Structural basis of CK2 regulation by autoinhibitory oligomerization

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Keywords:

Crystal structure; symmetric CK2 holoenzyme; asymmetric CK2 holoenzyme; trimeric CK2 holoenzyme; oligomerization; filaments; activity regulation; autoinhibition; crystal contacts; α-subunit; β-subunit; $\alpha_2\beta_2$ tetramer; α/β interfaces; CK2β acidic loop; CK2α p+1 loop; CK2β C-terminal tail; electrostatic interactions; polyamines; autophosphorylation.

Abstract

The mechanism of regulation of CK2 differs from those common to other eukaryotic protein kinases and is not entirely established yet. Nowadays several crystal structures of the tetrameric $\alpha_2\beta_2$ holoenzyme are available, supporting a structural model of an autoinhibitory regulation by oligomerization proposed several years before on the basis of biochemical, biophysical and functional data. Monoclinic crystal forms of the holoenzyme reveal the symmetric architecture of the "free" isolated active tetramers. The dimension and the nature of the α/β interfaces configure the symmetric holoenzyme as a strong complex that does not spontaneously dissociate in solution, in accordance with the low dissociation constant ($\approx$ 4 nM). Hexagonal crystal forms of the CK2 holoenzyme show an asymmetric arrangement of the two α catalytic subunits around the obligate β_2 regulatory subunits. These asymmetric $\alpha_2\beta_2$ tetramers are organized in trimeric rings and filaments that correspond to inactive forms of the enzyme, whereby the β-subunit plays an essential role in the formation of inactive polymeric assemblies. The derived structural model of (down)-regulation by aggregation contributes to the interpretation of many available biochemical and functional data, although awaits for a more comprehensive validation at cellular level. These findings pave the way for the design of novel strategies aimed at the modulation of the CK2 activity.

The first asymmetric α₂β₂ holoenzyme crystal structure

The first crystallographic structure of the CK2 holoenzyme was determined in 2001 (PDB 1JWH) [1] joining those already known of the isolated α and β subunits [2-3], a great achievement finally solving the puzzling CK2 quaternary arrangement. CK2 tetramer has a butterfly shape with a central β-dimer and the two α subunits one on each side and not in contact between them (Figure 1a). This was also the first structure including the full-length β subunit that can be divided into a body and a C-terminal tail, the last one not included in previous structures. CK2β C-terminal tail has no contact with the body of its own chain but binds both the other CK2β monomer and one of the α subunits (Figure 1a). This tail contributes significantly to the stability of the β-dimer. The β/β interface increases from 543 $Å^2$ in the structure of the isolated and truncated β-dimer to 1766 $Å^2$ in the structure of the holoenzyme, in agreement with the obligate nature of the β-dimer. The authors also highlighted that this increase in the interface requires the CK2β tail in a specific conformation, which is stabilised by the involvement of the same tail in large contacts with the α subunits and hence depends on the existence of the complete tetramer. The CK2β tail has then a synergistic character, stabilizing both the β/β and the α/β contacts. At the same time, the authors pointed out that this region was not well defined in the electron density and then the very C-terminal amino acids (from Asn206 to Arg215) were left out from the final model while in the preceding region (from Phe190 to Ser205) the conformations of peptide groups and side chains remain questionable.

Each α subunit binds both subunits of the β-dimer, the body of one chain and the tail of the other (Figure 1a), this last interaction exploring a larger interface (491 $Å^2$ vs 336 $Å^2$) and being considered the major determinant of the α/β stability. The structure of the α catalytic subunit is not significantly affected by the binding of the β-dimer, maintaining its active conformation and confirming previous observations that CK2β is an environment- and substrate-dependent modulator of CK2α activity rather than an on/off switch. It was also noted that the tetramer is asymmetric with a relative rotation of 16.4° at the two α/β interfaces for the two halves of the tetramer (Figure 1a). As a consequence, the two α/β interfaces are different (Figure 1b) and this was interpreted as an

intersubunit flexibility. In the basis of such observation, together with an average size for the α/β interface of 832 Å^2, authors proposed that the CK2 tetramer is a transient heterocomplex.

Finally, in the same paper it was pointed out that the inhibitory effect of the CK2β acidic loop (Asp55-Asp64) observed in previous works [4] cannot be exerted intramolecularly since the distance between this and the closest CK2α basic cluster (the potential substrate binding site) was >30 Å.

