1. Introduction

Single wavelength anomalous scattering (SAS) phasing has become a common tool for macromolecular structure determination as evidenced by the 245% increase in SAS versus multi-wavelength anomalous dispersion (MAD) structures deposited to the Protein Data Bank [Berman et al., 2000] over the last year [http://asdp.bio.bnl.gov/asda/Libraries/pdb_statis/latest/xme/SING.html].

The major problem in collecting SAS data is that the anomalous difference in Bijvoet pairs ($\Delta F_i = F_{+} - F_{-}$) is generally about one order of magnitude smaller than the difference between $|F_{ph}(hkl)|$ and $|F_{p}(hkl)|$ used in isomorphous replacement. Since SAS data collection requires measurement of small differences (typically 3–8%) in the considerably larger structure factor amplitudes, the signal-to-noise (S/N) level in the data is critical to the success of the experiment. Thus, the higher the S/N level in the data, the greater the probability of success in producing an interpretable electron density map. One way to increase the S/N in the data is to increase the anomalous signal, which is dependent on the X-ray energy used in the analysis and generally increases with wavelength as illustrated in Fig. 1. Thus, data collection using soft X-rays ($\lambda = 2.2909 \text{Å}$ for Cr Kα) for this purpose for over 20 years. Compared to copper X-rays ($\lambda = 1.5418 \text{Å}$ for Cu Kα), the anomalous scattering signal for most elements is nearly doubled with chromium X-radiation. Some exceptions are V, Cr, Mn, Fe and Co, which lie close to the chromium absorption edge. Indeed, doubling of the anomalous scattering signal for light atoms, such as sulfur, makes chromium X-rays an intriguing source for SAS phasing. However, X-ray absorption, which also increases with increasing wavelength, must be addressed (see Fig. 2).

In studies carried out in the early 1980's using a 1 kW sealed tube chromium source on a Picker FACS diffractometer, it was shown that problems with X-ray absorption could be overcome through the careful use of helium beam paths (Rose, unpublished results). Tests on porcine neurophysin I showed that vanadium-filtered Cr Kα X-rays gave equivalent or better intensities than those recorded using nickel-filtered Cu Kα X-rays for similar reflections from capillary mounted crystals of similar size and shape, provided the air path between source and detector was minimized. This was accomplished by purging the path from source to collimator exit with helium gas and by placing a helium-filled beam path between the beam stop position and the entrance to the scintillation counter. Unfortunately, three problems (source intensity, the lack of a good monochromator and crystal decay) limited the system’s usefulness for routine SAS structure determination using chromium X-rays.

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The source intensity problem was easily addressed through the use of a rotating anode generator. The crystal decay problem was essentially solved by the introduction of cryo-crystallography [Hope, 1988; Teng, 1990]. Again, studies carried out at the Institute for Molecular Biology [(IMB), Academia Sinica, Taiwan ROC] in 1990, using a chromium anode and a Rigaku RU-300 rotating anode generator with a Rigaku AFC5 diffractometer showed that, with careful use of helium beam paths, X-ray absorption could be minimized. However, inadequate monochromatization (a high degree of Kβ contamination) of the X-ray beam (Rose, unpublished results) continued to be a problem.

In 2002, Rigaku/MSC introduced the first confocal optic for chromium radiation (the Osmic CMF15-50Cr8). Crystallographers now had access to high intensity monochromatic chromium Kα X-rays [Yang et al., 2003]. The first commercial unit (CHROMOS I) was installed at the University of Georgia in January 2003 as part of the Southeast Collaboratory for Structural Genomics’ (SECSG) Direct Crystallography research program. Direct Crystallography, structure determination from underderivatized native protein crystals, relies on the accurate detection of the SAS signal from sulfur and/or metals present in the native protein. Initial tests indicated that sulfur anomalous scattering signal recorded using CHROMOS I was superior (see Beamline Optimization below) to the anomalous scattering signal observed in the best synchrotron data collected at longer wavelengths (\(\lambda>1.5\) Å). This observation is not surprising since most modern beamlines are optimized for data collection near the selenium absorption edge at \(\sim 0.97\) Å, dictated by the requirements of the seleno-met MAD (multi-wavelength anomalous dispersion) experiment [Hendrickson, et al., 1990]. Little attention has been paid to optimizing performance in the 1.5 to 2 Å wavelength range required for successful sulfur SAS phasing. Thus, in addition to providing intense monochromatic soft X-rays for phasing purposes, CHROMOS I also served as

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**Fig. 1.** A plot of \(\Delta f''\) as a function of atomic number for copper (blue) and chromium (purple) Kα radiation showing the doubling of the available anomalous signal for most elements when chromium radiation is used. The value of \(\Delta f''\) for selenium at the selenium absorption edge (0.9795 Å) is shown in orange for comparison.

