doi:10.2533/chimia.2014.54 Chimia 68 (2014) 54–59

Biocrystallography in Switzerland: Achievements and Future Perspectives

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Abstract: The first protein crystallography group in Switzerland was installed at the Biozentrum of the University of Basel approximately 40 years ago. Since then protein crystallography has grown and matured remarkably and is now established in the molecular biology, biochemistry or biological medicine departments of most major Swiss Universities as well as in the pharmaceutical industry and in biotech startup companies. Swiss X-ray biocrystallography groups have made remarkable contributions from the beginning and have brought Switzerland to the forefront in biostructural research during the last 5 to 10 years. Switzerland has now a leading position in the areas of supramolecular complexes, membrane proteins and structure-based drug design in pharmaceutical and biotech industries. Protein crystallography on the outer membrane protein ompF as well as the development of the lipidic cubic phase crystallization methodology has been pioneered at the Biozentrum. The latter found its somewhat late recognition through the recent explosion in structure determinations of the seven transmembrane helix G-coupled receptors. Highlights from Swiss structural biology groups in the field of supramolecular complexes include the structures of ribosomal particles, of the nucleosome and the pilus assembly complex of uropathogenic *E. coli*. On the membrane protein side advances in the field of ABC transporters and ion channels are world-recognized achievements of Swiss structural biology. Dedicated laboratories at many academic and industrial institutions, their current research programs, the availability of excellent infrastructure and the continuing efforts to build new facilities such as the SwissFEL indicate an even brighter future for structural biology in Switzerland.

Keywords: Protein crystallography

1. Introduction and Historical Overview

The first diffraction pattern of a protein crystal, namely that of pepsin, was recorded by Bernal and Crowfoot in 1934.[1] In 1959 the crystal structures of the first proteins, myoglobin and hemoglobin, were determined by Kendrew and Perutz, respectively.[2] With these achievements macromolecular crystallography has become an essential methodology in the field of modern molecular biology and has progressed in distinct steps. Until about the mid 1970s macromolecular crystallography was a technique that was practiced only by physicists and chemists in relatively few specialized research laboratories around the world. In those days macromolecular X-ray structure analysis was complex and slow because X-ray sources were weak, data collection on films required data processing with manual interventions and the storage and processing capacity of computers were limited given the large amount of data to be handled. Additionally, model building was a manual task that typically took several months and structure refinement was only possible for small proteins. The structures then analyzed were of proteins that naturally occurred in large quantities and could easily be purified from natural sources. Despite these limitations the contribution of macromolecular crystallography to the understanding of the structure/function relationship of macromolecules was already enormous.

Protein crystallography in Switzerland started in 1972/1973 at the Biozentrum of the University of Basel with the installation of the first protein crystallography group headed by Johan Jansonius. The research of the group focused on vitamin B6 (pyridoxal-phosphate, PLP) dependent enzymes. Structures of different states of the PLP-dependent enzyme aspartate aminotransferase were determined in collaboration with the group of Philip Christen at the University of Zurich. Together with enzymatic and spectroscopic experiments a detailed, general catalytic mechanism of this enzyme class was developed.[3]

At the same time crystallographic work toward the structure of the major coat protein hexon of the adenovirus type-2 was performed at the Biozentrum. This protein with a molecular weight of 360'000 Daltons was at the time the largest biomolecule for which crystals were available that could be studied by X-ray crystallography. New techniques of data collection, programs for data evaluation and model building using computer graphics had to be developed to be able to determine a structure of this size. Thanks to all these developments the structure was finally published in 1986 in *Science*.[4]

In the mid 1970s the group of Jürg Rosenbusch, also at the Biozentrum, was among the first groups worldwide to initiate biochemical and X-ray crystallographic investigations of membrane proteins. The group focused on the outer membrane protein porin ompF from *E. coli* and on bacteriorhodopsin. Porin ompF was one of the first membrane proteins crystallized and its structure was solved in 1992.[5] The group also studied membrane protein solubilization by performing thorough physicochemical analyses of detergent solutions. This research helped in the development of new techniques for the crystallization of membrane proteins and was the basis for the lipidic cubic phase technique of crystallization.[6] Recently this method gained much attention because it turned out to be – in combination with conformation stabilizing approaches – the method of choice for the crystallization and structure determination of several G-protein coupled receptor

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proteins.[7] Since 1992 the group of Tilman Schirmer has continued structural biology of membrane proteins at the Biozentrum of the University of Basel.

