INTRODUCTION

Molecular Biophysics at the Beginning of the Twenty-First Century: From Ensemble Measurements to Single-Molecule Detection

The ideal biophysical method would be capable of measuring atomic positions in molecules *in vivo*. It would also permit visualization of the structures that form throughout the course of conformational changes or chemical reactions, regardless of the timescale involved. At present there is no single experimental technique that can yield this information.

1 A BRIEF HISTORY AND PERSPECTIVES

Molecular biology was born with the double-helix model for DNA, which provided a superbly elegant explanation for the storage and transmission mechanisms of genetic information (Fig. 1). The model by **J. D. Watson** and **F. H. C. Crick** and supporting fiber diffraction studies by **M. H. F. Wilkins, A. R. Stokes**, and **H. R Wilson**, and **R. Franklin** and **R. G. Gosling**, were published in a series of papers in the **April 25, 1953** issue of *Nature*, and marked a major triumph of the physical approach to biology.

The Watson and Crick model was based only in part on data from X-ray fiber diffraction diagrams. The patterns, which demonstrated the presence of a helical structure of constant pitch and diameter, could not provide unequivocal proof for a more precise structural model. One of the "genius" aspects of the discovery was the realization that A–T and G–C base pairs have identical dimensions; as the rungs of the double-helix ladder, they give rise to a constant diameter and pitch. From a purely "diffraction physics" point of view, a variety of helical models was compatible with the fiber diffraction diagram, and other authors proposed an alternative model for DNA, the so-called "side-by-side model," coupling two single DNA helices. This shows that if molecular biology were ever to be established, it was important to obtain the structure of biological molecules in more detail than was possible from fiber diffraction. Considering the dimensions involved, about 1 Å (0.1 nm) for the distance between atoms, X-ray crystallography appeared to be the only suitable method. Major obstacles remained to be overcome, such as obtaining suitable crystals, coping with the large quantity of data required to describe the positions of all the atoms in a macromolecule, and solving the phase problem.

Protein crystals had already been obtained in the 1930s. It was not until 1957, however, that **Max Perutz** and **John Kendrew** found a way to solve the crystallographic phase problem by isomorphous replacement using heavy-atom derivatives. This permitted the structure of myoglobin to be solved in sufficient detail to describe how the molecule was folded. The difficulties encountered with protein crystallization, and the labor-intensive nature of the crystallographic study itself (this was before powerful computers, and long calculations were essentially performed by "post-doctoral hands") appeared to doom protein crystallography to providing rare, unique information on the three-dimensional structure of a very few biological macromolecules. Structural molecular biologists, therefore, continued the development and improvement of methods that could not provide atomic resolution but have complementary advantages for the study of macromolecular structures. These methods, at the boundary between thermodynamics and structure, had already played crucial roles in the century before the discovery of the double helix. The discovery of biological macromolecules is itself tightly interwoven with the application of physical concepts and methods to biology (biophysics).

The application of physics to tackle problems in biology is certainly older than its definition as biophysics. The *Encyclopædia Britannica* suggests that the study of bioluminescence by **Athanasius Kircher** in the **seventeenth century** might be considered as one of the first biophysical investigations. Kircher showed that an extract made from fireflies could not be used to light houses. The relation between biology and what would become known as electricity has preoccupied physicists for centuries. **Isaac Newton**, in the concluding paragraph of his *Principia* **(1687)**, reflected that

> *all sensation is excited, and the members of animal bodies move at the command of the will, namely, by the vibrations of this Spirit, mutually propagated along the solid filaments of the nerves, from the outer organs of sense to the brain, and from the brain into the muscles. But these are things that cannot be explained in few words, nor are we furnished with that sufficiency of experiments which is required to an accurate determination and demonstration of the laws by which this electric and elastic Spirit operates.*

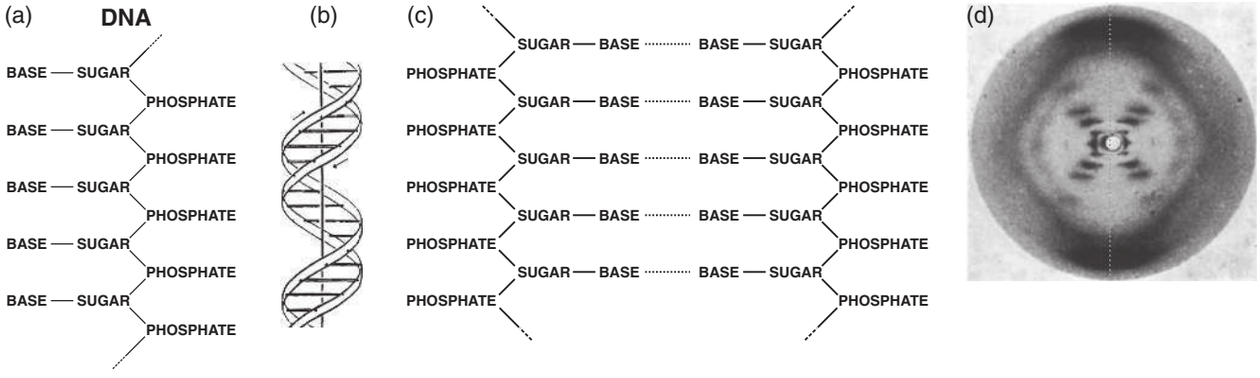

Fig. 1 (a) Chemical organization of a single chain of DNA. **(b)** This figure is purely diagrammatic. The two ribbons symbolize the two phosphate–sugar chains, and the horizontal rods the pairs of bases holding the chains together. The vertical line marks the helix axis. **(c)** Chemical organization of a pair of DNA chains. The hydrogen bonding is symbolized by dotted lines. **(d)** X-ray fiber diffraction of the B-form of DNA. The figures are facsimiles from the original papers of Watson and Crick (1953) and Franklin and Gosling (1953).

