New approaches to high-throughput phasing

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Recent progress in macromolecular phasing, in part stimulated by the high-throughput structural biology initiatives, has made this crucial stage of the elucidation of crystal structures easier and more automatic. A quick soak in various salts leads to the rapid incorporation of the anomalously scattering ions, suitable for phasing by MAD (multiwavelength anomalous dispersion), SAD (single-wavelength anomalous dispersion) or MIR (multiple isomorphous replacement) methods. The availability of stable synchrotron beam lines equipped with elaborate hardware control and sophisticated data processing programs makes it possible to collect very accurate diffraction data and to solve structures from the very weak anomalous signal of such atoms as sulfur or phosphorus, inherently present in macromolecules. The current progress in phasing, coupled with the parallel advances in protein crystallization, diffraction data collection and so on, suggests that, in the near future, the process of macromolecular crystal structure elucidation may become fully automatic.

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Abbreviations

MAD	multiwavelength anomalous dispersion
MIR	multiple isomorphous replacement
SAD	single-wavelength anomalous dispersion
SeMet	selenomethionine

Introduction

Multiple isomorphous replacement (MIR), the classic method of solving novel crystal structures of macromolecules, has been responsible for an enormous amount of the success of structural biology since the first days of protein crystallography. Over the past decade, multiwavelength anomalous dispersion (MAD) has been a locomotive of progress in phasing new crystal structures. The third classic method, that of molecular replacement, has also been used widely when appropriate models have been available.

Both MIR and MAD require the presence of appropriate heavy or anomalous scatterers — either naturally occurring or specifically introduced — in the macromolecule. The standard method of derivatization in MIR involves soaking (or crystallizing) the native crystals in diluted (low millimolar) solutions of heavy-metal reagents. The most popular MAD vehicle is selenomethionine (SeMet), which is introduced into proteins in place of normally occurring methionine by genetic engineering.

Although these approaches are used routinely in the current practice, they have some drawbacks. A common problem with heavy-atom derivatization is the resulting nonisomorphism between the native and derivatized crystals. Sometimes, several different derivatives are required to achieve success. The production of SeMet variants of bacterial proteins is relatively straightforward, but the expression of eukaryotic proteins containing SeMet is often difficult. Thus, the production of derivatized protein crystals, either by heavy-atom reagents or by SeMet, is hard to automate.

The aim of current structural genomic projects is to solve a large number of selected protein structures as rapidly as possible. This requires a high degree of automatization and standardization at every step of the whole process, and substantial efforts are directed towards these goals. Good examples of such efforts include the expression of proteins with various tags, which makes them easier to isolate and to purify, and the introduction of robotics into the process of crystallization, thereby allowing the automatic setup and scanning of numerous crystallization trials in various conditions.

As stated above, it is not easy to obtain automatically the crystal derivatives appropriate for phasing. But for this step of the crystal structure determination, new ideas have been also put forward that aim to make the phasing of novel structures easier and more susceptible to routine and automatic treatment. The most recent of these ideas, that most probably will gain wide popularity in the future, are described below.

SAD on sulfur and other weak anomalous scatterers

The solution of novel structures through MIR or MAD is based on an isomorphous or anomalous signal provided by heavy and/or anomalously scattering atoms, which are usually introduced to the protein sample (although they may be present inherently as, for example, in metalloproteins).

By contrast, sulfur is present in almost all proteins. It is heavier than any other element found in most proteins and displays some anomalous signal. Sulfur has 16 electrons (8 more than oxygen) and its X-ray absorption edge lies at a prohibitively long wavelength (5.02 Å), but its anomalous scattering contribution (f″) at the copper $K\alpha$ wavelength is 0.56 electrons (at a wavelength of 1.74 Å, f″ = 0.70 electrons). Nevertheless, the anomalous signal of sulfur is measurable and has been shown to be useful. For example, the structure of crambin [1], which contains six cysteines in 45 residues, was solved solely on the basis of the anomalous signal of sulfur within a single data set collected at the copper wavelength.

