Biomacromol. J. Vol. 1, No. 1, 19-45, July 2015.

Biomacromolecular Journal

www.bmmj.org

Structural Characteristics of Stable Folding Intermediates of Yeast Iso-1-Cytochrome-*c*

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(Received 26 November 2014, Accepted 9 February 2015)

ABSTRACT

Cytochrome-*c* (cyt-*c*) is an electron transport protein, and it is present throughout the evolution. More than 280 sequences have been reported in the protein sequence database (www.uniprot.org). Though sequentially diverse, cyt-*c* has essentially retained its tertiary structure or fold. Thus a vast data set of varied sequences with retention of similar structure and function makes it a primary candidate for studying molecular evolution, phylogenetics and sequence conservation. When amino acid sequences of mammalian cyts-*c* are aligned with the sequence of the yeast iso-1-cyt-*c* (y-cyt-*c*), it is observed that the yeast protein not only contains five extra N-terminal residues but it has only 60% sequence homology, *e.g.*, with the horse heart cyt-*c*. Structural and thermodynamic studies suggest that there are four states in the folding equation of y-cyt-*c*, *i.e.*, Denatured (D) state ↔ Pre molten globule (PMG) state ↔ Molten Globule (MG) state ↔ N (Native) state. This review summarises findings of structural and thermodynamic characteristics of these thermodynamic states of y-cyt-*c* and its folding mechanism.

Keywords: Cytochrome-*c*, Folding intermediates, Yeast iso-1-cytochrome-*c*, Pre-molten globule, Molten globule

INTRODUCTION

Cytochrome-*c* (cyt-*c*) is an electron transport protein that lies in the inter-membrane space and transfers electrons from bc_1 complex to cyt-*c* oxidase (CCO) [1,2]. It has been a model protein for studies in diverse aspects of science that varies from physiology to electrochemistry to biochemistry to apoptosis and many more. The ease to express, purify and extract considerable amount of cyt-*c* made it a favoured protein [3]. It is a universal protein present in almost every living organism ranging from prokaryotes to eukaryotes, and it carries out electron transfer throughout the evolution. More than 280 diverse sequences have been reported [4]. With its tertiary structure well conserved, it has remarkably diverse primary structure throughout the evolution. Ample amount of discrete sequences of cyt-*c* are known which possess similar structure and function, and makes it a primary candidate for studying molecular evolution, phylogenetics and sequence conservation. Amino acid residues that are conserved and significantly contribute to the structure, function and stability in eukaryotic cyst-*c* have been discussed in detail in our previous publication [4]. Another reason of it being intensively studied is its ability to accept genetic alteration in its genome that makes site-directed mutagenesis based studies in cyt-*c* easier [5-9]. Where small size and high solubility makes its purification easier, its high helical content and the presence of the heme cofactor have allowed mitochondrial as well as some bacterial cyts-*c* to be studied through a variety of spectroscopic techniques. These features have contributed in making cyt-*c* a model protein [10]. Being one of the earliest proteins to be crystallised [11-16], its structure is well known thus making its study easier.

Primarily, cyt-*c* is an electron transport protein. It is associated with the aerobic respiration in eukaryotes, shuttling electrons from the *bc* complex to CCO in mitochondria and from the cyt *bf* complex to photosystem I in chloroplast [17]. On the other hand, it is engaged in both aerobic and anaerobic respiration in prokaryotes [18,19].

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Covalent attachment of heme to the polypeptide and the heme conformation in cyt-*c*, accounts for the greater range of its reduction potentials displayed in different organisms [20]. Covalent attachment of heme in cyt-*c* also made it a model system to study cofactor containing proteins [21-23]. Thorough analysis of various cyt-*c* biogenesis systems also reveals the importance of heme in proper folding of holocyt-*c* emphasising the role of cofactor in cofactor containing proteins [20]. Cyt-*c* also holds importance in studying protein-protein interaction as it comes in contact with various physiological partners in course of commuting electrons from one protein to another [24-28].

Furthermore, their thermodynamic stability and capability to reversible denaturation make cyt-*c* an ultimate system to study protein folding [29-39]. Different opinions have been given regarding the folding mechanism of cyt-*c*. It has been proposed that, in spite of large differences in their physico-chemical properties and thermodynamic stability, cyts-*c* share a common folding mechanism [40]. On the contrary, various studies have also shown that some closely related species of cyt-*c* follow different folding pathways [41,42]. Where a complex stepwise folding process has been observed for horse cyt-*c* (h-cyt-*c*), [29,30,34,43-52], a different folding pathway has been observed for bovine cyt-*c* which differs from h-cyt-*c* at only three residue positions [41,42,53-55]. Furthermore, yeast iso-1-cyt-*c* (y-cyt-*c*) shows highly assorted denatured (D) state, composed of both extended and compact structures together [56-61]. Formation of partially structured intermediates, along the course of folding, has been reported for various cyts [62-66]. Various intermediates have been reported for cyt-*c* but molten globule (MG) is one of its most characterized intermediates [39,42,44,67-71]. Besides all these characteristics, cyt-*c* may also play an important role in studying protein import in mitochondria as it is located in the inter-membrane space [72-74]. Heme, present in cyts, makes its tracking easier during purification and this merit has been used to make fusion proteins. Proteins that are large in size with no prosthetic groups and are poorly antigenic such as trans-membrane proteins are difficult to be produced in large quantities. But fusion with cyt-*c* domain, enhances the visibility and simplifies tracking, quantification and determination of the orientation of the fusion polypeptides [75]. The commercially available Cherry ^{TM}Express (Eurogentec) claims that fusion to a cyt-*c* fragment also improve solubility of target protein.

Lately an imperative role in intrinsic pathway of apoptosis has also been assigned to cyt-*c* [76-80]. When metabolic stress, DNA damage or some other apoptotic stimulus is detected in a cell, the intrinsic apoptosis pathway gets initiated, and release mitochondrial cyt-*c* into cytosol. Cyt-*c*, in cytosol, binds with Apaf-1 to form apoptosome which activates procaspase-9 which in turn activates the effector caspase cascade that eventually cause cell death [81-91].

Another important function that was noticed lately, is that of radical scavenger or antioxidant [82]. Free radicals are generated in mitochondria along the electron transport chain. Complex I, complex III, and the reduced ubiquinol pool are the main sites where these radicals are produced. These are extremely reactive molecules which when come in contact with DNA or other molecules may cause damage which eventually may cause cell death. Frequently produced radicals in the cells are hydroxyl radicals (•OH), superoxide ($•O_2$) and hydrogen peroxide (H_2O_2). Cyt-*c*, being a part of respiration cycle in a living cell, is always in either of the redox forms. When oxidised, it neutralises superoxide ion by extracting the unpaired electron, reproducing the stable O_2 molecule and subsequently transfer the extracted electron to CCO to constructively utilise it in respiration and also regains its oxidised state [92,93]. On the other hand, it also acts as a scavenger for H_2O_2 in both redox forms. As its indispensable redox capabilities are responsible for its radical scavenging properties, cyt-*c* turns out to be an excellent antioxidant.