One hundred years later, **Luigi Galvani** and **Alessandro Volta** performed experiments on frogs' legs that would lead to the invention of the electric battery. They also laid the foundations of the science of electrophysiology, even though, because of the excitement caused by the electric battery, it was well into the **nineteenth century** before the study of animal electricity was developed further, notably by **Emil Heinrich Du Bois-Reymond**. Another nineteenth-century branch of biophysics, however, that dealing with diffusion and osmotic pressure in solutions, would later overlap with physical chemistry, and is more directly relevant to the discovery and study of biological macromolecules. The first papers published in *Zeitschrift für Physikalische Chemie* **(1887)** were concerned with reactions in solution, because biological processes essentially take place in the aqueous environment inside living cells.

The thermal motion of particles in solution ("Brownian" motion) was discovered by **Robert Brown (1827). Abbé Nollet**, a professor of experimental physics, first described osmotic pressure in the early **nineteenth century** from experiments using animal bladder membranes to separate alcohol and water. The further study and naming of the phenomenon is credited to the medical doctor and physiologist **René J. H. Dutrochet (1828)**, who recognized the important implication of osmotic phenomena in living systems and firmly believed that basic biological processes could be explained in terms of physics and chemistry. The theory of osmotic pressure was developed by **J. Van't Hoff (1880). George Gabriel Stokes** (middle of the **nineteenth century**) is best known for his fundamental contributions to the understanding of the laws governing particle motion in a viscous medium, but he also named and worked on the phenomenon of fluorescence. The laws of diffusion under concentration gradients were written down by **Adolf Fick (1856)**, by analogy with the laws governing heat flow.

Macromolecules, although large as molecules, are still much smaller than the wavelength of light. They could not be seen through direct observation by using microscopes, which had already shown the existence of cells in biological tissue and of structures within the cells such as the chromosomes (from the Greek, meaning "colored bodies"). From the knowledge gained from experiments on solutions it gradually became apparent that the biochemical activity of proteins, studied by **Emil Fischer (1882)**, is due to discrete macromolecules. In **1908, Jean Perrin** applied a theory proposed by **Albert Einstein (1905)** to determine Avogadro's number from Brownian motion. The theory of macromolecules is due to **Werner Kuhn (1930)**, after **Hermann Staudinger (1920)** proposed the concept of macromolecules as discrete entities, rather than colloidal structures made up of smaller molecules. The discovery of X-rays by **Wilhelm Conrad Röntgen (1895)**, and its application to atomic crystallography in the **1910s** through the work of **Peter Ewald, Max von Laue**, and **William H**. and **W. Laurence Bragg** laid the groundwork for the observation of atomic structural organization within macromolecules almost half a century later. **Theodor Svedberg (1925)** made the first direct "observation" of a protein as a macromolecule of well-defined molar mass by using the analytical centrifuge he had invented. In parallel, the atomic theory of matter became accepted as fact. There was rapid progress in X-ray diffraction and crystallography, electron microscopy and atomic spectroscopy. The novel experimental tools, provided by the new understanding of the interactions between radiation and matter, were carefully honed to meet the challenge of biological structure at the molecular and atomic levels. Physicists, encouraged by the example of **Max Delbrück**, who chose to study the genetics of a bacteriophage (a bacterial virus) as a tractable model in the **1940s**, and **Erwin Schrödinger's** influential book *What is Life?* **(1944)**, which discussed whether or not biological processes could be accounted for by the known laws of physics, turned to biological problems in a strongly active way.

At the beginning of the **twenty-first century**, biophysics is dominated by two methods, X-ray crystallography and nuclear magnetic resonance (NMR), which play the key role in determining three-dimensional structures of biological macromolecules to high resolution. But even if all the protein structures in different genomes were solved, crucial questions would still remain. What is the structure and dynamics of each macromolecule in the crowded environment of a living cell? How does macromolecular structure change during biological activity? How do macromolecules interact with each other in space and time? These questions can be addressed only by the combined and complementary use of practically all biophysical methods. Mass spectrometry can determine macromolecular masses with astonishing accuracy. Highly sensitive scanning and titration microcalorimetry are applied to determine the thermodynamics of macromolecular folding and stability, and are joined by biosensor techniques in the study of binding interactions. There has been a rebirth of analytical ultracentrifugation, with the advent of new, highly precise and automated instrumentation, and it has joined small-angle X-ray and neutron scattering in the study of macromolecular structure and interactions in solution and the role of hydration. A femtosecond time resolution has been achieved for the probing of fast kinetics by optical spectroscopy. Light microscopy combined with fluorescence probes can locate single molecules inside cells. Scanning force microscopy is determining the profile of macromolecular surfaces and their time-resolved changes. Electron microscopy is approaching atomic resolution and is most likely to bridge the gap between single-macromolecule and cellular studies. Neutron spectroscopy is providing information on functional dynamics of proteins within living cells. Synchrotron radiation circular dichroïsm can access a wider wavelength range in the vacuum ultraviolet for the study of electronic transitions in the polypeptide backbone.

Up to the **late 1970s**, biophysics and biochemistry had only dealt with large molecular ensembles for which the laws of thermodynamics are readily applicable. One hundred microliters of a 1 mg ml^{-1} solution of hemoglobin, for example, contains 10^{15} protein molecules; a typical protein crystal contains of the order of 10^{15} macromolecules. In their natural environment, however, far fewer molecules are involved in any interaction and exciting new methods have been developed that allow the study of single molecules. Single molecules can now be detected and manipulated with hypersensitive spectroscopic and even mechanical probes such as atomic force microscopy, with which a single macromolecule can even be stretched into novel conformations. Conformational dynamics can be measured by single-molecule fluorescence spectroscopy. Fluorescence resonance energy transfer can measure distances between donor and acceptor pairs in single molecules, in vitro or in living cells. Near-field scanning optical microscopy can identify and provide dynamics information on single molecules in the condensed phase.