An early and persistent advocate of the utility of the anomalous signal of sulfur is Wang, whose simulations based on

idealized data [2] led to the conclusion that a single disulfide bridge in a protein of about 120 residues might provide enough signal to solve the structure. This corresponds to a ratio of the average anomalous difference to the total protein amplitude ($<\Delta F^{\pm}>/<F>$; known as the Bijvoet ratio) of 0.6%; for crambin, the Bijvoet ratio is 1.4%. The anomalous difference Fourier peaks of sulfur can be also used as markers for tracing of newly solved proteins in experimental electron density maps [3,4].

In recent years, interest in using the signal of sulfur for phasing has been revived. The availability of accurate detectors and improved data processing algorithms now makes it possible to measure precisely the small Bijvoet differences, which can be used for single-wavelength anomalous dispersion (SAD) phasing by new efficient phasing programs. The recent works on S-SAD phasing include several novel structure solutions and some test results obtained on crystals of previously known structures.

The structure of the bioluminescent protein obelin [5] was phased at 3 Å resolution and then refined at 1.73 Å. This 22.5 kDa protein has 195 amino acids, including eight sulfur atoms (plus one solvent chloride) that, at a wavelength of 1.74 Å, provide a Bijvoet ratio of about 1.1%. The sulfur positions were identified by SOLVE [6] and refined by PHASES [7], and the protein phasing was achieved by the ISAS method [2].

The structure of the 16-residue macrocyclic antibiotic thiostrepton has been phased on a 2.5% Bijvoet ratio provided by its five sulfur atoms in diffraction data collected using the copper *K*α source to 1.33 Å resolution [8]. The sulfur positions were found with SnB [9], and phases calculated by MLPHARE [10] and improved by DM [11].

The asymmetric unit of crystals of α-crustacyanin contains two molecules of 181 residues, which each contain six sulfur atoms. The structure of this protein was solved by a combination of SAD identification of the positions of the anomalous scatterers from data collected to 2.5 Å with a wavelength of 1.77 Å, and phase estimation and refinement using atomic resolution data [12•]. So far, this is the largest structure (40 kDa) with the smallest signal (Bijvoet ratio 1.0%) that has been solved through the anomalous dispersion effect of sulfur. Among the 12 sites found by SnB, the first 6 were confirmed by SHARP [13] to correspond to a separate disulfide bridge. Even though the resolution of the data (used at only 2.6 Å) was lower than the length of the disulfide bond, the SHARP residual map clearly identified the second sulfur atom in each disulfide. The signal of the 12 sulfur atoms in the data at 2.5 Å resolution was not enough to obtain an interpretable map by any of the available phasing programs. But starting from the known sulfur sites and using (nonanomalous) data to 1.15 Å resolution, the protein phases were refined easily by the program ACORN [14] to give an excellent map.

Among the structures solved by other means, but independently phased by SAD, is that of hen egg-white lysozyme [15,16•]. It crystallizes from a 1 M solution of NaCl and has ten sulfur atoms and an additional eight chloride sites, so that the Bijvoet ratio of 1.5% in the data to 1.55 Å resolution was sufficient to find all anomalous scatterers by SHELXD [17], and to phase the protein by SHARP and SOLOMON [18]. The same data were used in a very successful application of the matrix method for solving the substructure of the anomalous scatterers [19••], which, instead of the classic triple phase relationships, utilizes the large Karle–Hauptman matrices for phase refinement. In addition, this method identified 17 anomalous scatterers from data at 1.66 Å resolution collected at the copper wavelength, which were then expanded to the complete protein model by MLPHARE, DM and wARP [20].

Similar approaches based on the anomalous signal of phosphorus have been used to phase the structure of a small oligonucleotide [19••,21•]. In contrast to the variable amount of sulfur in proteins, the content of phosphorus in nucleic acids is constant and provides a Bijvoet ratio of about 2% at the copper wavelength, in spite of a smaller anomalous scattering effect [$f''(P) = 0.43$ and $f''(S) = 0.56$ at a wavelength of 1.54 Å].

Two tetragonal crystal forms of tryparedoxin, both containing 16 sulfur atoms in 332 residues in the asymmetric unit, have been solved [22] on the basis of the anomalous signal in data collected with a wavelength of 1.77 Å and a Bijvoet ratio of 1.2%. The sulfur positions were found by SnB (first form, with data to 2.5 Å) or SOLVE (second form, with data to 2.7 Å), and phases estimated by SHARP and SOLOMON gave fully interpretable maps.

