Crystal structures of human CK2 α 2 in new crystal forms arising from a subtle difference in salt concentration

Masato Tsuyuguchi,^a Tetsuko Nakaniwa^b and Takayoshi Kinoshita^{a*}

Received 14 February 2018

Accepted 2 April 2018

Edited by A. Nakagawa, Osaka University, Japan

Keywords: CK2 kinase; catalytic subunit; crystal structure; human CK2 α 2; polymorphism; male contraceptives.

PDB references: human CK2 α 2, form B1, 5ywm; form B2, 5yf9; form B3, 5y9m

Supporting information: this article has supporting information at journals.iucr.org/f

^aGraduate School of Science, Osaka Prefecture University, 1-1 Gakuen-cho, Naka-ku, Sakai, Osaka 599-8531, Japan, and ^bInstitute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan. *Correspondence e-mail: kinotk@b.s.osakafu-u.ac.jp

The catalytic subunits of protein kinase CK2 are classified into two subtypes: CK2 α 1 and CK2 α 2. CK2 α 1 is an attractive drug-discovery target for various diseases such as cancers and nephritis. CK2 α 2 is defined as an off-target of CK2 α 1 and is a potential target in the development of male contraceptive drugs. High-resolution crystal structures of both isozymes are likely to provide crucial clues for the design of selective inhibitors of CK2 α 1 and/or CK2 α 2. To date, several crystal structures of CK2 α 1 have been solved at high resolutions of beyond 1.5 Å. However, crystal structures of CK2 α 2 have barely achieved a low resolution of around 3 Å because of the formation of needle-shaped crystals. In this study, new crystal forms were exploited and one provided a crystal structure of CK2 α 2 at 1.89 Å resolution. This result, together with the structure of CK2 α 1, will assist in the development of highly selective inhibitors for both isozymes.

1. Introduction

Protein kinase CK2 (previously called casein kinase 2) is a serine/threonine kinase that regulates miscellaneous cell signalling through the phosphorylation of various substrates (Meggio & Pinna, 2003). The CK2 holoenzyme consists of two catalytic subunits (CK2 α 1 and/or CK2 α 2) and two regulatory subunits (CK2 β) (Niefind *et al.*, 2001). The CK2 α 1 subunit is expressed in the whole body, while CK2 α 2 is specifically expressed in the testis and brain. CK2 α 1 is an attractive drug-discovery target for serious diseases such as cancers and nephritis (Dominguez *et al.*, 2009; Yamada *et al.*, 2005). CK2 α 2 is defined as an off-target for the development of CK2 α 1 inhibitors (Guerra *et al.*, 1999). CK2 α 2-knockout mice exhibited a pathological condition similar to globozoospermia in humans (Xu *et al.*, 1999). This result suggests that inhibition of CK2 α 2 is likely to cause testicular toxicity, and indicates that CK2 α 2-selective inhibitors might be suitable as male contraceptive drugs.

High-resolution crystal structures of both isozymes would be likely to provide crucial clues for the design of selective inhibitors of CK2 α 1 and/or CK2 α 2, although the high amino-acid sequence homology between the CK2 α isozymes makes it difficult to develop isozyme-selective inhibitors. We have previously determined a number of crystal structures of CK2 α 1 at high resolutions of beyond 1.5 Å, with a maximum resolution of 1.06 Å (Kinoshita *et al.*, 2013). CK2 α 2 crystals were obtained using a condition including polyethylene glycol 8000 and Tris–HCl buffer and had a thin needle shape. The

crystals barely diffracted to 3.0 Å resolution (Nakaniwa *et al.*, 2009). This enabled structure analysis, but it was difficult to obtain crystals suitable for the collection of high-resolution data. Here, we exploited new crystal forms obtained by rescreening and optimization. One of these crystal forms provided a 1.89 Å resolution structure of CK2 α 2. This structure, together with the CK2 α 1 structure, represents a basis for the development of novel CK2 α isozyme-selective inhibitors.

