Cellular and Molecular Biology™ **47** (5), 000-000
Printed in France.

0145-5680/01
2001 Cell. mol. Biol.™

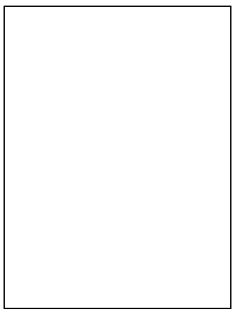
Review

WATER AND CELLULAR FOLDING PROCESSES

Peter CSERMELY✍

Department of Medical Chemistry, Semmelweis University, P. O. Box 260, H-1444 Budapest, Hungary
✍ H-1088 Budapest, Puskin str. 9., Hungary
Fax: +361 266 6550; E-mail: csermely@puskin.sote.hu

Received September 14, 2000; Accepted October 2, 2000



***Peter CSERMELY**, is Professor of the Semmelweis University (Budapest, Hungary). As a Fogarty Fellow he spent two years in the Joslin Diabetes Center (Harvard University, Boston USA) in 1989-1991. His major fields of study are molecular chaperones and diabetes. In 1995 he launched a highly successful initiative, which provides research opportunities for more than 1500 gifted high school students annually. He has published more than a hundred research papers and three books with a total citation over 900. Dr. Csermely is the secretary of the Hungarian Biochemical Society and Cell Stress Society International, is an International Research Scholar of the Howard Hughes Medical Institute and received several other national and international honors and awards.*

Abstract - Proteins require a unique, native structure to perform their functions. Water molecules play an important role to develop and maintain this three-dimensional structure. Water is also necessary for several forms of enzyme catalysis, and is a constituent of many protein-protein, protein-DNA, or protein-RNA interfaces. Larger proteins acquire their native structure in a complicated folding pathway having several folding traps. Recent data indicated a key role of water molecules in this process. Protein flexibility, structural rearrangements, conformational transitions all require the fluctuating changes in hydrogen bond structure provided by interacting water molecules. Besides proteins, RNA and DNA structure is also heavily influenced by the presence of water. This review summarizes the important aspects of these fields, and draws attention to several open questions and hypotheses.

Key words: Aging, catalysis, chaperones, DNA-binding proteins, extremophiles, folding, heat shock proteins, hydrogen bond, protein dynamics, ribozymes, water

INTRODUCTION

The form of life we experience on Earth cannot exist without the presence of liquid water. Water has numerous abnormal thermodynamic parameters (melting point, boiling point, heat of vaporization, etc.), a maximum density at 4°C, a decrease in viscosity with pressure up to ~1000 atm, and several other unique macroscopic parameters (5). Obviously there are several important structural peculiarities behind these anomalies. The complexity of liquid water is due to a combination of the small size and distinct polar charge distribution of the water molecules allowing them to participate in strong polar interactions with a high degree of spatial directionality. Water molecules form an extensive, fluctuating hydrogen-bridge network. Since water is a dipole, and has a non-regular tetrahedral shape, these fluctuations also change the actual geometrical arrangement and dipole properties. Moreover, water tetrahedra cannot occupy space in a close-spaced manner, and the occurring voids give an inherent instability of the overall structure. As a consequence, in the liquid state additional water molecules transiently "invade" the coordination sphere of others, and the mean number of nearest neighbors increases to 5.5 from the original 4

(22,32,44). These unique structural features of liquid water make it specially well-designed to complement various macromolecular structures and give them an exceptional degree of flexibility.

WATER AND PROTEIN STRUCTURE

Hydrated proteins accommodate a large number of water molecules bound to their structure. The usual weight of bound water varies between 20% to 60% of the dry weight of the protein (22,39,53). Even early nuclear magnetic resonance relaxation studies gave rather strong evidence for tightly protein-bound water molecules (29). These waters are mostly in the interior of proteins with residence times between 10^{-2} to 10^{-8} sec. Surface waters usually have residence times in the subnanosecond range even if they bind to well-defined sites (49). Bound water differs from bulk water in its kinetic properties, volume, and in almost all of the attributed macroscopical parameters such as vapor pressure, melting point, boiling point, etc. (39).

Peripheral water molecules

In globular proteins peripheral water molecules preferentially bind to carboxylate sites and to other charged or polar surface residues leaving the peptide backbone rather dehydrated (10,39). Structural surface-bound water molecules tend to "smooth" proteins by filling gaps on protein surfaces (25). The exact position and orientation of water molecules is influenced by their dipole-dipole interactions with N-terminally positive α-helices and other polar elements of proteins. Israelachvili and Wennerström (32) emphasized that the exact configuration of the strongly bound first layer of oriented water molecules and hydrated counterions is rather important, since their dipole-dipole interactions may give rise to various attractive and repulsive forces.

