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From phasing to structure refinement in-house: Cr/Cu dual-wavelength system and a loopless free crystal-mounting method

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The practical applicability of in-house structure determination using Cr $K\alpha$ X-rays (2.29 Å) and a loopless free crystal-mounting method was examined using five novel proteins. Proteins from 9.6 to 84 kDa have been solved using this method without any derivatization. In all cases, more than 90% of structures were constructed automatically with side chains by use of the Cr SAD method. The free crystal-mounting technique increases the accuracy of the anomalous differences between Bijvoet mates and makes the in-house single-wavelength SAD method with a Cr $K\alpha$ X-ray source a very useful tool for high-throughput structure determination. In addition, a Cr/Cu dual-wavelength system makes it possible to perform structure analysis from phasing to refinement of the structure in-house.

1. Introduction

Single-wavelength anomalous diffraction (SAD) phasing has become a useful tool for protein structure determination (Rice *et al.*, 2000; Dauter *et al.*, 2002; Blow, 2003). The choice of X-ray wavelength is one of the major decisions required for SAD data collection (Mueller-Dieckmann *et al.*, 2005). However, changing the wavelength is not possible for in-house X-ray sources. In the laboratory, we can only choose from several anticathode targets such as Cu $K\alpha$ (1.54 Å), Co $K\alpha$ (1.79 Å) and Cr $K\alpha$ (2.29 Å). By utilizing the longer wavelength, the small anomalous signals generated from small atomic weight or 'light' atoms such as sulfur can be enhanced to a level where they can be used to phase macromolecular structures. For example, the anomalous scattering factor of sulfur increases from 0.56 e^- at Cu $K\alpha$ to 1.14 e^- at Cr $K\alpha$. Considering the phasing using the anomalous signal of such weak anomalous scatterers in underivatized native protein, the longer wavelength of Cr $K\alpha$ X-rays may be the optimal choice for these experiments (Wang, 1985; Kwiatkowski *et al.*, 2000). Successful application of the sulfur SAD (S-SAD) technique has been reported using a longer wavelength from a chromium target (Chen *et al.*, 2004; Phillips *et al.*, 2004; Madauss *et al.*, 2004; Rose *et al.*, 2004) with an X-ray apparatus optimized for protein crystallography (Yang *et al.*, 2003). However, the Bijvoet difference is still very small and the development of a highly accurate data-collection method is essential for the routine use of a longer wavelength. One of the experimental difficulties in using longer wavelengths is increased X-ray absorption. Although sources of absorption can be minimized by providing a helium beam path and by changing the material of the detector window (Yang *et al.*, 2003; Kitago *et al.*, 2005), a large absorption problem remains for intensity measurements under cryo-conditions. In the standard crystal-mounting method, there is a lens-shaped

frozen buffer solution and cryoloop around the crystal. For example, the reduction in intensity is 20% if a thickness of 500 µm of water is assumed. This is three times greater than that for Cu $K\alpha$ radiation and results in large variations in scale factors. Taking into account the problems of absorption alone, measuring anomalous signals of the order of 1% is not trivial.

To minimize these absorption effects, we have developed a novel technique for mounting a protein crystal to eliminate absorption by the cryobuffer and cryoloop around the frozen crystal (Kitago *et al.*, 2005). This technique increases the accuracy of the anomalous differences between the Bijvoet mates and makes the in-house S-SAD method with a Cr $K\alpha$ X-ray source a very useful tool for high-throughput structure determination. The present study was performed to examine the practical applicability of in-house structure determination using a Cr target and the loopless free crystal-mounting method. In addition, a Cr/Cu dual-wavelength system that makes it possible to perform a series of structure analyses from phasing to refinement of the structure in-house is briefly described.