PROTEIN FOLDING

Studies on protein folding started in early 1960s with the work of Anfinsen who introduced 'thermodynamic hypotheses' [94]. According to this hypothesis, folding of a protein to its native (N) state is governed by its amino acid sequence by the virtue of the interatomic interactions and the conformation that it attains is a thermodynamically stable state with lowest Gibbs free energy. Later another idea that gave a new direction to the protein folding studies was that a real unfolded protein cannot fold by randomly searching its native state from among the infinite possible

conformations [95]. Base on his calculations, Levinthal [95] proposed that it would take million of years for a completely unfolded protein to finally attain its native state if the search is random or unsystematic, which is in absolute contradiction to the fraction of seconds time in which a protein actually folds, *in vivo* or *in vitro*. He concluded that an unfolded random coil attains its metastable native state following a well-defined sequence of events [95]. Though his conclusion "protein folding is speeded and guided by the rapid formations of local interactions which then determine the further folding of the peptide" [96] clearly indicated the existence of folding pathways. His conclusion revealed two perplexing targets that should be attained for a protein to fold, *i.e.*, to attain global minima and that too in fraction of seconds. Attaining a metastable state or global minima refers to the thermodynamic control which is a pathway independent phenomenon, while attaining the N state on biological time scale (*i.e.*, in seconds) is a pathway dependent kinetic process which may only attain a local minima [97]. Innumerable studies have been carried out till date to solve this paradox from the time it was proposed [97-111]. The solution to this problem is been sought under two major ideologies that are termed as classical and new view. Levinthal's paradox gave a new direction to the area of protein folding and introduced the concept of folding pathways which is termed as classical view. This view advocates that a protein folds in a systematic manner with the existence of intermediates along the pathway that limit the scale of conformational search and reduces the time of random searching to seconds [45,64,97,109,112-129]. The 'new view' is a statistical approach which describes folding in terms of energy landscapes. The new view considers folding as a diffusion-like process, where Brownian forces cause non-concurrent movement in each polypeptide chain resulting in different conformations, all of which eventually find their ways to the same native structure [56,97,122,130-140].

Classical View/Protein Folding Pathways

Three models emerged to define the path that a protein would follow to attain its N state. All these models share a common assumption that folding intermediates are structurally different. Folding intermediates are stabilised by hydrogen bonding and/or hydrophobic interaction.

Framework model. This is a hierarchical model of protein folding (*i.e.*, intermediate(s) on the folding pathway has (have) native like secondary and/or tertiary structure). It hypothesises that folding starts with formation of local H-bonded secondary structure which randomly diffuses in solution, collide and dock to form native tertiary structure [141-145]. Several proteins have shown to follow this folding/unfolding pathway. This model emphasizes that it is the local contacts and subsequent secondary structure formation that is the initial driving force for folding [146]. It has been shown in RNase A and RNase S that secondary structure is formed early in the folding while in the unfolding pathways of α-lactalbumin, penicillinase and carbonic anhydrase tertiary structures were found disrupted first [142,145,147]. All these examples favour framework model of protein folding.

Hydrophobic collapse model. Hydrophobicity has long been considered the dominant force for protein folding [148]. This model proposes sequestration of nonpolar groups, away from the aqueous environment into the core of the protein, to be the driving force of protein folding [143,149,150]. It presumes that an unfolded protein with its hydrophobic groups exposed to aqueous environment would rapidly collapse around its hydrophobic residues in a nonspecific manner. Hydrophobic collapse during burst phase of folding drives compaction of the protein, thereby drastically reducing the available conformational search-space so that folding can take place in a confined volume. Native-like tertiary interactions in the collapsed ensemble then directs the formation of secondary structures and stabilization of native tertiary contacts.

Early studies that show the significant contribution of hydrophobic driving force, produced as a result of expulsion of water from the burial of nonpolar surfaces, in protein folding strongly support the hydrophobic-collapse model [151,152]. Sequestration of hydrophobic residues into the core of the protein to avoid contact with aqueous environment can also be validated from the crystal structures of globular proteins [148,153-157]. Hydrophobic amino acid residues in the cores of proteins appear to be more strongly conserved and correlated with structure than other types of interactions [158-162]. It has also been established that apolar environment favours hydrogen bond (H-bond) formation, thus the hydrophobic environment of

the collapsed ensemble would favour the formation of H-bonded secondary structures and rearrange the random interactions of the ensemble to eventually attain the native conformation [160]. This model is also consistent with the 'new' folding funnel view which advocates the existence of multiple parallel folding pathways for a protein. Hydrophobic collapse is a nonspecific process that would give rise to heterogeneous intermediates that would eventually take different courses to eventually attain the N state thereby agreeing to the existence of parallel pathways [60,97,135,144,163-165].

Several proteins have been shown to follow hydrophobic collapse model [166,167]. Agashe *et al*. [166] have shown that barstar undergoes a nonspecific hydrophobic collapse in its burst phase (within 4 ms) to form a compact hydrophobic core with no concomitant secondary structure formation. Crowhurst *et al*. [167] have shown that the folding of SH3 domain has shown consistence with both hydrophobic collapse model as well as with the folding funnel view.

Nucleation condensation model. Several problems were encountered when the previous two models were taken into consideration. Framework model advocated the formation of stable native-like secondary structure to be the first step towards folding. It is practically unlikely to find conformational preferences that are strong enough to form secondary structures without any tertiary interactions, and most of the protein sequences that form regular secondary structures in proteins do remain disordered when taken as independent small peptides [107,108]. For many proteins, it was observed that it is the long range interactions that stabilise the secondary structures in those proteins [168,169]. While in case of hydrophobic collapse, the rearrangement of the secondary structure and the native tertiary contacts in the collapsed ensemble seems to be difficult task as the random interactions formed in the collapsed structure might create hindrance for restructuring. And the studies that validate the formation of molten globule (MG) also suggest that the secondary structure formation is parallel event to the collapse, suggesting a model that proposes a middle of the road pathway [170-172]. Both the previously mentioned models consider the denatured state of protein to be a random coil which is a rare phenomenon [173-180]. Generally the denatured state retains some residual structure that is composed of elements of secondary structure as well as hydrophobic clusters that predispose the folding towards the native structure. Several examples of two-state kinetics with no accumulation of any intermediate and φ-value analysis of the transition state of a protein that follows two-state kinetics suggested that the secondary and tertiary structure formation is a parallel event [181-188]. All these points directed the folding studies to search for another pathway that may solve the chicken-egg scenario of the secondary-tertiary structure formation.

Nucleation condensation mechanism envisages that folding of a protein starts with the formation of a marginally stable nucleus, formed by local secondary structure stabilized by long-range interactions, which in turn allows rapid condensation of further structure around it. This mechanism couples the features of both framework model and hydrophobic model by simultaneously forming the secondary and tertiary structure [107-193]. Wetlaufer *et al*. [194,195] introduced the idea of nucleation model at the first place in which they proposed that the formation of nucleus, composed of local secondary structure, is the rate limiting step followed by the growth of rest of the structure around this nucleus. Nucleation condensation mechanism extended this model by considering that secondary structures are not stable enough and concerted long-range interactions are required to make them stable and the formation of this diffused nucleus is altogether a rate limiting step along the folding pathway [190]. An important evidence in favour of this model is provided by the study of Uversky and his coworkers [196]. They analysed conformational characteristics of the native and partially folded states of more than 40 proteins and observed that hydrophobic collapse and secondary structure formation occurs simultaneously in early stages of folding. Nolting *et al*. [146,192,197-199] also have worked on around 15 proteins and confirmed that this mechanism unifies the features of both framework and hydrophobic collapse models. They assessed while formation and presence of higher content of secondary structure in transition states favours framework model, the hydrophobic collapse model is endorsed by the hydrophobic effect which is an important driving force for folding and simultaneous shrinking of molecular volume. They also suggested the existence of multiple pathways in the early stages of folding prior to nucleation, thus supporting the funnel model of energy

landscapes. This mechanism, instead of intermediates, emphasized on the study of transition states which can only be studied by kinetic measurements [181,189,200-203].