2. Materials and methods

2.1. Macromolecule production

Recombinant human CK2 α 2 was prepared according to a previously reported protocol (Nakaniwa *et al.*, 2009). The gene coding for human CK2 α 2 (1–334) was cloned into the pGEX-6P-1 vector (GE Healthcare) at the BamHI and EcoRI restriction sites. *Escherichia coli* strain HMS174 (DE3) cells (Novagen) were transformed with the cloned plasmid and cultured in LB medium containing 100 µg ml⁻¹ ampicillin. Protein expression was induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside and the cells were further incubated at 291 K for 18 h. The cell lysate including GST-fused CK2 α 2 was loaded onto Glutathione Sepharose 4B (GE Healthcare). The column was washed with buffer consisting of 20 mM Tris–HCl pH 8.0, 160 mM NaCl, 0.1% Tween 20, 1 mM EDTA, 1 mM dithiothreitol. The GST tag was cleaved by PreScission protease (GE Healthcare) on the column at 277 K for 18 h. The CK2 α 2 protein lacking the GST tag was eluted with 25 mM Tris–HCl pH 8.5, 1 mM dithiothreitol. The protein was further purified on a Mono Q 4.6/100PE anion-exchange column (GE Healthcare) with a linear salt gradient of 50–500 mM NaCl in 40 column volumes of buffer consisting of 25 mM Tris–HCl pH 8.5, 1 mM dithiothreitol using an ÄKTAexplorer system (GE Healthcare). The CK2 α 2 solution was concentrated to 3 mg ml⁻¹, as confirmed spectrophotometrically by the Bradford method (Bradford, 1976).

2.2. Crystallization

Purified CK2 α 2 in 25 mM Tris–HCl pH 8.5, 140 mM NaCl, 1 mM dithiothreitol was used for crystallization. New crystal forms of CK2 α 2 were explored using the sitting-drop vapour-diffusion method at 277 K against 50 µl reservoir solution from the commercially available screening kit Crystal Screen HT (Hampton Research). Trials were performed by mixing 0.5 µl CK2 α 2 solution with an equal volume of reservoir solution. Conditions that produced crystals were optimized by the sitting-drop vapour-diffusion method at 277 K against 500 µl reservoir solution by varying the reservoir composition. Optimization was performed by mixing 2 µl CK2 α 2 solution with an equal volume of reservoir solution.

2.3. Data collection and processing

Diffraction data were collected using synchrotron radiation on SPring-8 beamlines BL44XU and BL32XU, and Photon Factory beamline BL17A with an MX-300HE (Rayonix), EIGER X 9M (DECTRIS) or PILATUS3 S6M (DECTRIS)

Table 1

Data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

	Form B1	Form B2	Form B3
PDB code	5ywm	5yf9	5y9m
Data collection			
Diffraction source	BL44XU, SPring-8	BL17A, Photon Factory	BL32XU, SPring-8
Data processing	<i>XDS</i>	<i>HKL-2000</i>	<i>KAMO</i> with <i>XDS</i>
Wavelength (Å)	0.90	0.98	1.00
Temperature (K)	95	95	95
Detector	MX-300HE	PILATUS3 S6M	EIGER X 9M
Crystal-to-detector distance (mm)	300	344	160
Rotation range per image (°)	1	1	1
Total rotation range (°)	270	180	180
Exposure time per image (s)	1	1	1
Space group	<i>P</i> ₂ ₁ ₂ ₁ ²	<i>P</i> ₂ ₁	<i>P</i> ₂ ₁ ₂ ₁ ²
<i>a</i> , <i>b</i> , <i>c</i> (Å)	67.29, 101.02, 45.76	46.47, 102.42, 72.23	72.51, 91.67, 102.48
α , β , γ (°)	90, 90, 90	90, 95.05, 90	90, 90, 90
Mosaicity† (°)	0.45	0.38	0.11
Resolution range (Å)	50.00–1.94 (2.06–1.94)	50.00–1.89 (1.92–1.89)	49.92–2.01 (2.02–2.01)
Total No. of reflections	260508	234921	309736
No. of unique reflections	27566	53854	50687
Completeness (%)	99.8 (99.5)	99.9 (99.5)	99.6 (98.8)
Multiplicity	9.5 (11.3)	4.4 (4.2)	6.1 (6.4)
$\langle I/\sigma(I) \rangle$	12.0 (2.6)	14.6 (4.0)	7.0 (2.9)
CC _{1/2}	0.998 (0.867)	nd‡ (0.936)	0.990 (0.864)
<i>R</i> _{merge} (%)	8.3 (63.7)	11.9 (30.0)	15.6 (42.0)
Refinement			
Resolution range (Å)	45.80–1.94 (1.96–1.94)	46.30–1.89 (1.92–1.89)	49.93–2.01 (2.03–2.01)
No. of reflections			
Working set	23794	51088	46888
Test set	3772	2736	3799
Final <i>R</i> _{cryst} (%)	19.1 (45.8)	21.2 (23.1)	17.1 (23.1)
Final <i>R</i> _{free} (%)	24.4 (53.0)	26.3 (29.5)	20.5 (28.5)
No. of non-H atoms			
Protein	2666	5423	5392
Ion	10	40	20
Ligand	9	18	18
Water	132	997	818
Total	2817	6478	6248
R.m.s. deviations			
Bonds (Å)	0.008	0.008	0.008
Angles (°)	1.1	1.1	1.1
Average <i>B</i> factors (Å ²)			
Protein	34.8	16.5	15.2
Ion	59.2	31.2	27.4
Ligand	33.5	16.7	14.9
Water	40.2	24.7	24.9
Ramachandran plot			
Most favoured (%)	94.1	95.9	96.8
Allowed (%)	4.6	2.6	2.5