Buried water

Protein interiors are very well-packed and only seldom contain small empty cavities. Internal voids are filled by water molecules in most cases. For a long time it was thought that buried water molecules stabilize hydrophilic patches in the otherwise hydrophobic core of globular proteins. However, water-containing hydrophobic cavities have also been found (24,36,78). The dynamic behavior of proteins allows a ready access of water molecules to most of these internal sites. Buried water (especially in a hydrophobic environment) has numerous properties deviating from those of the bulk solvent (74). As an important example, its dielectric constant may well be almost half of the normal value (43). Internal waters are not only space-filling, stabilizing bricks, but significantly contribute to the kinetics of the protein by increasing the host protein's vibrational entropy, thus making the protein more flexible (26).

Water at contact surfaces

Water molecules are often found on the interfaces of protein domains, protein-protein, protein-DNA or protein-RNA complexes. Usually there is an increased number of "void-filling" water molecules in non-specific complexes compared to high affinity, specific interactions. Waters are also used to adapt quaternary changes in allosteric enzymes with fewer waters in the high-affinity state because of improved surface complementarity (18). However, the residual interfacial water molecules may significantly contribute to the binding strength of protein complexes even in the high affinity complexes comprising up to 25% of total binding energy (13). A good example of this is, the attraction between individual chains of the collagen triple helix, largely attributed to the formation of hydrogen-bonded water bridges between specific recognition sites (41). Water not only stabilizes contact surfaces, since water-lubricated cavities frequently occur near interdomain boundaries suggesting their contribution to enhance the flexibility of interdomain motion (58).

Bound water as a "lubricant" of structural transitions

Numerous experimental evidence indicates that water is not a simple building block of protein structure, but an essential and unique source of conformational flexibility (3). As mentioned above bound water increases the vibrational entropy of proteins (26) and may contribute to the interdomain flexibility (58). Further reasons to support the role of bound water as a source of protein flexibility are summarized below:

- fluctuating peripheral water-protein interactions influence protein dynamics in a global manner causing a progressive induction of mobility from the periphery toward the interior 79);
- water-binding increases the dielectric constant, which shields electrostatic interactions contributing to the stabilization of buried charges and providing another source of increased protein flexibility (51) ;
- anhydrous enzymes are "frozen" and retain a molecular memory (38);
- lipid bilayers preserve kinetically trapped polypeptide conformations (1).

WATER AND RNA, DNA STRUCTURE

Due to their phosphate backbones, RNA and DNA are twice as hydrated as proteins (62). Hydration of RNA G-C pairs is well defined, while hydration around A-U base pairs is more diffuse. The presence of the 2'-OH group in RNA polymers allows for hydration of the shallow groove of RNA helices (2). On the contrary, the minor groove of DNA lacks the 2'-OH group, and is barely hydrated (4). As an exception to this rule, narrowing of the minor groove traps adenine-bound water molecules, and causes a local hydration (42). A-DNA is less hydrated than the more physiological B-DNA, which explains the observed B→A transition at low humidity. In agreement with common sense, double helical DNA does not seem to contain buried water molecules with longer residence times (69). However, it is good to keep in mind that the hydration patterns of both RNA and DNA are significantly changed by the hydrate-shell of phosphate-bound counterions, and associating divalent cations.

Fig. 1 Role of water in protein folding *in vitro*. The unfolded protein first undergoes a fast hydrophobic collapse, where most of its original surfaces become dehydrated. The folding intermediate develops its final, native structure in a slower process, where water "lubrication" often plays an important role [Adapted from (Guo and Thirumalai, 1984 ,18)].

Similarly to protein-protein interfaces, water molecules are often found in the interface of protein-RNA and protein-DNA complexes. In agreement with this, the hydrated RNA shallow groove is a favorable protein-binding region, and extensive hydration of DNA may also enhance protein binding (6,75). When binding to their catalytic sites, restriction endonucleases sequester several water molecules. A mismatch of one base pair provides a worse fit, and increases the number of shape-adjusting water molecules by 70 (61,66). A similar phenomenon occurs when the *E. coli* cAMP receptor protein binds to its response element. Here, binding to the nonspecific site requires the assistance of an excess of 2 to 40 waters compared to binding to a specific cAMP response element (72). Molecular dynamics simulation indicated that "breathing" of protein-DNA complexes may be rather intense allowing an ultrafast exchange of bridging water molecules (6).

