Stability Studies of Proteinous Compounds 13

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Abstract

Protein stability is a subject of great interest to the pharmaceutical, biotechnological, and food industry, as well as, for academic researchers who are studying proteins on regular basis. The stability of proteins is important to understand the optimization of protein purification, composition, expression, storage, and conformational studies. This study concentrates on the factors affecting the protein stability and type of stability undergone by proteins and chemical kinetics, which provide the designs and modifications of chemical reactors to maximize the product yield of pharmaceuticals and to eliminate hazardous by-products. A brief introduction of conformational and compositional protein stability will be discussed for analyzing protein stability by key methods. The importance of the different methods followed for pharmaceutical product stability testing, guidelines given by the ICH, FDA, WHO, and other agencies for stability testing, and other aspects related to pharmaceutical product stability will be discussed in a concise manner in the present study.

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13.1 Introduction

Proteins are huge, complicated molecules which have central importance in the body. They do much of the work and are necessary to build, operate, and control the tissues and organs of the body. In 1938, the Swedish chemists Berzelius coined the Greek term proteins for "protein," meaning "the first class."

Proteins are amino acid polymers. All proteins, either from the most primitive lines of bacteria or from the most diverse life types, are covalently bound to form linear arrangements of 20 amino acids in the same basic range. Each of these amino acids has a side chain with different chemical characteristics. Proteins vary according to amino acids that constitute the backbone of polypeptides differ in the shape, number, and sequence. Their molecular structures, biochemical features, and physiochemical properties are therefore differed. In many combinations and sequences, cells can generate proteins with distinctly different properties. The amino acid sequence defines the specific three-dimensional structure and function of the protein. They act as catalysts, hold and store oxygen and other molecules, offer assistance in defense system, nerve impulse, and transmission, stimulate movement, and regulate differentiation and growth.

A protein's amino acids have been categorized as aliphatic, aromatic, polar, nonpolar, hydrophobic, hydrophilic, oxidative, and acidic. For example, a protein may attain a regular secondary arrangement such as coils, α-helix, β-turn, and β-pleated board. The secondary structures are folded further leading to the construction of a higher cognitive structure called tertiary structure which is stabilized primarily by hydrophobic, van der Waals and electrostatic interactions, and hydrogen bonding. Proteins aren't rigid molecules entirely. They endure conformational variations after binding of ligands. When binding to oxygen, myoglobin and hemoglobin undergo structural modifications, and they can carry oxygen to the lungs and various tissues. Thus these proteins represent a relationship between structure and function.

In certain circumstances, exposure to denaturing conditions makes it possible to duplicate the structural transformations of normal functional proteins into misfolded, aggregated, or denatured proteins. The in vivo pathways of normal functioning proteins misfold into aggregates are still not well-known. How distinct proteins may structurally transform into a highly ordered aggregate structure such as amyloid fibrils which is extremely thermodynamically stable is unclear [49]. Well-known and etiological diseases were linked with the misfolding of protein in aggregates during the last decade. Alzheimer's disease and diabetes are among the most crippling, economically destructive, and expensive disorders in the developed world. As civilization ages, they become largely reliant on new agricultural and nutritional techniques [17]. It is important to consider the processes and concepts underlying the

folding of proteins, denaturation, and malformation in order to cure or potentially avoid these human diseases. This chapter will primarily concentrate on the functional modifications in proteins and their importance to product production stability. Further guidelines of proteinous compounds for drug stability will also be discussed.

13.2 Essential and Nonessential Proteins

Proteins are macronutrients supportive of body tissue development and maintenance. Amino acids are the rudimentary protein elements. Amino acids derived from poultry, beef, and legumes are essential, while amino acids synthesized naturally in your body are nonessential.

13.3 Protein Structures

The four types of protein structure (Fig. 13.1) are characterized by the level of complexity within polypeptides (Table 13.1).

13.3.1 Primary Structure

The basic order in which amino acids are combined together forms a protein primary structure (Figure 13.1a). Proteins are consisting of collection of 20 amino acids. In

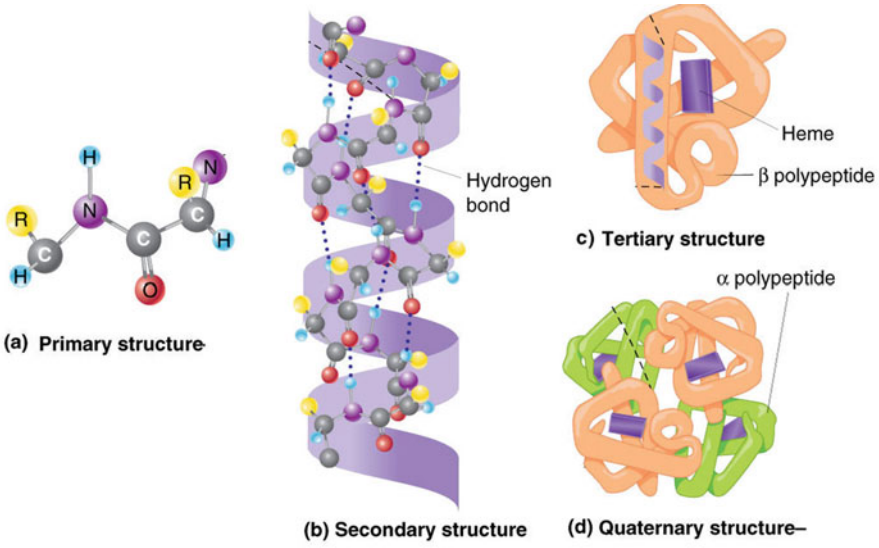

Fig. 13.1 Structures of protein

Table 13.1 *Common measures of protein stability*: Definitions of protein stability at each structural level are shown along with common methods used to analyze the degree of stability. Asterisks denote the relative merits of the three main structure determination techniques, with five asterisks denoting the optimal method. For example, NMR solution methods are often more favorable for studying dynamic processes and quaternary states as they are not influenced by crystal packing

Structural level	Definition of stability	Example biochemical processes or features	Common methods	Crystallography	NMR	EM
Primary	Primary change of amino acid sequence or modification of amino acids	PTM Proteolysis Protein splicing	Half-life analysis SDS-PAGE Mass spectrometry Eastern and Western blotting	*****	****	*
Secondary	Change of helix, sheet, and loop content	Secondary structure formation Racemization Aromatic side chain interactions Ligands	Circular dichroism (CD) Synchrotron radiation CD UV-CD FT-IR 2D-IR Deuterium exchange Mass spectrometry (DXMS)	*****	*****	*
Tertiary	Change of overall fold or protein Conformation	Hydrogen bonding Hydrophobic interactions Conformational change Disulfide bonding Topology	ITC DSC Thermofluor	****	****	*
Quaternary	Change in oligomeric state	Protein-protein interactions Oligomerization	Size exclusion chromatography Native gel electrophoresis	*	*****	*****

general, the following structural properties of amino acids have an amino group (-NH2), α carbon, an atom of hydrogen (H), a group of "unit" or "R," and a group of carboxyl (-COOH).

13.3.2 Secondary Structure

Two kinds of secondary structures are present in proteins (Figure 13.1b). One is an alpha (α) helix structure, is identical to a helical cord, and is protected by hydrogen bonding in the polypeptide chain. The pledged layer beta (β) is the second form of secondary protein structure.

13.3.3 Tertiary Structure

Tertiary structure leads to the 3-D intrinsic polypeptide chain structure of a protein. Its tertiary structure contains several types of bonds and forces which hold a protein (Figure 13.1c). Hydrophobic interactions make a major contribution to a protein's folding and formation. The "R" unit is hydrophobic or hydrophilic. Hydrophilic "R"-linked amino acids seek interactions toward aqueous environments. On other hand, the hydrophobic "R"-associated amino acids try to move toward protein center and away from the water.

13.3.4 Quaternary Structure

Quaternary structure means a macromolecule of protein containing several interactions between polypeptide chains. Polypeptide chains act as a subunit. Quaternary proteins may consist of more than one subunit of the same type of protein (Figure 13.1d). Example of a protein with a quaternary structure is hemoglobin. It comprises two alpha subunits and two beta subunits [53].

13.4 Domains of Protein

Most proteins are made up of some protein domains, i.e., protein sections which fold into distinct structural units. Usually, domains also have different functions. Binding modules, SH3 domain binds to proline-rich sequences in other proteins; kinase helps in enzymatic activities.

13.5 Classification of Proteins Based on Structure (SCOP)

The database Structural Classification of Proteins (SCOP) is a mostly manual classification of protein structural domains based on the similarity of their structures and sequences of amino acids. Setting the evolutionary relationship between proteins is a justification for this classification. Same shape proteins with no functional or sequence resemblance are put in separate superfamilies and are thought to have a shared very distant ancestor. Proteins with the same form and any sequence and/or function similarities are grouped in "families" and are believed to have a closer shared ancestor [59].

13.5.1 Chemical Composition-Based Classification of Protein

Based on their chemical structure, proteins can be divided into two groups (Fig. 13.2)

13.5.2 Simple Proteins

They are also known as homoproteins and consist only of amino acids. Examples include albumin, keratin, collagen, and plasma.

13.5.3 Conjugated Proteins

These are sometimes called homeoproteins; they contain a nonprotein component in their structure. Phosphoproteins, glycoproteins, and chromoprotein are examples of conjugated proteins.

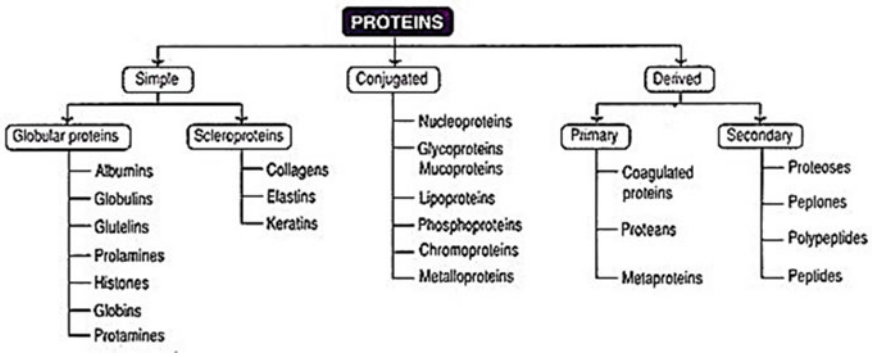

Fig. 13.2 Classification of proteins based on chemical composition

13.5.4 Phosphoproteins

These proteins bind the threonine and serine residues to phosphoric acid. They usually have a structural function, e.g., phosvitin of egg yolk, tooth dentin, and caseins of milk or reserve function.

13.5.5 Glycoproteins

These proteins are bind to polypeptide backbone with one or more units of carbohydrates covalently. The divisions typically comprise of more than 15–20 carbohydrate units comprising arabinose, galactose, 6-deoxygalactose (fucose), glucose, mannose, and N-acetylneuraminic acid (Neu5Ac or NANA). Examples are:

(1) Fibronectin binds the extracellular matrix to cells by interactions with fibrous or collagen proteins on one side while cell membrane on the other side.
(2) The best-known example of erythrocyte membrane glycoproteins is glycophorin.
(3) Immunoglobulins or antibodies and all plasma proteins in the blood, except albumin are the best examples of glycoproteins.

13.5.6 Chromoproteins

These proteins are containing colored prosthetic group. Myoglobin and hemoglobin binding with four and one class of hemes, respectively, rhodopsins binding retinal, and chlorophylls binding a porphyrin ring with its central magnesium atom.

