THE VERSATILITY OF PROTEINS: FROM BIOMOLECULES TO BIOPHARMACEUTICALS

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ABSTRACT

The vast majority of genes in any life form encode information for the production of proteins through the biosynthesis of polypeptide chains from amino acids having different side groups. Interactions among these side groups and the environmental conditions cause the proteins to adopt distinct three-dimensional structures or conformations that are critical to their functions. Proteins are essential biomolecules that are involved in almost all biological functions with unparallel versatility in performance: Numerous different protein molecules take part in a wide variety of task. Proteins are the main catalysts, structural elements, antibodies, signaling messengers and molecular machines of cells and biological tissues; moreover, proteins regulate gene expression for the differential production of all gene products. In addition, these are often mediated through various protein-protein interactions. In cancer, which is a disease of genes, certain key proteins get altered to affect the cellular growth-control machinery, causing uncontrolled proliferation of the cancer cells. Since biological and environmental factors influence the gene products, a proper understanding and use of the 'genome' information necessitate a comprehensive analysis of the gene products, the 'proteome'. Manipulation and improvement of proteins and enzymes for their use in chemical, pharmaceutical, food and other industries are a major application of biotechnology. Protein biopharmaceuticals which include engineered proteins and antibodies with improved properties developed and designed for human therapeutic use, have been synthesized by cloned genes in bacterial or other suitable host cell systems using modern biotechnology.

Keywords: Polypeptide; Conformation; Proteomics, Gene expression; Recombinant DNA technology; Biopharmaceuticals.

PERSPECTIVE

The vast complexity of biological cells, with their varied structures and ability to perform different tasks are linked to self replication, growth and development. These are vital to all living systems and are governed by the same laws of physics and chemistry that determine the behavior of nonliving systems. While the genetic information is carried by nucleic acids, the execution of tasks directed by that information is the responsibility of proteins, which are the most diverse of all macromolecules. A wide range of protein molecules take part in a variety of functions. At the chemical level, proteins are polymers of twenty different amino acids, linked through peptide bonds between the α-amino group of one amino acid and the α-carboxyl group of the adjacent one; therefore, each polypeptide chain has two ends, namely the amino terminus and the carboxy terminus and the length

ranging from 50 to more than 1,000 amino acid residues. The side chains of different L-α-amino acids determine the role of each amino acid residue in protein structure and function. Thus, depending on the interactions among these side chains along with the environmental conditions, proteins adopt distinct three-dimensional (3-D) structures or conformations that are critical to their functions.

The versatility of proteins is manifested by their remarkable ability to perform a wide variety of tasks namely, to act as enzymes, transporters, receptors, binding proteins, scaffolds, signaling molecules and many more, linking practically all aspects of the life process. Molecular biological techniques and evolution of modern biotechnology helped to realize the structure, function, synthesis and regulation of proteins, as well as to make proteins with desired properties for their use in the medicine, as biopharmaceuticals.

With an aim to emphasize on the multiple virtues of proteins, this article will review certain selected areas of cell functions that represent some major activities of living systems, to depict different roles proteins play and major attributes of these biomolecules

PROTEIN AS A CATALYST

A fundamental task of proteins is to act as biocatalysts, enzymes, which catalyze nearly all chemical reactions within the cells. Cells contain thousands of different enzymes and their activities determine which of the many possible chemical reactions actually occur within the cell; even so, the same basic principles apply to their action: Once a substrate molecule is bound to the active site of its specific enzyme, multiple mechanisms can accelerate the conversion of the substrate to the product of the reaction. The enzyme provides a template upon which the reactants are brought together and properly oriented to favor the formation of the transition state in which they interact. Further, this transition state is stabilized by its tight binding to the enzyme, thereby lowering the required energy of activation. The catalytic property of a protein resides in the 3-D structure or conformation resulting from the amino acid sequence encoded by the gene. Although some reactions can be catalyzed by certain RNAs, most biological reactions are catalyzed by proteins. [1]

