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Structural Determinants of Protein Kinase CK2 Regulation by Autoinhibitory Polymerization

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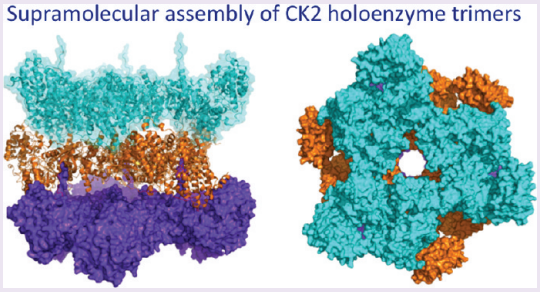
Supporting Information

ABSTRACT: CK2 is a Ser/Thr protein kinase essential for cell viability whose activity is anomalously high in several cancers. CK2 is a validated target for cancer therapy with one small molecule inhibitor in phase I clinical trials. This enzyme is not regulated by mechanisms common to other protein kinases, and how its activity is controlled is still unclear. We present a new crystal structure of the CK2 holoenzyme that supports an autoinhibitory mechanism of regulation 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 biochemical and functional data and paves the way for new strategies in the modulation of CK2 activity and for the design of non-ATP-competitive inhibitors targeting the interaction between the α catalytic and the β regulatory subunits.



The enzyme CK2 is a pleiotropic, acidophilic, highly conserved Ser/Thr protein kinase essential for cell viability.[1] CK2 is involved in many cellular processes such as cell cycle progression, gene expression, cell growth and differentiation, embryogenesis, circadian rhythms, and apoptosis.[2] Abnormal high levels of CK2 enzymatic activity have been found in a large variety of solid and hematological tumors.[3] Its oncogenic potential depends mainly on the capacity to act as an antiapoptotic agent.[4]

In vivo, the fully functional form of CK2 is considered the $\alpha_2\beta_2$ holoenzyme, a heterocomplex of 140 kDa composed of two catalytic α-subunits and two regulatory β-subunits. The structures of the isolated β_2 dimer and α-subunit are known.[5−9] One crystal structure is available for the holoenzyme, dated 2001, at 3.1 Å resolution (PDB code 1JWH[7]). It shows the constitutive β_2 dimer recruiting two catalytic α-subunits on opposite sides, in a "butterfly" shaped complex. Both isolated α-subunit and holoenzyme are catalytically active *in vitro*. The formation of the holoenzyme causes only minor conformational changes in the protomers, leaving unaltered the structural determinants for an effective catalysis, already present in the isolated α-subunit.

Protein kinases are strictly regulated by mechanisms such as phosphorylation or dephosphorylation events, second messenger binding, and reversible association with regulatory subunits. So far, none of these conventional mechanisms has been found applicable to CK2, and the regulation of CK2 activity is still a matter of debate.[10] On the basis of much evidence that CK2 can form high-order aggregates, it was proposed that its aggregation propensity could have a regulatory function.[11]

Herein we present a new crystal structure of the human CK2 holoenzyme (hereinafter $\alpha2\beta2^{new}$), of higher overall quality with respect to 1JWH, improved especially in important regions such as the α/β interface and the C-terminal tail of the β subunit, now entirely traced until the last Arg215.

The human CK2 holoenzyme $\alpha2\beta2^{new}$ is composed of the dimer of the full β-subunit and by two C-terminally truncated α-chains, carrying the point mutation Tyr125Arg, for a total molecular weight of 129.8 kDa. $\alpha2\beta2^{new}$ has been refined at the nominal resolutions of 3.0 Å in the same space group ($P6_3$) and unit cell of 1JWH. Most importantly, the quality of the final electron density map of $\alpha2\beta2^{new}$ is significantly higher than that of the published holoenzyme, allowing a better definition of some functionally relevant regions. Examples of the final electron density are reported in Supplementary Figure 1. The higher global quality of $\alpha2\beta2^{new}$ is also underlined by lower crystallographic R/R_{free} values (19.5/22.2 *vs* 26.7/33.8) and is mainly due to the better quality of the diffraction data used in the refinement process, in particular in the completeness (99.7 *vs* 78.7%), redundancy (19.1 *vs* 8.8), and $I/\sigma(I)$ (23.9 *vs* 16.7) (Table 1), also taking advantage of the highly performing ESRF ID23-2 Microfocus Beamline.