13.5.7 Classification of Proteins According to Shape

13.5.7.1 Fibrous Proteins

These proteins have primarily structural and mechanical functions, serving cells and the entire organism. The hydrophobic amino acids, both internally and on their surface, make these proteins soluble in water. In supramolecular structures, hydrophobic amino acids are present on their surface and make their packing simpler. Here, the long filaments or sheets of polypeptide chains in most instances can only be found and repeated in one type of secondary structure. The structural properties of these proteins actually preserve their stability, and they provide shape, support, and protect vertebrates. These proteins are partially hydrolyzed in gut-like, alpha-keratine. Some examples are given here.

13.5.7.2 Fibroin

It is made of insects and spiders. An example of this is the silkworm developed *Bombyx mori*.

13.5.7.3 Collagen

"Collagen" does not mean a single protein but a group of structurally linked protein (at least 29 different types), which is the common extracellular scaffolding of multicellular organisms and dominant protein in the connective tissue. It comprises around 25–30 percent of all proteins in vertebrates. They are found in high concentration in eye cornea, cartilage, organic bone matrix, and tendons and other various tissues and organs. They shape various structures of the tissue, each able to fulfill a specific need. For example, these are enticed and guided fibers in the skin, which maintain the strength of skin tensile. The molecules are in crystalline position in the cornea, so that they are almost transparent.

13.5.7.4 α-Keratins

Almost all dry weight of skin's outer layer, claws, beaks, nail, wool, fur, and hooves is made up of α-keratins. The specific rigidity and flexibility of these structures result from the number of disulfide bonds which contribute to the stabilization of the protein structure along with other binding forces. And this is why, in beak and claws, keratins are rich in disulfide bonds, while wool keratins are smooth, extensible, and flexible because of less disulfide bonds.

13.5.7.5 Elastin

The skin and blood vessels possess elasticity due to random coiled structure of elastin; it varies from α-keratin and collagen structures. .

13.5.7.6 Globular Proteins

Most of the proteins belong to globular family. These are more complex and spherical than fibrous proteins and have a compact construction. These are normally water soluble but can also be present in biological membranes that result in a hydrophobic environment (transmembrane protein). Globular proteins act like, hormones, enzymes, membrane conveyors and triglyceride conveyors, receptors, blood oxygen and fatty acids, antibodies or immunoglobulins, and legume protein and grain. Most animal-associated globular proteins are solubilized to amino acids almost entirely at the intestinal level.

Cytochrome c, myoglobin, and hemoglobin are examples of globular proteins.

13.5.8 Classification of Proteins According to Solubility

Proteins are categorized into the following categories based on their solubility in water

13.5.8.1 Globular Proteins

They're soluble in water. They include the proteins that are functional, such as hemoglobin, enzymes, etc.

13.5.8.2 Fibrous Proteins

These are insoluble liquids and comprise the structures of proteins. They are protective (e.g., fibrin and hair keratin) and/or supportive (e.g., collagen).

13.5.9 Protein Classification Based on Functions

Schematic representation of classification of proteins on the basis of their function has been described in Fig. 13.3.

13.5.9.1 Hormonal Protein

Hormones are protein-dependent chemical compounds that are secreted by cells in the endocrine gland. Usually blood-borne hormones act as chemical messengers to relay signal from cells to cells. They affect numerous target cells in your body. Such cells have special receptors linked to signals by the hormone. Insulin, which secreted by the pancreas to regulate the blood sugar levels, is an example of a hormone protein.

13.5.9.2 Enzymatic Protein

Enzyme proteins facilitate metabolic processes in your cells, including stomach absorption, liver functions, glucose conversion, and blood clotting. One example is digestive enzyme which breaks the food down into simple forms which your body can easily absorb.

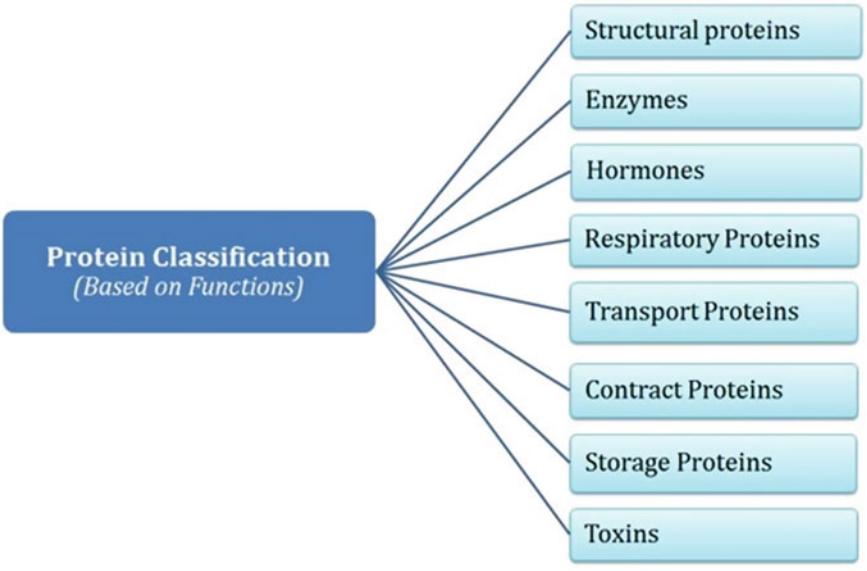

Fig. 13.3 Classification of protein on the bases of function

13.5.9.3 Structural Protein

The structural proteins of the body are also known as the fibrous proteins, which include elastin, collagen, and keratin. Collagen as a connective structure shapes the cartilage, tendons, bones, muscles, and skin. The nails, teeth, hair, and skin are mainly components of keratin.

13.5.9.4 Defensive Protein

Central parts of the immune system are immunoglobulins or antibodies, which carry a loop for diseases. In WBCs, antibodies are produced which targeted viruses, bacteria, and various other microorganisms and make them inactive.

13.5.9.5 Storage Protein

Proteins in the body mainly store ions including potassium in the mineral. Casein and ovalbumin, for example, are storage proteins found in breast milk and egg whites that play an important role in embryonic development, respectively. Iron is an ion needed to produce the main structural component of red blood cell, hemoglobin. Ferritin-protein storage controls and protects the body from adverse effects of excess iron.

13.5.9.6 Transport Protein

Transportation proteins provide the cells with important materials. For example, a transport protein, calbindin, facilitates the calcium absorption from intestinal walls. Serum albumin contains blood fats. Oxygen from the lungs into the tissues is transported by hemoglobin, while myoglobin absorbs oxygen from hemoglobin and transport that oxygen the muscles.

13.5.9.7 Respiratory Protein

These are colored proteins. All of them are conjugated proteins, and they contain pigments (chrome) as their prosthetic group, for example, hemoglobin and myoglobin.

13.5.9.8 Receptor Protein

Substance like nutrients and water that can enter and leave the cells are regulated by receptor proteins on external cells. Some receptors activate enzymes and glands. Endocrine gland stimulates epinephrine and insulin to regulate blood sugar level.

13.5.9.9 Contractile Protein

These are the motor proteins that control the speed and frequency of contractions in the muscles and heart. These contractile proteins are myosin and actin. Extreme contractions may cause heart problems (https://www.tuscany-diet.net/proteins/classification).

13.6 Stability of Proteins

The net energy balance which determines whether a protein is its original, folded or denatured (unfolded or prolonged) condition is protein stability. The net protein stability is extremely limited and is a contrast between two major contrasting forces. Various interactions (atomic / group), such as electrostatic, Van der Waals, disulfide, hydrophobic, and hydrogen bonding, stabilize the folded natural form of protein structures and regulate free entropic and non-entropically energies. In view of the relatively high complicated three-dimensional structure and rapid spontaneous folding of proteins, net stability is surprisingly low, usually between 5 and 10 kcal / mol. [72]. The word stability can be defined as the tendency of the protein structure to maintain the native (biologically active) conformation. Local proteins stabilize only slightly, shift to free energy (Δ G) in the 20–65 KJ/ mol range, separating plied and unfolded states under physiological conditions in typical protein.

Theoretically, a specific polypeptide chain may be able to take various configurations and thus describe the deployed protein condition with a high degree of confirmatory entropy. This interaction between entropy and hydrogen connection maintains the unfolded state in multigroup polypeptide chain. These effects revert and stabilize the folded native conformation by means of chemical interactions involve strong bonds including (non-covalent) peptide and disulfide bonds, hydrogen bonds, hydrophobic, and ionic interactions. 200–400 KJ/mol are required to break a single covalent bond, whereas a mere 4- KJ / mol can disrupt weak forces. Each covalent bond that results in native protein conformation such as disulfide bonds linking different parts of a single polypeptide chain is apparently much stronger than specific weak interactions. The protein conformation is the one that typically has the lowest free energy and the highest interplay intensity. Not only are there several weak interactions among the various molecules (intermolecular interactions), but they are also performed in just one molecule. Overall, intermolecular interactions are much less active than intramolecular. The stability of proteins isn't just the amount of free energy that comes from its many weak interactions. Inside a folded polypeptide chain, a hydrogen bonding group is bound to the water before folding, and with each hydrogen bond formed into a protein, a hydrogen bond (with equal strength) is broken between the same body and water.

In determining the net thermodynamic contribution of hydrogen bonding to folding, energy required to split the water hydrogen bond is distinguished from that extracted energy in folded proteins between two atoms in the formation of hydrogen bonds. The net stability that leads in the folded and unfolded states to a certain weak interaction or difference in energy is almost zero. However, that the power of a hydrophobic linkage is not due to strong intrinsic appeal between nonpolar groups. It is also important to note that the water solvent properties in which nonpolar groups are dissolved are the key cause for this interaction. A nonpolar residue dissolved in water creates a solvation shell in water that strongly order water molecules. As the polypeptide chain is folded into two nonpolar groups, the exposed surface area to solvent is decreased, and a portion of water highest order is released into the bulk solvent in the solvent shell. Entropy of water is also on the

rise. Entropy increases the driving force behind nonpolar solvents and enhances thermodynamically favorable process. In a protein, tightly packed core side chains of hydrophobic amino acid are usually inside. In aqueous solution, proteins enclose water and swell. As protein solution charged, they will colloidal emulsoids or micelles, and there is ring around each molecule. When a polypeptide loses its superior structure, denaturation of protein occurs which tends to minimize the biological activity.

13.7 Types of Stability

A structural biologist uses techniques such as cryo EM, crystallography, or NMR spectroscopy for evaluation of precise molecular structure of protein. A significant challenge to the crystallization of certain proteins is the reliance on crystallographic science, such as lack of or protein stability. Protein stability is a wide field addressing physical and chemical elements, entropy, thermodynamics, computational chemistry, dynamics, and protein folding [13]. Stability is essential to understanding the evolution of biological processes. It is the capacity of a sequence to persist over time [82]. Apparently, the particular nature of a protein molecule's "pattern" is rather unclear, but this "pattern" can turn by processes such as unfolding protein, degradation, denaturation, enzymatic alteration, conformation shift, and proteolytic cleavage. As regards the quality of the fully folded protein's primary and conformational structures, these transformations are usually considered or analyzed. Additionally, the stability of proteins means different things for different individuals. For example, the half-life activity of a protein may primarily be considered to be a test of its stability by a pharmacologist, biotechnologist, or food science. A protein chemist or a structure biologist, however, may use adjustments in all levels of protein structures as a test of its stability. Stability of proteins is a basic precondition for crystallization. At this stage, distinction between thermodynamic stability and conformal disorder of protein is important to consider, especially provided some specific parameters used for the characterization and analysis of structures by structural biologists.