This mechanism of folding was initially introduced to explain the two-state kinetics of small globular proteins but it eventually has evolved to explain folding of multi-domain proteins and existence of intermediates. For multi-domain or large proteins, it is envisaged that they are composed of modules or semi autonomous domains that initially form their independent local nuclei and form further structure around it and then dock over each other. Multi-nucleation has also been suggested by lattice calculations [204]. Folding of barnase is a very good example of folding of multi-domain protein where individual foldon forms independently and the rate limiting step is docking and rearrangement of the foldons [107,108,205-209]. And as far as intermediates are concerned, if the foldons are stable enough then they can also result in the formation of stable folding intermediates which will dock and consolidate the packing interactions to attain the native structure. If these intermediates had stable secondary structure and no tertiary interactions then the folding is more like framework model [107]. On the other hand, if hydrophobic interactions are formed too rapidly then the protein may form molten globule like intermediates [107,108,196]. Thus it can be said that nucleation-condensation mechanism is middle of the road pathway between the framework and hydrophobic collapse model. It can describe the folding mechanism of proteins independent of its complexity in terms of its size or its folding. Depending on the stability of the secondary structure or hydrophobic interactions in the transition state, the other two models may also be justified.

New View/Folding Funnel/ Energy Landscapes

New view is a statistical approach to solve protein folding problem in terms of folding funnels and energy landscapes [113,130,133,134,139,140,163,210-214]. Classical view or a folding pathway is a unidimensional attempt to explain multidimensional problem of protein folding. The denatured state is not a state with a single conformation but is an ensemble with undefined conformational properties. Thus, the folding of an ensemble cannot be described by any single pathway but needs a multidimensional approach. The new view explains folding by parallel multiple pathways and diffusion like processes [97,134]. And as Dill describes it (new view) "solution of Levinthal's paradox is funnels not tunnels" [215].

Energy landscape of a folding protein molecule is a plot of enthalpy (or free energy) of each conformation as a function of configurational entropy (the degrees of freedom). The vertical axis of the funnel represents the internal free energy (or enthalpy) of the polypeptide chain conformation. Broadly two kinds of energy landscapes can be obtained in nature: smooth and rough/rugged. The most rational model of a protein proposed by Onuchic *et al*. [134] is defined by a rugged energy landscape, with least frustrated heteropolymer, that tapers in a funnel like manner towards the N state. The local roughness of the funnel reflects transient trapping of the protein conformations in local free energy minima. Rugged landscapes have hills, which represent high energy conformations (unfavourable dihedral angles and/or buried polar groups in nonpolar environment), and valleys represents comparatively favourable conformations [139,140,213,216]. According to this model, as the polypeptide chain folds, the compactness increases and the free energy decreases, along with progressively limiting the entropy, *i.e.*, conformational freedom of the polypeptide, eventually narrowing down to unique native structure giving funnel its shape.

Transition state according to classical view is about specific structures, while landscapes view considers it an ensemble rather than a specific structure. Transition state, according to new view, is basically determined by rate limits and bottlenecks that involve all the conformations that come across the transition from the denatured (D) state to the unique N state [216,217]. Rate in turn depends on many factors such as temperature, solvent conditions, free energy barriers due to conformational entropy.

The rugged folding landscape of a small helical protein is funnel-like, with a preferred direction of flow toward the unique N state [140,210,218]. The ensemble of conformations in the upper part of the funnel takes into account the formation of secondary structure. Both the order parameters the solvent-averaged energy (E) and the fraction of native-like contacts (Q) describe the position of an ensemble of states within the funnel and stratify the landscape [134].

CYTOCHROME-*C*

The folding dynamics of cyt-*c* has been an area of extensive research. Opinions as different as hydrophobic collapse/sequential stabilization/nucleation condensation have been given to annotate the folding pathway of cyt-*c*. While folding studies have been extensively done on h-cyt-*c*, it has been shown that folding mechanism of h-cyt-*c* and y-cyt-*c* is similar [142,219-221]. Englander and co-workers [49,124,222-230] have done an extensive research to decipher the folding mechanism of h-cyt-*c*. They divided the whole process into four steps:

Burst phase chain contraction. The folding of the protein starts with the reorganization of the unfolded ensemble from a random coil in a good solvent (strong denaturant such as guanidinium chloride, GdmCl) to a more contracted state in less concentrated denaturant [231-233]. This chain contraction is an energetically downhill process that represents the submillisecond burst phase in h-cyt-*c* [187,188,230].

Refolding kinetics of denatured cyt-*c* and two of its fragment peptides, monitored by fluorescence and CD, provided evidence for the existence of burst phase contraction [188]. H-cyt-*c* fragments represented the unfolded state. H-cyt-*c* fragments (fragments with 1-80 and 1-65 amino acids) which lacked the C-terminal helix, an important component of the native structure and early folding intermediate [112,234], were found to be as unfolded at room temperature as thermally unfolded cyt-*c* and showed similar sensitivity to GdmCl as cyt-*c*, when monitored by CD. Thus, these fragments were taken as unfolded states. When the high GdmCl was diluted for both h-cyt-*c* and fragment peptides, they showed 50% increase in fluorescence quenching (5 Å decrease in Trp59-heme distance) and change in ellipticity in stopped flow dead time of less than 1 msec [188,230]. The decrease in fluorescence is suggestive of contraction in the unfolded ensemble in low denaturant concentration, reasonably due to strengthening of random interactions of hydrophobic side chains, increasing the stability of the unfolded protein [235]. This aspect is in good agreement with the concept that when a polymer chain is transferred from a good to poor solvent the chain tends to contract. The increase in ellipticity in the contracted chain at low denaturant may represent a secondary effect o f the chain compaction [236-238] or a solvent effect on the distribution of main chain φ and ψ rotations [239-241]. Absence of any H-bonded structure, as no hydrogen exchange protection could be observed in burst phase, denies the possibility of formation of any intermediate in this phase. This phase may be defined as a rearrangement of the polypeptide chain that leads a fully unfolded denatured state under the influence of concentrated denaturant to a compact, more stable but still unfolded state on dilution of the denaturant without accumulating any intermediate.

Collapse-Search-Nucleation. The second step in h-cyt-*c* folding is formation of transition state as a result of massive chain condensation. Contrary to the belief that rate-limiting barrier in two state folding occurs late in the process of folding [172,242,243], it was established that the initial chain collapse is the rate-limiting step for h-cyt-*c* in two-state and three-state folding [230]. It is an energetically uphill process that involves the time consuming long-range search for a native-like transition state which include some helix formation and burial of considerable polar and nonpolar residues. Formation of secondary structure in this transition state is also associated with subsequent hydrophobic interactions to stabilize those secondary structures. Roder and co-workers [244] have also advocated that chain condensation of h-cyt-*c* is driven by specific secondary structure formation, and that h-cyt-*c* follows a two-state folding mechanism.

Insertion of reorganization barrier which results in three-state folding (explained later), lead to the accumulation of first folding intermediate at the end of molecular collapse phase. Hydrogen-deuterium exchange experiments, at the end of collapse, suggested that almost all molecules have formed their two terminal helices, along with a few that proceeded a little further with some more structure and some attained N state [230,245]. Thus the transition state that precedes the collapsed intermediate may account for the formation of terminal helices along with the burial of significant polar and non-polar residues. The whole process of transition state formation qualifies the definition given for the process of nucleation [222,230,246].

Formation of nucleus is inherently a slow step in folding. The transition state once formed limits the dimensions of random search for the native conformation and facilitates local searches for favourable interactions that

proceed with secondary structure formation and tertiary packing in an energetically downhill manner.

Sequential stabilization. Transition state formation is followed by macroscopic folding of h-cyt-*c*, explained as sequential stabilization of foldons, cooperative unfolding/refolding units of a protein. The foregoing structure guides the formation and sequential accession of each consecutive foldon unit stabilized by native-like interactions in a sequential stabilization process to eventually assemble the native protein. In h-cyt-*c*, the unit composed of N- and C-terminal helices formed in the transition state, corresponds to the first foldon. Englander and co-workers used various techniques such as hydrogen exchange (HX) pulse labeling [224,225], by equilibrium native HX (NHX) [112,224,229,247-249], and kinetic NHX method [124,250,251] under diverse experimental conditions to identify different foldons of cyt-*c*. They, on the basis of unfolding free energy, characterized five discrete foldons in h-cyt-*c* and color coded them in order of increasing energy (under native conditions) and increasing *m*-value, as infrared (I; 40-57 Ω loop), red (R; 71-85 Ω loop), yellow (Y; 37-39, 58-61 short anti-parallel β strands), green (G; 60s α helix and 19-36 Ω loop) and blue (B; N- and C-terminal α helices) [228].

