New opportunities in biological and chemical crystallography

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Banerjee [*Proc. R. Soc.* (1933), **141**, 188–193] offered a new way of approaching the crystallographic phase problem which not only broke new ground beyond the 'trial and error' structure solution method of that time but also heralded the extremely powerful direct methods of crystallography of the modern era from the 1970s onwards in chemical crystallography. Some 200000 crystal structures are known today. More complex crystal structures such as proteins required new experimental and theoretical methods to solve the phase problem. These are still evolving, and new methods and results involving synchrotron radiation at softer X-ray wavelengths (2 Å) are reported. In addition, an overview is given of the new opportunities that are possible for biological and chemical crystallography, especially *via* harnessing synchrotron radiation and neutron beams.

Keywords: protein and chemical crystallography synergies; synchrotron radiation and neutron technique synergies; softer X-rays; Professor K. Banerjee; future directions.

1. Introduction and cross-disciplinary connections: from physics to biology and chemistry

New opportunities in biological and chemical crystallography arising from physics-based developments in synchrotron radiation and neutron beam production are at an exciting stage. The synergy between developments in one area of crystallography and another is of great interest. This article describes these synergistic developments. It is an honour to be awarded the Professor K. Banerjee Memorial Lecture Silver Medal as part of the birth centennial celebrations for this pioneer of crystallography (Fig. 1). To gauge the honour I need only cite his seminal paper on direct methods (Banerjee, 1933), *i.e.* pre-dating by nearly 20 years the next papers on direct methods and which led to the Nobel Prize in Chemistry in 1985 to H. Hauptmann and J. Karle [see Hauptmann (1992) and Karle (1992)]. The compendium of Banerjee's publications (Mukherjee, 2000) shows breadth too, covering experimental techniques in crystallography and diffraction, structural chemistry and, as referred to above, the mathematical and physical basis of diffraction and the crystallographic phase problem. Another famous connection for Professor Banerjee in the history of crystallography is that he worked with Sir W. H. Bragg in the 1920s. W. H. Bragg, winner of the Nobel Prize in Physics in 1915 with his son W. L. Bragg, invented the four-circle diffractometer, a most famous example of physics and instrumentation furthering crystallographic science. This historical context of synergies between scientific fields shows that cross-disciplinary connections in crystallography were common then as they are now. This article explores modern examples of such synergies. Fig. 2 shows photographs of Professor K. Banerjee.

2. Anomalous scattering and tunable synchrotron radiation: recent examples involving softer X-rays

The phase problem of crystallography arises because it is not possible to record the phase of a 'Bragg reflection' with respect to the incident beam. The desired experimental 'phase sensitive' detector at X-ray frequencies does not exist. Instead, some way has to be found from the measured intensities alone to obtain the phases which are then used in the Fourier series summation to obtain the electron density distribution in the crystal unit cell. Banerjee's insight (Banerjee, 1933) that an 'algebraic relationship' between the phases of several reflections might exist is the core idea of the 'direct methods'. Banerjee, in a review written in 1960 (Banerjee, 1960), described direct methods as either algebraic, *i.e.* like his approach, or statistical, *i.e.* probabilistic [like the methods of Karle and Hauptmann (Hauptmann, 1992; Karle, 1992)]. The two approaches are very closely connected in principle, but both suffer from being 'too weak' to be applied to large structures; Banerjee certainly did not pursue his approach, as he said (Banerjee, 1960), for that reason. Crystal structures involving the placement and assignment of up to 200 atoms are now routine; some 200000 chemical crystal structures have been solved today. Is there such a possibility for complex structures, *i.e.* proteins? Currently there are 15000 protein crystal structures in the Protein Data Bank, of which ~2500 are distinct structures (*i.e.* excluding genetic mutants or related crystal forms). The chemistry-based phasing approach of multiple isomorphous replacement (MIR) involving heavy-atom substitution of proteins and multiple data set measurements from several crystals was developed by Green *et al.* (1954), Harker (1956) and Blow & Crick (1959) through the mid- to late-1950s and was the mainstay of *de novo* protein crystal structure determination in the 1960–1990 period. This situation has changed with the advent of polychromatic synchrotron radiation where a multiple-wavelength anomalous-dispersion approach, based on

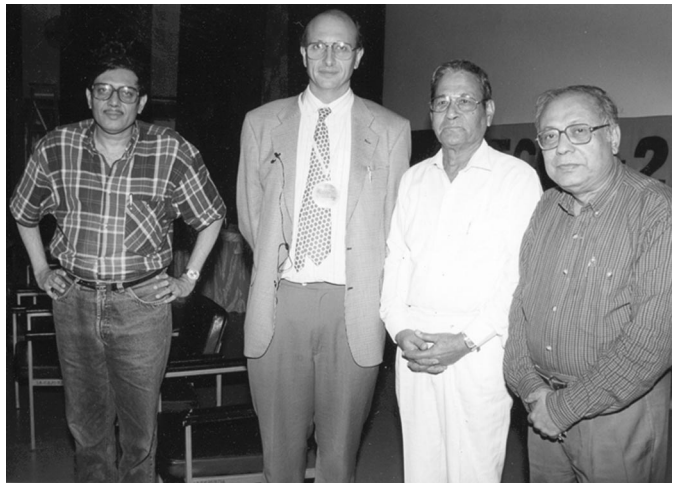

Figure 1
Professor Helliwell, proudly wearing the Endowment Medal, along with Professor S. P. Sen Gupta (Chairman, Organizing Comittee), Professor Debashis Mukherjee (Director, IACS) and Mr K. C. Banerjee (son-in-law of Professor K. Banerjee).