For instance, NMR spectroscopy experts often note dramatically divergent values within their ensemble structures of a given protein crystal model, whereas crystallographers are estimating positional uncertainty by documenting factors B. Each of parameter defines displacements and disorder within a system which may show the degree of conformation stability. Systemic genomics projects have much alleviated the bottlenecks of out mode systemic, but even researchers are too mindful to deal with difficulties of encoding the puzzling protein target and purifying it. The primary structure, architecture configuration, and expression conditions of host cells should all be taken seriously to reduce much of the problems observed in stability of protein like purification and expression. Several specific approaches will address on protein stability tests, including the analysis of the protein melting temperature (Tm), cryo-EM, and NMR strategies that are widely used to maintain proteins' stability.

13.7.1 Stability: An Important Precondition for Crystallization

The self-organization of macromolecules into a long-range, translational periodical structure can be described as biomolecular crystallization. To accomplish this function effectively and efficiently, the moieties of a crystal's asymmetric unit should have the similar kind and shape. A central and prevalent criterion for crystallization of a protein has not been fulfilled if these stable objects can't form, and no attempt is made at discovering appropriate kinetic and thermodynamic circumstances leading to these protein composition crystals.

13.7.2 Compositional Stability

In the crystallization experiment, the same species must be maintained during the crystallization processes; some sort of compositional stability must exist. This ensures that on a basic level, the protein molecules have to have the same chemical composition. A sample's chemical uniformity can also be calculated with mass spectrometry or an SDS-PAGE gel. The primary structures of proteins are usually impairing due to post-transitional changes like proteolysis and glycosylation, which generate compositional heterogeneity. As it takes time to crystallize the protein, the primary requirements of compositional stability should be preserved by a given duration and an appropriate variety of environmental circumstances. It is worth noting that absolute stability is nothing like that. For example, Protein compositionally stable is enough to produce SDS-PAGE gel single band for crystallization experiment which can still not stable enough over a time.

13.7.3 Conformational Stability

If the protein sample is compositionally uniform, it therefore would also not crystallize until it is stable conformationally [80]. A large number of conformationally disordered proteins are considered to be exhibiting less or no organizational conformation. A protein with major abnormalities or regions exhibiting complex variability is less able to autonomously shape a crystal [60]. This could be true only though the composition of the sample is completely stable. The rigorous criterion for minimum conformational variation is a specific challenge while seeking a sample of protein to crystallize confronting a crystallographer. The puzzling problem is that a context-driven property is the conformation of flexible protein regions. Conformations can be somewhat specific in a macro-molecular crystal, for example, in an NMR. Although it is important to remember that the findings may only be applicable in relation to the nature and circumstances of such a specific methodology, structural approaches are used to evaluate conformational homogeneity. For example, the studies of conformational stability and dynamics, as crystal packaging can impede these motions, are sometimes limited using crystallographic methods. Complementary details can be given in such cases by NMR solution methods. By X-ray

crystallography, structures assessed provide scant evidence about the protein structure dynamics. Nevertheless, the atomic model contains some complex knowledge in the style of the factor B or atomic displacement parameter (ADP).

The factor B functions as statistically reflects atom's probability on the structure at mean specific location, and it is presented in A^o2 [110]. When a given atom's B factor is high, then it means that the likelihood of having the atom in the structure at that location is small. In crystals, high-B-factor regions atoms may be moved because of complex disturbances in the polypeptide chain or a long or short-range disruption. Such dynamic or flexible regions can also be formed in a crystal and planned for better conformation stable protein samples during cloning. These improvements will not only help to improve the stability by purification and expression but also raise the likelihood of molecules being able to stack more organized in the crystal lattice. Consequently, these efforts also result in improved diffraction of crystals and X-ray data with higher resolution. In root-mean-square variance between the corresponding atoms of the members of the ensemble, it is possible for a comparison of structures produced by NMR spectroscopy to obtain a measure close to a crystallographic factor of B. This calculation can be used to determine the heterogeneity of stability, disorder dynamics, or stereochemistry through a range of conformational designs. The crystallographic factor B is equivalent to the meaning r.m.s.d. and is not disrupted or affected by crystal packing because the structure is in solution [85].

A broad class of proteins, called IDPs, includes large amounts of confirmatory disruption and, in some instances, has no apparent three-dimensional structure overall. 40% of the human protein is considered to have a minimum of one section, and 25% is disorganized fully [96]. Owing to its predicted problems in crystallization, the crystallographic group has fully prevented such proteins. However, NMR techniques were used to reveal the functions of these unstructured proteins [111]. Recent experiments introduced a profound shift of protein function and structure. Modern theory suggests that protein works in a lock and key manner which binds to ligand (or protein) by adopting a pre-shaped rigid structure. However, the NMR studies of Mujtaba et al. [68] and Sugase et al. [93] on p53, 14–3-3 and CREB (IDP proteins) indicated that these disordered territories provide for plasticity and versatility and often shape structure only when the partner protein is binding. These core proteins interact in sensitive context with several protein patterns, which is a feasible way due to initial loss of conformational stability and elasticity [102]. These large "central protein" complexes will have high-resolution crystal structures necessary to understand their role in human diseases such as Alzheimer's and Parkinson's diseases. While the low conformable stability of such protein poses problems, cellular IDPs provide several benefits relative to more conventional single structure folded proteins [56]. Besides IDPs, some proteins include APRs, which typically include 5 to 15 amino acids that appear to shape expanded board structures. Their usage is not restricted to IDPs. APR parts present in macroglobulin are responsible for amyloidosis disorders due to aggregated amyloid fibers [3]. Another protein named IIPs cannot be replenished with conventional buffer solution and is completely insoluble. Normal V22-SH3 and Mutant SH3 are resurrected and

solubilized in pure water but intractable in the presence of ions, allowing NMR spectroscopy to study further unstructured proteins in solution [36, 57].

13.7.4 Structure Level Stability of the Proteins

One clear approach to conceptualize protein stability from a theoretical point of view is to find stability at increasing protein structure level.

13.7.4.1 Primary Structure

Post-translational modifications (PTM) severally modified the sequence of primary protein or amino acid in peptide chain. PTMs are the central debate of protein stability which results in alteration of function and structure of protein. Conformational and compositional stabilities can be affected by instability and dynamic of protein and can be in complete or defective, respectively, by PTM. This freedom of conformation contributes to increased disorder, thus intermolecular crystal formation preserving the charged residues on the surface of protein. Although the heterogeneity of glycosylation appears to delay crystallization, its divergence may have significant functional consequences for a protein. Proteolysis, protein splicing, and inclusion of other function group of amino acid arise PTMs in the primary structure of protein. These types of PTMs modify the enzyme's activity and specificity and directed a wrong protein to particular area. For example, ethanolamine phosphoglycerol, hypusine, and carboxylate are the functional groups used to regulate protein operations [75, 100, 105]. Isoprenoid, palmitate, myristate, and glycosylphosphatidylinositol (GPI) are functional groups which often used for membrane targeting by attaching itself to proteins [10]. ISG15 [61], SUMO [39], ubiquitin, and PUP [92] are large peptides which can covalently bound to proteins.

Many PTMs play role particularly regard the protein's half-life and turnover within the cell, important for protein stability. PTMs include acylation, butyrylation, ADP-ribosylation, malonylation, iodination, succinylation, s nitrosylation, S-glutathionylation, glycosylation, and oxidation. All of these PTMs have vital role in the function and structure of target protein. Proteolytic and protein splicing like certain PTMs have a significant effect on the primary level of protein structures and lead to dramatic alterations in the compositional stability. A polypeptide precursor is processed to form a functional and mature protein. An auto-catalytically excised protein precursor is intein, which is of particular interest to protein stability, and they are also concern for a protein engineering point of view [2]. Sniping exteins are used to produce two new peptides ligated together [66].

13.7.4.2 Secondary Structure

Secondary structure of protein is a chain of tridimensional compact structure of polypeptide. In terms of stereochemistry pattern, polypeptide backbone of carbonyl O atom and amide H atom between hydrogen bonding describes the secondary structure [76]. The principal hydrogen bonding and hydrophobic behaviors are fairly

behind the formation of a secondary structure and in turn tertiary structure at simplified stage. -helix is the primary element of a secondary structure containing around 1/3 of all components. Residues including leucine, glutamate, and alanine are typically present in helices when the first crystal structure was examined [91]. By comparison, glycine, aspartic acid, and proline were detected less gradually. According to popular method of Chou and Fasman [12], many algorithms for protein secondary structure prediction were used to construct. The destabilizing impact of proline on helix (G 3.16 kcal/ mol vs. alanine 0.1 kcal/ mol) is a dominant consensus in the amino acid trends. This is a consequence of the absence of the amide H atom in the backbone that averts proline from contributing in the stability. Due to steric hindrance, proline thick cyclic side chain of 30 percent kink in backbone helix is obtained.

Glycine has the second lowest tendency to shape -helices due to greater strength of conformation when folding to shape α –helix. It's necessary to know all such propensities of are context based [40]. Proline, for example, is very popular in integral membrane protein transmembrane helixes and under these conditions is stable for helixes [55]. These findings clearly support the hypothesis that the folded protein stability is mainly determined by the composition of the amino acids, and the primary design results as a normal, realistic minimum of free energy. Easy algorithms for the construction of both stable helices and sheets have been applied to these simple principles [50]. Relative modeling can also be used to construct thermal stability proteins that are of a higher level, and associated models can be used to describe the tertiary structure.

13.7.4.3 Tertiary Structure

Overall folded form of polypeptide chain of protein is tertiary structure of protein. Primary and secondary structure, composition and conformation, cellular environment factors, pH, temperature, solvents, salt bridge, ligand binding, hydrogen bonding, van der Waals forces, hydrophobic forces, cofactors, ion bindings, PTMs, and chaperone are some factors influencing the protein folding process [16]. Many of these effects have recently been quantified by a series of Pace and colleagues experiments [74]. Hydrophobic reactions contribute 60% to protein stability and 40% to hydrogen bonding [73]. By conformational entropy, a single methyl group loss 2.4 kcal/mol to net instability and 1.1 kcal/mol to net stability of protein [73]. The net input of hydrogen binding to the total safety of proteins stability is 1.1 kcal/ mol 1. In addition, hydrophobic reactions normally do nothing for small protein stability [74]. For thermally protein stability for industrial usage, for example, proteases and biofuels for washing detergents of the shielding fold are particularly critical.

The deep hot vents of the sea Sargasso need protein, in those extreme conditions, in order to maintain the fold and structure of thermophilic species such as *Thermotoga maritima*. A thermophilic protein analysis indicates that protein compositions are essentially mesophilic, and small variations in amino acid composition imply thermal stability. There are some anomalies as applied to thermophilic proteins, including an upsurge in salt bridges, hydrophobicity, and aromatic deposits

[31, 113].Unlike the IDPs, the integrity of the tertiary protein structure is also treated as important to preserving protein function. However, as part of its mechanism of action, several proteins experience complete alteration in protein fold [104]. For example, serpins when interacting with the proteinase, endure a change after a longer, stable native stressed (S) to a more compact folded shape relaxed (R). These structural reorganizations include inserting a ring in core sheet or in the strand to generate a dimer that can cause polymerization [112]. Big conformational modifications like this are normal and are found in other proteins including lymphotactin, HA influenza, intracellular channel chloride LIC1, and protein at the Mad2 spindle checkpoint. It is therefore critical that every examination of stability takes careful account of the structure of the protein being studied, as certain protein folds are engineered to be unstable inherently [7].

