Crystal structure of human protein kinase CK2: insights into basic properties of the CK2 holoenzyme

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The crystal structure of a fully active form of human protein kinase CK2 (casein kinase 2) consisting of two C-terminally truncated catalytic and two regulatory subunits has been determined at 3.1 Å resolution (Protein Data Bank code: 1JWH). In the CK2 complex the regulatory subunits form a stable dimer linking the two catalytic subunits, which make no direct contact with one another. Each catalytic subunit interacts with both regulatory chains, predominantly via an extended C-terminal tail of the regulatory subunit. The CK2 structure is consistent with its constitutive activity and with a flexible role of the regulatory subunit as a docking partner for various protein kinases. Furthermore it shows an inter-domain mobility in the catalytic subunit known to be functionally important in protein kinases and detected here for the first time directly within one crystal structure.

Keywords: casein kinase 2/CK2 holoenzyme/constitutive activity/protein kinase CK2/X-ray crystallography

Introduction

Protein kinase CK2 (casein kinase 2) is one of the most unspecific eukaryotic protein kinases: first, >160 *in vitro* protein substrates of CK2 have been described to date (Pinna and Meggio, 1997); secondly, CK2 shows the rare ability to use either ATP or GTP as phosphoryl donor (dual-co-substrate specificity) (Niefind *et al.*, 1999); thirdly, although known as a serine/threonine kinase for several decades, the capability of CK2 also to phosphorylate tyrosine has been reported repeatedly in recent years (Chardot *et al.*, 1995; Wilson *et al.*, 1997; Marin *et al.*, 1999); and fourthly, CK2 activity was found not only with the natural cofactor Mg²⁺ but also with other divalent cations such as Mn²⁺ and Co²⁺ (Gatica *et al.*, 1993).

These biochemical properties and its biomedical significance (Guerra and Issinger, 1999) make CK2 a favourite research subject. The enzyme has been found in all eukaryotic cells investigated so far and is highly conserved in evolution, indicating its critical function in cellular life. Within the phylogenetic tree of the protein kinases the catalytic core of CK2 belongs to the CMCG group (Hanks and Hunter, 1995) meaning that its nearest

neighbours are key regulatory enzymes, including the cyclin-dependent kinases (CDK) or the mitogen-activated protein kinases. CK2 is essential for the viability of a cell (Padmanabha *et al.*, 1990), and overexpression of its catalytic subunit is correlated with lymphoma development in transgenic mice (Seldin and Leder, 1995).

Not least due to its unspecific activity profile and its wide occurrence in tissues and cell compartments, the exact cellular functions of CK2 remain elusive. The metabolic roles of the >160 *in vitro* substrates of CK2 identified so far (Pinna and Meggio, 1997) have led to many hypotheses about the involvement of CK2 in carcinogenesis, viral tumorigenesis, transcriptional control, apoptosis, cell cycle, signal transduction and other key biological processes (Guerra and Issinger, 1999). It is generally accepted that CK2 plays an important role in cell proliferation and embryogenesis.

In parallel with its functional diversity CK2 also presents a complex picture concerning its quaternary structure. *In vivo* it exists mainly as a holoenzyme composed of two catalytic subunits (CK2 α) and two regulatory subunits (CK2 β). Two isoforms of the catalytic subunit (CK2 α and CK2 α') have been found in human and many other sources and CK2 complexes of $\alpha_2\beta_2$, $\alpha\alpha'\beta_2$ and $\alpha'\beta_2$ stoichiometry can occur (Chester *et al.*, 1995). Moreover there is increasing evidence that the isolated subunits can exist *in vivo* under certain circumstances and possibly have specific functions (Pinna and Meggio, 1997). *In vitro* the CK2 holoenzyme forms spontaneously from the individual subunits by a self-assembly mechanism mediated by dimerization of the two CK2 β chains (Graham and Litchfield, 2000). The formation of higher oligomeric states of CK2 was also observed *in vitro* (Glover, 1986).

At the level of tertiary structure the current knowledge about CK2 is restricted to the individual subunits. Crystal structures have been published for recombinant maize CK2 α (rmCK2 α ; Niefind *et al.*, 1998) and for recombinant and C-terminally truncated human CK2 β (rhCK2 β^A ; Chantalat *et al.*, 1999), while for the CK2 holoenzyme only a theoretical model derived from the crystal structure of maize CK2 α in complex with a short peptide of human CK2 β exists (Battistutta *et al.*, 2000). This model suggests a direct contact of the two CK2 α subunits within the CK2 complex and is therefore in conflict with yeast two-hybrid system studies showing that CK2 α molecules are able to bind to CK2 β but not to each other (Gietz *et al.*, 1995; Boldyreff *et al.*, 1996).

To clarify this conflict and to rationalize some of the specific biochemical properties of the CK2 holoenzyme on a structural level we describe here for the first time the crystal structure of a complete CK2 tetramer. For this work we used recombinant human CK2. Human CK2 α is normally 391 amino acids long (Figure 1A) and has a

A **B**

Fig. 1. Sequence overview of human CK2 α (A) and CK2 β (B). Small characters in the sequence indicate residues that are truncated or disordered in both copies of the chains and are therefore left out in the final rhCK2 α^A structure. Positions at which the final $2F_o - F_c$ electron density is badly defined are printed in italics. The contact residues are assigned with black bars. The α/β contacts normally occur twice in the rhCK2 α^A complex; if a certain residue was detected as a contact partner only in one chain it is marked with a grey bar instead of a black one. For human A-Raf and *Xenopus laevis* c-Mos partial sequences are included in (A).

molecular mass of 45.1 kDa. During purification a 5 kDa peptide was spontaneously cleaved from the catalytic subunit leaving a degraded form of ~40 kDa (rhCK2 α^A ; Niefind *et al.*, 2000), which is around the typical size of CK2 α subunits from various sources. The resulting stable CK2 holoenzyme comprising two molecules of rhCK2 α^A and two molecules of recombinant human CK2 β (rhCK2 β) is fully active with several typical CK2 substrates (Niefind *et al.*, 2000). It is called rhCK2 α^A throughout this paper. Whether this partial degradation of human CK2 α occurs also in natural host cells and

whether the cleaved peptide has a biological function is not known to date.

