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ISSN: 0975-7538
Research Article

# Structural stability and folding pathways of proteins under native conditions as monitored by hydrogen/deuterium (H/D) exchange methods

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**ABSTRACT**

The conformational stability and dynamics of proteins are indispensable to address the structure-function relationships of proteins and also to design novel proteins for therapeutic purposes. At molecular level, conformational stability of a protein can be monitored in presence of external denaturing agents by using traditional biophysical techniques, whereas at residue level, conformational stability of a protein can be measured by hydrogen/deuterium (H/D) exchange methods in conjunction with nuclear magnetic resonance (NMR) techniques. In this review, stability and folding of proteins probed at residue level resolutions by using H/D exchange methods to date have been exclusively covered, systematically classified and discussed in detail. Moreover the merits and limitations of the exquisite methods and computational alternatives to the proteins H/D exchanges have also been discoursed.

**Keywords:** H/D exchange; Protein folding; Residue-specific stability; Unfolding kinetics

## PROTEIN FOLDING PROBLEM

Proteins play vital roles in carrying out most of the biological functions such as immune system reactions, signal transduction, gene expression, storage, translocation and many more. In general, proteins are synthesized in ribosomes of eukaryotic organisms as linear polypeptide chains, which then acquire unique biologically active three-dimensional (3D) structures in a few seconds or so (Cabrita et al., 2010). The mechanisms by which these functional proteins are folded from their unstructured conformations are still puzzling, though much has been learnt and this problem is known as the 'protein folding problem' (Dill and MacCallum, 2012). Unlocking this puzzle will help on understanding the mechanisms of the protein misfolding/aggregation and also on stimulating drug designing process (Uversky, 2003; Luheshi et al., 2008). Christian Anfinsen, who was awarded Nobel prize in 1972 for his studies on protein folding, demonstrated that proteins fold spontaneously to their active conformations in ambient conditions and the primary structures of proteins tightly govern their folding processes (Anfinsen, 1973). However, it is still unclear so as to how a polypeptide chain folds to its native conformation within a reasonable time frame. In principle, a polypeptide chain, which is composed of 100 amino acids, needs to sample out about $8^{99}$ conformations before acquiring its thermodynamically stable state and the process may require about $10^{66}$ years, if one assumes that only $10^{-13}$ seconds are required for interconverting one conformation to other. Interestingly, proteins fold in the time span of sub-seconds, in general. This puzzle is the crux of the 'protein folding problem' and it is popularly called as the 'Levinthal paradox' (Levinthal, 1968). Interestingly, growing evidences on the studies of protein folding through experimental and as well computational methods suggest that proteins could presumably fold to its global minimum in a stepwise manner either through a defined pathway or multiple parallel pathways (Maity et al., 2005).

Precise estimation of the stabilization free energy of a protein at ambient conditions is important for understanding folding mechanism of the protein. Moreover, the structural stability of the protein provides insights about the forces governing conformations of the protein. Conformational stability measured in terms of free energy of unfolding of proteins ($\Delta G_U$) is defined as the free energy difference between their unfolded and folded states. The $\Delta G_U$ of a protein is conventionally estimated at molecular level using classical biophysical methods which monitor the protein folding mechanism in the presence of thermal or chemical denaturants. On the other hand, residue-specific stability of proteins can be measured by NMR-assisted hydrogen/deuterium exchange (H/D) methods (Huyghues-Despointes et al., 1999) and it is an only method for delineating stability and folding of proteins under native conditions. In this review, we present a compre-

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Received on: 09-11-2013
Revised on: 11-12-2013
Accepted on: 13-12-2013

hensive list of all the proteins for which H/D exchange studies have been carried out to date and the proteins could be classified into various categories on the basis of purposes of experiments carried out. Furthermore, merits of H/D exchange methods over conventional studies on understanding stability and folding of proteins and constraints which restrict the applications of the methods to certain proteins are also brought into fore. We have also concisely discussed various *in silico* tools that are being used to probe structural stability and folding pathways of proteins.