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measurements from a single crystal of a protein containing a suitable anomalous scatterer, becomes practical. Such a formalism, based on a minimum of two wavelengths (λ_1 and λ_2) and three experimental measurements ($F\lambda_1^+$, $F\lambda_1^-$ and $F\lambda_2^+$ or $F\lambda_2^-$, where '+' refers to an *hkl* reflection and '−' refers to its inverse reflection (or their crystal symmetry equivalents), was described as early as 1956 by Okaya & Pepinsky (1956). An 'algebraic approach' by Karle (1989, and references therein) and Hendrickson (1991) or a treatment analogous to MIR (see Helliwell, 1979, 1992) have made the 'one crystal' approach very effective. This is readily applicable due to two factors: firstly some 30% of all proteins are metalloproteins, where the metal anomalous X-ray dispersion can be harnessed, and, secondly, there is the possibility of preparing selenomethonine-substituted proteins, where the Se *K* edge is well placed at $\lambda = 0.98$ Å (Hendrickson *et al.*, 1990). These two factors, along with the steady concerted development of instruments at synchrotron radiation facilities capable of MAD measurements, has opened up the prospect of very large numbers of protein structures being determined, even on a genome scale, *i.e.* one beamline alone already delivering hundreds of protein structures per year (Cassetta *et al.*, 1999; Chayen & Helliwell, 1998).

These developments have been facilitated *via* the low emittance of the electron storage ring allowing high-brilliance X-ray emission. This yields a finely focused beam. Freezing of a protein crystal preserves the crystal sample against irradiation, although not indefinitely, usually allowing a full set of MAD data from one crystal. However, with too big a crystal the sample can break up on freezing and the diffraction pattern is then spoiled (Fig. 3). Hence, a fine-focus beam from a high-brilliance synchrotron radiation source is important to allow use of small crystals (<0.2 mm). Small crystal size is also emerging as a regular feature of high-throughput 'structural genomics' protein crystallography pilot projects where 'microcrystals' are cited as a frequent problem. Third-generation high-brilliance synchrotron radiation X-ray sources such as ESRF in Grenoble, APS in Chicago and SPring-8 in Japan have reduced the sample size that can be used to the 20 µm or less range when working at 1 Å wavelength. Further optimization *via* the wavelength is possible, *i.e.* to allow even smaller crystals to be studied; the diffraction energy, E_{hkl}, into a reflection can be described by the following expression, for the monochromatic rotating crystal case (Woolfson, 1970),

$$E_{hkl} = \frac{e^4}{m^2c^4\omega}\, I_0\lambda^3 LPA\, \frac{V_x}{V_0^2}\, |\underline{F}(h)|^2. \qquad (1)$$

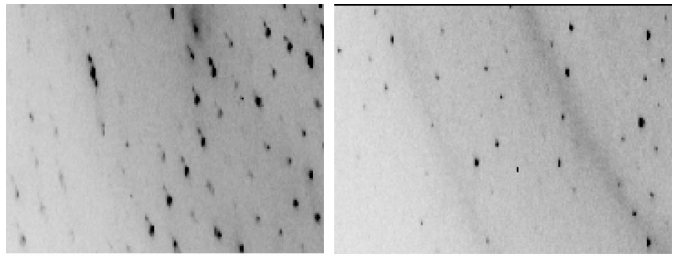
So, with the Lorentz factor, L, being approximately proportional to $1/\lambda$, for small θ, the overall proportionality on the wavelength, λ, is $\sim\lambda^2$. Thus, softer X-rays can favourably enhance the diffraction from small crystals (*i.e.* small crystal volume V_x), and also where absorption is not a problem (A is close to 1.0). There is a small penalty for utilizing a longer wavelength in having a less favourable monochromator transmission polarization correction, but overall an increase from 1 Å to 2.5 Å wavelength offers an order of magnitude increase in E_{hkl} and a smaller crystal volume by an equivalent factor (Helliwell, 1993). Chayen *et al.* (2000) and Weiss *et al.* (2001) document the routine use of softer X-rays in data collection. More experience, of course, needs to be gained for many more proteins of their sample radiation damage tolerances at softer wavelengths.

Use of a softer X-ray wavelength to enhance the diffraction from small crystals (Fig. 4) is not then at, or even near to, the Se *K* edge (0.98 Å), and is well beyond the *K* absorption edges of the commonly occurring transition metals (Fe, 1.743 Å; Zn, 1.283 Å; Cu, 1.380 Å; Mn, 1.896 Å *etc*) and beyond the *L* edges of the common heavy-atom

(*a*)

(*b*)

Figure 2
(*a*) Professor K. Banerjee, born 15 September 1900, 30 died April 1975. (*b*) Professor K. Banerjee in 1958 with, on his left, Pandit Nehru and, next left, Indira Ghandi, and others at the time of his Directorship of the Indian Association for the Cultivation of Science Physical Science Laboratories in Calcutta.



