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Crystallization and preliminary characterization of crystals of human protein kinase CK2

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The heterotetrameric recombinant holoenzyme of human protein kinase CK2 was purified to homogeneity. It degraded spontaneously to a stable and fully active state in which the catalytic subunit was about 5 kDa smaller than the wild type. The degraded enzyme was crystallized using polyethylene glycol 3350 as precipitant. The crystals belong to the hexagonal space group $P6_3$. They have unit-cell parameters $a = b = 176.0$, $c = 93.6$ Å and diffract X-rays to at least 3.5 Å resolution. The calculated crystal packing parameter is $V_M = 3.22$ Å^3 Da^{-1}, suggesting that one CK2 tetramer is contained in the asymmetric unit and that the solvent content of the unit cell is 62%.

1. Introduction

Protein kinase CK2 (former name casein kinase 2) is a highly conserved Ser/Thr kinase found in all eukaryotic cells so far investigated. Its characteristic and often unusual properties have been described in a number of excellent reviews (Issinger, 1993; Allende & Allende, 1995; Pinna & Meggio, 1997; Guerra & Issinger, 1999; Blanquet, 2000).

In natural sources, CK2 predominantly occurs as a heterotetramer composed of two catalytic subunits (CK2α or CK2α') and two regulatory β subunits (CK2β). Human CK2α has 391 amino acids and a molecular mass of 45.1 kDa, while the accompanying β-chain is 215 amino acids long, corresponding to a molecular mass of 24.9 kDa. Hence, the complete human CK2 holoenzyme has a molecular mass of 140 kDa.

Two CK2 entities have so far been structurally characterized by X-ray crystallography: CK2α from *Zea mays* (Niefind *et al.*, 1998) and a C-terminally truncated form of human CK2β (Chantalat *et al.*, 1999). A large amount of information concerning the interactions of CK2α and CK2β in the CK2 holoenzyme exists.

(i) The two catalytic subunits within the CK2 complex are linked by the CK2β dimer and make no direct contact with each other (Gietz *et al.*, 1995; Boldyreff *et al.*, 1996).

(ii) CK2α is enzymatically active alone, but CK2β has a significant impact on this activity. Normally CK2β enhances the activity of CK2α (Boldyreff *et al.*, 1994), but under special circumstances such as the presence of calmodulin an inhibitory effect was observed (Meggio *et al.*, 1994).

(iii) The previously published CK2β structure (Chantalat *et al.*, 1999) lacks the C-terminal part; however, this sequence was shown to be important for interaction with CK2α (Boldyreff *et al.*, 1993; Kusk *et al.*, 1998; Graham & Litchfield, 2000).

(iv) In CK2α the contact region to CK2β is less clear. Sarno *et al.* (1998) assumed that CK2β binds to the activation segment of CK2α, substituting the N-terminal segment of CK2α, which was found to be in close contact with the activation segment in isolated CK2α from *Z. mays* (Niefind *et al.*, 1998).

(v) Jakobi & Traugh (1995) demonstrated that the CK2α–CK2β interaction effects the dual co-substrate specificity of the enzyme, *i.e.* its ability to use either ATP or GTP as a cosubstrate.

(vi) CD spectroscopic data (Issinger *et al.*, 1992) suggest that the formation of CK2 complexes from isolated subunits results in an increase of the α-helical content.

In summary, a large amount of biochemical and biophysical data about the CK2 holoenzyme and the subunit interactions within the holoenzyme exist that await rationalization on a structural level. We addressed this problem using recombinant human CK2 (rhCK2 composed of rhCK2α and rhCK2β) as a research subject. As a first step, we describe in this paper the production of rhCK2 and its subsequent crystallization.

2. Experimental procedures

2.1. Purification of rhCK2

The purification of rhCK2 was carried out essentially as described by Guerra *et al.* (1997). In brief, the subunits rhCK2α and rhCK2β were separately expressed. Equal amounts of the bacteria harbouring the recombinant proteins were combined and broken by soni-

cation. After phosphocellulose chromatography as an initial purification step, the material was further purified by gel filtration.