Results and discussion

Structure determination

The rhCK2 α^A structure was solved with crystals grown in the presence of Mg²⁺ ions and the non-hydrolysable ATP analogue adenylyl imidodiphosphate (AMPPNP). The crystals belong to space group $P6_3$ with lattice constants $a = b = 176.0$ Å, $c = 93.7$ Å. The asymmetric unit of the

crystals contains one rhCK2 Δ tetramer and has a solvent content of 61%. We hereafter designate the two rhCK2 α Δ chains as A1 and A2 and the two rhCK2 β chains as B1 and B2.

The crystals were of limited X-ray diffraction quality. The best resolution obtained with a rotating copper anode was 6 Å. At a synchrotron we measured a complete data set to 3.1 Å resolution (Table I), merging data from two

crystals. The single diffraction frames showed a significant anisotropy, which was also detected by the program TRUNCATE (CCP4, 1994) for the complete data set. A considerable internal disorder in the crystals was indicated by high mosaicities of the two crystals (1.3 and 1.4°) and an overall *B*-factor of 92.2 Å² calculated by TRUNCATE from a Wilson plot.

The structure was solved by a combination of molecular replacement and phase refinement techniques (see Materials and methods) and refined with all reflections from 59.3 to 3.1 Å to a final model with acceptable stereochemical parameters (Table I). A Ramachandran graph for each subunit is shown in Supplementary figure 1, available at *The EMBO Journal* Online.

Table I. Crystallographic analysis

Characteristic data of the rhCK2 Δ synchrotron data set	
Space group	<i>P</i> 6 ₃
Lattice constants	<i>a</i> = <i>b</i> = 176.0 Å, <i>c</i> = 93.7 Å
No. of crystals used	2
Temperature of data collection	100 K
Resolution range	59.3–3.1 Å
No. of observations	268 848
No. of rejections	6073
No. of independent reflections	29 935
Wilson-plot <i>B</i> -factor	92.2 Å ²
Multiplicity	8.8
Multiplicity for last shell (3.2–3.1 Å)	6.0
Average of (<i>I</i> / σ _{<i>i</i>})	16.7
Average of (<i>I</i> / σ _{<i>i</i>}) for last shell (3.2–3.1 Å)	2.6
Completeness for whole range	98.4%
Completeness for last shell (3.2–3.1 Å)	90.4%
<i>R</i> _{sym} for whole range	9.6%
<i>R</i> _{sym} for last shell (3.2–3.1 Å)	42.0%
Overview of the final structure model	
Resolution range included in refinement	3.1–60.0 Å
<i>R</i> _{free} (4% of all reflections) ^a	33.8%
<i>R</i> _{work} (96% of all reflections) ^a	26.7%
Real space <i>R</i> -value ^a	5.3%
Average <i>B</i> -factor	91.0 Å ^b
R.m.s.ds for bonds ^a	0.009 Å
R.m.s.ds for angles ^a	1.5°
Quality of Ramachandran plot	
% residues in most favoured ^b regions	69.5
% residues in additional allowed ^b regions	28.1
% residues in generously allowed ^b regions	2.2
% residues in disallowed ^b regions	0.2
Cross-validated sigmaaa estimate of mean coordinate error ^a	1.04 Å
Cross-validated Luzzati estimate of mean coordinate error ^a	0.67 Å

$R_{\text{sym}} = \sum_h \sum_j |I_{h,j} - \langle I_h \rangle| / \sum_h \sum_j I_{h,j}$, where $I_{h,j}$ is the intensity of the *j*th observation of unique reflection *h*, and $\langle I_h \rangle$ is the mean intensity of that reflection. Reflection intensities related by Friedel symmetric were merged in this data set.

^aCalculated with CNS (Brünger *et al.*, 1998).

^bAccording to PROCHECK (CCP4, 1994).

Quality of the structure

Most parts of rhCK2 Δ are defined by good electron density (see Figure 2 for examples), while in some sections the quality of the density is reduced as indicated by increased real space *R*-factors (Supplementary figure 2). Finally there are regions lacking any ordered electron density. Among these, as indicated in Figure 1, the N-terminus of B2 and the zone Asn206 to Arg215 of B1 and B2 were left out from the final model whereas the zone Asp55 to Asn65 of B1 was modelled as a copy of its equivalent in B2.

Some parts of the chains missing in the initial models, for instance the C- and N-termini of A1 and A2 or the functionally critical zone around Tyr188 of B1 and B2, were added during the refinement process. Model building at 3.1 Å resolution and with model-biased phases is difficult and susceptible to errors. We cannot exclude such errors, which will become apparent with higher-resolution diffraction data, but in view of the final electron densities we are nevertheless confident that we have identified the correct structure from Gly3 to Pro6 of A1 and A2, from Arg333 to Ser337 of A1 and from Pro176 to Gly189 of B1 and B2.

In some other parts (C-terminus of A2, N-terminus and zone Asp55 to Asn65 of B2, zone Phe190 to Ser205 of B1 and B2) the rough trace of the main chain was clear enough, but the conformations of peptide groups and side chains remain questionable. Within such a zone, namely from Met195 to Gln200 of rhCK2 β , we modelled an α -helix (α G; Figures 1B and 3D) in agreement with a secondary structure prediction (Korn *et al.*, 1999) and with circular dichroism spectroscopic data, indicating that the CK2 complex formation is accompanied by an increase in the α -helical content of the protomers (Issinger *et al.*, 1992).