Using HX pulse-labelling, structural stability, folding rate, structural and biophysical properties of trapped intermediates were determined [225]. It has been reported that the first step in folding, of all those proteins which follows two-state folding, essentially involve docking of the secondary structures of N- and C-terminals [227]. In refolding experiments of h-cyt-*c*, HX pulse-labelling results showed that the first foldon to fold was the one with N- and C-terminal helices [222,230,246]. Amides along the entire length of these two helices showed protection against HX, suggesting their involvement in H-bond formation. The site of interaction of these terminal helices in the native protein was found to be highly stable as observed in HX pulse-labeling experiments suggesting their native-like conformation [224,252]. Folding and unfolding rates obtained by HX measurements as well as stopped flow fluorescence were same, confirming this foldon to be the first on pathway intermediate [223]. Stopped flow circular dichroism (CD) also confirmed the formation of helical content equivalent to that present in terminal helices in this first foldon/intermediate. The unfolding reaction monitored by NHX experiment found N/C helices foldon to be the last foldon to unfold thus validating all the above mentioned observations.

Besides this foldon, small protection was observed for Met65, Leu68 (core hydrophobic residues of the 60s helix), His33 and Thr78 which form main chain to side chain bends in the native protein and retain some protection even in the unfolded protein. Little other H-bonded structure is found. Some core hydrophobic residues of the 60s helix (Met65, Leu68) have small protection suggesting burial but not helix formation. Some minimal protection is seen for His33 and Thr78 which form main chain to side chain bends in the native protein and retain some protection even in the unfolded protein. Thus, N/C bi-helical foldon is described as the first kinetic (HX pulse labelling, stopped-flow experiments) as well as equilibrium (NHX experiments) intermediate along the folding of h-cyt-*c* [45,49,222,230]. The next foldon to fold is the green one. The green foldon composed of a loop and helix, was initially thought to represent a single foldon but it has shown to unfold separately and their unfolding order is optional at low pH [229]. Either the green loop or the green helix can unfold first, and is then joined by the other. Folding of green foldon is followed by the folding of yellow foldon which in turn is followed by the folding of red and infra red foldons [225]. Stability labeling experiments carried out as a function of decreasing pH or mutation show that the infrared and red foldons can unfold in either order. Either one can unfold first and the other then joins [228,253].

Both the concept of foldons and stabilization of complementary structure justify the stepwise nature of the h-cyt-*c* folding pathway. However, it is not known how the first pathway step is predetermined [254,255]. It has been suggested that the initial search for the nucleating transition state may similarly be directed by the sequential stabilization principle [256,257].

Misfold-reorganization barrier. H-cyt-*c* has shown to follow both two-state and three-state folding pathways. When a reorganizational barrier is inserted, the two-state folding switches to three-state folding. Englander and co-workers [124,223,228] observed that N/C bi-helical foldon is always the first intermediate along the folding pathway whether cyt-*c* follows two/three state mechanism of folding.

It is after this intermediate, that cyt-*c* switches between two-state or three-state mechanism. This switching arises as a result of misligation of one of the peripheral histidines to the heme iron. These histidines (His26 or His33), the non-native ligands, are part of the next foldon in queue to fold that misligate to block the further folding, accumulating intermediates, making an otherwise two-state folding process to three-state process. At neutral pH, the histidines misligate to heme, holding green loop on the wrong side. The folding initiates with burst-phase followed by chain condensation by forming the N/C bi-helical foldon but get blocked thereafter as the next foldon (green foldon) is held wrongly on the other side. Undoing this misligation is a slow error-repair process that allows the accumulation of intermediates. Englander and co-workers [49,118,124,223, 224,226,229,251] are of the opinion that it follows a predetermined pathway-optional error (PPOE) model. On the other hand, histidines when protonated (at acidic pH) or mutationally altered do not form non-native interactions thus skipping the error-repair process allowing h-cyt-*c* to undergo fast two-state folding [118,124]. So it was concluded that though the entire ensemble folds through the same pathway and intermediates, it is the slow error/misfolding-repair step that makes a two-state process of h-cyt-*c* folding to a three-state folding process. Heterogeneous folding may also be observed if different fractions of the denatured ensemble follow two/three state mechanism.

Hydrophobic Collapse Model

Akiyama *et al.* [155] showed that y-cyt-*c* follows hydrophobic collapse model for its initial folding mechanism. They observed significant quenching of Trp-59 fluorescence with trivial formation of secondary structure in the initial phase of folding that made them conclude that y-cyt-*c* follows hydrophobic collapse model where it is assumed that condensation of hydrophobic elements precedes the formation of secondary structure. Later when they carried out sub-millisecond time resolved small angle X-ray scattering (SAXS) measurements, they concluded that it is not exactly the hydrophobic collapse that initiates folding of y-cyt-*c* but there is simultaneous secondary structure formation along with the hydrophobic collapse [258]. Their results were in accordance with work of Guo *et al*. [259] who showed similar results for another α-helical protein showing synchronized collapse and secondary structure formation.

Funnelled Landscape of cyt-*c*

As discussed previously, H-exchange experiments showed that cyt-*c* follows a particular order during its folding, so obtaining a funnelled landscape for cyt-*c* was considered a challenge [112,251,260,261]. But it has been shown that energy landscape for cyt-*c* folding has a narrow central funnel with a flat upper rim on which collapsed and extended polypeptides interchange rapidly in a search for the native structure [38,56]. And it was also shown later that energy landscapes obtained for h-cyt-*c* confirms the order of the folding attained by classical pathways [135]. Weinkam *et al.* [135], from the free energy plots, observed that heme acts as a nucleation site for the collapse of the polypeptide and native heme contacts are formed early in the folding process. They also obtained the same order of the folding of different foldons as suggested by H-exchange experiments. The N/C bihelical foldon showed dependence on heme contacts and together with heme form the nucleation site. Rest of the foldons showed similar order of folding as was obtained by H-exchange experiments. Like H-exchange experiment results, the topological frustration of N- and C-terminal helices with respect to heme slow down the folding otherwise the rest of the folding goes uninhibited downhill. Energetics of h-cyt-*c* folding has also shown that the kinetic traps for h-cyt-*c* folding is the non-native His-heme ligation which takes place during or just after the burst phase and the breakdown of this misligation limits the rate of folding of the polypeptide [164]. Panda *et al.* [164] also showed that the barrier to folding is largely entropic, and purely entropic kinetic barrier to folding is consistent with a smooth funnel folding landscape.

In another study, stable folding intermediates were found to be induced when different concentrations of KCl were added to the acid-induced D state of h-cyt-*c*. These intermediates were still energetically separated (*i.e.* high energy barrier in between intermediates and native conformation) from the N state [38]. This study is also in agreement with the MG state formation from acid-denatured state on addition of salt [62,262]. Zhong *et al.* [38] interpreted that the charge distribution on the polypeptide

chain is an important factor to decide the order of folding. Accumulation of intermediates due to KCl alters the shape of the energy landscape by raising the slope of the upper rim and introduces a rugged energy surface toward the bottom of the folding funnel.

All these studies suggest that sequential assembly in cyt-*c* proposed by classical pathways is indeed quite consistent with the modern energy landscape theory of protein folding.

