FOR THE RECORD

The interaction of CK2 α and CK2 β , the subunits of protein kinase CK2, requires CK2 β in a preformed conformation and is enthalpically driven

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Abstract

The protein kinase CK2 (former name: “casein kinase 2”) predominantly occurs as a heterotetrameric holoenzyme composed of two catalytic chains (CK2 α) and two noncatalytic subunits (CK2 β). The CK2 β subunits form a stable dimer to which the CK2 α monomers are attached independently. In contrast to the cyclins in the case of the cyclin-dependent kinases CK2 β is no on-switch of CK2 α ; rather the formation of the CK2 holoenzyme is accompanied with an overall change of the enzyme’s profile including a modulation of the substrate specificity, an increase of the thermostability, and an allocation of docking sites for membranes and other proteins. In this study we used C-terminal deletion variants of human CK2 α and CK2 β that were enzymologically fully competent and in particular able to form a heterotetrameric holoenzyme. With differential scanning calorimetry (DSC) we confirmed the strong thermostabilization effect of CK2 α on CK2 β with an upshift of the CK2 α melting temperature of more than 9°. Using isothermal titration calorimetry (ITC) we measured a dissociation constant of 12.6 nM. This high affinity between CK2 α and CK2 β is mainly caused by enthalpic rather than entropic contributions. Finally, we determined a crystal structure of the CK2 β construct to 2.8 Å resolution and revealed by structural comparisons with the CK2 holoenzyme structure that the CK2 β conformation is largely conserved upon association with CK2 α , whereas the latter undergoes significant structural adaptations of its backbone.

Keywords: protein kinase CK2; casein kinase 2; catalytic subunit CK2 α ; regulatory subunit CK2 β ; X-ray crystallography; isothermal titration and differential scanning calorimetry; thermostabilization of CK2 α by CK2 β ; binding affinity between CK2 α and CK2 β

Protein–protein interactions are fundamental for protein function. They increasingly attract attention as potential targets for small molecules in chemical biology and pharmaceutical chemistry (Wells and McClendon 2007), as subjects for bioinformatic and statistical analyses

(Jones and Thornton 1996), or as a challenge for theoretical prediction (Lensink et al. 2007).

The Ser/Thr protein kinase CK2 (former name: “casein kinase 2”) provides a prime example of a physiologically relevant protein–protein interaction. In vertebrates, CK2 predominantly occurs as a heterotetrameric holoenzyme, i.e., as a complex of two catalytic subunits (CK2 α) attached to a stable central dimer of two noncatalytic subunits (CK2 β) (Niefind et al. 2001). CK2 α is a close relative of the cyclin-dependent kinases and the MAP kinases. It is catalytically active already as a monomer,

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but the association with CK2 β significantly changes its enzymological properties, e.g., its substrate specificity (Pinna 2003), its ability to dock to membranes (Sarrouilhe et al. 1998), or even to penetrate them (Rodriguez et al. 2008). Moreover, CK2 α -independent roles for CK2 β have been suggested (Bibby and Litchfield 2005; Bolanos-Garcia et al. 2006). However, the recruitment of CK2 α for the CK2 holoenzyme complex is so far the only validated function. Therefore, the lack of the modulatory impact of CK2 β on CK2 α may be the reason for the nonviability of CK2 β knockout mice (Buchou et al. 2003). To test this hypothesis and to reveal more details of the physiological role of the CK2 α /CK2 β interaction, small molecule antagonists of CK2 β are desirable.

First attempts to develop such substances have been published recently (Laudet et al. 2007; Raaf et al. 2008). These efforts can be supported by structural and biophysical insights about the CK2 α /CK2 β interaction. Therefore, we present here the first unbound three-dimensional (3D) structure of a CK2 β construct that is fully capable of CK2 α recruitment and we quantify its affinity to CK2 α thermodynamically. This structure supplements existing structures of unbound human CK2 α (Ermakova et al. 2003; Niefind et al. 2007; Raaf et al. 2008) and of the “bound case,” i.e., the human CK2 holoenzyme (Niefind et al. 2001); thus, it allows a comprehensive view on the conformational changes that occur at the CK2 α /CK2 β interface upon association.