(*a*) (*b*)

Figure 3
The correct choice of protein crystal sample size is important for freezing. Diffraction pattern (*a*) involved too big a crystal (0.5–0.8 mm in size, minimum to maximum) and yielded high mosaicity and split spots. In (*b*), sharp single spots (not split) from a crystal of size 0.2 mm are shown and from which a full data set could be recorded and processed to 1.2 Å. Sample: disaccharide-bound concanavalin A (see Sanders *et al.*, 2001). Methods to reach atomic resolution but at room temperature, which is, after all, closer to physiological temperature, seek to improve the reflection peak-to-background ratio by combining the synchrotron radiation beam near parallel collination with low-mosaicity crystals (Snell *et al.*, 1999). Hence, the link between crystal growth, data collection and structural detail is made (Boggon *et al.*, 2000). One can readily see how high-brilliance synchrotron radiation X-ray sources and their gradual evolution over these last 20 years (Ealick & Helliwell, 1999, and articles therein) has had such a profound effect on the data-collection capability in protein crystallography structural studies.

derivatives (Hg L_{III}, 1.000 Å; Au L_{III}, 1.04 Å; Pt L_{III}, 1.073 Å *etc*). The crystallographic phase problem is likely to require new ideas in the 'structural genomics' small-crystal era to supplement the seleno MAD approach. Two new approaches are possible and offer exciting prospects.

The incorporation of xenon into a protein crystal under high pressure is possible (Schoenborn *et al.*, 1965). Apparatus for freezing xenon in the protein crystal has been developed (Sauer *et al.*, 1997). The 'xenon' diffraction data can then be recorded, *e.g.* by MAD or by combining with a native protein crystal diffraction data set; a hybrid phasing approach SIROAS (single isomorphous replacement with optimized anomalous scattering) involving one suitably chosen wavelength is also feasible. Optimization of the xenon anomalous signal using softer X-rays *via* the xenon *L* edge (L_I = 2.27 Å) is possible (Chayen & Helliwell, 1998). This method has now been applied to the lobster apocrustacyanin A1 protein using diffraction data recorded on Daresbury SRS station 7.2 at 2 Å wavelength, the first protein crystallography station on the first dedicated synchrotron radiation source (Helliwell *et al.*, 1982), now used in a novel way (Fig. 5). Fig. 5(*a*) shows the xenon anomalous difference Patterson section and has peaks that are comparable with, even superior than, the xenon isomorphous difference Patterson section (Fig. 5*b*) by virtue of the 2 Å X-ray wavelength. A second benefit of softer X-rays is that the sulfur anomalous dispersion signal is enhanced over that at 1.5 Å by a factor of two even though the sulfur *K* edge optimization at 5 Å wavelength is remote.

The first novel protein structure to be solved using softer X-rays has now been realised (Cianci *et al.*, 2001). Owing to the limited geometric aperture of the experiment the resolution limit at 2 Å X-ray wavelength was 2.3 Å. The data to 1.4 Å was collected using a short wavelength (0.87 Å). Our next steps in further developing this technique in crystallography, protein and chemical, include higher θ measurements up to full backscattering.

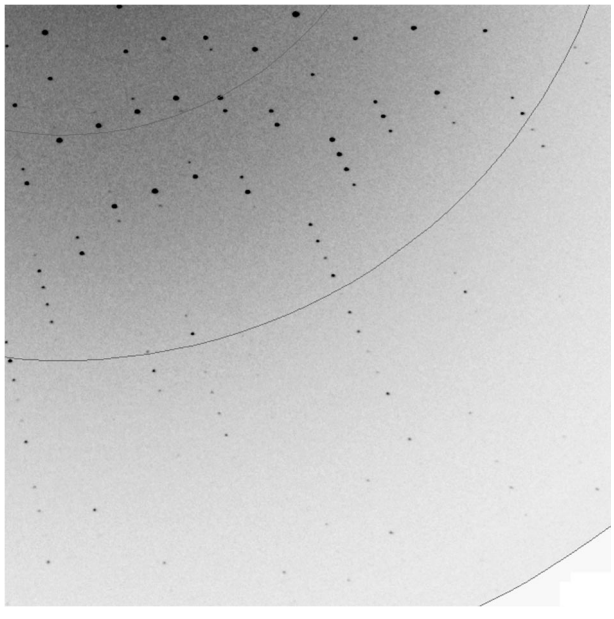


Figure 4
Softer X-rays have great potential in protein crystallography, especially 'structural genomics' projects. Very small crystal size is a common occurrence in current pilot projects. High-quality diffraction data can be recorded using, for example, a wavelength of 2 Å, evidenced here for apocrustacyanin A1 needle crystals (~0.1 mm thick). From Chayen *et al.* (2000). See also Cianci *et al.* (2001).

Determination of an S atom substructure using the anomalous differences from disulphide atoms (at cysteine–cysteine covalent bridges between the polypeptide chain) was made by Gordon *et al.* (2001) on the protein apocrustacyanin C1, from which the whole structure was then developed. In general, determination of a substructure of the whole protein structure could be combined with atomic-resolution protein crystal diffraction data and follow on from the successes of direct methods with metalloproteins (Mukherjee, 1999) or solvent-flattening-based approaches (Dauter *et al.*, 2000). Determination of the substructure of the anomalous scatterers is a pivotal step. A seminal paper was that of Mukherjee *et al.* (1989) who used anomalous differences and the direct-methods program *MULTAN* for finding such substructures. A popular method for this step today, involving larger and larger constellations of anomalous scatterers, is the real- and reciprocal-space-based program *Shake-and-Bake* (Howell *et al.*, 2000). Again, use of softer X-rays, with back-scattering capability, would enhance the sulfur signal significantly.