13.7.4.4 Quaternary Structure

A quaternary arrangement is the organization into a multi-subunit matrix of the subunits of folded proteins. The stability of the complexes is crucial in regulating allostery and cooperation, often the product of the improvements in conformation in each polypeptide chains. The Monod–Wyman–Changeux (MWC) model is one of the standard models to explain protein's allosteric transformations [67]. In this model, there are two states in which protein exist: one is (R) relax, and other is (T) tense. Ligands can be bound to T or R any one state, but if bound with R, the affinity of protein will be improved. If ligands are attached to state T, affinity is decreased, and an allosteric modulator substance is established. Hemoglobin, deoxyhemoglobin in the state of R, and oxyhemoglobin in the state of T, are the possible candidates [6, 78].

As discussed above, recognition of protein stability in isolation from action is not appropriate for IDPs and metastable proteins [71]. Many proteins experience major conforming changes affecting both the secondary and the tertiary structures, and the compositional or conformational stability of each state is different [35, 103]. Abbott drug Navitoclax is an example of destabilizing, which destabilizes the relationship between the Bad / Bid / Bak and Bcl-2 an antiapoptotic protein. Roche drug Nutlin-3 is another destabilizer which inhibits interaction between MDM2 and p53, two tumor suppressors [42]. Cyclosporin A is a natural product, which stabilizes the bond between cyclophilin and calcineurin and increases protein-protein interaction. Tafamidis, a synthetic product, binds to transthyretin packet at dimer boundary are the antidote of destabilization [84]. Tafamidis stabilizes the transthyretin dimerized component and prevents the aggregation and misplacement seen with transthyretin amyloidosis disorders [8] as shown in Fig. 13.4.

13.8 Factors Affecting Protein Stability

The optimal requirements for keeping each human protein stable have to be empirically determined. Protein solutions are, however, usually kept cold (< 4oC) for experiments and other processes involving different temperatures. Several proteins

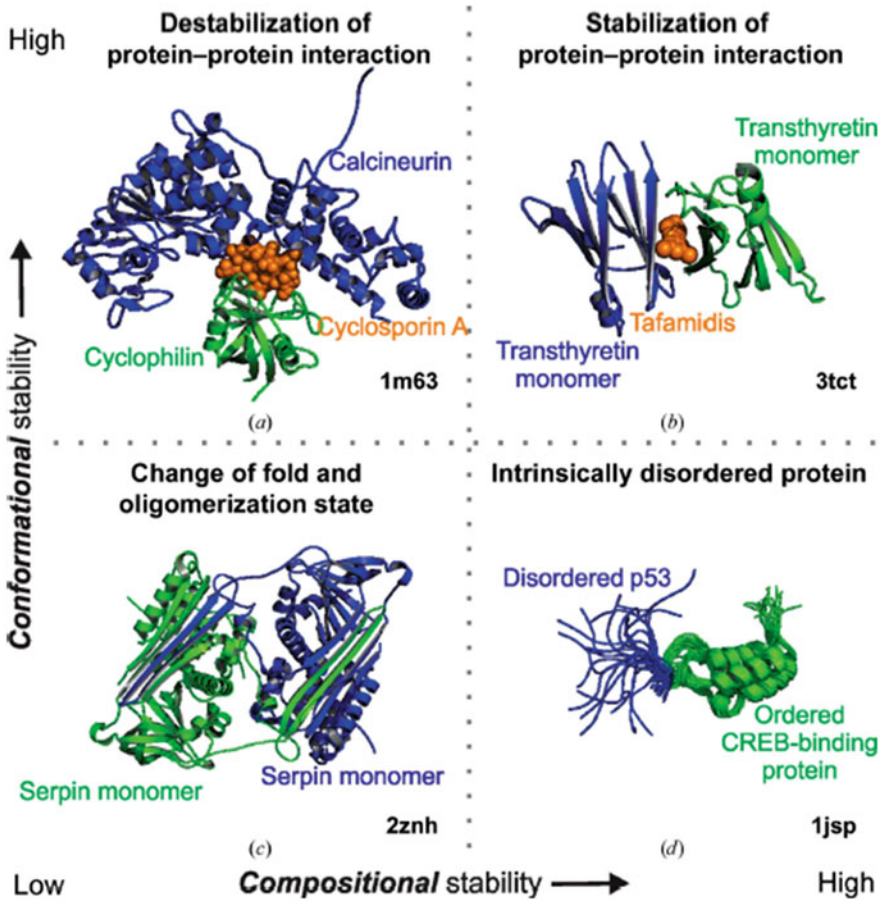

Fig. 13.4 *Matrix of examples of protein stability and disorder*. (**a**) Examples of proteins with high conformational stability include the protein-protein destabilizing compound cyclosporin in complex with calcineurin and cyclophilin, (**b**) the protein-protein stabilizing drug Tafamidis in combination with transthyretin, (**c**) examples of proteins with low conformational stability include the serpins, which undergo large changes in fold and oligomerization state, and (**d**) intrinsically disordered proteins (IDPs) such as the tumor suppressor protein p53

are highly suggestible which should be kept at −20 °C or − 80 °C. Moreover, continuous freezing and defrosting of protein solutions are also damaging. For the preservation of buffer, glycerol (50%) is added which lowers the freezing point and enables storage at −2 °C. Protein solutions can also comprise proteins such as heavy metal chelating agents and/or antioxidants. During cell destruction, proteases may be emitted, and thus protease inhibitors may need to be used.

13.8.1 Temperature

The unfolding free energy of a protein is defined as thermodynamic equilibrium, and it is calculated as differential equilibrium by its folded and unfolded positions. Therefore, knowledge about structural and functional dimensions that contribute to the stabilization of unfolded conditions is important in order to understand the diverse interconnectedness between enthalpic and entropic contributions to the stabilization of proteins. Latest experimental findings suggest that unfolded protein states are arrays of quickly interconverting, structurally, and dynamically distributed intermolecular interactions that can be relatively compact and exhibit significant nonrandom structure quantities. Leftover the secondary and tertiary structure are maintained in unfolded positions relating various long-term interactions, some are native, and many are found in the (folded) natural state. Temperature variations will break the hydrogen bonds and hydrophobic interactions. The explanation is that kinetic energy is increase by heating that the bonds are broken deeply by quickly wobble the molecules. This is one of the reasons in the use of heat for sterilization, as high temperatures lead by killing the bacteria to denaturation of bacterial cell proteins.

Example: Coagulation of protein and egg while frying an egg. Preserving protein solutions on ice and avoiding high temperatures can limit temperature-caused denaturation. Higher temperature is a major stress for protein which produces damaged globular structure. A temperature plot is sigmoidal to the unfolded protein fraction, and the medium point (standing for the melting temperature) is indicated as Tm value. Increasing Tm value can assume the improvement in conformity stability. This could be exact in the thermal transfer to compare the reversibility degree between folded and unfolded form of protein.

Over the past 20 years, however, reversibility comparison has proved to be an even stronger indicator than Tm value for notice storage stability. Subsequently, other measures of conformation stability, such as those obtained from the studies of chemical denaturation, may be more efficient in decisions guiding on formulation. Using DSC, it is noted that denaturation induced thermally is an irreversible phenomena as molecules of unfolded protein easily combine and aggregate. Ever after the study by Sanchez-Ruiz et al. [81], there are frequent Tm′s rate dependence reports of DSC to examine rate of aggregation by adjusting the scan rate. More recent attempts to create more generalized kinetic schemes have been made. Mathematics assistance can be very much involved in overcoming some of the restrictions on the order of reaction of previous methods.

13.8.2 Freeze-Thaw

Freezing and thawing can interact with indigenous protein conformation and pH variability and buffer portion precipitation all of which can lead to protein denaturation. Freezing effects are determined by freezing to avoid the denaturation caused by freezing. Keeping aliquot of 50% glycerol so it would be easy to remove an

aliquot rather than freezing and deal with the whole solution. There are only few reports on cold denaturation of protein because this process has been recorded since 1961. Denaturation of protein is not of major significance because it is noted that majority of proteins denatured by cold only below than water freezing point. Tg' is glass transition temperature of fully freeze state (< -20 °C). It means that proteins may have flexibility similar to that of a fluid solution in a frozen state of −20 °C. Cold denaturation potential may therefore be higher than what is expected. For instance, a recent IL-1ra study found that the temperature for cool denaturation is −10 °C and reachable easily in frozen condition except the temperature is < −30 °C in storage.

13.8.3 Physical Factors

Physical instability refers to the process by which by altering the chemical composition, the protein changes its physical structure. Owing to protein storage, physical denaturation happens, shaking triggers protein accumulation, vortex shaking, etc. Do not agitate, shake, or churn violently (don't foam protein solutions) and help prevent denaturation of protein. To avoid protein denaturation due to solution effects, replicate the cell environment and maintain ionic composition and neutral pH. Maintaining as much as possible protein concentration (> 1 mg / ml) dilution effects can be prevented.

13.8.4 Oxidation

Oxidation of proteins can disrupt protein stability and may use strong reducing agents such as DTT (or β-ME) in buffers to prevent oxidation-induced denaturation [15]. Proteins, often of major biological effect, have been known to be resistant to oxidize damage. Oxidized proteins have also been shown to increase in the aging of animals, thereby adding to the probability of protein oxidation leading to aging [33, 90]. Methionine has been shown to be oxidated into the form of sulfoxides in a broad variety of proteins and sometimes inhibits or prevents biological activities. As proteins are used as medicines, methionine oxidation is of particular concern [4]. Many atmospheric oxidants were also shown to produce the methionine sulfoxide in proteins and peptides [5, 58].

Oxidize methionine affects the organic role, in certain instances; it is known as destabilizer of native protein structure with apparent effects on activity [14]. It is well-known that substitution in the lateral chain – has major effects on stability. Further oxidation can be regarded as chemical mutagen, which substitutes the methionine side chain to methionine sulfoxide a larger mutated side chain. Surprisingly, the structure and stability of proteins would not be greatly affected. A potential way of protecting to methionine oxidation consequences, code a protein with other

residues of side chains which is known to resist the oxidation process. Altering active or binding sites is another attempt to avert the methionine oxidation which primarily leads to inactivity of proteins. Subtilisin, anti-trypsin, and D-amino acid oxidase are all oxidative inactivators that impact on methionine substitution [27, 79]. However, within just one case that we are aware of, the effect of methionine oxidation on stability combined with mutagenesis has been measured to anesthetize the protein toward oxidation. This distinction is significant; subsequently, a side chain substitution effects can be equally sensitive as this site is mutated by oxidation.

Eventually methionine oxidation can influence the protein structure and stabilization by reducing side chain hydrophobicity, increasing the hydrogen binding ability, and modifying methionine size and shape. Relative value of these variables depends on a given methionine's particular atmosphere. It can also be assumed that if the source of instability will change in side chain polarity after oxidation, then the results of nonpolar side chain substitution could be minimal. At oxidation of a given methionine residue, it is difficult to estimate the degree of stability changes, much less whether the side chain substitution is permanent.

13.8.5 Heavy Metals

Heavy metal salts typically contain Hg2 +, Pb2 +, Ag2 +, Ti1 +, Cd2 + and other weighed atomic metals. Since salts are ionic, salt connections break in proteins; the contact between heavy metal salts and protein usually leads to unsolvable metal protein salt. By reducing the denaturation of proteins, EDTA in buffers helps subsides heavy metals there.

