}$ (M0) mutant the decrease of activity at concentration of 50 mM was 10%, while at 100 mM NaCl concentration it was only 30%. Not mutated CK2α′ showed at this salt concentration almost 100% of its activity, while CK2α only 37%. Obviously, CK2α containing the two additional α-helical fragments is much more salt-sensitive than CK2α′. This special structural feature may display a "salt-sensitivity activity" (Figs. 1 and 2). At this point it should be noted that CK2α activity can be stabilized by addition of the regulatory subunits and the $\alpha_2\beta\beta'$ holoenzyme has a much stable structure. At 200 mM NaCl its activity drops only by 10–15% (not shown).

Polyamines, like spermine, spermidine, and polylysine, have been shown to stimulate CK2 activity and this unique effect is often used as diagnostic of this protein kinase (Tuazon & Traugh, 1991; Riera *et al*., 2003; Litchfield, 2003).

Treatment of CK2α or CK2α′ with increasing concentrations of spermine (0–5 mM) (Fig. 4B) led to an almost 3-fold increase of CK2α′ activity at 0.2 mM spermine. Higher concentrations of spermine had a slightly lower stimulatory effect, about 2.5-fold at 5 mM. This is in contrast to the CK2α subunit, on whose activity polyamines had no effect. But here also, as in the case of NaCl, spermine recovered its stimulatory influence when instead of CK2α the M0 mutant was used in the assay. In this case 0.5 mM spermine increased the activity of the enzyme about 75%. At higher concentrations of polyamine, no fur-

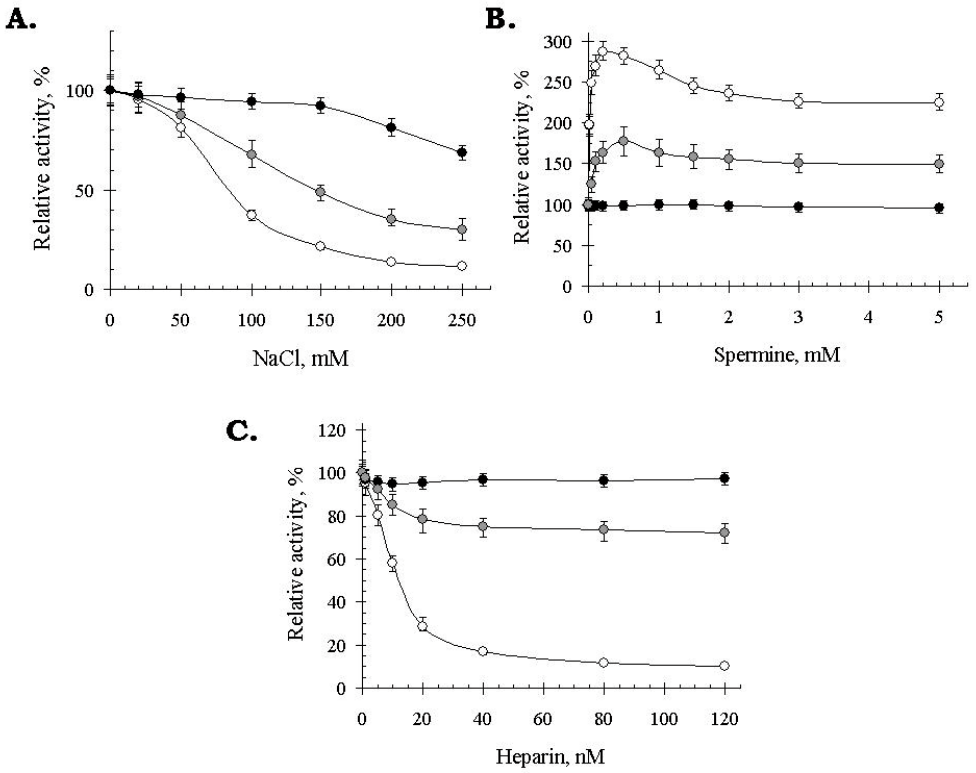

Figure 4. Biochemical characterization of yeast catalytic subunits CK2α′, CK2α and mutant CK2α-M0.
Activity measurements of CK2α′ (black circles), CK2α (white circles) and mutant CK2α-M0 (grey circles) with increasing concentrations of NaCl (A), spermine (B) and heparin (C). Each point shows average of three independent experiments, with standard deviations.

ther increase in M0 activity was observed, similarly as in the case of CK2α′.