$\alpha2\beta2^{new}$ and 1JWH show the same architecture, with rmsd values over all Cα atoms of 1.0 Å. However, we noticed some significant differences in the α/β interfaces (Figure 1). In 1JWH, β-Phe190 leans on the outer surface of the α N-terminal lobe, nearby the secondary or allosteric binding site, a

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Table 1. Data Collection and Refinement Statistics

Data Collection[a]	
space group	$P6_3$
unit cell *a*, *b*, *c* (Å)	175.65, 175.65, 96.26
resolution (Å)	44.9−3.0 (3.16−3.0)
total observations	649580
unique observations	34036
R_{sym}	0.122 (0.990)
R_{meas}	0.125 (1.016)
R_{pim}	0.029 (0.231)
completeness (%)	100 (100)
multiplicity	19.1 (19.2)
mean $I/(\sigma)I$	23.9 (3.1)
solvent content (%)	62.7
B_{Wilson} (Å^2)	82.9
Refinement	
total no. of atoms	8877
no. of waters	12
resolution (Å)	44.9−3.0
R_{work}/R_{free}	0.195/0.222
av *B*-factor (Å^2)	92.2
rmsd bond (Å)	0.014
rmsd angle (deg)	1.597

[a]Numbers in parentheses refer to the highest resolution shell.

hydrophobic cavity filled with a water molecule. In $\alpha 2\beta 2^{new}$, β-Phe190 inserts into this deep cavity, increasing and strengthening the α/β interaction, in accordance with the almost complete abrogation of the CK2α binding for the single-point mutant β-Phe190Ala.[13] Moreover, just downstream from Phe190, the β C-terminus (from residue 194 on) was built differently from 1JWH, with an upstream shift in sequence of two amino acids and extending helix-αG (residues 196−200 in 1JWH) to residues 194−207, again with better energetics at the α/β interface.

Prompted by those discrepancies, we checked the 1JWH structure against its deposited structure factors and noticed that both β-Phe190 and the β C-terminus could be better modeled, in accordance to our structure, with an increase in the average α/β interface from 832 to 946 Å^2. These α/β interfaces are mostly hydrophobic, as indicated by the PISA *P*-values of the solvation energy gain below 0.5 (around 0.1−0.15).[14] Design of new β-competitive small molecule inhibitors will benefit from the $\alpha 2\beta 2^{new}$ enhanced α/β interface.

Unlike in 1JWH, in $\alpha 2\beta 2^{new}$ we could trace the entire C-terminus (until Arg215) of one β chain. This crosses a large section of the C-terminal lobe of one α-subunit in the neighboring tetramer, ending in its ATP-binding pocket (Figures 2a and 3a). The interaction is quite extended (interface area 753 Å^2), involving elements from both the N-terminal and the C-terminal lobes of the α-subunit. The interface is mainly hydrophobic, but some polar interactions are also presents (*P*-value 0.467) (Figure 2b). This interaction is complemented by another contact between the same two tetramers that involves exclusively the C-terminal and the N-terminal lobe of two α chains (Figure 2a), with an additional interface of 501 Å^2 and a *P*-value of 0.567, for a total interaction area of 1254 Å^2. In 1JWH, the eight extra amino acids newly build downstream of Ser205 take a different route because of the presence of a molecule of AMPPNP in the active site, whose γ-phosphate is in close proximity to β-Thr213 (Figure 2C). Consequently, this interface is slightly smaller (680 Å^2) than that seen in $\alpha 2\beta 2^{new}$, while the α/α interface is instead very much similar, for a total interface of 1181 Å^2.

This interaction (interface area 1254 Å^2) is much more extended than those in normal crystallographic contacts that have a mean interface area of 285 Å^2 [15] and rarely exceed 500 Å^2.[16] This indicates that such interaction, with an interface even greater (albeit less hydrophobic) than the intratetramer α/β one, can take place also in solution and can have a functional significance.[17,18]

The acidic loop of the β-subunit (Asp55−Asp64) is in contact with the P+1 loop of the α-subunit (Arg191−Lys198) of another tetramer (acidic/P+1 interaction). The total interface is 651 Å^2, larger than in 1JWH (538 Å^2) and again greater than a common crystallographic contact. Noteworthy, the α-subunit in contact with the β acidic loop simultaneously interacts also with the β C-terminal tail of a third tetramer, as shown in Figure 3a. Consequently, its catalytic site is completely occupied by two beta elements from two different tetramers, the acidic loop and the C-terminal tail. The acidic/P+1 interaction is essential for the organization of circular trimers of tetramers, as indicated by Niefind and Issinger [12] (Figure 3b), with a positive cooperative contribution for their