Bio-X-ray crystallography at the ETH Zürich started with the appointment of Timothy Richmond in 1987. He elucidated the structure of the nucleosome particle and until to date his research focuses on the chromatin structure, chromatin remodelling and gene expression. To be successful with this extremely challenging project the group had to advance the methods for the biochemical preparation of homogeneous material, for crystallization and for data collection from small crystals with large unit cells. The latter impacted the design and setup of the microfocus biocrystallography beamline at the Swiss Light Source significantly (see also below). With these advances the nucleosome particle structure could be determined and was published in 1997 in *Nature*.[8] This work represents a hallmark in biology, biochemistry and biocrystallography.

In the early 1980s the situation regarding macromolecular crystallography and target selection completely changed. Due to the groundbreaking new development of recombinant DNA technology it now became possible to overproduce, isolate and purify essentially every protein with the help of bacterial host cells, as well as eukaryotic cell systems such as yeast, insect cells and mammalian cells. Likewise computer technology advanced and molecular dynamics simulations eliminated crystallographic computing, model building and refinement as the limiting time consuming factor.

At about the same time macromolecular X-ray crystallography became useful for structure-based drug design in the big pharmaceutical industries. Biocrystallography groups in Sandoz, Ciba-Geigy and Roche were established almost simultaneously and made critical contributions to a number of drug development projects. Today structure-based drug design combined with biophysical characterizations of protein–drug complexes represent an integral part in the drug development process: essentially any disease relevant target protein can be subjected to structure determination, binding of ligands or synthetic inhibitors in the active site of enzymes can be experimentally determined and docking of compounds in the ligand binding site by various computational methods can be performed. Popular protein classes currently investigated in the pharmaceutical industry are kinases, proteases, DNA modifying enzymes and G-protein coupled receptors. The latter are involved in numerous different signaling pathways and other biological processes that are affected in diseases such as cancer, cardiovascular diseases, cell death deregulated diseases or diseases of the nervous system.

A very important and specific driver for the further progress of biocrystallography was the availability of highly intense, extremely monochromatic and focused synchrotron radiation. Such radiation allows the acquisition of data from small crystals with much higher accuracy and at a much faster rate than with conventional X-ray sources. To optimally exploit synchrotron radiation freezing the biocrystals to liquid nitrogen temperature is essential. It was very fortunate for the field of biocrystallography in Switzerland that in the late1990s the Swiss Light Source (SLS) was built. SLS designed, installed and operates one of the world's best fine-focussed beamlines making it one of the preferred places to collect data and determine crystal structures not only of single proteins and protein-nucleic acid or protein- small molecule complexes but also of membrane proteins as well as large macromolecular machines.

From around 1990 to 2000 X-ray crystallography of bio-macromolecules has undergone further significant technological advances mainly as a consequence of the massive effort by the structural genomics research initiatives that were started primarily in the United States of America, in Japan and in Europe.[9] These programs provided, among other things, today's high-throughput methodologies for the production, crystallization, data collection and structure determination of macromolecules. These developments certainly helped in the decision of the Universities Zurich and Bern to engage in bio-X-ray crystallography. The two protein crystallographers, Markus Grütter (Zürich) and Ulrich Baumann (Bern) were appointed in 1998.

2. Swiss Structural Biology Initiative in the Postgenomics Era

In the postgenomics era linking biological data with related structural and functional information has become a new challenge. Although the structural genomics initiatives could fulfill expectations only to a limited extent, they have nevertheless, turned structural biology into one of the central disciplines for the explanation, linking and exploitation of biological data in the life sciences. This is true in academic research as well as for applications envisaged in the biotechnological, agricultural and pharmaceutical industry.

Structural biology relates the function and interaction of biological macromolecules to their three-dimensional structure and provides the physical basis of biological activity. Swiss scientists working in the field in the late 1990s realized that a network of specialists would be essential to be able to optimally study the complex problems in macromolecular structure and interactions. As a consequence and with the support of the University of Zurich, the ETH Zurich and the Swiss National Science Foundation, a Swiss national center of excellence (NCCR) was established in 2001 and was funded for the following 12 years. It brought together specialists in experimental structure determination by X-ray crystallography, NMR spectroscopy and electron microscopy/crystallography, in protein biophysical chemistry, in modern molecular biology, and in computational biology. The project has provided and will continue to provide a platform for Swiss interdisciplinary projects of world-class standing and high efficiency, which extends the leverage of individual research groups. In this article the following topics from this program will be briefly reviewed: (i) application of new methods for macromolecular structure determination by X-ray crystallography, (ii) structural biology of membrane proteins and (iii) structural biology of large supramolecular complexes.

