A structural perspective on protein–protein interactions

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Structures of macromolecular complexes are necessary for a mechanistic description of biochemical and cellular processes. They can be solved by experimental methods, such as X-ray crystallography, NMR spectroscopy and electron microscopy, as well as by computational protein structure prediction, docking and bioinformatics. Recent advances and applications of these methods emphasize the need for hybrid approaches that combine a variety of data to achieve better efficiency, accuracy, resolution and completeness.

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Introduction

Genome sequencing has provided nearly complete lists of the macromolecules present in many organisms (e.g. [1,2]). However, these lists reveal comparatively little about the function of biological systems because the functional units of cells are often complex assemblies of several macromolecules [3]. Such complexes vary widely in their activity and size [3–7], and play crucial roles in most cellular processes. They are often depicted as molecular machines [3], a metaphor that accurately captures many of their characteristic features, such as modularity, complexity, cyclic functions and energy consumption [8]. For instance, the nuclear pore complex, a 50–100 MDa protein assembly, regulates and controls the trafficking of macromolecules through the nuclear envelope [9]; the ribosome is responsible for protein biosynthesis; RNA polymerase catalyzes the formation of RNA [10]; and ATP synthase catalyzes the formation of ATP [7]. Macromolecular assemblies are also involved in transcription control (e.g. the IFNβ enhanceosome) [6,11] and the regulation of cellular transport (e.g. microtubulins in complex with the molecular motors myosin or kinesin) [12–14], and are crucial components in neuronal signaling (e.g. the post-synaptic density complexes) [15]. A structural description of the protein interactions within such complexes is an important step toward a mechanistic understanding of biochemical, cellular and higher order biological processes [16–18,19•].

There are currently about 12 000 known structures, from a variety of organisms, of assemblies involving two or more protein chains (http://pqs.ebi.ac.uk/pqs-doc.shtml) (April 2004) [20]; these complexes can be organized into about 3500 groups based on sequence similarity [19•]. Just how many complexes exist in a particular proteome is not easy to deduce because of the different component types (e.g. proteins, nucleic acids, nucleotides, metal ions) and the varying life span of the complexes (e.g. transient complexes, such as those involved in signaling, and stable complexes, such as the ribosome). Until recently, the most comprehensive information about protein–protein interactions was available for the *Saccharomyces cerevisiae* proteome, consisting of approximately 6200 proteins. This information has been provided by methods such as the yeast two-hybrid system and affinity purification followed by mass spectrometry [21–27,28•,29]. The lower bound on binary protein interactions and functional links in yeast has been estimated to be in the range of approximately 30 000 [30,31]; this number corresponds to about nine protein partners per protein, although not necessarily all direct or interacting at the same time. The human proteome may have an order of magnitude more complexes than the yeast cell and the number of different complexes across all relevant genomes may be several times larger still. Therefore, there may be thousands of biologically relevant macromolecular complexes whose structures are yet to be characterized [32].

We review here recent developments in the experimental and computational techniques that have allowed structural biology to shift its focus from the structures of individual proteins to the structures of large assemblies [19•,33,34]. We also illustrate these developments by listing their application to the determination of the structure of specific assemblies of biological importance. In contrast to structure determination of individual proteins, structural characterization of macromolecular assemblies usually poses a more difficult challenge. We stress that a comprehensive structural description of large complexes generally requires the use of several experimental methods, underpinned by a variety of theoretical approaches to maximize efficiency, completeness, accuracy and resolution [19•,35].

X-ray crystallography and NMR spectroscopy

X-ray crystallography has been the most prolific technique for the structural analysis of proteins and protein complexes, and is still the 'gold standard' in terms of accuracy and resolution (Figure 1a). Structures of several macromolecular assemblies have recently been solved by X-ray crystallography: RNA polymerase [36], the ribosomal subunits [37–41], the complete ribosome and its functional complexes [42], the proteasome [43], the GroEL chaperonin [44], various complexes of the cellular transport machinery [12,13], the Arp2/3 complex [45], photosystem I and the light-harvesting complex of photosystem II [46,47], the SRP (signal recognition particle) complex involved in nascent protein targeting [48•], and various viral capsid and virion structures [49–51]. However, the number of structures of macromolecular assemblies solved by X-ray crystallography is still quite small compared to that of the individual proteins and it will probably be many years before we have a complete repertoire of high-resolution structures for the hundreds of complexes in a typical cell. This discrepancy is due mainly to the difficult production of sufficient quantities of the sample and its crystallization.