Subsequently, rhCK2 was re-buffered and concentrated by dialysis against a solution of 20%(*w*/*v*) PEG 20000, 25 m*M* Tris–HCl, 300 m*M* NaCl, 1 m*M* dithiothreitol (DTT) pH 8.5. The resulting rhCK2 stock solution contained 5.0 mg ml^{-1} protein [determined by the Bradford method (Bradford, 1976) using bovine serum albumin as a reference] in 25 m*M* Tris–HCl, 300 m*M* NaCl, 1 m*M* DTT pH 8.5.

2.2. Formation of rhCK2 with a 40.1 kDa form of rhCK2α

During the course of the purification a partial degradation process of rhCK2α could be observed. For this reason, the concentrated enzyme was left at room temperature for 10 d in order to obtain a partially degraded but stable enzyme suitable for crystallographic studies. Subsequently, the protein stock solution was frozen without any further purification step. The enzyme preparation was checked for purity by SDS–PAGE and by MALDI mass spectrometry as described below.

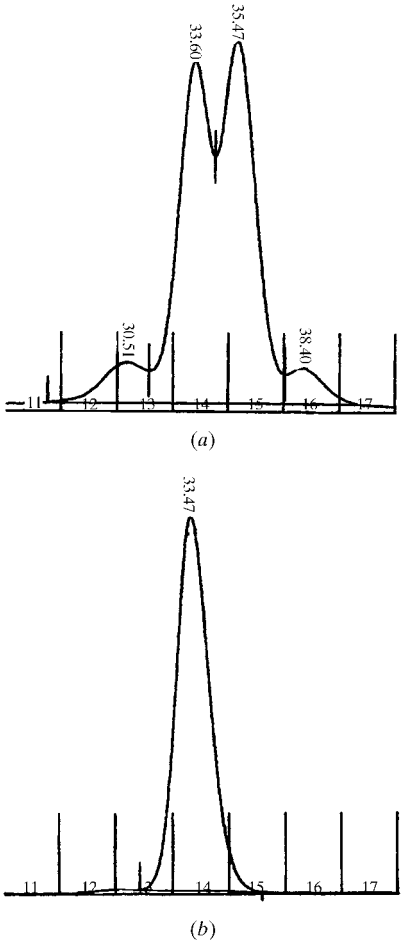

Figure 1
Gel-filtration chromatograms of CK2 holoenzyme. (*a*) Chimeric CK2 composed of maize CK2α and human CK2β; (*b*) rhCK2 after partial degradation of CK2α.

2.3. Protein kinase assay

The activity test was essentially performed as described by Guerra *et al.* (1998). RhCK2 was tested before and after incubation at room temperature. 1 U of enzymatic activity is defined as the amount of rhCK2 necessary to transfer 1 µmol of phosphate per minute into the synthetic peptide substrate (sequence RRRDDDSDDD) at 310 K.

2.4. Phosphorylation assay with calmodulin and casein

To investigate the activity profile of rhCK2 before and after the partial degradation step, a phosphorylation assay with two natural proteins (calmodulin and casein) as substrates was performed. The phosphorylation assay mix contained either 100 ng calmodulin or 50 ng casein, 0.03 mU CK2 in a final volume of 30 µl in the presence of 25 m*M* Tris–HCl pH 8.5, 150 m*M* NaCl, 5 mM $MgCl_2$, 1 m*M* DTT, 50 µ*M* [^{32}P]-γ-ATP (specific activity 1000 counts min^{-1} pmol^{-1}). As indicated in the results, when required 50 ng of polylysine or 1.2 ng heparin were added to the mixture. The assay was performed at 310 K for 30 min and the reaction was stopped by adding SDS sample buffer.

The samples were subjected to SDS–PAGE. The dried gel was then exposed to autoradiography and radioactivity was measured in an Instant Imager apparatus (Packard).