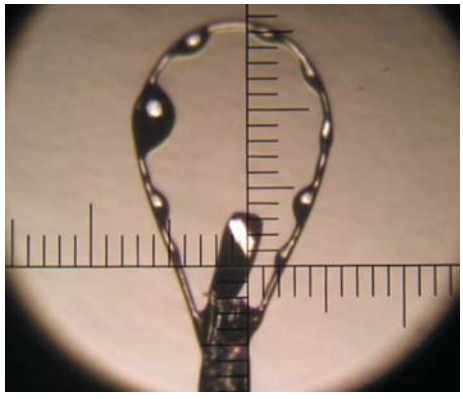

Figure 1
A TT0570 crystal mounted on the tip of the mounting capillary. The photograph was taken after aspirating the cryobuffer and freezing. One division of the scale is 50 µm.

2. Crystal-mounting technique for Cr S-SAD method

The new crystal-mounting method is based on the easy free crystal-mounting method for dehydration of protein crystals reported by Kiefersauer *et al.* (2000). They used a micropipette for holding a protein crystal without a loop. The potential disadvantages of their device for routine use for longer wavelength experiments are that a humid air system is necessary to avoid dehydration and the final crystal orientation becomes perpendicular to the micropipette or the φ axis of the diffractometer. We have overcome these problems by using a micropipette capillary that has a loop at the tip (Kitago *et al.*, 2005). Using this mounting tool, the crystal is kept in the cryobuffer before freezing, which prevents unwanted dehydration, and it can be frozen without buffer and the loop. It is easy to fabricate this mounting capillary. A glass capillary of 1 mm in diameter is pulled to make long taper and then cut and ground at a suitable diameter for each protein crystal. The nylon loop is then glued in place, carefully aided by the use of a microscope. It takes only several minutes to make this mounting capillary if we can use apparatus for cellular biology such as a micropipette puller, a microforge and a micropipette grinder. The most difficult or annoying part of making this device is gluing the nylon loop to the capillary and an easier method for making this device is under development. The mounting capillary is used as follows. Firstly, a crystal is picked up from the cryobuffer as in the standard cryoloop method. The buffer around the crystal is then aspirated through the capillary immediately before the blocked cryostream is flushed for freezing. Finally, the nylon loop is removed with a small hook or forceps under a microscope. Fig. 1 shows an example of a TT0570 crystal from *Thermus thermophilus* standing on the tip of the capillary. It is possible to mount crystals of different shapes free from buffer and loop using our crystal-mounting method. Photographs of the crystals whose structures were solved by Cr SAD are shown in Fig. 2. Movies of the crystal-mounting