**Fig. 2.** A plot of X-ray absorption by air (80% N\textsubscript{2}/20%O\textsubscript{2}) for copper (magenta) and chromium (blue) Kα radiation as a function of distance.
an ideal test bed for soft X-ray beamline optimization for the SER-CAT (www.ser-cat.org) insertion device and bending magnet beamlines at the Advanced Photon Source, Argonne National Laboratory.

The UGA CHROMOS I in-house phasing system, SAS data collection protocols, initial results related to Direct Crystallography and beamline optimization are described below.

2. Experimental Setup

CHROMOS I (Fig. 3a) was based on an existing Rigaku rotating anode area detector system (RU-300, MSC Blue confocal optics, MSC X-stream, inverted phi axis, R-AXIS IV image plate detector). For the upgrade, a chromium target, purchased in 1990, was borrowed from IMB. After replacing the magnetic seal, the target was installed in the RU-300. The existing MSC Blue confocal optic was removed and replaced with the chromium optic. Since the footprint of the two optics is similar, little modification to the initial R-AXIS IV placement was necessary and the whole transformation was accomplished within a day. The new collimator was machined to allow the beam stop to slide as close to the crystal as possible in order to minimize the collimator-crystal-beam stop air path. In addition, all components of the optic/collimator unit are purged with helium to reduce X-ray absorption. Finally, the original paper light barrier at the detector’s entrance was replaced with a lower absorbing material. A 100 mm helium beam path added to the front of the detector completed the transformation. The measured flux on sample, as measured with a pin diode, is similar to that measured for the Cu Kα X-rays focused using MSC Blue confocal optics.

The large (30×30 cm) active area of the R-Axis IV detector is well suited for chromium data collection since the diffraction pattern is expanded by about 50% when compared to a copper source. Image plate efficiency however is reduced by a factor of 1.5 since Cr Kα X-rays produce 33% fewer color centers upon interaction with the phosphor compared to the number of color centers produced in the phosphor when Cu Kα X-rays are used.

In practice, the total air path of the system is kept to a minimum. The collimator-crystal-beam stop air path is typically 15 mm to allow for cooling of the crystal without ice formation on the crystal, collimator or beam stop (see Fig. 3b). The detector is placed in line with the beam with 2θ=0. The crystal-to-detector distance (typically 120 mm) is set such that the Mylar entrance window to the diffracted beam path is placed as close to the beam stop as possible. This arrangement gives a total air path of under 20 mm and allows for data collection to 2.5 Å at the detector’s edge, which is more than sufficient for SAS phasing experiments. An example of a diffraction pattern collected using CHROMOS I is shown in Fig. 4.

3. Data Collection

Over the past half-decade, SAS data collection protocols for both in-house [Chen et al., 1991; Wu et al., 2000] and synchrotron [Liu et al., 2001; Ramagopal et al., 2003] have been es-
established. These protocols rely on data redundancy to improve the accuracy of the measured structure factors, thus providing better estimates of the Bijvoet differences. However, X-ray induced crystal decay [Gonzalez and Nave, 1994], which increases with increasing wavelength can become a problem in collecting highly redundant data sets even at cryogenic temperatures. Radiation damage has implications to the success of Direct Crystallography since in addition to radiation-induced decarboxylation of acidic amino acid residues, disulfide bond breakage and disulfide radical formation also occurs [Weik et al., 2000]. Thus radiation damage (unobserved in the diffraction pattern) can perturb the sulfur substructure that is the basis for the SAS signal. This must be taken into account when designing the data collection strategy, i.e. “more data is not necessarily better data”.