2.5. Analytical gel-filtration chromatography

The aggregation state of rhCK2 in the final preparation was analysed by analytical gel-filtration chromatography. For this purpose, a Superose 6 column from Amersham Pharmacia was mounted on a SMART chromatographic system (Amersham Pharmacia). The running buffer had the composition 25 m*M* Tris–HCl pH 8.5, 500 m*M* NaCl.

2.6. MALDI mass spectrometry

A MALDI spectrum was measured to characterize the purification state of rhCK2 and to obtain the molecular masses of the subunits within the holoenzyme. For this purpose, 5 µl of the rhCK2 stock solution was mixed with 15 µl 0.1%(*v*/*v*) trifluoroacetic acid (TFA) and 30 µl of a saturated solution of sinapinic acid in a mixture of two volumes of 0.1%(*v*/*v*) TFA and two volumes of acetonitrile. 0.6 µl of the resulting rhCK2 solution was applied to a Bruker Biflex III mass spectrometer. Trypsinogen from bovine pancreas served as a mass standard for calibration.

2.7. Crystallization

All crystallization experiments were performed according to the sitting-drop variant of the vapour-diffusion method (Ducruix & Giegé, 1999) using Cryschem crystallization plates. For each setup, temperatures of 293 and 277 K were tested in parallel. Generally, a 3 µl droplet of rhCK2 stock solution was mixed with 1.5 µl of reservoir solution, 3 µl 2.0 m*M* $MgCl_2$ and 3 µl 1 m*M* adenylyl imidodiphosphate (AMPPNP), which is a non-hydrolysable analogue of ATP. Sometimes further additives such as detergents were added to the crystallization drops. Each well contained 0.4 ml of reservoir solution.

Initial crystallization conditions were established by several fast-screen and grid-screen approaches purchased from Hampton Research, Laguna Niguel, USA. Subsequently, the initial conditions were refined by systematic variation of the pH value, the temperature and the concentration of the precipitating agent and by testing 72 different detergents from detergent-screen kits (Hampton Research, Laguna Niguel, USA).

2.8. X-ray diffraction experiment

RhCK2 crystals were characterized by X-ray diffraction at the BW7b beamline of the EMBL Outstation at DESY, Hamburg. A MAR 345 imaging-plate detector and

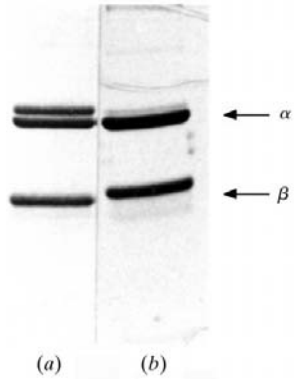

Figure 2
SDS–PAGE showing rhCK2 preparation (*a*) before and (*b*) after degradation.

X-ray radiation of 0.8428 Å wavelength were used. For the diffraction experiments, a cryo-protectant solution containing 25%(*w*/*v*) polyethylene glycol 3350 (PEG 3350), 25%(*v*/*v*) glycerin, 0.2 *M* K_2HPO_4, 0.1%(*w*/*v*) polyethylene glycol 400 dodecyl-ether (THESIT) was added in 2 µl portions to the crystals in their original mother liquors. After each addition, the solutions were cautiously mixed with a cryo-loop and tested for the formation of ice crystals in a stream of cold nitrogen (100 K). Finally, an rhCK2 crystal together with cryo-buffer was transferred to the cold stream.

In a first run, 130 frames, each with a rotation angle around the spindle axis ($\Delta\varphi$) of 0.5°, were measured. Subsequently, 90 frames of a low-resolution run with the same crystal and a $\Delta\varphi$ of 1° per frame were added. All data were processed with the *HKL* package (Otwinowski & Minor, 1997) and *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

2.9. Self-rotation calculations

Self-rotation calculations were performed with *GLRF* (Tong & Rossmann, 1997) using reflections in the resolution range 20–3.5 Å.

