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X-ray crystallography at the heart of life science

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X-ray crystallography is the fundamental research tool that shaped our notion on biological structure & function at the molecular level. It generates the information vital to understand life processes by providing the information required for creating accurate three-dimensional models (namely mapping the position of each and every atom that makes up the studied object). The use of this method begun in the middle of last century following Max von Laue discovery of the phenomenon of diffraction of X-rays by crystals, and the successful application of this discovery for the determination of the electronic distribution within simple inorganic molecules by Sir William Henry Bragg and his son, William Lawrence Bragg. The idea of extension of this method to biological molecules met initially with considerable skepticism. For over two decades many respected scientists doubted whether it could be done. Yet, despite its bottlenecks (some of which are described below), the superiority of X-ray crystallography over all other approaches for shedding light on functional aspects at the molecular level became evident once the first structure was determined. The power of this method inspired continuous efforts and spectacular innovations, which vastly accelerated its incredible expansion. Consequently, over the last six decades biological crystallography has produced a constantly growing number of structures, some of which were considered formidable. This remarkable advance yielded numerous new insights into intricate functional aspects. Owing to space limitation this article focuses on selected studies performed recently and highlights some recent exciting developments.

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X-ray scattering from a single molecule is continuous. In crystals (namely entities composed of the studied compound arranged periodically along three dimensions), a huge amount of molecules are arranged in the same orientation, so that the signal is significantly increased. Moreover, the scattered waves are convoluted with the lattice contribution and the diffraction becomes discrete, hence measurable. However, growing useful crystals is a major bottleneck in determining the three dimensional structures of all proteins owing to their inherent internal flexibility and the acute requirement for being immersed in supporting solvent in order to maintain their functional meaningful conformations. Membrane proteins are particularly difficult. Most of these proteins control the transport through the membranes that are water-impermeable barriers encapsulating cells or their internal cellular specialized compartments. As these proteins are embedded within the membranes, they are hardly soluble in aqueous media, and hence their crystallization is significantly more complex than the crystallization of soluble proteins. Therefore, compared to the large number (over 80,000, around half of them deposited during the last three years) of structures of citosolic proteins currently known, only about 280 structures of membrane proteins have been deposited in the protein data bank (PDB) during the last 26 years, since the first membrane protein structure has been determined [1].

Impressive increase in resolution limits has recently been reached in the membrane field. Among the structures determined recently are those of the DesT-oleoyl-CoA-DNA and DesT-almitoyl-CoA complexes (determined at 2.3–2.65 Å resolution), which shed light on key issues in transcriptional regulation of genes controlling the ratio of unsaturated/saturated fatty acid available for membrane lipid synthesis [2••]; the sulfide:quinone oxidoreductase and its complexes, a protein thought to be buried about 12 Å within the bilayers, which were determined at 2.0–2.9 Å resolution [3••] and the multimeric beta-barrel membrane PorB outer membrane protein in complex with various sugars and nucleotides, which were determined at 2.2, 2.3 and 2.9 Å resolution [4•].

Additional high resolution structures are the beta-barrel membrane Pla Plasminogen activator and its mutant that was determined at 1.9, 2.30 and 2.55 Å [5•]; the 1.97 Å structure of the Omp85-N polypeptide transport-associated domains from *Thermosynechococcus elongates* [6•]; metarhodopsin II, the G-protein-coupled receptor that transduce signals into living cells by binding extracellular ligands [7••]; the human mono acyl glycerol lipase that recruit its substrate by interacting with membranes and plays a key role in endocannabinoid signaling [8•]; the secreted lysophospholipaseD, which generates the lipid mediator lysophosphatidic acid that interacts with cell-surface integrins through its N-terminal B-like domains and its complex with an inhibitor [9••]; the actin regulatory complex (WAVE) that controls cytoskeletal

dynamics by promoting actin filament nucleation [10••] the estradiol derived metal chelate and the estrogen receptor–ligand binding domain complex [11•] and the PagP outer membrane palimitoyl transferease, which was determined at 1.4 Å resolution [12•].