Results and Discussion

Thermodynamic background

For our analysis we used *hsCK2 β ^{1–193}*, a C-terminally truncated mutant of human CK2 β , in order to avoid aggregation problems as reported for the 215-amino-acids-long wild-type protein (Chantalat et al. 1999b). *HsCK2 β ^{1–193}* was among a set of 21 CK2 β mutants characterized by Boldyreff et al. (1993). This study demonstrated that the ability of CK2 β to bind CK2 α critically depends on the C-terminal segment: While *hsCK2 β ^{1–180}* retained this competence only to a significantly reduced amount, *hsCK2 β ^{1–193}* was the shortest construct to be similarly functional as the wild type. In other words, the CK2 β segment from Asn181 to His193 is a key region for the formation of the CK2 holoenzyme.

We confirmed these results as far as they refer to *hsCK2 β ^{1–193}* with micro-calorimetric methods. First, we used differential scanning calorimetry (DSC) to determine the melting temperatures of *hsCK2 β ^{1–193}*, of *hsCK2 α ^{1–335}*—a fully active C-terminal deletion mutant of human CK2 α (Ermakova et al. 2003)—and of a tetrameric complex of both. The resulting temperature scans (Fig. 1A) demonstrate that the melting point of *hsCK2 β ^{1–193}* changes only marginally

by complex formation, while that of *hsCK2 α ^{1–335}* increases from 45.6°C to 54.7°C. This strong thermostabilizing impact of *hsCK2 β ^{1–193}* on *hsCK2 α ^{1–335}* is fully consistent with former results obtained with the wild-type proteins using circular dichroism spectroscopy (Issinger et al. 1992), and it demonstrates a high affinity between both proteins.

To quantify this affinity we used isothermal titration calorimetry (ITC) (Fig. 1B). Assuming that a *hsCK2 β ^{1–193}* dimer possesses two equivalent binding sites for *hsCK2 α ^{1–335}*, we determined a dissociation constant of 12.6 nM at 35°C. This value falls in a range typically reported for transient heterocomplexes (Nooren and Thornton 2003). It is in the same order of magnitude as the K_d value of 5.4 nM, measured for the interaction of full-length CK2 β with a CK2 α /glutathion-S-transferase fusion protein using surface plasmon resonance (Martel et al. 2002). This equivalence demonstrates that the final 22 amino acids of human CK2 β contribute only marginally to the affinity to CK2 α .

Remarkably, the association of *hsCK2 α ^{1–335}* and *hsCK2 β ^{1–193}* is strongly exothermic. The standard free enthalpy ΔG° for the complex formation amounts to –46.6 kJ/mol and stems exclusively from the enthalpic term ($\Delta H^\circ = -49.0$ kJ/mol), while the entropic contribution ($-T\Delta S^\circ = +2.4$ kJ/mol) slightly disfavors the binding (Fig. 1B). This result is surprising since, according to the CK2 holoenzyme structure (Niefind et al. 2001), the critical patch on the CK2 α surface—which is located at the outer surface of the β -sheet typical for the N-terminal domain of eukaryotic protein kinases (indicated in Fig. 2A)—is characterized by a cluster of hydrophobic side chains (Fig. 1C). Therefore, one would expect the formation of hydrophobic interactions between the subunits, a release of ordered water molecules, and correspondingly, a significantly positive entropic term.

A structural comparison of bound and unbound human CK2 α suggests a possible explanation of this paradox. In all unbound structures (Ermakova et al. 2003; Niefind et al. 2007; Raaf et al. 2008) the just mentioned surface patch is not directly accessible for CK2 β ; rather the neighboring β 4 β 5-loop occurs in a closed and CK2 β incompatible conformation (see two representative examples in Fig. 1C). In this state the hydrophobic patch is reduced to a cavity that at best can harbor flat-shaped small molecules (Raaf et al. 2008). As a consequence unbound human CK2 α cannot release a major amount of ordered water molecules during CK2 β association.