2.1 *Application of New Methods for Macromolecular Structure Determination by X-ray Crystallography*

2.1.1 *Protein Production*

An important prerequisite for structural studies by X-ray crystallography is the availability of sufficiently large amounts of highly purified macromolecular sample. Cloning, expression in bacteria, yeast, insect cells or mammalian cells or even in cell-free systems, as well as purification, biochemical and biophysical characterization of proteins are performed as a matter of routine in structural biology before the samples are submitted to crystallization experiments. For a review see ref. [10].

2.1.2 *Crystallization*

Today crystallization experiments are routinely carried out using the vapor diffusion method. To increase the throughput and to reduce consumption of purified protein, crystallization is performed at the nanoliter scale with pipeting robots that can reproducibly dispense nl amounts of solution. With the enormous increase in the number of crystallization experiments examination of their outcome is also automated. Each crystallization experiment is photographed following a given protocol and evaluated by the human eye. Such a facility has been established within the NCCR program and is available to all scientists in Switzerland who need a high-throughput facility for crystallization (con-

tact address: *http://www.structuralbiology.uzh.ch*). The ultimate test of the quality of a crystal is the quality of its diffraction pattern. For this test crystals are removed from the crystallization droplet with a small loop of a fine fiber and subsequently flash frozen to liquid nitrogen temperature. At this temperature the crystals are much more resistant to radiation damage by the strong synchrotron radiation beams.[11] The world-class protein crystallography beamlines at the SLS have undoubtedly and markedly promoted biocrystallography in Switzerland. In addition, physicists at the Paul Scherrer Institute (PSI) in Villigen have developed new detectors for rapid data collection, the Pilatus detector among them. Meanwhile these detectors have become the gold standard in X-ray photon detection and are installed at synchrotron beamlines throughout the world.

2.2 *Membrane Proteins*

Membrane proteins are a particularly challenging class of proteins for structural studies due to their relatively hydrophobic surface and their flexible and unstable structure. These properties make all the steps from protein expression and purification to data collection and structure resolution more difficult than for other types of proteins. NCCR scientists have contributed in a major way towards understanding the molecular structure of several classes of proteins.

2.2.1 *Ligand-gated Ion Channels*

Pentameric ligand-gated ion channels (pLGICs) are key players in the early events of electrical signal transduction at chemical synapses. Characteristic of this family of channel proteins is a structurally conserved scaffold that opens in response to the binding of neurotransmitter molecules. All proteins share a pentameric organization of identical or related subunits that consist of an extracellular ligand-binding domain followed by a transmembrane channel domain. Raimund Dutzler's group has resolved the crystal structure of two prokaryotic pLGICs (Fig. 1a, b), one (ELIC) in a non-conducting and the other (GLIC) in a conducting conformation. Their studies revealed the first high-resolution structures of a pLGIC and provide an important model system for the investigation of the general mechanisms of ion permeation and gating within the family. Studies are ongoing to find the endogenous ligands for these pLGICs.[12]

2.2.2 *ABC Transporters*

ATP-binding cassette (ABC) transporters are a large superfamily of membrane proteins with diverse functions. They convert the energy gained from ATP hydrolysis into trans-bilayer movement of substrates either into the cytoplasm (import) or out of the cytoplasm (export). The considerable efforts directed at understanding the detailed mechanism of ABC transporters is in part motivated by the fact that there are several clinically relevant representatives. Two NCCR groups have been working on this important class of membrane proteins. Kaspar Locher's group published the first crystal structure of an ABC exporter, a bacterial multidrug ABC transporter, revealing the architecture of these proteins and suggesting a drug extrusion mechanism (Fig. 2a).[13] The group also described the crystal structure of a complete bacterial ABC importer in complex with the cognate binding protein. Markus Grütter's group solved the structure of a bacterial heterodimeric ABC exporter at high resolution. This ABC transporter exhibits an inward-facing conformation in which the nucleotide binding domains (NBDs) interact *via* an interface involving highly conserved structural motifs which provide a functional link between the two asymmetric ATP hydrolysis sites (Fig. 2b).[14]