3. Laue diffraction and polychromatic synchrotron radiation also leading to synergy with neutrons

Unlike conventional X-ray sources which emit emission lines on a weak continuum, the synchrotron X-radiation universal curve is a

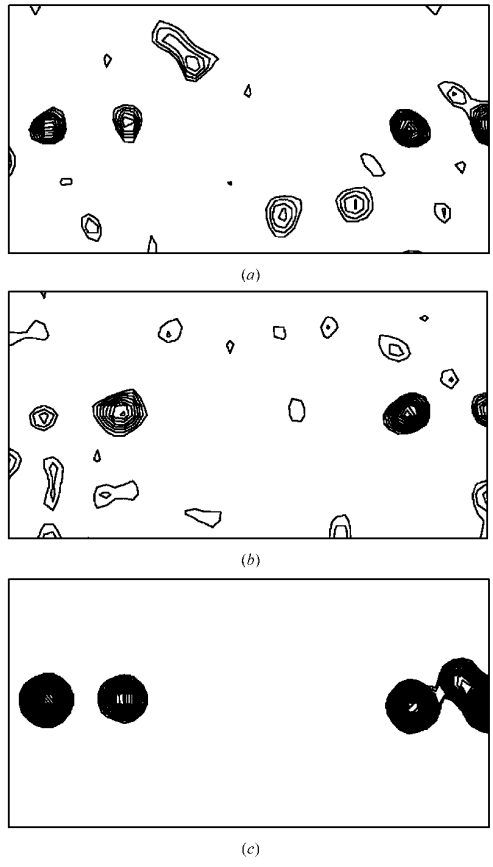


Figure 5
Softer X-rays also yield optimized anomalous scattering from the xenon *L* edge, and where xenon incorporated into a protein crystal under high pressure is now a commonly obtainable 'heavy atom' derivation. (*a*) Anomalous difference Patterson Harker section. (*b*) Isomorphous difference Patterson Harker section for apocrustacyanin A1 as an example. Data were recorded on station 7.2 at the Daresbury SRS, λ = 2 Å. The high quality of (*a*) is evident and quite comparable, superior even, with respect to the isomorphous case (*b*). (*c*) Calculated Harker section peaks from the Xe atom positions involved. Based on Cianci *et al.* (2001).

strong continuum of radiation. Hence, extremely strong monochromatic beams can be extracted, for which the wavelength can be tuned (as referred to in the previous section). In this section, however, consideration is given to how to utilize all the wavelengths simultaneously. This is the well known original Laue method of crystallography of 1912. In this way, ultrashort X-ray exposure times can be realised, as short as subnanosecond time resolution (Bourgeois *et al.*, 1996; Ren *et al.*, 1999). An example of a time-resolved Laue enzyme structural study is described in §4.3.

An analogous situation to synchrotron radiation exists with neutron beams from a reactor source where a polychromatic spectrum of neutrons is emitted. The neutron Laue method allows the sample size barrier to be transcended, rather than achieving ultrashort exposure which is the purpose of the synchrotron radiation Laue method. With neutrons the size of a single protein crystal has been severely rate-limiting. Alternatively, the molecular weight is restricted [see Kalb (Gilboa) *et al.*, 2001]. The harnessing of a broader band of wavelengths at neutron sources is an important development (Helliwell & Wilkinson, 1997). At reactor sources the wavelength bandpass that can be used is ∼33%. A vital next step for proteins beckons using the time-of-flight Laue approach, feasible at a proton synchrotron source. This type of source employs accelerated proton pulses which strike a target such as mercury. Neutrons are ejected (spallated) out of the target also in pulses and over a broad continuum of wavelengths. Laue spots containing overlapping Bragg reflections can be resolved by the time of arrival at the detector and, likewise, noise pile-up is excluded. A better signal-to-noise ratio can be realised in the broad-band case. This approach has been used so far in chemical crystallography (Wilson & Smith, 1997). Enhanced proton synchrotron power is coming, suitable for time-of-flight Laue protein crystallography with neutrons, *via* the proposed ISIS second target station (Rutherford Appleton Laboratory, 1997) and, ultimately, the proposed European Spallation Source (5 MW *versus* 160 kW for ISIS) (European Science Foundation, 1997). The importance of these developments for the future lies in the definition of the H atoms. While synchrotron radiation X-rays have opened up the chance to visualize H atoms for relatively well fixed atoms, more mobile H atoms have a better chance to be defined when exchanged with deuterium and studied with neutrons. Hence, those catalytic H atoms on more mobile pieces of polypeptide can be determined. Moreover, bound water molecules in ligand binding sites can be studied in more detail by neutron protein crystallography (Habash *et al.*, 2000) and thus the chance to further the understanding of the thermodynamics of ligand binding to protein receptor sites *via* structure might be advanced (Bradbrook *et al.*, 1998, 2000; Pratap *et al.*, 2001). Structure-based drug design is a pivotal method in finding new lead compounds for drug design, and the science on which it is based is a vital topic in molecular biophysics and biophysical chemistry.

4. Synergy between protein and chemical crystallography

Three specific areas interfacing between large- and smaller-molecule crystallography will be highlighted *via* case studies.