DIFFERENT THERMODYNAMIC STATES

Native State

Cyt-*c* has been one of the earliest proteins whose crystal structure has been deduced and analysed [11,13-16,263-269]. Crystal and solution structures of wild type (WT), mutant forms and complexes of y-cyt-*c* with its physiological partners (cyt-*c* peroxidase and cyt-bc_1) have also been reported [26,27,270-272]. Here, we are going to discuss the more abundant isozyme of cyt-*c* present in yeast, *i.e.*, y-cyt-*c*. Many crystal structures of y-cyt-*c* at different resolutions varying from 2.8 Å to 1.23 Å and different oxidation states have already been reported [11,13,16].

Y-cyt-*c* is a 108 amino acid long protein with a typical cyt-*c* fold where heme prosthetic group is enclosed in hydrophobic pocket formed from a series of α-helices and turns. It is a small globular protein composed of three major and two minor α-helices interconnected with β-turns. The heme moiety is rigidly held in the core, bound by two thioether covalent bonds with cysteine at positions 14 and 17 and two co-ordinate axial linkages with side chains of His18 and Met80. Covalent attachment of heme to polypeptide is the characteristic feature of *c*-type cyts, making it different from other cyts (*e.g.* cyt-*b*) [1,2]. Heme group is not absolutely planar but is distorted with respect to the plain defined by the pyrrole N atoms [16].

Cys14 and Cys17 form thioether bonds to second and fourth vinyl side chains of heme, respectively [16,269]. Both are a part of a highly conserved pentapeptide motif -Cys-Xxx-Xxx-Cys-His- (CXXCH) [273-276]. The covalent attachment of heme is the critical step of cyt-*c* maturation. It enhances axial strength of heme, contributes in electronic environment of heme, sets up redox potential, and adds robustness to the structure of cyt-*c* preventing the loss of heme during structural changes [20,277,278].

His18 and Met80 form out of plane co-ordinate bonds with heme and lie nearly perpendicular to the pyrrole N plane. Substitution or mutation at position 18 abolishes holo-protein formation, preventing the cell to survive [279]. Met80 is also a highly conserved intrinsic ligand of heme and binds axially to the iron atom. The coordination bond that is present between Met80 and heme is fragile but is responsible in preserving the structure, function (oxidation-reduction potential, electron transport, *etc.*) and stability of cyt-*c* [280-283]. Met80 has been found liable for the attainment of comparably high reduction potential under physiological conditions [280,284-286]. And any disruption of Met80-Fe axial bond leads to rearrangement in the protein that leads to severe reduction in protein's redox potential [280,285,287-289]. 695-nm absorption band which is a spectroscopic diagnostic tool for this Fe-Met80 coordination bond, also provides valuable information about heme pocket region and redox properties of cyt-*c* [290].

Most of the residues that line the heme pocket are nonpolar with some exceptions of solvent inaccessible polar residues (Tyr48, Thr49, Trp59, Asn52, Thr78) that are H-bonded to the propionate group of pyrrole ring. Sequence of y-cyt-*c* when compared with mammalian cyt-*c* shows five extra N-terminal residues. Measurement of mean isotropic temperature factors of atoms of all residues suggested that the atoms of heme and that of hydrophobic side chains in the heme pocket are least flexible (lowest thermal motion) while atoms of the N-terminal extension show highest thermal motion. Interaction of this N-terminal extension with the bulk of the protein and its contribution in the stability of the protein has also been discussed in our previous publication [291,292].

Another notable feature of y-cyt-*c* is trimethylation at Lys72 which is considered to increase the ability of the protein to bind to mitochondria, facilitate its transportation from cytoplasm to inner mitochondria and play a protective role against proteolytic attack [293-297]. Lys72 which is located in the highly conserved region is important for binding to redox partner's cyt-*c* oxidase and cyt-*c* bc_1 complex [2,298-301]. In contrast to higher animals, methylation of Lys72 in fungi and plants has been well understood. The biological function and importance of cyt-*c* methylation have not been clearly established [297,302,303]. As various studies involving mutant forms of y-cyt-*c* lacking trimethylated Lys72 showed to preserve

nearly full T_m activity *in vivo* [303,304]. Moreover, in higher animals which have unmethylated Lys72, this residue plays a significant role in the apoptogenic activity of cyt-*c* [77,91].

Various studies in yeast have reported the function of trimethylated Lys72 [80,305-308]. When Arg was substituted in place of Lys at position 72 (mutant Lys72Arg; unmethylated cyt-*c*) the binding capacity of cyt-*c* to cyt-b_2 decreased, though it did not alter the conformation and stability of the mutant [296,304,309]. Thus, it was concluded that trimethylated Lys72 may have a significant function in detection and binding to the physiological partners of cyt-*c* [304]. Another role that has been assigned to methylation of the Lys72 is that it lowers the isoelectric point of the apo form of cyt-*c*, thereby decreasing its Stokes radius and thus, fecilitate the import of apocyt-c into the mitochondria [297,310].

Unlike many eukaryotic cyts-*c*, y-cyt-*c* has one free sulfhydryl group, Cys102. It is a solvent inaccessible residue situated towards the inner side of the C-terminal helix. If left unaltered or in oxidised state, it may lead to protein dimerization and thus destabilises the protein or undergo autoreduction and effect the electrochemical properties of the protein [311,312]. Therefore, in most of the studies, this Cys102 is rendered inactive by either completely reducing it or mutating it to some other residue as Thr, Ala or Ser [220,313-325]. Most eukaryotic cyts-*c* have Thr at position 102, so Pielak and co-workers [220,313,314] substituted Cys102 with Thr in y-cyt-*c* to eliminate the problems associated with the presence of free sulfhydryl group. Substitution with Thr did not alter conformation of the protein as it was able to grow, transported into the mitochondria and get methylated as a WT protein. The electrochemical property was also comparable with that of the WT protein. Thus, it can be concluded that Cys102Thr mutation did not alter structure and function of the protein albeit, it prevents dimerization and autoreduction of the protein [313]. NMR studies have also shown that the solution structure of the mutant with Thr at position 102 is similar to that of eukaryotic cyts-*c* [220,314]. Replacement with Ser also did not show any change in physical or functional properties of the mutant [319-323]. An increase in stability has often been reported for substitutions with Thr/Ser/Ala at position 102 [316,318].

Interface formed by the perpendicular pairing of N- and C-terminal helices is the most conserved structural motif in all cyts-*c* and represents an early event in cyt-*c* folding. The residues of these helices interact in a specific manner. Absence of side chain in Gly6 allows side chain of Leu94 to fit in, whereas Phe10 and Tyr97 share protein stabilizing aromatic-aromatic interaction. Leu94 has a role in the formation of early and late intermediates along the folding pathway of cyt-*c*.

Trp59 is a highly conserved residue, mostly present as a single entity in the whole sequence of most of the cyts-*c*. Its unique location in the protein allows it to make H-bonds with heme propionate and simultaneously also contribute to the hydrophobicity of the heme crevice [13,16,268,269, 326]. Most of the substitutions at position 59 rendered the protein non-functional or cause loss in stability and activity of the protein [327-334]. Tyr67 is another conserved aromatic residue which is crucial in maintaining the redox potential of the protein on account of the extensive H-bonding network that it forms in the heme crevice. It also contributes to the pro-apoptotic activity of cyt-*c* [12,263, 318,335-338]. Phe82 plays important role in recognising and interacting with redox partners and thus contribute in electron transport conducted by the protein. Its role owe its origin to its unique position on the protein surface along with its proximity to heme. Its aromatic side chain also contributes to the hydrophobic environment of heme crevice [14,15,339-343].

Prolines are highly conserved, contributing to the conformational integrity of cyt-*c* by virtue of their unique cyclic structure. Pro30/71 residues contribute in maintaining the proper axial ligation of heme, thus indirectly contribute to structure and function of cyt-*c*. Pro76 also contributes to structural stabilization [344-350].