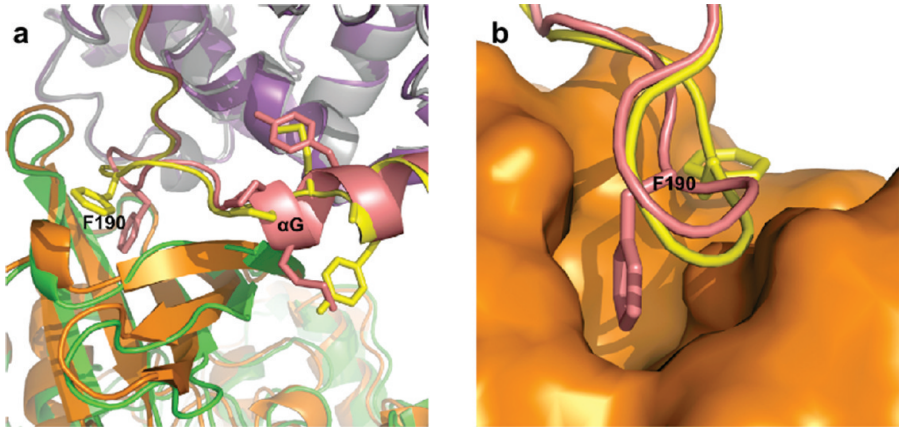

Figure 1. New details in the intratetrameric α/β interface. (a) $\alpha 2\beta 2^{new}$ (α in orange, β dimer in salmon and violet) superimposed on 1JWH (α in green, β dimer in yellow and gray). Position of F190 and registry of αG in the β subunit have been optimized. (b) β-F190 inserts into the α allosteric binding site, covering a relevant role in defining the α/β interface.

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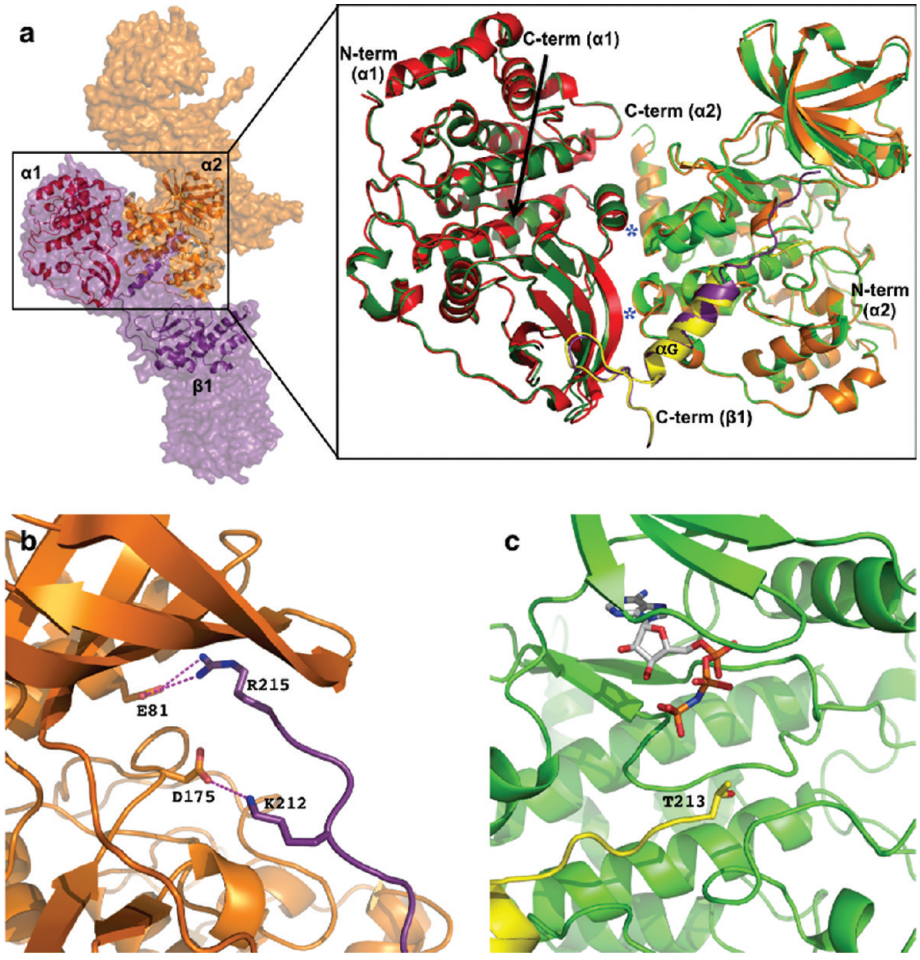