**H/D EXCHANGE METHODS**

In traditional methods, protein is denatured using high temperature or chemical denaturants (such as urea, thiourea, GdmHCl) and then the population of the unfolded/folded states are monitored using techniques such as fluorescence spectroscopy, circular dichroism or infrared spectroscopy (Huyghues-Despointes et al., 1999; Bartlett and Radford, 2009). Free energy of unfolding ($\Delta G_U$) is measured from the melting curves by using linear extrapolation method when chemical denaturants are used and by Gibbs-Helmholtz equation when thermal denaturant methods are used. The data analyses yield a single $\Delta G$ value representing overall stability of those proteins. These methods require long extrapolation from high denaturant concentration region to region of no denaturant in order to estimate $\Delta G_U$ under given experimental conditions. This methods suffer due to following facts: (i) denatured state of proteins at high temperature or high concentration of denaturants may not be as same as the unfolded states existing under native conditions of the proteins (ii) signals monitored by these methods are primarily dominated by the folded conformations of the proteins which often obscures the detection of partially unfolded states that are immeasurably populating (insensitive to conventional methods) in the unfolding transition of proteins under native conditions.

Unfolding free energy exchange of proteins can be studied at residue level under native conditions (either in absence or low concentrations of denaturants) by using NMR-assisted H/D exchange methods (Englander et al., 1972; Mayne and Englander, 2000). In a typical H/D exchange method, a protein is dissolved in deuterium oxide ($D_2O$) following which the backbone amide protons of the protein residues exchange with deuterium, when they are exposed through an ephemeral conformational opening events as described in the following equation (Hvidt and Nielsen, 1966):

$$\text{Closed (NH)} \underset{k_{cl}}{\overset{k_{op}}{\rightleftharpoons}} \text{Open (NH)} \xrightarrow{k_{rc}} \text{Exchanged (ND)}$$

In the above equation, Closed (NH) and Open (NH) denote folded and unfolded conformations of proteins, respectively. The rate constant of unfolding is $k_{op}$ and $k_{cl}$ is the folding rate constant. The intrinsic rate of exchange is $k_{rc,}$ which can be predicted using the method reported by Bai et al (1993). In ambient conditions, native states of proteins are always in equilibrium with the number of microstates defined by the Boltzmann relationship. Unlike traditional methods, signal of the microstates are not swamped by the predominant native state in the H/D exchange methods as the exchange reactions of labile protons of proteins are happening through unfolded states and the reactions are independent of population of native states.

There are two limiting factors in the H/D exchange reactions of proteins: EX1 limit and EX2 limit as mathematically represented in the equations 2 & 3, respectively. The free energy of exchange ($\Delta G_{HX}$) for each residue of proteins can be calculated as shown in equation 4 (Englander et al., 1972).

$$k_{ex} = k_{op} \quad (2)$$

$$k_{ex} = (k_{op}/k_{cl})*k_{rc} = K_{HX} * k_{rc} \quad (3)$$

$$\Delta G_{HX} = -RT \ln K_{HX} = -RT \ln (k_{ex}/k_{rc}) \quad (4)$$

Wherein $k_{ex}$ is an extrinsic exchange rate constant, R is the gas constant, T is the absolute temperature and $K_{HX}$ is the residue-specific equilibrium constant for labile protons in proteins. The free energy of exchange of a protein is averaged out to three largest residue-specific $\Delta G_{HX}$ of the protein (Huyghues-Despointes et al, 1999).

Another potential advantage of the H/D exchange methods is detection of short-lived partially unfolded states known as cryptic intermediates (CIs) accumulating in the unfolding kinetics of proteins under native conditions. The CIs are usually insensitive to the optical probes of conventional methods. These CIs may act as the prime sources of discrepancy between $\Delta G_U$ and $\Delta G_{HX}$ of proteins (Bai et al., 1994; Mayne and Englander, 2000). In order to identify CIs, H/D exchanges of proteins are monitored in presence of mild denaturant concentrations (without perturbing the native state) well below the transition/melting region (Bai et al., 1994; Bai, 2006). The methods are robust not only in identifying the CIs structurally and energetically but also on defining the folding pathways of proteins. In the following section, we present an exclusive list of proteins which have been characterized by using H/D exchange methods in conjunction with multi-dimensional NMR techniques to date.

**PROTEINS PROPED BY USING H/D EXCHANGE METHODS**

The thermodynamics and kinetics of 83 proteins have been studied using NMR-assisted H/D exchange methods to date. The exchange data of these proteins can be used to examine their local, sub-global and global energetic conformations. A comparison of $\Delta G_{HX}$ (globally exchanging residues) and $\Delta G_U$ of proteins under identical conditions will also provide valuable insights into the unfolding mechanisms of proteins (Bai et al., 1994; Bai and Englander, 1996) and also on understanding the axioms of H/D exchange mechanisms of proteins (Skinner et al., 2012a; Skinner et al., 2012b).