4.1. Protein crystal structure analysis of concanavalin A with small-molecule accuracy

Small-molecule accuracy means that the protein crystal diffraction pattern extends to a resolution limit where the measurable number of unique reflections exceeds the number of atomic parameters (with full anisotropic ellipsoid refinement) by around an order of magnitude. Hence, for a protein with 1800 atoms, some 180000 X-ray reflections are needed to satisfy that criterion. There is a significant difference, however, between small- and large-molecule analysis because the protein has some mobile loops and side chains, which do not diffract well. These atoms require the benefit of standard model geometry restraints such as planar peptide bond and standard bond distances and angles. With this caveat in mind, small-molecule accuracy protein refinement is possible today with the advent of cryoprotection of the protein crystal against radiation damage, strongly focused synchrotron radiation beams and sensitive automatic area detectors. The crystal structure analysis of concanavalin A, initially at 0.94 Å (with 117000 unique X-ray data; 20000 model restraints and 18500 parameters to be refined) (Deacon *et al.*, 1997) and extended to 0.92 Å (with 20000 more X-ray reflections), reached an X-ray data/parameter ratio of around 8/1 [Hunter *et al.*, 1999; Kalb (Gilboa) *et al.*, 2000]. Full matrix inversion using *SHELXL* (Sheldrick, 1996) allowed standard uncertainties (s.u.) on bond distances to be determined and, for well ordered parts of the protein (some 60%), s.u.'s of 0.005 to 0.01 Å were obtained. Thus, the precise metal (Mn and Ca) bioinorganic coordination was determined and also the protonation states of well tethered (*i.e.* not too mobile) carboxylic acid side chains were revealed from the X-ray analysis (typically the province only of neutron protein crystallography previously). Concanavalin A is a tetrameric protein which binds saccharide (Fig. 6) and is involved in cell-to-cell cross-linking and is thought also to serve as an antifungal protection agent in jack beans.

4.2. Multiple wavelength anomalous dispersion (MAD): Mn, Ca concanavalin A and CoZnPO

The MAD method is proving to be very effective as a means of protein crystal structure determination especially involving selenomethionine-expressed variants of a protein (Hendrickson, 1991; Hendrickson *et al.*, 1990; Cassetta *et al.*, 1999; Chayen & Helliwell, 1998). Since a large percentage (estimated as high as 30%) of proteins are metalloproteins, then the MAD method's potentiality to be applied widely to access the intrinsically present metal atom or atoms

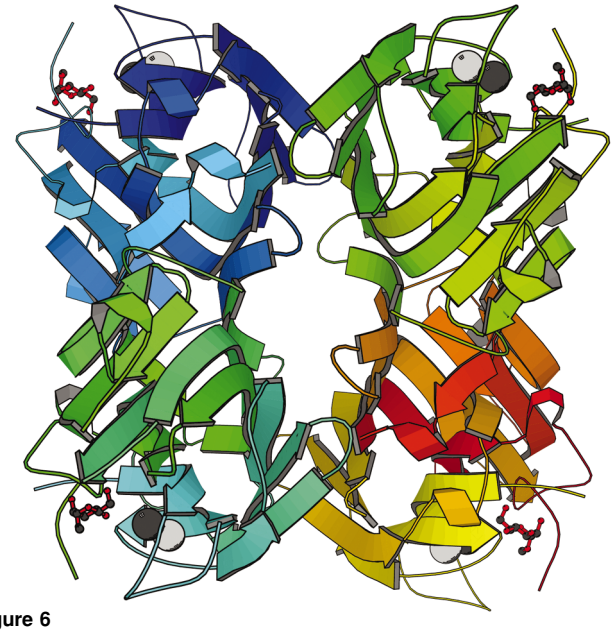


Figure 6
Structural studies on concanavalin A from jack bean reveal a tetramer of protein subunits shown here in 'ribbon format', with two metal ions as small spheres and nearby glucose molecule per subunit. From Kalb (Gilboa) *et al.* (2000), based on Bradbrook *et al.* (1998) and Harrop *et al.* (1996).

is possible. For metalloproteins quite a range of X-ray wavelengths are of interest, *e.g.* from the Mo *K* edge at 0.62 Å to, say, the calcium *K* edge at 3 Å. Working with longer wavelengths does mean that X-ray absorption becomes a concern and smaller crystals need to be selected. Otherwise the principles of the method are similar. In the analysis two different procedures exist. One, the algebraic MAD method (Karle, 1989, and references therein), relies on measurement redundancy and least squares (MADLSQ; Hendrickson, 1985). The second method is a variant of MIR, with one wavelength serving as a native data set and the other remaining wavelengths (one, two or more) as derivatives, two wavelengths being the minimum (see §2). The map quality from adding more wavelengths has been evaluated with respect to a brominated oligonucleotide (Peterson *et al.*, 1996). In such a case of a rather concentrated anomalous scatterer (two bromines in 400 atoms), two wavelengths proved very satisfactory. The evaluation of map quality for the Mn, Ca metalloprotein concanavalin A has been undertaken by us using Mn *K*-edge MAD data recorded at ELETTRA in Trieste, Italy (Hunter *et al.*, 1999). With one Mn in 1800 atoms, this was a more challenging case. The anomalous difference and dispersive difference Patterson maps both clearly revealed the Mn atom position and, from phasing on that alone, the Ca atom position was found. The Mn MAD (including a small but significant calcium anomalous signal) with density modification phasing quality had a mean figure of merit of 0.58 for three wavelengths (mean phase error of 55°) and the best two-wavelength-derived phase set had a mean figure of merit of 0.43 (mean phase error of 65°). Hence, the need for three wavelengths was found to be necessary for such a case but the best two-wavelength case was promising. Moreover, it was concluded that since frozen crystals of concanavalin A on the CHESS Cornell multipole wiggler had yielded diffraction data to 0.92 Å resolution, then a combined future structure solution approach involving MAD to ∼2 Å resolution and phase extension to 0.92 Å resolution suggests itself for the future. Moreover, since an ultra-intense and rapidly tunable capability is now being combined in one beamline, *e.g.* at ESRF (S. McSweeney & A. W. Thompson, personal communication), this will be feasible in one experimental data-collection run. Also, electron density maps will be calculable 'on the fly' as each data set at each wavelength and at the highest resolution are collected. High-performance computing on the beamline is a necessity for that. This case study comments on issues regarding numbers of wavelengths and efficiency of beam-time usage posed over 20 years ago (Helliwell, 1979). In terms of strong user centres worldwide for MAD, the NSLS X4C beamline (Hendrickson, 1999), CHESS and ESRF BM14 (Cassetta *et al.*, 1999), LURE (Fourme *et al.*, 1999) and EMBL Hamburg (Pohl *et al.*, 2001) are examples. On BM14 at ESRF, for example, over 100 MAD protein structures are solved typically per year. Very recent developments include a new multipole-wiggler-based beamline for MAD at SRS ('MAD MPW10'; see www.nwsgc.ac.uk) and three undulator-based PX MAD beamlines on DIAMOND, the new 'medium energy' synchrotron radiation source under construction at the CCLRC Rutherford Appleton Laboratory.