GENE EXPRESSION AND PROTEIN SYNTHESIS

Genomic DNA can be viewed as the set of genetic instructions governing all cellular activities. From a molecular perspective, the genetic information residing in the sequence of the four types of nucleotides in the DNA molecule is expressed via synthesis of RNA by 'transcription' process, to the synthesis of protein by 'translation' process. Transcription is catalyzed by RNA polymerase enzymes and controlled by a number of protein molecules, called 'transcription factors', which ensure faithful copying of the genetic message from DNA to RNA. Different types of RNA are created by transcription: these are messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). Transcription constitutes the primary level of regulation of gene expression. In eukaryotic organisms, these three types of RNA are each transcribed by a different RNA polymerase. The process of translation involves decoding of the codes in mRNA for the synthesis of polypeptide chains in three distinct phases namely, initiation, elongation and termination. Translation of mRNA takes place on ribosomes consisting of specific ribosomal protein (rProtein) and rRNA species and needs amino acids, tRNA and many specific protein factors for achieving various tasks in protein synthesis, which is the final step in gene expression. Regulation of gene expression at protein synthesis level occurs mainly in the initiation phase and involves interplay of multiple protein factors.[1, 2]

Exact replication of genomic DNA is crucial for all cells and organisms. When a cell divides, its entire genome must be duplicated by copying the large DNA molecule. This requires DNA polymerase enzymes in

complex enzymatic machinery involving many specific protein factors; these are essential for maintaining the fidelity of replication and integrity of the newly formed DNA molecules, which must be identical to the parent DNA molecule. The machinery can also rectify any mistakes during DNA replication. Additional proteins are needed to repair DNA damage caused by environmental agents. A failure to maintain the high fidelity of DNA replication can result in genetic problems including cancer. In all these processes of replication, transcription and translation, proteins play major roles through the action of specific enzymes and regulatory factors.

PROTEIN FOLDING AND QUALITY CONTROL

The flow of genetic information within the cell results in the synthesis of polypeptide chains with amino acid sequence as directed by the nucleotide sequence of DNA. However, for the formation of active enzymes or other functional proteins, the polypeptide chains fold into precise 3-D conformations. A protein folds into a particular shape depending on the location of its specific amino acid residues and the overall amino acid composition. Many functional proteins consist of two or more polypeptide chains as subunits and are termed as homomeric (with same subunits) or heteromeric (with different subunits) protein molecules. Often large protein complexes are made up of many different protein components to perform important cellular functions.

Cells have mechanisms to detect whether the newly synthesized polypeptide chains are properly folded to their native structure for their necessary function; those polypeptides which fail are either refolded or degraded. The central players in this post-translational quality control process for proteins are chaperons, a class of proteins and proteases. Chaperons facilitate the initial folding of other proteins by interacting at nascent polypeptide stage, prevent protein aggregation due to misfolding and stabilize the released completed protein molecule for folding into its proper 3-D shape. If a protein does not fold properly, it is recognized as misfolded; in eukaryotic cells it is degraded by the ubiquitin-proteasome system. Ubiquitin is a polypeptide involved in the major pathway of selective protein degradation. Proteins destined for degradation are marked by ubiquitination (attachment of ubiquitin); the polyubiquitinated proteins are recognized and degraded by the proteasome, which is a large multisubunit protease complex; ubiquitin is released and reused. [3, 4]

Disorders in protein folding are the basis of Prion diseases such as scrapie in sheep, Creutzfeld Jacob disease (CJD) in humans and bovine spongiform encephalopathy (BSE). The agents that cause these transmissible diseases are called prions, which are neither cells e.g., bacteria, nor viruses. Prions are a malfolded form of a cellular protein (PrPc), which has been remodeled into a protease-resistant, aggregation-prone and infectivity-associated protein (Prpsc). Prion diseases arise by errors in PrPc folding due to a templated conformational remodeling initiated by exposure to PrPsc. [5]

Programmed cell death plays a key role in the maintenance of adult tissues and embryonic development. It involves a process, known as 'apoptosis', featuring distinct morphological change due to shrinkage and cleavage of the cell and its chromosomes into fragments, called 'apoptotic bodies', which are subsequently removed by the surrounding cells. The orderly execution of apoptosis is orchestrated by a family of conserved cysteine proteases, called caspases, which undergo a cascade of catalytic activation at the onset of apoptosis and are subject to regulation by yet other proteins. [6]