The historical development of each of the biophysical methods outlined above is discussed in more detail in the corresponding section of this book.

2 LANGUAGES AND TOOLS

Physike in Greek is the feminine of *physikos* meaning *natural*. Physics is the science of observing and describing Nature. When one of the authors (J. Z.) was a student at Edinburgh University, physics was taught in the department of Natural Philosophy. The word *philosophy*, love of wisdom, conveyed quite accurately how the wisdom of the observer is brought to bear in science. The observer plays his role through the tools he uses in his experiments and the language he uses to describe his results. Modern science covers so many diverse areas that it is impossible to master an understanding of all the tools and languages involved. Biophysics students are familiar with the language difficulties of trying to communicate with "pure" physicists, on the one hand, and "pure" biologists, on the other, despite decades of interdisciplinary teaching and research in universities. Rather than bemoaning this fact, we should recognize that it reflects the richness and depth of each discipline, expressed in its own sophisticated language, and developed in its own set of observational tools. Clearly, physics and biology have different languages, but it is important to appreciate that within each discipline also there are different languages. Language influences tool development, which in turn contributes to refining the concepts described by language. Biophysicists have to be fluent in the various languages of physics and biology and be able to translate between them accurately. This is a very difficult and sometimes impossible task, as any good language interpreter can testify, each language having its own specificity and viewpoint.

Biophysics deals, to a large extent, with the structure, dynamics, and interactions of biological macromolecules. What are biological macromolecules? Their biological activity is described in the language of biochemistry and molecular spectroscopy; they were discovered through their hydrodynamic and thermodynamic properties; they are visualized by their radiation scattering properties, and their pictures are drawn in beautiful color as physical particles. To each language there corresponds a set of tools, the instruments and methods of experimental observation. Progress in probing and understanding biological macromolecules has undoubtedly been based on advances in the methods used. Physical tools capable of ever-increasing accuracy and precision require a parallel development in biochemical tools (often themselves of physical basis, like electrophoresis or chromatography, for example) to provide meaningful samples for study. The word *meaningful* is a key word in the previous sentence. It refers to the relevance of the study with respect to *biology* (from the Greek *bios*, life, and *logos*, word or reason), i.e., biophysics has

the goal of increasing our understanding of life processes. It should be distinguished from *biological physics*, which deals with the properties of biological matter, for example to design nanomachines based on DNA.

3 LENGTH AND TIMESCALES IN BIOLOGY

Biological events occur on a wide range of length and timescales – from the distance between atoms on the ångström scale to the size of the Earth as an ecosystem, from the femtosecond of electronic rearrangements when retinal absorbs a photon in the first step of vision to the 10^9 years of evolution. Observation tools have been developed that are adapted to the different parts of the length and timescales. The cell represents a central threshold for biological studies (Fig. 2). With a usual size of the order of 1–10 µm, cells can be seen under the light microscope. Also, the durations of cellular processes, which are of the order of seconds to minutes, can be observed and measured with relative ease. If we imagined diving into a eukaryotic cell through its plasma membrane, we would see other membrane structures that separate distinct compartments like the nucleus or mitochondria, large macromolecular assemblies such as chromatin, ribosomes, chaperone molecular machines or multienzyme complexes. Looking for progressively smaller structures we would find RNA and protein molecules, then peptides and other small molecules, water molecules and ions, and finally the atoms that make them up (Fig. 3).

The smaller the length, the shorter the time, the heavier is the implication of sophisticated physical instrumentation and methods for their experimental observation.

The femtosecond (10^{-15} s) is the shortest time of interest in molecular biology; it corresponds to the time taken by electronic reorganization in the light-sensitive molecule, retinal, upon absorption of a photon, in the first step in vision. Time intervals of this order can be measured by laser spectroscopy (the distance covered by light in 1 fs is 3×10^{-7} m, or 300 nm, about one half the wavelength of visible light). Thermal fluctuations are in the picosecond (10^{-12} s) range; DNA unfolds in microseconds; enzyme catalysis rates are of the order of 1000 reactions per second; protein synthesis takes place in seconds, etc. The longest time of interest in molecular biology is, in fact, geological time, corresponding to the more than 1000 million years of molecular evolution (Fig. 4).

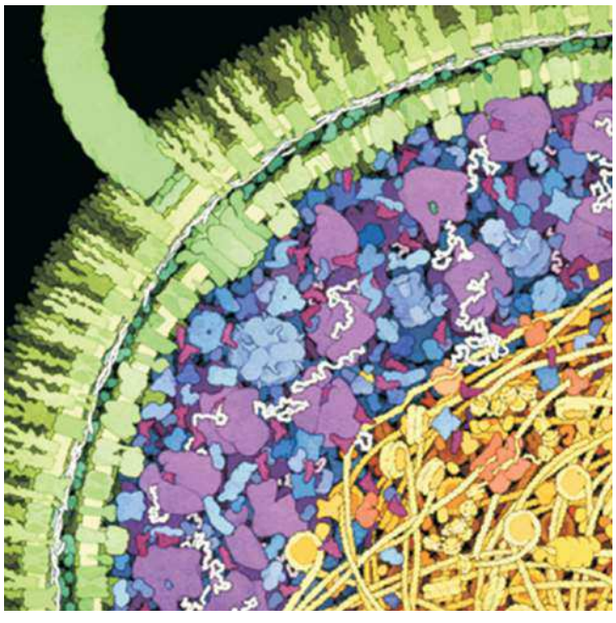

Fig. 2 A "realistic" drawing of the bacterium *Escherichia coli*, based on available experimental data. A flagellum, the double cell membrane and its associated proteins and glycoproteins are shown in hues of green; ribosomes and other protein and nucleic acid cytoplasmic components are in violet and blue; nascent polypeptide chains are in white; DNA and its associated proteins are in yellow and orange. The scale is given by the size of the bacterium of about 1 µm, or the double membrane thickness of about 10 nm. (www.scripps.edu/mb/goodsell)