The signal of sulfur in diffraction data collected on the laboratory sources was also found very useful in the presence of other anomalous scatterers, such as iron in myohemerythrin [23] or calcium in trypsin [24•]. At longer wavelengths, the signal of sulfur increases, but the absorption effects become more detrimental. Nevertheless, tests carried out on crystals of HIV-1 integrase [25] showed that, at the chromium *K*α wavelength (2.29 Å), the signal of sulfur is considerably enhanced in comparison with data collected at the copper *K*α wavelength. A series of synchrotron data sets were collected at wavelengths ranging from 1.5 Å to 2.64 Å from crystals of thermolysin, which contains one zinc, five calcium and three sulfur atoms [26••]. The best substructure solution and phasing results were achieved with data collected at a wavelength of 1.9 Å, which seems to be the optimal compromise between the increasing anomalous signal and larger absorption effects at long wavelengths. Even at a wavelength of 2.6 Å, however, the data collected on the crystal of factor XIII [27] displayed a strong anomalous signal of calcium and sulfur.

Chlorine has one more electron than sulfur and shows slightly stronger anomalous scattering. Its anomalous

signal has been used successfully for phasing the structure of the aglycon of vancomycin — a medically important antibiotic that contains two covalent chlorine atoms [28]. Eight chlorine atoms in four molecules were found by SnB on the basis of data collected with a wavelength of 1.9 Å, where $f'' = 1.0$ electron units for chlorine. The chlorine substructure was then expanded to the full structure using data at atomic resolution collected at a shorter wavelength.

The structure of a similar antibiotic, decaplanin, has been solved by SAD phasing with data collected at a wavelength of 1.54 Å, where $f'' = 0.70$ electrons for chlorine [29]. Decaplanin contains one chlorine atom and SHELXD found eight independent chlorines, corresponding to four molecules of decaplanin and four chloride ions in the asymmetric unit. SHARP and DM run against the same data produced a very clear map.

The above examples show that even weak anomalous scatterers, such as the second-period atoms phosphorus, sulfur and chlorine, provide useful phasing information if the diffraction data are evaluated with high enough accuracy.

Quick soak procedures

Classic MIR derivatization relies on the soaking of native crystals in diluted, millimolar solutions of heavy-atom reagents for a prolonged time, ranging from several hours to days. The chemical reactions involved in this procedure require a long time for the typical transition metal coordination compound to hydrolyze or to exchange its ligands from small ions to certain protein binding sites. By contrast, small molecules and ions can diffuse into macromolecular crystals quite rapidly; for example, glycerol molecules have been often observed bound to the protein surface after the protein has been soaked for only a few seconds in cryoprotecting solution. This property can be used for a very quick derivatization of protein crystals by ions that do not form specific and stable bonds with a macromolecule.

The two heavier halides, bromide and iodide, have been proposed for such a purpose [30,31] because they display a significant anomalous scattering effect. Bromine is a good vehicle for MAD phasing [32] because it has one more electron than selenium and an analogous X-ray K absorption edge at 0.92 Å, whereas iodine has about seven anomalous electrons at the copper $K\alpha$ wavelength and is appropriate for SAD or SIRAS (single isomorphous replacement with anomalous scattering) phasing. Even more anomalous signal can be expected from the incorporation of polyiodide by quick soaks in a potassium iodide/iodine solution [33•].

Several novel structures of various sizes have been solved recently by using cryosoaked halides coupled with a MAD, SAD or MIR approach. These molecules have been crystallized in various conditions and at different pH, using salts, polyethylene glycol or MPD as precipitants. The halide sites in crystals are located in the ordered solvent around the protein surface. They can form van der Waals interactions with hydrophobic patches at the protein surface and can also accept hydrogen bonds from polar groups or water molecules in the solvent. There are usually many sites with various occupancies that are shared with solvent water molecules. Sufficient occupation of sites and successful phasing have been achieved with the solution concentration of the halide salt ranging from 0.2 to 1.0 M. Although some crystals do not withstand soaking in a relatively high concentration of halide salt solution, there have been reports of improved diffraction of crystals after such treatment [34] and even a crystal symmetry transformation [35].

