**Insights from soft X-rays:
the chlorine and sulphur sub-structures of a CK2 α /DRB complex**

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Abstract

The diffraction pattern of a protein crystal is normally a product of the interference of electromagnetic waves scattered by electrons of the crystalline sample. The diffraction pattern undergoes systematic changes in case additionally X-ray absorption occurs, meaning if the wavelength of the primary X-ray beam is relatively close to the absorption edge of selected elements of the sample. The resulting effects are summarized as “anomalous dispersion” and can be always observed with “soft” X-rays (wavelength around 2 Å) since they match the absorption edges of sulphur and chlorine. A particularly useful application of this phenomenon is the experimental detection of the sub-structures of the anomalous scatterers in protein crystals. We demonstrate this here with a crystal of a C-terminally truncated variant of human CK2 α to which two molecules of the inhibitor 5,6-dichloro-1- β -D-ribo-furanosyl-benzimidazole (DRB) are bound. The structure of this co-crystal has been solved recently. For this study we measured an additional diffraction data set at a wavelength of 2 Å which showed strong anomalous dispersion effects. On the basis of these effects we detected all sulphur atoms of the protein, the two liganded DRB molecules and a total of 16 additional chloride ions some of them emerging at positions filled with water molecules in previous structure determinations. A number of chloride ions are bound to structural and functional important locations fitting to the constitutive activity and the acidophilic substrate specificity of the enzyme.

Key words: casein kinase 2, anomalous dispersion, soft X-rays, kinase inhibitors

Introduction

Soft X-rays and their use in protein crystallography

In the last years the use of so-called “soft X-rays” with relatively long wavelengths ($\lambda = 1.5\text{-}3.0 \text{ \AA}$) has got increasing interest in macromolecular crystallography [1]. Such wavelengths are relatively close to X-ray absorption edges of elements like sulphur (K-edge at $5.016 \text{ \AA}$), calcium (K-edge at $3.070 \text{ \AA}$) or chlorine (K-edge at $4.393 \text{ \AA}$), which are either inherent components of protein molecules or often – in the form of ions – attached to them. As a consequence X-ray diffraction patterns collected at such wavelengths are slightly, but significantly modified by absorption effects – a phenomenon, called as “anomalous dispersion”. This means that the diffraction intensities I_{hkl} and I_{-h-k-l} , which are related by an inversion operation (a couple of so-called Bijvoet partners) and which are normally equal, differ now systematically.

These anomalous differences can be exploited in two ways:

- (i) They can serve for the *de novo* solution of protein crystal structures. The traditional anomalous dispersion approach (MAD [2]) required the artificial derivatization of the protein by soaking the crystal in heavy atom containing solutions or – more elegantly - by incorporation of selenomethionine during gene expression. With soft X-rays, however, the anomalous signal of the native protein’s sulphur atoms can be strong enough to allow phase determination which makes derivatization unnecessary [3].
- (ii) The second application is the determination of sub-structures in which the positions and identities of different ions can be reliably established. Isolated and spherical electron density maxima close to crystalline protein molecules are routinely interpreted as water molecules even if they are essentially due to chloride, sulfate, phosphate or metal ions. Some of these water assignments are revised in the sense of such ions during final refinement steps depending on experience and care of the crystallographer. A recent anomalous dispersion study [4], however, showed that often such pseudo water molecules persist and are present in more than 90 % of all deposited protein crystal structures.

In the currently available structures of protein kinase CK2 entities a number of anions and metal ions have been identified by conventional indications like low temperature factors, electron density height or charge distribution in the local environment. We show here that the application of soft X-rays and the resulting anomalous signal of chlorine reveals several more chloride sites – sometimes with an interesting structural and functional background.

Probing crystals of a CK2 α /DRB complex with soft X-rays

Recently we published [5] the co-crystal structure of *hsCK2 α ¹⁻³³⁵* – a C-terminally truncated but fully active mutant of human CK2 α [6] – with 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB) which is to our knowledge the oldest ATP-competitive CK2-inhibitor in the literature [7]. Surprisingly in this structure we found DRB not only bound to the canonical ATP-binding site but also to an allosteric site located at the interface region to the enzyme's non-catalytic subunit CK2 β (Fig. 1).