The native structure of cyt-*c* is highly conserved and there are many residues that are highly conserved throughout the evolution. The key residues that play significant role in structure, function and stability of the protein have been discussed in detail in our previous publication [4].

Besides crystal structures of y-cyt-*c* in different oxidation states and mutated forms, solution structures are also available for the protein [13-16,263,264,317,324,351]. All of the secondary structure and the overall folding

revealed by X-ray diffraction [11,16], are completely maintained in the solution structure as observed in the case of h-cyt-*c* [352] and for the cyanide adduct of the Met80Ala mutant [317]. An additional helix involving residues 15-18, which was not reported for the crystal structure, is detected in solution.

INTERMEDIATE STATES

Intermediates can be differentiated into two classes, *i.e.*, thermodynamic/equilibrium and kinetic intermediate. A thermodynamic intermediate is a state that accumulates under mild denaturing conditions with distinct spectroscopic and hydrodynamic properties, intrinsic volume and H-exchange rates other than that of the native and fully denatured states. It undergoes a phase transition with other thermodynamic states (native or denatured state) coupled with at least one of the first derivatives of free energy like enthalpy [171,172,243,353-356]. The intermediates that are found during studies of kinetics of refolding of a protein are said to be kinetic intermediates. Studying both kinds of intermediates is necessary to completely elucidate the folding pathway of a protein. Equilibrium intermediates give structural information about protein folding. Though stable, these intermediates are not sufficiently populated. On the other hand, the kinetic intermediates though well populated but are highly unstable with folding steps lasting 1-100 msec [48,112,171,172,357].

MOLTEN GLOBULE (MG) STATE

The term MG was introduced by Ohgushi and Wada [358] for an equilibrium intermediate state which has native-like secondary structure, compact molecular size but distorted tertiary structure. Later it was shown that MG state is a common physical state that comes across the folding of every globular protein [358,359,360,361,362]. The common structural features of MG state are: (a) the presence of almost all of the native secondary structure, (b) the absence of most of the native tertiary structure produced by tight packing of side chains, (c) presence of loosely packed hydrophobic residues that increase the hydrophobic surface area accessible to the solvent, and (d) hydrodynamic volume is 1.5 times more than that of the native protein [363,364,365]. Study of MG or MG-like intermediates can provide valuable information about complex and poorly understood folding and unfolding pathways [366]. Besides initially referred as equilibrium intermediate, it was shown to be a productive on-pathway folding intermediate. MG state in protein folding would be expected to limit the search for conformations leading to the unique N state hence responsible for rapidity in folding of U, the unfolded state [243,355,367]. MG state is said to be productive when it lies along the pathway, *i.e.*, in between N and D (unfolded) state (D ↔ MG ↔ N) as evident by the lag phase during the refolding from U to N, otherwise if it lies off pathway (MG ↔ D ↔ N), it would be a kinetically trapped, probably misfolded specie [142,368-370].

Cyt-*c* is one of the first protein which showed MG state when acid-denatured in the presence of high salt concentration [358]. Acid-induced MG state of cyt-*c* is often also known as A-state. A-state of WT cyt-*c* is shown to be an equilibrium analogue of late folding intermediate. On the other hand, A-state of some mutants of h-cyt-*c* has shown to be analogous to early kinetic intermediate [48]. It is an on-pathway folding intermediate with secondary structure content same as that of the native and with almost no native tertiary structure. The A-state in mammalian cyts has been reviewed intensively [71]. MG state of h-cyt-*c* is analogous to the late folding intermediate. The kinetic refolding of cyt-*c* ($D \leftrightarrow I_C \leftrightarrow I_{NC} \leftrightarrow I^* \leftrightarrow N$) at neutral pH has been shown to accumulate three intermediates: I_C, the burst phase intermediate; I_{NC}, the intermediate accumulated due to misligation of non-native ligands to heme, it prevents further folding of the protein; and I*, the penta-*c*oordinated intermediate formed after the dissociation of non-native ligand, its formation is the rate limiting step and it is analogous to the equilibrium MG state [33, 230,234,246,371]. Rapid formation of Met80-heme coordination marks the last step of folding, forming the N state [33,230].

A-state in y-cyt-*c* has not been studied in detail. Unlike mammalian cyts, MG of y-*c*yt-*c* has been studied under a few conditions. Like mammalian cyts, most of the times A-state in y-cyt-*c* is obtained at low pH in the presence of salt [62,262,372]. The A-state forms because low pH (~2) denatures y-cyt-*c* by increasing its positive charge, while added salt screens charge repulsion so that the protein

collapses to a compact form. At pH 2 in the absence of salt, most proteins are acid denatured. For some proteins, decreasing the pH to <1.5 creates such a high acid concentration that the dissociated ions act as a salt to form the A-state [373]. A-state fulfils all the above mentioned criteria that is required for a specie to be a MG.

To explore the tertiary interactions that stabilise the A-state, Marmorino *et al*. [62,262] studied A-state of WT y-cyt-*c* as well as its mutants. They studied mutants of the residues that form the interface between the N- and C-terminal helices. The interface formed as a result of perpendicular pairing of N- and C-terminal helices is a remarkable feature and is a highly conserved motif of cyt-*c* fold throughout the evolution [48,252,374]. As discussed earlier, formation of this interface represents an early event in the folding pathway of h-cyt-*c* [188,222-224,227-230, 234,245,246]. So the mutation studies of interface forming residues revealed the significance of this interface in the stabilization of MG state of y-cyt-*c*. The residues that are involved in the formation of this interface are Gly6 and Phe10 from N-terminal helix and Leu94 and Tyr97 from C-terminal helix. The mutations (Phe10Tyr/Trp, Leu94Ile/Thr/Ala/Val, Tyr97Ala/Phe) that disrupted the interface destabilized the protein as well. The degree of destabilization of each mutant obtained for native protein ($\Delta G_{N\leftrightarrow D}$) was in accordance with that obtained for respective A-states ($\Delta G_{A\leftrightarrow D}$). While mutation in residue (Ala7Leu/Tyr/Phe/Gly) that lie very close to the interface forming residue does not make much difference to the stability of either N state or A-state. Thus it was concluded that the evolutionary conserved tertiary interaction that was playing an important role in the folding of the native protein is also present in the A-state. It also strengthened the explanation that confirms the native-like MG characteristics of this A-state. It also strengthens the explanation given by Sosnick *et al*. [230] for the folding of A-state of h-cyt-*c* to its N state in one kinetic phase with a submillisecond time constant.

Fluorescence energy transfer (FET) kinetics is another technique that was used to probe the structural features and heterogeneity of MG state [57,246,375,376]. Winkler and co-workers [57,58,375-378] used dansyl fluorophore (DNS) to label and study cyt-*c* with FET. The DNS fluoresces strongly when the bound protein is in unfolded condition, while in compact conformations its florescence is considerably quenched by energy transfer to the heme. Analysis of the fluorescence decay profile gives information about the distribution of distances between dansyl fluorophore (D) and heme (A) in the labelled polypeptide. In Forster model, the rate of energy transfer is equal to the decay rate of unquenched fluorophore when distance between D and A is equal to the critical length. Unlike other probes that report on the average properties of the ensemble (absorbance, CD, fluorescence *etc*.), FET kinetics give information about the conformational fluctuation of the polypeptide [56,379].

Lyubovitsky *et al*. [375] dansylated Cys102 of y-cyt-*c* and monitored the acid-induced MG state with different techniques. While far-UV CD showed unaltered secondary structure in the MG state with reference to WT protein, the tertiary structure was found to be disrupted as shown by near-UV CD. Absorption bands in the visible region (*i.e.*, at 410 nm, Q-bands and 695 nm) for acid-induced denatured state shows blue shift with respect to absorption bands of the protein in the N state. On the other hand, bands for MG state lie between the bands of native and acid-denatured proteins. The heme in MG state of the protein is believed to be mixed spin. Where His18 was invariably ligated to iron in both spin states, Met80 was axially ligated in only low-spin state [380-382].