Figure 2. Inter-tetrameric interaction mediated by the β C-terminus. (a) Two interacting tetramers in $\alpha 2\beta 2^{new}$ are shown as violet (tetramer 1) and orange (tetramer 2) surfaces; on the right is an enlarged view with $\alpha 2\beta 2^{new}$ ($\alpha 1$ in red and $\alpha 2$ in orange, $\beta 1$ in violet) superimposed to revised 1JWH (in dark and light green $\alpha 1$ and $\alpha 2$, respectively; $\beta 1$ in yellow). αG of the β subunit of tetramer 1 interacts with the C-terminal lobe of the α subunit of tetramer 2. The $\beta 1$ C-terminus takes a different route in $\alpha 2\beta 2^{new}$ with respect to 1JWH. Blue stars indicate the $\alpha 1$-$\alpha 2$ interfaces. (b) In $\alpha 2\beta 2^{new}$, the $\beta 1$ C-terminus (violet) inserts into the $\alpha 2$ (orange) ATP binding site. Interactions β1Arg215/α2Glu81 and β1Lys212/α2Asp175 disrupt the correct orientation of alpha residues involved in nucleotide binding. (c) In revised 1JWH, $\beta 1$ C-terminus (yellow) reaches $\alpha 2$ (green) catalytic residues. β1Thr213 is in proximity of the AMPPNP γ-phosphate.

formation and a total interface of 1953 Å^2 (651 Å^2 × 3). This ring organization is in accordance with the well documented existence of an electrostatic interaction between the β acidic loop and the α P+1 loop and with its dependence on salt concentration.[12]

In trimeric oligomers the β N-terminus is close to an α-catalytic site (Figure 3a), suggesting that this organization greatly favors the well-known *in vivo* autophosphorylation of Ser 2 and 3 of the β-chain. This is supported by the following data: (a) autophosphorylation is crucially dependent on the formation of oligomers and is abolished at high ionic strength (>0.3 M)[19] when trimers dissociate; (b) neutralization of residues in the β-acidic loop have a strong inhibitory effect on autophosphorylation;[20] (c) although clearly in trans, between different tetramers, it shows a intramolecular kinetic,[21] compatible with a catalytic event taking place at the level of the trimeric organization; (d) no β autophosphorylation occurs in the case of the holoenzyme with the paralog α' instead of the α subunit, a holoenzyme that does not show any tendency to form aggregates, even at low salt.[22] Once phosphorylated, the very acidic β N-terminus (MS^{P}S^{P}SEEVS) could move toward the nearby region of the αC basic cluster (Figure 3a), with the formation of further electrostatic interactions that strengthens the ring-like organization. Importantly, CK2 is found extensively autophosphorylated when obtained from cells,[23] autophosphorylation has a strong positive impact on the stability of the enzyme *in vivo*,[24] and the treatment with protein phosphatases of native CK2 from liver cytosol enhances its specific activity,[25] all observations consistent with the existence of this kind of trimeric organization *in vivo*.

In the trimeric ring, three out of six catalytic subunits are essentially hindered to substrates due to the acidic/P+1 interaction, with a consequent downregulatory effect. Moreover, the N-terminal portion of the β-subunit (until the acidic loop), which presents docking sites for several CK2 substrates[26] and is exposed in the tetramer, is mostly inaccessible in oligomeric trimers, locating in their interior. Consistently, holoenzyme mutants with neutralization of acidic loop residues are hyperactive and show a salt dependence activity profile similar to that of isolated α,[27] suggesting the inability to form trimers.