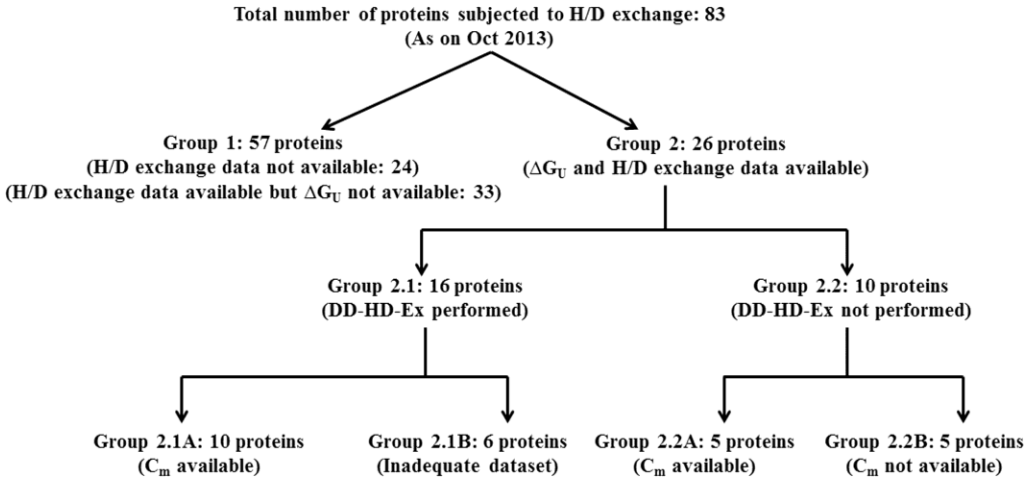


**Figure 1: A schematic diagram depicting protein classifications based on their thermodynamic and kinetic data reported in the literature**

Table 1 depicts a complete list of proteins which have been subjected to native-state H/D exchange to date as reported in the literature, along with essential experimental conditions, main purposes of the studies and corresponding references. Proteins of varying sizes ranging from 53 residues (Thermophilic Rubredoxin (Hiller et al., 1997)) to as large as 370 residues (Maltose binding protein (Merstorf et al., 2012)) have been studied by the methods. Streptomyces subtilisin inhibitor was the first protein to be studied (1985) using NMR-H/D exchange method under EX2 conditions (Akasaka et al., 1985). Mayo and Baldwin were the first to study the unfolding kinetics of RNase A equilibrated with chemical denaturants under native conditions (Mayo and Baldwin, 1993). However, cytochrome c was the first protein in which CIs have been unambiguously identified and structurally characterized by using denaturant-dependent H/D exchange methods (Bai et al., 1995). Interestingly, the detected CIs of cytochrome c were in close resemblance of kinetic intermediates trapped in refolding kinetics of the protein and similar trends have been observed for a several proteins, though there are a few exceptions (Bai, 2006; Chamberlain and Marqusee, 1997).

Proteins listed in Table 1 have been divided into many groups based on types of H/D exchange experiments to which the proteins were subjected and availability of their thermodynamic ($\Delta G_U$ and $C_m$) and kinetic (residue-specific $k_{ex}$) parameters acquired at similar experimental conditions as reported in the literature (Fig. 1). The 83 proteins could be broadly divided into two main groups: Group 1 comprises of 57 proteins of which residue specific exchange rate constants were not provided for 24 proteins and thermodynamic parameters under the solution conditions used to monitor H/D exchange reactions were not given to the rest of 33 proteins. Group 2 comprises 26 proteins for which both kinetic data from H/D exchange methods and thermodynamic data from macroscopic methods have been well-furnished in the literature. The PDB IDs, $\Delta G_U$, $\Delta G_{HX}$ and experimental solution conditions of the 26 proteins have been enumerated in Table 2. Of 26 proteins, 4 are all-α protein, 4 are all-β proteins and 18 are mixed-αβ proteins. Overall sizes of proteins were varied from 56 to 162 residues in length and most of them were of single domain proteins. The H/D exchange experiments have been performed with wide pH ranges between 2.1-7.5 and narrow range of temperature from 295 K to 313 K. Although the experimental conditions of both H/D exchange and macroscopic methods are well matching to each other, the $\Delta G_{HX}$ and $\Delta G_U$ of the proteins were not in good agreement (varied from 0.3 to 4.7 kcal/mol). Smaller discrepancy between the two thermodynamic parameters of proteins may be either due to the effect of *cis-trans* proline isomerisation or/and the effect of baselines of melting curves, whereas larger differences may indicate possible accumulation of either cryptic intermediates or metastable states in the unfolding kinetics of proteins (Huyghues-Despointes et al., 1999; Mayne and Englander, 2000; Yadav and Ahamad 2000).

