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Lab Source Anomalous Scattering Using Cr *Kα* Radiation

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ABSTRACT

High-throughput crystallography requires a method by which the structures of proteins can be determined quickly and easily. Experimental phasing is an essential technique in determining the three-dimensional protein structures using single-crystal X-ray diffraction. In macromolecular crystallography, the phases are derived either by Molecular Replacement (MR) method using the atomic coordinates of a structurally similar protein or by locating the positions of heavy atoms that are intrinsic to the protein or that have been added (MIR, MIRAS, SIR, SIRAS, MAD and SAD). Availability of in-house lab data collection sources (Cu *Kα* and Cr *Kα* radiation), cryo-crystallography and improved software for heavy atom location and density modification have increased the ability to solve protein structures using SAD. SAD phasing using intrinsic anomalous scatterers like sulfur, chlorine, calcium, manganese and zinc, which are already present in the protein becomes increasingly attractive owing to the advanced phasing methods. An analysis of successful SAD phasing on three proteins, lysozyme, glucose isomerase and thermolysin based on the signal of weak anomalous scatterers such as sulfur atom and chloride ion have been carried out. This analysis also proves that even the anomalous signal provided or present naturally in a macromolecule is good enough to solve crystal structures successfully using lab source chromium-generated X-ray radiation.

Keywords: Anomalous Scattering; SAD Phasing; Lysozyme; Glucose Isomerase; Thermolysin

1. Introduction

The sequencing of complete genomes has spurred a growing need for structural information on the encoded gene products that has driven the development of high-throughput crystallography [1-7]. Anomalous scattering methods are currently widely used for phasing macromolecular structures [8]. Experimental phasing is an essential technique in determining the three-dimensional protein structures using single-crystal X-ray diffraction. The power of Single-wavelength Anomalous Diffraction (SAD) was illustrated even 25 years ago and the anomalous signal from six sulfur atoms was used to solve the structure of crambin, a 4.8 kDa protein [9,10]. In recent years, SAD has become the choice for phasing [11-13] after gaining advantage over the Multi-wavelength Anomalous Diffraction, MAD [14]. The SAD approach shares advantage with MAD that all measurements can be made on the same single crystal so that lack of isomorphism does not occur and a single data set at single wavelength is enough [15]. Availability of synchrotron radiation, cryo-crystallography and improved software for heavy atom location and density modification have increased the ability to solve protein structures using SAD. In the absence of synchrotron radiation, SAD method using rotating anode generator (Cu *Kα* and Cr *Kα*) lab source data is mostly used to solve the phase problem in X-ray crystallography. Using high redundant data, sufficiently accurate anomalous data can now be collected in the laboratory using Cu *Kα* and Cr *Kα* radiations [16-20].

Longer wavelengths than Cu *Kα* (1.54 Å) would produce a larger signal, but at the same time experimental difficulties may increase as does the noise level in the data [19]. It has been recently reported that data collection wavelengths in the range of $\lambda = 1.5 - 3.0$ Å are fairly easy to handle in a diffraction experiment and even at home sources using instant Cr *Kα* radiation [20]. The use of chromium-anode X-ray radiation is very useful for SAD experiments. The anomalous scattering signal is more than doubled for various metals when compared to conventional copper characteristic wavelength. Further-

more, naturally bound metals and atoms from crystallization solutions tend to show a significant increase in anomalous scattering with chromium radiation [21,22]. Improved data quality helps in exploiting the weak anomalous signal derived only from the sulfurs or in particular from halide ions incorporated by soaking [23].

Most of the sulfur SAD data have been collected on tunable synchrotron beamlines in order to make use of the appreciably larger f″ of sulfur at longer wavelengths in the range between 1.7 - 2.5 Å [24-30] although Cu *Kα* is also feasible for sulfur SAD phasing [31-33]. Both copper and chromium anode wavelengths (1.54 and 2.29 Å) have been increasingly employed for the same purpose in lab X-ray sources with success [34-36]. Using in-house copper wavelengths and synchrotron beamlines, phasing of protein like lysozyme, glucose isomerase and thermolysin have been carried out [27,28,31].