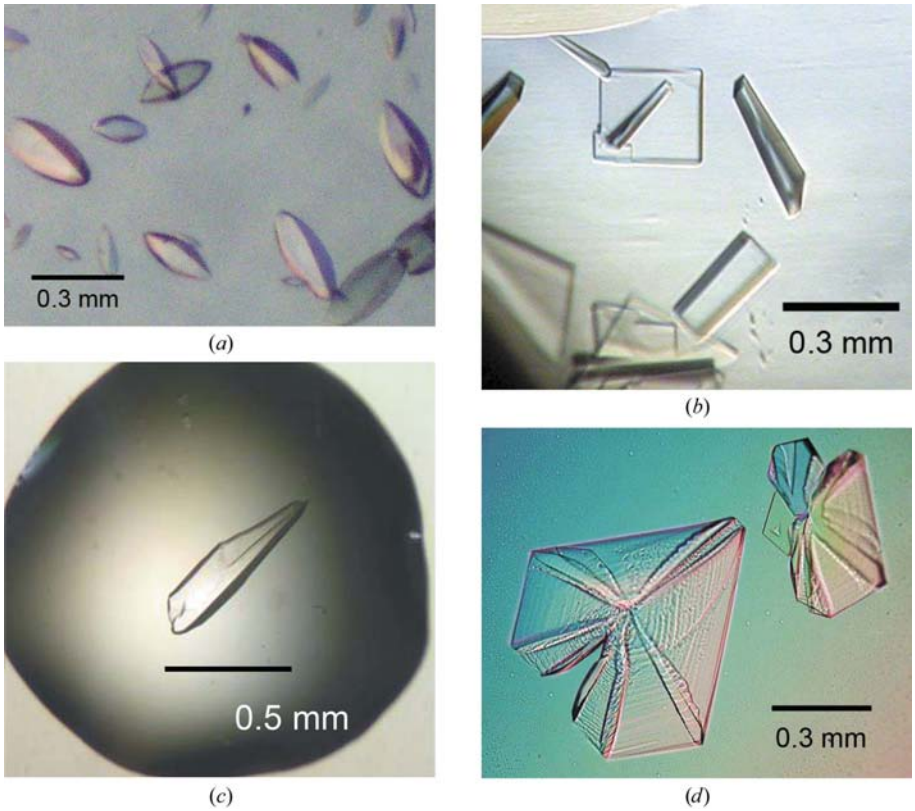

Figure 2
Micrographs of crystals solved by the Cr SAD method. (*a*) PHS023, (*b*) RNA-binding protein, (*c*) TT0570 and (*d*) glucosidase.

Table 1
Summary of the novel structures solved by the Cr SAD method.

Proteins	PHS023	PH1109	RNA-binding protein	TT0570	Glucosidase
No. of residues	83	144	241	603	738
Molecular weight (Da)	9667	16721	26918	68624	84310
Sulfurs per molecule	6 Met	4 Met, 2 Cys	4 Met	8 Met, 2 Cys (S—S)	23 Met, 6 Cys
Estimated Bijvoet ratio† (%)	2.45	1.86	1.11	1.09	1.68
Space group	$P3_221$	$P6_522$	$P2_1$	$P2_12_12$	$P2_1$
Unit-cell parameters (Å, °)	a = 61.7, c = 76.7	a = 70.0, b = 144.1	a = 44.3, b = 73.9, c = 44.3, β = 109.0	a = 100.3, b = 109.0, c = 114.6	a = 75.6, b = 112.4, c = 102.5, β = 100.6
Solvent content (%)	45.1	58.0	46.5	45.7	51.6
Molecules per ASU	2	1	1	2	2
Other anomalous scatterers per molecule‡	1 K^+	1 Ca^{2+}, 2 Cl^-, 2 P	2 Cl^-	None	1 Ca^{2+}
Software used	*SHELXD*, *OASIS*, *DM* *ARP/wARP*	*SHELXD*, *SHELXE*, *RESOLVE*	*SHELXD*, *SOLVE*, *RESOLVE*, *OASIS*-2004, *DM*, *ARP/wARP*	*SHELXD*, *SOLVE*, *RESOLVE*, *OASIS*-2004, *DM*, *ARP/wARP*	*SHELXD*, *SOLVE*, *RESOLVE*, *ARP/wARP*
No. of autobuilt residues with side chains	154 (93%)	79 (55%)§	223 (93%)	1167 (97%)	1378 (93%)

† Calculated after Hendrickson & Teeter (1981) using only S atoms as anomalous scatterers. ‡ Confirmed by final structures. § In the case of PH1109, we did not use *ARP/wARP* because coenzyme A binds to the protein.

process can be seen at http://www.sci.hokudai.ac.jp/~nobuhisa/XtalMount/. The success rate of this mounting method becomes close to that of the standard cryoloop with a little practice. The advantage of the method is that the X-ray absorption can be accounted for by that of the protein crystal itself. Comparison of the data quality of our free mounting method with standard loop mounting has been reported previously (Kitago *et al.*, 2005). As the mounting base used initially was large and it was not possible to store or recover frozen crystals before and/or after exposure, we have developed a modified Hampton CrystalCap Copper with the mounting capillary at its centre (Fig. 3). With a special magnetic base for this cap, it becomes possible to store or recover crystals as with the standard CrystalCap, if necessary. At synchrotron beamlines, this loopless free-mounting method also aids analyses of microcrystals that are difficult to centre in the lens-shaped frozen buffer in the cryoloop.