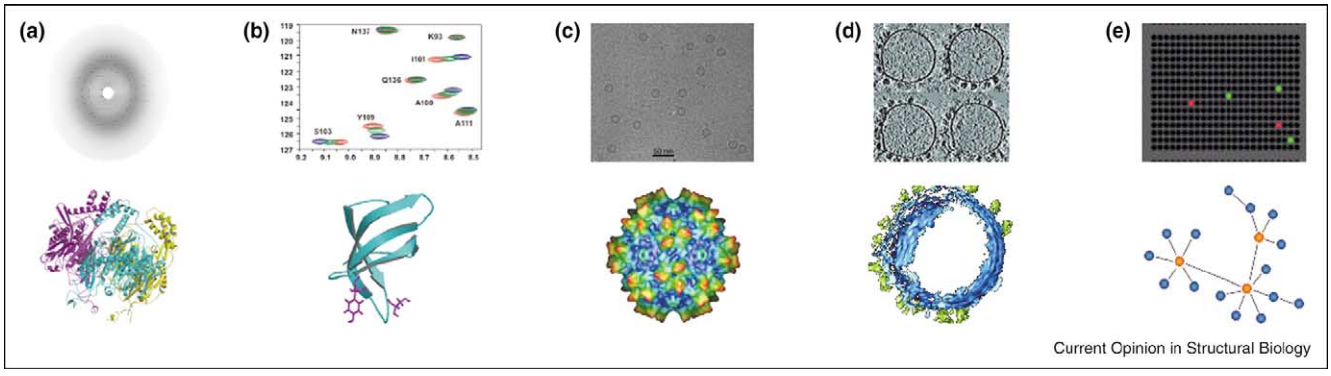
NMR spectroscopy allows the determination of atomic structures of ever-larger subunits and even their complexes [52–54]. Increasingly, it is used to identify residues involved in protein interactions (Figure 1b) [55–58]. Recent technical advances have allowed its application to systems as large as the 900 kDa GroEL–GroES complex [52]. Also, it was recently used to describe structural differences between interactions among different LIM and SH3 domains [59].

Electron microscopy and electron tomography

There are several variants of electron microscopy (EM), including single-particle EM (Figure 1c) [60], electron tomography (Figure 1d) [61] and electron crystallography of regular two-dimensional arrays of the sample [62].

For particles with molecular weights greater than 200–500 kDa, single-particle cryo-EM can determine the

Figure 1



Methods for the structural characterization of macromolecular assemblies. **(a)** Electron diffraction map and three-dimensional structure of the bacterial degradosome component PNPase (polyribonucleotide phosphorylase) determined by X-ray crystallography [183]. X-ray crystallography integrates the diffraction patterns collected after bombarding a crystallized protein or complex with X-rays to construct its three-dimensional structure. In principle, there is no size limit on the structures studied using this technique, although it is often difficult to obtain sufficient material for crystallization. This technique provides atomic-resolution structures and thus molecular details of how the interactions between the different components occur. **(b)** Three-dimensional structure and plot showing chemical shifts upon association of the human survival motor neuron (SMN) tudor domain solved by NMR spectroscopy [184]. NMR spectroscopy extracts distances between atoms by measuring transitions between different nuclear spin states within a magnetic field. These distances are then used as restraints to build three-dimensional structures. NMR spectroscopy also provides atomic-resolution structures, but is generally limited to proteins of about 300 residues. It plays an increasingly important role in studying interaction interfaces between structures determined independently. **(c)** EM micrograph and three-dimensional reconstruction of adeno-associated virus type 2 empty capsids [185]. EM is based on the analysis of images of stained particles. Different views and conformations of the complexes are trapped and thus thousands of images have to be averaged to reconstruct the three-dimensional structure. Classical implementations were limited to a resolution of 20 Å. More recently, single-particle cryo techniques, whereby samples are fast frozen before study, have reached resolutions as high as approximately 6 Å. EM provides information about the overall shape and symmetry of macromolecules. **(d)** Slice images and rendered surface of a ribosome-decorated portion of endoplasmic reticulum [73]. In electron tomography, the specimen studied is progressively tilted upon an axis perpendicular to the electron beam. A set of projection images is then recorded and used to build a three-dimensional model. This technique can tackle large organelles or even complete cells without perturbing their physiological environment. It provides shape information at resolutions of approximately 30 Å and promises to reach higher resolutions soon. **(e)** Yeast two-hybrid array screen and small network of interacting proteins [124••,186]. Interaction discovery comprises many different methods whose objective is to determine spatial proximity between proteins. These include techniques such as the two-hybrid system, affinity purification, FRET, chemical cross-linking, footprinting and protein arrays. These methods provide very limited structural information and no molecular details. Their strength is that they often give a quasi-comprehensive list of protein interactions and the networks they form.