The proteins in Group 2 have been further divided into two more groups: Group 2.1 comprises 16 proteins ((Barnase (Clarke and Fersht, 1996), Chymotrypsin Inhibitor 2 (CI2) (Itzhaki et al., 1997), FAT Domain (Dixon et al., 2004; Zhou et al., 2006), Barstar (Bhuyan and Udgaonkar, 1998), Hisactophilin (Houliston et al., 2002), RNase A (Neira et al., 1999), RNase H (Chamberlain et al., 1996), β-lactoglobulin (Ragona et al., 1999), PDZ third domain (Feng et al., 2005), Thioredoxin (Bhutani and Udgaonkar, 2003), T4 Lysozyme (Llinas et al., 1999), Src SH3 (Grantcharova and Baker, 1997), Cytochrome $C_{551}$ (Michel and Bren, 2008), Apocytochrome $b_{562}$ (Fuentes and Wand, 1998), Src SH2 (Wildes et al., 2006) and Cytochrome C (Milne et al., 1998)) for which energy landscapes of their unfolding kinetics have been examined using denaturant dependent H/D exchange (DD-HD-Ex) methods and 10 proteins (N-terminal of ribosomal protein L9 (NTL9) (Vugmeyster, 2001), RNase T1 (Huyghues-Despointes et al., 1999),

**Table 1: This table lists proteins that are analyzed by using H/D exchange methods in conjunction with NMR techniques under equilibrium conditions**

| S. No. | Proteins | pH | Temperature | References | Purpose of the study |
|---|---|---|---|---|---|
| 1 | Streptomyces Subtilisin inhibitor | 7-11 | 303K - 333K | Akasaka et al., 1985 | Identification of stable regions |
| 2 | Apomyoglobin | 6 | 278K | Hughson et al., 1990 | Characterization of stable intermediates |
| 3 | IL-1β | 5.4 | 309K | Gronenborn, 1990 | Structure analysis |
| 4 | HEWL | 7.5 | 303K | Radford et al., 1992 | Structure analysis |
| 5 | Ubiquitin | 3.5 | 295K | Pan and Briggs, 1992 | Characterization of stable intermediates |
| 6 | Guinea Pig α-Lactalbumin | 2 | 298K | Chyan et al., 1993 | Characterization of stable intermediates |
| 7 | BPT1 | 3.5 | 303K | Kim et al., 1993 | Identification of stable regions |
| 8 | GB1 | 5.7 | 298K-333K | Orban et al., 1995 | Stability analysis |
| 9 | GB2 | 5.7 | 298K-333K | Orban et al., 1995 | Stability analysis |
| 10 | Human α-Lactalbumin | 6.3 | 288K | Schulman et al., 1995 | Characterization of stable intermediates |
| 11 | Acyl carrier protein | 6.2 | 303K | Andrec et al., 1995 | Structure analysis |
| 12 | Jun Leucine Zipper domain | 3.6 | 310K | King, 1995 | Structure analysis |
| 13 | Putidaredoxin | 7.4 | 290K | Lyons et al., 1996 | Structure analysis |
| 14 | RNase H | 5.1 | 298K | Chamberlain et al., 1996 | Characterization of cryptic intermediates |
| 15 | Barnase | 4.5 | 298K | Clarke and Fersht, 1996 | Characterization of cryptic intermediates |
| 16 | OMTKY3 | 5 | 303K | Swint-Kruse and Robertson, 1996 | Stability analysis |
| 17 | Tendamistat | 3 | 323K | Schönbrunner et al., 1996 | Characterization of stable intermediates |
| 18 | Variants of Yeast L cytochrome 6 | 4.6 | 298K | Betz et al., 1996 | Stability analysis |
| 19 | CI2 | 5.3 | 306K | Itzhaki et al., 1997 | Characterization of cryptic intermediates |
| 20 | CheY | 6.3 | 298K | Lacroix et al., 1997 | Structure analysis |
| 21 | SH3 | 6 | 295K | Grantcharova and Baker, 1997 | Characterization of cryptic intermediates |
| 22 | ScFv | 6.5 | 300K | Freund et al., 1997 | Stability analysis |
| 23 | Equine Lysozyme | 4.5 | 298K | Morozova-Roche et al., 1997 | Characterization of stable intermediates |
| 24 | Groel | 6 | 295K | Goldberg et al., 1997 | Binding interface analysis |
| 25 | Ribosomal Protein L9 | 7 | 305K | Lillemoen et al., 1997 | Structure and stability analysis |
| 26 | Thermophilic Rubredoxin | 6.5-9.4 | 333K | Hiller et al., 1997 | Characterization of cryptic intermediates |
| 27 | Protein A-B domain | 7 | 293K | Bai et al., 1997 | Characterization of cryptic intermediates |