In addition, here we show that the α subunit in contact with the β acidic loop can simultaneously accommodate the C-terminus of the β subunit from a third neighboring tetramer in

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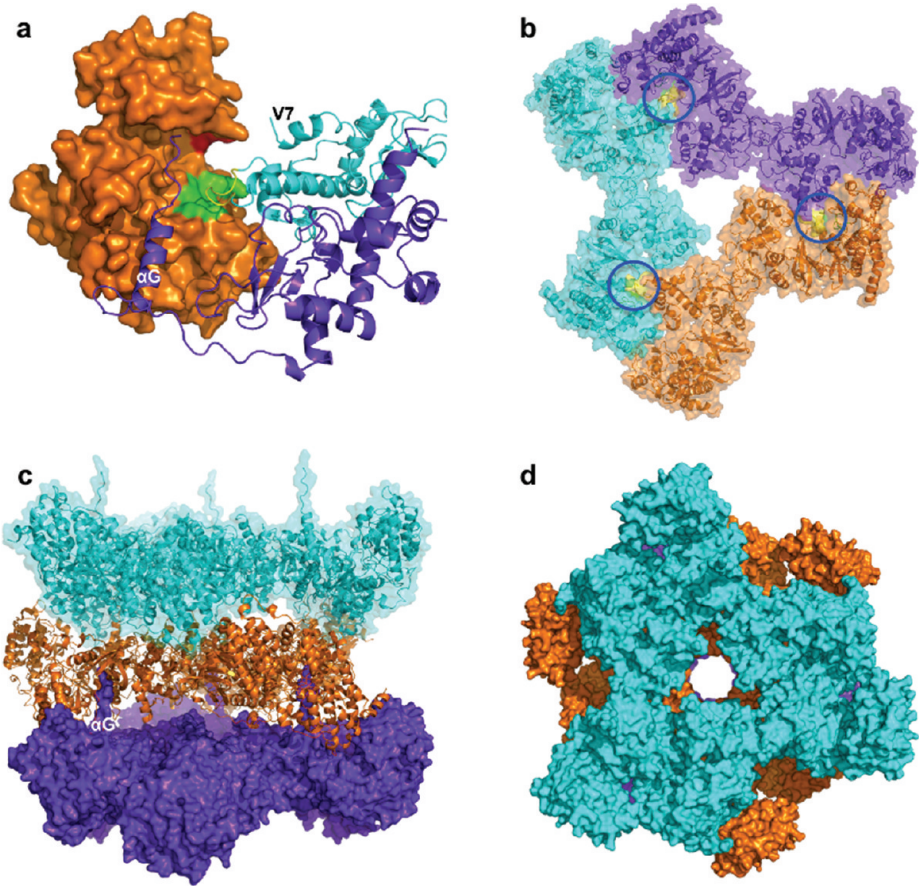

Figure 3. CK2 autoinhibitory mechanism. (a) CK2α (orange) is inhibited by the αG of a β subunit (violet) that brings its C-terminus in α nucleotide binding site and by the acidic loop (yellow) of another β subunit (cyan) interacting with α P+1 loop (green). Position of the first amino acid visible at the β N-terminus (Val7) is compatible with the autophosphorylation of the upstream residues Ser2 and Ser3. Phosphorylated residues could then interact with the basic cluster of the α subunit (red). (b) Trimeric organization of CK2 tetramers. The trimeric assembly is mainly based on the acidic/P+1 interaction (circled in blue). (c) Portion of the filamentous assembly. C-termini of three β subunits are plugged into the following trimer. (d) Hexagonal geometry along the piling axis. Each trimer is rotated of 60° with respect to the following one.

its ATP-binding site (Figure 3a). In $\alpha2\beta2^{new}$ the β C-terminus competes with ATP for the binding to the α subunit. Further, once inserted in the ATP-binding pocket, it stabilizes a nonproductive conformation of residues involved in catalysis. β-Arg215 replaces α-Lys68 in its interaction with α-Glu81 from the αC helix (the Lys68-Glu81 ion-pair is essential for an efficient catalysis and is fully conserved in protein kinases), while β-Lys212 interacts with α-Asp175, which is not more available for Mg^{2+} binding as in the fully functional state (Figure 2b).