NaCl and KCl are part of almost every protein buffer and crystallization cocktail. Sulfur atoms and chloride ions are present in almost all known proteins and already been successfully utilized for the SAD phasing of many proteins. On an average, a protein contains 2.4 methionine and 1.7 cysteine residues per 100 amino acids [36], corresponding to an estimated *Bijvoet* ratio of 1.4% at 2.0 Å wavelength (assuming 7.46 non-hydrogen atoms per amino acid). Many other heavy atoms have also been successfully derivatized for SAD phasing [37-47]. Only rarely are intrinsic S atoms the sole source of the anomalous signal [16,18,48]. However, heavy atom derivatives suffer from nonspecific binding, which results in low occupancy of the heavy-atom sites leading to weak anomalous signal and disruption of the crystal lattice and failure in derivatization.

Exploiting the anomalous signal already present in the native protein or in the solvent would eliminate the extra experimental work *via* derivatization and would also eliminate the risk of lack of isomorphism. The anomalous signal from different anomalous scatterers present within the SAD data (native lysozyme, native glucose isomerase and native thermolysin) collected at chromium wavelength is used for successful structure solution.

2. Methods

2.1. Crystallization

Hen Egg White Lysozyme (HEWL), Glucose Isomerase

(GI) and Thermolysin (TL) were purchased from Hampton Research Ltd., USA and used without further purification. Crystallization was performed based on the earlier established protocols [49-51]. Crystals were obtained in two days by hanging-drop vapour diffusion method. HEWL crystallized in tetragonal space group, GI crystallized in orthorhombic space group and TL crystallized in hexagonal space group. Before data collection, the crystals were quickly soaked in mother liquor containing cryoprotectant and then mounted into a loop of the required size using a cold nitrogen stream at 100 K. Crystallization, cryoprotectant and soaking conditions are shown in **Table 1**.

2.2. Data Collection

Three datasets (native HEWL; native GI; native TL) were collected separately using Rigaku R-Axis IV++ image plate detector [52] equipped with Cr *Kα* (2.29 Å) anode X-ray generator, at CCMB, Hyderabad, India. The crystals were mounted in a random orientation. Crystals diffracted upto 2.53 Å and 360 frames were collected with crystal to detector distance being 110 mm at 0.5˚ oscillation steps and 120 seconds exposure time per frame in each case. The intensity data were collected and analyzed for anomalous signal, substructure solution and binding sites. The intensities were integrated using *HKL*2000 [53], refining all parameters including crystal mosaicity.

2.3. Substructure Solution, Phase Determination and Model Building

Anomalous scatterers were located using the dual-space recycling algorithm enabled in *SHELXD* [54-56]. Patterson seeding was used in all cases, resulting in an appreciable improvement in the success rate. 100 cycles of dual-space recycling were performed for the peak search and structure factor calculation. For the location of substructures of anomalous scatterers with *SHELXD*, only the internal loop which relies on the strongest E magnitudes was used. The best solution obtained with the highest correlation coefficient was directly fed as an input to *SHELXE* [57] for phase calculation and improvement without further refinement of the atomic positions and their occupancies. The high resolution limit for each data was used for phasing. The poly ala model building

Table 1. Crystallization, soaking concentration and cryoprotectant details.

	Native HEWL	Native GI	Native TL
Crystallization Condition	50 mM Sodium Acetate and 1 M Sodium Chloride pH 4.7	200 mM Magnesium chloride and 100 mM Tris, pH 4.7	1.4 mM Calcium Acetate, 10 mM Zinc Acetate, 1 mM Sodium Nitrate and 50 mM Tris; pH 7.3
Cryoprotectant	30% Glycerol	25% MPD	25% Ethylene Glycol

was carried out using *SHELXE beta* version. The final quality *SHELXE* phases were fed as input to *ARP/wARP* [58] for main-chain tracing and side-chain docking. Clearly interpretable maps were obtained after the automated model building. Manual rebuilding was carried out using *COOT* [59] and the final model was refined using *REFMAC*5.0 [60] available in *CCP*4*i suite* [61]. Figures were prepared using *PyMOL* software [62].