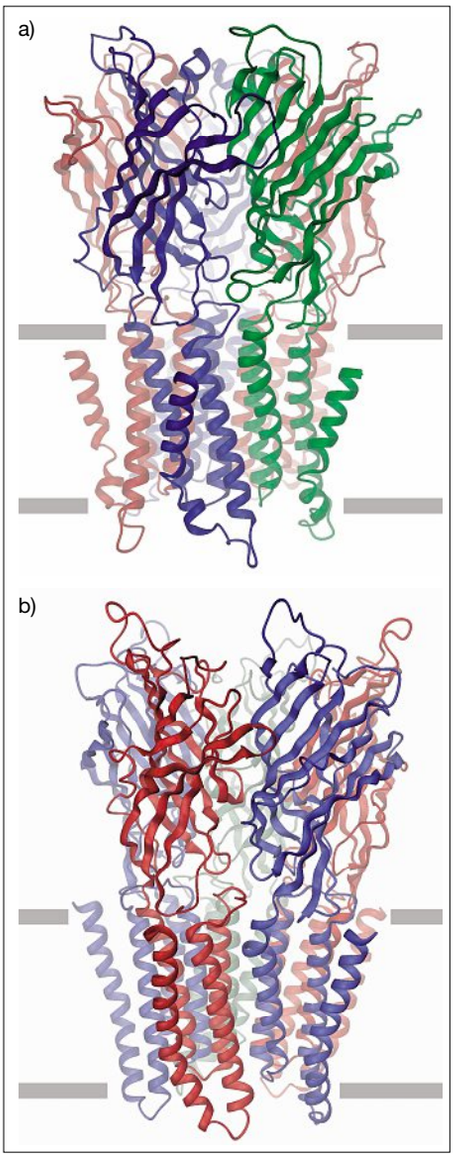

Fig. 1. a) X-ray structure of the pLGIC ELIC, a member of an important family of neurotransmitter receptors. The structure has provided the first view of this ion channel family at high resolution and shows a non-conducting conformation of the channel. b) Structure of a potentially open state of the proton-activated pentameric ligand-gated channel GLIC. The structure provides the first insight at high resolution into a conducting conformation of this ion channel family.

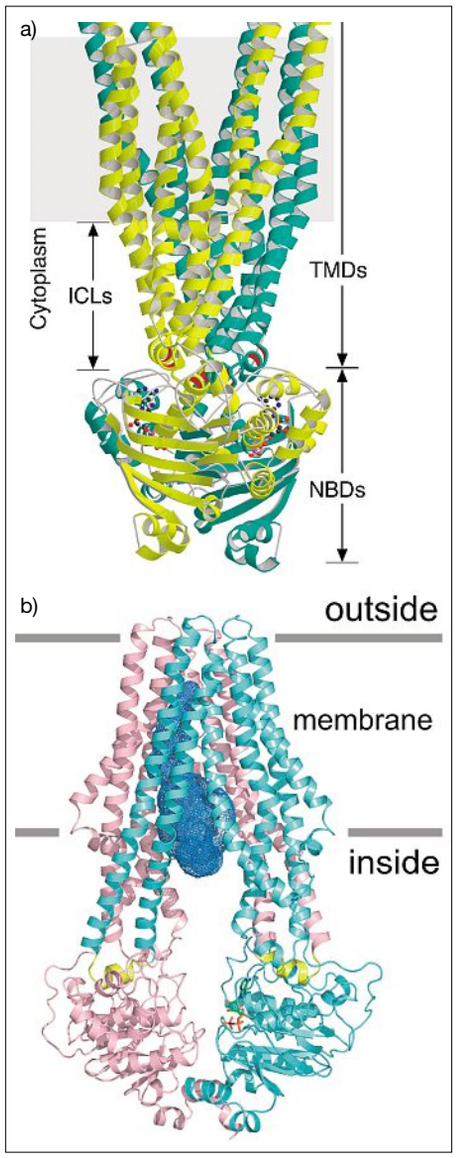

Fig. 2. a) The first structure of an ABC exporter. These proteins are relevant in multidrug extrusion and (glyco-)lipid flipping. The study revealed the architecture of these proteins and suggested a drug extrusion mechanism that exploits the binding and hydrolysis of cellular ATP. b) Crystal structure of a bacterial heterodimeric ABC exporter at very high resolution. This ABC transporter depicts an inward-facing conformation with the nucleotide binding domains (NBDs) interacting *via* an interface involving highly conserved structural motifs which provide a functional link between the two asymmetric ATP hydrolysis sites.