Fig. 2. Electron densities in selected regions of rhCK2 Δ . All maps apart from that covering the AMPPNP molecule in (D) are sigma-weighted $2F_o - F_c$ electron densities contoured with a 1 σ cutoff. The red map in (D) around AMPPNP is an $F_o - F_c$ omit density (4 σ cutoff) calculated after a 5000 K simulated annealing run excluding the AMPPNP coordinates. (A) Stereo figure of the CK2 β tail as part of the β/β contact. The two rhCK2 β subunits are distinguished by different colours (red and blue) for the electron densities and the labels. His165 and Met169 of rhCK2 β ^a (Chantalat *et al.*, 1999) and some corresponding water molecules displaced by the formation of the β/β contact are drawn in black. (B) Stereo figure of the hydrophobic core of the α/β contact. The colour coding for electron densities and labels is black for the CK2 β tail with Tyr188 as the central residue of the whole core, red for the body of the second rhCK2 β chain and blue for the participating rhCK2 α Δ subunit. (C) The N-terminal segment of rhCK2 α Δ attached to the activation segment (yellow). For comparison the backbone courses of both segments in rmCK2 α (Niefind *et al.*, 1999; PDB code: 1DAW) after superimposing on rhCK2 α Δ are drawn in black. Further colour coding for activation segments after three-dimensional fits: red for the partially active CDK2 in complex with a cyclin A fragment (Jeffrey *et al.*, 1995; PDB code: 1FIN) and violet for the inactive CDK2 in isolated form (De Bondt *et al.*, 1993; PDB code: 1HCL). (D) Stereo figure of AMPPNP bound to the active site of rhCK2 Δ chain A1. To compare the γ -phosphate positions, AMPPNP as bound to rmCK2 α (Niefind *et al.*, 1999) is drawn in black after superimposition of the protein matrices.

In the active site of subunit A1 a bound AMPPNP molecule—but no accompanying Mg^{2+} ions—was detected while A2 was free from a co-substrate analogue.

Several peaks of residual electron density were filled with water molecules and phosphate ions in accordance with a phosphate concentration of ~0.2 M in the crystallization

Fig. 3. Various aspects of the rhCK2 Δ structure. (A and B) Overall shape of rhCK2 Δ in a view perpendicular to the local C2 axis (A) and along this axis (B). The two rhCK2 β chains are drawn in blue and red, the two rhCK2 α subunits in yellow and grey. (C) Structural overview of rhCK2 α chain A1 with bound AMPPNP and interdomain flexibility. The hinge axis and the bending residues of the domain closure motion as detected by DYNDOM (CCP4, 1994) are included. To illustrate the interdomain flexibility, the N-terminal domains of rmCK2 α (PDB code: 1DAW) and of rhCK2 α chain A2 are shown in yellow and black, respectively, after three-dimensional alignment of the corresponding C-terminal domains. (D) Structural overview of rhCK2 β . The human CK2 β peptide bound to rmCK2 α (black) was taken from PDB file: 1DS5 (Battistutta *et al.*, 2000) after superimposition of the corresponding CK2 α subunits. (E) Intersubunit flexibility at the α/β contact. Subunit A1 is drawn with yellow colour for the C-terminal domain and grey for the N-terminal domain. Subunit B1 bound to A1 by an α/β contact is sketched in red. Subunit B2 is shown in blue after a three-dimensional fit of the N-terminal domain of subunit A2 (not drawn) on that of A1.

drops (Niefind *et al.*, 2000). In agreement with rhCK2 β Δ (Chantalat *et al.*, 1999) a zinc ion was found in both regulatory subunits.

With 91.0 Å² the average *B*-factor of the structure is relatively high, but it corresponds well with the *B*-factor obtained from a Wilson plot (92.2 Å²; Table I). Furthermore the *R*-factors are relatively large (Table I). While crystal twinning was excluded as a reason, this is likely to be a consequence of the conservative refinement

strategy and of the disorder in the crystals, which is indicated by zones of diffuse electron densities and by the drawbacks of the data set mentioned above (low resolution, anisotropy, high mosaicity, high overall *B*-factor).

Overall architecture of rhCK2 Δ and its subunits

Shape of the complex. The rhCK2 Δ complex has the shape of a butterfly in a view from above perpendicular to the molecular 2-fold axis (Figure 3A). Its approximate

dimensions in the directions of the principal axes are $155 \times 90 \times 66$ Å. The second of these axes coincides with the molecular dyad.

Figure 3A and B shows that the central building block of the rhCK2 Δ complex is the rhCK2 β dimer bridging the space between both rhCK2 α chains. This happens in such a way that each of the two rhCK2 β monomers touches both rhCK2 α subunits, which in contrast make no contact with each other. In fact the centres of mass of the two rhCK2 α molecules are 98.5 Å, their active sites ~80 Å and their nearest atoms 32.4 Å distant from one another (Figure 3A and B).

This central result confirms former findings based on the yeast two-hybrid system (Gietz *et al.*, 1995; Boldyreff *et al.*, 1996) and is consistent with the monomeric state generally found for isolated CK2 α subunits. In contrast it disproves the theoretical CK2 holoenzyme model of Battistutta *et al.* (2000) in which the two catalytic subunits are postulated to be in close contact and the active sites only 13 Å apart from one another.

The shape of rhCK2 Δ in the view of Figure 3B resembles the quaternary structure models that Zhao *et al.* (1998) derived for the holoenzyme of cAMP-dependent protein kinase (CAPK) from neutron small angle scattering experiments. In these models, as in rhCK2 Δ , the catalytic C subunits do not touch and have a centre-of-mass distance of as much as 122 Å, while the R₂C₂ complex is formed by the central dimer of two regulatory R subunits. Hence the organization of the rhCK2 Δ complex presented here may also be representative for CAPK and other protein kinases.