4 THE STRUCTURE–FUNCTION HYPOTHESIS

This book describes the application of classical and advanced physical methods to observe biological structure, dynamics, and interactions at the molecular level. Intensive research since the 1950s has emphasized the fundamental importance of biological activity at this level. The

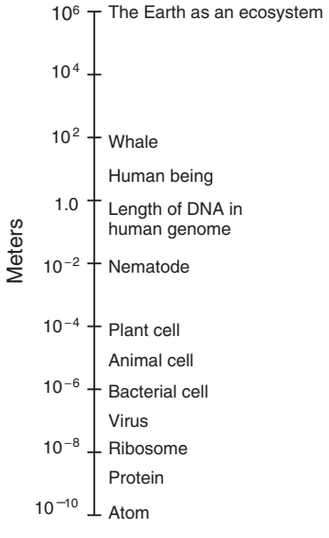

Fig. 3 Length scales in biology.

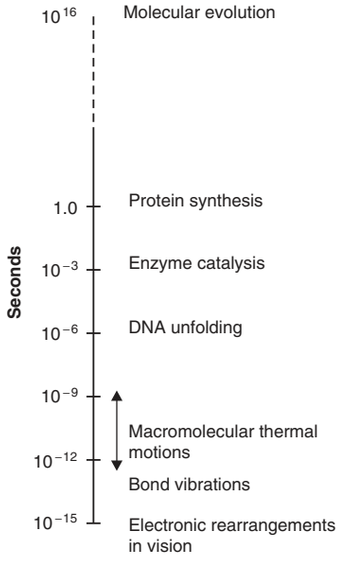

Fig. 4 Timescales in biology.

structure–function hypothesis is the foundation of molecular biology. One of its implications is that if a protein exists today in an organism, it is because it fulfills a certain biological function and its "structure" has been selected by evolution. The discovery and study of nucleic acids and proteins as macromolecules with well-defined structures has allowed an unprecedented understanding of processes such as the storage and transmission of genetic information, the regulation of gene expression, enzyme catalysis, immune response or signal transduction. In parallel, it became apparent that we could act on biological processes by acting on macromolecular structures, and powerful tools were developed not only to further fundamental scientific understanding but also to apply this knowledge in biotechnology or in drug design pharmacology.

The concept of "structure" should be understood in the broadest sense. The three-dimensional organization of a protein is not rigid but can adapt to its ligands according to the hypothesis of "configurational adaptivity" or "induced fit." Also, many proteins have been found that display a highly flexible random-coil conformation under physiological conditions. An intrinsically disordered protein could adopt a permanent structure through binding, but there are cases of proteins with intrinsic disorder that are biologically active while remaining disordered. A large proportion of gene sequences appear to code for long amino acid stretches that are likely either to be unfolded in solution or to adopt non-globular structures of unknown conformation.

Events taking place on the ångström and picosecond scales have profound consequences for life processes over the entire range of length and timescales – from the length and time associated with a cell, via those associated with an organism to those associated with the relation between an organism and its environment. The development of high-throughput techniques for whole-genome sequencing, for the analysis of genomic information (bioinformatics), for the identification of all the proteins present in a cell (functional proteomics), for determining how this population responds to external conditions (dynamic proteomics), and for protein structure determination (structural genomics) has opened up a new era in molecular biology whose revolutionary impact still remains to be assessed.

Biological macromolecule structures usually appear in pictures as static structures. A more precise definition would be "ensemble and time-averaged" structures. The atoms in a macromolecular structure are maintained at their average positions by a balance of forces. Under the influence of thermal energy, the atoms move about these positions. Dynamics, from the Greek *dynamis* meaning strength, pertains to forces. Structure *and* motions result from forces. It is common usage in biophysics, however, to separate *structure* from *dynamics*, considering the first as referring to the length scale (i.e., to the time-averaged configuration) and the second as referring to the timescale (i.e., to energy and fluctuations). The separation into two separate concepts is validated by the fact that the methods used to study structure and dynamics are usually quite separate and specialized. Modern experiments, however, often address both an average structure and how it changes with time.

5 COMPLEMENTARITY OF PHYSICAL METHODS

We know of the existence of macromolecules only through the methods with which they are observed. No single method, however, provides all the information required on a macromolecule and its interactions. Each method gives a different view of the system in space and time: the methods are *complementary*.

Biological macromolecules take up their active structures only in a suitable solvent environment. The forces that stabilize them are weak forces (of the order of kT, where k is Boltzmann's constant and T is absolute temperature), which arise in part from interactions with the solvent. The study of biological macromolecules, therefore, cannot be separated from the study of their aqueous solutions. The macromolecules are usually studied in dilute or concentrated solutions, in the lipid environment of membranes, or in crystals. Protein molecules or nucleic acid molecules in the unit cell of a crystal are themselves surrounded by an appreciable number of solvent molecules, and there are aqueous layers on either side of membranes. According to the experimental method used, we shall consider biological macromolecules in solution as "physical particles" (mass spectrometry, single-molecule detection …), "thermodynamics particles" (osmotic pressure measurements,