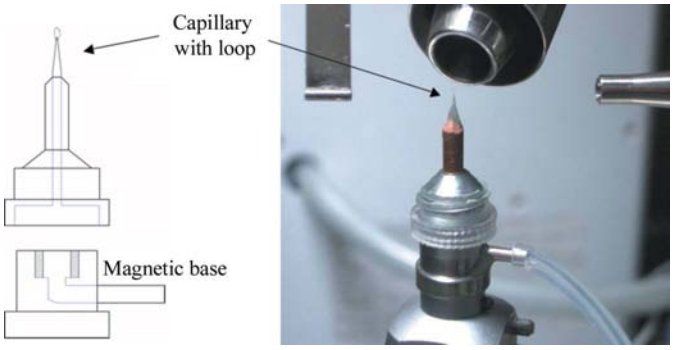

Figure 3
Modified Hampton CrystalCap for use in the loopless crystal-mounting method and magnetic base that allows aspiration. This modified CrystalCap allows one to store or recover crystals mounted by the loopless method.

The loopless free-mounting method has been shown to improve data quality for the Cr SAD method. However, attempts have also been made to develop an easier crystal-mounting method for highly accurate data collection at longer wavelengths. One possibility is the use of laser processing of protein crystals (Kitano *et al.*, 2004; Kitano, Matsumura *et al.*, 2005; Kitano, Murakami *et al.*, 2005). If crystals large enough for laser processing can be obtained, it will be possible to make a perfect spherical crystal as shown in Fig. 4 without inducing damage by the laser irradiation. These laser-shaped crystals also gave us anomalous signals that were as good those obtained using the loopless free-mounting method (manuscript in preparation).

Figure 4
Example of a laser-processed protein crystal. A lysozyme crystal frozen in the standard cryoloop (*a*) was laser-processed to make a spherical ball-shaped crystal (*b*). The diameter of the sphere is 250 µm.

3. Cr/Cu dual-wavelength system

The Cr/Cu dual-wavelength system used is a modified Rigaku FR-E SuperBright (maximum power and voltage 2 kW and 60 kV, 70 × 70 µm focal spot) with a Cr/Cu zebra-stripe target anode, similar to

that described by Kwiatkowski *et al.* (2000). It is operated at 40 kV, 40 mA for the Cr target and 45 kV, 45 mA for the Cu target. Switching between the two wavelengths can be performed easily by rotation of the cathode assembly inside the vacuum chamber. The Osmic Confocal MaxFlux optics optimized for chromium (Cr CMF) are exchanged for the Red optics when using Cu $K\alpha$. This allows use of both wavelengths through one of the two anode ports with a Rigaku R-AXIS VII imaging-plate detector. The resolution limit at the edge of the detector imaging plate is 2.2 Å for Cr $K\alpha$ and 1.5 Å for Cu $K\alpha$ radiation at a crystal-to-detector distance of 80 mm. To reduce X-ray absorption, the black paper of the detector window of R-AXIS VII was replaced with a carbon-filled thin polymer film. A post-sample helium path and a collimator extension cap were also used to reduce X-ray absorption and scattering by air. A 0.5 mm collimator is used to ensure that the whole crystal is always bathed in the X-ray beam. The only disadvantage of our system is that the beam displacement at the φ-axis position between two wavelengths is as large as 7 mm and alignment of the diffractometer is necessary each time the wavelength is switched. If a second detector is used, it is possible to use Cr $K\alpha$ radiation with one of the anode ports and Cu $K\alpha$ radiation with the other in alternate shifts to provide an in-house multi-wavelength anomalous diffraction method. The same system is now commercially available as FR-E DW SuperBright with integrated dual-wavelength VariMax mirror.

4. Examples of structure solution by the Cr SAD phasing technique

By use of the loopless free crystal-mounting method, proteins with molecular weights ranging from 9.6 to 84 kDa have been solved by Cr SAD using native protein crystals without any modification or derivatization. Table 1 lists these examples. In all cases the resolution limit of the data sets was 2.2 Å, which was limited by the detector dimensions.