The recent crystallographic activity in signaling, stress handling and recognition pathways includes the plant self activating G-protein [13•]; the catalytic domain of the human Ca^{2+}/calmodulin-dependent protein kinase complexed with its selective inhibitor [14•] and the 1.65 Å crystal structures of the sHspA chaperon from the plant pathogen Xanthomonas [15•]. Significant progress has also been made in the identification of diverse binding modes between proteins and DNA. Among those are the high resolution structures of the core domain of the tumor suppressor protein p53 that binds as a tetramer to DNA [16••] and of p63 DNA binding domain in complexes with half-site and spacer-containing full response elements [17••].

Of special interest is a series of studies aimed at the design of improved enzymes by direct evolution, namely by substitutions of amino acids predicted to optimize the enzyme function. During the last three years these studies yielded not only up to 400 fold improvement in the catalytic efficiency, but also an impressive number of structures, most of which were determined at 1.2–2.0 Å resolution (e.g. [18••,19••,20••]).

In parallel to the intensive interest in proteins' functions, the regulatory and catalytic properties of non-coding RNA molecules have been in the focus of modern biology during the last two decades. RNA molecules are readily deteriorating and therefore hard to crystallize. Consequently, so far this group has been hardly represented in the Data Bank. Nevertheless, this field underwent fascinating expansion recently. Examples are the 3.0 Å structure of the aptamer domain of the yitJ S-box riboswitch bound to S-adenosyl-L-methionine [21•], which joins the previously determined (in 2008) crystal structure of the 174-nucleotide sensing domain of the *Thermotoga maritima* lysine riboswitch in the lysine-bound (at 1.9 Å) and free (at 3.1 Å) states [22••]. Noteworthy, in terms of size two extreme limits were reached during the last two years: from the smallest artificial nano-object made entirely of double-stranded RNA that diffracts to 2.2 Å [23•] via the eukaryotic small ribosomal subunit from the eukaryotic protozoan *Tetrahymena thermophila* in complex with a translation initiation protein [24••] to the eukaryotic complete ribosome from the yeast *Saccharomyces cerevisiae* [25••]. Considering the much higher complexity of eukaryotic ribosomes, which are 40% larger than their bacterial counterparts, this is indeed a remarkable progress in the ribosome field, as during three decades of ribosomal crystallography, until the end of 2010 the only available ribosome structures were of ribosomes from prokaryotes.

Resolution is one of the major issues in crystallographic structure determination. Aiming at illuminating the mechanisms of biological processes at the molecular level, atomic resolution is desired. Mandatory for diffraction that can yield electron density map showing the exact position for each atom is that all atoms do not move and all crystallized molecules are in identical conformations. Because proteins are fairly flexible, and because crystals of biological macromolecules contain significant amount of solvent, the lattice is somewhat disordered and, therefore, the diffraction pattern cancels out at lower than the desired resolution limits. Nevertheless, an impressive result was documented in 2011. The structure of crambin (a very small protein) was determined at 0.48 Å resolution [26•].

Resolution is not the only bottleneck in biological crystallography. Throughout, the horizons of the crystallographers exceeded the at-the-time so-called realistic expectations. Therefore it has been experiencing numerous bottlenecks and obstacles. Consequently biological crystallography can be characterized by intensive attempts at pursuing problems that require means beyond the availability. Challenging these hurdles inspired not only major innovation and outstanding technological advances but also a conceptual revolution in the philosophy of the experimental design. The installation of the advanced technologies and innovative computational tools stretched the bio-crystallographic limits beyond expectations.