A simple data collection strategy has been developed for use with CHROMOS I that has proven to be very effective for crystals belonging to space groups with orthorhombic or higher symmetry. Essentially, a crystal is mounted in a fiber loop that is matched, as closely as possible, to the dimensions of the crystal to limit the X-ray absorption by the mother liquor and flash-cooled to 77 K in liquid nitrogen (LN2). The detector is moved away from the goniometer to allow access and the crystal is transferred to the goniometer in LN2 aided by the inverse φ axis. After carefully centering the crystal, the detector is repositioned to give a crystal-to-detector distance of 120 mm. The helium gas supply and flow rates are checked to insure an adequate flow and supply. Data collection consists of collecting 360 one-degree oscillation images with a typical exposure time of 300 s or longer as determined by the diffraction quality of the crystal. If more data is desired, a second crystal is mounted as described above and another set of 360 images are collected. Data reduction is carried out using either d*TREK [Pflugrath, 1999] or HKL2000 [Otwinowski and Minor, 1997].

4. Example of CHROMOS I Structures

Using the above setup, six structures, as described below, have been determined. In each case, only one crystal (the first one mounted)

![Diffraction Pattern](image)

**Fig. 4.** An example of a diffraction pattern recorded with CHROMOS I. The crystal to detector distance was 100 mm.

| Table 1. Experimental parameters for the CHROMOS I structures. |
|-----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                 | Len 1801964    | Q15691         | Aequorin Sso10a | 838710         |
| Figure          | 5a             | 5b             | 5c             | 5d             | 5e             | 5f             |
| Molecular weight (kD) | 12.6          | 34.1           | 30.0           | 22.5           | 11.2           | 20.4           |
| Number of residues | 114           | 302            | 153            | 185            | 95             | 171            |
| Anomalous scatterers | 2S            | 8S             | 7S             | 8S, 3Ca        | 7S             | 2Pt, 1S        |
| Number of disulfides | 1             | 0              | 0              | 0              | 0              | 0              |
| Space group     | P422          | P222           | P422           | P222           | P222           | P321           |
| a (Å)           | 65.3          | 68.5           | 58.3           | 54.3           | 57.6           | 97.2           |
| b (Å)           | 65.3          | 68.5           | 61.2           | 54.3           | 72.3           | 97.2           |
| c (Å)           | 51.4          | 150.7          | 67.5           | 135.1          | 30.0           | 127.6          |
| Molecules per asymmetric unit | 2            | 1              | 2              | 1              | 1              | 1              |
| Data resolution (Å) | 2.5           | 2.5            | 2.5            | 2.5            | 2.5            | 2.7            |
| Completeness of data | 99.7          | 94.2           | 98.0           | 98.8           | 95.7           | 98.8           |
| Bijvoet redundancy | 11.4          | 6.1            | 8.5            | 9.4            | 12.3           | 11.7           |
| Rsym (%)        | 4.6            | 4.1            | 4.3            | 5.3            | 3.9            | 5.9            |
| Rsym (high resolution shell) | 6.8          | 6.1            | 13.1           | 11.2           | 15.0           | 31.6           |
| PDB ID          | 1NNH          | 1VKA           | 1SL8           | 1RJ7           |                  |                 |

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was required for successful structure determination. The six examples (Table 1 and Fig. 5a–f) illustrate the quality of the experimental electron density maps produced from SAS data collected with chromium X-rays. It should be noted that data collection for phasing purposes at SECSG is kept separate from data collection for high-resolution refinement, which is generally carried out using the high intensity SER-CAT undulator beamline 22-ID. This approach avoids making compromises that either effect S/N of the phasing data or in the recording of reflections at high-resolution.

Of the six examples described here, Bence Jones (Fig. 5a) Protein Len and Pfu-1801964 (Fig. 5b) represent a redetermination of the structure based on the sulfur anomalous scattering signal recorded using CHROMOS I. The purpose of these analyses was to test the quality of data produced by CHROMOS I and the quality of the resulting electron density maps. The remaining four examples [Q15691 (Fig. 5c), aequorin (Fig. 5d), Ss010a (Fig. 5e) and Pfu-838710 (Fig. 5f)] represent de novo structures determined as part of SECSG’s structure deter-

![Fig. 5a.](image1) (Left) a section of the 3 Å experimental ISAS-phased electron density map for Bence Jones protein Len contoured at 1σ with the refined coordinates for the structure superimposed and (Right) a ribbon drawing of the Len structure. Len was chosen because its molecular weight and sulfur content (3 sulfurs per 114 residues with one disulfide) are nearly identical to that of Rhe (2 sulfurs per 114 residues with one disulfide) used in the initial S-SAS simulations by Wang [1985]. In this analysis, data to 2.5 Å resolution was collected and processed with HKL2000, keeping anomalous pairs separate during scaling. Sulfur positions were determined using SHELXD [Sheldrick, 1998] using 3.0 Å data. Protein phases were calculated using ISAS [Wang, 1985]. Three filters, each with four cycles of iteration, were used in the ISAS noise filtering process.