DNA hydration may also occur at unusual sites. Interestingly, a profound hydration has been found at the methyl-groups of methylated cytosines using C-H···O hydrogen bonds (45), which may also serve as a special recognition signal for maintenance methylases. Mismatches increase the hydration of the otherwise "dry" minor groove, which may be a reason why repair enzymes recognize DNA damage-sites (4). DNA hydration anomalies seem to emerge as efficient "binding-traps" of various DNA-binding proteins.

WATER AND PROTEIN FOLDING

Protein folding is characterized by two major events *in vitro* (Fig. 1). In the *initial and fast steps* most of the secondary structure is already formed. In most cases folding starts with the formation of α-helices, since here the participation of only adjacent amino acids is required. β-sheet formation establishes hydrogen bonds between amino acids which are far from each other in the primary sequence; therefore, a greater decrease of entropy occurs than in the formation of α-helices (8,20,21,70). In the end of this first step the unfolded protein is collapsed, and a (more-less) stable intermediate, the molten globule is formed. The partially folded state of molten globules can be characterized by a developed secondary structure, which is mostly un-organized showing almost no tertiary structure. Molten globules still have large unburied hydrophobic surfaces and often lead to extensive aggregation. The volume of molten globules, however, is close to that of the final, folded protein (40,55). The *closing steps* of protein folding are the slow, rate-limiting steps. Here the inner, hydrophobic core of the protein is organized and unique, high-energy bonds are formed, such as disulfide bridges, ion-pairs, and the isomerization of proline cis/trans peptide bonds occurs. The free energy gain of these processes enables the formation of local, thermodynamically unstable, "high-energy" protein structures, which are stabilized by thermodynamically favorable conformation of the rest (bulk) of the protein. These "high-energy" segments of proteins can stabilize themselves by forming complexes with another molecule; thus, they often serve as active centers of enzymes or as contact surfaces between various proteins involved *e.g.* in signal transduction (8,16,17,20,21).

Protein folding is accompanied by massive dehydration (39). Most of this dehydration occurs during the initial, fast step of protein folding, during the hydrophobic collapse. Though a "wet" folding intermediate, where hydrophobic side chains were separated by one or more water layers would be energetically, and sterically favorable (52), the expulsion of most water molecules (the agent, which catalyzes rapid conformational fluctuations as described above) seems to be necessary to stabilize the transient structures during and after the hydrophobic collapse (3). Experimental evidence from unfolding studies indicates that, indeed, this might be the case: exclusion of most water molecules precedes formation of the correct side-chain contacts (31,37,50). However, the exclusion is not complete: the usual folding intermediate, the molten globule, preserves most of the native internal hydration sites, and has a native-like surface hydration (19). Intrusion of excess water molecules, and the consequent break in peptide hydrogen bonds is a key step in the reverse process, in protein denaturation (9,37).

WATER AND CHAPERONE ACTION

Folding of larger proteins is not a straightforward process, but often requires assistance. Aggregation of unfolded proteins and of molten globules is a great danger, which would drive the majority of folding intermediates to a non-productive side-reaction, much before reaching their fully folded, competent state. Molecular chaperones serve to prevent this. They recognize and cover hydrophobic surfaces successfully competing with the aggregation process. Chaperones also assist protein folding by rescuing misfolded proteins from their folding traps. This is performed by unfolding the incorrectly folded proteins, thus giving them another chance for spontaneous refolding. These two processes may go in parallel, or may be characteristic of different chaperone-target pairs (7,30).

Unfolding studies indicate that the folding intermediate, the molten globule, is "dry" (31,37,50), as predicted by Shaknovich and Finkelstein (65). However, the molten globule is not *completely* dry (19). Without the residual, and periodically exchanging internal waters in the molten globule its conformational transitions would slow by a factor up to 10^3 (3,77).

Setting the exact amount of internal water molecules seems to be a rather important parameter of a "smooth" protein folding process. Too much buried water would allow a structural uncertainty, while a complete exclusion of internal waters would freeze any further conformational transitions. In agreement with this intricate balance, the internal hydration is often readjusted by molecular chaperones. Those chaperones (often called as Anfinsen-cages), which have an internal cavity to sequester partially folded proteins, grab their targets by multiple interactions. After binding of ATP a conformational change occurs, where the inner walls of the expanding chaperone-cavity draw away, thus partially unfold the target protein by a preferential mobilization of its internal, hydrophobic core (17,54,63). As evidenced by hydrogen-deuterium studies which show a massive increase in the amount of exchangeable protons during chaperone action in parallel with a rather high residual structure of the target protein, expansion of the hydrophobic core is accompanied by a chaperone-facilitated entry of additional water molecules to the hydrophobic core of the target. Chaperones behave as "water-percolators" or, in other words: as "washing machines" they allow a transient increase of buried water molecules of the target protein, and allow these water molecules to catalyze conformational transitions necessary to rescue the misfolded target from its folding trap (Fig. 2) (17).