| S. No. | Proteins | pH | Temperature | References | Purpose of the study |
|---|---|---|---|---|---|
| 28 | Peptostreptoccal Protein L | 7 | 295K | Yi et al., 1997 | Characterization of cryptic inter-mediates |
| 29 | 434Cro | 6 | 293K | Padmanabhan et al., 1997 | Structure and stability analysis |
| 30 | Cytochrome c | 7 | 303K | Milne et al., 1998 | Structure and stability analysis |
| 31 | Apocytochrome b562 | 4.5 | 298K | Fuentes and Wand, 1998 | Characterization of cryptic inter-mediates |
| 32 | Barstar | 6.7 | 305K | Bhuyan and Ud-gaonkar, 1998 | Characterization of cryptic inter-mediates |
| 33 | Apoflavodoxin | 6.2 | 303K | Steensma et al., 1998 | Structure and stability analysis |
| 34 | RNase H1 | 5.5 | 300K | Yamasaki et al., 1998 | Structure and stability analysis |
| 35 | Acyl coenzyme A | 5.2-8.1 | 298K | Kragelund et al., 1998 | Structure and stability analysis |
| 36 | Haemoglobin | 7.4 | 273K | Englander et al., 1998 | Analysis of allosteric mechanism |
| 37 | T4 Lysozyme | 5.6 | 298K | Llinas et al., 1999 | Characterization of cryptic inter-mediates |
| 38 | RNase A | 6 | 313K | Neira et al., 1999 | Stability analysis |
| 39 | RNase T1 | 7.3 | 298K | Huyghues-Despointes et al., 1999 | Stability analysis |
| 40 | CTXIII | 3.2 | 298K | Sivaraman, 1999 | Structure and stability analysis |
| 41 | Bovine β-Lactoglobulin | 2.1 | 310 | Ragona et al., 1999 | Characterization of cryptic inter-mediates |
| 42 | CBTX | 3.2 | 298K | Sivaraman, 1999 | Structure and stability analysis |
| 43 | T. thermophilus RNase H | 5.5 | 298K | Hollien and Marqusee, 1999 | Characterization of cryptic inter-mediates |
| 44 | RNase S | 6 | 298K | Neira et al., 1999 | Stability analysis |
| 45 | LysN | 6 | 293K | Alexandrescu et al., 1999 | Characterization of cryptic inter-mediates |
| 46 | Transerythrin | 5.7 | 313K | Liu et al., 2000 | Identification of stable regions |
| 47 | Equine β-lactoglobulin | 4 | 298K | Kobayashi et al., 2000 | Characterization of stable inter-mediates |
| 48 | Canine Milk Lyso-zyme | 4.5 | 298K | Grinberg et al., 2000 | Characterization of stable inter-mediates |
| 49 | E. Coli CspA | 5.4 | 278K | Jaravine et al., 2000 | Stability analysis |
| 50 | Hpr | 5.5 | 303K | Peterson, 2001 | Structure and stability analysis |
| 51 | NTL9 | 5 | 303K | Vugmeyster, 2001 | Structure and stability analysis |
| 52 | Intestinal Fatty acid binding protein | 6.5 | 298K | Hodsdon and Frieden, 2001 | Characterization of cryptic inter-mediates |
| 53 | N-terminal domain of rat CD2 | 5.5 | 298K | Parker and Mar-qusee, 2001 | Characterization of cryptic inter-mediates |
| 54 | Plastocynin | 6.5 | 295K | Bertini et al., 2001 | Structure analysis |
| 55 | Hisactophilin | 7.8 | 290K | Houliston et al., 2002 | Characterization of cryptic inter-mediates |
| 56 | Human growth harmone | 7 | 305K | Kasimova et al., 2002 | Characterization of stable inter-mediates |
| 57 | Human acid fibrob-last growth factor | 6 | 298K | Chi Y et al., 2002 | Stability analysis |