Structure of the catalytic subunits. Both rhCK2 α subunits show the typical bilobal architecture of the catalytic core of eukaryotic protein kinases with a β -sheet-rich N-terminal domain, an α -helical C-terminal domain and the active site between them (Figure 3C). Structural superpositions of the two rhCK2 α subunits and of isolated rmCK2 α (Niefind *et al.*, 1998), including virtually all C α atoms, lead to root mean square deviations (r.m.s.ds) of ~0.8 Å (Table II). These values demonstrate the overall similarity of the superimposed structures, although a closer inspection discloses an interdomain flexibility, which is discussed below.

One of the most conspicuous structural features of rmCK2 α , a strong attachment of the N-terminal segment to the activation segment from Asp175 to Glu201 (Niefind *et al.*, 1998), is also found in the catalytic subunits of rhCK2 Δ and hence is not affected by rhCK2 β (Figure 2C). This observation disproves the hypothesis of Sarno *et al.* (1998) that CK2 β and the N-terminal segment of CK2 α are competitive for attachment to the activation segment.

The conservation of the activation segment conformation in isolated and complex-bound CK2 α is in remarkable contrast to CDK2, where binding of a cyclin A fragment changes the conformation of the activation segment dramatically and brings CDK2 to a partially active conformation (Figure 2C; Jeffrey *et al.*, 1995). In this way cyclin A is a molecular switch for CDK2 activity similar to the R-subunit in the case of CAPK. In contrast CK2 α is catalytically active both in isolated and in complex-bound form. As an isolated molecule it has a basal activity, but this is elevated for most substrates

Table II. Minimal r.m.s.ds after three-dimensional fits of C α atoms

Catalytic subunits	rhCK2 Δ , chain A1 ^a	rhCK2 Δ , chain A2 ^a
rhCK2 Δ , chain A1	0.0 Å (337)	0.92 Å (331)
rhCK2 Δ , chain A2	0.92 Å (331)	0.0 Å (336)
rmCK2 α (1DAW ^b)	0.91 Å (327)	0.76 Å (326)
Regulatory subunits	rhCK2 Δ , chain B1 ^a	rhCK2 Δ , chain B2 ^a
rhCK2 Δ , chain B1	0.0 Å (200)	0.25 Å (199)
rhCK2 Δ , chain B2	0.25 Å (199)	0.0 Å (204)
Isolated CK2 β , chain A (1QF8 ^b)	0.82 Å (165)	0.85 Å (165)
Isolated CK2 β , chain B (1QF8 ^b)	0.67 Å (163)	0.76 Å (163)
Dimers	rhCK2 Δ , CK2 β dimer	
Isolated CK2 β , full dimer (1QF8 ^b)	0.83 Å (328)	

^aThe values in brackets are the numbers of matched C α atoms.

^bRCSB Protein Data Bank code.

significantly by CK2 β , for example, for the synthetic peptide RRDDSDDD by a factor of four (Boldyreff *et al.*, 1994). Calmodulin, however, serves as a much better substrate for CK2 α than for the CK2 holoenzyme. More complicated still, these stimulatory and inhibitory effects of CK2 β on CK2 α strongly depend on the salt concentration and on the presence of effector molecules such as polyamines or polybasic peptides (Pinna and Meggio, 1997). Taken together, CK2 β is an environment- and substrate-dependent modulator of CK2 α activity rather than an on-off switch (Boldyreff *et al.*, 1994). This constitutive activity fully agrees with the structural similarity between isolated and complex-bound CK2 α .

Structure of the regulatory subunits. The r.m.s.ds in Table II show that the structure of the CK2 β body changes only a little as a consequence of the formation of the rhCK2 Δ complex, and further that the two rhCK2 β molecules of the complex are more similar to one another than the two rhCK2 α subunits. This difference is certainly due to the intense interactions between the two rhCK2 β chains imposing constraints in favour of structural similarity, whereas the rhCK2 α subunits are not in contact with one another and hence much more free for conformational variations.

Each of the rhCK2 β molecules can be divided into a body comprising an α -helical N-terminal domain and a Zn²⁺-containing domain on the one hand and a C-terminal tail on the other (Figure 3D). While the CK2 β body has been characterized by Chantalat *et al.* (1999), the C-terminal tail of CK2 β is a new structural element. It points away from the body, forms a 90° knee with a β -hairpin loop with Tyr188 at its top and becomes more and more disordered afterwards (Figure 2A). The importance of this loop is indicated by the fact that the sequence zone R¹⁸⁶LYGFKI¹⁹² is highly conserved. Gly189 and Phe190 are present in all CK2 β sequences currently known, while at position 188, apart from tyrosine, phenylalanine also occurs. The CK2 β tail has no contact

with the body of its own monomer (Figure 3D) but is stabilized mainly by hydrophobic interactions with the second rhCK2 β (Figure 2A) and with one of the rhCK2 α^A subunits (Figure 2B). These interactions are described below.

The conformation of the CK2 β tail is partially consistent with the 3.1 Å resolution structure of rmCK2 α in complex with a peptide comprising positions 181–203 of human CK2 β (Battistutta *et al.*, 2000; PDB code 1DS5). These authors correctly identified the β -hairpin loop (Figure 3D) and its binding site at CK2 α . However, the structure of the rmCK2 α -bound peptide deviates more from rhCK2 α^A the larger the distances are from the hairpin loop (Figure 3D). This drawback and the absence of any structural overlap with the rhCK2 β^A structure of Chantalat *et al.* (1999) are certainly the reasons for the wrong architecture of the CK2 holoenzyme model derived by Battistutta *et al.* (2000). This failure demonstrates the limits of a peptide-based approach for the detection of the architecture of an oligomeric protein complex.

