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Maturation of MAD phasing for the determination of macromolecular structures

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The method of multiwavelength anomalous diffraction (MAD) is having a large and ever-increasing impact in macromolecular crystallography. Synchrotron radiation is essential for the practical implementation of this concept for solving crystal structures, but the method was slow to evolve despite two decades of synchrotron availability. Other factors critical in the maturation of MAD analyses have included the development of area detectors for the accurate and rapid recording of diffraction patterns, techniques for cryopreservation against radiation damage, the systematic incorporation of selenomethionine into recombinant proteins, and new beamlines equipped for optimized MAD experiments. Continued expansion of MAD applications is anticipated since several new beamlines designed to accommodate MAD experiments have recently become operational.

Keywords: anomalous scattering; multiwavelength diffraction; protein structure; selenomethionine; X-ray crystallography.

1. Introduction

Synchrotron radiation is making a qualitative difference in the practice of macromolecular crystallography. Multiwavelength anomalous diffraction (MAD) experiments are major players in this transformation. With MAD, the data from one single crystal can suffice for a structure determination, and this is now performed with amazing speed and rather routinely, even for very complicated molecules. To be sure, other factors such as recombinant expression of selenomethionyl proteins, cryoprotection, and effective analytical procedures have also facilitated the MAD method, but it is the continuous tunability and high brightness of synchrotron X-ray beams that is crucial.

Phase evaluation and structure determination from MAD measurements (MAD phasing) have become widely employed only in the past few years (Fig. 1). Although the first completed MAD experiments were reported in 1985 and a number of significant applications to molecules of unknown structure were published in the next decade, for various reasons the development from novelty to the routine was rather slow. This gradual maturation associated in part with the technical complexity in developing synchrotron instrumentation but it also reflected an understandable reluctance of macromolecular crystallographers to move away from the efficacious traditional methods already in place. In any case, the acceptance of MAD phasing by the community is now unquestioned with impressive activity at each of several newly commissioned beamlines.

The theoretical and technical elements of MAD phasing have been reviewed quite thoroughly (Fourme & Hendrickson, 1990; Hendrickson, 1991, 1994; Smith, 1991; Hendrickson & Ogata, 1997), and this aspect will not be repeated here. Instead, the course of development for this methodology will be discussed and characteristic traits of current applications analyzed. Suffice it to state here that MAD phasing exploits the element-specific wavelength dependence of X-ray scattering, first to extract the atomic positions of the anomalously scattering centers and then to use the calculated contribution to the diffraction from these centers as reference waves for the evaluation of phases associated with the observable diffraction amplitudes.

2. Origins of multiwavelength phase evaluation

The concepts behind MAD phasing predate synchrotron radiation as used in X-ray experimentation. Bijvoet (1949) first noted the potential use of anomalous scattering in phase evaluation, and Okaya & Pepinsky (1956) elaborated a theoretical analysis for exploiting measurements made at two wavelengths. The earliest such experiments were carried out by Ramaseshan *et al.* (1957) on $KMnO_4$. Multiwavelength experiments with vacuum-tube sources were barely practical, however, and the idea lay virtually dormant. The one exception in protein crystallography was a test by Hoppe & Jakubowski (1975) who obtained a few hundred phases for *Chironomus* hemoglobin from the small anomalous effects of iron at Co $K\alpha$ and Ni $K\alpha$ wavelengths. On the other hand, the structural analysis of crambin from sulfur anomalous scattering of Cu $K\alpha$ radiation clearly showed the power of such signals even at the 1.5% level (Hendrickson & Teeter, 1981).

The importance of synchrotron radiation for the development of MAD phasing was not just in providing a bright tunable source of X-rays but also in demonstrating the striking 'white-line' resonant character of anomalous scattering such as seen at transition-metal *L* edges by X-ray absorption spectroscopy (Lye *et al.*, 1980) and at caesium *L* edges also by diffraction (Templeton *et al.*, 1980). Moreover, the monochromator optics developed for EXAFS experiments would prove appropriate for MAD experiments (see Hasnain & Hodgson, 1999; this issue). The strength of anomalous scattering at white lines prompted a numerical simulation of multiwavelength diffraction data for phase calculations in an approach like that used for multiple isomorphous replacement (MIR) data (Phillips & Hodgson, 1980). In an independent vein, spurred by the crambin experience, a unique algebraic solution was formulated for phase evaluation from multiwavelength data (Karle, 1980; Hendrickson, 1985).