13.8.6 Microbial Growth

Sanitized procedures that can include antimicrobials may be used to inhibit microbial production and/or frozen and to help prevent microbial growth.

13.8.7 Proteases

Proteases split the protein and thus absence the structure of the protein, leading to denatured proteins including protease inhibitors. Holding protein solutions on ice contributes to reducing protease effect.

13.8.8 Denaturation

Denaturation means that most proteins tolerate the loss of the globular or 3D shape. This globular configuration is called the native state, but it is well recognized to be merely a multiplicity of microstate. Denaturation can lead to loss of the secondary or tertiary structure (or both). Subsequently, it will denature the physical form, but chemical structure remains the same.

13.8.9 Chemical Denaturation

Chaotropes are the compounds that damage the globular structure of proteins by another technique of denaturation to unwind the protein; among these, guanidinium hydrochloride and urea are the most common. Urea also helps to prevent hydrophobic collapse linked to the development of a globular native structure. In comparison with absent solutes, chaotropes tend to bind proteins, which reduces their chemical potential. The protein unfolds to smaller than native state, so native state has usually much surface area than unfolded structure, and the chemical potency of unfolded state is further reduced. The pKa of polypeptide side chain has been reported to be modified by 0.3 to 0.5 units using high concentrations of GnHCI or urea. Through enhanced electrostatic repulsion, it alone can affect the protein's conformation stability.

13.8.10 Pressure-Induced Denaturation

The molecular ground for denaturing caused by pressure has recently been identified. Interestingly, the potential of osmolytes applied to stabilize proteins also tends to strain-caused denaturation. Furthermore, unlike other unfolding protein pressures, the pressure-induced denature is reversible. The intermediate pressures of 1000–1500 bar can be used to separate aggregates and enable the aggregated protein to be easily replunged.

13.8.11 Solid State Denaturation

As discussed above, at high temperature, proteins are denatured, but the mobility of proteins may presume in solid state. For dried proteins, the recorded denaturation temperatures are mostly very high, i.e., above 150 °C. Like Tg value, the Tm value seems to scale with humidity level. For example, for hGH, denaturation occurs just above Tg temperature, is largely irreversible and cooperative.

13.8.12 Intrinsically Denatured Proteins

There has been a discovery over the past decade that many random coiled proteins function as unfolded structure, under native conditions. These proteins are intrinsically denatured proteins (IDPs), whose certain pharmaceutical value proteins especially belong to superfamily of fibroblast growth factor. Proteins may also be interactive, without denaturation occurs in their globular structure, in the normal sense.

13.9 Limitations for Stability Studies

- Stability studies are valid only when the breakdown depends on temperature.
- Stability studies are accurate for only 10 to 30 kcal / mol activation energy. Most reactions have activation heat ~10 to 30 k cal/mole in solution phase. If the activation energy is <10 kcal / mol, its rate at room temperature would be rapid. Elevated temperature has slight effect on denaturation in these cases. If the activation energy is >30 kcal / mol, it needs very high temperatures for degradation. Reactions may be meaningless at such a high temperature, because they do not represent the state of ambient storage.
- The result reported for one set of formulation condition cannot be extended to another of the same product preparations.
- Stability prediction at high temperatures is of little benefit as diffusion, microbial contamination, and photochemical reaction result in degradation.
- Stability experiments are useless if the substance loses its physical integrity at higher temperatures such as suspending agent coagulation and protein denaturation.
- The prediction would be incorrect if the order changes at high temperatures, as in the case of a suspension (zero order) which at higher temperatures is converted to a solution following the first order.

13.10 Chemical Kinetics

It is the study of the rate of chemical processes or reactant transformations occurring in the products according to the basic mechanism, i.e., the mechanism of reaction.

13.10.1 History of Chemical Kinetics

By the implementation of law of motion and mass, Cato Guldberg and Peter Waage in 1864 stated the chemical kinetic studies. According to this, the speed of a reaction is directly proportional to available quality of reacting materials. Van't Hoff was awarded the First Nobel Prize in 1901 in chemistry. He published "Études de

dynamique chimique" and deliberated on chemical mechanics in gratitude of the strange amenities while researching on the law of osmotic strains and chemical kinetics. For zero-order reactions, simple rate laws exist, which require reaction rates to be taken irrespective of first-order reactions and the second-order reactions. Basic reactions obey the rule of mass movement, but the rule of the rate reactions step by step can lead to a mix of the rate laws from the different basic phases and can become very complicated. The first is van't Hoff wave, related to thermodynamic kinetics which is general chemical reaction laws. The second is focus on reaction mixture and called as Semenov Hinshelwood wave. Third wave is related entirely to mathematical science of chemical reaction networks.

13.10.2 Rate of Reaction

The sum of a chemical change occurring per unit time is called reaction rate. The rate is usually indicated as the increase or reduction in the concentration of a reactant or drugs, where the reactant concentration is dx / dt at any point [86].

13.10.3 Order of Reaction

The number of absorption conditions that depend on the speeds of the reaction is called reaction order. If reaction rate depends on the first reactant concentration, then the rate of reaction is = KC1. A chemical reaction whose intensity does not depend on the attention of the reactant is called a zero-order chemical reaction. So the reaction is presumed to be of first order. The second-order reaction presumed when the intensity is equal to the sum of absorptions of reactants or the square of absorption of a reactant.

13.10.4 Molecularity of Reaction

The molecularity of a reaction is known as the amount of molecules or atoms that create a part in a process of chemical change. According to one, two, or three molecules, the reaction is called unimolecular, bimolecular, and trimolecular. The term unimolecular was used in the first order for all reactions, the term bimolecular for second order reactions, etc.

13.10.5 Types of Chemical Reactions

13.10.5.1 Instantaneous Reactions

Such reactions occur very quickly once the reactants are recognized. These reactions are called ionic reactions as they contain ionic compounds. It takes between 10–14

and 10–16 seconds for such reactions to initiate. Regulating the frequency of such reactions is typically unbelievable. There are two examples of this:

- When in aqueous solutions, an acid mixed with base, the reaction is called neutralization:

$$HCl + NaOH \rightarrow NaCl + H_2O$$

- When silver nitrate and sodium chloride solutions are combined, precipitation of AgCl occurs.

$$AgNO_3 + NaCl \rightarrow AgCl + NaNO_3$$

- By reaction of sulfuric acid and barium chloride, precipitation will occur [70].

$$BaCl_2 + H_2SO_4 \rightarrow BaSO_4 + 2HCl$$

13.10.5.2 Slow Reactions

There are very slow realistic reactions. It takes months to reveal any quantifiable change at room temperature. The kinetics of these reactions, too, are difficult to understand.

Some examples are:

- Hydrogen and oxygen reaction at room temperature. Atmospheric H2S clearly leads acetate reaction.
- Carbon and oxygen reaction: at 298 K, CO2 is more thermodynamically stable than carbon and oxygen, but coke remains unreacted for year and spontaneously does not catch fire in the soil.
- Carbon monoxide and hydrogen reaction: in actual practice, the reaction at 298 K bur is thermodynamically feasible [26].

13.10.5.3 Moderate Reactions

There are a number of reactions between the above two excesses that occur at room temperature at appropriate and evaluable rates, and in chemical kinetics, these reactions are studied. These reactions are normally molecular type. Below are some examples of this:

- Hydrogen peroxide decomposition: $2H_2O_2 \rightarrow 2HO + O_2$
- Decolorization of acidified potassium permanganate with sodium oxalate.
- Nitric oxide and chlorine reaction: $NO + Cl_2 \rightarrow NOCl_2$
- An ester hydrolysis: $CH_3COOC_2H_5 + NaOH \rightarrow CH_3COONa + C_2H_5OH$
- Nitrogen dioxide and carbon monoxide reaction. $NO_2 + CO \rightarrow NO + CO_2$
- Reaction between ferric chloride and stannous chloride.

$$2FeCl_3(aq) + SnCl_2(aq) \rightarrow 2FeCl_2(aq) + SnCl_4(aq)$$

- Cane sugar inversion in aqueous solution: $C_{12}H_{22}O_{11} + H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6$
- Nitrogen pentoxide decomposition: $2N_2O_5 \rightarrow 2N_2O_4 + O_2$

Changing conditions under which they occur can decelerate or paced chemical reactions, e.g., the reaction is very slow. By maintaining temperature about 400oC and pressure about 300 atmospheres, CO + 2H2 CH3OH can be increased by using a ZnO and Cr2O3 containing catalyst. Decline in foodstuffs can be decelerated by refrigerating them [109].

13.10.5.4 Fast Reactions

For rapid reactions, the time needed may be similar or longer than reaction's half-life to combine the reactants and get them to a defined temperature. Specific solutions will minimize the time to the millisecond level for twitching rapid reactions. Flash photolysis, without a slow mixing step, include stopped flow techniques, where a laser pulse produces super excited speed.

13.11 Factors Affecting the Reaction Rate

13.11.1 Physical State

The physical state of a reactant (solid, liquescent, or vapor) is also a significant factor in the degree of alteration. Once reactants are as like as in aqueous solution, they may bring into contact by thermal movement. However, when they are in separate phases, the reaction to the interface between the reactants is incomplete. In the case of a liquid and a gas, reaction may only occur in interaction area. To complete the reaction, strong shaking and agitating may be appropriate. It means differentiation of the solid or liquid reactant, the greater the surface area per unit volume, and the greater the interaction with that other reactant, the more rapidly the reaction occurs. For example, you use wood chips and tiny branches to make a comparison, when you start a fire, you don't start with big logs right away.

13.11.2 Surface Area of Solid State

A reaction in a solid may involve only those particles that are on the surface. When a solid crushed into smaller particles, the reaction occurs more quickly. Because of the collision, probability between these smaller reactant particles was increased. For example, a mixture between a very fine weak organic acid malic acid and sodium hydrogen carbonate is sherbet (liquid).These chemicals dissolve and react rapidly in the mouth upon contact with the saliva, providing the taste of citrus and releasing

carbon dioxide. Fireworks manufacturers also regulate the rate at which the fireworks oxidize the fuels by adjusting the surface area of the solid reactants, to produce different effects. For example, finely split aluminum is exploding violently enclosed in a shell. The reaction is slower when larger aluminum parts are used, and sparks that is burning metal parts are shown as removed.

13.11.3 Concentration

The reactant species collide in these reactions. The frequency at which molecules or ions react depends on whether they occur faster is 21% O_2 (the pure oxygen). The more crowded these molecules are, the more likely they will collide with each other and react. As a result, a rise in concentrations of reactants will normally have an inverse effect usually consequence in decrease in concentrations and increase in rate of reaction. The rate equation explains the extensive reaction rate dependence on the reactants and other present species concentrations. The reaction mechanism depends upon the mathematical expression. For a given reaction, the actual rate equation is determined experimentally and describes the mechanism of reaction. The rate of mathematical equation is given by rate of constant reaction which is the molar concentration and partial order of reactant. A reactant's partial order could be experimentally calculated, and even its stoichiometric coefficient is not indicated.

13.11.4 Temperature

In general, temperature has a significant influence on the amount of a chemical reaction. They have higher temperatures and more thermal energy. While the incidence of collisions at higher temperatures is greater, this rise the reaction rates by a very small proportion. Most importantly, the proportion of reactant molecules with sufficient energy to react (energy higher than activating energy: $E > Ea$) is substantially higher and is explained in detail by molecular energy. A common misunderstanding is the "general rule" which doubles the rate of chemical reactions for every 10 ° C temperature rise. This may have been generalized from the biological systems' special case, where the A is often between 1.5 and 2.5. The method of temperature jumping may be used to study rapid reaction kinetics. This includes the use of a sudden temperature increase and measure of the time of stress relief to come back to equilibrium. A shock tube is a particularly useful form of temperature jumping system, which can quickly increase a gas's temperature by over 1000 degrees.