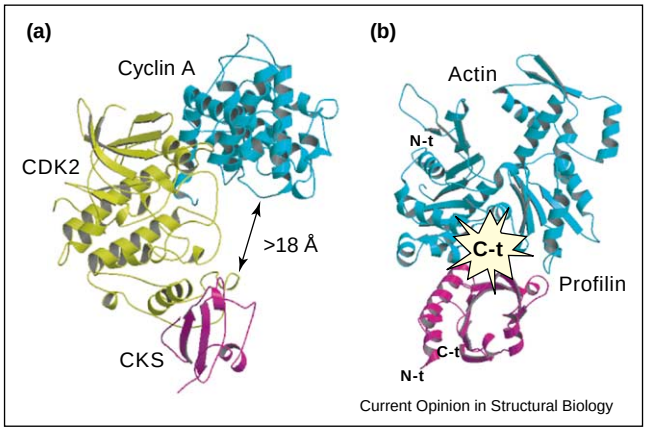
electron density of an assembly at resolutions as high as approximately 5 Å [63•,64–66,67•,68•,69,70•]. The full three-dimensional structure of the particle is reconstructed from many two-dimensional projections of the specimen, each showing the object from a different angle. Imaging by cryo-EM requires neither large quantities of the sample nor the sample in a crystalline form. Therefore, single-particle cryo-EM is a powerful tool to investigate the structure and dynamics of macromolecular assemblies for which X-ray structure determination is very difficult. Although it is generally impossible to build atomic models solely from cryo-EM density maps, the maps give valuable insights into the structure and mechanism of large complexes (e.g. [63•]). They are particularly useful when combined with atomic-resolution structures of the subunits, as reviewed in the section on hybrid methods below.

One of the most exciting developments in structural biology is the new generation of tomography methods based on multiple tilted views of the same object [33,71]. Although electron tomography can be used to study the structures of isolated macromolecular assemblies at a relatively low resolution of a few nanometers, its true potential lies in visualizing the assemblies in an unperturbed cellular context [72]. These data sets provide fascinating three-dimensional images of entities as large as a small cell at approximately 5 nm resolution [73]. To widen the scope of cellular tomography, it is necessary to improve the resolution of the tomographic images, as well as identify the structures in these images [73–75]. Theoretical considerations [76] and ongoing improvements in the instrumentation make a resolution as high as 2 nm a realistic goal [77].

Low-resolution experimental methods

Several experimental techniques can provide structural information about protein interactions at low resolution (Figure 1e). This information may be used to infer the configuration of the proteins in a complex. Methods for the mapping of protein interactions may provide contact or proximity restraints for pairs of proteins that are useful in the modeling of higher order complexes. Such methods include new implementations of the two-hybrid system [78–81], tagged affinity chromatography [82•,83] and the combination of phage display with other techniques [84], such as synthesis of peptides on cellulose membranes (SPOT) [85••]. Because of the low-resolution nature of these biochemical characterizations, care is needed in their interpretation. For example, comparing biochemically derived interaction sets against known three-dimensional structures of complexes revealed potential sources of systematic errors in interaction discovery, such as indirect interactions in two-hybrid systems, the obstruction of interfaces by molecular labels and artificial promiscuity in the detected interactions (Figure 2) [86].