Heparin is a classical inhibitor of CK2 (Tuazon & Traugh, 1991; Litchfield, 2003). This highly-sulfated glycosaminoglycan was used to study its influence on yeast CK2α, its mutated form M0, and CK2α′ (Fig. 4C). A 50% inhibition of CK2α activity was seen with 10 nM heparin whereas CK2α′ activity was not sensitive to this polyanionic compound. Again, the mutated M0 form of CK2α has lost its sensitivity to this inhibitor in which it resembled CK2α′ more than its parallel CK2α: the highest inhibition obtained was in the range of 25% when heparin was present at 120 nM. The observed effect was probably due to the structural changes shown in Fig. 2 caused by deletion of the α-helical structures marked as αX and αY.

Influence of mutations in the CK2α hydrophobic pocket on the binding of ATP and ATP-competitive inhibitors

We investigated the importance of hydrophobic interactions in the binding of specific inhibitors by mutating CK2α residues whose side chains contribute to reducing the size of the hydrophobic pocket. Site-directed mutagenesis was used to replace Val67 and Ile213 (homologues of Val66 and Ile174 in human CK2α) with Ala. The mutated Val67 residue is located in the middle of β3 strand (belonging

Table 3. Kinetic parameters of wild and mutated forms of yeast CK2α and CK2α′

Parameter	CK2α	M0	M1	M2	M3	CK2α′
$K_{m\ ATP}$ (μM)	7.2±0.4	44.3±2.1	10.0±0.45	19.0±0.8	19.0±0.7	3.6±0.18
$K_{i\ TBBt}$ (μM)	1.2±0.04	4.9±0.15	14.1±0.48	12.4±0.37	14.3±0.3	0.1±0.03
$K_{i\ TBBz}$ (μM)	2.0±0.07	12.1±0.38	6.5±0.18	9.5±0.42	11.6±0.33	19±0.76
k_{cat} (s^{-1})	4.0±0.15	2.2±0.07	1.5±0.05	0.35±0.12	5.8±0.2	2.1±0.06
k_{cat}/K_m ($s^{-1} \times \mu M^{-1}$)	0.55±0.04	0.05±0.007	0.15±0.02	0.02±0.003	0.3±0.04	0.58±0.06

K_m and K_i values were determined using the computer program GraphPad Prism4. The kinetic data are average results obtained from 3–5 individual experiments, ±S.D.

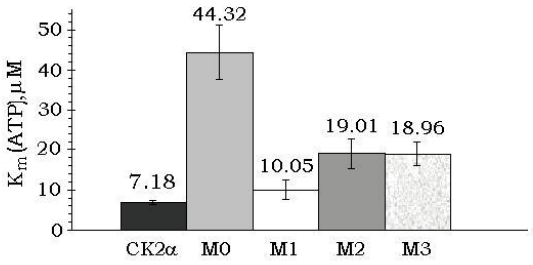

Figure 5. Comparison of K_m values for CK2α and its mutants CK2α-M0, CK2α-M1, CK2α-M2, and CK2α-M3.
Each point shows the average of three independent experiments, with standard deviations.

to the ATP-binding loop) and on the opposite side of the hydrophobic pocket engaged in ATP binding Ile213 located on the C-terminal end of the β8 strand (Table 1 and Fig. 1). All kinetic properties of the single mutants CK2α^{V67A} (M1), CK2α^{I213A} (M2) and the double mutant CK2α^{V67AI213A} (M3) were studied with respect to ATP, the competitive inhibitors TBBt and TBBz and Elf1 protein as a substrate (Table 2).