During this same period, macromolecular crystallographers began to take synchrotron radiation seriously, with the greatest pressure being for applications like those conducted at home sources. Many of the beamlines that were developed to meet this need featured bent single-crystal monochromators (*e.g.* SSRL VII-1, CHESS A1, DESY X11, PF 6A2, SRS 9.6), which produce intense and well focused X-ray beams but are not readily tunable. They also depended initially on photographic film, which is not well suited for high accuracy. A few beamlines were developed with readily tunable double-crystal monochromators and accurate electronic area detectors, notably at LURE D23 (Kahn *et al.*, 1982) and at SSRL I-5 (Phizackerley *et al.*, 1986). Because of detector count-rate limitations, these beamlines were used without focusing. They did prove effective for pilot studies, but both aperture and counting restrictions of the multiwire detectors limited the range of possible studies. Thus, even as synchrotron radiation grew increasingly important in crystallography, both self-imposed and intrinsic technical limitations impeded its expansion into MAD phasing.

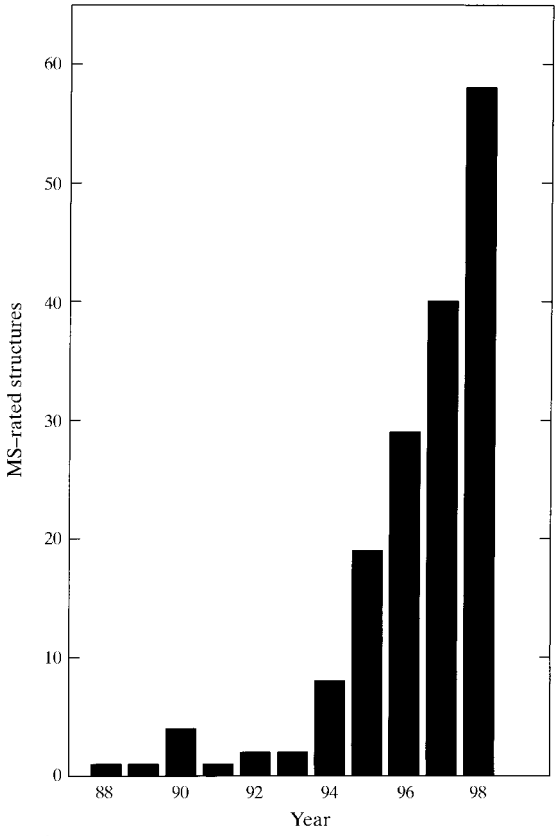

Figure 1
Production of MAD-phased structures as a function of time. Entries give the number of structures, published in a given calendar year, that met the criteria for inclusion as an abstract in the annual macromolecular structures compilation (Hendrickson & Wüthrich, 1998) and for which MAD data were actually used in the structure determination.

3. Practical development of MAD phasing

Effective implementation of phase determination from MAD measurements required suitable test problems and optimized experimental protocols as well as the appropriate instrumentation. This first came together in pilot studies described in 1985. One of these was a three-wavelength study on a parvalbumin, from the fish *Opsanus tau*, wherein calcium had been replaced with terbium (Kahn *et al.*, 1985). The experiment was conducted at LURE D23 near the Tb L_{III} edge, and the data were analyzed by an MIR-like formulation (Phillips & Hodgson, 1980) which produced an electron-density map in good agreement with a molecular replacement solution of the structure. Another was a four-wavelength study of lamprey hemoglobin carried to 5.5 Å resolution in measurements taken at SSRL I-5 from the Fe *K* edge, and the third was a four-wavelength Se *K*-edge experiment on selenolanthionine complete to 0.98 Å resolution that was performed with X-rays from the bremsstrahlung continuum of a molybdenum target (Hendrickson, 1985). The algebraic approach as implemented in the MADSYS programs was used for the MAD analyses in these latter two cases. Besides reporting the first successfully completed MAD experiments, these papers also introduced into print the MAD acronym. In addition, they anticipated several aspects of contemporary applications including the exploitation of white lines, experimental designs to control systematic errors, the potential for selenomethionyl proteins, and the use of direct methods to solve for anomalous substructures with large numbers of sites (megasite structures).