Small angle X-ray scattering (SAXS) method was used to measure the size or radius of gyration (R_g) of the N, D and MG state of the protein, for SAXS is sensitive to conformation of the scattering macromolecule [56]. SAXS gives measure of R_g, which for y-cyt-*c* was in the increasing order of N state < MG state < acid-denatured state, showing MG state to be more compact than unfolded state. Lyubovitsky also showed that R_g for y-cyt-*c* and h-cyt-*c* were identical in the respective conformations [56,375].

Measurements of FET kinetics of DNS(C102)y-cyt-*c* were carried out at low pH in the presence of Na_2SO_4 [375]. At low concentrations of the salt (100 mM), polypeptide ensemble showed an equilibrium between heterogeneous conformations with half of it being compact (D-A distance < 35 Å) and the other half showed extended conformation (D-A distance > 35 Å). As the salt concentration was increased, the equilibrium started shifting towards compact state until the salt concentration was ≥ 700 mM, where the

heterogeneous ensemble was completely converted to the compact state with D-A distance of 25-30 Å. Thus, FET clearly showed the clear transformation of acid-denatured state into compact MG state, which ensemble averaged spectroscopic probes such as spectroscopic techniques (UV-Vis absorption, CD, Trp-fluorescence) are unable to do.

To further ascertain their findings obtained for DNS(Cys102)y-cyt-*c*, Winkler and co-workers [57,58,376] labelled other residues of the polypeptide to make sure that the results could be generalized for the entire cyt-*c* folding landscape [57,376]. In this study they linked DNS fluorophore to mutant Cys at positions 4, 39, 50, 66 and 99. These residues are present in different structural elements in the natively folded protein and the results corresponding to these residues individually would give an overall view of the folding landscape of y-cyt-*c*. Like DNS(Cys102)y-cyt-*c*, spectroscopic (absorbance and far-UV CD) studies confirmed the transition of acid-denatured states of four out of five variants to their respective A-states as the concentration of salt is increased. DNS(Lys4)y-cyt-*c* showed no changes in its kinetics on account of closely positioned DNS label which never drifted apart in any state whether it was native, A or acid-denatured state. Though it has been established that MG state of cyt-*c* is much native like as in structure and native interactions but there are subtle differences that demarcate its identity as a state other than native the [56-58,62,246,262,375,383,384]. FET kinetics results show that though the D-A distances (measure of compactness of the protein molecule) measured for the A-state of variants is in close similarity to that of the their respective N states, A-states unlike the N state demonstrated highly heterogeneous conformations on account of flexibility and equilibration between different heme ligation states [57,376]. Kinetics results also confirmed much structural heterogeneity in loop region as has been previously reported in NMR studies of cyt-*c* [384,385]. FET kinetics as well as SAXS studies also confirmed that GdmCl-denatured state is much unfolded than acid-denatured state [57,353].

Rupturing of Met80-Fe(III) bond is one of the important steps that precedes the formation of MG [32,50, 381,382,385-391] but this event is not responsible to trigger the acid-induced unfolding of y-cyt-*c* [372]. Instead, it is the unfolding of the segment containing Met80, due to the enhanced mobility of the Ω loops (20s and 40s loops). 20s and 40s loops provide rigidity to the tertiary structure of the protein by forming inter-segmental H-bonds. 40s loop (residues 40-57) is known to be the least stable segment of cyt-*c* and is mainly responsible to prompt acid-induced unfolding of the protein [219,225,392]. At lower pH, the H-bonds are disrupted thus increasing the fluctuation in loops which in turn increase the mobility in the heme pocket and disrupt the heme-Met80 interaction [225,393].

Other than acidic pH, there are several point mutations that have shown to trigger the formation of MG. Mutation at position 26 in y-cyt-*c*, reported by Sinibaldi *et al.* [393] has shown to produce a MG at pH 7.0. His26 form intraloop H-bond with Asn31 while it forms an interloop H-bond with Glu44, linking two Ω loops (*i.e.* 20s and 40s loops) together stabilising the tertiary structure of the protein in the N state. This interaction has been reported to be absent in acid-induced MG state of y-cyt-*c* [62,262]. At low pH, protonation of side chain of His26 renders it unable to make H-bond with Glu44 providing non-native flexibility thus disturbing the tertiary structure without intervening secondary structure. The fluctuation in tertiary structure in MG state has been ascribed to the movement in the loops [50,62,381,382,384,387].

Sinibaldi *et al*. [393] mutated His at position 26 to Tyr to investigate the effect of the mutation on tertiary structure. Far-UV CD spectrum of the mutant was comparable to that of the WT protein suggesting unchanged α helical content. While the Soret CD spectrum of the mutant showed weakened 416 nm band. Cotton effect at 416 nm is attributed to the heme-Met80 and Phe82-heme interaction [394,395], and any alteration in this band suggests structural transition [394,396] due to enhanced mobility of the segment that includes residues at position 70-85 [393]. Near-UV CD spectrum of the native y-cyt-*c* which is attributed to the interaction of Trp59 with one heme propionate [1,397] was also found to be weakened implying increase in the distance between Trp59 and heme. Absorption spectrum of the mutant blue shifted along with the weakening of the band at 695 nm which is again a diagnostic of Met80-heme interaction [290,398,399]. Changes in Resonance Raman (RR) frequency also suggested relaxed heme distortion [393,400,401]. All these observations suggest enhanced peptide flexibility, altered

heme pocket and disrupted tertiary structure. RR spectrum also suggested the presence of low spin specie with either Lys-heme-His18 ligation or bis-His-heme ligation in equilibrium with low spin specie with Met80-heme-His18 ligation but redox studies suggested that the latter specie forms the major component [393]. For the mutant His26Tyr, decrease in ΔG_D^0 and m (dependence of ΔG_D^0 on the denaturant concentration) showed destabilization and decreased structural compactness, respectively. All these observations suggested that like acid-induced A-state, mutant His26Tyr has all the properties of MG. Sinibaldi *et al*. [388,393] also measured all the above mentioned parameters for another mutant His33Tyr but could not observe any significant change in its conformation or stability with respect to WT protein.

Another mutation at position 67 also triggered the formation of MG. Battistuzzi *et al*. [372] through their studies on y-cyt-*c* established that Met80 is not responsible for the triggering acid induced unfolding. Battistuzzi *et al*. [372], on the pretext of determining the amino acids that are involved in low-pH conformational equilibrium of cyt-*c* and MG formation, made mutants of Met80 and Tyr67. They studied the WT and the mutant proteins from pH 7 to 2 at low ionic strength through electronic absorption, magnetic CD and resonance Raman spectroscopies. It was already established that Tyr67 that plays a key role in maintaining the H-bond between Ω loop containing Met80 and another Ω loop extending between residues 40-57 [283,335,336, 338,402,403,404]. Through their studies they established that it is the disruption of H-bond network between these loops that facilitate acid-induced denaturation and stabilization of MG formation in the protein [372]. They showed that mutation of Met80 does not make much difference to the mechanism of acid-induced denaturation. On the contrary, mutant Tyr67Ala w completely disturbed the hydrogen bonding pattern, altered the mechanism of acid-induced denaturation by stabilising MG at higher pH.