POST-TRANSLATIONAL MODIFICATION FOR PROTEIN FUNCTION

After the synthesis of a polypeptide chain in eukaryotic system, it may reach the site of its intended job or may

have to undergo some covalent modification(s) for its specific job. Such modifications include phosphorylation, glycosylation, attachment of lipids, disulfide (-S-S-) bond formation between cysteine residues and partial cleavage of the peptide chain to smaller chains with distinct functions. The activities of many cellular proteins are regulated by reversible phosphorylation at specific sites namely, serine/ threonine residues or tyrosine residues by the action of specific protein kinases and protein phosphatases. Protein kinases often function in signal transduction pathways by cascading action such that one kinase activates another, which in turn may activate or inactivate a third one or a cellular protein. Such sequential action can transmit a signal received at the cell surface to target proteins within the cell, causing alterations in cell behavior in response to external stimuli. The dynamic nature of such phosphorylation steps involving multiple sites contributes to precise tuning of the protein factor(s) as key to the signal incorporation and intricate cellular control. [1, 7]

MEMBRANE PROTEINS

Membrane proteins reside and function at the interface to the adjacent medium. They play vital roles as cell receptors, transporters, channels and as essential components of respiratory complexes. The cell membrane is heterogeneous and is composed mainly of phospholipid bilayer; while the polar heads define the lipid-water interphases, its hydrophobic part forms the core of the membrane. Because of constraints in this complex hydrophobic environment, the transmembrane domains of the membrane proteins fold and the α-helical type proteins are most abundant. Different classes of membrane receptor proteins take part in the transmission of extracellular signals inside the cell for regulating the cell activities. Many such receptors belong to the super family of G-protein coupled receptors (GPCRs) that mediate taste, smell, vision, and the effects of most hormones and neurotransmitters. They are important for biomedical and pharmaceutical research as potential targets for therapeutic intervention; they are the primary site of action of many life-saving drugs and are target for future development. [8-10]

ANTIBODIES

Antibodies are immunoglobulin proteins produced in animals as a defence mechanism in response to foreign materials, called antigens. Immunoglobulins consist of mainly two types of polypeptides, called heavy (H) chains and light (L) chains, which form Y-shaped molecules. The amino terminal regions of both H and L chains of an antibody molecule bear highly variable sequence, evolved to bind an antigen molecule very specifically and tightly. Injection of an antigen in experimental animals can produce antibodies with the capacity to bind the antigen molecules with exquisite specificity and high affinity. Owing to their specificity, affinity and ease of production, antibodies are critical reagents in many experiments as well as for analytical, preparative and diagnostic procedures. Monoclonal antibodies, which can recognize and bind specific single minute chemical parts of an antigen, have been developed for application in research and medicine. [10]

CYTOSKELETAL PROTEINS

The cytoskeleton consists of a network of protein filaments extending throughout the cytoplasm of all eukaryotic cells and is composed of microfilaments, intermediate filaments and microtubules with diameters 7-9.5 nm, 10-12 nm and 25 nm respectively; these are held together and linked to subcellular organelles and plasma membrane with a variety of accessory proteins. It provides a structural frame work as a scaffold for the cell.

The major cytoskeletal protein of most cells is actin, which polymerizes to form microfilaments. Actin bundles arise when small rigid proteins, called actin-bundling proteins force the filaments to align closely

with one another. Conversely, the proteins that organize actin filaments into network appear to be large flexible modular proteins that can crosslink perpendicular filaments. Many types of cell movements are due to actin filaments, usually in association with myosin, as the prototype of a molecular motor and play a central role in cell biology. Intermediate filaments are composed of a variety of proteins expressed in different types of cells. They appear to play a structural role by providing mechanical strength to cells and tissues.