The development and application of MAD techniques in inorganic (smaller molecule) chemical crystallography has been reviewed recently (Helliwell, 2000). From the Manchester Laboratory, again using the ELETTRA 'XRD' station, a five-wavelength MAD analysis has been conducted on the zincophospate CoZnPO (CZP) (Helliwell *et al.*, 1999). This analysis allowed the site of incorporation of cobalt to be settled, *i.e.* at which of the two possible (or both) zinc sites. The most sensitive wavelength pair to the cobalt occupancy was the 1.45 Å reference wavelength and the f^1 minimum wavelength (1.608 Å) between which difference structure factor amplitudes were calculated. A complication *versus* a protein MAD typical case is that there were sufficiently strong variations in the structure factor amplitudes, owing to the zinc atoms' contributions, to complicate the scaling of the data sets between the wavelengths. However, since an atomic model for the crystal structure was available (only the cobalt substitution effect was to be settled), it was feasible to calculate which structure factor amplitudes had a small contribution from the zinc atoms (indeed there were not so many of these reflections). Hence, this small subset of reflections could be used to put the data sets on a common scale. The analysis concluded by estimating that such a MAD analysis would be sensitive down to 12–15% cobalt substitution levels. Overall, these techniques are applicable to settling details of a small-molecule crystal structure rather than structure solution *per se* (which is usually straightforward by conventional Patterson or direct methods). However, such special cases are not uncommon, for instance, in metal-substituted aluminophosphates as well as compounds of several heavy atoms, of close atomic number, and where wavelength tuning leads to unambiguous atom identification. Yet more sophisticated analyses combining MAD with change of temperature have been reported and for which metal ion migration can be tracked in the crystal (Coppens *et al.*, 1999).

4.3. Time-resolved and temperature-resolved crystallography: hydroxymethylbilane synthase (HMBS) enzyme and a liquid crystal study

The study of the evolution of structural intermediates with time of reactive protein molecules, such as enzymes like HMBS, and the perturbation of smaller molecules with temperature as a variable is a modern growth trend in crystal structural analyses. These developments have been made feasible due to the hugely expanded capabilities for fast repeated data set collections. Thus a structure-to-function relationship can be explored directly by experiment. A particular impetus in time-resolved protein crystallography has arisen from harnessing the Laue method with focused polychromatic synchrotron radiation beams where even subnanosecond exposures are possible (Bourgeois *et al.*, 1996). Thus the range of time resolutions reaches well beyond freeze trapping of structural intermediates. Time-resolved techniques have been applied in biological crystallography, *e.g.* see the ten case studies over the last decade highlighted by Ren *et al.* (1999). In chemical crystallography, where the scattering strength (equation 1) is higher due to the smaller unit-cell volume, V_0, ultrafast monochromatic techniques have been preferred so far over the polychromatic technique (Coppens *et al.*, 1999). In a crystal, both reversible and irreversible structural change can be stimulated. Reversible cases are amenable to stroboscopic (cyclic) data collection.

HMBS (Fig. 7) catalyses the polymerization of four molecules of porphobilinogen to form hydroxymethylbilane. The evolution of the reaction in crystals of a Lys 59 Gln mutant was studied by repeated data collection *via* Laue diffraction snapshots. A progression of Laue exposures with pre-set lengthening time gaps after the substrate solution was passed over a crystal held in a flow cell were recorded at ESRF on the 'Laue beamline', ID09. The experimental Laue difference maps revealed an elongated difference electron density, most prominent after 2 h, commencing after ∼8 min, and by which time the initially colourless enzyme crystal had become red/pink (Helliwell, Nieh *et al.*, 1997). This elongated density commenced at the position of ring C2 of the oxidized cofactor of the enzyme, the putative binding site for the substrate, and directly above the critical carboxyl side chain of Asp 84 involved in the first ring coupling reaction step. The electron density then extended past amino acid residues that are known from protein engineering to affect later stages of the catalysis,