Subunit interactions

Overview and designations. The two principal types of protein–protein complexes are homocomplexes between identical subunits, which are usually permanent and evolutionary optimized, and heterocomplexes between non-identical chains, which can be also permanent but are quite often non-obligatory, that is they are made and broken according to the environment and external factors. The size of a protein interface allows an estimation of the permanent or non-obligatory character of a protein–protein contact (Jones and Thornton, 1996).

The rhCK2 α^A complex is a mixture of both complex types. (i) An rhCK2 β homodimer formed by an interface that we call ' β/β contact' further on is at the centre of rhCK2 α^A around the molecular 2-fold axis (Figure 3A and B). (ii) There are two ' α/β contacts' between the rhCK2 β dimer and either rhCK2 α^A chain, respectively. Each of these contacts is heterotrimeric, meaning that the interface is composed of one rhCK2 α^A chain and both rhCK2 β subunits (Figure 3A and B). To distinguish the individual contributions we use the designations ' α/β body contact' and ' α/β tail contact' further on. An ' α/β body contact' connects an rhCK2 α^A subunit and the body of an rhCK2 β monomer, while an ' α/β tail contact' connects an rhCK2 α^A subunit and the tail of an rhCK2 β monomer.

The β/β contact. An r.m.s.d. of 0.83 Å between isolated and complex-bound CK2 β dimers (Table II) demonstrates that the whole CK2 β dimer arrangement is conserved during CK2 complex formation. Consequently the basic principles of the β/β contact in the isolated CK2 β dimer (Chantalat *et al.*, 1999) are also valid for the CK2 β bodies in rhCK2 α^A . (i) The interface is formed by the Zn $^{2+}$ -binding motif (Figure 1B). (ii) The interface is mainly hydrophobic with a central area composed of Pro110, Val112, Leu124, Val143 and the hydrophobic parts of Tyr113 and Tyr144. (iii) Well defined intersubunit salt bridges (Arg111 with Asp142; Figure 2A) and hydrogen bonds (Pro110 O with Thr145 N; Val143 O with Val112 N) provide a further stabilization.

Chantalat *et al.* (1999) argue about the fact that the buried surface at the β/β contact in dimers of C-terminally

truncated CK2 β amounts to only 543 Å 2 , which is less than a third of the typical value for protomers of that size (Jones and Thornton, 1996). In rhCK2 α^A this paradox is solved because here the β/β interface, with 1766 Å 2 per subunit, is more than three times as big. The reason for this increase is that in rhCK2 α^A not only the CK2 β body contributes to the β/β contact but in an extensive manner so does the tail (Figures 1B and 2A). For example, a novel hydrogen bond is formed from the peptide oxygen of Arg186 in one rhCK2 β subunit to the side chain of Thr161 of the other (Figure 2B). And above all in the centre of the new contact region a hydrophobic cluster of Met141, Val143, Tyr136, Phe168, His165 and Met169 from one chain and Phe183, Pro185, Leu187, Ile192 and Met195 from the other forms. Figure 2A illustrates how the side chains of His165 and Met169 change their conformations to participate in this hydrophobic zone and how water molecules found in isolated rhCK2 β^A dimers (Chantalat *et al.*, 1999) are displaced from it. In particular His165 manages in this way to become a member of the aromatic core of the hydrophobic cluster together with Tyr136 and Phe168 from its own and Phe183 from the other subunit (Figure 2A).

The remarkable increase in the β/β contact by the CK2 β tail, however, requires a specific conformation of this tail, which also depends—as discussed below—on its participation in the α/β contact and hence on the existence of the complete tetramer. In contrast, for isolated CK2 β the region Val170 to Ala180 at the beginning of the tail attenuates the dimerization (Boldyreff *et al.*, 1996). This demonstrates the synergistic character of the CK2 β tail: it causes aggregation and stability problems in isolated CK2 β and was therefore removed for its structure determination (Chantalat *et al.*, 1999), but within the CK2 tetramer it stabilizes the β/β and α/β contacts.

The compact nature of the rhCK2 β dimer and the extended β/β contact compared with isolated CK2 β dimers fully agree with the central role of CK2 β dimer formation as the initial step of CK2 holoenzyme assembly (Graham and Litchfield, 2000).

The α/β contacts. The contacts of either catalytic subunit with the CK2 β dimer are restricted to the N-terminal lobe of the common core (Figure 1A) typical for all eukaryotic protein kinases (Hanks and Hunter, 1995). More precisely parts of the outer surface of the central β -sheet within the N-terminal domain form the interface with the rhCK2 β dimer. Minor contact zones in rhCK2 α^A are found at strand β_3 , at the subsequent loop and further at the loop connecting the strands β_4 and β_5 . The main contact region is one of the most important for catalytic activity, namely the strands β_1 and β_2 and the glycine-rich ATP-binding loop in between (Figure 1A). This fact is especially conspicuous in view of the modulating influence of CK2 β on CK2 α activity, although it does not allow its detailed structure-based rationalization because the rhCK2 α^A structure presented here contains no substrate molecules.

The observation that no CK2 α -specific structural elements but a part of the common protein kinase core forms the α/β interface fits well with the finding that CK2 β is able to interact with other protein kinases such as A-Raf (Boldyreff and Issinger, 1997) and c-Mos (Chen *et al.*, 1997) as well as with CK2 α . These interactions are

activating (A-Raf) (Hagemann *et al.*, 1997) or inhibiting (c-Mos) and they are competitive with CK2 α so that the same parts of the N-terminal domains are probably involved. Some of the residues in the α/β contact regions including Leu41 or Val42 are conserved between CK2 α and A-Raf or c-Mos (Figure 1A), although the fact that CK2 β interacts exclusively with A-Raf but not with B-Raf and c-Raf-1 (Hagemann *et al.*, 1997) cannot be explained on the primary structure level. In any case the rhCK2 Δ structure supports the view that free CK2 β dimers may play a role *in vivo*, interacting with the N-terminal core domains of a certain set of protein kinases leading to specific activations or deactivations.