3. Results and Discussion

3.1. Data Analysis

The data was processed using *HKL*2000, by separately merging the anomalous pairs as I^+ and I^-. The overall redundancy of the data collected to 2.53 Å for the complete datasets varied between 10.3 and 43.49 at high resolution bin. The intensity data were analyzed for the presence of anomalous signal using *HKL*2MAP [63]. The full resolution range of the data was found to have anomalous signal measurability greater than 14%. The mean signal to noise ratio for all the datasets were found between 19.9 and 57.6.

The completeness of the datasets varied between 98% and 100%. Both the redundancy and completeness play a major role in model building after substructure determination. The substructure solution, phasing and refinement statistics are shown in **Table 2**. The overall *figure of merit* ranged from 0.53 to 0.58 for all the datasets. The

map correlation coefficient was also found to be higher for all the datasets. The model obtained was manually corrected using *COOT* and refined using *REFMAC*5.0. The B-factor (thermal factor) in all the cases or datasets ranged from 10.3 to 39.7. The final model of all the datasets were refined to reasonable R_{work} and R_{free} values.

All the three datasets were processed and scaled using *HKL*2000. The scala output obtained was given as input for *SHELXC/D/E* phasing using *CCP*4*i*. At the chromium wavelength of 2.29 Å, a sulfur atom has an f'' value of 1.14 $\overline{e}$ and for a chlorine atom, $f'' = 2.01$ $\overline{e}$ as estimated from program *CROSSEC* [64]. During the substructure solution of HEWL dataset using *SHELXD*, fifteen peaks were identified out of which eight peaks correspond to sulfur as there are four disulphide bridges in HEWL. Similarly, seven chloride ions were identified by comparing the chloride ion sites in the earlier reported HEWL structure [31]. The density modified phases were used for model building using the *ARP/wARP* program web server [53] and refined using *REFMAC*5.0 of *CCP*4*i* software. For the dataset with redundancy 10.6, the automated solution and the model building by *ARP/wARP* resulted in building 126 amino acid residues, in which 110 were with side chains indicating that the dataset with reflections collected upto 2.53 Å was sufficient for structure solution and 10 fold redundancy is enough for substructure solution. Final refined model has $R_{work} = 19.4\%$ and $R_{free} = 23.6\%$. The disulphide bonds

Table 2. Crystal Data Statistics, phasing and model building details.

	Native HEWL	Native GI	Native TL
Cell Parameters	a = b = 77.67 Å, c = 37.43 Å	a = 92.78 Å, b = 97.31 Å, c = 102.64 Å	a = b = 92.76 Å, c = 128.36 Å, γ = 120˚
Space Group	P4$_3$2$_1$2	I222	P6$_1$22
Mosaicity	0.76	0.49	0.6
Resolution range	27.48 - 2.53 Å	27.48 - 2.53 Å	38.33 - 2.53 Å
Redundancy	10.6 (9.98)	10.3 (9.73)	43.49 (42.84)
Completeness (%)	99.5 (97.8)	98.1 (94.8)	100.0 (100.0)
I/σ(I)	57.6 (13.3)	17.4 (4.8)	19.9 (7.1)
Anomalous signal (%)	17.1	18.3	14.6
FOM	0.56	0.58	0.53
Map Correlation	0.76	0.81	0.79
B$_{Wilson}$	10.53	12.79	39.69
Total Residues Built	122	388	316
Side Chains	110	383	314
R$_{work}$ (%)	19.4	18.2	18.6
R$_{free}$ (%)	23.6	22.8	21.9
Number of solvent molecules	102	140	145

showed clear elliptical density and Met sulfurs showed spherical density. Anomalous map of monomeric HEWL with eight sulfur peaks and seven chloride ions at 5σ with water molecules is shown in **Figure 1**.