A fully detailed description of the test of MAD phasing as applied to lamprey hemoglobin, now extended to 3.0 Å resolution, was given a little later (Hendrickson *et al.*, 1988). This study provided a pattern for MAD experiments to come by setting out the essential ingredients of an appropriate experimental design. The use of fluorescence-measured absorption spectra was described for the selection of wavelengths corresponding to the extremes of anomalous scattering and for monitoring energy stability of

the X-ray beam, and procedures were given for extracting anomalous scattering factors from these measurements. Wavelengths were chosen to optimize signals by including both near-edge and remote points. A diffraction measurement protocol was established to reduce systematic errors by recording all data pertinent to a given phase close together in time and with equivalent absorption paths for Bijvoet mates. Local scaling procedures were used to reduce further the errors in common for Bijvoet or dispersive differences, thereby giving these differences at a 3% error level despite only an 8–9% level of absolute accuracy. Finally, a fitting procedure was devised to extract fundamental wavelength-independent parameters from the measured data. These fitted variables, $|^{\circ}F_T|$, $|^{\circ}F_A|$, $\Delta\varphi = {}^{\circ}\varphi_T - {}^{\circ}\varphi_A$, derive from structure factors $^{\circ}F_T$ and $^{\circ}F_A$ that are solely due to the normal scattering, f°, of the total structure and the anomalous subset, respectively. The extracted values were then used to evaluate the desired phases, $^{\circ}\varphi_T$, by using the $|^{\circ}F_A|$ data to solve for the structure of anomalous scatterers $\{\mathbf{r}_A\}$ from which the $^{\circ}\varphi_A$ intermediates could be calculated.

The procedures established in the lamprey hemoglobin study were used in the first applications to previously unknown structures. The structure of a basic 'blue' copper protein from cucumbers was determined at 3.0 Å resolution from four-wavelength MAD data associated with the Cu *K* edge as measured with the multiwire detector at SSRL I-5 (Guss *et al.*, 1988), and the structure of streptavidin complexed with selenobiotin was determined at 3.1 Å resolution from three-wavelength MAD data measured near the Se *K* edge with a single proportional counter at Photon Factory beamline 14 A (Hendrickson *et al.*, 1989). The analysis in each case included two additional features: phase probability distributions (Blow & Crick, 1956; Pähler *et al.*, 1990) and selection between the alternative enantiomeric maps (Hendrickson & Teeter, 1981). The cucumber basic protein is related to plastocyanin and azurin, but similarity was not sufficient for a solution by molecular replacement; streptavidin is unrelated to any previously known structure. Other early applications performed at low resolution or from otherwise limited data, which fail to qualify as novel atomic level results, are noted in an earlier review (Fourme & Hendrickson, 1990).

4. Nurturing influences and innovations

The first tests and applications of MAD phasing showed quite convincingly that the technique could be effective (Moffat, 1988). Indeed, despite being based on signals of 2.5% at best, the accuracy of the streptavidin model, built directly into the unmodified MAD map at 3.1 Å resolution (Hendrickson *et al.*, 1989), suggested a great potential for prospective applications with larger signals. Instrumentation of the time was clearly limiting, however. Additional experiments were conducted with the equipment of the initial beamlines at LURE, SSRL and PF, and the effort also expanded to other sources including the Au *L*-line emissions from a rotating anode and horizontally deflected single-crystal beamlines PF 6A2 and CHESS F1. Still, the applications were confined to molecules of only moderate complexity and MAD experiments were tried by only a few investigators (see review by Hendrickson, 1991). Progress remained slow through the early 1990s in terms of published results (Fig. 1). This was nevertheless an important period in the evolution of MAD phasing as pertinent innovations inspired by the early successes and other technical advances of the time were being deployed. The most important of these developments concerned imaging-plate area detectors, selenomethionyl proteins, new beamlines dedicated to MAD applications, and cryopreservation techniques. Much as a consequence, the production of MAD structures finally began to grow substantially starting in 1994.

A major impact on the maturation of MAD phasing was felt with the introduction of imaging-plate (IP) detector systems. These detectors provided sufficient accuracy for weak-signal MAD experiments while being able to sustain essentially unlimited counting rates. The initial applications were with a non-commercial IP scanner developed at the Photon Factory (Amemiya *et al.*, 1988). It was first used at PF14A on a chromomycin:brominated DNA complex (Ogata *et al.*, 1989) and its first published result was on ribonuclease H (Yang *et al.*, 1990). Later, off-line IP scanners built by Fuji were widely used, and then ultimately the MAR detector/scanner systems became commonplace.

The studies on selenobiotinyl streptavidin and selenolanthionine inspired the possibility of using selenomethionyl proteins as a general MAD phasing vehicle. Systematic incorporation of selenomethionine into recombinant proteins was required, and this was shown to be feasible in tests on thioredoxin (Hendrickson *et al.*, 1990). The first actual applications in MAD structure determinations were to interleukin 1-α (Graves *et al.*, 1990) and ribonuclease H (Yang *et al.*, 1990). Other such applications followed soon after, and selenomethionyl proteins rapidly became the dominant subjects for MAD phasing experiments.