A few years later, the same authors analysed the crystal packing in the previous structure showing that the contact between the β-acidic loop and the α-basic cluster was indeed present, but between different tetramers [5]. They identified a new CK2 organisation, composed by self-consistent ring-like trimers of tetramers, where the core of the interaction between different tetramers in $(α_2β_2)_3$ is formed by the interface between the β-acidic loop and the α p+1 loop (Arg191-Lys198) at the entrance of the α active site (Figure 1c). As a consequence, three out of six catalytic subunits of the trimeric ensemble are blocked by the β acidic loop.

This organisation in trimers of tetramers was able to explain a number of previous observations not easily interpretable solely on the basis of the tetrameric structure. Mutations in the β-acidic loop or in the α p+1 loop generate hyperactive CK2 holoenzyme [4, 6], since trimers of tetramers cannot form anymore. Polyamines like spermine activate CK2 [7-9] by interacting with the CK2β acidic loop and then disrupting the trimers of tetramers. The CK2 holoenzyme exhibits maximum activity at about 300 mM NaCl or KCl, a property of the holoenzyme and not of the isolated CK2α [10]. The holoenzyme also needs a minimal NaCl concentration of 250 mM NaCl to bind to phosphocellulose, while it aggregates under low salt conditions [11]. Since the interaction between the β acidic loop and the α p+1 loop is electrostatic in nature, it is strongly affected by ionic strength. Finally CK2 holoenzyme has been observed in different ring-like and linear polymeric forms connected via reversible equilibria affected by ionic strength and spermine [12-13].

The second asymmetric α2β2 holoenzyme crystal structure

In 2012 we presented a new structure of the holoenzyme (4DGL) crystallized in the same space group and unit cell of the previous 1JWH structure, but with higher overall quality [14]. While confirming may features of the 1JWH structure, the 4DGL structure shows significant improvements in important regions such as the α/β interface and the C-terminal tail of the β subunit. In particular, β-Phe190 leans on the outer surface of the a N-terminal lobe in 1JWH, while in 4DGL it inserts deeply into a hydrophobic cavity in the α subunit, known as the secondary or allosteric binding site (Figures 2a and 2b). The α/β interaction is then significantly strengthened in accordance with the almost complete abrogation of the binding for the mutant β-Phe190Ala [15]. Also the β C-terminus was build differently with an upstream shift in sequence of two amino acids and extended the αG helix from residues 196-200 in 1JWH to residues 194-207 in 4DGL (Figure 2b). This again contributes to refine the α/β interface to a better energetic profile (interface increased from 832 Å^2 in 1JWH to 1010 Å^2 in 4DGL (Figure 2c). Finally the β C-terminus was build to the last Arg215 amino acid.

As already noted by Niefind and Issinger [5], the acidic loop of the β-subunit from one tetramer is in contact with the p+1 loop of the α subunit from another tetramer (Figure 3a). The interface area is 651 Å^2, larger than in 1JWH (538 Å^2) and greater than normal crystallographic contacts, which have a mean interface of 285 Å^2 and rarely exceed 500 Å^2 [16]. This interaction is essential for the organization of circular trimers of tetramers as previously observed (Figure 1c), with a positive cooperative effect for their formation and a large interface of 1953 Å^2 (651 Å^2 x 3).

The newly traced C-terminus of CK2β is instead responsible of a different inter-tetrameric interaction. This region protrudes from one tetramer and contacts the C-terminal lobe of an α subunit of a neighbouring tetramer ending in its ATP pocket (Figures 3a and 3b). The interface is large (753 Å^2) and is complemented by the interaction between the N-terminal lobe and the C-terminal lobe of two α chains from the same two tetramers. The overall interface of 1254 Å^2 is then quite extended and again much larger than a normal crystallographic contact. It shows a number of polar contacts, which again makes this interaction susceptible to ionic strength changes. This new

interaction is fundamental for a piling organization of trimers of tetramers (Figures 3c) and the newly identified polymeric form can represent the filamentous CK2 aggregates described in literature [12-13]. Moreover, the β C-terminus inserts into the ATP binding pocket of a catalytic subunits, competing with ATP and stabilizing a non-productive conformation of the α catalytic residues (Figure 3b). This polymeric form is then expected to be completely inactivated by the combination of the interactions of the α subunits with the acidic loops and C-termini of β subunits (Figure 3a), and by steric hindrance.