Microtubules are hollow cylindrical and dynamic structures, assembled from their building block protein tubulin, and undergo assembly and disassembly depending on necessity within the cell. Microtubules constitute the mitotic spindle and therefore, play a central role in mitosis. They also execute in cell shape, cell movements, intracellular transport of organelles, separation of chromosomes during mitosis, and the beating of cilia and flagella. The dynamic behavior of microtubules within the cell is influenced by interactions with several proteins. Some cellular proteins (stathmin in certain cancers) can disassemble microtubules, while some other proteins, called microtubule-associated proteins (MAPs) bind to microtubules and increase their stability. Certain antimitotic drugs for cancer therapy act by interfering with the microtubule system. [1, 10, 11]

PROTEIN FUNCTION AND PROTEIN-PROTEIN INTERACTION

Protein function typically depends on a subset of its amino acid residues and is commonly mediated by regions on the surface that interact with external factors such as substrates, ligands, or specific nucleic acid sequences. The concept of protein function and its understanding can vary largely depending on the functional level under consideration (molecular, cellular, physiological etc.). The classic view of protein function focuses on the action of a single protein molecule, either in the catalysis of a given reaction, or in the binding of a small or large molecule. This is sometimes termed as 'molecular' function of the protein, in an attempt to distinguish it from an expanded view of function, termed as 'contextual' or 'cellular' function, where a protein is an element in the network of its interactions. Each protein in living matter functions as part of an extended web of interacting molecules. Since most cellular processes are regulated by multiprotein complexes, the interactions among proteins play a vital role in determining all the biological events in organisms. Several approaches have so far been made to understand these protein-protein interactions. Genomic studies and high throughput methods of the postgenomic era may shed a new light on the protein function. The analysis of large protein-protein networks also may permit the emergence of a more integrated view of protein functions. [12, 13]

The complete genomic sequencing of a number of species ranging from bacteria and viruses to plants and human has yielded vast data on genetic information, genetic maps, nucleotide sequence and markers. Yet, such genetic information does not necessarily match by quantity or quality at the protein levels, because biological and environmental factors influence the gene products. So, a comprehensive analysis of the gene products, meaning a study of the 'proteome', is essential for a proper understanding and application of the genome information. The proteome may be defined as the time- and cell-specific protein complement of the genome and it covers all proteins that are expressed in a cell at one time, including isoforms and protein modifications. Thus, organism complexity is generated more by a complex proteome than by a complex genome. While genome is constant and largely identical for all cells of an organism, the proteome is more variable with time and significantly differs between cell types, in response to external

stimuli. Proteomics or proteome analysis in general involves methods of protein analysis viz., 2-D gel electrophoresis, mass spectrometry; protein identification using databases; biochemical characterization of proteins of unknown function by amount, localization, structure, post-translational modification, antibody binding, etc. [10, 13, 14]

CANCER AND CELL TRANSFORMATION

The fundamental abnormality resulting in the development of cancer is the continual uncontrolled proliferation of cancer cells with lack of inhibition by cell-cell contact, defective differentiation and reduced requirements for extracellular growth factors. Cancer is a disease of genes, which is caused by diverse factors or carcinogens, viz., certain chemicals, radiations and specific viruses; these affect the host's genetic system through mutations with rearrangements or deletions of specific genes to result in uncontrolled growth of cells or tissues to produce tumors and metastasis. The characteristic immortality of cancer cells is due to their failure in apoptosis that contributes substantially to tumor development. Cancer cells typically display defects in the regulation of cell proliferation, differentiation and survival. These abnormalities arise due to activation of cancer causing genes (oncogenes) and/or inactivation of tumor suppressor genes and their respective products, called oncoproteins and tumor-suppressor proteins. The uncontrolled proliferation of cancer cells *in vivo* is mimicked by their behavior in cell culture. Tumor viruses, which cause cancer in humans and experimental animals, have played a critical role in cancer research by serving as models for cellular and molecular studies of cell transformation i.e., the conversion of normal cells to tumor cells in culture. These have been contributing to our current understanding of cancer at the molecular level. [15]
Progress in molecular biology research is driving the pharmaceutical R&D towards the development of new inhibitors of the cell division cycle, where most factors are controlled by protein-phosphorylation / dephosphorylation events. The main players in such controls are a group of related protein kinases (specifically, protein-serine/threonine kinases) named 'cyclin-dependent kinases' (CDKs) and their regulatory subunit proteins, called 'cyclins'. The activities of CDKs are regulated in several ways e.g., by differential phosphorylation at several sites on the CDK proteins, expression and degradation of respective cyclins, and by the binding of inhibitory proteins, called CDK inhibitors. Cyclins and CDKs are among the most extensively studied potential targets for developing drugs against cancer since the tumor cells evade the cell cycle control and exhibit false checkpoints in favor of their proliferation. [10, 14]