![Fig. 5b.](image2) (Left) a section of the 3 Å experimental ISAS-phased electron density map for ORF 1801964 from *Pyrococcus furiosus* contoured at 1σ with the refined coordinates for the structure superimposed and (Right) a ribbon drawing of the 1801964 structure. The protein was chosen in order to investigate whether the lack of disulfide bonds affected the success rate of sulfur SAS phasing using chromium X-rays. Data collection, processing and phasing was carried out using the programs and procedures described previously for Len. It is interesting to note that initial attempts at sulfur SAS phasing using data collected at SER-CAT and other beamlines (λ ~ 1.74 Å) failed to produce an interpretable electron density map.
mination efforts. These structures were determined using the SECSG SCA2Structure high-throughput structure determination pipeline [Liu et al., 2002]. Sulfur positions and protein phases were determined using SOLVE/RESOLVE [Terwilliger and Berendzen, 1999] and ISAS respectively. In most cases, native data collected to high resolution at SER-CAT was used together with the SAS phases generated from the CHROMOS I data to auto-trace the experimental electron density map.

**Beamline optimization**—An obvious source of soft X-rays is synchrotron radiation since the wavelength of the X-rays produced is in the range of 0.6 to 2.3 Å depending on beamline configuration. Since the value of $\Delta f''$ for sulfur is relatively small (0.90) for data recorded using 2 Å X-rays compared to the value of $\Delta f''$ for selenium (3.84) for data recorded at the selenium absorption edge (0.97 Å), it is important to know whether the data collection system (source, optics, goniometer and detector) is optimally tuned for data collection at the chosen wavelength. Researchers at SECSG have developed a series of tests based on the anomalous scatter-
ing signal of zinc-free cubic insulin that reflects the ability of the data collection system to record the weak sulfur anomalous scattering signal [Liu et al., 2002]. These tests were used to analyze system performance for 22-ID, the SER-CAT undulator beamline for data collection using 1.54 Å and 1.74 Å X-rays. The results of one of the more sensitive tests (ISAS map correlation) are shown in Fig. 6. From Fig. 6, it is apparent that 22-ID, as initially configured (October 2002), is suboptimal compared to CHROMOS I. This comparison led to the identification of a problem in the 22-ID optics caused by radiant heating within the monochromator. Tests conducted in April 2003, after additional shielding was installed on the first crystal holder, show that the problem has been corrected.

5. Conclusions
In order for any method to be considered routine, it must be easy to use, robust and have a high potential for success. The initial results with the CHROMOS I in-house phasing system reported here, as well as other studies [Yang et al. 2003; Phillips et al., 2004], show that routine data collection with chromium X-rays is possible, the experimental design is simple and the potential for successful sulfur SAS structure de-
termination is high for crystals of orthorhombic symmetry or higher. As with other sources, success of the structure determination is dependent on the diffraction quality of the crystals used in the analysis. The Pfu-838710 example, however, shows a successful structure determination from moderately diffracting crystals can be achieved by soaking the crystal with stronger anomalous scatterers such as platinum using the quick-soak technique [Dauter et al., 2000]. In addition, CHROMOS I has proven to be a useful tool for optimizing synchrotron beamlines for data collection at longer wavelengths since it can provide an externally derived performance benchmark for comparison. Finally, chromium radiation offers an in-house solution for data collection on large unit cells as shown in Fig. 7 where a 600 Å axis is clearly resolved using a crystal to detector distance of 175 mm.

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References


Fig. 6. Results of the SER-CAT beamline performance tests (ISAS map correlation) conducted in October 2002 during 22-ID beamline commissioning (blue) and after beamline optimization in April 2003 (green) based on comparisons to the CHROMOS I benchmarks (red).

Fig. 7. An example of a diffraction pattern recorded with chromium X-rays (using a system similar to CHROMOS I at Rigaku/MSC) showing resolution of a 600 Å using a 175 mm crystal-to-detector distance (Figure courtesy of Cheng Yang, Rigaku/MSC). Note: the image was taken without a proper helium beam path.
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