13.11.5 Catalysts

Generic energy potential diagram showing the catalyst effect in a hypothetical chemical endothermic reaction. The catalyst's presence opens a new reaction path

with lower energy activation. The end result is the same as for thermodynamics in general. A substance which is chemically unchanged but increases the chemical reaction is called catalyst. In lower activation energy, the catalyst increases the reaction rate by supplying a new mechanism for reaction to occur with. In a reaction, product itself is an autocatalysis with positive feedback. Enzymes are called proteins which function in biochemical reactions as catalysts. A catalyst does not affect the equilibrium path, because the catalyst accelerates the reactions backward and forward equally. Related substituents in some organic molecules may influence the rate of reaction in the involvement of neighboring group.

13.11.6 Pressure

The number of reactant collides, and the rate of reaction increases by increasing the pressure in a gas reaction. This is because a gas's activity is directly proportional to the gas's component pressure. That is similar to the product of a solution's rising concentration. Beyond clear effect of mass action, the coefficients of the rate itself change because of strain. The rate coefficients and products of such high-temperature gas-phase reactions alter when an inert gas is added to the mixture; changes to this effect are called fall-off and chemical activation. Such results are due to exothermic or endothermic reactions occurring faster than heat transfer, causing nonthermal distributions of energy for the reacting molecules. By increasing pressure, the rate of heat transfer between reacting molecules and the rest of system increases. Thus, pressure can also affect the coefficients of the condensed-phase scale by reducing this effect, but a measurable effect requires very high pressure because the ions and molecules are not very compressible. This effect is studied also using diamond anvils. The kinetics of a reaction can be studied with a pressure jumping method which includes time recovery and quick adjustment in pressure to be back to normal.

13.11.7 Absorption of Light

Study of light-initiated reactions with photosynthesis being one of the prominent examples of photochemistry. When one reactant is pushed to an excited state by absorbing an appropriate wavelength of light, the activation energy can be provided to it.

13.11.8 Free Energy

Generally speaking, the free energy transfer of a reaction determines that there is going to be a chemical shift; however, kinetics describes how quick the reaction is. A reaction may be very exothermic and can result in a very positive increase in entropy, but in reality, it will not occur if the reaction is too late. If a reactant may produce two

products, it typically forms the most thermodynamically stable one, except in particular circumstances where it is stated that the reaction is under kinetic reaction. Predictions can be produced for a reaction from free energy relationships on constants in reaction rate. The kinetic isotope effect is the difference in the rate of a chemical reaction when one of the isotopes in one of the reactants replaces an atom. Awareness of heat transfer and residence time in chemical engineering in a molar mass distribution and nuclear reactor in polymer chemistry are given by chemical kinetics. It also includes the knowledge of corrosion engineering [70].

13.11.9 Stability Testing

A drug product can undergo changes in consistency, appearance, uniformity, clearness (solution), moisture content, package integrity, pH, particle size, and shape all affecting its stability. These physical changes can occur due to variations in impact, vibration, abrasion, and temperature such as freezing, thawing, shearing, etc. The chemical reactions that occur in pharmaceutical products such as solvolysis, oxidation, reduction, racemisation, etc. can lead to degradation of drug formation, loss of active pharmaceutical ingredient potency (API), loss of excipient activity such as antimicrobial preservative action and antioxidants, etc. [9]. Microbiological development such as non-sterile microorganism and improvement of preservative performance affected the pharmaceutical product stability [65]. Table 13.2 illustrated possible adverse effects of volatility on pharmaceutical products.

13.12 Need and Purpose of Guidelines for Stability Studies

In order to ensure that good drugs are developed that may be sufficiently potent to last until their duration of stability is well promoted and reaches people on time. In many countries, authorities have concerned that the manufacturers should make available information about the power or consistency of the same drug or the length of its shelf life. The goal was to bring all manufacturers to similar test methods. The guidance embodies the simplest drug / stability-related problems. It includes documentation on the way to qualify for drug manufacture by supplying requisite details on shelf life, efficacy, and durability of a drug and strategies for applying it. Such kinds of recommendations emerged first in the 1980s (www.ICH.org).

13.12.1 Brief History

In 1987, the FDA provided its initial guidelines (Huynh 2010).

Table 13.2 Potential adverse effects of instability in pharmaceutical products

Potential adverse	Potential adverse	Potential adverse	Potential adverse
Loss of active ingredient	Degradation of API in product resulting in less than 90% drug as claimed on label – unacceptable quality	Nitroglycerine tablets	Time elapsed before the drug content no longer exceeds 90%
Increase in concentration of active ingredient	Loss of vehicle perfusion bags sometimes Allow solvent to escape and evaporate so That the product within the bag shows an increase in concentration.	Lidocaine gel, products in perfusion bags	Stability in final container
Alteration in bioavailability	Changes in rate and extent of absorption on storage	_	Dissolution/ release studies
Loss of content uniformity	Loss of contents as a function of time	Suspension	Ease of re-dispersion or sedimentation volume
Decline of microbiological status	Increase in number of viable microorganisms already present in the Product. Contamination because of compromised package integrity during Distribution/ storage	Multiuse cream	Total bioburden after storage
Loss of pharmaceutical elegance and patient acceptability	Speckling caused by the interaction of the drug containing amine group with a minor component in the lactose resulting in the formation of a chromatophore	Slight yellow or brown speckling on the surface of tablet containing spray dried lactose	Visual examination
Formation of toxic degradation products	Degradation of the drug component	Formation of epianhydrotetracycline from tetracycline, protein drugs	Amount of degradation products during shelf life
Loss of package integrity	Change in package integrity during storage or distribution	Plastic screw cap losing back-off-torque	Specific package integrity tests
Reduction of label quality	Deterioration of label with time and cause the ink to run and thus adversely affect legibility	Plasticizer from plastic bottle migrates into the label	Visual examination of the label
Modification of any factor of functional relevance	Time-dependent change of any functionally relevant attribute of a drug product that adversely affects safety, efficacy, or patient acceptability or ease of use	Adhesion aging of transdermal patches	Monitoring changes

13.12.2 Guidelines by the Food and Drug Administration

- Incorporate medication safety designs, correct expiry dates, storage methods, and precautions to be taken during medication storage.
- To apply data on the investigational stability analysis of synthetic products, biologics, experimental drug applications, and the application for a biological product license have stressed upon.

Later, various regulatory bodies from different countries established their own strategies. Because of discrepancies on these guidelines, the need to harmonize the guidelines was felt strongly. In the International Conference on Harmonization (ICH), regions, such as the USA, Japan, and Europe in the 1990s, make efforts in stability practice to bring uniformity. They made uniform products data in the ICH at a later date to register and promote in various regions. The ICH was a union where the regulatory and manufacturing industries of ICH regions made daily suggestions. It also extended earlier ICH guidelines for veterinary products [52].

13.12.3 Benefits of Regulatory Harmonization

For each changing regulatory authority, regulatory harmonization provides many direct advantages, as well as the manufacturing trade with useful advantages required to protect people's health. The key benefits are preventing the duplication of human clinical trials, thus reducing animal testing and without sidelining shelter and efficiency, controlling, formulating the procedure for testing new applications of drugs, and reducing development period and cost of drug production [52]. Slow but great developments have taken place in the International Conference on Harmonization since its participation in 1990, and the ICH has seen significant growth in its initial period of entry into action, in particular in the areas of health, safety, and effectiveness. There have been studies on multidisciplinary topics, including Common Technical Document (CTD) and Medical Dictionary for Regulatory Studies (MedDRA) [11, 89].

13.12.4 ICH Guidelines

In November 2005, the International Conference on the steering committee for harmonization assigned codification for ICH Guidance. The goal of allocating new codes was to make it easier to enforce in practice, and sure there is ambiguity in it. The allocated codes are based on a number of revisions such as (R1), (R2), and (R3). This was done to make guideline codification for ICH easier for all. Several secessions have also been applied to the guidelines, and the key or core guidelines are called revisions (e.g., R1) [11, 89].

13.12.5 General Categories of ICH Guidance

The following groups and codes were assigned according to www. ICH.org.

13.12.6 "Q" Guidance

Efficiency guidance within the main area of harmonization encompasses critical achievements, as do methods of understanding drug stability studies, setting minimum thresholds needed to check impurities in the studies' products and to determine the quality of the goods produced in accordance with good manufacturing practice (GMP) risk management [89]. The ICH-released "Q" guidelines are listed in Table 13.3.

13.12.7 "S" Guidance

These are health recommendations that provide safety precautions to reject serious problems such as gene toxicity, cancer, and kidney toxicity. The ICH-issued "S" recommendations are specified in Table 13.4.

13.12.8 "E" Guidance

These guidelines regulating the manner of planning trials, trails performing, and the shelter measures taken and reporting on the clinical trials that have been carried out. This also regulates the various vital forms of medicinal products which are attained by the adoption of specific biotechnological procedures. Additionally, the use of pharmacokinetics and pharmacogenomics methods are applied in the development of the best medicines. The ICH-released "E" Guidelines are listed in Table 13.5.

Table 13.3 Showing ICH "Q" guidelines. Source: www.ICH.org

Q1 A –Q1 F	Stability guidelines
Q2	Analytical validation
Q3 A –Q3 D	Impurities
Q4 –Q4 B	Pharmacopoeias
Q5 A –Q5 E	Quality of biotechnological products
Q6 A –Q6 B	Specifications
Q7	Good manufacturing practices
Q8	Pharmaceutical development
Q9	Quality risk management
Q10	Pharmaceutical quality system
Q11	Development and manufacture of drug substances
Q12	Lifestyle management

Table 13.4 Showing ICH "S" guidelines. Source: www.ICH.org

S1 A –S1 C	Carcinogenicity studies
S2	Genotoxicity studies
S3 A –S3 B	Toxicokinetic and pharmacokinetics
S4	Toxicity testing
S5	Reproductive toxicology
S6	Biotechnological products
S7 A –S7 B	Pharmacology studies
S8	Immunotoxicology studies
S9	Nonclinical evaluation for anticancer pharmaceuticals
S10	Photosafety evaluation
S11	Nonclinical safety testing

Table 13.5 Showing ICH "E" Guidelines. Source: www.ICH.org

E1	Clinical safety for drugs used in long-term treatment
E2 A-E2 F	Pharmacovigilance
E3	Clinical study reports
E4	Dose response studies
E5	Ethnic factors
E6	Good clinical practice
E7	Clinical trials in geriatric population
E8	General considerations for clinical trials
E9	Statistical principals of clinical trials
E10	Choice of control group for clinical trials
E11	Clinical trials in pediatric population
E12	Clinical evaluation by therapeutic category
E14	Clinical evaluation
E15	Definitions in pharmacogenetics and pharmacogenomics
E16	Qualifications for genomic biomarkers
E17	Multiregional clinical trials
E18	Genomic sampling methodologies

13.12.9 "M" Guidance

These are multidisciplinary guidelines, in which cross-cutting disputes do not activate clearly within the reference classes of health and effectiveness community. It includes the International Conference on the Common Technical Manual (CTD) and Medical Terms of Harmonization (MedDRA) and the establishment of Electronic Standards for the Transmission of Regulatory Knowledge (ESTRI). Table 13.6 lists the "M" Guidelines established by the ICH.