2.2.3 *Multidrug Exporter AcrB*

Bacterial resistance to antibiotics is a major challenge for the current treatment of infectious diseases. One way bacteria can escape destruction is by pumping out administered drugs through specific transporter proteins that span the cell membrane. Markus Grütter's group used designer proteins that bind to and stabilize AcrB, a protein of interest in the study of the major drug efflux pump of *Escherichia coli*. After selecting for designed ankyrin repeat proteins (DARPins) that inhibit this pump, the crystal structure of AcrB was determined in a complex with the DARPin inhibitor. This study confirmed that the AcrB is built from three subunits, each of which exhibits a distinctly different conformation (Fig. 3). The structure also offers an explanation for how substrate export is structurally coupled to simultaneous proton import – thus significantly improving current understanding of the mechanism of action of AcrB. This study is the first report of the selection and co-crystallization of a DARPin with a membrane protein and demonstrates the potential of DARPins not only as inhibitors but also as tools for the structural investigation of integral membrane proteins.[15]

2.2.4 *Ammonium Transport Proteins*

The Amt/Mep/Rh family of integral membrane proteins comprises ammonium transporters of bacteria, archaea and eukarya, as well as the Rhesus proteins found in animals. They play a central role in the uptake of reduced nitrogen for biosynthetic purposes, in energy metabolism, or in renal excretion. The group of Fritz Winkler resolved the crystal structure of the bacterial ammonium transporter AmtB that revealed a trimeric structure with three pores lined by hydrophobic residues suggesting that the transported species is the neutral ammonia molecule rather than the charged ammonium ion.[16]

2.2.5 *Oligosaccharyltransferase*

N-linked glycosylation in which sugars are attached to the side chain of the amino acid asparagine is one of the most common protein-modification reactions in the cells of eukaryotes (organisms that include plants, animals and fungi). The modification has diverse roles in protein folding and stability, intracellular trafficking and cell–cell interactions and is catalyzed by the enzyme oligosaccharyltransferase (OST). It has been unclear how OST recognizes the glycosyl-acceptor sites and the associated amino-acid sequences and how it activates the normally unreactive amide nitrogen in the side chain of asparagine for glycosylation. Kaspar Locher's group has provided remarkable insight into these issues with their report of the X-ray crystal structure of PglB, a bacterial OST. The crystal structure of PglB in complex with a peptide substrate provides invaluable information about PglB–peptide binding and the enzyme's catalytic mechanism (Fig. 4).[17]

2.2.6 *G-protein-coupled Receptors*

Nearly all physiological processes in higher organisms involve G-protein-coupled receptors (GPCRs), which represent the largest class of membrane proteins in the human genome. Rhodopsin is the archetypal class A GPCR and structurally the best characterized. Its atomic structure serves as a model for structure prediction of other GPCRs. Gebhard Schertler's group has described the crystal structure of an active rhodopsin mutant in complex with a peptide derived from the carboxy-terminus of the α-subunit of the G protein transducin, showing how an agonist ligand can activate its GPCR.[18]

2.3 *Supramolecular Assemblies*

The structure elucidation of assemblies or complexes of biological macromolecules provides essential information towards the understanding of biological processes at the supramolecular level. A number of systems are under investigation in this research area including DNA structure, protein–protein interactions, and ribosome complexes showing RNA–protein interactions. Results of this research provide fundamentally new insights into the regulation, functioning and macromolecular interaction mechanisms at the molecular level.

2.3.1 *Chromatin Structure and Organization*

The DNA of higher organisms from yeast to man is organized as chromatin. The hierarchical packaging of DNA is reversible and fundamental to the genetics and epigenetics of natural and disease gene regulating and transcription processes. The crystal structure of the nucleosome which represents the first level or DNA organization, has been solved to atomic resolution by the group of Timothy Richmond (Fig. 5a).[19] The chromatin fiber is the second level of organization. The structure of a representative tetranucleosome was elucidated (Fig. 5b).[20] The position of nucleosomes along DNA can be altered by energy-dependent chromatin remodelling factors affecting gene regulation. The structurally based mechanism for such a factor was determined.[21]

2.3.2 *Eukaryotic Ribosome*

Ribosomes are the sites of protein synthesis in all living organisms. A ribosome particle is a complex of proteins and RNAs, thus called a ribonucleoprotein. Ribosomes are composed of a large and a small subunit. Nenad Ban's group has

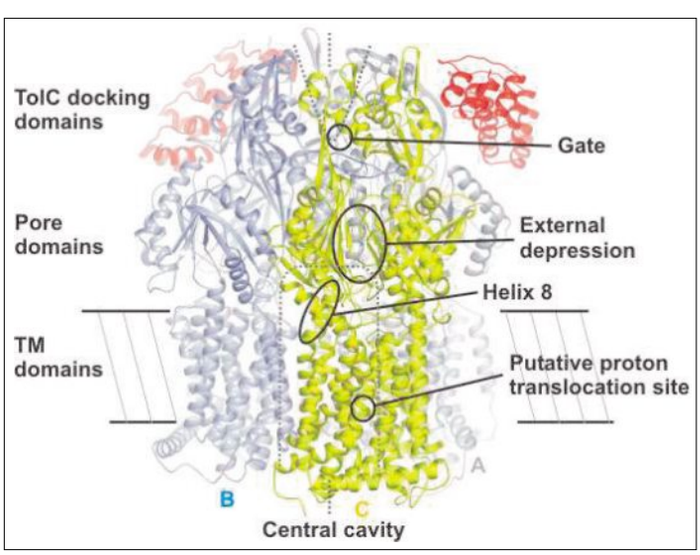

Fig. 3. First high-resolution crystal structure of a DARPin - membrane protein (AcrB) complex. The structure shows an asymmetric trimeric exporter and allows insight into the transport mechanism.