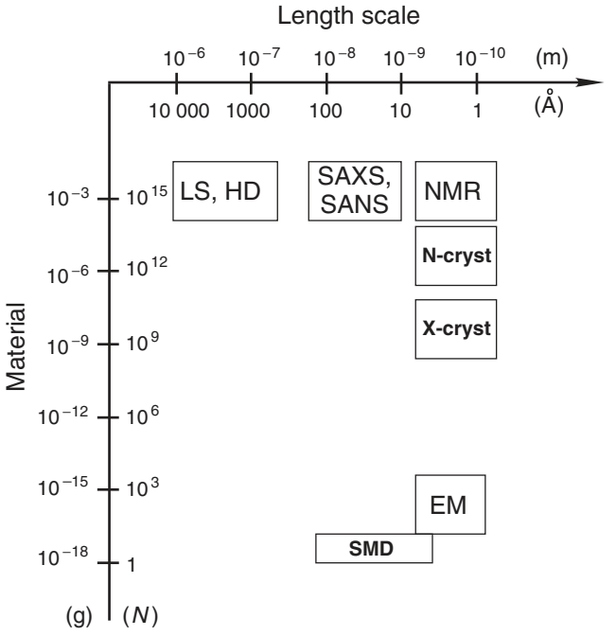

Fig. 5 Length resolution achieved and amount of material required for the sample for experiments using different physical methods to determine structure. Abbreviations: g, grams; *N*, number of molecules (assuming a molecular weight of the order of 100 000); LS, light scattering; HD, hydrodynamics; SAXS, SANS, X-ray and neutron small-angle scattering, respectively; NMR, nuclear magnetic resonance in solution; N-cryst, neutron crystallography; X-cryst, X-ray crystallography; EM, electron microscopy; SMD, single-molecule detection methods.

calorimetry . . .), "hydrodynamics particles" (viscosity, diffusion, sedimentation . . .) or "radiation interaction particles" (spectroscopy, diffraction and microscopy).

The length resolution scale achieved, the techniques involved, and the sample mass required for some biophysical methods are illustrated in Fig. 5.

6 THERMODYNAMICS

It is a result of classical thermodynamics that many properties of solutions, such as an increase in boiling point, freezing point depression, and osmotic pressure, depend on the number concentration of the solute. At constant mass concentration, therefore, these thermodynamics parameters vary sensitively with the molecular mass of the solute. Thus, for example, macromolecular masses and interactions have been determined from osmotic pressure measurements.

Macromolecular folding itself and the stabilization of active biological structures follow strict thermodynamics rules in which interactions with solvent play a determinant role. Sensitive calorimetric measurements of heat capacity as a function of temperature showed very clearly that stabilization free energy presents a maximum at a temperature close to the physiological temperature, the stability of the folded particle decreasing at lower as well as higher temperatures. The interpretation is the following. The behavior of the chain surrounded by solvent is much more complex than if it were in a vacuum. Enthalpy may rise, decrease, or even not change upon folding, because bonds can be made equally well within the macromolecule and between the chain and solvent components. Similarly for entropy, the loss of chain configuration freedom upon folding may be more than compensated for by a loss of degrees of freedom for the solvent molecules around the unfolded chain, for example through the exposure of apolar groups to water molecules. A water molecule in bulk has the freedom to form hydrogen bonds with partners in all directions. Apolar groups cannot form hydrogen bonds, so water molecules in their vicinity lose some of their bonding possibilities; their entropy is decreased.

In a protein solution, the heat capacity is strongly dominated by the water, and that of the macromolecules represents a very small part of the measured total. High-precision microcalorimeters were built to allow experiments on protein solutions to be performed. Nevertheless, early calorimetric studies on biological macromolecules concentrated on relatively large effects such as sharp transitions as a function of temperature. They led to a fundamental understanding of the energetics of protein folding. There are now important modern developments in the field. Very sensitive nanocalorimeters have been developed as well as analysis programs to treat the thermodynamics information and relate it to structural data. The energetics of intramolecular conformational changes, of complex formation and of interactions between partner molecules can now be explored in detail for proteins and nucleic acids. We should recall, however, that calorimetry (like all thermodynamics-based methods) provides measurements of an ensemble average over a very large number of particles (typically of the order of 10^{15}), even if results are usually illustrated in a simple way in terms of changes occurring in one particle.

7 HYDRODYNAMICS

The first hints of the existence of biological macromolecules as discrete particles came from observations of their hydrodynamic behavior. The language of macromolecular hydrodynamics is the language of fluid dynamics in the special regime of low Reynolds numbers. The Reynolds number in hydrodynamics is a dimensionless parameter that expresses the relative magnitudes of inertial and viscous forces on a body moving through a fluid. Bodies with the same Reynolds number display the same hydrodynamics behavior. Because of this, it is possible, for example, to determine the behavior of an airplane wing from wind-tunnel studies on a small-scale

model. The Reynolds numbers of a small fish and a whale are 10^5 and 10^9, respectively.

Reynolds numbers in aqueous solutions for biological macromolecules and their complexes, from small proteins to large virus particles and even bacteria, are very small. For example, it is 10^{-5} for a bacterium swimming with a velocity of about 10^{-3} cm s^{-1}. Inertial forces are negligible under such conditions, so that the motion of a particle through the fluid depends only on the forces acting upon it at the given instant; it has no inertial memory. Particle diffusion through a fluid under the effects of thermal or electrical energy, and sedimentation behavior in a centrifugal field can be predicted by relatively simple equations in terms of macromolecular mass and frictional coefficients that depend on shape. The *resolution* defines the detail with which a particle structure is described. Hydrodynamics provides a *low-resolution* view of a biological macromolecule, for example as a two- or three-axis ellipsoid, but it is also very sensitive to particle flexibility and particle–particle interactions. Modern hydrodynamics includes a number of novel experimental methods. In addition to the classical approaches of analytical ultracentrifugation to measure sedimentation coefficients and dynamic light scattering to measure diffusion coefficients, we now have free electrophoresis to measure transport properties in solution, fluorescence photobleaching recovery to monitor the mobility of individual molecules within living cells, time-dependent fluorescence polarization anisotropy and electric birefringence to calculate rotational diffusion coefficients, fluorescent correlation spectroscopy and localized dynamic light scattering to measure macromolecular dynamics.