† The definition of mosaicity differs according to the processing program. ‡ The CC_{1/2} value for full data for form B2 is not available.

detector. X-ray diffraction data sets were processed and scaled using *HKL-2000* (Otwinowski & Minor, 1997), *XDS* (Kabsch, 2010) or the *KAMO* system (<https://github.com/keitaroyam/yamtbx/blob/master/doc/kamo-en.md>) with *XDS*. We determined the high-resolution cutoff based on $I/\sigma(I) < 2$ or $R_{\text{merge}} > 1.0$ in the resolution shell. After the cutoff, final scaling was performed. Data-collection parameters and statistics are shown in Table 1.

Figure 1
CK2 α 2 crystals. (a) Crystals obtained under the condition including 0.1 M lithium sulfate (form A). (b) Crystals obtained under the condition including 0.15 M lithium sulfate (form B). (c) Crystals obtained under the condition including 0.25 M lithium sulfate (form C). The scale bars in (a), (b) and (c) indicate lengths of 0.1 mm.

2.4. Structure solution and refinement

Initial phasing was performed by the molecular-replacement method with *MOLREP* (Vagin & Teplyakov, 2010) or *Phaser* (McCoy *et al.*, 2007) using a previously reported CK2 α 2 structure (PDB entry 3e3b or 3ofm; Nakaniwa *et al.*, 2009; Bischoff *et al.*, 2011). Refinements, electron-density map calculations and model improvements were performed using *REFMAC5* (Murshudov *et al.*, 2011), the *PHENIX* program package (Adams *et al.*, 2010) and *Coot* (Emsley *et al.*, 2010). The refinement statistics are shown in Table 1.

3. Results and discussion

3.1. New crystallization conditions obtained by screening and optimization

As a result of the screening, clusters of needle-shaped CK2 α 2 crystals were obtained in several batches, including condition B5 from Crystal Screen HT consisting of 0.2 M lithium sulfate, 0.1 M Tris-HCl pH 8.5, 30% (w/v) PEG 4000. The crystals obtained from condition B5 were slightly thicker than those obtained from the other screening batches or from the original condition containing PEG 8000 (Nakaniwa *et al.*, 2009). Therefore, condition B5 was optimized by varying the concentrations of PEG 4000 and lithium sulfate. Three apparent crystal forms were obtained from this optimization. Surprisingly, a slight difference in the concentration of lithium sulfate led to crystal polymorphism in 24% (w/v) PEG 4000 (Fig. 1). A large number of tiny crystals were obtained from the condition including 0.1 M lithium sulfate (Fig. 1a, referred to as form A). Plate-shaped crystals were grown using a reservoir solution containing 0.15 M lithium sulfate (Fig. 1b, form B). Needle-clustered crystals were obtained with 0.25 M lithium sulfate (Fig. 1c, form C). The appearance of the form C crystals resembled that of the crystals obtained with 0.2 M lithium sulfate as the hit condition in screening, but they were much thinner. The form A crystals were too small for X-ray experiments. Thus, the crystals of form B were most suitable

for X-ray crystallography because of their plate-shaped appearance and crystal size.