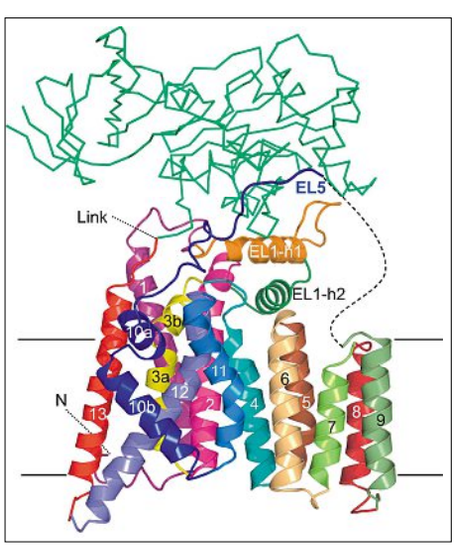

Fig. 4. The first crystal structure of an oligosaccharyltransferase. These proteins catalyse protein N-glycosylation, an essential cellular process. The study not only revealed the architecture of this protein and the molecular basis of substrate recognition, but also suggested the mechanism of glycan transfer.

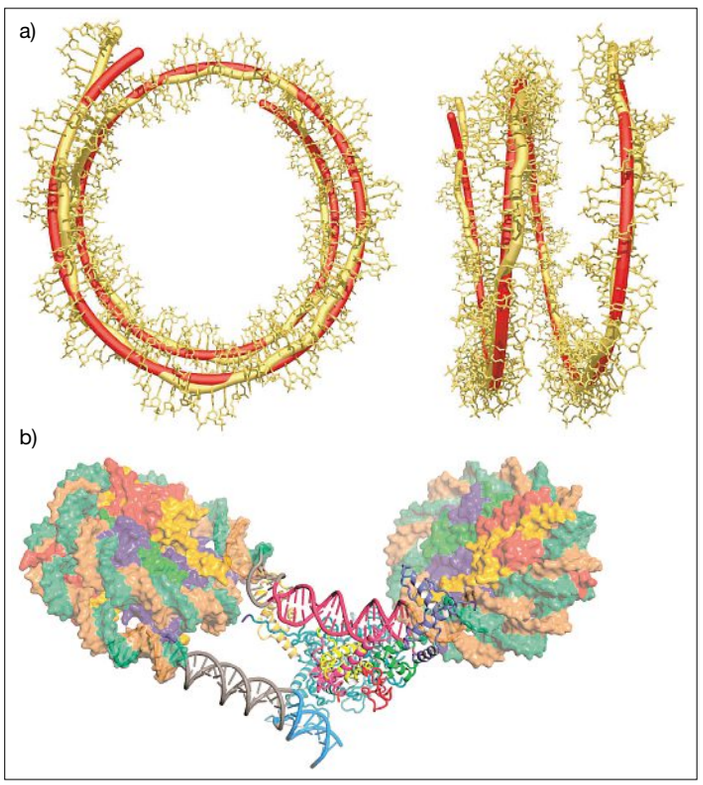

Fig. 5. a) Crystal structure showing the DNA portion of the nucleosome, the first level of hierarchical DNA packaging. b) X-ray structure of a tetranucleosome and its implications for the chromatin fibre. The figure shows the structure of a tetranucleosome.