WATER AND RNA FOLDING

The mechanism of RNA folding has been only partially explored in recent studies. These experiments suggest a surprising similarity with protein folding in the sense that RNA molecules also undergo a rapid collapse followed by a slow search for the active structure (64). Similarly to protein folding, folding of RNA may also be accompanied by a release of water molecules. Since RNA does not contain a well-defined hydrophobic core, the extent of this release might be smaller. Protein folding is a highly cooperative process, while the cooperativity of RNA folding might involve a smaller degree (16). Since protein folding cooperativity is significantly enhanced by participating water molecules (67,77), a smaller folding-mediated dehydration of RNA may contribute to its smaller folding cooperativity.

WATER AND ENZYME ACTIVITY

Most enzyme actions require large conformational changes, which are unimaginable in the absence of bound water. In agreement with this general statement, dehydration usually results in a complete loss of enzyme activity (53). Those enzymes which are able to work in anhydrous solvents acquire a "molecular memory" by "remembering" the conformation of bound substrates or inhibitors due to their frozen structure (38). Water molecules help interdomain and catalytic site mobility, and their binding fluctuations greatly affect the course of the catalytic event (62). In many cases the hydrophobic catalytic center of the enzyme shields the substrate from the polar aqueous environment and allows a "catalysis by desolvation". In other cases (e.g. in hydrolytic reactions)

Fig. 2 Water in the mechanism of chaperone action. Molecular chaperones, which surround their target proteins exert a periodic multidirectional pulling of the target using their periodic conformational changes governed by the hydrolysis of ATP. In the pulling process the hydrophilic exterior of the target protein becomes immobilized, while its hydrophobic core becomes mobilized and gets another chance to rearrange itself. During the chaperone-mediated expansion of the target, water molecules enter to its hydrophobic core and facilitate its rearrangement further. This model is supported by fluorescence anisotropy, electron spin resonance measurements and by hydrogen/deuterium exchange studies [Adapted from (18)].

water molecules are required and can be found in the catalytic center itself, directly participating in the enzyme action. Here enzyme-mediated positioning of the substrate ensures a better catalytic efficiency than that of the bulk water (76). Moreover, the hydrophobic environment provides a lower local dielectric constant, which increases the strength of electrostatic interactions, ensuring a more efficient catalysis (34). Waters of the catalytic cleft may also regulate substrate specificity allowing the binding of one, while restricting the binding of another potential substrate (56). In more complicated arrangements, such as the case of cytochrome *bf* complex or bacteriorhodopsin, internal water molecules may form hydrogen-bonded networks inside the hosting protein, providing an efficient proton channel, which may span the accommodating membrane together with the transmembrane host protein (53). A more general occurrence of hydrogen-bonded networks is suggested by the work of Jesior (35) finding a hydrophilic structural framework in a set of 511 proteins.

Restriction endonuclease-mediated DNA cleavage induces the binding of 30 water molecules at specific sites, while a release of 40 water molecules occurs when the cleavage is performed at single mismatches (61). This phenomena can be generalized: transformation of a high-affinity macromolecular substrate may result in a worse fit requiring more waters to adapt. On the contrary, enzymatic action on a low-affinity substrate may increase binding efficiency, which is accompanied by water release. For example oxygen-binding to hemoglobin is accompanied by the association of 60 water molecules, while electron-transfer of cytochrome oxidase/cytochrome-a operates a "water cycle" of 10 water molecules (12). Hexokinase releases about 100 water molecules in the process of binding glucose. The expulsion of these water molecules (a mass ten times than that of the substrate molecule) was invoked to acquire the dehydration needed to prevent water phosphorylation instead of glucose (57).

Ribozyme substrate binding and release are also accompanied by changes in ribozyme hydration (48). Hydration and structure of RNA are stronger, and much more regular than those of proteins, allowing a smaller chance for the formation of an extensive hydrophobic core. In agreement with this idea, a reduction of the hydration of a pre-tRNA by a micellar environment enhances its self-cleavage by a factor of 100 (60). The more efficient utilization of water-mediated catalytic enhancement may have been another factor why evolution selected proteins over ribozymes as general catalysts (16).