This new structure and the hypothesized organization in trimers of tetramers and subsequently in filaments allow expanding the experimental data previously reported in support of the trimeric organization by Niefind and Issinger [5]. FRET experiments revealed the existence *in vivo* of two subpopulations of "fast" and "slow moving" CK2 and showed that different CK2 β-dimers can be in close molecular proximity [17]. The down-regulating effect proposed for the CK2β C-terminal tail is in accordance with other *in vivo* observations. CK2 activity is increased in prostate cancer cells by CDK1 phosphorylation of β-Ser209 [18] that engages α-Glu230 in the filamentous organization, which will then be weakened by the phosphorylation event. Phosphorylation of β-Thr213 by Chk1 is observed in the free β subunits but not in the tetrameric holoenzyme [19], where the residue is masked in the filamentous oligomers. The down-regulatory polymerization mechanism mediated by the β subunits is also supported by the observation that transfection of HeLa cells with a degradation-resistant CK2β strongly inhibits proliferation and counteracts promotion of proliferation exerted by the isolated CK2α [20].

It is well known that CK2 autophosphorylates on residues Ser2 and Ser3 of the β-chain [21]. As already observed by Niefind and Issinger [5] and further discussed in [14], the β N-terminus is close to an α catalytic site in the trimeric organization, so that one tetramer could phosphorylate the neighbouring one (Figure 3a). Observations in favour of this trans-autophosphorylation mechanism inside the trimeric organization are the following: autophosphorylation (a) is abolished at high ionic strength [22], (b) is strongly inhibited by neutralization of residues in the β-acidic loop [23], (c)

although being in trans, it shows an intramolecular kinetic [24], (d) no autophosphorylation occurs in the holoenzyme with the α′ paralog, a holoenzyme that does not form oligomers [25]. We also proposed that the β N-terminus, once phosphorylated, could engage the nearby αC basic cluster of the α subunit from the neighbouring tetramer, further stabilizing the ring-like organization. Autophosphorylation is (e) extensively observed in cells [21], (f) stabilizing the enzyme *in vivo* [26] and (g) having an inhibitory effect, since treatment of CK2 with phosphatases increases its activity [27].

The symmetric α₂β₂ holoenzyme crystal structures

Very recently, we added to the above picture three additional crystal structures of the $\alpha_2\beta_2$ holoenzyme [28]. In both the 1JWH and 4DGL structures, the $\alpha_2\beta_2$ tetramer is asymmetric. Notably, in the new structures (4MD7, 4MD8 and 4MD9), obtained in monoclinic (rather than hexagonal) space groups, the tetramer is symmetric (Figure 4a). This reveals that the asymmetry observed in the previous structures is not an intrinsic structural property of the monomeric holoenzyme, but rather is generated by the incorporation of tetramers into trimeric rings, whose assembly is not compatible with the symmetric architecture (Figure 4b). The analysis of the crystal packing of the monoclinic form of the holoenzyme does not seem to support the possibility of stable oligomeric or filamentous forms. Unlike in the hexagonal lattice, where discrete trimeric rings of tetramers are clearly recognizable, in the monoclinic form all contacts between different tetramers have a pure crystallographic nature. Indeed there is one interaction between the acidic loop of the β-subunit and the basic clusters of the α-subunit of a symmetric tetramer with a significant interface area (around 750 Å^2). However, this contact is not responsible for the formation of any closed, self-consistent, oligomeric form of tetramers, nor of a filament of tetramers. Rather it is responsible for the propagation of two parallel layers of tetramers in two dimensions for the entire crystal lattice (i.e. infinitively), giving raise to a sort of infinite two-dimensional bilayer of tetramers. Hence, it has the characteristic of a crystal lattice contact, making highly improbable, in our opinion, the existence of

stable discrete oligomeric or filamentous forms based on this interaction. We then concluded that the new symmetric structures represent the organization of the "free", active, monomeric CK2 holoenzyme, the previous 1JWH and 4DGL showing instead the structure of inactive trimers of tetramers. However, it has to be noted that some of the inter-tetrameric interactions typical of the monoclinic crystal packing, observed also for the CK2[andante] holoenzyme mutant [29], have been postulated to be at the basis of other possible (linear) assemblies with physiological significance [30].