Only when CK2 β binds, the β 4 β 5-loop adopts an open conformation as it is found in the case of human CK2 α so far exclusively in both CK2 α chains of the CK2 holoenzyme (Fig. 1C; Niefind et al. 2001). Possibly this open state of human CK2 α is energetically more relaxed than the unbound one with the closed β 4 β 5-loop, meaning that the conformational change of CK2 α is a potential source

Figure 1. Calorimetric and structural analysis of the CK2α/CK2β interaction. (A) DSC curves of *hsCK2α*¹⁻³³⁵ (red), *hsCK2β*¹⁻¹⁹³ (blue), and the corresponding holoenzyme [(*hsCK2α*¹⁻³³⁵)₂(*hsCK2β*¹⁻¹⁹³)₂] (black). One representative example out of three repetitions, respectively, is drawn; the indicated melting points are the corresponding average values. (B) ITC profile of the *hsCK2α*¹⁻³³⁵/*hsCK2β*¹⁻¹⁹³ interaction. A representative example out of three ITC runs is documented. The upper half shows the original heat production upon injection and the lower one the integrated and dilution corrected peaks. The final thermodynamic parameters in the inset are average values over three repetitions. (C) The CK2β binding region of human CK2α. The figure is based on the structure of *hsCK2α*¹⁻³³⁵ in complex with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (2RKP) (Raaf et al. 2008). After structural superimposition, the β4β5-loops of *hsCK2α*¹⁻³³⁵ in complex with sulfate ions (1PVR) (Niefind et al. 2007) (black color) and of a *hsCK2α* chain within the CK2 holoenzyme (1JWH) (Niefind et al. 2001) (blue color) are drawn. Some hydrophobic side chains of the interface (yellow) and of the tip of the β4β5-loop (gray: closed conformation; blue: open conformation) are added. The figure was prepared with BRAGI (Schomburg and Reichelt 1988).

of the heat released upon complex formation (Fig. 1B). This notion is currently speculative but it fits conspicuously to the observation that CK2α from *Zea mays* has an open β4β5-loop in nearly all of its crystal structures (Niefind et al. 1998), and simultaneously a significantly higher tendency to exist in vivo in a monomeric (unbound) state than its orthologs from higher animals (Dobrowolska et al. 1992). Further structures together with site-directed mutagenesis and ITC data are required to decide whether the β4β5-loop really plays a key role in the context of the CK2 holoenzyme formation.

Crystal structure of *hsCK2β*¹⁻¹⁹³

Does CK2β—similar to CK2α—change its conformation at the interface region upon CK2 holoenzyme formation? So far this question could not be answered, since for CK2β no true unbound case structure existed. Rather the only available structures of free CK2β dimers (Chantalat et al. 1999a; Bertrand et al. 2004) were obtained with C-terminal deletion mutants without the aforementioned key region from Asn181 to His193, so that they miss the ability to form a CK2 holoenzyme. To close this gap we