Another advance that proved to be critical for the ultimate proliferation of MAD phasing applications was the widespread adoption of techniques for cryopreservation and rapid freezing of macromolecular crystals (reviewed by Rodgers, 1994). A great strength of MAD phasing is the possibility to measure all necessary data from one single crystal, but radiation damage tended to frustrate MAD data acquisition from all but the most robust of crystals. The resistance to damage that freezing afforded, even to the point of virtual immortalization, was of special benefit to MAD experiments. Several frozen MAD experiments were conducted as early as 1993, and the first published result on a frozen crystal phased by MAD was that on human chorionic gonadotropin (Wu *et al.*, 1994). Since that time, nearly all MAD experiments have exploited cryoprotection.

Table 1
Evolution of MAD-phased structures as a function of anomalous-scattering element.

Entries are by calendar year for the number of published structures meeting the criteria for inclusion as an abstract in the annual macromolecular structures compilation (Hendrickson & Wüthrich, 1998) for which MAD data were actually used in the structure determination. For four structures that used MAD data from experiments involving two absorption edges, both are included.

	88	89	90	91	92	93	94	95	96	97	98	Total
Fe			1		1		1		1	4	3	11
Cu	1							1	1	1	1	5
Zn								1	1	2	2	6
Se		1	2		1	2	4	8	18	27	42	105
Br									1	2	4	7
Gd									2			2
Sm										1	1	2
Ho				1							1	2
Yb								2				2
W								1				1
Os									1	1		2
Pt			1						3			4
Au										1	1	2
Hg							2	5	2	2	3	14
Pb									1		1	2
U							1	1				2
Total	1	1	4	1	2	2	8	19	31	41	59	169

Table 2
Evolution of MAD-phased structures as a function of X-ray source.

Entries are by calendar year for the number of published structures meeting the criteria for inclusion as an abstract in the annual macromolecular structures compilation (Hendrickson & Wüthrich, 1998) for which MAD data were actually used in the structure determination. For the one structure that used MAD data from two sources, both are included.

	88	89	90	91	92	93	94	95	96	97	98	Total
SSRL I-5	1				1		1	1		2	4	10
PF 14A		1	2				1				1	5
PF 6A2			1						1			2
Rotating anode			1									1
LURE D23				1								1
CHESS F1					1							1
NSLS X4A						1	4	10	17	12	19	63
NSLS X12C						1		2	2	6	6	17
CHESS F2							1	3	2	4	8	18
SRS 9.5							1		1			2
NSLS X25								1		2	1	4
ESRF ID10								1				1
DESY BW6								1	1	2	1	5
PF 18B									2	2		4
ESRF BM14									2	5	8	15
ESRF BM2									1	1		2
DESY X31										2	3	5
APS 19-ID										1	1	2
ELETTRA										1		1
NSLS X9B											1	1
NSLS X12B											1	1
NSLS X8C											2	2
LURE DW21b											1	1
ALS 5.0.2											2	2
Total	1	1	4	1	2	2	8	19	29	40	59	166

Perhaps the single most important advance to the cause of MAD phasing came with the innovation of new beamlines designed expressly to accommodate optimized MAD experiments. The first of these was NSLS beamline X4A built by the Howard Hughes Medical Institute (HHMI). This facility was designed to deliver focused radiation that could be readily tuned at high energy resolution and to provide appropriately matched detectors and the automation for facile changes of wavelength and geometry (Staudenmann *et al.*, 1989). The first MAD experiments to be conducted at NSLS X4A were carried out at the end of 1992, and the first to be published was on selenomethionyl DsbA (Martin *et al.*, 1993). Thereafter, X4A quickly became highly productive and for the next few years it accounted for at least half of the published MAD structures. During this time, several other new beamlines were designed for MAD phasing experiments, and existing beamlines with double-bounce monochromators were also adapted to MAD phasing with the incorporation of appropriate detectors. In particular, NSLS X12C had its first MAD structure with histone H5 (Ramakrishnan *et al.*, 1993), CHESS F2 and SRS 9.5 had their firsts with BamH1 (Newman *et al.*, 1994) and OppA (Tame *et al.*, 1994), respectively.