3.2. New crystal forms

The largest form B crystals grew to maximum dimensions of $0.1 \times 0.05 \times 0.02$ mm in three months. Surprisingly, data-collection experiments indicated that form B was further divided into three crystal forms, referred to as form B1, form B2 and form B3 (Table 1). It was not possible to distinguish these three crystal forms based on their crystalline appearance (Fig. 1b). Form B1 was nearly identical to the original crystal form obtained using PEG 8000, although the appearances of the crystal forms were quite different: the form B1 crystals were plate-shaped, whereas the original crystals were needle-shaped. The sulfate ions were likely to have impacted the crystal growth of form B1. However, the other two forms were dissimilar from the original crystal form. Differences in the sulfate ion-binding sites are likely to have altered the crystal

Figure 2
Superimposed structures of three crystal forms. Form B1 is presented as a green ribbon, form B2 in blue and form B3 in magenta. The ATP-binding site is located around the hinge region connecting the N- and C-lobes. The CK2 β binding site is located at the top of the N-lobe and the α D helix is followed by the hinge region.

forms. Sulfate ions were found at two sites in form *B1*, eight sites in form *B2* and four sites in form *B3* in each asymmetric unit. These sulfate ions bound to CK2 α 2 at dissimilar sites, apart from one. Therefore, the polymorphism of form *B* was possibly caused by subtle differences in the concentration of sulfate ions owing to their incorporation into the crystals during the crystallization process. Therefore, it might be difficult to control the crystal forms of form *B* crystals. Fortunately, all of the crystal forms diffracted to approximately 2.0 Å resolution. Form *B1* contained one molecule, and forms *B2* and *B3* both contained two CK2 α 2 molecules in the asymmetric unit.

3.3. Overall structures of the new crystal forms

The crystal structures of CK2 α 2 in forms *B1*, *B2* and *B3* have a typical kinase fold (Fig. 2) and closely resemble each other, with C^α r.m.s.d. values of <0.4 Å. The folding of CK2 α 2 involved the N-terminal (residues 1–114) and C-terminal (residues 120–334) lobes and the hinge region (residues 115–119) jointing the lobes (Fig. 2). The N-terminal lobe is mainly constructed of β -sheet and the C-terminal lobe is mainly constructed of α -helices. The ATP-binding site is located in the cleft between the N- and C-terminal lobes (Fig. 2). Some residues in the N-terminus, C-terminus and Gly-rich loop (residues 47–50), which serve to tether the phosphate groups of ATP, were disordered in all form *B* structures. Two differing residues between the isozymes (His115–Val116 in CK2 α 1 and Tyr116–Ile117 in CK2 α 2) in the hinge region had no significant effect on the main-chain folding of both isozymes, including the disordered regions. The region forming the ATP-binding site (Fig. 2) in forms *B1*, *B2* and *B3* did not participate in

Figure 3
Nicotinic acid bound to the ATP-binding site of CK2 α 2 with an electron-density map.

Table 2

Averaged *B* factors ($\text{\AA}^2$) overall, for the CK2 β binding site and for the α D helix of CK2 α 2.

	Form <i>B1</i>	Form <i>B2</i>		Form <i>B3</i>	
		Chain <i>A</i>	Chain <i>B</i>	Chain <i>A</i>	Chain <i>B</i>
Overall	34.8	14.2	18.7	15.0	15.5
CK2 β binding site	52.1	23.0	17.4	17.8	20.3
α D helix	45.3	16.3	21.3	17.2	18.2

crystal packing. The solvent channel connecting to the ATP site observed in these crystal forms may be useful in structure-based drug discovery *via* soaking experiments.