Figure 2. Structure of *hsCK2β*¹⁻¹⁹³. (A) Overall ribbon presentation of the *hsCK2β*¹⁻¹⁹³ dimer. Each *hsCK2β*¹⁻¹⁹³ monomer consists of a body comprising two domains (subunit A: blue/red; subunit B: green/yellow) and a C-terminal tail. The β -sheets of the N-terminal CK2 α domains of the CK2 holoenzyme structure (Niefind et al. 2001) were drawn as black C α traces after structural superimposition to indicate the location of the CK2 α /CK2 β interface. Two sulfate ions found at the juxta-dimer interface region are covered with FoFc-omit density (cutoff level 3 σ above the mean). The figure was drawn with BOBSCRIPT (Esnouf 1997) and Raster3D (Merritt and Bacon 1997). (B) Stereo picture of the C-terminal two-stranded β -sheet (β 4 β 5) of a *hsCK2β*¹⁻¹⁹³ monomer. For reasons of clarity some side chains were left out. The *hsCK2β*¹⁻¹⁹³ structure segment is covered by electron density drawn with a contour level of 1 σ . The purple dotted lines indicate hydrogen bonds; distances are given in angstroms. For comparison the backbone of the equivalent region of the CK2 holoenzyme together with some side chains are drawn in black. The figure was prepared with BOBSCRIPT (Esnouf 1997) and Raster3D (Merritt and Bacon 1997). (C) Stereo picture of the anion binding sites at the juxta-dimer interface region. The sites are occupied by sulfate ions in the *hsCK2β*¹⁻¹⁹³ structure, by phosphate ions (black) in the CK2 holoenzyme structure (Niefind et al. 2001), and by negatively charged side chains from a crystallographic neighbor (ball-and-stick representation of reduced size) in one of four dimers per asymmetric unit of the CK2 β ²⁻¹⁸² structure (Bertrand et al. 2004). The sulfate ions are covered with blue, the protein parts of *hsCK2β*¹⁻¹⁹³ with green pieces of electron density (all taken from the final 2Fo - Fc density map and contoured with a cutoff level of 1 σ). Some hydrogen bonds to the sulfate ions are indicated by dotted lines in magenta color. The figure was prepared with BOBSCRIPT (Esnouf 1997) and Raster3D (Merritt and Bacon 1997).

crystallized *hsCK2β*^{1–193} and determined its structure to 2.8 Å resolution (Table 1).

The asymmetric unit of the crystals contains two *hsCK2β*^{1–193} chains that are arranged as a dimer via a zinc-stabilized interface as described for the first time for human CK2β^{1–182} (Fig. 2A; Chantalat et al. 1999a). Similar to previously published CK2β structures (Chantalat et al. 1999a; Bertrand et al. 2004) the N-terminal segment (residues Met1 to Glu5) and parts of the so-called “acidic loop” (Fig. 2A; residues Glu60 to Asp64) are flexible and undefined by electron density.

Like CK2α-bound CK2β within the CK2 holoenzyme (Niefind et al. 2001), each CK2β monomer consists of a “CK2β body” (Glu6–Ala180) composed of two folding domains and a “CK2β tail” (remaining C-terminal segment) (Fig. 2A). This tail is well defined by electron density up to the C terminus of the construct (His193; Fig. 2B). The CK2β tail of each monomer makes no contact to its own CK2β body; rather it crosses the dimer interface and is closely attached to the body of the second chain (Fig. 2A). Together with helix αF (and some smaller parts) of that body it forms the surface patch that can recruit a CK2α chain (Fig. 2A).

A remarkable detail of the *hsCK2β*^{1–193} structure is two sulfate ions bound at the so-called juxta-dimer inter-

face region (Fig. 2A), whose function as a preferred anchor for several other proteins has been predicted bioinformatically (Bolanos-Garcia et al. 2006). Conspicuously, the CK2β dimer within the CK2 holoenzyme structure (Niefind et al. 2001) contains phosphate ions at very similar locations (Fig. 2C). Finally, in the CK2β^{2–182} structure published by Bertrand et al. (2004), one of a total of four CK2β^{2–182} dimers per asymmetric unit uses the equivalent positively charged surface patch to coordinate acidic side chains from a crystallographic neighbor (Fig. 2C). This coincidence reveals an anion binding capability of the juxta-dimer interface region that may in fact be utilized by some of the many interaction partners of CK2β (Bibby and Litchfield 2005).

The CK2β conformation is preformed for CK2 holoenzyme formation

To check the adaptability of CK2β at the interface region to CK2α we fitted the *hsCK2β*^{1–193} dimer structure on the CK2β dimer of the CK2 holoenzyme. For this purpose we used the LSQ_improve subroutine of the program O (Jones et al. 1991) that optimizes an initial least-squares fit of equivalent C_α atoms by iterative inclusion of more residues. The result of this fit was an RMS deviation of 1.1 Å for 361 C_α atoms which are 98.6% of the ordered residues. The only deviations occur in the flexible acidic loop regions, while the tail zones from Asn181 to His193 match well to each other as well as the helices αF, which also contribute to CK2α binding.