When during that study this unexpected result emerged we decided to confirm it with a so-called anomalous difference Fourier density depending on an additional diffraction data set with a soft X-ray beam. In such a density we expected to see – together with the enzyme's sulphur atoms – the chloro substituents of the DRB molecules as parts of the chlorine sub-structure. This was in fact the case [5] (Fig. 1), but additionally the anomalous density showed a number of chloride ions, partly at functionally or structurally interesting locations.

Materials and Methods

Enzyme production, crystallization and structure determination

The experimental procedures of the *hsCK2 α ¹⁻³³⁵*/DRB structure determination have been described

in detail elsewhere [5]. Briefly bacterially expressed $hsCK2\alpha^{1-335}$ [6] was purified in a three-step chromatographic procedure. The resulting pure $hsCK2\alpha^{1-335}$ stock solution contained among others 0.5 mole/L sodium chloride. This stock solution was incubated with DRB for at least 30 min and subsequently used for crystallization. $hsCK2\alpha^{1-335}$ /DRB crystals grew with ammonium sulfate as a precipitant. For cryoprotection the ammonium sulfate concentration in the crystal mother liquid was increased to 3.9 mole/L.

The $hsCK2\alpha^{1-335}$ /DRB crystals were initially characterized with CuK_{α} radiation from a Bruker-Nonius FR591 rotating copper anode diffractometer. The crystallographic space group was determined to be $P4_32_12$ with lattice constants of $a=b=71.5 \text{ \AA}$, $c=125.8 \text{ \AA}$. A high-resolution diffraction data set was collected at beamline X12 of the EMBL outstation in Hamburg, Germany. This data set was complete to $1.56 \text{ \AA}$ and led finally to the second highest resolution structure among all $CK2\alpha$ structures currently available (PDB code 2RKP).

Data collection with a soft X-ray

After measuring the high-resolution diffraction data corresponding to the deposited $hsCK2\alpha^{1-335}$ /DRB structure we changed the wavelength of EMBL-beamline X12 to $2 \text{ \AA}$ and collected a second data set with the same $hsCK2\alpha^{1-335}$ /DRB crystal. In this case a full 360° sweep was performed to get a complete set of anomalous differences. The data collection temperature was 100 K. The crystal-detector distance was set to the relatively low value of 60 mm to minimize absorption of the diffracted beams in the air.

The soft-X-ray diffraction data set was processed with the HKL-software package [8]. During scaling with SCALEPACK [8] the “anomalous” flag was set to keep the anomalous signal. The scaled diffraction intensities were transferred to the CCP4 program suite [9] and converted to structure factor amplitudes with TRUNCATE [9].

Calculation and analysis of an anomalous Fourier density

To calculate an anomalous Fourier density in which the anomalous scatterers peak up Fourier coefficients of the type $\text{DANO}_{\text{hkl}} * \exp[i(\varphi_{\text{hkl}}+90^\circ)]$ are required. DANO_{hkl} are the anomalous differences between the structure factor amplitudes of the Bijvoet partners coming from the soft-X-ray diffraction data set. The terms φ_{hkl} are the phases of the beams which were calculated from the refined $hsCK2\alpha^{1-335}/\text{DRB}$ structure. Both types of data were combined with the CCP4 program CAD [9] and afterwards applied to the Fourier transform program FFT [9]. The calculated map covered one asymmetric unit of the crystallographic unit cell. A systematic peak analysis was performed with PEAKMAX [9] which calculated statistical parameters [mean value, root-mean-square deviation (RMSD)], assigned pseudo atoms to each peak above a given threshold, ranked them according to peak height and output them to a separate file in Protein Data Bank (PDB) format. This pseudo atom file together with the original anomalous Fourier density, with the $hsCK2\alpha^{1-335}/\text{DRB}$ structure and with the corresponding 2Fo-Fc electron density were loaded to the graphic program O [10] for further inspection.

Results and discussion

Quality of the soft-X-ray diffraction data set

The soft-X-ray-diffraction data set of the $hsCK2\alpha^{1-335}/\text{DRB}$ complex was collected to a high resolution limit of 2 Å (Tab. 1). On average the intensity of each X-ray diffraction maximum was measured 25.2 times independently. In spite of this large multiplicity the overall R_{sym} value of the data set is 6.3 % reflecting a high inherent quality.