8 RADIATION SCATTERING

We see the world around us because it scatters light, which is detected by our eyes and analyzed by our brains. In a diffraction experiment, waves of radiation scattered by different objects interfere to give rise to an observable pattern, from which the relative arrangement (or structure) of the objects can be deduced. The interference pattern arises when the wavelength of the radiation is similar to or smaller than the distances separating the objects. In some cases, the waves forming the pattern can be recombined by a lens to provide a direct image of the object. Atomic bond lengths are close to one ångström unit (10^{-10} m or 0.1 nm), and three types of radiation are used, in practice, to probe the atomic structure of macromolecules by diffraction experiments: X-rays of wavelength about 1 Å, electrons of wavelength about 0.01 Å, and neutrons of wavelength about 0.5–10 Å. Visible light scattering, with wavelengths in the 400–800 nm range, provides information on large macromolecular assemblies and their dynamics. X-rays, however, because they permit studies to atomic detail, provided the foundation on which structural biology has been built and is developing.

Neutron diffraction studies of biological membranes, fibers, and macromolecules and their complexes in crystals and in solution became possible in the 1970s with the development of methods that make full use of the special properties of the neutron.

Following the limitations of staining techniques, cryo-electron microscopy was developed to visualize subcellular and macromolecular structures to increasing resolution.

In the last decade of the twentieth century, the availability of intense synchrotron sources caused a revolution in macromolecular crystallography by greatly increasing the rate at which structures could be solved. Efficient protein modification, crystallization, data collection, and analysis approaches were developed for macromolecular crystallography. Extremely fast data-collection times made it possible to use time-resolved crystallography to study kinetic intermediates in enzymes. In parallel, field emission gun electron microscopes were applied and new methods developed to solve single-particle structures. Spallation sources for neutron scattering promise highly improved data-collection rates.

Light, X-rays, and neutrons are scattered weakly by matter and require samples containing very large numbers of particles in order to obtain good signal-to-noise ratios. Experiments provide ensemble-averaged structures. Modern electron microscopy methods, on the other hand, allow single macromolecular particles to be visualized.

9 SPECTROSCOPY

In spectroscopy, the radiation has exchanged part of its energy with the sample, through absorption effects or excitations due to particle internal or global dynamics, resulting in a change in the wavelength (frequency or color) of the outgoing beam with respect to the incident beam. Since absorption depends on the location of an atom in a structure, certain types of spectroscopic experiment may also be used to study structure. Nuclear magnetic resonance spectroscopy is sensitive to close to atomic resolution. The frequency of absorbed radiation can be measured as a function of time with an accuracy better than one part in a million. The precise nature of the signal depends on the chemical environment of the nucleus; hence structural information is obtained. In magnetic resonance imaging (MRI), millimeter resolution is obtained with meter wavelength probes by placing the body to be observed in magnetic field gradients and by focusing on nuclei in a given chemical environment; an absorption resonance then corresponds to a given field value and therefore to a precise location. As with diffraction, for which the wavelength matches the structural resolution required, the beam energy in spectroscopy is chosen so that differences due to sample excitations or absorption can be measured readily. In general, therefore, radiation of different wavelengths is used for diffraction and for spectroscopic experiments.

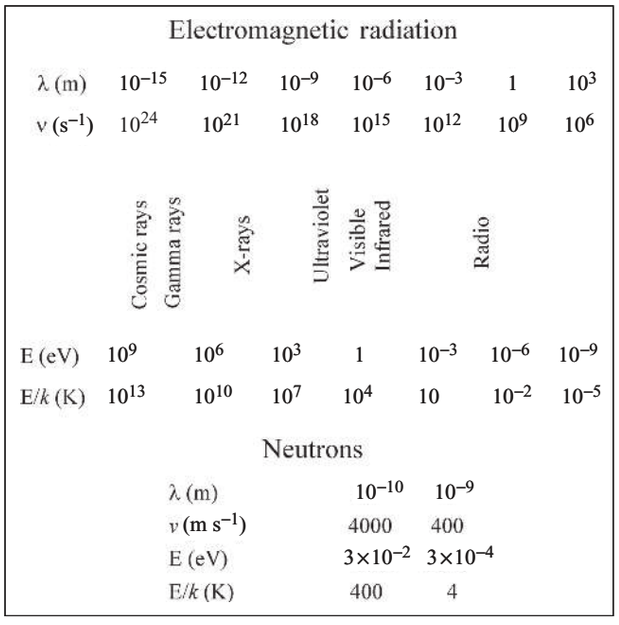

Fig. 6 Wavelength, energy, and frequency for electromagnetic and neutron radiation. The scales in the figure give approximate orders of magnitude. The precise values for the constants are obtained from: $\nu\lambda = c$ where $\nu\lambda$ are the frequency and wavelength, respectively, of electromagnetic radiation and c is the speed of light (3×10^8 m s^{-1}); $E = h\nu$ (where E is energy and h is Planck's constant ($6.626 = 10^{-34}$ J s $= 4.136 \times 10^{-15}$ eV s); the temperature equivalent of energy, 1 eV/k = 11604.5 K, where k is Boltzmann's constant. In the neutron case, $\lambda = h/mv$ (where v m s^{-1} is neutron speed), and $E = \frac{1}{2}mv^2$, where m is neutron mass (1.6726×10^{-27} kg).