The replacement of the two hydrophobic residues by alanines still produced a catalytically active kinases. The K_m of the analysed mutants for ATP did not change or was very close to that of the parental kinase; it was 7.2, 10, 19 and 19 μM, respectively for CK2α, M1, M2 and M3 for ATP (Table 3, Fig. 5). The phosphorylation efficiency (k_{cat}) of the double substituted CK2α mutant M3 was the highest, little surpassing that of the parental kinase, while both single mutants showed decreased k_{cat} values. Especially the mutation of Ile213 decreased the enzymes efficiency over 10-fold when compared with wild-type CK2α. A similar influence of the mutations was found for k_{cat}/K_m. The highest, almost 30-fold, drop of k_{cat}/K_m (0.02 $s^{-1}\times\mu M^{-1}$) was observed for the M2 mutant with Ile213 replaced by Ala213. Surprisingly, the effect of this mutation was abolished when the second mutation, Val67Ala, was introduced, to give a value of 0.3 $s^{-1}\times\mu M^{-1}$ for the double mutant M3 (Table 3).

As previously shown, halogenated benzimidazoles and benzotriazoles, compounds competitive with respect to ATP/GTP binding, are specific and effective inhibitors of protein kinase CK2 (Szyszka *et al*., 1995; Zień *et al*., 2003a; 2003b). The CK2α mutants analysed displayed a markedly lower affinity for the ATP-competitive inhibitors TBBz and TBBt, comparing to that of wild-type CK2α (Fig. 6, Table 3). The K_i of the analysed mutants M1, M2 and M3 for TBBt increased about 10-fold when compared with the K_i of wild-type CK2α. The K_i values were 2.0, 6.5, 9.5 and 11.6 μM, respectively, for CK2α, M1, M2 and M3. This weaker influence of the investigated CK2α mutations on TBBz binding may be due to the fact that the polarization energy of an imidazole ring is lower than that of a triazole ring in which a carbon atom is replaced by a more electronegative N2 atom.

As can be seen in Table 3 and Figs. 5 and 6, deletion of the 38-amino acid-long loop from CK2α decreases the affinity of the mutant for ATP as well as TBBz 6-fold, but the binding of TBBt decreases only 4-fold.

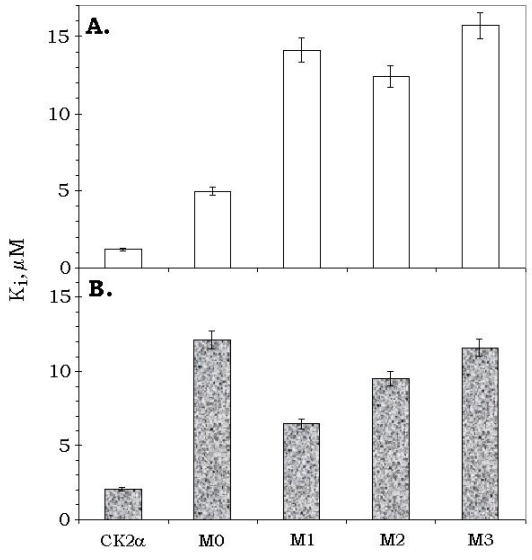

Figure 6. Comparison of K_i values for CK2α and its mutants with TBBt (A) and TBBz (B).
Each point shows the average of three independent experiments with standard deviations.

DISCUSSION

Protein kinase CK2, a member of the protein kinase CMGC subfamily, is ubiquitously distributed in eukaryotic organisms where it exists as a tetrameric complex consisting of two catalytic α and two regulatory β subunits (Pinna, 1990; Issinger, 1993; Litchfield, 2003; Filhol *et al*., 2004). In *Saccharomyces cerevisiae* two catalytic (α and α′) and two regulatory (β and β′) CK2 subunits are present (Glover, 1998; Kubiński *et al*., 2007), and they can associate to form distinct tetramers (αα′ββ′, α_2ββ′ and α′$_2$ββ′) *in vivo* (Domańska *et al*., 2005).