5. MAD applications in the mature state

Has MAD phasing reached maturity? Yes and no. Arguing in the affirmative, MAD experiments are being conducted in increasing numbers on diverse subjects by a multitude of investigators at dozens of beamlines around the world. On the other hand, MAD phased structure still account for a minor, albeit increasing, fraction of all new structures. To analyze the impact of MAD phasing, I have compiled a list of those structures for which MAD data were used in the phase determination, whether exclusively or in combination with other information, and which also qualify for inclusion in our annual compilation of macromolecular structures (MS) abstracts (Hendrickson & Wüthrich, 1998). Briefly stated, the criteria for inclusion in MS are atomic resolution, experimental derivation, novelty and macromolecular character. The results of this listing of MS-rated MAD structures (tentative and possibly underestimated for 1998) are shown graphically in Fig. 1 and they are tabulated in breakdowns by element and by beamline in Tables 1 and 2.

A dramatic expansion in MAD-phased structures began in 1994 when suddenly eight structures were published after an annual average of under two in the preceding six years (Fig. 1). This surge correlated strongly with the adoption of innovations discussed in the preceding section: six of the eight used IP detectors, four used selenomethionyl proteins, four used frozen crystals, and four used NSLS X4A. Moreover, it reflected an increasing dissemination of MAD technology with results from five different facilities by seven different research groups. Output more than doubled again in the next year (19 new structures), and a compilation of the MAD structures published through 1995 (Hendrickson & Ogata, 1997) documented

that MAD was now addressing a diverse set of larger structures at the frontiers of structural biology. Vigorous growth has continued in the following years such that at least 58 new MAD structures were published in 1998 (Fig. 1).

Despite this impressive progress, MAD phasing still remains a relatively minor player in structure determination. An analysis of the abstracts published in MS-1998, which covers calendar year 1997, shows that the 40 MAD structures represented 7% of all X-ray structures that met the MS criteria. This compares with the 58% that used molecular replacement, 31% that used MIR and 6% that used single isomorphous replacement with anomalous scattering (SIRAS). Note that some analyses included multiple sources of information, including five of the 40 MAD structures. MAD constituted a greater fraction (20%) of the truly novel structures, ones for which there is no known structural precedent (type A MS abstracts). In addition, many of the MIR and SIRAS analyses were MAD-like in selecting wavelengths to optimize the anomalous scattering or in including the selenomethionyl protein as a derivative. Structure determinations from single-wavelength anomalous diffraction (SAD) data also have a MAD-like character. Though none were reported in MS-1998, such applications may increase. SAD experiments are optimal when tuned to maximize the Bijvoet differences as for rusticyanin (Harvey *et al.*, 1998), but some are necessarily conducted at wavelengths remote from resonance as for crambin (Hendrickson & Teeter, 1981).

There has been, of course, a persistent overall growth in structure determination as well as in the MAD phasing component. Nevertheless, the fraction of structures solved by MAD phasing is increasing year by year. Thus, whereas the eight MAD structures in 1994 comprised just 2% of all X-ray structures, the 29 in 1996 had grown to 6% and, by a preliminary estimate, the 58 in 1998 (double the number from 1996) will correspond to 8%. No abatement is in sight.

Even if MAD phasing is still evolving, the method has certainly matured to the point that successful experiments are routinely conducted at a number of synchrotron beamlines on a variety of problems. Trends in the demographics of this evolution may be useful in projecting future developments. Tables 1 and 2 document the progression by anomalous element and by synchrotron beamline as discussed below. Other trends are also evident. The use of IP detectors dominated until recently, but these have now been replaced by CCD detectors at most beamlines. For some time, nearly all MAD structures have been carried out with frozen crystals. The trend toward larger structures (Hendrickson & Ogata, 1997) has continued, and large megasite selenium structures have become commonplace, as noted below. Finally, the analysis of data is becoming increasingly automated and effective. The repertoire of software now includes comprehensive program systems for MAD analyses such as *MADSYS* (Hendrickson *et al.*, 1988; Wu & Hendrickson, in preparation) and *SOLVE* (Terwilliger & Berendzen, 1999); MIR-based phasing programs such as *MLPHARE* (Otwinowski, 1991; Ramakrishnan *et al.*, 1993), *PHASES* (Furey & Swaminathan, 1997), *X-PLOR/CNS* (Burling *et al.*, 1996) and *SHARP* (de la Fortelle & Bricogne, 1997); and programs used for site identification such as *MULTAN* (Germain *et al.*, 1970), *SHELX* (Sheldrick, 1990), *PATSOL* (Tong & Rossmann, 1993) and *SnB* (Miller *et al.*, 1994; Smith *et al.*, 1998).