Table 13.6 Showing ICH "M" Guidelines. Source: www.ICH.org

M1	MedDRA terminologies
M2	Electronics standards
M3	Nonclinical safety studies
M4	Common technical document
M5	Data elements and standards for drug dictionaries
M6	Gene therapy
M7	Genotoxic impurities
M8	Electronic common technical document (eCTD)

13.13 Guidelines for Stability Studies

13.13.1 ICH and FDA Stability Regulatory Guidelines

One of the subject areas protected by the guideline documents of the ICH is checking stability for drug registration. The ICH mutually administers managers and pharmaceutical companies from the USA and Japan converging all the technological measures for drug-containing medical products. In the early 1990s, this organization was launched, and one of the first topics was stability monitoring to be carried out by regulatory bodies from all three regions through the stepwise method of recognition [19].

Currently, with in manufacturing industries, stability studies are a significant approach approved for emerging brand new drugs and products. Stability analysis is also used to recommend desirable situations for storage of the imports and emphasize the fact for marketing purposes the expiry date and potency details of the drug must be specified on the cover of the outer packaging of the product. Specifying that the drug is harmless and effective up to its expiry date definite on suggested storage conditions is added to the outer packaged of the product. It is certified on the label inside that the medication is harmless and operative for its entire shelf life. Monitoring requirements being made tighter and tighter to touch the optimum target of each possible situation so that the medication can be put during its shelf life. Therefore, after the adoption of good science principles, the stability studies can be performed by accurately understanding the standing regulations regulating the same and also taking into account the environment sectors [89].

13.13.2 Guidelines

To precisely illuminate the details of stability required to record the new drug and products, a series of guidance documents were created within the ICH regions. These guidance documents will adhere the stability practice conducted for registration of products. There are currently five online guidance documents.

13.13.3 Q1A

Stability testing of new drug materials and goods lays down the basic procedure for registration stability studies (www. ICH.org). In both materials and goods of new drug, this monitor sets out types and number of lots, stabilization vessel closing schemes, and time arguments, and storage situations are revised to endorse registry. The ICH guidance documents reference for more comprehensive details on specifications and impurities. Correct measurements, diagnostic procedures, and proposed acceptance requirements should be used. ICH document reports pressure testing and essential assertions to offer details on new drug material and its stability to three product lots at least, during the recommended period of expiry or retesting [47].

13.13.4 Q1B

The four remaining documents are general guidelines for the protocol. There is the Q1B guideline and photostability checking for new drug drugs and products (www. ICH.org). This article provides recommendations for photostability research on new drugs and drug products that demonstrate that light exposure does not impact materials adversely. The work outlined is performed in a single batch from the stabilization analysis of registration and constitutes a step-by-step process involving exposed medication content and exposed formulation of the drug, shortly following packaging and packaging for distribution product formulation [45].

13.13.5 Q1C

Guidance Q1C provides information about testing stability of new dosage (www. ICH.org) was written by the holder of the initial submission to explain the criteria for a new dosage form or line extension. In this case, Q1A criteria were followed, but at the time of submission, less data may be needed. In the Q1A parent guidance, the use of brackets or matrix is stated to minimize the amount of testing for registration associated with the stability program.

13.13.6 Q1D

Guidance Q1D offers more comprehensive matrixing and bracketing proposals for drug substance and drug product stability testing [46] on the subject. The guide addresses when to use each of these methods and offers examples of them. This also addresses the short prototype possible dangers for testing.

13.13.7 Q1E

The fifth guidance, Q1E, Stability Data Assessment [47], offers additional details regarding the method of assessing and analyzing the produced details, statistically following the Q1A guideline. This guide includes a step-by-step method for evaluating the data on stability and extrapolating the knowledge obtained to indicate the product's expiry date. It addresses applying linear regression, pool capacity checks, and statistical modeling to registration stability results.

13.13.8 Q5C

An additional guidance has been drawn up to complement these guidance documents for the study of biotechnology products [44]. The guidelines set out only key stability registration system requirements. All the information required for designing and maintaining the stability system to facilitate the registration of new goods. In addition, the brief applications for the registration of generic medicinal products go beyond the scope of the ICH documents, but the guidance documents may be supplemented by general guidelines in order to facilitate registration studies. A paper published by the FDA in 1987 offers further guidance and recommendations for sending reports on the stability of human drugs and biologics [28]. In 1998, the FDA developed a draft guideline for industry following this paper: stability testing of drug substances and medicinal products [29].

13.13.9 ICH Q1AR2

It merged ICH Q1AR2 with several different guidelines from the ICH guidelines. For those who are carrying out stability studies, this guideline acts as basic guide. In the year 2004, the International Conference on Harmonization provided Q1F guidelines, which recommended ongoing stability research programs to facilitate third and fourth zones. The Association of Southeast Asian Nations (ASEAN) pronounced the requirements needed to be met and enforced for the extremely hot and humid climate.

In June 2006, both documents of the FDA, one is the stability guidance and the other is the draft guidance, were removed. As a result, in July 2006, the ICH revoked the ICH Q1F guideline. As part of the agency's implementation, sustainable production practices in pharmaceutical current (cGMPs) for the twenty-first century emerged ([30]; www.fda.gov/cder/gmp).

The QbD, quality by design principles established by the Food and Drug Administration in drug development, has been the most debated subject of all time. For easy reference, Table 13.7 shown the ICH assigned names and their respective codes of Harmonization guidelines (www.ICH.org; [51]). In addition to different factors that influenced the stability, the guidelines took account of the climatic regions. Specific

Table 13.7 Codes and titles used in ICH Guideline. Source: www.ICH.org

Q1A	Stability testing of new drug substances and products (second revision)
Q1A (R2) 2	Stability testing of new drug substances and products
Q1B	Stability testing: Photo stability testing of new drug substances and products
Q1C	Stability testing of new dosage forms
Q1D	Bracketing and matrixing designs for stability testing of drug substances and products
Q1E	Evaluation of stability data
Q1F	Stability data package for registration applications in climatic zones III and IV
Q5C	Stability testing of biotechnological/biological products

guidelines for the particular drug delivery system were drafted for each product. In addition, guidelines were also developed from the accelerated studies for inferences.

13.14 CPMP Stability Guidelines

Under the European Agency for Assessment of Medicinal Products, the Committee on Planted Medicinal Products (CPMP) published a set of guidelines relating to stability studies in contribution of seeking market authorization for medicines beyond their acceptance of the ICH instructions [19, 52].

These guidance documents are as follows:

13.14.1 Guidance CPMP/QWP/122/02

Checking stability of known active substances and associated finished products [21].

13.14.2 Guidance CPMP/QWP/576/96

Stability testing for applications for marketing authorization variations [20] offers the information required to perform studies on product stability that should be produced in support of variations made to marketing authorization. It gives some examples of variations and the stability data types and quantities that can be required to sustain them.

13.14.3 Guidance CPMP/QWP/2934/99

In-use stability testing of human medicinal products [23] offers the requisite details required to perform stability studies to assess the length of time a multidose product should be used after it has been first opened.

13.14.4 Guidance CPMP/QWP/159/96

Maximum shelf life for human use sterile products after first opening or subsequent reconstitution [22] notes that research should be carried out to confirm the realistic usage of sterile product. It also includes sample wording to provide the correct hold times and storage conditions in consumer labeling until the product is opened, diluted, or reconstituted.

13.14.5 Guidance CPMP/QWP/609/96

Accordingly, the declaration of storage conditions has been defined as follows: A: in the stock details of medicinal products and B: for active substances [25], document providing standard commodity storage status declaration shall contain more details (Annex) to the ICH Stability Guidelines. On the basis of the stability data generated according to the guidelines given by the ICH, an appropriate storage labeling statement and, if applicable, further storage statements are recommended.

13.14.6 Guidance CPMP/QWP/072/96

The beginning of a pharmacological shelf life [24] explains how a pharmaceutical product's expiry date can be calculated and assigned depending on the period of release or manufacture. For easy reference, the guidelines are mentioned in Table 13.8.

Table 13.8 Codes and titles used in ICH guidelines. (CPMP/QWP/122/02 2003)

CPMP/QWP/576/96 Rev. 1	Guideline on stability testing for applications for variations to a marketing authorization
CPMP/QWP/6142/03	Guideline on stability testing for active substances and medicinal products manufactured in climatic zones III and IV to be marketed in the EU
CPMP/QWP/609/96 rev. 1	Note for guidance on declaration of storage conditions for medicinal products particulars and active substances
CPMP/QWP/122/02 rev. 1	Note for guidance on stability testing of existing active substances and related finished products
CPMP/QWP/072/96	Note for guidance on start of shelf life of the finished dosage form
CPMP/QWP/2934/99	Note for guidance for in-use stability testing of human medicinal products
CPMP/QWP/576/96	Note for guidance on stability testing for a type 2 variation to a marketing authorization
CPMP/QWP/159/96	Note for guidance on maximum shelf-life for sterile products after first opening or following reconstitution

13.15 WHO Stability Guidelines

The World Health Organization (WHO) has provided guidelines on stabilization research [19]. The research on stability studies started in the year 1988 by the World Health Organization. In the technical expert, list No. 863 of Annex-V of the WHO was given guidelines for stability studies of drug substances in traditional dosage to prepare pharmaceutical requirements to expert committee [106]. Refusal to follow ICH guidelines to improve extreme weather conditions in many countries and announce only new medicines and products and no medicine recommendations already available on the markets of World Health Organization countries [52]. In the year 1996, the World Health Organization made some amendments to the International Conference on Harmonization. This advice was adopted in 2003 and 2006 to benefit for zone IV climatic areas for owing long-term storage conditions [107, 108]. In the year 2004, the World Health Organization also released guidelines on stability checking in the global environment [52].

The new World Health Organization's first draft, Stability Guidelines, were published in the year, April 2007, for feedback and suggestions. In October 2007, on the basis of the guidelines for stability of the eastern Mediterranean region of the WHO, the second draft was available. In addition, technical monograph of testing stability of new drugs and products has been published by Indian Drug Manufacturer's Association. In addition, other checking criteria for drug formulation and active pharmaceutical substance have also been set out in the guideline [52]. As other not specifically mentioned countries, many have accepted the ICH or WHO criteria for their stability assessment criteria [19].

13.16 Stability of Climatic Zones

Based on the environmental conditions, the entire planet has been divided into four zones to which pharmaceutical products for stability purpose are likely to be exposing in their storage period.

For these areas, these parameters were measured on the basis of mean annual temperature and relative humidity. On the basis of that data, long-term or real-time stability test conditions and accelerated stability test conditions have been established. The traditional climate zones for use in stabilization studies of pharmaceutical products were presented in Table 13.9. The rise up of ambient conditions in each zone and the resulting long-term stability test storage conditions as given by the WHO were also presented. The stability needs have also been harmonized to make the implementation of industry more practical and reliable for widespread use [48].