The sulphur sub-structure of the $hsCK2\alpha^{1-335}$ /DRB complex

To assess the reliability of the method and the information content of the anomalous data we checked whether the positions of the sulphur atoms – as known from the $hsCK2\alpha^{1-335}$ /DRB complex structure – could be successfully reproduced. Therefore we calculated and analysed an anomalous difference Fourier density in the resolution range from 1.95 to 21 Å.

The peaks of the highest significance (RMSD above the mean) are illustrated in Fig. 1; moreover they are listed in Table 2 together with corresponding atoms of the $hsCK2\alpha^{1-335}$ /DRB complex structure. The construct $hsCK2\alpha^{1-335}$ contains 11 methionine and 2 cystein residues. One of them – Met1 – was never seen in any crystal structure even if the remaining N-terminus was well defined. This N-terminal methionine was certainly lost by post-translational processing in the bacterial host cell. Thus we expected to see only ten methionine sulphur atoms and two cystein sulphur atoms. Table 2 shows that all of these twelve sulphur atoms are present in the list. All of them have relative peak heights higher than 10. This result clearly demonstrates the power of the method.

One of the methionine residues – Met334 – deserves a special note. Typically maize and human CK2 α structures end around position 330 because the final residues are flexible and not covered by electron density. This is, however, not the case with the $hsCK2\alpha^{1-335}$ /DRB complex which is structured upto the C-terminus of the construct. In particular the position of Met334 is clearly indicated by a peak in the anomalous difference Fourier density (Fig. 1, Tab. 2). Remarkably the side chain of Met334 is in direct contact to the $\beta 7\beta 8$ -loop (Fig. 1). This observation may indicate that the genuine C-terminal segment of human CK2 α – which is structurally completely uncharacterized so far due to its instability - folds back to the N-terminal domain of the enzyme in a similar fashion as it is known for the C-terminal segment of the cAMP-dependent protein kinase [11].

The chlorine sub-structure of the hsCK2 α ¹⁻³³⁵/DRB complex

Apart from the protein sulphur atoms the two chloro substituents of two DRB molecules are visible in the anomalous difference Fourier density (Fig. 1, Tab. 2) so that the identity and the orientation of the two DRB molecules are confirmed. All further significant peaks of the anomalous Fourier density map must be due to non-protein and non-DRB substances. An inspection of the list of ingredients of the crystallization setup revealed that the only candidates were chloride and sulfate ions. The latter, however, were excluded since there was no piece of the conventional 2Fo-Fc electron density fitting in size and shape to a tetrahedral anion.

Thus, we assigned all remaining peaks of the anomalous Fourier density with a relative peak height above 5.5 to chloride ions (Tab. 2). In all cases the plausibility of the assignment with the chemical environment (atomic distances, charge distribution) was assessed. In this way a total of 16 chloride ions were added to the structure and are now present in PDB file 2RKP [5]. Some of them are bound to structurally or functionally important sites of CK2 α as we will show in the following discussion.

A structural chloride ion

The strongest peak of the anomalous difference Fourier density (Tab. 2) is assigned to the chloride ion Cl⁻1. The following reasons favour the notion that this ion is not a transient binding partner with access to the solvent but an inherent stabilizing part of the CK2 α structure:

- (i) peak height: with more than 24 the relative height of the corresponding peak is by far stronger than those of all other chloride ions and even stronger than those of the inherent sulphur atoms (Tab. 2).
- (ii) structural environment and importance: Cl⁻1 is completely buried and located at a strategic position (Fig. 2A) where it – together with some highly conserved water molecules (Fig. 2B) - connects the N-terminal segment, the helix α M from the C-terminal segment, the activation segment and the catalytic

loop. The ion is coordinated by two nitrogen atoms of the protein matrix (backbone nitrogen of Ala315 and side chain of the conserved residue His148 from the C-terminal part of helix α E; Fig. 2B) and two water molecules (W377 and W395 in Fig. 2B) which themselves participate in a conserved cluster of buried water molecules (Fig. 2B). This water cluster is part of the most conspicuous feature of all CK2 α structures, namely the close contact of the N-terminal segment and the activation loop that forms the structural basis of the enzyme's constitutive activity by keeping the enzyme in an active conformation.