Reflections were indexed and integrated with *HKL*2000 (Otwinowski & Minor, 1997). The anomalous scattering substructures were solved using *SHELXC* (Sheldrick, 2003) and *SHELXD* (Sheldrick *et al.*, 2001). Primary phasing, phase improvement and auto model building were performed by *SOLVE* (Terwilliger & Berendzen, 1999), *OASIS* (Hao *et al.*, 2000), *OASIS*-2004 (Wang *et al.*, 2004), *DM* (Cowtan, 1994),

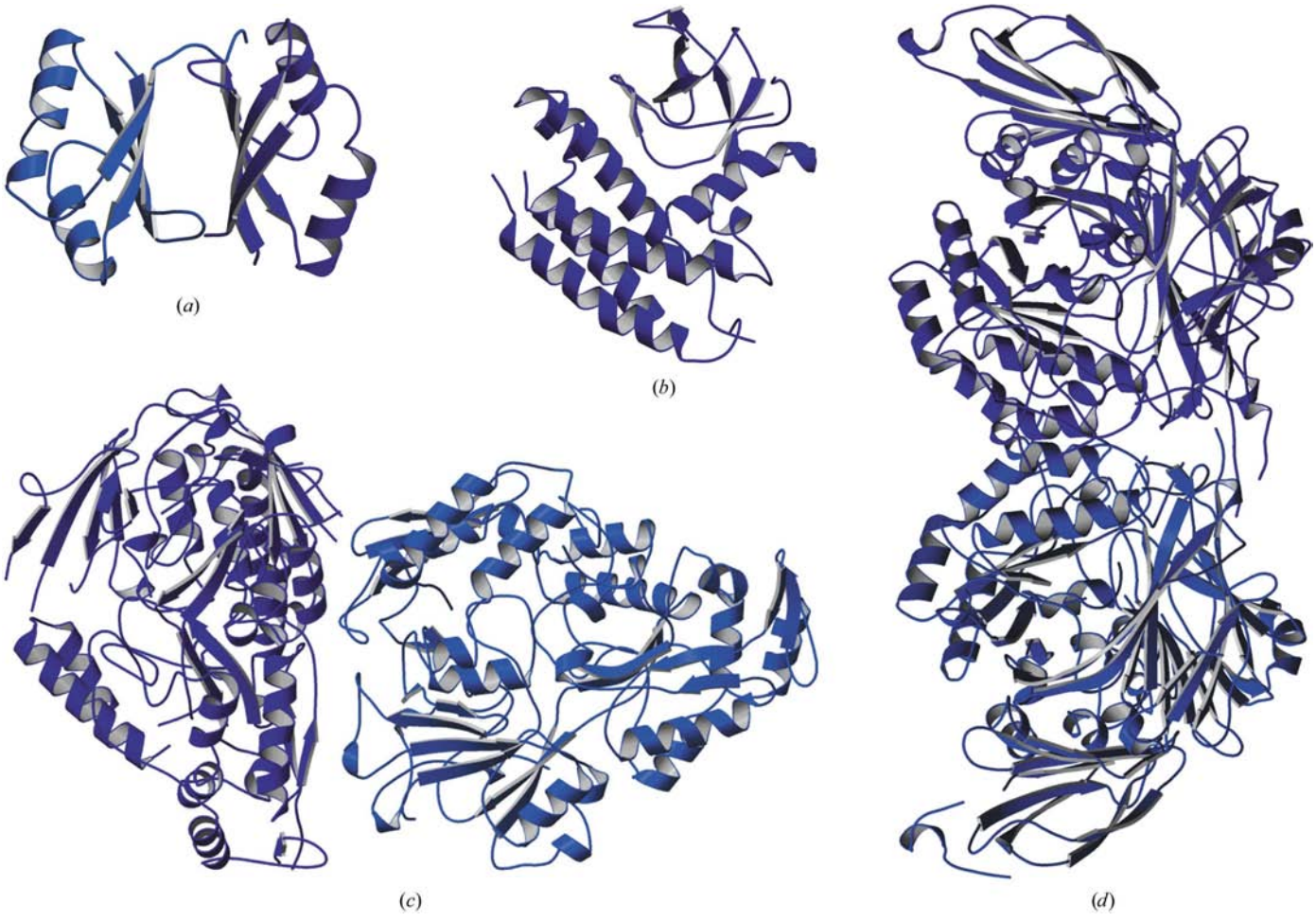

Figure 5
Autobuilt structures constructed by *ARP*/*wARP*. (*a*) PHS023, (*b*) RNA-binding protein, (*c*) TT0570 and (*d*) glucosidase. All ribbon models were plotted using *PyMOL* (DeLano, 2002).

RESOLVE (Terwilliger, 2003*a*,*b*), *ARP*/*wARP* (Perrakis *et al.*, 1999) and *REFMAC* (Murshudov *et al.*, 1997) as shown in Table 1. In all cases except for the PH1109 protein, more than 90% of the structures were automatically built with side chains. Auto-built structures of these examples are shown in Fig. 5. Phase improvement using solvent modification is essential for the Cr native SAD method (Watanabe *et al.*, 2005). In the case of the two larger proteins, NCS averaging was also used. The current success rate of our native Cr SAD phasing is 100% when the data set was collected with the loopless free-crystal mount.

With our dual-wavelength system, it is possible to perform a series of structure analyses from phasing to refinement in-house. A partial model built with Cr native SAD can be extended and refined with higher resolution data collected with Cu $K\alpha$ radiation. For example, the structure of glucosidase with a molecular weight of 84 kDa was refined using a 1.77 Å Cu data set to final R and free R factors of 0.194 and 0.224, respectively.