Figure 2



Examples of potential errors in biochemical interaction discovery techniques, as revealed by a structure-based analysis [17]. **(a)** Indirect interactions between cyclin-dependent kinase regulatory subunit (CKS) and cyclin A detected by the yeast two-hybrid system. Several interactions between CKS domains and cyclins were reported in genome-scale two-hybrid studies [21,187]. However, analysis of three-dimensional structures suggests that the endogenous cyclin-dependent kinase 2 (CDK2) probably mediates the interaction, as combining the CDK2–CKS and CDK2–cyclin A structures places the CKS and cyclin domains 18 Å apart [86]. **(b)** An example of an interaction that is not detected by any screen, possibly because molecular labels (e.g. affinity purification tags, or two-hybrid DNA binding or activation domains) are interfering with the interaction. The X-ray structure of the actin–profilin complex reveals that the actin C terminus (C-t) lies at the interaction interface (the other N and C termini are also labeled).

Biophysical, biochemical and molecular biology methods can also be used to derive low-resolution information about the relative position and orientation of domains in a larger complex. These methods include: site-directed mutagenesis, which can identify the residues that mediate the interaction [87]; various forms of footprinting, such as hydrogen-deuterium exchange [88,89•] and hydroxyl radical footprinting [90], which can identify surfaces buried upon complex formation; chemical cross-linking [91–93], which can identify interacting residues; fluorescence resonance energy transfer (FRET) [94,95], which can determine the distance between labeled groups on the interacting proteins; and Fourier transform IR spectroscopy (FTIR), which describes structural changes upon complex formation [96]. Small angle X-ray scattering (SAXS) is another biophysical method that can provide low-resolution information about the shape of a complex. For instance, SAXS has recently been used to study the dynamics of conformational change in Bruton tyrosine kinase [97,98].

Computational protein–protein docking

When atomic structures of the individual proteins involved in an interaction are known, either by experiment or by modeling, several computational methods are available that suggest the structure of the interaction [99].

Most of these docking methods aim to predict the atomic model of a complex by maximizing the shape and chemical complementarity between a given pair of interacting proteins [99–102]. Docking strategies usually rely on a two-stage approach: they first generate a set of possible orientations of the two docked proteins and then score them in the hope that the native complex will be ranked highly. Katchalski-Katzir, Vakser and co-workers [103] have pioneered a fast Fourier transform (FFT)-based method for rapidly searching through the space of possible docked configurations. Due to its computational efficiency, it has also been incorporated into programs such as FTDock and 3D-Dock [104–106], GRAMM [107], DOT [108], ZDOCK [109] and HEX [110]. Other docking programs include ICM [111] and ROSETTA [112]. The searches may be restrained by other considerations, such as the known binding site location. These methods differ in protein representation, in the scoring of different configurations and in the search for the best solutions. Some methods boldly model the actual diffusion/collision trajectories involved in the docking process [113,114].

Although docking methods are not sufficiently accurate to predict whether or not two proteins actually interact with each other, they can sometimes correctly identify the interacting surfaces between two structurally defined subunits [115]. Docking methods are systematically assessed through blind trials in the Critical Assessment of PRedicted Interactions (CAPRI) [101,116•,117]. Predictions are made just before the structures are solved experimentally, followed by the assessment of the models at the CAPRI meetings. The best of the methods assessed in the last CAPRI experiment correctly predicted three of the seven target complexes [116•].

Methods that are able to work with comparative protein structure models [118] instead of experimentally determined subunit structures would extend the applicability of docking to many more biological problems, but would probably have poorer performance. Currently, docking is often applied in concert with experimental techniques, including site-directed mutagenesis [119], amide hydrogen-deuterium exchange [89•] and NMR spectroscopy [120•,121], as well as solid-state binding and surface plasmon resonance [122].

Inferring interactions by homology

Protein interactions can also be modeled by similarity [123,124••,125]. If a complex of known structure comprising homologs of a pair of interacting proteins is available, it is usually possible to build a model by comparative modeling [126]. There are now approximately 2000 distinct interaction types of known structure (i.e. whereby interacting domains sharing 30% or greater sequence identity are considered to be a single type; P Aloy, RB Russell, unpublished).