Such a role of CK2 β would require free CK2 β dimers in the cell. For a long time CK2 was thought to exist exclusively as a tetrameric complex *in vivo*. Meanwhile, however, several observations indicate that unbound CK2 α monomers and CK2 β dimers can occur at certain times or in certain compartments (Pinna and Meggio, 1997). Presumably they stem from a spatial or temporal excess of one of the subunits after translation.

Alternatively CK2 holoenzyme could form a pool from which the subunits could be released by dissociation. Generally this possibility is considered improbable because of the spontaneous formation and stable nature of the CK2 tetramers *in vitro* (Pinna and Meggio, 1997). However, some properties of the α/β contact in rhCK2 Δ indicate that an association–dissociation equilibrium *in vivo* should not be completely excluded. (i) Interface size: with an average size of 832 Å² the α/β interface is relatively small. Although the precision of this value is reduced at 3.1 Å resolution, it can be compared with the average interface sizes reported by Jones and Thornton (1996), namely 1722 Å² for permanent and 804 Å² for non-obligate protein–protein complexes. This comparison shows that the α/β contact clearly belongs to the second class. (ii) Intersubunit flexibility: to compare the two α/β contacts of the rhCK2 Δ tetramer we reduced it to two trimers, one of which contained the subunits A1, B1 and B2 and the other the subunits A2, B1 and B2. These trimers were overlaid by superimposing the N-terminal domain of subunit A2 on that of A1 and taking the CK2 β dimer along accordingly. This procedure disclosed the flexible character of the α/β contact (Figure 3E). A quantitative analysis of this feature with DYNDOM (CCP4, 1994) revealed a 16.4° pseudo rotation around an axis lying at the outer β -sheet surface of the N-terminal CK2 α domain (Figure 3E).

These findings suggest that the CK2 holoenzyme is a transient heterocomplex, which is formed and dissociates *in vivo* for specific functional and regulatory reasons. This idea is speculative at the moment, but if supported by further data in the future it would shed new light on the often addressed problem of regulation of CK2 activity.

Neither the α/β -body nor the α/β -tail contact alone is sufficient to establish a stable α/β interface. However, a value of 491 Å² for the α/β -tail contact and only 336 Å² for the α/β -body contact demonstrate the major contribution of the α/β -tail contact for the stability of the CK2 holoenzyme, while the α/β -body contact is probably the main determinant for activation. This distinction between activating and stabilizing contacts agrees with the properties of CK2 β mutants lacking the C-terminus after Ala180,

which retain the capability to activate significantly CK2 α but are unable to form stable CK2 holoenzymes (Boldyreff *et al.*, 1994).

The cooperativity of the two rhCK2 β subunits in rhCK2 α^A binding can also be seen at an atomic level. At the heart of the contact region a hydrophobic cluster is found that comprises residues from all three contact partners (rhCK2 α^A : Leu41, aliphatic part of Glu52, Phe54, Val67, Ile69, Ala110; first rhCK2 β chain: Leu89, Met166, Met169; second rhCK2 β chain: Leu187, Tyr188, Ile192). Figure 2B demonstrates the central role of the rhCK2 β tail around Tyr188 in this contact zone. After Ile192 both rhCK2 β chains are increasingly disordered. They are obviously not important for the holoenzyme formation, fitting the observation that a CK2 β mutant lacking the C-terminus after Pro194 behaves biochemically like the wild type (Boldyreff *et al.*, 1994).

In contrast to the β/β contact, salt bridges and hydrogen bonds play no important role at the α/β contact region. Unless interface hydrogen bonds are detected in better resolved structures in the future, the absence of specific attachments may be the reason for the inter-subunit mobility at the α/β interface mentioned above (Figure 3E).

Comparison with other protein kinase complex structures. Presently only a limited number of protein kinases have been structurally characterized together with regulatory molecules. Most information exists about cyclin-dependent protein kinases in complex with various cell cycle regulatory proteins: CDK2 was co-crystallized with a cyclin A fragment (Jeffrey *et al.*, 1995), with CksHs1 (Bourne *et al.*, 1996) and with both a cyclin A fragment and the inhibitory domain of p27^{Kip1} (Russo *et al.*, 1996); while for CDK6 complex structures together with the cell cycle inhibitors, p19^{INK4d} (Brotherton *et al.*, 1998) and p16^{INK4a} (Russo *et al.*, 1998) have been studied. According to these structures the four groups of ligands bind to different regions of the catalytic kinase core: both the cyclins and the INK4 proteins bind to both kinase domains and bridge the active site cleft between them. However, while the cyclins as CDK activators do this in such a way that the activation segment is fixed in an active conformation, it is the other way round with the INK4 proteins. A different inactivation mechanism is found with p27^{Kip1}, which directly blocks the active site of a CDK2–cyclin A complex without inducing major conformational changes in the kinase. Unlike these activators and inhibitors the Cks proteins are targeting molecules. Consequently they bind exclusively to the C-terminal kinase domain far away from the active site cleft.

The α/β contact of rhCK2 Δ represents a valuable new member of the list of interfaces to the catalytic core of protein kinases. To our knowledge the CK2 β dimer is the first kinase ligand that binds only to the N-terminal domain. Moreover the α/β contact is restricted to a structurally conserved part of this domain, suggesting that it might represent an interaction site not only used in CK2 α .

The fact that CK2 β neither blocks the active site nor affects the activation segment agrees with its proposed role as a moderate modulator of CK2 α activity rather than an on–off switch. Furthermore, the parallel with the Cks proteins that the interaction site lies completely in one of

the two protein kinase domains suggests that intracellular targeting might be an important function of CK2 β , as with the Cks proteins.