In the native GI data, the anomalous difference map showed significant density for nine sulfur atoms within the protein and an additional peak corresponding to one manganese ion. At the chromium wavelength of 2.29 Å, a manganese ion has a f″ value of 0.97 $\bar{e}$ as estimated from program *CROSSEC*. Out of nine sulfur atom peaks, eight were of MET sulfurs and one peak was of the CYS. The position of the manganese ion peak was identified by comparing the manganese position or occu-pied site in the earlier reported GI structures [27]. They were given as input into *SHELXE* for obtaining the electron density maps. The final maps obtained after density modification were subjected to analysis by *ARP/wARP* web server for automatic chain tracing and model building. The electron density map allowed *ARP/wARP* program to build 388 residues, in which 383 were built with side chains. The dataset has a redundancy of 10.3. The dataset with reflections collected upto 2.53 Å was sufficient for structure solution and 10 fold redundancy is enough for substructure solution. Final refined model has R_{work} = 18.2% and R_{free} = 22.8%. Anomalous map of monomeric GI with nine sulfur atoms and one manganese ion at 5σ with water molecules is shown in **Figure 2**.

For native TL data, anomalous signals for three calcium ions, one zinc ion and two sulfur atoms were located using *SHELXD*. At the chromium wavelength of 2.29 Å, a calcium ion has a f″ value of 3.62 $\bar{e}$ and for a zinc ion, f″ = 2.05 $\bar{e}$ as estimated from program *CROSSEC*. The two sulfur atom peaks belonged to the MET sulfurs present in the TL. The positions of the four calcium ion peaks and one zinc ion peak were identified by comparing their respective positions in the earlier

reported TL structures [28]. The density modified phases were used for model building using the *ARP/wARP* program web server and refined using *REFMAC*5.0 of *CCP*4i software. TL dataset had a redundancy of 43.49. Final refined model has R_{work} = 18.6% and R_{free} = 21.9%. 316 residues were built totally, in which 314 were built with side chains, by the iterative free-atom density modification and model building procedure. Anomalous map of monomeric TL with four calcium ions, one zinc ion and two sulfur atoms at 5σ with water molecules is shown in **Figure 3**. Crystal data statistics, phasing and model building details are given in **Table 2**.

SAD phasing has been carried out earlier on proteins like HEWL, GI and TL using synchrotron beamlines and in-house copper wavelengths [27,28,31]. Using in-house chromium X-ray source, HEWL structure was attempted using different mounting techniques [29]. Using in-house chromium wavelength, glucose isomerase structure was also attempted using different solving techniques like *ARP/wARP* and *OASIS*2004 [30], but the complete structure could not be solved. Our attempt to collect en-

(a)   (b)

Figure 2. (a) Anomalous map and (b) Cartoon representation of monomeric GI with nine sulfur atoms and one manganese ion at 5σ with water molecules.
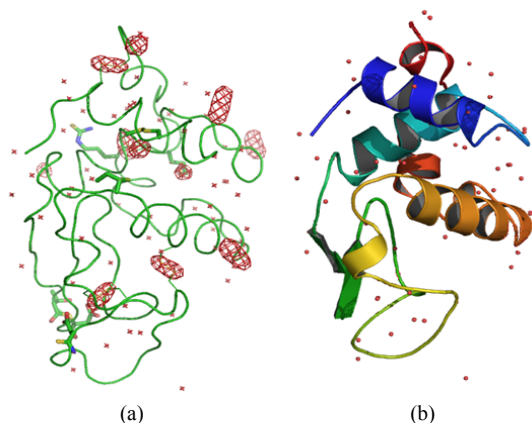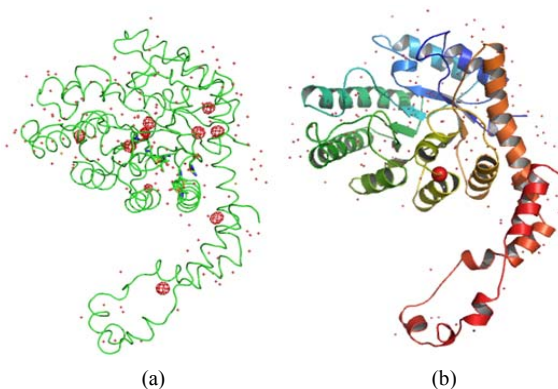
(a)   (b)