(iii) Conservation: a very similar cluster of water molecules is found in all highly resolved CK2 α structures (see Fig. 2C with maize CK2 α [12] for comparison). It is very likely that also in these cases one of the assigned water molecules (V31 in Fig. 2C) is in fact a chloride ion. In the case of the highly resolved CK2 α mutant structure 1YMI, for instance [12], which we reinspected in the context of the *hs*CK2 α ¹⁻³³⁵/DRB structure determination ([12]; new PDB code – replacing 1YMI – is 3BW5), this was very obvious: in the final difference electron density a strong peak remained if a water molecules was placed to the respective position, meaning an oxygen atom has not enough electrons to fill the density completely. Only after replacing the water molecule by a more electron-rich chloride ion the density was interpreted satisfyingly.

Taken together, the structural chloride ion Cl⁻1 is a further member in the list of conspicuous elements stabilizing the constitutively active structure of CK2 α . Previously identified elements – described already in the first CK2 α structure publication [13] - are the N-terminal segment with its aforementioned contact to the activation segment and the DWG motif at the beginning of the activation segment. The DWG motif replaces the canonical DFG motif of most eukaryotic protein kinases and forms one additional hydrogen bond across the magnesium binding loop thus preventing inactive “DFG-out” conformations as found for instance for MAP kinases [14]. Thus the structural chloride ion fits perfectly to our picture of CK2 α as a protein kinase evolutionary optimized for activity [15].

Chloride at the positive electrostatic area of the ATP-binding site

The chloride ion with the second highest peak in the anomalous difference Fourier density is located at the canonical ATP-binding site (Cl⁻3 in Fig. 3) which is mainly occupied by a DRB molecule. The ion is hydrogen-bonded to two water molecules (W385 and W595) which on their part are anchored to highly conserved side chains of the enzyme (Glu81, Asp175). Cl⁻3, W385 and W595 fill the so-called “hydrophobic region I” [16] and lie in-plane with the benzimidazole group of DRB (Fig. 3).

The detection of a chloride ion at this site demonstrates the power of the anomalous dispersion method since intuitively - given the distribution of charges in the environment - one would not assign a negative charge to this site. There is only one further case of a CK2 α /inhibitor complex – maize CK2 α in complex with 4,5,6,7-tetrabromo-N,N-dimethyl-benzimidazole ([17]; PDB file 1ZOE) - with a chloride ion at an equivalent location. In that case it was the strength of the conventional 2Fo-Fc electron density which prompted the authors to add a chloride ion rather than a water molecule [17]. Nevertheless, the assignment is somewhat obscure since the final coordinate file harbours a water molecule (HOH1344) in such a close distance to the chloride ion (1.73 Å) that it is stereochemically impossible. Thus, the Cl⁻3 ion of the *hsCK2 α* ¹⁻³³⁵/DRB structure is the first unambiguous assignment of a chloride ion to the hydrophobic region I of CK2 α .

The ensemble of the Cl⁻3 ion with two water molecules is a relatively conserved arrangement of ligands at the hydrophobic region I. It fits nicely into a scheme introduced recently by Battistutta et al. [18] on the basis of comparative structural analysis. According to this scheme W385 (Fig. 3) is the absolutely conserved “water 1” and Cl⁻3 fills a region with a positive electrostatic potential occupied by various anionic species in CK2 α structures [18].

Chloride ions mark the substrate binding site

A striking chain of chloride ions (Cl⁻2, Cl⁻10, Cl⁻8, Cl⁻14, Cl⁻15, Cl⁻6; Fig. 4A) – some of them like Cl⁻2 with a very significant peak height in the anomalous difference Fourier density (Tab. 2) - fills the potential binding and recognition region for peptide and protein substrates. Given the decidedly acidophilic substrate specificity of CK2, the obvious interpretation of this finding is that the chloride ions mark recognition sites for negatively charged side chains.