The comparison of symmetric and asymmetric tetramers in terms of their α/β interfaces and then of their stability is of particular relevance since the possibility that the CK2 holoenzyme coexists in equilibrium with its free constituent subunits can represent a regulatory mechanism for its biological function. In 1JWH, the two α/β interfaces are significantly different in size, one being larger (960 $Å^2$) and the other significantly smaller (771 $Å^2$) [1] (Figure 1b). This suggested that the CK2 holoenzyme might be a transient heterocomplex, spontaneously dissociating *in vivo*. This seems in contrast with the well-known elevated stability *in vitro* of the complex ($K_D \approx 4$ nM) [31-32]. In 4DGL, the better-traced β C-terminal tail generated larger interfaces with similar sizes (1009 and 1011 $Å^2$); however, due to the asymmetric assembly, the two interfaces are different in nature with different residues involved [14] (Figure 2b).

The new symmetric structures have very similar α/β interfaces both in size (1099 and 1073 $Å^2$) and chemical nature (Figure 4c). This was also confirmed by PISA ('Protein interfaces, surfaces and assemblies' service at the European Bioinformatics Institute [33]) that predicted a dissociation pattern in $2\alpha + \beta_2$ from monoclinic structures, but in $\alpha\beta_2 + \alpha$ for both 1JWH and 4DGL as a consequence of their asymmetry. The interface size is the most commonly used parameter to infer the stability of a complex; indeed also its chemical and sterical nature (hydrophobicity and complementarity) needs to be considered [34]. Evaluating a number of parameters (reported in Table 1), we proposed that the CK2 holoenzyme configures as a non-obligate permanent heterocomplex. In obligate complexes, the constitutive protomers are not stable in the isolated form

and they simultaneously fold and bind to their partner [35-36], as in the case of the CK2β dimer. Obligate complexes have interface areas generally much larger than 1000 Å^2 [37]. The CK2 holoenzyme is obviously a non-obligate complex. Permanent protein-protein interactions are strong, with nanomolar or subnanomolar dissociation constants, and irreversible, normally only perturbed by proteolysis [35-36]. Transient complexes can instead dissociate in their constituent subunits and can be distinguished in weak or strong [35-36]. In strong transient complexes, a molecular trigger (i.e. phosphorylation or effector molecule) drives the equilibrium through a large conformational change between a high affinity state (low or sub nanomolar K_D) and a low affinity state (micromolar K_D). Weak transient complexes have instead micromolar dissociation constants and are characterised by a dynamic equilibrium where interaction is formed and broken continuously; their interfaces are small (mean interface for weak transient complexes was determined in 740 Å^2 by Nooren and Thornton [37] and in 810 Å^2 by Dey and co-workers [34]). Given those data, with a $K_D \approx 4$ nM and an interface area of 1086 Å^2, CK2 cannot be considered a weak transient complex. It is however impossible to distinguish between permanent and strong transient complexes solely on the basis of the dissociation constant and the interface paramenters [34]. Despite that, since no molecular triggers able to alter the affinity of the CK2 protomers have been identified so far and no significant structural rearrangements of the individual subunits are observed during the formation of the CK2 holoenzyme complex, the $\alpha_2\beta_2$ tetramer should be considered a permanent complex, until proven otherwise.

Indeed isolated α and β subunits have been observed in cells [38]. However, as underlined above, crystallographic data, the K_D and the stability of the enzyme *in vitro* strongly argue against the possibility that the CK2 $\alpha_2\beta_2$ holoenzyme can spontaneously dissociate in solution. Instead, the existence of "free", isolated α and β_2 subunits can be associated to different factors such as unbalanced expression, differential localization or transport mechanism and to competitive binding with other partners, like the A-Raf, c-Mos and Chk1 kinases all reported to interact with CK2β possibly using the same interface explored by CK2α [39]. As already stated, molecular triggers

capable to dissociate the complex have not been identified so far. We also excluded by crystallography that this molecular switch could reside in the phosphorylation of the CK2α C-terminal tail (phosphorylated by Cdk1 at mitosis [40]).

Working model of CK2 regulation by autoinhibitory oligomerization

The overall regulatory model emerging from structural studies and in accordance with many biochemical, biophysical and activity data, is depicted in Figure 5. When co-localised in time and space, CK2α and CK2β-dimer spontaneously assemble into an active, symmetric, and permanent complex with a $K_D \approx 4$ nM. Enzymatic activity is turned on/off by the equilibrium between the active tetramers and the inactive asymmetric trimers of tetramers that can further polymerize in higher supramolecular assemblies. These represent the latent form of the kinase in accordance with the observation that most of CK2 in crude liver extract is inactive [41]. As for the majority of protein kinases, activity is restored only upon necessity, in this case by a depolymerization process generating active free tetramers. This process can be triggered by different events like β dephosphorylation, physical-chemical variations of the environment (pH, ionic strength, etc.) and/or action of substrates themselves or other effectors (highly charged macromolecules or low-molecular-weight compounds). When the stimuli end, CK2 tetramers are assembled back in trimers and then in fibrous polymers, with the turning off of the catalytic activity. The β subunit is fundamental in this process driving both the assembly in trimers of tetramers (via the acidic loop) and in filaments (via its C-terminus), fully deserving the name of "regulatory" subunit.