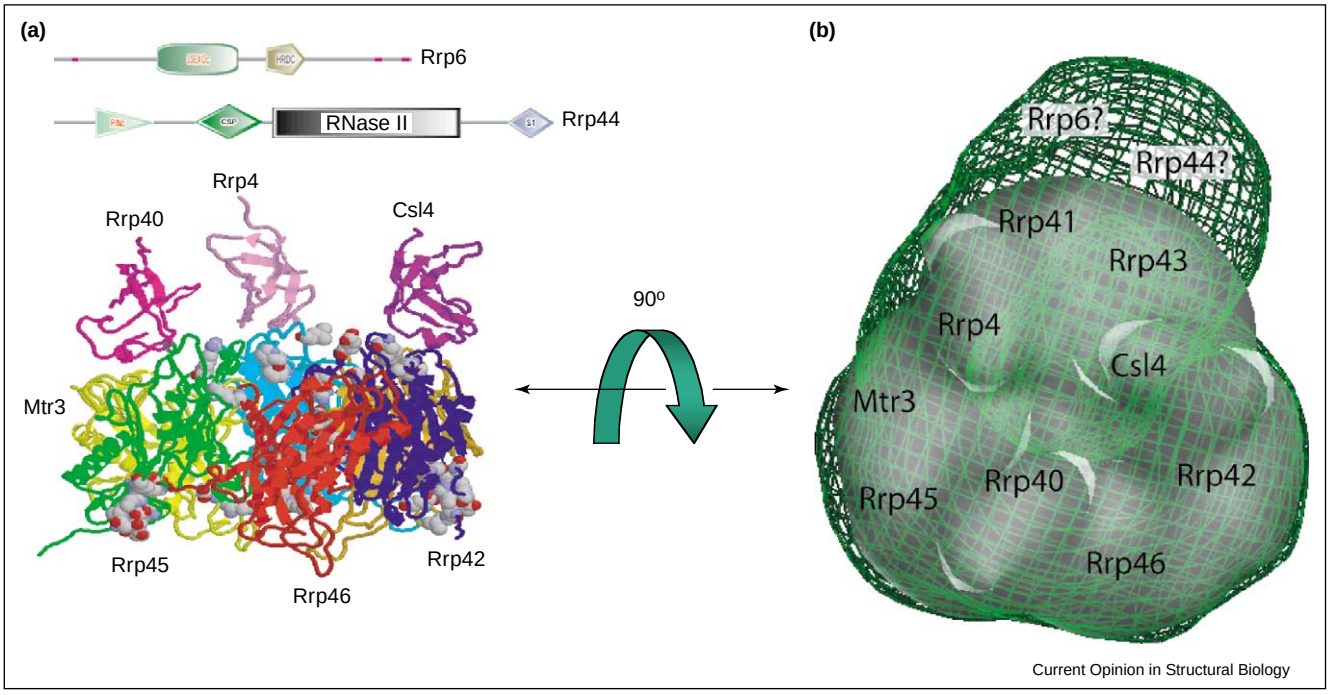
Building a model of the interaction between a pair of proteins based on the known structure of the complex between interacting homologs raises the question of whether or not homology of the subunits implies similarity of interaction. It was found that interactions between proteins of the same fold tend to be similar when the sequence identity is above approximately 30% [127•]. Below this cutoff, there is a twilight zone where interactions may or may not be similar geometrically.

Given a template, it is possible to model an interaction using standard comparative modeling techniques [126]. However, frequently there are multiple templates for the same interaction type. In addition, a single interaction template can be used to model many putative interactions in a single organism. Therefore, it is important to assess the likelihood of these potential interactions, particularly in the absence of experimental validation [128••]. For example, each of the dozens of fibroblast growth factors (FGFs) interacts with one or more of seven receptors with different affinities [129]. Two approaches have been developed recently that attempt to predict specificity by modeling interactions. The first approach, implemented by InterPReTS [123,130] and ModBase [125], uses empirical pair potentials derived from interfaces of known structure to score how well a pair of homologous proteins fits a known complex structure. The second approach, MULTIPROSPECTOR, is similar, although it attempts to study more distantly related protein sequences by threading sequences onto a library of interacting templates, followed by scoring how well the individual sequences fit their proposed folds and the interface between them [131]. Both approaches have since been applied to study large collections of sequences and interactions [124••,125,132••].