3. Results and discussion

The critical step in order to obtain rhCK2 crystals was the production of a pure and stable protein solution without aggregation into oligomers. The tendency of CK2 from various sources to form oligomers in solution is well known and was first observed by Glover (1986), who described a filamentous form of *Drosophila* CK2 which induced the formation of linear polymers or filaments. Valero *et al.* (1995) explored this phenomenon in more depth showing *in vitro* evidence for the existence of ring-like structures.

We also observed aggregation effects when we tried to use CK2 chimeras composed of maize CK2α and human CK2β for crystallization. Maize CK2α is easier to purify (Guerra *et al.*, 1998), is fully active and is more stable in solution than its human counterpart. However, the chimera of maize CK2α and human CK2β led to spontaneous precipitation in all our crystallization experiments. Subsequent gel-filtration experiments demonstrated that this chimeric CK2 aggregates to oligomers of various sizes (Fig. 1*a*).

A solution to this problem was the use of rhCK2 which was homogenous with respect to its oligomerization state according to the final gel-filtration profile (Fig. 1*b*). However, a difficulty with rhCK2 was the partial instability of its catalytic subunit. Fig. 2(*a*) shows the purified holoenzyme directly after purification, where a part of the enzyme fraction had already lost a fragment of about 5 kDa so that in the final SDS–PAGE after all chromatographic steps the rhCK2α band was split into a doublet of about 45 and 40 kDa (Fig. 2*a*).

Deliberate incubation of such a preparation at room temperature for several days led to the almost complete conversion of the 45 kDa form to the stable 40 kDa state of the CK2 holoenzyme (Fig. 2*b*). The degradation of the catalytic subunit within the holoenzyme has been described by many authors over the past 30 years when working with native CK2 from various mammalian sources, *e.g.* porcine, mouse, rat *etc*. (Münstermann *et al.*, 1990; Zandomeni *et al.*, 1988). In our case, the degradation process of the recombinant enzyme started as soon as the expression was initiated by the addition of IPTG. Despite the presence of protease inhibitors (the routinely performed Twinings test for protease activity was always negative), rhCK2α was virtually completely shortened without any loss of activity. Therefore, we hypothesize that the degradation may be an autoproteolytic event independent from external proteases, as is the case for the well studied protein nucleolin (Suzuki *et al.*, 1993).

The degradation of rhCK2α stopped at the level of about 40 kDa. The specific activities of the rhCK2 before and after degradation were 2.8 and 2.5 U mg^{-1}, respectively. Hence, the 40 kDa cleavage product of rhCK2α does not show any significant loss of activity with a synthetic peptide as substrate. A more refined test is the phosphorylation of calmodulin and casein. Normally, calmodulin is not a substrate for the holoenzyme because CK2β acts as a negative regulator with respect to this particular protein. Only in the presence of polylysine is the inhibitory effect of CK2β overcome and calmodulin becomes heavily phosphorylated by the holoenzyme. The autoradiograph in Fig. 3 demonstrates that rhCK2 with the 40 kDa form of rhCK2α is also capable of phosphorylating calmodulin and casein. Moreover, the stimulating and inhibitory effects of polylysine and heparin, respectively, on casein phosphorylation do not show significant differences between the normal catalytic subunit and the 40 kDa cleavage product (Fig. 3). Hence, we conclude that with the present knowledge on CK2 specificity, the degraded enzyme can be considered to match the properties of the intact holoenzyme.

A MALDI spectrum of the partially degraded rhCK2 proved that the full-length CK2α had completely disappeared (Fig. 4). The molecular mass of rhCK2β obtained from the MALDI spectrum was 24.826 Da (Fig. 4), which is in good agreement with the theoretical value (24.942 Da) calculated from the amino-acid sequence (Jakobi *et al.*, 1989) if the N-terminal methionine is supposed to be excized after translation (Hirel *et al.*, 1989). The degraded rhCK2α