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Table 1. α/β interface parameters in symmetric $\alpha_2\beta_2$ holoenzyme

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Parameter	Value	Comment
Mean Interface Area	1086 Å^2	740-810 Å^2 for weak transient complexes
f_{np} (non polar fraction of interface area)	70.5%	Largely hydrophobic
P-value (PISA solvation energy gain)	0.109	Largely hydrophobic
R_p (residue propensity score)	9.8	Typical of "strong" complexes
f_{bu} (fraction of fully buried atoms)	30%	High shape complementarity
S_c (shape complementarity score)	0.68	High shape complementarity
I_{gap} (gap volume index)	1.6	High shape complementarity
L_D (atomic density index)	38.7	High shape complementarity

Figure legends

Figure 1. Structure of the CK2 holoenzyme (PDB: 1JWH) [1]. a) The CK2 holoenzyme has a butterfly shape with the two catalytic α-subunits not in contact between them. The CK2β tails have a fundamental role in the holoenzyme both by strengthening the β-dimer and by largely contributing to the α/β interface. The holoenzyme is asymmetric with a relative rotation of 16.4° for the two halves of the tetramer. b) The asymmetric assembly generates two different α/β interfaces. Adapted with permission from [28]. Copyright (2014) American Chemical Society. c) The holoenzyme assembly in circular trimers of tetramers has been identified in the crystallographic packing [5]. The core of the interaction is between the β acidic loop and the α p+1 loop at the entrance of the catalytic site.

Figure 2. Details of the α/β interaction in the improved structure of the CK2 holoenzyme (4DGL) [14]. a) In 4DGL, β-Phe190 (salmon) inserts into a deep hydrophobic pocket on the CK2α surface (green), differently from the 1JWH structure (orange). Adapted with permission from [14]. Copyright (2012) American Chemical Society. b) In 4DGL, β C-terminus was built to the last Arg215 and region 194-205 was better modelled in respect to the 1JWH structure; color code as in a). Adapted with permission from [14]. Copyright (2012) American Chemical Society. c) In the improved 4DGL structure, the two α/β interfaces are larger than in 1JWH (Fig. 1b). Adapted with permission from [28]. Copyright (2014) American Chemical Society.

Figure 3. Oligomeric organization in the 4DGL structure. Adapted with permission from [14]. Copyright (2012) American Chemical Society. a) Different inter-tetrameric contacts can be identified. The interaction between the β acidic loop (β subunit in cyan with its acidic loop in yellow) and the α P+1 loop (α subunit in orange with its P+1 loop in green) is responsible for the assembly in circular trimers of tetramers. The interaction between the αG and the C-terminal tail of

a different β subunit (purple) and the α C-terminal lobe and active site (orange) is responsible for a piling organization of trimers of tetramers. Position of the first amino acid visible at the β N-terminus (Val7) is compatible with the autophosphorylation of the upstream residues Ser2 and Ser3. Phosphorylated residues could then interact with the α basic cluster (red) b) Insertion of the β C-terminus into the α active site disrupts the correct orientation of α residues involved in nucleotide binding. c) In the filamentous organization, C-termini of three β subunits are plugged into the following trimer.

Figure 4. Structural features of the symmetric tetrameric holoenzyme. Adapted with permission from [28]. Copyright (2014) American Chemical Society. a) Structure of symmetric tetramer in salmon (4MD7) [28] superposed to the asymmetric 4DGL tetramer (cyan). b) In 4DGL the assembly of three tetramers (green, cyan and purple) in trimers of tetramers induces the distortion not present in the symmetric tetramer (salmon). c) In the symmetric tetramer, the two α/β interfaces are very similar in size and nature.

Figure 5. Proposed model of CK2 regulation. Reprinted with permission from [28]. Copyright (2014) American Chemical Society. CK2 α and β subunits readily assemble into active and symmetric tetramers. Activity is turned off through organization in inactive trimers and filaments. Activity is restored upon a depolymerazion process induced by variations in the physical-chemical environment, action of substrates or effectors, or other events.