5. Conclusions

Wang (1985) predicted that proteins with a Bijvoet ratio of 0.6% could be solved by the SAD method if accurate data sets were available. From our experience with proteins solved using our Cu/Cr dual-wavelength system and the loopless free crystal-mounting method, an estimated Bijvoet ratio of 1% is sufficient for routine native SAD phasing once well diffracting crystals have been obtained. A structure of TT0570 protein from *T. thermophilus*, whose estimated Bijvoet ratio was 1.09%, was solved without difficulty. Recently, the putative transcriptional regulator SCO7518 from *Streptomyces coelicolor* A3(2), which had an estimated Bijvoet ratio was 0.89%, was also solved using this system (PDB code 2dg8). In addition, four of the five examples shown here had extra anomalous scatterers, such as Cl^-, K^+, and Ca^{2+}, as shown in Table 1 and these extra scatterers aided phasing. Therefore, the estimated Bijvoet ratio of 1% from the sequence is not a crucial threshold for the native SAD phasing. Bacteria and archaea have lower frequencies of sulfur-containing residues than eukaryotes (Micossi *et al.*, 2002). The histograms in Fig. 6 show the distribution of estimated Bijvoet ratio of proteins of *Pyrococcus horikoshii*, *Escherichia coli* and *Caenorhabditis elegans* calculated from the amino-acid composition of their ORFs. If an estimated Bijvoet ratio of 1% is sufficient for primary phasing, the blue portion of the histogram will be able to be solved with the native SAD method. Using Cr $K\alpha$ radiation and our loopless free-mounting method, almost all