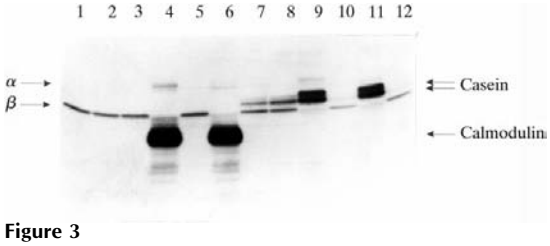

Figure 3
Autoradiograph showing the phosphorylation of calmodulin and casein using rhCK2 before and after degradation. Lanes 1 and 2 show the autophosphorylation of the β-subunit of rhCK2 before and after degradation, respectively. Lanes 3 and 4 show phosphorylation of calmodulin in the absence and presence of polylysine catalysed by rhCK2 before degradation, respectively. Lanes 5 and 6 correspond to lanes 3 and 4, respectively, in the presence of the degraded rhCK2. Lanes 7 and 8 show phosphorylation of casein catalysed by rhCK2 before and after degradation, respectively. Lanes 9 and 11 show phosphorylation of casein in the presence of polylysine by rhCK2 before and after degradation, respectively. Lanes 10 and 12 show phosphorylation of casein in the presence of heparin by rhCK2 before and after degradation, respectively.

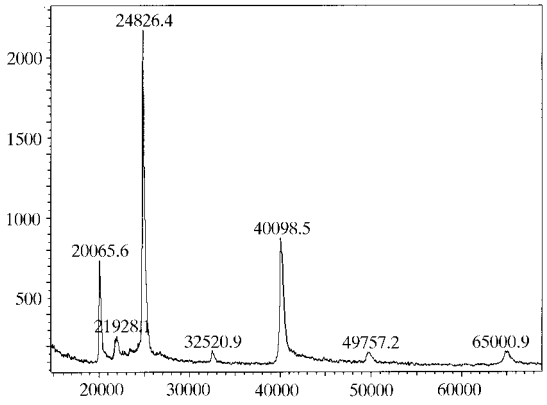

Figure 4
MALDI spectrum of rhCK2 after partial degradation of the catalytic subunit.

Table 1
Native data-set statistics of rhCK2 crystals.

Total number of observations, 566 334; number of outlier rejections, 3424.

Resolution shell (Å)	No. of unique reflections	$I/\sigma(I)$	Completeness (%)	Multiplicity	R_{sym} (%)
99.00–7.33	2387	32.2	99.6	9.2	5.5
7.33–5.81	2358	26.5	100	9.6	9.4
5.81–5.08	2306	20.2	100	9.3	14.8
5.08–4.61	2329	16	100	8.3	18.5
4.61–4.28	2167	10.1	93.7	3.4	16.7
4.28–4.03	2023	6.8	87.2	1.8	21.1
4.03–3.83	1850	4.6	80.3	1.6	21.8
3.83–3.66	1868	4.1	81	1.6	21.6
3.66–3.52	1827	2.5	79.8	1.6	25.6
3.52–3.40	1806	1.1	78.7	1.5	47.3
99.00–3.40	20921	13.8	90.1	5.2	8.9

has a mass of 40.098 Da (Fig. 4). Provided that the degradation took place at the C-terminal side of rhCK2α (an assumption which is suggested by the fact that the catalytic activity is preserved), this molecular mass corresponds to a molecule of 337 amino acids ending with the sequence motif VVKDQARMGSS (Meisner *et al.*, 1989).

Interestingly, apart from the prominent rhCK2α and rhCK2β peaks the MALDI spectrum shows lower but significant peaks corresponding to a rhCK2β dimer and an adduct of rhCK2α and rhCK2β but not to a rhCK2α dimer (Fig. 4). This result is fully consistent with knowledge of the intersubunit contacts in the tetramer (Gietz *et al.*, 1995; Boldyreff *et al.*, 1996).

Crystallization experiments with rhCK2 led to two crystal forms: crystals of the first form have a cuboid-like morphology (not shown) and do not diffract beyond 10 Å resolution in a synchrotron beam. However, with crystals of the second form (Fig. 5) we measured a diffraction data set to 3.5 Å resolution (Tab. 1).