These results together with the structural overlay in Figure 2B demonstrate the preformed character of the CK2β dimers, which contrasts strongly with the aforementioned adaptability of CK2α in the contact region (Fig. 1C). At least up to Ile192 (Fig. 2B) no significant backbone adaptations occur in CK2β as a consequence of or as a condition for CK2α binding. Like in the CK2β dimer of the holoenzyme the final section of *hsCK2β*^{1–193} forms a small two-stranded antiparallel β-sheet (β-strands 4 and 5) with Tyr188 at the tip of the connecting loop (Fig. 2A,B); however, due to the higher resolution the β-strand geometry is better defined here than in the CK2 holoenzyme structure.

The only distinct conformational changes in that region refer to the C-terminal His193 and to the side chain of Phe190 (Fig. 2B). In the case of His193, this is probably a crystal packing artifact, since this residue additionally has different (but well defined) conformations in the two *hsCK2β*^{1–193} monomers. Phe190, however, is known as one of the “hot spots” of the CK2α/CK2β interaction from the CK2 holoenzyme structure (Niefind et al. 2001) and from a recent kinetic and site-directed mutagenesis study (Laudet et al. 2007). This crucial role of Phe190 fits nicely to the change of its side-chain rotamer after CK2α attachment, illustrated in Figure 2B.

Table 1. Data collection and refinement statistics of the *hsCK2β*^{1–193} crystals

Data collection	
Space group	<i>P</i> 4 ₁ 2 ₁ 2
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	116.46, 116.46, 76.09
α, β, γ (°)	90.0, 90.0, 90.0
Resolution (Å) (highest shell)	29.7–2.8 (2.9–2.8)
<i>R</i> _{sym} (%) (highest shell)	9.9 (61.8)
<i>I</i> / <i>σI</i> (highest shell)	29.9 (4.6)
Completeness (%) (highest shell)	100.0 (100.0)
Redundancy (highest shell)	13.1 (13.2)
Wilson-plot <i>B</i> -factor (Å ²)	71.0
Refinement	
Resolution (Å)	29.7–2.8
No. of reflections	13,382
<i>R</i> _{work} / <i>R</i> _{free} (%)	17.2/21.5
No. of protomers per AU	2
Missing (disordered) residues	Met1–Glu5, Glu60–Asp64
No. of atoms per AU	
Protein	3000
Zinc and sulfate ions	17
Water	82
Average <i>B</i> -factors (Å ²)	
Protein	62.0
Zinc and sulfate ions	103.2
Water	45.4
RMS deviations	
Bond lengths (Å)	0.011
Bond angles (°)	1.123

Taken together, while CK2 α undergoes large conformational changes in the contact region upon docking, the critical part of CK2 β is preformed. The reduced adaptability of CK2 β ensures specificity of its interactions with CK2 α and possibly a few other protein kinases (Bibby and Litchfield 2005); moreover, it increases the prospects of theoretical docking calculations with the *hsCK2 β ¹⁻¹⁹³* structure and the chance to develop CK2 β antagonists of high selectivity.

Materials and Methods

Protein expression and purification

HsCK2 α ¹⁻³³⁵ and *hsCK2 β ¹⁻¹⁹³* were expressed recombinantly in *Escherichia coli* BL21(DE3) cells. To generate the CK2 holoenzyme, mixed lysates of the bacterial cells containing the expressed *hsCK2 α ¹⁻³³⁵* and *hsCK2 β ¹⁻¹⁹³* proteins were incubated overnight at 4°C. The three proteins—*hsCK2 α ¹⁻³³⁵*, *hsCK2 β ¹⁻¹⁹³*, and the holoenzyme (*hsCK2 α ¹⁻³³⁵*)₂(*hsCK2 β ¹⁻¹⁹³*)₂—were purified with a two-step chromatographic procedure. The first purification step in all three cases was a phosphocellulose chromatography. The column was equilibrated with 300 mM NaCl, 25 mM Tris/HCl, pH 8.5. After protein application and washing, a gradient elution was performed using 1 M NaCl, 25 mM Tris/HCl, pH 8.5, as a high-salt component. The second step for *hsCK2 β ¹⁻¹⁹³* was an anion exchange chromatography with a HiTrap Sepharose Q column (GE HealthCare). The equilibration and low-salt solution of the gradient was 150 mM NaCl, 25 mM Tris/HCl, pH 8.5, and the high-salt component was again 1 M NaCl, 25 mM Tris/HCl, pH 8.5. For *hsCK2 α ¹⁻³³⁵* and the holoenzyme, the second purification step was affinity chromatography with a HiTrap Heparin HP column (GE HealthCare). The equilibration and low-salt solution of the gradient was 400 mM NaCl, 25 mM Tris/HCl, pH 8.5, and the high-salt component was 1 M NaCl, 25 mM Tris/HCl, pH 8.5. Finally, the proteins were concentrated and rebuffed in 500 mM NaCl, 25 mM Tris/HCl, pH 8.5 by ultrafiltration using AMICON Ultra-15 tubes.