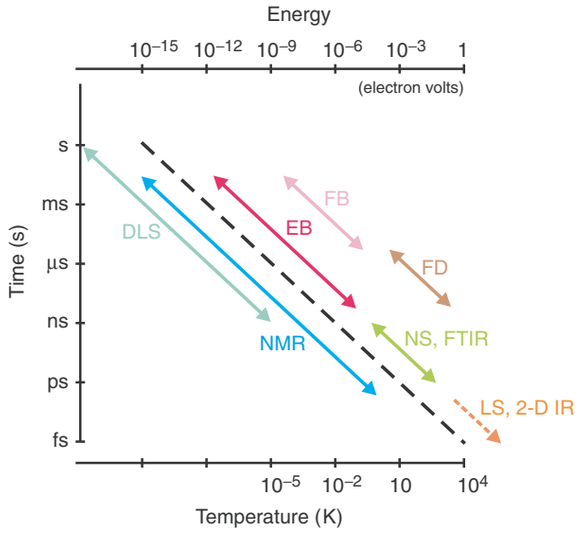

Fig. 7 Molecular timescales, associated energies and temperatures of various biophysical methods. The range follows the dashed black diagonal but the arrows have been displaced horizontally for clarity. Abbreviations: DLS, dynamic light scattering; NMR, nuclear magnetic resonance; EB, electric birefringence; NS, neutron spectroscopy; FTIR, Fourier transform infrared spectroscopy; LS, laser spectroscopy; 2-D IR, two-dimensional infrared spectroscopy; FD, fluorescence depolarization.

Coherent spectroscopy, in which radiation fields of well-defined phase are used, created unprecedented opportunities to study dynamics and time-evolving structures. The "spin echo" method, applied to NMR and neutron spectroscopy, was extended by the "photon echo" method when coherent lasers became available. Two-dimensional spectroscopy, first developed for NMR, measures the coupling within networks of vibrational modes. It has been applied to the infrared region to determine the structure of small molecules. The most exciting aspect of two-dimensional infrared spectroscopy is the combination of its sensitivity to structure and time resolution down to the femtosecond.

Taking electromagnetic radiation as an example, atomic diffraction requires X-ray wavelengths, while intramolecular vibrations correspond to infrared energies (Fig. 6). In NMR spectroscopy, the probing electromagnetic radiation is in the radio-frequency range, corresponding to meter wavelengths. Note that with neutron radiation, wavelengths of about 1 Å (corresponding to interatomic distances and fluctuation amplitudes) have associated energies of about 1 meV (corresponding to the energies of atomic fluctuations), so that diffraction and spectroscopy experiments can be performed simultaneously to measure atomic amplitudes and frequencies of motion in macromolecules. Molecular timescales and corresponding energies and temperatures are shown in Fig. 7 for different biophysical methods.

10 SINGLE-MOLECULE DETECTION

Until the 1980s, biochemical and biophysical studies of biological macromolecules suffered the fundamental disadvantage of always having to deal with very large numbers of particles, whereas under in-vivo conditions they function as single particles in a dynamic heterogeneous environment. Structures, dynamics, and interactions were (and predominantly still are) observed and measured as ensemble averages. Furthermore, enzymatic, binding, or signaling reactions are in general stochastic, so that the kinetics of a protein activity measurement, for example, is also hidden in an ensemble average when measured in a large molecular population, even if the reaction is triggered contemporaneously for the entire sample.

Single macromolecules had been visualized by electron microscopy, but only in the last decade have methods become available to observe them while they were active. The development of single-molecule detection (SMD)

techniques now allows the observation as well as the manipulation of single macromolecules in action. SMD is based on the two key technologies of *single-molecule imaging under active conditions* and *nanomanipulation*. Single-molecule signals that are detectable with good signal-to-noise ratios are given by fluorescent labels, which are observed using fluorescent optical microscopy. Applying total reflection and evanescent field techniques, the resolution of the method is several-fold better than the diffraction limit given by the wavelength of light. Single-molecule nanomanipulation techniques include capturing biomolecules using a glass needle or beads trapped by the force exerted by a focused laser beam (optical tweezers), and probing molecular forces with atomic force or scanning probe near-field microscopy. The forces involved are in the piconewton range, comparable to the thermal forces stabilizing the active macromolecular structures.

Erwin Schrödinger wrote in 1952 that we would never be able to perform experiments on just one electron, one atom, or one molecule. In the early 1980s, however, scanning tunneling microscopy was invented by G. Binning and H. Rohrer and radically changed the ways scientists view matter. Mechanical experiments to measure the piconewton forces that structure a single macromolecule became possible (Comment 1).

In optical tweezers instruments (Fig. 8a) one or two laser beams are focused on a small spot, creating an optical trap for polystyrene beads. One end of a single molecule (DNA, for example) is attached to a bead, while the other end is attached to a moveable surface, which, in this example, is another bead on a glass micropipette. The opposing force is measured as the molecule is stretched by moving the micropipette.

In magnetic tweezers instruments (Fig. 8b), one end of the single molecule is attached to a glass fiber, while the other end is attached to a magnetic bead. A magnetic field exerts a constant force on the bead. The extension and rotation of the molecule as a function of the applied force is then measured.

In an atomic force microscopy experiment (Fig. 8c), one end of the molecule is attached to a surface and the other to a cantilever. As the surface is pulled away, the deflection of the cantilever is monitored from the position of a reflected laser beam.