Bertini *et al*. [68] have shown that N state of y-cyt-*c* transforms into MG state with altered axial ligation in the presence of 100 mM sodium dodecyl sulphate (SDS). NMR spectrum of the protein in the presence of SDS shows large changes in the backbone and suggests the presence of two protein forms, high spin specie with broken Met80-heme axial bond and low spin specie with His39 as the sixth ligand. Results also suggest loss of native structure along with the change in axial coordination. Bertini *et al*. [68] interpreted distortion in the helical structure and the presence of two misligated forms as results of equilibrium between MG state and random coil. They also described the dynamic features of protein in the presence of SDS as MG globule state of y-cyt-*c*.

Moosavi-Movahedi and co-workers [405-408] have studied the effect of several n-alkyl sulphates on the acid denatured h-cyt-*c* (pH 2.0). They showed that these salts induce MG state in the acid denatured protein. They carried out extensive studies to characterize the thermodynamic and structural characteristics of the MG state of h-cyt-*c* induced by n-alkyl sulphates using various techniques (ITC, DSC, spectroscopy and cyclic voltametry). They for the first time called these salts as hydrophobic salts [406].

PRE-MOLTEN GLOBULE

Jeng and Englander introduced the term ‘‘pre-molten globule’’ (PMG) who observed that acid-induced unfolding of cyt-*c* in D_2O is not a three-state process but involves a fourth state that is more expanded than the MG state but still helical [260]. Characteristics of the PMG state are that it (a) retains about 50% native secondary structure, (b) looses all the native tertiary structure, (c) is more compact than unfolded state (approximately 3-4 times that of the N state in terms of hydrodynamic volume), and (d) binds ANS approximately 4-5 times more weakly than MG state [409-411]. Thus, PMG can be defined as a relatively compact denatured form in which protein molecule has a very significant amount secondary structure but no globular structure. PMG probably represents a ‘squeezed’ and partially ordered form of a denatured state [363].

A two-state transition between PMG and MG has been shown using size exclusion liquid chromatography [412]. A structural transition between PMG and completely unfolded state has also been reported [413] but phase of this transition was not demonstrated. Thus these observations validate the existence of PMG as a distinct state. Fink *et al*. [373] have shown an intermediate state between the MG and unfolded states at low pH for more than 10 proteins. The examples include cyt-*c*, β-lactamase, staphylococcal nuclease, myoglobin and subtilisin as well as papain,

parvalbumin, and ribonuclease A [373,410].

PMG state for y-cyt-*c* is obtained under the influence of weak salt denaturant, LiCl [414]. Denaturation of y-cyt-*c* and its N-terminal deletants induces biphasic transition, N (native) state ↔ X (intermediate) state ↔ D (denatured) state. The intermediate (X) was characterized by the far-UV, near-UV and Soret circular dichroism, ANS (8-anilino-1-naphthalenesulfonic acid) binding and dynamic light scattering measurements. These measurements led to the conclusion that X state of each protein has structural characteristics of PMG state.

DENATURED STATE

Traditionally, understanding the N state was the main focus to solve the protein stability problem. Lately the role of D state in understanding the refolding code and the stability of protein has also been established [174,415-421]. The D state, unlike the N state of a protein which has a unique structure, is comprised of a broad structural ensemble and is considerably more difficult to characterize [174,415,422,423]. Earlier it was believed that GdmCl-induced D state of a protein is a random coil [424-427] but later various conformational and NMR studies established that D state often retains some residual structure [141,174, 177-179,417,423,428-435]. Different D states for the same protein under the influence of different denaturants have been reported on several occasions [174,231,421,423,426, 428,434,436-440].

Chemical-Induced Denatured States

Bowler and co-workers have shown that GdmCl-induced D state of y-cyt-*c* is not a random coil [61,176,319-322,441-444]. It retains some residual structure. They chose site-directed mutagenesis of conserved surface residues to confirm if the GdmCl-induced D state is a random coil or does it have a role in controlling the equilibrium between N and D states of a protein. Residues accessible to solvent usually have relatively less interaction with the folded part of the native protein. Thus there interference with the normal function or structure of the folded or native protein is minimal but they primarily affect the D state of the protein [445]. Surface mutations thus provide a way to probe mutational effects on D state energy with less complication from N state energy effects than would be expected for mutations at buried sites. Upon denaturation, if the protein attains random coil conformation then any mutation on the solvent exposed residue would not make a difference but if the D state is not a random coil then there could be some steric constraints and alteration in the stability of the denatured state. They chose Lys73 a solvent exposed and conserved residue, to be the site for site directed mutagenesis [319-322,443].

Bowler *et al*. [319], in one of their studies, replaced hydrophilic Lys73 with some hydrophobic residues (Met, Tyr, Phe and Trp). Fourier transform infrared (FTIR) spectral analyses of all mutants showed insignificant effect on the native fold of the protein. The stability measurements of GdmCl-induced denaturation of each protein monitored by CD spectroscopy demonstrated reduction in the stability. The Gibbs free energy change in the absence of denaturant for these proteins shows linear relationship with the hydrophobicity of the amino acid at position 73 of the sequence. Stability was found to be inversely proportional to the hydrophobicity of the substituted residue. Decrease in the stability of mutants suggested that the D state was a compact state whose compactness increased due to hydrophobic clustering when hydrophilic residues were replaced with hydrophobic residues. Infrared spectroscopy also confirmed that such mutations did not alter the N state of such mutants but do alter the compactness of the D state [320].

In another study [443], Lys73 was replaced with non-polarisable aliphatic residues (Gly, Ala, Val, Leu and Ile). In this study also the hydrophobic clustering and decrease in the stability of the mutants was observed suggesting D state to be non-random compact state. The difference with the previous study was that in this study the stability of the D state was affected by α-helix propensity along with the hydrophobic effect.

It has been established that Met80-heme interaction is lost when the protein is denatured and is replaced by non-native His [446,447]. Bowler and co-workers [61,176,322, 441,442] showed that if the position of the non-native His ligands is changed then it affects the energetic of the D state of the protein or it can be said that residual structure in denatured proteins can make important thermodynamic contributions to changes in the free energy of unfolding caused by mutations.

In another study, introduction of a novel disulfide bond

in y-cyt-*c* resulted in a more compact denatured state, leading to stabilization of this state [448].

Heat-Induced Denatured State

Heat-induced denatured state of various proteins have been reported to have considerable amount of residual structure which when treated with strong denaturant undergo further transition [449-451]. Similar case has been reported for y-cyt-*c* as well [448], where heat-induced D state retained more structure that GdmCl induced D state and addition of GdmCl to the thermally denatured protein further unfolded the protein. Similar results have also been obtained in our laboratory for WT y-cyt-*c* and its N-terminal deletant proteins (unpublished results). On the contrary, Herrmann *et al*. [443] suggested that GdmCl-induced D state of cyt-*c* and its Lys73 mutants is less random than the heat-induced D state of respective proteins. Though CD spectra of D state induced by heat demonstrated more negative ellipticity than that in GdmCl-induced D state but they explained this aspect as a result of lack of positive ellipticity contributed by β-turns. They clarified their point on the pretext that β-turns present in the proteins contribute positive ellipticity at 220 nm [452,453], which during thermal denaturation also get denatured thus the loss of that positive ellipticity renders heat-induced D state apparently with more negative ellipticity.

CONCLUSIONS

This review summarises all the possible aspects or virtues of cyt-*c* that make it a model protein. Being universally present and multitasking, cyt-*c* has been a protein of choice to study evolution, physiology, electron transport, biochemical reactions, apoptosis, etc. Its protein folding mechanism has not only been studied in detail but it also shows an agreement between classical and new view of protein folding problem. The thermodynamic states with special reference to y-cyt-*c*; N (crystal/solution structures), intermediates (MG and PMG) and D states have been discussed in details.

ACKNOWLEDGEMENTS

We sincerely thank Department of Science and Technology, Government of India for financial support. SZ thanks Indian Council of Medical Research, Government of India for the award of fellowship.

Conflict of Interest: Authors declare no conflict of interest.

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