and into open solvent. There is a missing stretch of amino acid residues (47–58) in all current HMBS structures undertaken at ambient temperature (time-resolved Laue or static monochromatic). The elongated density referred to above resides between where that loop is likely to be and the position of the C2 ring, in the reduced active cofactor conformation (Hädener *et al.*, 1998). Thus the time-evolution of this reaction in the HMBS (Lys 59 Gln mutant) crystal in structural and functional terms has been established over a period of seconds to hours (Helliwell *et al.*, 1998). Structural puzzles remain in the study of this fascinating and important enzyme. For example, repeated ring coupling is required in the tetrapyrrole formation but there are not four conserved carboxyl side chains (akin to Asp 84). Domain-to-domain reorganization has been proposed (Louie *et al.*, 1992). Further studies involving time-resolved small-angle scattering in solution of the enzyme have been suggested (Helliwell *et al.*, 1998) to follow the enzyme radius of gyration with time as the substrate is mixed with the enzyme. In the crystal, of course, any domain-to-domain rearrangements would be inhibited.

A temperature-resolved chemical crystallography study utilizing synchrotron radiation sources has been undertaken on a thermotropic liquid crystal, 1,4,8,11,15,18,22,25-octahexylphthalocyaninatonickel (Helliwell, Deacon *et al.*, 1997). This study was made in two parts (Helliwell, Deacon *et al.*, 1997; M. Helliwell, personal communication) and involves a sequence of crystal structures at 100, 293, 323, 328 and 353 K, there being a sudden structural change between 323 and 328 K. A complication of the study at 100 K was that there was a crystal space-group transition between 195 and 205 K. As expected, the most precise structure 'snapshot' is that at the lowest temperature. The evolution of these structures with temperature increase shows an increased thermal motion of the hexyl groups. These motions presumably become so large as the actual liquid crystal transition temperature of 428 K is approached that free relative rotation of the molecules can take place, to give the discotic columnar mesophase, *i.e.* where there is random rotational orientation of the molecules about the symmetry axis of the phthalocyanine ring. Conversely, at the lower temperatures the phthalocyanine cores are locked together by the ordering of these hexyl groups. Hence, one sees directly here a neat explanation of the structural basis of this important phase transition from the crystal to liquid crystal molecular phases. The data collection for these studies involved the use of the 24 pole wiggler beamline station F2 at the CHESS synchrotron (for the most precise low-temperature structure at 100 K, as well as at 293 K). The elevated temperature studies were undertaken on the SRS Daresbury wiggler beamline station 9.8 (Cernik *et al.*, 1997). The crystal space-group transition was determined on SRS Daresbury station 7.2 with a CCD time-slicing detector (Helliwell, Deacon *et al.*, 1997).

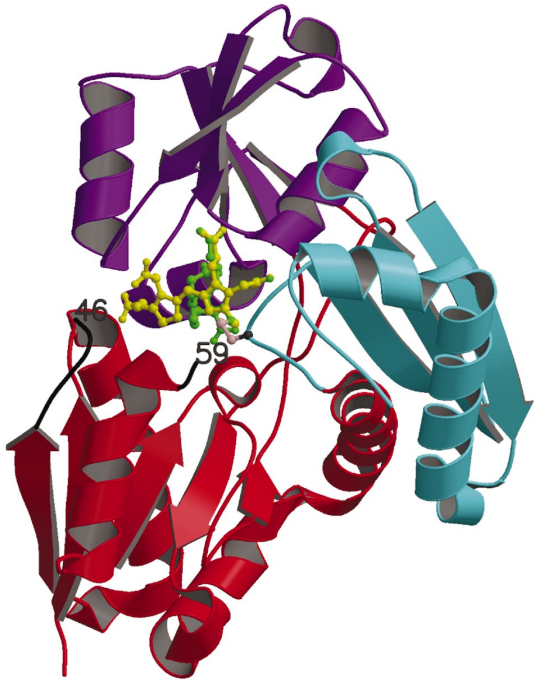


Figure 7
The enzyme hydroxymethylbilane synthase (HMBS) in ribbon format with the enzyme cofactor also shown in its active conformation (green) and its inactive conformation (yellow) [based on Hädener *et al.* (1998) and Louie *et al.* (1992), respectively].

5. Future directions

In macromolecular crystallography there are important current trends involving the elucidation of large multimacromolecular complexes, structural genomics and a growth in neutron protein crystallography.

The investigations of the structures of the whole ribosome are being reported after many years of effort in overcoming technical difficulties (Yonath *et al.*, 1998; Ban *et al.*, 1998; Wimberly *et al.*, 2000). Symmetrical viruses have been the subject of intense study from the late 1970s onwards [for an overview, see Rossman (1999)]. These structural studies are inspirational. Recently, in Manchester, with our collaborators in London, Peter Zagalsky and Naomi Chayen, we have begun to study a most interesting multimacromolecular complex. This is the 16mer protein subunit complex involved in the bathochromic spectral shift of bound carotenoid in the crustacyanin from lobster and Vellela vellela. This has apparently evolved as a camouflage mechanism of marine crustacea against predators. The elucidation of the crystal structures is underway (Snell *et al.*, 1997; Chayen *et al.*, 1999, 2000; Cianci *et al.*, 2001; Gordon *et al.*, 2001). This work has made intensive use of the Daresbury SRS, with our collaborator there, Pierre Rizkallah, and of especial note is the softer X-rays approach referred to in §2 to obtain the apocrustacyanin A1 subunit structure (Cianci *et al.*, 2001). Very recently this has been used as the molecular replacement search model for the 'beta dimer' part of the full complex. The beta structure is a dimer of A1 and A3 proteins with bound carotenoid and thus the structural chemistry of a 100 nm bathochromic shift has been revealed (Cianci *et al.*, 2002). The alpha full complex has a bathochromic shift of 150 nm and whose structural investigation now commences in earnest in Manchester.