The conformation in the moderately flexible regions, the CK2 β binding site (Fig. 2) and α D helix (residues 122–128), of CK2 α 2 is conserved in forms *B1*, *B2* and *B3*. The CK2 β binding site is located at the top of the N-terminal lobe of CK2 α (Fig. 2) and shows flexibility in the CK2 α 1 crystal structures (Niefind *et al.*, 2001). The CK2 β binding site of CK2 α 2 seems to be rather flexible because of its higher *B*-factor values compared with the averaged value for all atoms (Table 2), although the value for chain *B* of form *B2* is slightly low because of interaction with the adjacent molecule. The α D helix is considered to be an adjuster that enables the use of not only ATP but also GTP as a phosphate-group resource in CK2 α 1, switching between an ‘out’ structure (α D-out) and an ‘in’ structure (α D-in) (Yde *et al.*, 2005). CK2 α 1 has various α D-helix configurations depending on the crystallization condition and bound compounds (Guerra *et al.*, 2015). In addition, the α D helices in the form *B* structures are also rather flexible, with high *B*-factor values (Table 2), but are only present in the α D-out configuration observed in CK2 α 1. Therefore, the α D-in configuration is probably noteworthy for the production of selective CK2 α 1 inhibitors.

3.4. Structural dissection of the active site of CK2 α 2

Conformations, including those of the side chains around the ATP-binding site, are highly conserved among forms *B1*, *B2* and *B3* (Fig. 2). Nicotinic acid bound to the ATP-binding site in forms *B1*, *B2* and *B3* (Fig. 3), as is likely in the highest resolution structure of CK2 α 1 (Kinoshita *et al.*, 2013). Nicotinic acid interacts with Phe114, Ile175, Val54, Val67 and Lys69, and makes water-mediated interactions with Glu82 and Asp176 in these forms. The manner of this interaction is completely conserved between CK2 α 1 and CK2 α 2.

15 water molecules were found in the ATP-binding site of CK2 α 2 in form *B2*, which provides the highest resolution structure of the form *B* structures (Fig. 4*b*). However, eight water molecules were identified in the ATP-binding site of the nicotinic acid–CK2 α 1 complex (Kinoshita *et al.*, 2013; Fig. 4*a*). Among them, four water-molecule-binding sites are conserved between CK2 α 1 and CK2 α 2 (Fig. 4). One is located near the hinge region and forms hydrogen bonds to the main-chain backbone atoms. Another is located in the depths of the ATP-binding pocket and forms hydrogen bonds to the backbone atom of Trp177 and the side-chain atoms of Lys69 and Glu82. One of the remaining two water molecules interacts with

Lys69, Asp176 and nicotinic acid, and the second only interacts with Lys159. These conserved water-binding sites are largely available for increasing the inhibitory activity against both isozymes, but are unhelpful for developing selective CK2 α 1 inhibitors. Conversely, the unique water molecules in CK2 α 1 and CK2 α 2 would provide crucial clues for the design of CK2 α 1-selective inhibitors. It is noteworthy that three water molecules tightly bind to CK2 α 2 at Lys69, Lys97 and His161 (Fig. 4*b*), while one water molecule interacts with the hinge region of CK2 α 1 in addition to the conserved water molecule (Fig. 4*a*).

Alternative conformations of His161 of CK2 α 2 were observed in two molecules in the asymmetric unit of form *B*2

(Fig. 5*b*) and indicate the conformational flexibility of His161 as well as the corresponding His160 of CK2 α 1. To date, several conformations of His160 of CK2 α 1 have been reported and are likely to allow various inhibitors to bind to the ATP site (Fig. 5*a*; Ferguson *et al.*, 2011). The alternative conformations of CK2 α 2 imply that His161 is possibly an adjuster for inhibitor binding, similar to His160 of CK2 α 1.

In conclusion, we discovered new crystal forms (forms *B*1, *B*2 and *B*3) of CK2 α 2 and considerably improved the crystallographic resolution of CK2 α 2 to a maximum of 1.89 Å. In the new crystal forms, the conformation of CK2 α 2 was highly conserved without any influence from crystal packing. These structures conferred precise structural insights into the

Figure 4
Water molecules found in the ATP-binding site of (*a*) CK2 α 1 and (*b*) CK2 α 2. Water-binding sites that are conserved between isozymes are shown with red circles. Noteworthy unique water-binding sites are shown as blue circles.