This new interaction is fundamental for a piling organization of trimers, as shown in Figure 3c and d, with each trimer rotated of 60° with respect to the preceding one, to give a final hexagonal geometry along the piling axes. In this filamentous organization, half of the α-subunits are inhibited by the presence of the C-terminus and the acidic loop of the β subunit in the α active site, while the remaining half are inhibited by steric hindrance. This polymeric form is therefore expected to be completely inactive and can well represent the filamentous CK2 aggregates described in literature.[11,28] Like the acidic/P+1 interface, also the interface between the β C-terminal and α active site has a relevant polar character, and consequently both interactions and the polymeric organization are favored at low ionic strengths. It is well-known that the holoenzyme can be kept nonaggregated only at high ionic strengths, around 0.5 M, and starts to aggregate below 0.25 M. Hence, at physiological ionic strengths, the holoenzyme is expected to be essentially in some aggregated form. It is conceivable assuming that these autoinhibited, ordered polymeric aggregates can have physiological relevance and that variations in the local environment can modulate their equilibrium. Indeed, FRET-based experiments in living cells showed the presence of high-order structures of the CK2 holoenzyme *in vivo* and revealed the existence of two subpopulations of fast and slow moving forms of CK2,[29] supporting our model of CK2 oligomerization and polymerization. Interestingly, it was early observed that most of CK2 in crude liver cytosol was inactive, its activity being triggered (unmasked) by ion exchange chromatography at high ionic strength,[30] hampering electrostatic interactions. In accordance with a down-regulatory polymerization mechanism mediated by the β-subunit, transfection of HeLa cells with a degradation-resistant CK2β strongly inhibits proliferation, counteracting the promotion of proliferation exerted by isolated CK2α.[31] The functional role of the newly described, down-regulating interaction of the β C-tail is in accordance with other *in vivo* observations. Phosphorylation of β-Ser209 by Cdk1/cyclin B enhances CK2 activity in prostate cancer cells.[32] This can be explained by the weakening of the interaction in trimers between β-Ser209 and α-Glu230, residues at only 3.0 Å in $\alpha2\beta2^{new}$. Moreover, phosphorylation of β-Thr213 by Chk1

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affects the free β-subunit but not the holoenzyme[33] where, in the trimeric organization, this residue is not accessible.

The existence of aggregated forms of CK2 is largely supported by *in vitro* and *in vivo* biochemical and functional data accumulated in decades of CK2 research. The polymeric assembly of tetramers disclosed here accounts for all those experimental data. The $\alpha 2\beta 2^{new}$ structure reveals the structural basis of a polymerization mechanism responsible for the complete down-regulation of CK2 catalytic activity. The following model can be postulated: α and β_2 protomers readily associate to give the tetrameric $\alpha_2\beta_2$ holoenzyme that, in the cellular environment, cooperatively self-organizes in trimeric rings thus favoring the autophosphorylation of β, which in turn stabilizes the trimeric interaction. Due to a significant structural complementarity, trimers can pile one over another, giving rise to a polymerized form of CK2 that constitutes the latent, fully inactive form of this kinase. As for most protein kinases, CK2 activity is restored only upon necessity, in this case by a depolymerization process originating fully functional free tetramers. This can be triggered by a variety of events such as β dephosphorylation, variations in the physical-chemical local environment (pH, ionic strength, *etc.*), action of substrates or effectors (*e.g.*, highly charged macromolecules or low-molecular-weight compounds) or a combination of those. When the stimuli end, CK2 activity is turned off by the incorporation of free tetramers into fibrous polymers. The β-subunit plays an essential role in the assembly of both trimers (*via* the acidic loop) and the polymers (*via* the C-terminus), eventually fully deserving the name of "regulatory" subunit.

Our data and the derived model do not exclude the presence of other functional oligomeric forms of the CK2 tetramer, structurally different from the inactive trimeric association discussed here. For instance, sedimentation velocity and electron microscopy studies on recombinant CK2 from *Drosophila* indicate the existence *in vitro* of catalytically active ring-like structures, which were assumed to be composed by four tetramers.[28]

Our model provides the structural framework for the molecular mechanisms underlying the "environment-specific" functions of CK2 and for the design and analysis of further experiments aimed at clarifying the diverse aspects of CK2 assembly, functionality, and regulation.

METHODS

CK2α and CK2β were expressed in *E. coli* independently. Holoenzyme was reconstituted by mixing pellets before lysis and was purified by affinity chromatography on Heparin-Sepharose and by size-exclusion chromatography. $\alpha 2\beta 2^{new}$ was crystallized by vapor diffusion at 20 °C in 18% PEG 3350, 0.2 M sodium malonate, pH 7.0. Diffraction data were collected at ID23-2 (ESRF). Structure was solved by molecular replacement using as search models human CK2β (1QF8) and human CK2α (3BQC). Data collection and refinement statistics are reported in Table I. Full details are given in the Supporting Information online.

ASSOCIATED CONTENT

Supporting Information

Supplementary figures and experimental methods. This material is available free of charge *via* the Internet at http://pubs.acs.org.

Accession Codes

Atomic coordinates and structure factors have been deposited in the PDB with accession code 4DGL, with the designation "for immediate release upon publication" (HPUB).

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Notes

The authors declare no competing financial interest.

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