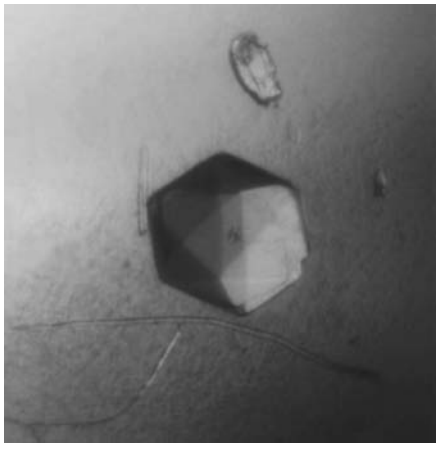

Figure 5
Hexagonal crystal of rhCK2.

After optimization, these crystals grew within 2 d at 285 K in sitting drops equilibrated against reservoirs of composition 20%(*w*/*v*) PEG 3350, 200 m*M* K_2HPO_4. The pH of the reservoir was approximately 9.3 and was not further adjusted. The crystallization drops consisted of 3 µl rhCK2 stock solution, 1.5 µl reservoir solution, 3 µl 1 m*M* AMPPNP, 3 µl 2 m*M* $MgCl_2$ and 2 µl 10%(*w*/*v*) THESIT. Typically, we observed the separation of two liquid phases under these conditions; the crystals tended to grow at the border of these phases.

The crystals were damaged when they were soaked in typical cryo-solutions containing glycerol and other common cryo-protectants. For this reason, the glycerol cryo-buffer could not be used directly but was successively added to the mother liquid as described in §2.

In accordance with the hexagonal morphology of the crystals (Fig. 5), the data could be indexed with a primitive hexagonal lattice and unit-cell parameters $a = b = 176.0$, $c = 93.6$ Å. By scaling and merging the integrated data the space group could be unambiguously determined to be $P6_3$. The mosaicity after post-refinement with *SCALEPACK* (Otwinowski & Minor, 1997) was 1.2°. While some frames showed diffraction spots near 3.2 Å resolution, the data set was only of reasonable quality to 3.5 Å resolution (Table 1).

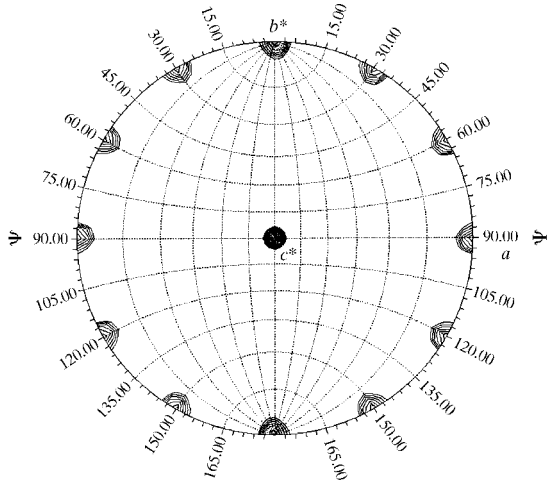

Figure 6
180° self-rotation function calculated with *GLRF* (Tong & Rossmann, 1997) using reflections in the resolution range 20–3.5 Å.

Assuming one rhCK2 tetramer per asymmetric unit, the determined space group leads to a plausible V_M value of 3.22 Å^3 Da^{-1}, corresponding to a solvent content of 62% of the crystallographic unit cell (Matthews, 1968). This result is in accordance with a 180° self-rotation function (Fig. 6), which shows one non-crystallographic dyad per asymmetric unit lying perpendicular to the crystallographic sixfold screw axis. The peak pattern of Fig. 6 suggests 622 as crystallographic point group, but this possibility had been ruled out during scaling of the data set.

The resolution of the collected diffraction data set is high enough to elucidate the quaternary structure of rhCK2 unambiguously by Patterson search calculations (molecular replacement) using maize CK2α (Niefind *et al.*, 1998) and truncated human CK2β (Chantalat *et al.*, 1999) as search models. Moreover, we are confident in being able to collect a data set to about 3 Å resolution or better and in determining the rhCK2 structure to atomic resolution in the future.

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