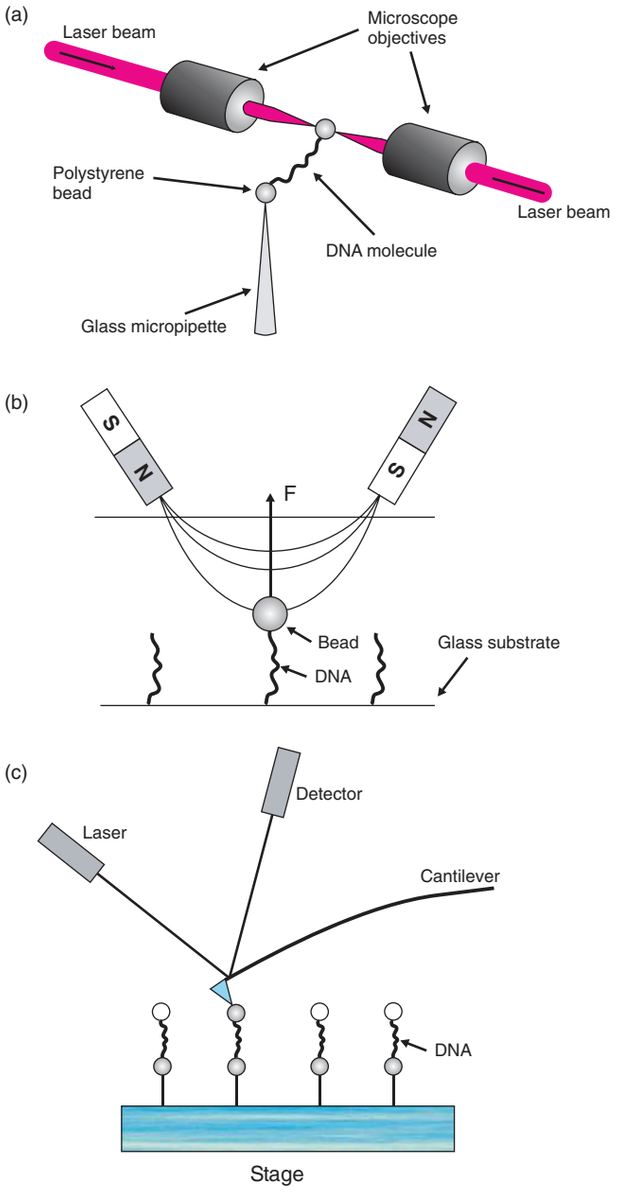

Fig. 8 A schematic view of three main techniques used in single-molecule force studies: **(a)** optical tweezers, **(b)** magnetic tweezers, and **(c)** atomic force microscopy (Carrion-Vazquez *et al.*, 2000).

COMMENT 1 ENTROPIC FORCE

The typical energy scale for a macromolecule is thermal energy: $k_BT = 4 \times 10^{-21}$ J. Since the length scale of biomacromolecules is of order of 1 nm, the force scale is on the order of the piconewton (10^{-12} N). Therefore an entropic force can be calculated as $k_BT/(1\,\text{nm})$, which is equal to 4 pN at 300 K.

The experiments allow a new structural parameter to be accessed within a single molecule: *force* (Table 1). The upper boundary for force measurements in micromanipulation experiments is the tensile strength of a covalent bond (in the eV/Å range or about 1000–2000 pN). The smallest measurable force limit is set by the Langevin force (about 1 fN), which is responsible for the Brownian motion of the sensor (size of the order of 1 μm).

Note that the total range of forces in Table 1 covers only three orders of magnitude. Until single-molecule

TABLE 1 THE RANGE OF FORCES IN MACROMOLECULES	
Tensile strength of a covalent bond	1000–2000 pN
Deformation of a sugar ring	700 pN
Breaking of double-stranded DNA	400–580 pN
Unfolding the β-fold immunoglobulin domain of the muscle protein titin	180–320 pN
Adhesive force between avidin and biotin	140–180 pN
Structural transition of uncoiling double-stranded DNA upon stretching	60–80 pN
Structural transition of double-stranded DNA upon torsional stress	~20 pN
Individual nucleosome disruption	20–40 pN
Unfolding triple helical coiled-coil repeating units in spectrin	25–35 pN
RNA–polymerase motor	14–27 pN
Structural transition of RNA hairpin in ribozyme under stretching (folding–unfolding)	~14 pN
Separation of complementary DNA strands (room temperature, 150 mM NaCl, sequence-specific)	10–15 pN
Stall force of the myosin motor	3–6 pN
Force generated by protein polymerization in growing microtubules	3–4 pN

techniques became available, information on protein stability could only be obtained by measuring the loss of structure under denaturing conditions (by using temperature, chemical agents, or pH) from which folding free energy could be calculated for an ensemble average of molecules. Free energy, however, does not provide direct information on mechanical stability. For mechanical stability, it is important to know how the total energy varies as a function of spatial coordinates. Several proteins were studied to measure the force required to unfold a single molecule. These studies revealed very large differences in magnitude (which can reach the order of a factor of 10) between the unfolding forces for different protein domains whose melting temperatures are very similar. These results demonstrated that the mechanical stability of a protein fold is not directly correlated with its thermodynamic stability. We expect the analysis of the mechanical properties of macromolecules to set the foundation of a new field of study, *mechanochemical biochemistry*.

11 BIOPHYSICS AND MEDICINE

The unprecedented insights into biological function gained from biophysics have created opportunities to rationally manipulate biological responses. In the most optimistic and perhaps simplistic case, once characterized, the molecular basis of a disease can be addressed. For example, evolving mutations on viral replication have led to the continuous structure-guided development of anti-HIV drugs.

This aspect of biophysics is complemented by the direct application of biophysical methods to the diagnosis and even treatment of disease. The same physical principles that underlie methods to observe molecules in vitro can also be applied in vivo, in the context of an organism. The practice of medicine has consequently been transformed by the advent of sonograms, MRI, CAT, and PET scans, which provide astonishing information about the workings of the human body, as well as the progression of disease. Fetal health can be monitored; cardiovascular disease and the spread of cancerous cells, for example, can be detected and measured prior to the appearance of symptoms.