BIOTECHNOLOGICAL APPROACHES

Biotechnology is a multidisciplinary science and has many applications. It offers the tools for manipulation and improvement of proteins and enzymes through the use of gene cloning or recombinant DNA technology. A major application involves the use of enzymes in chemical, pharmaceutical, food and other industries. By virtue of their specificity and ability to catalyze chemical reactions under relatively mild conditions, enzymes have found many industrial applications, helping to avoid the use of harsh chemicals and process patterns. Enzyme production in large amounts and with required properties has been revolutionized by the developments and applications of recombinant DNA technology, which have paved the path of modern biotechnology. [16, 17]

HETEROLOGOUS PROTEINS BY GENE CLONING

Numerous proteins of heterologous origin have been effectively expressed in genetically engineered prokaryotic host cells. Nevertheless, many proteins of eukaryotic sources need to undergo specific post-translational modifications (such as glycosylation), to be functional. For production of such recombinant

proteins in large quantities for research or industrial use, good expression systems have been devised using a number of eukaryotic hosts e.g., yeast, filamentous fungus, insect, plant and mammalian cells in culture and organisms. Each of these systems offers distinct advantages and disadvantages. In practical terms, however, there is no single eukaryotic expression system that is capable of producing an authentic protein from every cloned gene.

Although the primary objective of gene cloning is the expression of the cloned gene in a selected host organism, the insertion of a gene into a cloning vector does not necessarily ensure that it will be successfully expressed. The production of a protein requires that the gene be properly transcribed and the mRNA be translated. High expression vectors have been created with genetic elements for controlling transcription, translation, protein stability and secretion of the product of the cloned gene from the host cell. [14, 16, 18]

PROTEIN ENGINEERING

A major reason for the relatively low number of industrially usable enzymes is that an enzyme, evolved for a particular function in biological cell under natural condition, may not be suitable for a specialized industrial application. This is because most enzymes are prone to denaturation due to loss of their 3-D structure or conformation on exposure to relatively harsh conditions viz., high temperature, extreme pH or organic solvents that are used in many industrial processes.

The unique properties of a protein reside in its correctly folded, 3-D structure, which in turn is an outcome of the sequence of its amino acids encoded by the gene for the protein. Certain amino acids at some specific positions in a protein chain can play important role in determining the specificity, thermostability and other properties of a protein. Changing even a single nucleotide of the gene can cause incorporation of a different amino acid than the original one. This may result in either disruption of the normal activity, or enhancement of a specific property of the protein. Protein engineering techniques that involve directed mutagenesis and recombinant DNA technology can selectively alter or replace any nucleotide of a cloned gene for producing proteins with specific amino acid replacement at the chosen site. The selection of amino acid for replacement is based on the knowledge of the amino acid's role in the functional protein and this knowledge is gained through genetic studies, or X-ray crystallographic data of the 3-D structure of the protein. Applications of protein engineering in the pharmaceutical industry have led to the development of engineered proteins and antibodies with improved properties e.g., immunotoxins and immunolysins for the treatment of cancer, with an objective that these drugs will attack only the cancer cells and reduce the unpleasant side effects. [1, 14]

BIOPHARMACEUTICALS

The term "biopharmaceuticals" denote biotechnologically derived drug products. The 20th century saw the golden age for the discovery and synthesis of chemical entities, drugs and medicines; the present century is seen as era of drugs from biomaterials, particularly from biotechnology. The foundation of modern biotechnology was built by enhanced understanding of protein structure, metabolic regulation and gene expression that led to the discovery and use of restriction enzymes in recombinant DNA technology, and the invention of monoclonal antibodies (MAbs) through hybridoma technology.