For some large complexes, the specificity of interactions within a family of homologous subunits is an important determinant of complex assembly. For instance, the chaperonin CCT consists of eight homologous subunits that are all similar to the single subunit comprising the thermosome [133]. Thus, building CCT using the thermosome requires the conversion of a seven-subunit ring into an eight-subunit ring, and then choosing the correct arrangement from the 5040 (8!/8) possibilities. It is possible to guide this process by experiment, such as the detection of subcomplexes that reveal preferred interacting pairs [134] or the application of the two-hybrid system [135]. InterPReTS was also applied to select one of the 120 possible arrangements of six exosome subunits (Figure 3) [136], with mixed results.

In eukaryotes, many of the protein–protein interactions in regulatory signaling networks are mediated by modular protein interaction domains. Such domains appear to have been used in a modular fashion throughout evolution to generate novel connections between proteins.

Figure 3



Putative structure through modeling and low-resolution EM. **(a)** Exosome subunits. The top of the panel shows the domain organization of two subunits present in the complex, but lacking any detectable similarity to known three-dimensional structures. The model for the nine other subunits (bottom) was constructed by predicting binary interactions using InterPReTS [130] and building models based on a homologous complex structure using comparative modeling. **(b)** EM density map (green mesh) with the best fit of the model shown as a gray surface and the predicted locations of the subunits labeled. The question marks indicate those subunits for which no structures could be modeled.

However, the repeated use of such domains presents the problem of specificity: how are biologically unique connections made? Experimental evidence suggests that some of these interactions are remarkably specific [137], whereas others show overlapping specificity [85••]. These findings highlight the need for the development and validation [128••] of accurate computational methods that capture the structural principles of protein interaction specificity.

Low-resolution computational methods

Even when docking or modeling is not feasible, it may still be possible to get some structural insight into a protein–protein interaction using other computational approaches. Various methods combine structures with sequence alignments and phylogenetic trees to identify sites on the surface that are likely to be involved in function or specificity [138–145]. Other computational methods perform alanine scanning to identify 'hot spots' at known protein interfaces. A comparative analysis of such hot spots may reveal determinants of specificity and cross-reactivity [146–148]. There are also many computational methods for the prediction of protein–protein interactions when no structural information is available (see the review by Bork *et al.* in this section).

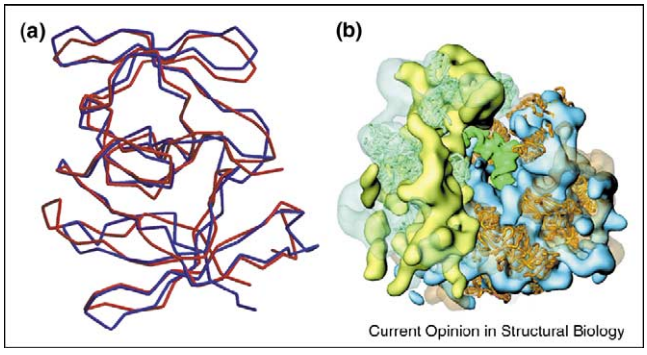
Hybrid methods

In the absence of atomic-resolution data, approximate atomic models of assemblies can be derived by combining low-resolution cryo-EM data on complete protein assemblies with computational docking of atomic-resolution structures of their subunits [149–156]. It has been estimated that using such fitting techniques improves the accuracy to up to one-tenth the resolution of the original EM reconstruction.

Hybrid approaches involving the fitting of subunits into EM maps are illustrated by pseudo-atomic models of the actin–myosin complex [157], the yeast ribosome [158,159] (Figure 4), the bacteriophage T4 baseplate [160••], pre-mRNA splicing complex SF3b [161], the RAD51 system involved in homologous recombination and DNA repair [162•], and complex virus structures [163,164].