The two catalytic subunits of CK2 are paralogous proteins and their presence in the cell is typically combined with a functional specialization. There is evidence showing that CK2α and CK2α′ have different functions in the cell (Faust & Montenarh, 2000). The C-terminal domain of CK2α is phosphorylated by p34^{cdc2} during cell cycle progression while CK2α′ is not phosphorylated (Messenger *et al*., 2002). Further support for the existence

of functional differences between CK2α and CK2α′ comes from the identification of isoform-specific interacting proteins. Cellular proteins such as Hsp90, PP2A, CKIP-1 and Pin-1 can interact with CK2α but not with CK2α′ (Litchfield, 2003).

CK2 is a highly conserved enzyme implicated in various cellular processes like cell proliferation, differentiation, and apoptosis and has been shown to be essential for yeast survival (Glover, 1998; Wang *et al*., 2001). Genetic studies in *S. cerevisiae* have indicated that knockout of the *CKA1* (CK2α) or *CKA2* (CK2α′) genes result in lethality and provided the first evidence to support the opinion that both subunits have independent cellular functions (Olsten & Litchfield, 2004). Yeast with temperature-sensitive alleles of *CKA1* or *CKA2* exhibit distinct phenotypes. The loss of CK2α results in cell polarity loss while of CK2α′ resulted in cell cycle arrest (Hanna *et al*., 1995; Rethinaswamy *et al*., 1998). Nevetheless most of the literature involving CK2 holoenzyme has not made any distinction between the different isoenzymes of CK2.

In this report we showed that differences in the N-terminal parts (Table 1) of the yeast catalytic subunits influence on the tertiary structure of the enzyme (Figs. 1 and 2) and the catalytic properties. A number of compounds have been proposed to regulate CK2 (Pinna, 1990; Tuazon & Traugh, 1991; Litchfield, 2003). It has long been known from *in vitro* studies that CK2 is inhibited by negatively charged compounds such as heparin, and is activated by positively charged compounds such as polyamines or basic polypeptides. At concentrations below 1 μg/ml heparin has little effect on protein kinases other than CK2 (Tuazon & Traugh, 1991). CK2 kinase is also rather not sensitive to salt at physiological or lower concentration.

We have investigated the behaviour of the yeast CK2 catalytic subunits and the CK2α M0 mutant in the presence of monovalent salt, spermine, and heparin. The effect of investigated compounds towards the three forms of CK2 catalytic subunits was completely different. In the case of CK2α and NaCl and heparin (Fig. 4A and 4C, respectively), we observed strong inhibition of the enzyme activity. The inhibitory effect (IC_{50}) was determined to be about 80 mM for NaCl and 10 nM for heparin. In contrast, the activity of CK2α′ subunit was inhibited by very high salt concentrations (>300 mM) only and was not sensitive to nanomolar concentrations of heparin. More telling are the results obtained with the CK2α M0 mutant, which shows that deletion of the 38-amino acid-long loop from CK2α reduces the sensitivity of this subunit to salt, spermine, and heparin, and makes its properties more similar to those of CK2α′ rather than of CK2α. This indicates that the structure of the CK2α subunit may differ in certain respects from that of CK2α′ and the known structure of the human enzyme (Niefind *et al*., 2001). In fact, modeling of both yeast catalytic subunits and the CK2α M0 mutant (Figs. 1 and 2) shows that the structural features of CK2α and CK2α′ are different (Fig. 2). In addition, the results obtained from modeling show that after removing two α-helical fragments (marked as αX and αY) (Fig. 2B) CK2α is structurally more similar to CK2α′ than to CK2α (Figs. 2C and 2A, respectively). It has been shown that the basic cluster of amino acids ($K^{75}KKKIKR^{81}$ in yeast CK2α) within the αC region of CK2α (Table 1, Figs. 1 and 2) serves as a binding site for the acidic determinants of CK2 substrates and for the strong inhibitor heparin (Vaglio *et al*., 1996). The observed lack of inhibition of yeast CK2α′ activity by heparin is probably due to different tertiary conformation of the enzyme caused by the absence of the α-helical structures αX and αY. They form an additional loop (present only in yeast CK2α) connecting the ATP-binding loop and the basic cluster KKKKIKR with the catalytic and activation loops (Table 1), both crucially implicated in catalysis (Sarno *et al*., 2002b; Kannan & Neuwald, 2004).