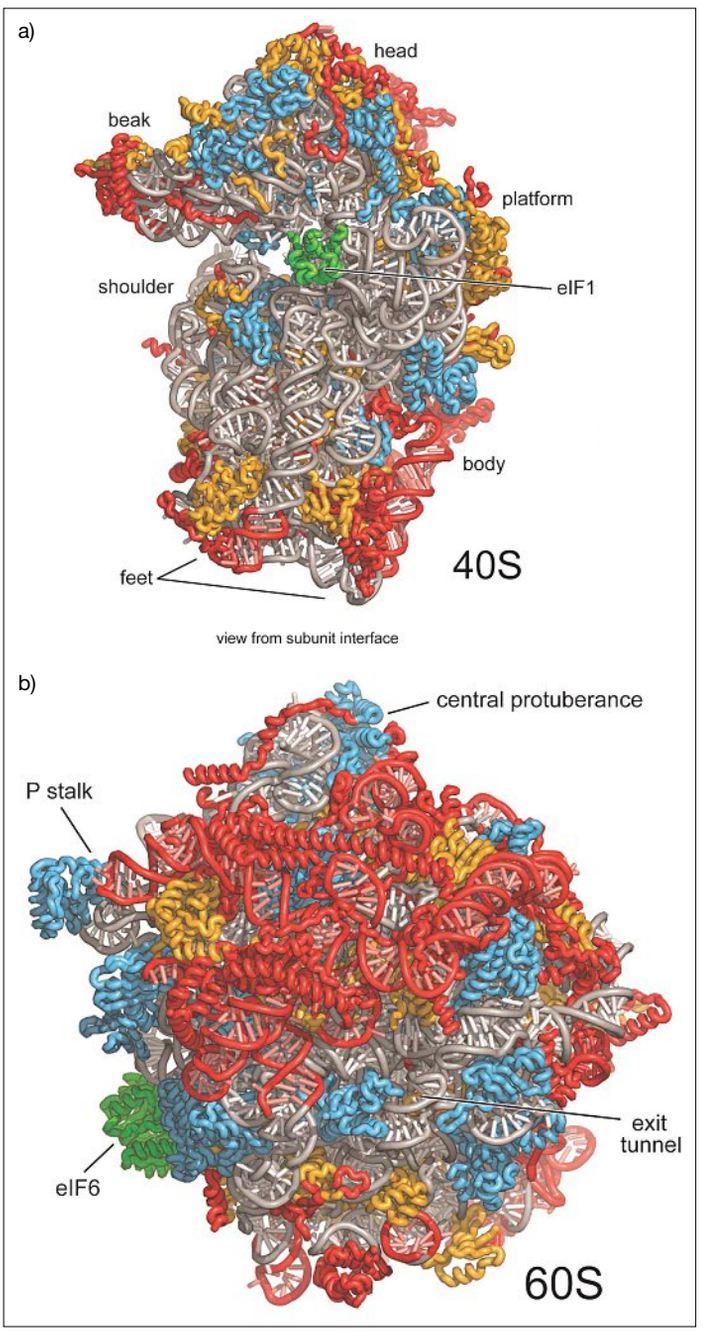

Fig. 6. Atomic resolution X-ray structures of the eukaryotic small 40S (a) and large 60S (b) ribosomal subunits colored according to evolution between kingdoms of life. The universally conserved core of the rRNA is depicted in grey, while eukaryote-specific rRNA expansion segments are shown in red. Ribosomal proteins occurring in all kingdoms are shown in blue, those present only in archaea and eukaryotes are colored orange, and eukaryote-specific proteins and protein extensions are displayed in red. Both structures were solved in the presence of initiation factors (shown in green). Architectural landmarks are highlighted.

obtained detailed structural information on eukaryotic ribosomes, which are significantly larger and more complex than their prokaryotic counterparts. They have determined the first complete structures of both small and large eukaryotic ribosomal subunits each in complex with an initiation factor (Fig. 6a, b).[22]

2.3.3 *Signal Recognition Particle*

The signal recognition particle (SRP) is an abundant, cytosolic, universally conserved ribonucleoprotein (protein–RNA complex) that recognizes and targets specific proteins to the endoplasmic reticulum in eukaryotes and the plasma membrane in prokaryotes. In eukaryotes, SRP binds to the signal sequence of a newly synthesized peptide as it emerges from the ribosome. Pioneering structural and mechanistic studies of various ribosomal complexes involved in co-translational protein processing, folding and targeting, have been carried out by Nenad Ban's group; they provide critical insights into this aspect of ribosomal function.[23]

2.3.4 *Fatty Acid Synthase*

Fatty acid synthase is a multi-enzyme protein that catalyzes fatty acid synthesis. It is not a single enzyme but a whole enzymatic system composed of two identical multifunctional polypeptides, in which substrates are handed from one functional domain to the next. Its main function is to catalyze the synthesis of the long-chain saturated fatty acid palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH. Work carried out in Nenad Ban's group offers first mechanistic insights into substrate shuttling and delivery in such megasynthases, with direct implications for our understanding of polyketide synthases and non-ribosomal peptide synthases (Fig. 7).[24]