Unfortunately, experimentally determined atomic-resolution structures of the isolated subunits are frequently not available. Even when available, the induced fit can severely limit their utility in the reconstruction of the whole assembly. In such cases, it might be possible to get useful models of the subunits by comparative protein

Figure 4



Hybrid assembly of the 80S ribosome from yeast [158]. **(a)** Superposition of a comparative protein structure model (red) of a domain from ribosomal protein L2 from *Bacillus stearothermophilus* with the actual structure (blue) (PDB code 1RL2). **(b)** A partial molecular model of the whole yeast ribosome calculated by fitting atomic rRNA (not shown) and comparative protein structure models (ribbon representation) into the electron density of the 80S ribosomal particle.

structure modeling [126,165–168]. The number of models that can be constructed with useful accuracy is already two orders of magnitude greater than the number of available experimentally determined structures. Models with at least the correct fold can be constructed for domains in approximately 58% of known protein sequences [125]. Comparative modeling will be increasingly more applicable and accurate because of structural genomics initiatives [169]. One of the main goals of structural genomics is to determine a sufficient number of appropriately selected structures from each domain family, such that all sequences are within modeling distance of at least one known protein structure [170,171].

Structural genomics may in fact contribute to a comprehensive and efficient structural description of complexes in an additional way. Although structural genomics currently focuses on single proteins or their domains, it could be expanded to the sampling of domain–domain interactions [127•,172,173]. Such an effort would provide a repertoire of templates for binary interactions, which would facilitate the building of higher order complexes.

Although X-ray crystallography and EM in combination with atomic structure docking have been successfully employed to solve the structures of protein assemblies, they are not capable of efficiently characterizing the myriad of complexes that exist in a cell. For example, most transient complexes cannot be addressed at all with these approaches. Therefore, there is a great need for the additional development of hybrid methods through which accuracy, high throughput, completeness and resolution are improved by integrating information from all available sources [19•,136,174].

The dynamics of complexes

By trapping complexes in different conformations and configurations, hybrid methods can be used to study the functional role of assembly dynamics. For instance, models of the two different functional states of the *Escherichia coli* 70S ribosome demonstrated that the complex changes from a compact to a looser conformation, and showed rearrangements of many of the ribosomal proteins [63•]. Similarly, T antigen double hexamers (a replicative helicase of simian virus 40) were assembled at the origin of replication using 27.5 Å cryo-EM maps at different degrees of bending along the DNA axis [175]. Fitting the crystal structure of the Tag helicase domain [176] into the three-dimensional cryo-EM density map ascertained that the C-terminal domains are rotated relative to each other in the complex. The results were combined with the available biochemical data to propose an integrated model for the initiation of viral DNA replication. Such a comparison also revealed details that are key to understanding filament function. Fitting atomic models of actin and the myosin cross-bridge into 14 Å cryo-EM maps showed that the closing of the actin-binding cleft upon actin binding is structurally coupled to the opening of the nucleotide-binding pocket [67•].

The dynamics of assembly models can also be studied by theoretical calculations [177–180]. A vibrational analysis of elastic models was employed to capture the essential motions of clamp closure in bacterial RNA polymerase, the ratcheting of the 30S and 50S subunits of the ribosome, and the dynamic flexibility of chaperonin CCT [181]. Also, a quantized elastic deformational model provided a basis for the simulation of conformational fluctuations related to the expansion and contraction of the truncated E2 core from the pyruvate dehydrogenase complex [182].

Conclusions

There is a wide spectrum of experimental and computational methods for the identification and structural characterization of macromolecular complexes. These methods need to be combined into hybrid approaches to achieve greater accuracy, coverage, resolution and efficiency than any of the individual methods. New methods must be capable of generating possible alternative models consistent with information such as stoichiometry, interaction data, homology to known structures, docking results and low-resolution images. There is a need to describe the structures and dynamics of both stable and transient complexes.

Structural biology is a great unifying discipline of biology. Thus, structural characterization of many protein complexes may be the way to bridge the gaps between genome sequencing, functional genomics, proteomics and systems biology. The goal seems daunting, but the prize will be commensurate with the effort invested,

given the importance of molecular machines and functional networks in biology and medicine.

Acknowledgements

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