DSC measurements

For DSC data collection we used a VP-DSC differential scanning calorimeter. For each of the three proteins three temperature scans were performed from 20°C to 80°C at a scan rate of 25°C/h. The protein concentrations varied between 64 to 96 μ M for *hsCK2 α ¹⁻³³⁵*, between 30 and 69 μ M for *hsCK2 β ¹⁻¹⁹³*, and between 30 and 68 μ M for the holoenzyme (*hsCK2 α ¹⁻³³⁵*)₂(*hsCK2 β ¹⁻¹⁹³*)₂. In all cases the protein buffer was 500 mM NaCl, 25 mM Tris/HCl, pH 8.5. Processing of the raw data was performed with ORIGIN software (version 7), Origin Lab.

ITC measurements

All experiments were performed with a Microcal VP-ITC at 35°C. *HsCK2 β ¹⁻¹⁹³* was provided in the sample cell at concentrations between 9 and 23 μ M. *HsCK2 α ¹⁻³³⁵* was regarded as the “ligand”; it was present in the injection syringe at concentrations between 98 and 230 μ M. Both proteins were diluted with 500 mM NaCl, 25 mM Tris/HCl, pH 8.5, to the required concentrations and subsequently degassed. Each ITC experi-

ment consisted of 25 injections of 10 μ L. The injections were made over a period of 20 s with a 300-s interval between subsequent injections.

The raw ITC data (Fig. 1B, upper panel) were processed with ORIGIN software (version 7), Origin Lab, assuming a binding model of a single set of two equivalent sites (meaning two *hsCK2 α ¹⁻³³⁵* ligands bind to one *hsCK2 β ¹⁻¹⁹³* dimer). The final values (inset of Fig. 1B) are averages of three repetitions.

Crystallization

All crystallization experiments with *hsCK2 β ¹⁻¹⁹³* were performed at 20°C with the vapor diffusion technique. The optimized crystallization condition was 0.17 M ammonium sulfate, 0.085 M sodium cacodylate, pH 6.3, 25.5% w/v PEG 8000, and 15% v/v glycerol for the reservoir. The crystallization drop contained 2 μ L of a *hsCK2 β ¹⁻¹⁹³* solution (6.6 mg/mL) and 3 μ L reservoir. The crystals were directly transferred from the mother liquor to liquid nitrogen for cryo-protection.

Diffraction data collection and structure determination

X-ray diffraction data (Table 1) were collected at a temperature of 100 K at the beamline X12 of the EMBL outstation in Hamburg. The wavelength was 0.9 Å. All diffraction data were processed with the HKL package (Otwinowski and Minor 1997). The structure of *hsCK2 β ¹⁻¹⁹³* was determined by molecular replacement using MOLREP (Collaborative Computational Project, Number 4 1994) and refined with REFMAC (Collaborative Computational Project, Number 4 1994). Manual corrections were performed with O (Jones et al. 1991). The structure was validated with PROCHECK (Collaborative Computational Project, Number 4 1994).

Protein Data Bank Deposition

The final atomic coordinates and structure factor amplitudes of *hsCK2 β ¹⁻¹⁹³* are available from the PDB (accession code 3EED).

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