The rate of determination of protein crystal structures has improved to such an extent that high-throughput approaches are being considered on the genome numbers scale. Amongst the first such programmes, in effect a pilot project, is that of Sung Ho Kim in Berkeley, USA. The feasibility of this has rested on MAD beamline availability to use the intrinsic metal atoms in metalloproteins and/or the selenium introduced *via* selenomethionine bacterial growth. A bottleneck reported in these pilot programmes, which are based principally in the USA, Japan and Germany at present (but see www.nwsgc.ac.uk), is the occurrence of microcrystals for many of the expressed proteins. This is understandable since the time to optimize conditions is reduced compared with 'standard' protein crystallographic projects. An additional way, besides the high brilliance of synchrotron radiation, to overcome small-crystal weak diffraction is to use softer X-rays and thus enhance the scattering efficiency (equation 1). This is then beyond the Se *K* edge and the transition

metal absorption edges as well as the L edges of the standard isomorphous derivatives used in MIR phase determination. Instead, the xenon derivatization approach allows for optimization of the anomalous scattering *via* the softer X-rays too and/or enhanced signal for the S atoms (as discussed in §2). Overall, high-throughput protein crystallography should lead to a large increase in the database of precise protein structures in the Protein Data Bank. This will help provide a platform of protein structures as data for many areas of molecular biophysics, biophysical chemistry, folding and structure prediction, and not least in opening new avenues in lead compound discovery towards new pharmaceuticals. The large-scale data mining of such information is akin to that which already exists in chemical crystallography with its current database of over 200000 chemical crystal structures. This is, then, another example of the intersection of chemical crystallography and protein crystallography.

The potential of neutron protein crystallography is strong, with a number of initiatives underway. At the neutron reactor source in Grenoble, the most powerful in the world, a coordinated millennium instrument refurbishment is taking place. This includes upgrades to the relevant instruments, namely D19 [a monochromatic neutron diffractometer with enlarged area detector coverage (thus improving data-collection efficiency)], and the Laue diffractometer (LADI) which will have a new neutron image-plate reader and a higher flux location yielding gains of three to five times in sensitivity + flux (thus reducing data-collection time or allowing smaller crystal samples to be used or larger molecular-weight proteins to be investigated, or combinations thereof). Thus, the determination of hydrogen-to-deuterium exchange details can be applied to a wider variety of proteins than ever before. The determination of the H atoms themselves is now feasible with ultrahigh/atomic-resolution protein synchrotron radiation X-ray crystallography where these H atoms are well defined. Mobility of H atoms can kill their diffraction signal, however, but, since neutron protein crystallography determination of deuterium atoms at around 2 Å or better resolution matches that at 1.0 Å by synchrotron radiation X-rays, then more mobile H atoms are determinable by the neutron approach. The bound solvent is a whole category of deuterium atoms which are more efficiently sought by neutron techniques (Habash *et al.*, 2000). Full deuteration through appropriate microbiological expression of proteins for bacteria grown on deuterated media is possible; it was recently shown (Shu *et al.*, 2001) how this worked for deuterated myoglobin, where a 1.7 Å neutron study was more effective than a 1.5 Å X-ray study in finding even the relatively static H atoms (as deuteriums). In the short to medium term, there is the proposed UK ISIS second target station (Rutherford Appleton Laboratory, 1997) which is set to produce longer-wavelength neutrons which scatter more strongly (*i.e.* as per equation 1), and included in that development is a plan for a large-molecule crystallography time-of-flight instrument (LMX). In the USA, the Spallation Neutron Source (SNS) under construction at Oak Ridge which, at 2 MW, is much more powerful than ISIS at 160 kW and *pro rata* delivers more flux. A neutron protein crystallography time-of-flight station on the SNS is being discussed. In Europe there is the proposed 5 MW European Spallation Neutron Source (ESS) envisaged for construction beginning in 2010 (European Science Foundation, 1997). The proposed ESS would reach the same average flux (in fact 0.5) as the Grenoble reactor neutron beam flux but with the addition of the benefit of the time-of-flight neutron approach. Hence, the detailed dissection of those enzyme mechanisms involving H atoms, of which there are many, is set for an expansion in examples.

Overall, the experimental capabilities for protein crystallography analyses have changed dramatically in scope through a combination of technology and advances in methods (Chayen *et al.*, 1996), from both the physical sciences and the biological sciences. Trends seen in chemical crystallography of efficient structure determination and the vast expansion of structural databases are set to occur for proteins. The application of developments in protein crystallography to chemical crystallography such as small crystals, synchrotron radiation MAD and perturbation crystallography is an exciting cross fertilization (Helliwell & Helliwell, 1996). Crystallographic science is a highly cross-disciplinary science. The years of Professor Banerjee, as witnessed by his compendium of publications in crystallography (Banerjee, 2000), show a predominant interaction between physics and chemistry. In the last decades that has expanded strongly also to include biology.

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