Mutational [19-21] and statistical [22] studies have revealed many determinants of substrate recognition by CK2 α . However, in contrast to several other protein kinases in the case of CK2 α no structure of an enzyme/substrate complex has been solved so far. The most reliable structural conception of substrate recognition has been derived from two sulfate ions bound to the P+1 loop and the activation loop of *hsCK2 α ¹⁻³³⁵* [15]. These two sulfate ions formed constraints to be maintained when a complex between *hsCK2 α ¹⁻³³⁵* and a peptide with the sequence DSDDD was modelled [15].

We superimposed that *hsCK2 α ¹⁻³³⁵/DSDDD* model on the *hsCK2 α ¹⁻³³⁵/DRB* structure. This led to nice matches of some of the negatively charged aspartate side chains with chloride ions, namely the P-1 aspartate with Cl⁻10, the P+1 aspartate with Cl⁻8 and the P+3 aspartate with Cl⁻14 and Cl⁻15 (Tab. 2) while the P+2 position is known to be less important for substrate recognition [21]. Due to the success of this approach we extend that modelling concept and enlarged the peptide substrate.

The ion Cl⁻2 can be easily matched by an aspartate at the P-2 position (Fig. 4B). Two positively charged residue in that region – Lys122 and His160 – are in fact known for their role in recognizing negative side chains at position P-2 [23].

At the C-terminal side an extension of the peptide substrate model is more difficult. Like in other CK2 α structures the characteristic lysine quartet (Lys74-Lys77) known for recognizing negative side chains in the region P+4 to P+5 [20-21] is partly disordered in the *hsCK2 α ¹⁻³³⁵/DRB* structure, and accordingly no chloride ions are bound here. Merely the ion Cl⁻6 is found in this

region (Fig. 4B); it interacts with Lys76 and His183, a residue which was not described to be involved in substrate binding so far.

Conclusions

Our anomalous dispersion study with a *hsCK2 α* ¹⁻³³⁵/DRB complex crystal confirms a previous study [4] with 19 different proteins that many hidden ions can be detected in protein crystal structures if soft X-rays are consequently applied. The result is not only a better structure model in a crystallographic sense; often the ions – in the case of the acidophilic enzyme CK2 α in particular the chloride ions – provide valuable structural insights into important biochemical properties of the proteins. Therefore, any X-ray diffraction data collection at a tunable synchrotron beamline should be accompanied by a carefully collected additional soft-X-ray diffraction data set.

Acknowledgments

We are grateful to Dr. Manfred Weiss and Dr. Annette Faust for assistance at beamline X12 of the EMBL outstation in Hamburg, Germany. This work was funded by the Deutsche Forschungsgemeinschaft (grant NI 643/1-3) and by the Forskningsråd for Natur og Univers (grant 272-07-0257).

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Tables

Table 1. X-ray diffraction data collection statistics of a *hsCK2α*¹⁻³³⁵/DRB crystal with a soft X-ray

Temperature [K]	100
Crystal-detector distance [mm]	60
Wavelength [Å]	2
Space group	P4 ₃ 2 ₁ 2
Cell dimensions	
a, b, c [Å]	71.6, 71.6, 125.9,
α, β, γ [°]	90.0, 90.0, 90.0
Resolution range [Å]	21-1.95 (1.98 – 1.95) ²
<i>R</i> _{sym} [%] ¹	6.3 (31.9) ²
Signal-to-noise ratio (<i>I</i> / <i>σ</i> _{<i>I</i>})	59.9 (10.3) ²
Completeness [%]	98.6 (95.9) ²
Redundancy	25.2 (21.1) ²

¹ $R_{\text{sym}} = \sum_h \sum_j |I_{h,j} - \langle I_h \rangle| / \sum_h \sum_j I_{h,j}$, 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. To keep the anomalous signal Bijvoet partners were not included in merging.

² The values in brackets refer to the highest resolution shell.

Table 2. Peak list of the anomalous difference Fourier map.