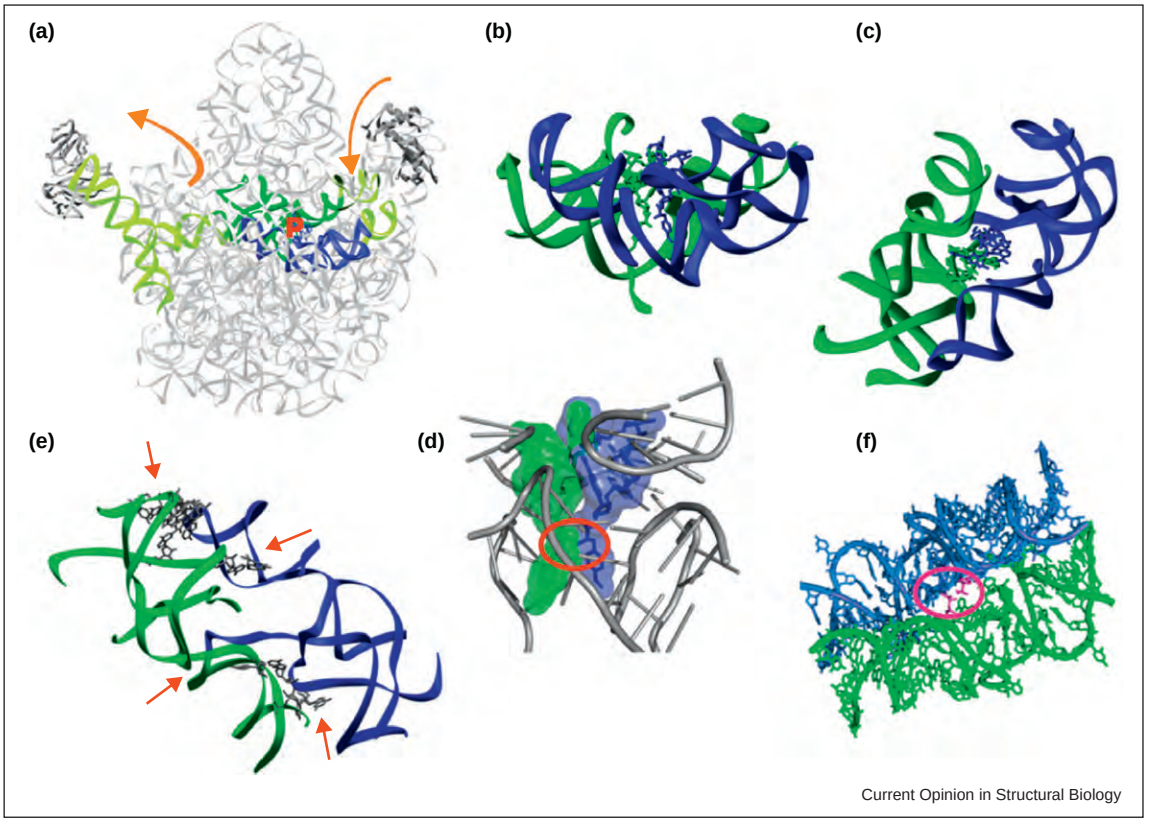
Four decades ago a striking progress followed the identification of the specific properties of synchrotron radiation [27], of which the beam brightness, beam collimation and wavelength tunability triggered its exploitation for accurate structure determination. Originally, using synchrotron radiation met with several severe technical problems, of which most, but not all, could be handled. For example, the damage of most biological crystals caused almost instantaneously by the high beam brightness that is required for obtaining measurable data from those crystals, could be reduced to tolerable level by the introduction of a novel procedure for conducting data collection at cryo temperature [28].

Despite the shortcomings, it was soon found that synchrotron radiation provides unprecedented experimental tools for the determination of the crystallographic phases, which are required for constructing electron density maps, but cannot be directly measured. For filling in this missing link, resonant diffraction methods capable of direct phase determination were pioneered, based on discovering the critical connection between the needed phases and discrete points in the synchrotron radiation continuous wavelength spectrum, alongside benefiting from the incredible development in genetic manipulations that occurred at the same time. This phasing

method utilizes selected wavelengths that span the absorption edge of specific atoms serving as anomalous scatterers. The genuine and overwhelming payoff of this method, called multi/single-wavelength anomalous dispersion (MAD/SAD), is the benign introduction of selenium instead of sulfur into proteins, as a vehicle for direct protein structure determination [29]. Importantly, over 90% of the currently available atomic-level de novo structures were determined by MAD/SAD. Among them nearly 70% used selenomethionine. Thus, this method, originally developed in the end of the eighties, became routine and therefore incorporated into software that converts each decision-making step into an optimization problem [30]. Indeed, during the last few years we are witnessing increased sophistication in crystallographic algorithms as well as increased automation of all steps in structure determination [31].