| S. No. | Proteins | pH | Temperature | References | Purpose of the study |
|---|---|---|---|---|---|
| 58 | Rd Apocytochrome | 7 | 298K | Chu et al., 2002 | Characterization of cryptic intermediates |
| 59 | Borrelia Burgderferi OspA | 6 | 310K | Yan et al., 2002 | Characterization of cryptic intermediates |
| 60 | Thioredoxin | 7 | 298 | Bhutani and Udgaonkar, 2003 | Characterization of cryptic intermediates |
| 61 | Bergrac Src Homology 3 variants | 2.6 | 298K | Viguera et al., 2003 | Characterization of cryptic intermediates |
| 62 | FAT Domain | 6 | 310K | Zhou et al., 2006 | Characterization of cryptic intermediates |
| 63 | PDZ | 6.3 | 298K | Feng et al., 2005 | Characterization of cryptic intermediates |
| 64 | MerP | 4.7 | 298K | Bororsson et al., 2006 | Characterization of cryptic intermediates |
| 65 | Src SH2 | 7 | 298K | Wildes et al., 2006 | Characterization of cryptic intermediates |
| 66 | Cyclophilin | 6.2 | 298K | Shi et al., 2006 | Stability analysis |
| 67 | FHA Domain from Arabidopsis Receptor Kinase-associated Protein Phosphatase | 6.3 | 293K | Ling et al., 2006 | Characterization of cryptic intermediates |
| 68 | MICAL-1 CH | 6.5 | 293K | Jin et al., 2007 | Characterization of cryptic intermediates |
| 69 | FF Domain | 5.7 | 298K | Korzhnev et al., 2007 | Characterization of stable intermediates |
| 70 | Maize ferrodoxin NADP Reductase | 6 | 313K | Lee et al., 2007 | Structure and stability analysis |
| 71 | Cytochrome c551 | 6 | 299K | Michel and Bren, 2008 | Characterization of cryptic intermediates |
| 72 | SNase | 7-9.5 | 293K | Skinner et el., 2012b | Structure analysis |
| 73 | α-Synuclein | 7.4 | 288K | Croke et al., 2008 | Structure analysis |
| 74 | Alpha subunit of Trp Synthetase | 7.4 | 298K | Vadrevu et al., 2008 | Characterization of cryptic intermediates |
| 75 | DCL8 | 7 | 293K | Mohan et al., 2009 | Characterization of cryptic intermediates |
| 76 | β2 microglobulin | 6.6 | 301-315K | Rennella et al., 2009 | Structure and stability analysis |
| 77 | Aminoglycoside phosphotransferase | 7.5 | 300K | Norris et al., 2009 | Structure analysis |
| 78 | Ribosomal protein S6 | 6.3 | 298K | Haglund et al., 2009 | Folding pathway analysis |
| 79 | Lysozyme from Bacteriophage λ | 5.6 | 293K | Di Paolo et al., 2010 | Characterization of stable intermediates |
| 80 | C-terminal domain of the Fas-associated death domain | 4.8 | 303K | Greene et al., 2012 | Structure analysis |
| 81 | Maltose Binding Protein | 7 | 310K | Merstrof et al., 2012 | Characterization of cryptic intermediates |
| 82 | HisF TIM barrel subunit of the heterodimeric Thermotoga maritima imidazole-3 glycerol phosphate synthase | 6.8 | 313K | Gangadhar et al., 2013 | Structure and stability analysis |
| 83 | *Escherichia coli* Co-Chaperonin GroES | 6.5 | 298K | Chandak et al., 2013 | Structure analysis |