The peaks are ordered according to their relative heights. Sulphur and chlorine of the *hsCK2 α ¹⁻³³⁵*/DRB crystal structure that match the peak positions are given in the third column. The numbers in brackets after the Cl⁻ ions indicate the residue number in the PDB file 2RKP [5].

rank	Relative peak height (RMSD above the mean)	corresponding atom (PDB residue number)	Function/position
1	24.16	Cl ⁻ 1 (336)	structural chloride ion
2	18.80	Cys220-SG	inherent sulphur
3	18.73	Met150-SD	inherent sulphur
4	18.59	Cys147-SG	inherent sulphur
5	18.36	Met215-SD	inherent sulphur
6	17.96	Met319-SD	inherent sulphur
7	16.23	Met137-SD	inherent sulphur
8	15.81	Met153-SD	inherent sulphur
9	13.61	Met225-SD	inherent sulphur
10	13.51	Met163-SD	inherent sulphur
11	12.55	Cl ⁻ 3 (338)	ATP-binding site
12	12.09	Cl ⁻ 2 (337)	P-2 substrate position
13	11.52	Cl ⁻ 6 (341)	P+X substrate position
14	10.88	Met208-SD	inherent sulphur
15	10.62	Met221-SD	inherent sulphur
16	10.14	Met334-SD	inherent sulphur
17	9.94	Cl ⁻ 15 (350)	P+3 substrate position
18	9.34	DRB 1 Cl2	chloro substituent of DRB 1
19	8.74	Cl ⁻ 4 (339)	-
20	8.30	Cl ⁻ 14 (349)	P+3 substrate position
21	8.23	DRB 1 Cl1	chloro substituent of DRB 1
22	7.74	Cl ⁻ 8 (343)	P+1 substrate position
23	7.58	Cl ⁻ 11 (346)	-
24	7.20	Cl ⁻ 9 (344)	-
25	7.05	Cl ⁻ 12 (347)	-
26	6.89	Cl ⁻ 10 (345)	P-1 substrate position
27	6.73	Cl ⁻ 7 (342)	-
28	6.59	Cl ⁻ 16 (351)	-
29	6.23	Cl ⁻ 13 (348)	-
30	6.05	Cl ⁻ 5 (340)	-
31	5.54	DRB 2 Cl2	chloro substituent of DRB 2
32	4.40	DRB 2 Cl1	chloro substituent of DRB 2

Figures

Fig. 2:

A

B

C

The structural chloride ion.

(A) Stereo picture of the *hsCK2 α* ¹⁻³³⁵ chain with the structural chloride ion at a central position linking the N-terminal segment (blue), the activation segment (yellow) and the helices α M (magenta) and α E. (B) Stereo picture of the structural chloride ion Cl⁻1 and its neighbouring water cluster in the *hsCK2 α* ¹⁻³³⁵/DRB complex. The 2Fo-Fc electron density of the protein is drawn in green or in blue for the water molecules (contour level 1.0 σ). The anomalous Fourier density around Cl⁻1 is colored red and drawn with a contour level of 6 σ . Purple dotted lines indicate hydrogen bonds. Distances are given in Å. (C) Stereo picture of the equivalent region in maize CK2 α (PDB file: 1LP4; [12]). The water molecule V31 corresponds to the structural chloride ion Cl⁻1 and has a relatively low B-factor of V31 (17.7 Å²) which indicates that it might be replaced with a chloride ion. All parts of the figure were drawn with BOBSCRIPT [24] and Raster3D [25].

Fig. 3:

Stereo picture of DRB and the chloride ion Cl⁻3 in the nucleotide binding pocket. The 2Fo-Fc electron density (contour level 1.0 σ) is coloured either in green (protein, two water molecules and one chloride ion) or in blue (DRB molecule). The Cl⁻ ion and the chloro substituents of the DRB molecule are covered by red anomalous difference Fourier density (contour level 6 σ). Purple dotted lines indicate hydrogen bonds. Distances are given in Å. The figures were drawn with BOBSCRIPT [24] and Raster3D [25].

Fig. 4:

A

B

Chloride ions along the substrate-binding site. (A) Surface of the *hsCK2 α* ¹⁻³³⁵/DRB complex coloured according the electrostatic potential (blue: positively charged; red: negatively charged). The purple balls represent bound chloride ions. (B) Detailed view of the substrate binding site. A modelled phosphoacceptor substrate peptide (Asp_(P-2)-Asp_(P-1)-Ser_(P)-Asp_(P+1)-Asp_(P+2)-Asp_(P+3)) and residues implicated in substrate recognition are drawn. Both parts of the figure were drawn with BRAGI [26].