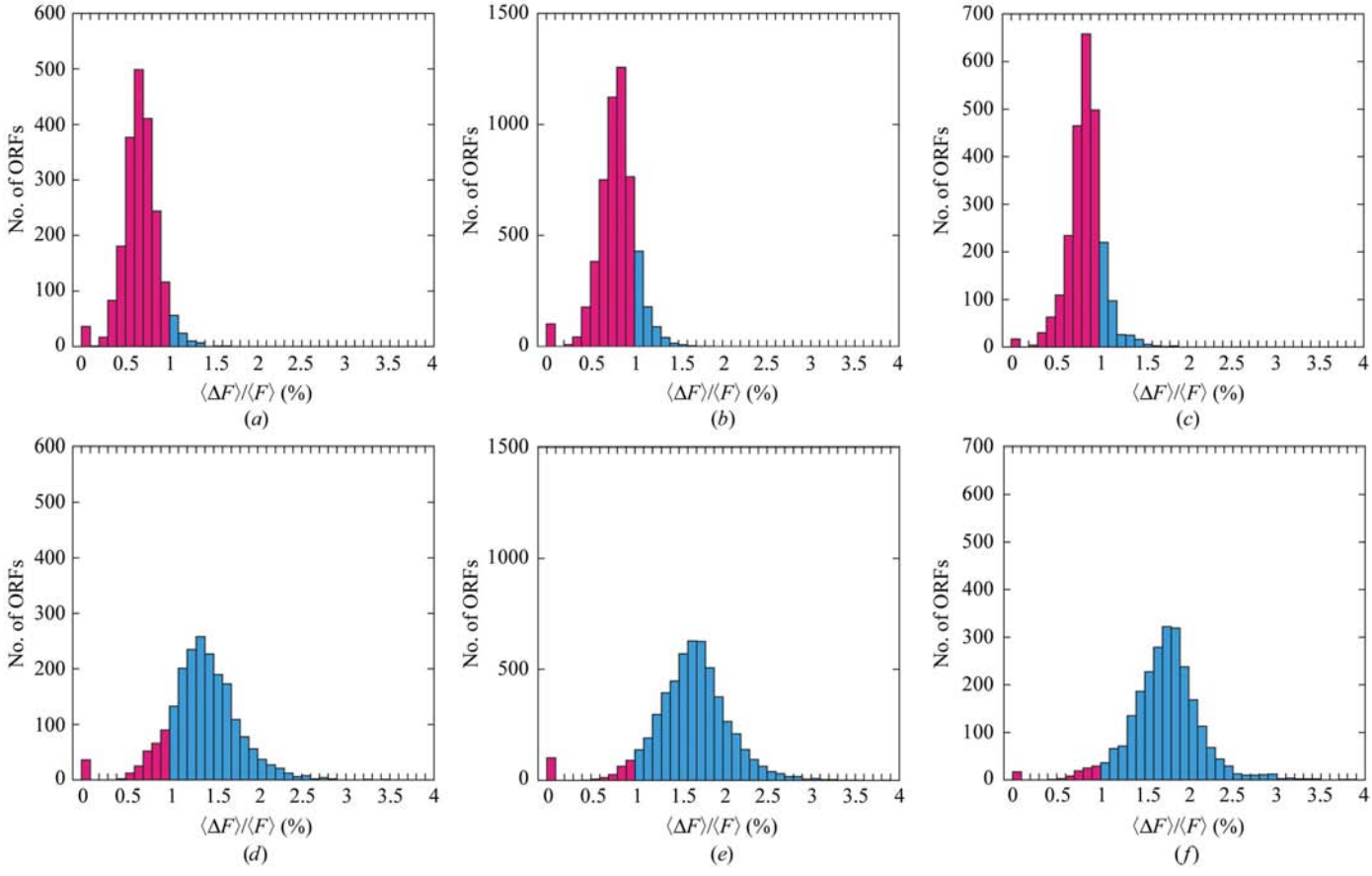

Figure 6
Histograms showing the distribution of the number of open reading frames as a function of percentage of the Bijvoet ratio. (*a*), (*b*) and (*c*) are the distributions for Cu $K\alpha$ and (*d*), (*e*) and (*f*) are those for Cr $K\alpha$. (*a*) and (*d*) are for *P. horikoshii*, (*b*) and (*e*) are for *E. coli* and (*c*) and (*f*) are for *C. elegans* chromosome I. Columns in which the Bijvoet ratio is higher than 1% are coloured blue.

proteins could be solved in-house without any modification or derivatization. This will allow greater flexibility when choosing sample-preparation systems.

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