**Table 2: Proteins for which free energy of exchange (ΔGHX) and free energy of unfolding (ΔGU) are available at similar, if not identical, solution conditions**

| Sl. No | Proteins | PDB ID | Length | Class | Experimental Conditions | | $\Delta G_U$ (kcal/mol) | $\Delta G_{HX}$ (kcal/mol) |
|---|---|---|---|---|---|---|---|---|
| | | | | | pH | Temp. (K) | | |
| 1 | Barnase | 1BNR | 110 | Mixed | 4.5 | 298 | 9.8 | 10.1 |
| 2 | Hisactophilin | 1HCD | 118 | All β | 7.8 | 290 | 10.8 | 11.1 |
| 3 | NTL9 | 1DIV | 56 | Mixed | 5 | 303 | 4.7 | 5 |
| 4 | Thioredoxin | 1XOA | 108 | Mixed | 7 | 298 | 9.6 | 9.9 |
| 5 | CI 2 | 2CI2 | 64 | Mixed | 5.3 | 306 | 7 | 7.6 |
| 6 | FAT Domain | 1PV3 | 146 | All α | 6 | 310 | 6.3 | 7 |
| 7 | HEWL | 1E8L | 129 | Mixed | 7.5 | 303 | 11.7 | 12.4 |
| 8 | CheY | 1CEY | 129 | Mixed | 6.3 | 298 | 5.6 | 6.5 |
| 9 | RNase H | 1F21 | 155 | Mixed | 5.1 | 298 | 9.9 | 10.9 |
| 10 | GB1 | 1PGA | 56 | Mixed | 5.7 | 298 | 6.6 | 7.7 |
| 11 | β-lactoglobulin | 1BSQ | 162 | Mixed | 2.1 | 310 | 7 | 8.2 |
| 12 | Barstar | 1BTA | 89 | Mixed | 6.7 | 305 | 5 | 6.2 |
| 13 | Hpr Protein | 1POH | 85 | Mixed | 5.5 | 303 | 4.6 | 5.8 |
| 14 | OMTKY3 | 2OVO | 56 | Mixed | 5 | 303 | 7.2 | 8.6 |
| 15 | PDZ third domain | 1BE9 | 115 | Mixed | 6.3 | 298 | 7.4 | 8.9 |
| 16 | SH3 | 1SRL | 64 | All β | 6 | 295 | 4.7 | 6.4 |
| 17 | Cardiotoxin III | 2CRT | 60 | All β | 3.2 | 298 | 4.9 | 6.7 |
| 18 | Ubiquitin | 1UBQ | 76 | Mixed | 3.5 | 298 | 5 | 7.1 |
| 19 | Cobrotoxin | 1COD | 62 | All β | 3.2 | 298 | 2.3 | 4.4 |
| 20 | Apocytochrome $b_{562}$ | 1APC | 106 | All α | 4.5 | 298 | 3.3 | 5.5 |
| 21 | Cytochrome $C_{551}$ | 351C | 82 | All α | 6 | 299 | 6 | 8.3 |
| 22 | Src SH2 | 1SPR | 103 | Mixed | 7 | 298 | 7.8 | 10.2 |
| 23 | Cytochrome C | 1HRC | 104 | All α | 7 | 303 | 10 | 12.6 |
| 24 | RNase T1 | 1YGW | 104 | Mixed | 7.3 | 298 | 7.9 | 10.7 |
| 25 | RNase A | 2AAS | 124 | Mixed | 6 | 313 | 7 | 9.8 |
| 26 | T4 Lysozyme | 1L63 | 162 | Mixed | 5.6 | 298 | 13 | 17.7 |

turkey ovomucoid third domain (OMTKY3) (Swint-Kruse and Robertson, 1996), B1domain of streptococcal protein G (GB1) (Orban et al., 1995), Hpr (Peterson, 2001), Chemotactic y (Che Y) (Lacroix et al., 1997), Cardiatoxin III (CTX III) (Sivaraman, 1999), hen egg white lysozyme (HEWL) (Radford et al., 1992), Ubiquitin (Pan and Briggs, 1992) and Cobrotoxin (CBTX) (Sivaraman, 1999)) in the Group 2.2 have not yet been characterized by the DD-HD-Ex methods. Proteins belonging to the both groups were further divided into two groups based on the availability of $C_m$ data for the proteins. $C_m$ which is midpoint of the melting curve is indispensable thermodynamic parameter for estimating population of various cryptic intermediates accumulating in the unfolding pathways of proteins (*http://sblab.sastra.edu/sw.html*). The group named 'inadequate dataset' (Group 2.1B) represents proteins (Barnase, Barstar, Hisactophilin, RNase A, RNase H and Thioredoxin) for which DD-HD-Ex experiments have not been systematically carried-out or/and H/D exchange data are acquired from skeptical set-up of experiments.