Figure 5
Observed multiple conformations of (*a*) His160 of CK2 α 1 (PDB entries 3war, 3u9c, 4uba and 3wil; Kinoshita *et al.*, 2013; Klopffleisch *et al.*, 2012) and (*b*) His161 of CK2 α 2.

ATP-binding site of CK2 α 2, involving conserved and unique water molecules compared with CK2 α 1. The results, together with the CK2 α 1 structures, could provide crucial clues for the development of selective inhibitors of CK2 α isozymes.

Acknowledgements

Preliminary experiments and diffraction data collection were carried out on beamline BL17A at Photon Factory (Proposal No. 2016G665) and on the Osaka University beamline BL44XU and the RIKEN beamline BL32XU at Spring-8 (Proposal Nos. 2015A6518 and 2015B6518). We thank Edanz Group (<http://www.edanzediting.com/ac>) for editing a draft of this manuscript.

Funding information

This work was supported by the Platform for Drug Discovery, Informatics and Structural Life Science of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

- Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
- Bischoff, N., Olsen, B., Raaf, J., Bretner, M., Issinger, O. G. & Niefind, K. (2011). *J. Mol. Biol.* **407**, 1–12.
- Bradford, M. M. (1976). *Anal. Biochem.* **72**, 248–254.
- Dominguez, I., Sonenshein, G. E. & Seldin, D. C. (2009). *Cell. Mol. Life Sci.* **66**, 1850–1857.
- Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). *Acta Cryst.* **D66**, 486–501.
- Ferguson, A. D., Sheth, P. R., Basso, A. D., Paliwal, S., Gray, K., Fischmann, T. O. & Le, H. V. (2011). *FEBS Lett.* **585**, 104–110.
- Guerra, B., Rasmussen, T. D. L., Schnitzler, A., Jensen, H. H., Boldyreff, B. S., Miyata, Y., Marcussen, N., Niefind, K. & Issinger, O. G. (2015). *Cancer Lett.* **356**, 751–761.
- Guerra, B., Siemer, S., Boldyreff, B. & Issinger, O. G. (1999). *FEBS Lett.* **462**, 353–357.
- Kabsch, W. (2010). *Acta Cryst.* **D66**, 125–132.
- Kinoshita, T., Nakaniwa, T., Sekiguchi, Y., Sogabe, Y., Sakurai, A., Nakamura, S. & Nakanishi, I. (2013). *J. Synchrotron Rad.* **20**, 974–979.
- Kloppfleisch, K., Issinger, O.-G. & Niefind, K. (2012). *Acta Cryst.* **D68**, 883–892.
- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- Meggio, F. & Pinna, L. A. (2003). *FASEB J.* **17**, 349–368.
- Murshudov, G. N., Skubák, P., Lebedev, A. A., Pannu, N. S., Steiner, R. A., Nicholls, R. A., Winn, M. D., Long, F. & Vagin, A. A. (2011). *Acta Cryst.* **D67**, 355–367.
- Nakaniwa, T., Kinoshita, T., Sekiguchi, Y., Tada, T., Nakanishi, I., Kitaura, K., Suzuki, Y., Ohno, H., Hirasawa, A. & Tsujimoto, G. (2009). *Acta Cryst.* **F65**, 75–79.
- Niefind, K., Guerra, B., Ermakowa, I. & Issinger, O. G. (2001). *EMBO J.* **20**, 5320–5331.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Vagin, A. & Teplyakov, A. (2010). *Acta Cryst.* **D66**, 22–25.
- Xu, X., Toselli, P. A., Russell, L. D. & Seldin, D. C. (1999). *Nature Genet.* **23**, 118–121.
- Yamada, M. *et al.* (2005). *Proc. Natl Acad. Sci. USA*, **102**, 7736–7741.
- Yde, C. W., Ermakova, I., Issinger, O. G. & Niefind, K. (2005). *J. Mol. Biol.* **347**, 399–414.