2.3.5 *Pilus Assembly*

Bacterial proteinaceous filaments termed pili or fimbriae are nonflagellar, hair-like structures protruding from the cell surface; they are critical for bacterial virulence and fitness. Rudolf Glockshuber's group studies the mechanism of the assembly of adhesive type-1 pili from *E. coli*. These are large, hetero-oligomeric protein filaments of uropathogenic *E. coli* strains that are required for the attachment

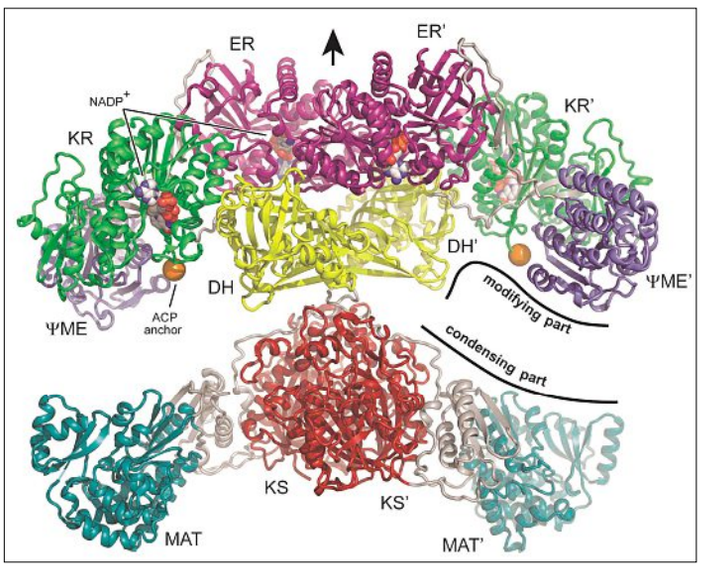

Fig. 7. Atomic resolution X-ray structure of the mammalian fatty acid synthase (FAS) showing the domain organization of the multifunctional enzyme. Saturated C16 fatty acids are synthesized in an iterative cyclic reaction. At the beginning of each reaction cycle, a C2 carbon unit is added to the growing acyl chain by the malonyl/acetyl transferase (MAT) and ketoacyl synthase (KS) domains. The unsaturated reaction intermediate is shuttled between the catalytic sites by the acyl carrier protein (ACP) domain, to which it remains covalently attached, and is further modified by ketoacyl reductase (KR), dehydratase (DH) and enoyl reductase (ER) to form the saturated primer substrate for the next reaction cycle. The 2-fold symmetry axis of the FAS is depicted with an arrow. The pseudo-methyl transferase (YME) is likely an inactive remnant of an ancestral domain, which is still functional in related polyketide synthases.

of the bacteria to host cell surfaces. Of particular interest is the function of FimC, a periplasmic assembly factor which is not a structural component of the pili but is required for pilus assembly *in vivo*. A major contribution is the identification and description of bacterial pilus assembly chaperones as a previously unknown class of protein folding catalysts.[25] The determination of nuclear magnetic resonance and X-ray protein structures of the N-terminal substrate recognition domain of FimD ($FimD_N$) before and after binding of a chaperone–subunit complex is a fine example of inter-disciplinary collaboration and shows how $FimD_N$ specifically interacts with both FimC and the subunit to be translocated (FimH). The structural work together with functional studies explains how $FimD_N$ discriminates between loaded and unloaded FimC molecules.[26]

3. Outlook

X-ray crystallography has undergone major developments during the last 70 years of its existence. It has established itself as a key method in biology for describing the architecture of proteins and protein assemblies. The structural information obtained from X-ray crystallography is the basis for a detailed understanding of the function of these molecules. For membrane proteins we currently experience a marked increase of new structures appearing in the protein structure database. With the advances already achieved and additional developments in the future, we can expect to see a further increase in the complexity of the molecules studied.

As outlined above the Swiss biocrystallography community has made spectacular contributions especially in the areas of membrane proteins and supramolecular complexes. The future for structural biology in Switzerland looks bright and its place at the forefront of the field is assured for the coming years. A Zurich center for molecular structure and mechanism is currently being planned as a platform for education, research and exchange of know how and for translational research. This center is seen as a key element of the efforts towards maintaining a leading position for Swiss structural biology. Another element is progress in technology. The development of the free electron laser sources in Stanford and Hamburg are probably the most exciting prospect. These facilities have the potential of revolutionizing the field again, since they may allow snapshots of different states of macromolecules at the femtosecond time scale, thus opening a new dimension in understanding their function. The SwissFEL under construction at the PSI in Villigen will undoubtedly have a positive effect on Swiss biocrystallographic research when it starts to operate in 2016. The discoveries that lie ahead will once again change the paradigm of our understanding of the molecular life sciences.

Received: December 7, 2013

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