## EXPERIMENTAL LIMITATIONS OF H/D EXCHANGE METHODS

Notwithstanding the advantages of the methods on delineating the structural stability and unfolding pathways of proteins under native conditions at residue level, the methods are laborious, expensive, technically challenging, time consuming (several months to years) and also require sound experimental knowledge. The methods may not be suitable to the proteins which cannot withstand their folded structures in the solution condition throughout the course of experiments and also to the proteins which are highly prone to get into aggregations or degradations. Apart from these limitations, locally unfolding residues often interfere with the successful identification of CIs and even the narrow stability differences of various CIs may also complicate their detection and structural characterizations (Englander et al., 2007). In these contexts, computational tools will be an excellent alternative to the H/D methods provided the tools are robust and reliable on probing stability and folding pathways of proteins independent of their topology.

## COMPUTATIONAL TOOLS FOR ANALYZING H/D EXCHANGE DATA

There are a few computational tools for predicting various parameters of proteins H/D exchange reactions

and for analyzing data derived from NMR-H/D exchange experiments: CIntX and CamP are useful for calculating exchange rate constants of labile protons in proteins (Richa and Sivaraman, 2012a; Tartaglia et al.,2007); COREX/BEST determines stability of various regions of proteins (Vertrees et al., 2005); OneG predicts energetic landscapes of proteins (Richa and Sivaraman, 2012b). CIntX estimates $k_{rc}$, intrinsic exchange rate constant, of all labile protons (from backbone, side chains, C-terminus & N-terminus regions) in proteins at defined conditions (such as pH, temperature and ionic strength) in a fully automated manner. In order to complete the task, the program requires only the atomic coordinates of three-dimensional (3D) structures of proteins. The webserver of the program is publicly available at *http://sblab.sastra.edu/cintx.html.* The computational tool, CamP, (*http://www-vendruscolo.ch.cam.ac.uk/camp.new.php*) predicts $k_{ex}$, extrinsic exchange rate constant, of backbone labile protons on the basis of their 'local structural contacts' in proteins. However, the prediction accuracy (about 50% only) of the tool is not quite impressive. COREX/BEST (*http://best.bio.jhu.edu/BEST/)* is a statistical thermodynamic tool which identifies the most stable and flexible regions within a protein structure by generating ensemble conformations of the protein. In these contexts, we have recently developed a novel computational tool, OneG, for detecting cryptic intermediates accumulating in the unfolding kinetics of proteins under native conditions. The program prerequisites four inputs (PDB file of the protein under interest, $k_{ex}$ of backbone amide protons in the absence of denaturant, $\Delta G_U$ and $\Delta G_U^*$ of the protein) and completes the task in a few minutes. The program is user-friendly and prediction accuracy of the tool is well-authenticated (Richa and Sivaraman, 2012b). Structures of predicted CIs and fitted/predicted parameters can be downloaded from the webserver (*http://sblab.sastra.edu/oneg.html*) in 'pdb' and 'txt' formats, respectively. In addition to the 4 computational tools discussed above, though there are a few databases and *in silico* tool that are relevant to H/D exchange experiments in one way or other, they were not discussed, herein, either to avoid redundant discussions or to make the review in forthright manner on the focused targets of structural stability and folding of proteins.

## FUTURE PERSPECTIVES

To our best knowledge, this is first review article which listed out all proteins characterized by H/D exchange methods in conjunction with NMR techniques to date (1985 – Oct 2013) and also systematically grouped them based on types of H/D experiments to which they were subjected under native conditions. We have also concisely discussed merits and limitations of the H/D exchange methods and possible computational alternatives to the methods as well. Quite a large amount of H/D exchange data on 83 proteins belonging to all types of classes brings an excellent platform where one can use the wealthy data to figure-out various types of structural and dynamic information that were mostly eluded in the macroscopic experiments. In these backgrounds, we trust that the review will be useful for structural biologists to trigger exciting research on 'protein folding paradigm' in near future. Also, we strongly feel that there are great scopes to develop unprecedented experimental strategies and computational tools for addressing various structural excursions of protein molecules, especially characterized by H/D exchange methods, under conditions favoring folded conformations.

## ACKNOWLEDGEMENTS

This work was partly supported by research grants from the Council of Scientific & Industrial Research (09/1095/(0004)/2013/EMR-I), New Delhi, India and T. R. Rajagopalan memorial research grant (TRR/13/Aug/2010), SASTRA University, India.

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