Recombinant technology enabled the production of a wide range of natural and modified proteins in large quantities. Previously, the dependence on the extraction from natural sources severely limited the range and quantity of proteins available for clinical use. The earliest recombinant products were replacements for existing protein products, which were extracted from animal sources. Insulin was the first recombinant

protein (approved in 1982); next were growth hormone, blood-clotting factor VIII, cytokines, growth factors, receptor antagonists, enzymes and antibodies. Hybridoma technology created new proteins namely MAbs that provided an alternative approach to treat many diseases.Gene technology has also made it possible to produce proteins, engineered with improved characteristics. Insulin analogs with altered amino acid sequences have been developed to affect the speed of hormone action: For example, insulin lispro and insulin aspart are fast acting analogs in which specific amino acids at the C-terminal were changed to reduce the self-association properties of insulin; insulin glargine is a long-acting variant that dissociates slowly from microcrystalline precipitate. These analogs enable better control of insulin availability based on clinical circumstances. [19, 20]

The second generation of products include recombinant proteins and MAbs which represent the largest and fastest growing category of biopharmaceutical proteins with applications in the cancer field; immune disorders and infectious diseases. Various approaches have been used to modify therapeutic activity of proteins, improve their stability, or reduce the rate of their clearance. These include amino acid substitutions, domain removal, fusion of peptide sequences from different proteins, and glycosylation engineering. Protein engineering techniques have been applied to MAbs for antibody humanization. PEGylation has been used to modify protein properties and to prevent proteolytic degradation and the rate of in vivo clearance.

Stabilizing the protein's native folded structure during storage is essential to maintaining the biologic activity. Ideally, the purpose of formulation development of biopharmaceutical protein therapeutics is to provide a final dosage form that offers sufficient *ex vivo* stability during processing, handling and long-term storage, and also provide adequate *in vivo* stability in terms of bioavailability that meets the pharmacokinetics / pharmacodynamics (PK/PD) therapeutic requirements. [20, 21]

Proteins are the most important class of receptors which are also excellent targets for drugs. Molecular cloning of receptor proteins is useful for gaining an insight of fundamental structural motifs; identifying novel receptors and isoforms of receptors; expression and purification of various classes of receptors, transducers and effector proteins; and understanding their biochemical mechanisms. The future of drug discovery depends on an understanding the genetic basis of the disease

A better understanding of biological systems leads to a dramatic increase in targets for drug development. Genomic revolution has been a key factor for driving the future developments through innovations in modern biotechnology. In the future, as 'drugs' become more complex and difficult to deliver – to cross the biological membranes to reach the target – novel drug delivery becomes very important as a critical component of drug research. New approaches to drug targeting are developed using monoclonal antibody, or a carrier-system or device for transporting the drug to a specific target site by means of liposomes, nanoparticles etc.

Perhaps the greatest beneficiary of the advancement of biotechnology has been the pharmaceutical industry. There is a rise in biopharmaceutical sales and the requirement of protein based drugs. The development of such products places increased emphasis on improving existing technologies, process efficiencies and yields.

CONCLUDING REMARKS

The vast majority of genes in any life form encode information for the production of proteins through the biosynthesis of polypeptide chains. Proteins are essential biomolecules that are involved in almost all biological functions with unparallel versatility in performance: They catalyze chemical reactions; take part in cell-cell communication and signal transduction; transport molecules within and between cells; provide support to cells, organs and body structures; cause movement; render protection against infectious agents

and toxins; and regulate gene expression for the differential production of all gene products, including their own !

Modern protein research seems to put major thrusts on: i) Understanding proteins as molecular machines; this needs detailed analysis of protein structure and function at the molecular level. ii) Identifying the spectrum of proteins synthesized by the time- and situation-specific differential expression of genes in the genome; the emergence of proteomics has attracted those involved in the drug discovery. iii) Manipulation and improvement of enzymes; optimization as commercial biocatalysts for industrial application. iv) Developments in biopharmaceutical proteins and their expression for human therapeutic use; expression systems with modified lower eukaryotic cells (as substitute of mammalian cells in culture). Nevertheless, the ultimate objective of all biotechnology research is the development of commercial products/processes and is therefore, driven to a great extent by economics.

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