The breakdown as a function of anomalous-scattering element is shown in Table 1. Categories of MAD applications have been suggested (Hendrickson, 1991) to include metalloproteins (*e.g.* Fe, Cu, Zn), metal replacements (*e.g.* lanthanides for Ca^{2+} of Mg^{2+}), ligand analogs (*e.g.* Se in selenobiotin), heavy-atom complexes (*e.g.* Pt, Hg, U), and systematic incorporations (*e.g.* Se in selenomethionyl proteins, Br in brominated nucleic acids). While there have been applications of all such kinds, the distribution is far from uniform. The most striking feature of Table 1 is the overwhelming dominance of selenomethionyl proteins in MAD applications (62% over all years). Heavy-atom complexes account for 16% of the total overall, more than half of which are mercurial derivatives. Natural metalloproteins consistently comprise a substantial fraction (13% overall). The fraction of selenomethionyl MAD structures has grown from 42% in 1995 to 71% in 1998, and amazingly complex selenium substructures are being solved (Smith *et al.*, 1998; Terwilliger & Berendzen, 1999).

The breakdown of MAD-phased structures as a function of synchrotron beamline is shown in Table 2. Two things are particularly notable here. One is the rapid proliferation of beamlines performing successful MAD experiments, and the other is the effectiveness of the MAD-dedicated beamline NSLS X4A. During each of the past few years, three or more beamlines produced their first MAD structures, including five new ones in 1998, and X4A which generated its first structure in 1993 produced 19 in 1998 and a lifetime total of 63 (38% of all published MAD structures). Several of the new beamlines also have MAD as their major mission and others have adopted this focus. Beamlines such as ESRF BM14 (see Cassetta *et al.*, 1999; this issue) are on course to recapitulate or surpass the performance of NSLS X4A. The gestation period for new facilities remains long. Notably, the first MAD results from undulator experiments were reported some time ago on ytterbium N-cadherin at ESRF ID10 (Shapiro *et al.*, 1995) and on selenomethionyl Fhit at APS 19-ID (Lima *et al.*, 1997), but additional undulator-based MAD structures are just beginning to appear (one in 1998). What we know from beamline reports, however, is that several new undulator beamlines at the APS, ESRF and SPring-8 are now conducting MAD experiments at a rapid pace. Additional beamlines designed for MAD experiments are also being implemented, both at third-generation sources and at the earlier sources. For example, a new wiggler station at the SRS is due to come on-line later this year to cater for MAD work at the Se edge (see Hasnain & Hodgson, 1999; this issue).

6. Prospects

By all indications, MAD phasing continues to be in a growth phase. One still finds papers that report having used MAD phasing only as a last resort, after efforts to find isomorphous derivatives or a molecular replacement solution had failed. But for a large and increasing body of investigators, MAD is already the method of choice. This community of users has also grown increasingly proactive in the implementation of beamlines appropriate for MAD experiments. Consequently, MAD phasing has reached maturity in the sense of routine accessibility.

Technical advances and user experience have made once heroic experiments now seem commonplace. The bright sources and CCD detectors of present-day beamlines turn MAD experiments into hour-long exercises (Walsh *et al.*, 1999). With such facility at command, new modes of operation can be anticipated. For example, one can expect more commonly to extend accurate MAD measurements to high resolution whereby automated chain tracing, such as with *wARP* (Perrakis *et al.*, 1997), becomes readily feasible. One can also realistically expect to screen a battery of prospective heavy-atom complexes in full MAD experiments, just as screening was at one time performed by precession photography. At another scale, MAD experiments, particularly on selenomethionyl proteins, are expected to be the mainstay of efforts in structural genomics, which are gaining in momentum for a high-throughput analysis of gene products without structural precedents. Even as MAD experimentation becomes routine, new kinds of applications can be foreseen based on uranyl *M* edges, xenon *L* edges and perhaps other systematic replacements of polymer building units analogous with selenomethionine and brominated bases. Regarding instrumentation, the intrinsic brightness of undulator sources can be exploited with X-ray optics to provide higher energy resolution and more extreme anomalous scattering effects.

MAD phasing has emerged as an important contributor to structural biology. The achievements of this once arcane methodology are accentuated by the sharpness of recent increase when contrasted against the slow earlier evolution. Nevertheless, beyond most expectation, this proves to be a richly potent methodology. We have only begun to realise the fullness of its potential.

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