13.16.1 Mean Kinetic Temperature

A single temperature at which the total degradation in a given time is equal to cumulative degradation of individual degradations occurring in a different higher or

Table 13.9 ICH climatic zones and long-term stability conditions. [37, 48]

Climatic zone	Climate/ definition	Major countries/ region	MAT[a]/Mean annual partial water vapor pressure	Long-term testing conditions
I	Temperate	United Kingdom, Northern Europe, Russia, United States	<15 °C/<11 hPa	21 °C/45% RH
II	Subtropical and Mediterranean	Japan, Southern Europe	>15–22 °C />11–18 hPa	25 °C/60% RH
III	Hot and dry	Iraq, India	>22 °C/<15 hPa	30 °C/35% RH
Iva	Hot and humid	Iran, Egypt	>22 °C/>15–27 hPa	30 °C/65% RH
IVb	Hot and very humid	Brazil, Singapore	>22 °C/>27 hPa	30 °C/75% RH

[a]MAT – Mean annual temperature measured in open air

lower time span is mean kinetic temperature (MKT). MKT is an isothermal storage temperature which simulates the non-isothermal effects of variations in storage temperature. MKT takes frequent and seasonal in temperature over a year. It reflects the accumulated thermal stress a commodity undergoes during storage and delivery at various temperatures. The definition of MKT is applied to ensure that the real storage conditions do not negatively impact the product's shelf life and stability. It focuses on the fact that the degradation rate constants are temperature dependent. Thermostatically adjusted room temperature to the usual operating range of 20 °C–25 °C results in a kinetic average temperature to not exceed 25 ° C. It includes stores, clinics, dealers, and storage areas, as well as vehicles and warehouses. Articles can be defined either by the term "restricted room temperature" or by a term/phrase of some other suitable definition based on the same kinetic temperature. The distribution in four different climatic zones of world countries and regions was focused on mean cinematic temperatures ([38, 48]; USP 1995). To calculate a mean kinetic temperature, i.e., two processes are used; USP program and FDA process MKT in the USP cycle are based on average storage temperatures over the course of 1 year, while the actual working weekly average for the preceding 52 weeks is high and low. This resulted in 52 data points, and the Hayne equation is calculated, which derives from the Arrhenius equation and relates the deterioration rate constants to activation energy at different temperatures.

$$MKT = \frac{\frac{-\Delta H}{R}}{ln\left(\frac{e^{-\Delta H_{RT}1} + e^{-\Delta H_{RT}2} + \ldots + e^{-\Delta H_{RT_n}}}{n}\right)}$$

Where the MKT = mean kinetic temperature; R = universal constant of gas, i.e., 83.14 kJ/mol;

ΔH = the energy of activation; T_1 = arithmetic mean of lowest and highest temperature recorded during first week; T_2 = arithmetic mean of lowest and highest temperature recorded during second week; n being the total number of average storage temperatures recorded during the annual observation period; and all temperatures T being absolute temperatures in degrees Kelvin (K). MKT calculating approach by entering the actual temperature values (rather than average values) is an equation proposed by the FDA. This result in oppose of point 52 and in favor of 104 points of USP. If temperatures are electronically registered during a day in several times, then there is no difference between the FDA and USP system.

13.17 Importance of Protein Stability

The main reason for the stability test is the interest of the patient with the condition for which the medications are intended. In addition to itemization of the unstable drug into toxic decomposition products, loss of operation up to the label stage of 85% may lead to therapy failure resulting in deaths such as angina and cardiac arrests by nitroglycerine tablets. Because of this issue, the supply of data for some forms of stability checks to regulatory agencies until approval of a new product has become a legal necessity. The second important issue is to maintain the manufacturer's image by ensuring that the product is kept fit for use in relation to all technically related features as long as they are on the market. Some advantages of stability studies at the manufacturing stage or of the products being sold are to provide a database of interest in the collection of suitable ingredients, excipients, and container closure systems for the production of a new product; to assess the shelf life and storage conditions for the production of a new product; to prepare the registration dossier; to substantiate the claimed shelf life for the registration dossier; and to verify that no changes have been made to the formulation or manufacturing process which could adversely affect the product's stability [9, 87].

The field of protein stability made groundbreaking progress in the last 20 years. Chemical instability pathways can now be better understood for better adjustment of the conditions of the solution. It in turn leads to reducing chemical degradation. Instability of proteins can be divided into two main groups, namely, physical instability and chemical instability. The word protein physical instability involves modification of the protein's physical state by precipitation (insolubility), aggregation, adsorption, and denaturation. Chemical instability involves certain processes that break or render covalent bonding, resulting in the creation of new, novel chemical entities.

13.17.1 Chemical Instability

Deamidation is a very popular chemical degradation pathway for proteins and peptides. This process includes the hydrolysis of Gln and Asn amides side chain [18]. Deamidation of Asn takes place under acidic conditions via direct hydrolysis of Asn side chain to make Asp. So the deamidation undergoes acid catalysis [34]. This mechanism occurs at pH 3 and does not involve cyclic imide formation at all [95]. Deamidation of Gln residue is far less common than Asn residues and is generally seen in larger proteins like monoclonal antibodies (Mabs) and crystallins. Cyclization method involves in Gln residues and generates a six-membered ring structure and is considered as less thermodynamically stable than Asn residual five-membered ring structure [83].

13.17.2 Physical Instability

Physical instability refers to the change in physical state of proteins without any change in its chemical composition. Some important physical instability methods are described below.

13.17.3 Denaturation

An important physical instability method is denaturation, which is basically the loss of three dimensional or globular structure of protein. It may also include the loss of secondary and/or tertiary structure of proteins, or may be both [41]. Denaturation may be thermal, cold, chemical, and pressure induced. Thermal denaturation means distortion of globular structure of proteins by elevated temperature. In general, an increase in temperature results in an increase in conformational stability, and also thermal denaturation is irreversible, as the protein structure rapidly changes from folded to unfolded ones, resulting aggregate formation [69].

Cold denaturation refers to the denaturation of proteins by employing "free concentrated states." In this regard, the recommended cold denaturation temperature is approximately 10 °C [94]. Another method of denaturation of proteins is chemical denaturation, which involves the unfolding of globular structure of protein by adding some chemical compounds like chaotropes, urea, and guanidinium hydrochloride (GnHCl) [72]. The mechanism by which these compounds destabilize or distort the globular structure is still not known very well [1]. Pressure-induced denaturation is a reversible process, involving the unfolding of proteins by applying pressure greater than 2000–4000 bar (atmosphere).

13.17.4 Aggregation

Protein aggregation is one of the most cited challenges in the development and manufacturing of protein therapeutics. The term protein aggregation refers to the many types of molecular assemblies. Aggregation is a reversible process and occurs either from covalently or non-covalently linked species [101]. Some causes of protein aggregation are misfolding during protein expression, freeze-drying, freeze-thawing, perturbation during protein purification, ultrafiltration, syringe filling, transportation, diafiltration, storage, etc. [62, 63]. All of these processes affect the product stability by directly exposing proteins to damaging/adverse conditions like extreme non-native protein aggregation has gained much attention because of increased adverse immunogenic effects during therapy, reduced efficacy of biological molecules, and turbidity caused by aggregated proteins and hence reducing the pharmaceutical elegance.

Despite the great potential of protein aggregation, it is very important to control protein aggregation during storage and processing. In this regard, protein-protein interaction and intrinsic conformational stability of protein play a key influential factor in modulating protein aggregation [54].

13.17.5 Precipitation

Precipitation basically refers to particle or particulate formation. This is a reversible behavior, in which proteins are either partially or completely unfolded. Protein solubility serves as an important factor in precipitation of proteins and is completely reversible upon dilution [64].

13.17.6 Surface Adsorption

Since the protein encounters many surface interactions during bioprocessing, interfacial stability is considered as a key factor. As adsorption itself is considered as physical instability, interfacial stress is even more problematic. Thus, interfacial stability greatly depends on several factors like property of protein molecule, available surface area, surface tension, and structural stability. Proteins are adsorb to various surfaces in aqueous form as well as in partially unfolded form due to increased exposure of hydrophobic amino acid chains [98].

13.17.7 Improving Protein Stability

Several protein stabilization methods have been well identified. Some of which are chemical modification, drying including lyophilization, stabilization by ions, and site-directed mutagenesis. By chemically modifying proteins with glycosylation, post-translational modifications may provide some means of altering physical and

chemical stability of a protein [77]. Among synthetic processes, PEGylation (addition of polyethylene glycol PEG) is very well-known to modify or alter the protein and also to improve physical and conformational stability of a protein [99].

Another method for improving protein stability is freeze-drying, which provide advantages of long-term storage stability, improved shipping, and also improved stability to varying degrees of temperature. Drying methods may also include spray drying, air drying, vacuum drying, film drying, and supercritical fluid drying [51]. Another important and well-known method to improve the solubility, chemical stability, as well as physical stability of proteins is "site-directed mutagenesis" which is a specific method to create targeted changes in double-stranded plasmid DNA [32].

13.18 Chemical Kinetics of Proteinous Compounds

Chemical kinetics basically deals with the rates of chemical processes. A chemical process is usually broken into a series of steps known as elementary reactions. These elementary processes involve a transitional state between two molecular and atomic states through a separated potential barrier. This potential barrier consists of activation energy of the process and is also responsible for determining the rate of reaction. This potential barrier is also an important source of temperature dependence of the reaction rate. Chemical kinetics of a reaction is usually performed to find out the reaction mechanism of a proteinous compound and also to determine absolute rate of reaction and individual elementary steps involved in proteinous compounds [97].

13.19 Applications and Advantages of Stability Studies

Chemical kinetics of protein compounds provide chemical engineers and chemists with different methods and tools to better describe the chemical processes like microbial growth, chemistry of biological systems, food decomposition, and stratospheric decomposition. Chemical kinetics can also be used to design and modify chemical reactors to optimize pharmaceutical product yields and also to eliminate environmental dangerous by-products. Kinetic methods may also be used to calculate temperature and pressure ranges for the maximum yield of hydrocarbons into gasoline.

There are several advantages of using chemical kinetic methods of proteinous compounds. Firstly, we can easily identify the transition intermediates and their concentration rates. In addition, they also allow large number of chemical reactions that can be easily used for analytical purposes. We can easily determine factors affecting rate of reaction, and the mechanism of chemical reaction can also be easily determined by using chemical kinetic methods of proteinous compounds [88].

13.20 Role of Pharmacists in Protein Stability

It is very necessary for a pharmacist or a pharmaceutical scientist to understand, to study, as well as to interpret the conditions of instability of any pharmaceutical product and also able to offer possible solutions to stabilize these pharmaceutical products. It is also the responsibility of a pharmacist to properly define the reaction rate, molecularity, and reaction order while applying zero order kinetics to the routine practice of pharmacy. In addition, a pharmacist should also be able to calculate shelf life and half-life of any pharmaceutical drug or product. It is very critical to interpret pH profile rates, kinetic data. So, maintenance of stability testing protocols, stabilization techniques, and regulatory requirements should be updated by a pharmacist. Therefore, stabilization of pharmaceutical agents is very essential to make acceptable products in community and the industrial pharmaceutical setting.

13.21 Conclusion

Stability testing is now the key procedural components in the pharmaceutical development for a new drug as well as new formulation. Stability test are carried out so that recommended storage conditions and shelf life can be included on the label to ensure that the medicine is safe and effective throughout its shelf life. There are several advantages of using chemical kinetic methods of proteinous compounds. We can easily identify the transition intermediates and their concentration rates. Thermodynamics also establish the necessary conditions for crystallization, and the kinetics dynamics of the processes determine whether a possible scenario actually becomes reality. The present study sums up the important land marks in the development of the guideline for stability studies. It is hoped that a ready to start reference is generated by the FDA, ICH, WHO, and CPMP guidelines to specific conditions. Therefore, stability tests should be carried out by following proper scientific principles and understanding of the current regulatory requirements as per the climatic zone.

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