A fundamental weakness of X-ray crystallography is the limited information concerning the dynamic of natural processes, although such information can, at times, be inferred from crystal structures that inspired subsequent investigations. Examples are the single-molecule analysis based on the high resolution information [32], which revealed the fascinating dynamic mechanisms of the Hsc70 driven clathrin uncoating [33••]; as well as the integration of a few crystal structures that led to the creation of motion pictures (http://www.youtube.com/

Figure 1



This figure shows the presumed remnant of a prebiotic apparatus within the modern ribosome. It is based on the assumption that the proto-ribosome was an actual pocket-like bonding machine, with a defined shape. This apparatus evolved to the much larger contemporary ribosome by the incorporation of the ribosomal proteins and the extension of its RNA chains and. Therefore, in the figure the ends of the proto-ribosome appear truncated from their extensions. However, in the original machine they may have been connected. For all panels the coordinates of the large ribosomal subunit from *Deinococcus Radiodurans* (PDB accession code 1NJP) were used. **(a)** The shape of the bacterial large ribosomal subunit is delineated by the backbone of the ribosomal RNA and 2 ribosomal proteins (L1 and L7/12), shown in grey. P shows the approximate location of the site for peptide bond formation. Each of the blue and green ribbons indicates the backbone of half of the hypnotized semi-symmetric prebiotic RNA machine that is still embedded and functions in the contemporary ribosome, and the yellow ribbons indicate the contemporary non-symmetrical extensions of it. The orange arrows indicate the directions for the entrance and exit of the contemporary ribosomal substrate, namely tRNA. **(b)** and **(c)** are two perpendicular views (side and top, respectively) of the ribosomal pocket suggested to represent the remnant of the proto-ribosome and proposed locations of the 3′ ends of the tRNA molecules within it (in blue and green). **(d)** A small portion of the RNA backbone of the proto-ribosome, located around the ribosomal active site (in grey) with the two tRNAs 3′ ends (in blue and green). The exact location of peptide bond formation is encircled in red. **(e)** A slightly different top view highlighting, by red arrows, the chemical connections (base pairing or covalent boons) between the two halves of the proto-ribosome. **(f)** Top view of all atoms of the proto-ribosome, indicating its internal crowdedness, with two amino acids (in pink) that may represent minimal substrates in its active site.

watch?v=Jml8CFBWcDs, http://www.youtube.com/watch?v=dDxdFHAr2ys&feature=related, http://www.mrc-lmb.cam.ac.uk/ribo/homepage/). In addition, obtaining useful crystals is a major bottleneck in determining the 3D structures. The essentiality of suitable crystals hampered many exciting structural studies. Therefore, alongside the impressive latest progress in growing crystals form difficult to crystallize molecules and assemblies (e.g. membrane proteins and eukaryotic ribosomes), massive attempts are being made to rid this hurdle. Consequently, an exciting conception for offering new opportunities in experimental structure determination, using the free electron laser (FEL), was recently developed. As this technology exploits extremely bright and coherent X-ray pulses (a billion times brighter than conventional SR sources), it is capable of extracting accurate diffraction data at very high resolution from the easier to prepare nano-crystals [34••]. This bean can also be utilized for many other applications, such as constructing rapid single-shot three-dimensional images of complex macromolecules, observing short-lived intermediates, following role of radiation damage of sensitive macromolecules and deciphering the interior structure of partially organized systems, such as intact huge viruses [35••].

Conclusions

The future of biological X-ray crystallography seems now to be several orders of magnitude brighter than expected even a decade ago, when statements such as 'all what could be done by X-ray crystallography is done, and the rest, although significant and meaningful, cannot be achieved', were widely made. Thus, the ingenious introduction of advances in synchrotron radiation alongside crystallographic algorithms have yielded a huge number of structures of biologically relevant macromolecules and their complexes, and the pioneering of cutting edge approaches in X-ray sources opened new routes in structural studies. Furthermore, the realization that a huge number of photons allow structure determination form nano crystals alongside visualization of the internal organization of non crystallized molecules in vacuum before they explode, paves the way to better understanding of extremely complex systems with minimal or no periodic organization (e.g. chromosomes, membranes, cellular organelles, etc.). This also provided means for following snapshots of dynamic biochemical processes, such as catalysis, membrane transport and signal transduction. Furthermore, the examination of the recently determined structures of the huge macromolecular assembly, the ribosome, yielded truly unexpected gains. An example is the identification of a ribosomal universal pocket-like semi symmetrical internal feature, accounting to 3–4% of the ribosomal RNA that appears to be a remnant of an RNA prebiotic bonding apparatus (Figure 1). Remarkably, this presumed prebiotic RNA machine, called proto-ribosome, is still functioning in all contemporary ribosomes and forms the peptide bonds [36,37••].

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