In our previous reports we have shown that the isoenzymatic forms of yeast CK2 holoenzyme exhibit substantial differences in protein substrate and phosphate donor specificity as well as sensitivity to the ATP-competitive inhibitors TBBt and TBBz (Zień *et al*., 2003a; 2003b; Domańska *et al*., 2005). The structural basis for the selectivity of ATP-binding-site-directed inhibitors is provided by a hydrophobic pocket adjacent to the ATP/GTP-binding site. Crystallographic studies together with mutational analyses done with maize and human CK2 have shown that the selectivity of these inhibitors depends on the size and shape of the hydrophobic ATP-binding pocket. In CK2 this space is smaller than in the majority of other protein kinases (Battistutta *et al*., 2001; Niefind *et al*., 2001; Sarno *et al*., 2005a; 2005b). Mutational analyses of human CK2 have shown that two hydrophobic residues, Val66 and Ile174, are critical for the interaction with TBBz (Sarno *et al*., 2002a).

In this report we investigated the importance of hydrophobic interactions in the binding of TBBz and TBBt by mutating the yeast CK2α residues whose side chains project into the hydrophobic pocket. Site-directed mutagenesis was used to replace Val67 and Ile213 (homologous to Val66 and Ile174 in human CK2α) with Ala. The kinetic properties of the mutant M0 ($CK2\alpha^{\Delta 91-128}$), single mutants M1 ($CK2\alpha^{Val67Ala}$) and M2 ($CK2\alpha^{Ile213Ala}$), and the double mutant M3 ($CK2\alpha^{Val67Ala\ Ile213Ala}$) (listed in Table 2) show that their reduced affinity for the phosphate donor ATP as well as lower sensitivity to the inhibitors TBBt and TBBz (Table 3). The highest influence on ATP binding was seen for the M0 mu-

tant where K_m increased 6-fold, while for the other mutants it had a similar value (M1) or increased 2-fold (M2 and M3) (Table 3, Fig. 5). Consequently, the yeast CK2α mutants in which either Val67 or Ile213 or both had been replaced by alanine displayed reduced affinity for the inhibitors with the increments of K_i ranging between 10- and 12-fold for TBBt, and 3- and 6-fold for TBBz (Table 3, Fig. 6). This different effect towards the two inhibitors probably reflects the different modes of their binding connected with the presence of an additional nitrogen atom in the benzotriazole structure. A similar decrease of affinity for emodin, TBBz and IQA was observed with mutants of human CK2α (Sarno *et al*., 2002a; 2005a; 2005b). All the mutations studied (with the exception of the M3 mutant) of yeast CK2α decreased k_{cat} and k_{cat}/K_m. The highest, almost 30-fold decrease of k_{cat}/K_m ($0.02\ s^{-1} \times \mu M^{-1}$) was observed with M2 mutant in which Ile213 was replaced by Ala213. Surprisingly, substantially different effects were observed for the double M3 mutant, where k_{cat} increased by about 50% while k_{cat}/K_m decreased by only 45%.

From the perspective of the present work, it will be interesting to study in detail the differences in the molecular structure of all forms of CK2 and the mechanisms of their regulation in the cell as well as their cellular targets. The availability of effective and specific inhibitors usable under *in vivo* conditions, on the one hand, and of active CK2 mutants insensitive to them, on the other hand, will help us in understanding the cellular functions of CK2. This may also provide interpretation for implication of protein kinase CK2 in cancer and viral infections.

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