Further functional implications

Interdomain and intersubunit flexibility. The CK2 β body consists of two domains (Figure 3D) that have a fixed interaction with each other. In contrast in rhCK2 α^A the two main domains are significantly flexible as illustrated in Figure 3C. A quantitative analysis of this feature with the automatic domain detection program DYNDOM (CCP4, 1994) led to some interesting results. (i) The N-terminal residues Ser2 to Arg10 belong structurally to the C-terminal domain (Figure 3C), which underlines the importance of the attachment between the N-terminal segment and the activation loop. (ii) The 'bending residues' between the two domains are firstly Arg10 to Val11 and secondly Asn118 to Thr119. (iii) The interdomain displacement consists of a rotation of 7.8° around an axis nearly perpendicular to the line connecting the centres of mass of the two domains (Figure 3C). Accordingly DYNDOM regards this rotation as a pure 'closure motion' (Hayward and Berendsen, 1998). (iv) The rotation axis is close to the second zone of bending residues (Figure 3C). Hence, the dipeptide Asn118/Thr119 is classified as an 'effective mechanical hinge' (Hayward and Berendsen, 1998).

From comparisons between protein kinase crystal structures the interdomain mobility within the catalytic core and its importance for the catalytic mechanism are well known (Cox *et al.*, 1994). However, we checked all protein kinase entries of the Protein Data Bank and found that rhCK2 Δ provides the first case in which two significantly different opening states of the active site cleft are seen in the same crystal.

The functional implications of the aforementioned intersubunit flexibility at the α/β interface (Figure 3E) are, however, less clear. One consequence of this mobility is that certain parts of rhCK2 β approach the active site of rhCK2 α^A . Examples are helix αB and the unusual 95° knee at the transition from helix αD to αE pointed out already by Chantalat *et al.* (1999) (Figure 3E). Whether this is functionally important and correlated with the conserved character of the amino acids in this region of CK2 β remains open at the moment.

Co-substrate binding. Conspicuously, only the active site of chain A1 is occupied by the co-substrate analogue AMPPNP while that of A2 is not. To rationalize this difference we superimposed the two rhCK2 α^A subunits on each other and on rmCK2 α in complex with AMPPNP (Niefind *et al.*, 1999) (Figure 3C; Table II). The comparison reveals the unexpected fact that the opening of the active site cleft is distinctly larger in the AMPPNP complexed form of rhCK2 α^A than in the other, and that in the rmCK2 α -AMPPNP complex the cleft is more similar to the empty and less open form of rhCK2 α^A . Obviously not only the opening state of the interdomain crevice but also other, more subtle structural adaptations determine the affinity of the active site for co-substrates.

A comparison of the AMPPNP molecules bound to rmCK2 α and to rhCK2 Δ demonstrates that the conformations of the triphosphate regions and the positions of the

γ -phosphate groups are very different (Figure 2D). While in rmCK2 α the bound AMPPNP is in an active conformation (Niefind *et al.*, 1999) this is obviously not the case in rhCK2 Δ . A possible reason for this difference is the loss of bound Mg $^{2+}$ ions, which are usually necessary to stabilize the cosubstrate conformation.

The autophosphorylation site. The main autophosphorylation sites of CK2 are Ser2 and Ser3 at the N-terminus of CK2 β (Figure 3D). The extent of autophosphorylation is strongly affected by mutations in the acidic loop from Asp55 to Asp64 (Boldyreff *et al.*, 1994), supporting the notion that in unphosphorylated CK2 the N-terminus of CK2 β is attached to the acidic loop and that this contact is broken by autophosphorylation. The rhCK2 Δ structure neither shows such a contact nor does it rule out this hypothesis: although the acidic loop and the N-terminus of rhCK2 β are >25 Å apart from one another (Figure 3D), large *B*-factors in both elements indicate a high flexibility, making conformations plausible in which they are attached to one another.

There has been speculation that autophosphorylation of CK2 may be a potential regulatory mechanism for the enzyme. However, according to Bodenbach *et al.* (1994) the consequences of autophosphorylation for the kinetic parameters are negligible. This observation is confirmed by the rhCK2 Δ structure, which shows that the distance from the autophosphorylation site to the nearest active site is >40 Å.

The acidic loop. The acidic loop (Asp55–Asp64) as part of an extended acidic groove (Figure 4) plays an important role in the modulation of CK2 activity. Essentially this loop down-regulates the activity of the enzyme, but neutralization or shielding of its negative charge by mutation of the negative residues to alanine (Boldyreff *et al.*, 1994), by addition of polylysine or by increasing the ionic strength, hyperactivates CK2. The negatively charged character of this loop is well conserved as is the basic cluster K 74 KKKIKR 80 within the αC region of CK2 α (Figure 1A), which is the potential binding site for the acidic determinants of CK2 substrates and for the strong inhibitor heparin. An interaction between these two regions within the CK2 holoenzyme was the first direct α/β contact to be postulated (Lozeman *et al.*, 1990).

This idea was supported by crosslinking and immunoprecipitation experiments (Krehan *et al.*, 1996), but biochemical and mutagenesis investigations (Boldyreff *et al.*, 1993) suggested a more complex picture. According to these studies CK2 β can protect CK2 α partially against inhibition by heparin. This protection is much less effective with CK2 β mutants in which the negative charges of the acidic loop are removed, supporting the notion that heparin and the acidic loop of CK2 β compete for binding to the basic cluster of CK2 α in agreement with the hypothesis outlined above. Additionally, however, Boldyreff *et al.* (1993) have shown that a hyperactivating CK2 β mutant with a neutralized acidic loop still retains the full potential to form a stable CK2 holoenzyme. Hence the ability of the acidic loop to down-regulate the activity of CK2 is not coupled to its integration in the α/β interface. This conclusion is supported by the rhCK2 Δ

Fig. 4. Electrostatic surface of rhCK2 Δ . The surface is coloured according to the electrostatic potential ranging from deep blue (positive charge) to red (negative charge). Atomic charges were assigned by GRASP (Nicholls *et al.*, 1991) using default values.

structure showing that the acidic loop of rhCK2 β is >30 Å distant from the nearest basic cluster of rhCK2 α .