Figure 1. Anomalous map (a) and Cartoon representation (b) of monomeric HEWL with eight sulfur peaks and seven chloride ions at 5σ with water molecules.
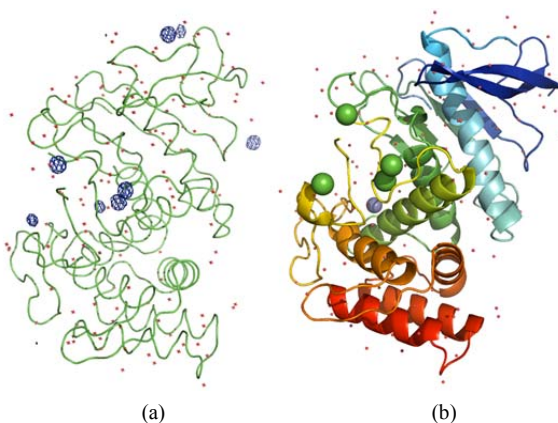
(a)   (b)

Figure 3. (a) Anomalous map and (b) Cartoon representation of monomeric TL with four calcium ions, one zinc ion and two sulfur atoms at 5σ with water molecules.

hanced anomalous scattering data of TL using Cr *Kα* radiation has not been attempted before.

The obstacle so far has been the quality of the data required to take advantage of the vanishingly small signal of the sulfur atom and chloride ions. The location of the S atoms and other metal ions with the integrated Patterson and direct methods implemented in *SHELXD* is relatively straightforward. The results reveal that anomalous signal enhancers like chlorine and sulfur, which are naturally bound metals can very well contribute and allow easier substructure solution for sulfur SAD phasing. Access to in-house chromium generated X-ray radiation makes it easier to solve protein structures using bound lighter atoms and metals.

4. Conclusions

High-throughput crystallography requires a method by which the structures of proteins can be determined quickly and easily. SAD phasing using intrinsic anomalous scatterers like sulfur, chlorine, calcium, manganese and zinc becomes increasingly attractive owing to the advanced phasing methods. Due to the large anomalous scattering coefficient of the heavy metal ions used with respect to copper wavelength, they prove to be excellent heavy atom markers for automated phasing. Metal ions present in the soaking solution in molar concentrations are well enough to rapidly diffuse or enter into protein crystals. The anomalous scattering signal obtained from such crystals through soaking or co-crystallization within the diffraction data can be utilized for structure solution. The approach is quick as well as efficient. In all the cases discussed above, it was possible not only to locate the anomalous scatterers, but also subsequently to solve the protein model by SAD phasing.

All the collected datasets are of good quality and they have close to 100% completeness. Longer wave- lengths not only provide an increased anomalous signal for phase determination, but also allow a much clearer definition of substructures their positions and occupan- cies, which may turn out to be very important for eluci- dating the function of a molecule. Data quality is deci- sive for successful location of the anomalous substruc- ture. The example of successful SAD phasing based on the signal of weak anomalous scatterers such as sulfur atom and chloride ion prove that even the anomalous signal provided or present naturally in a macromolecule is good enough to solve crystal structures successfully using lab source chromium-generated X-ray radiation.

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