Some simple (i.e. noncomplex) metal ions can also serve as convenient anomalous heavy atoms. Such metal ions are not very strongly coordinated by the protein carboxyl and carbonyl groups or the solvent water molecules, and also diffuse rapidly through solvent channels in the crystal. For example, rubidium has an absorption edge at 0.82 Å and can be used for MAD phasing [36]. Similarly, quick-soaked lanthanide ions [37] are suitable for MAD phasing work. Like the iodide anion, the caesium cation shows a significant anomalous signal at long wavelength radiation and can be used for MIR or SAD phasing [37]. The recently proposed combinatorial counterion replacement approach [33•] involves a quick soak in various salt solutions containing sodium or caesium cations, and chloride or iodide anions.

The classic heavy-atom reagents can also incorporate into protein crystals after a relatively short soaking if the concentration of the appropriate reagent is higher than 10 mM [38•,39]. Because these atoms bind covalently, the time required for successful derivatization ranges from 10 min to 2 h, but is not as long as that required at low concentrations of heavy-atom reagents. Such quick-soaked heavy-atom derivatives display better isomorphism and diffraction than do those obtained after standard, prolonged soaks.

The protocols of soaking with various ions are very simple and lead quickly to either a success or a failure of the phasing attempt. They may well qualify as the trials of first choice in the high-throughput X-ray crystallography structural projects.

New algorithms and programs

The advancement of the high-throughput structural genomics initiatives is paralleled by the recent progress in crystallographic computing. In fact, these two fields are interconnected and largely depend on each other: the very rapid elucidation of a multitude of protein crystal structures relies crucially on the availability of fast, robust and automatic methods of structure solution; simultaneously, part of the support for genomics projects directly benefits the development of new methods.

Several 'older' programs have been improved substantially and new programs have been written. Generally, the programs became more powerful, integrated and user-friendly. The new versions of direct methods programs, SnB [40] and

SHELXD [17], are able to identify heavy or anomalous atoms from a very small signal. The program CRUNCH, which is based on the newly introduced to macromolecular crystallography matrix method of phase evaluation, has proved to be very powerful for locating weak anomalous scatterers [19••].

Programs for evaluating and refining protein phases, such as SHARP [13] and SOLVE [6], have been improved and have become more powerful. RESOLVE [41], the maximum-likelihood density modification program, now has the option of automatic map interpretation, and the SOLVE–RESOLVE suite can produce almost automatically the atomic model from diffraction data. Newly introduced SHELXE can evaluate very quickly and automatically protein phases from the anomalous atom substructure found by SHELXD. The wARP program [20] interprets electron density maps automatically and often leads automatically to almost complete protein models with largely correct amino acid sequence. The program ACORN [14], which uses a new dynamic density modification algorithm, can solve a complete structure from a very small fragment or even a single heavy atom [42••] if data to atomic resolution are available, and also can locate heavy or anomalous scatterers from data at lower resolution.

Advancements in molecular replacement include, for example, implementation of the maximum-likelihood-based algorithms in BEAST [43], the spherically averaged phased translation function in MOLREP [44] and the six-dimensional evolutionary search in EPMR [45,46]. The pace of improvements in molecular replacement, coupled with the increasing speed of computers, makes feasible a generalized search of all unique protein domains present in the Protein Data Bank to solve numerous crystal structures automatically in the high-throughput mode [47].

Conclusions

The recent progress in methodologies for solving protein crystal structures is, in large part, pushed forward by the requirements of the high-throughput initiatives but is, of course, of benefit to all macromolecular crystallographers. The main driving force has been the mutual involvement of the more theory-inclined creators of algorithms and the structural biologists, who are interested in the structures of proteins encoded by the multitude of newly sequenced genes. In the near future, it can be expected that newer ideas and more powerful programs will emerge, fulfilling further the prophecy of apotheosis — not apocalypse — expressed at the Workshop on the Automation of Structure Determination held at the Brookhaven National Laboratory in June 1999 [47].

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