If not by a direct attachment within the holoenzyme, how else does the acidic loop affect the activity of CK2? The electrostatic surface shown in Figure 4 illustrates the strong differences in the charge distribution at the outer surface of the rhCK2 Δ complex. In particular the interior of the active site cleft is highly positive, not only because of the basic cluster but also from the R⁴³KLGRGK⁴⁹ motif of the ATP binding loop. In contrast the acidic CK2 β loop forms a negatively charged, prominent wedge at one of the borders of the cleft. This charge distribution suggests two ideas: (i) the acidic loop might disturb the access of negatively charged substrates to the active site, or (ii) the interaction between the acidic loop and the basic cluster might involve subunits from different complexes, by plugging the negatively charged wedge of one CK2 complex into a positive active site cleft of a second one. The latter idea would be consistent with the propensity of CK2 to form higher-order aggregates (Glover, 1986).

Both these hypotheses are speculative at the moment. Structures of CK2 complexes including substrate mole-

cules or heparin would be helpful to obtain further insights into the functional roles both of the acidic loop and of the basic cluster.

Materials and methods

Production and crystallization of rhCK2 Δ

rhCK2 Δ was prepared and crystallized as described by Niefind *et al.* (2000). Briefly, optimal crystals grew at 12°C by vapour diffusion in sitting drops in which 3 μ l of an rhCK2 Δ preparation with 5.0 mg/ml protein in 25 mM Tris-HCl, 300 mM NaCl, 1 mM dithiothreitol pH 8.5, had been mixed with 3 μ l of 2.0 mM MgCl₂, 3 μ l of 1 mM AMPNP, 1 μ l of 10% (w/v) polyethylene glycol (PEG) 400 dodecylether and 1.5 μ l of reservoir solution composed of 20% (w/v) PEG 3350, 200 mM K₂HPO₄ pH 9.3.

X-ray diffraction data collection

X-ray diffraction data were collected at the EMBL beamline BW7b at DESY, Hamburg, using a MAR345 imaging plate detector. For the diffraction experiments a cryo-protectant solution containing 25% (w/v) PEG 3350, 25% (v/v) glycerol, 0.2 M K₂HPO₄, 0.1% (w/v) PEG 400 dodecylether was added in small portions to the crystallization drops until the resulting mixture was a suitable cryo buffer for the crystals.

The diffraction data (Table I) were collected from two crystals and processed with the HKL package (Otwinowski and Minor, 1997), followed by TRUNCATE (CCP4, 1994) to obtain structure-factor amplitudes. The processed data set was checked for merohedral twinning with CNS (Brünger *et al.*, 1998) but no indications of it could be detected.

Structure determination and refinement

The structure was determined by molecular replacement in combination with phase refinement techniques. Various search models for cross rotation and translation searches were extracted from the coordinates of rmCK2 α (Niefind *et al.*, 1999) and rhCK2 β ^A (Chantalat *et al.*, 1999).

Numerous molecular replacement searches were performed with AMoRe (CCP4, 1994) and CNS (Brünger *et al.*, 1998) at various resolutions. A significant solution was detected only with a poly-alanine backbone of rmCK2 α as search model and the locked [non-crystallographic symmetry (NCS) constrained] rotation option of AMoRe for the cross rotation step.

As we were unable to find a molecular replacement solution for CK2 β we started the refinement of the structure on the basis of the rhCK2 α ^A sub-structure. All subsequent refinement and density modification calculations were performed with CNS (Brünger *et al.*, 1998) while model building was performed with program O (Jones *et al.*, 1991).

To locate the CK2 β subunits, the initial phases based on the rhCK2 α ^A sub-structure alone were refined by NCS averaging and solvent flattening. In this way an improved electron density was achieved in which a long α -helix could be found in a region not occupied by the rhCK2 α ^A sub-structure. This helix was correctly interpreted as helix α D of rhCK2 β (Figure 3D) and served as a starting point for the final insertion of the complete molecule.

As it was impossible to apply the NCS operations of the rhCK2 α ^A sub-structure to the growing model of the rhCK2 β subunit, we located the second rhCK2 β chain independently of the first one, calculated the NCS matrix for the rhCK2 β sub-structure approximately and refined the NCS mappings for both sub-structures on the electron density level using DM (CCP4, 1994).

The refinement was governed strictly by following the R_{free} value (Brünger, 1992). Strong NCS restraints for atomic coordinates and B -factors were applied. Individual temperature factors were refined after a CNS run with the script 'optimize_rweight.inp' (Brünger *et al.*, 1998) had provided a suitable weighting factor to lower R_{free} significantly. During model building the coordinates of rmCK2 α (Niefind *et al.*, 1998) and rhCK2 β ^A (Chantalat *et al.*, 1999) were used as matrices as far as possible.

In retrospect we confirmed the orientation and position of CK2 β relative to CK2 α using the refined rhCK2 β monomers as search models in molecular replacement searches with CNS (Brünger *et al.*, 1998).

Structure interpretation and documentation

We used some CCP4 programs (CCP4, 1994) for structure interpretation and documentation purposes: PROCHECK to validate the refined complex, AREAIMOL to calculate solvent-accessible surfaces and

DYNDOM to detect domains and hinge axes. R.m.s.ds (Table II) were calculated with the least-squares-fit options of O (Jones *et al.*, 1991).

Figures 2 and 3 were prepared with BOBSCRIPT (Esnouf, 1997) and Raster3D (Merritt and Bacon, 1997) and Figure 4 with GRASP (Nicholls *et al.*, 1991). The coordinates and the structure factors have been deposited at the RCSB Protein Data Bank (ID code 1JWH).

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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