WATER AND FOLDING PROCESSES IN LIVING CELLS

Macromolecules have a much higher concentration in cells than in the usual *in vitro* preparations. As a consequence, molecular crowding develops, and the excluded volume effect may change activity coefficients by magnitudes (80). Most of the water in cells has macroscopical parameters, and exhibits kinetics which differ appreciably from those of the pure liquid (11). Molecular crowding increases the chances of protein aggregation, which requires more efficient chaperone function. *In vivo* chaperone function is all the more important, since a relatively small unfolding of cellular proteins may immobilize a rather significant amount of water. After a severe stress the massive unfolding may cause water scarcity, which makes refolding attempts less efficient. Aging-induced dehydration and chaperone-damage (68) may aggravate the situation further.

WATER IN EXTREME CONDITIONS

Fluctuations in osmotic strength give rise to various prompt responses, initiating signaling cascades (73), the acquirement of ions and osmolites (71) and change water availability. However, there are organisms (called extremophiles), which constantly live in an environment far from the usual conditions experienced by ourselves. In these organisms adaptation to the scarcity of water developed various survival strategies.

Halophiles

Halophilic microorganisms experience constant osmotic stress. As a response to this, they accumulate a large amount of potassium chloride and/or osmolytes to build up the osmotic balance and to preserve their water content. Osmolytes (similarly to cryoprotectants) are excluded from the protein surface and – by inducing a more efficient hydration of protein surfaces – also act as structure stabilizers (71). High ionic and osmolyte content immobilizes most of the internal water as a hydrate shell. Therefore, halophilic proteins need to fight for water by accommodating a large number of charged glutamic acids on their surface (33). On the other hand, lowered water activity is also helpful, since it strengthens the association of high affinity protein-protein, protein-DNA and protein-RNA complexes having a small number of connecting water molecules at their interfaces. Scarcity of cytoplasmic water decreases water concentration, which, in turn, increases the concentration of cytoplasmic biopolymers.

This results in an increased macromolecular crowding and efficiently balances the effects of high salt, or osmolyte concentrations (59).

Desiccation – anhydrobiosis

The most obvious lack of water can be experienced when the organism is partly or almost completely desiccated. The ability to reversibly lose a majority of intracellular water (anhydrobiosis) is widespread among microorganisms, represented in most animal groups and occurs in many plants (14,15). Organisms in anhydrobiosis are more resistant to heat- and cold-shock, than their hydrated counterparts. Part of the defense system is the accumulation of "compatible solutes" such as polyhydroxy alcohols and certain non-reducing sugars like trehalose which have a large overlap with the osmolytes mentioned above. These molecules replace water molecules in structural term, however, largely lack the dynamism of the fluctuating hydrogen bonds of water molecules and, therefore, induce a "dormant" state of the anhydrobiotic organism. As another defense, a set of proteins, called hydrophilins is synthesized. These proteins have a high hydrophilicity to ensure a residual hydration, and a high percentage of glycines, to maintain conformational flexibility even in the absence of water (27).

Psychrophiles

Freezing induces dehydration of proteins, since protein-water hydrogen-bonds are slightly weaker than those of bulk water (39). Most enzymes can not work in a dehydrated state, therefore psychrophiles have to defend themselves from ice-formation. To accomplish this, these organisms often accumulate a large concentration of cryoprotectants. Cryoprotectants are small molecules, which are preferentially excluded from the protein surface and – by inducing a more efficient hydration of protein surfaces – act as structure stabilizers (71).

Hyperthermophiles

At elevated temperatures the dehydration penalty for formation of salt bridges is markedly reduced. This allows the stabilization of hyperthermophil proteins by excessive salt bridges (23). Salt bridges and excessive hydrogen bonds help to stabilize protein structure against the rapid fluctuation and "invasion" of water molecules close to 100°C.

Hyperbaric organisms

Electrostatic interactions and hydrophobic contacts become weaker, while hydrogen bonds and stacking interactions between aromatic rings increase their strength at elevated pressures. Since hydration energy becomes more favorable, protein hydration is increased, which leads to a higher perturbation of protein structure. Water penetration is a major cause of pressure-induced protein denaturation (46,47). Pressure denaturation is further enhanced by the fact that due to the reduced "invasion" of waters to each other's coordination sphere, peripheral water molecules occupy volumes smaller than those in the bulk solvent by up to 20% (28). This results in the "forced hydration" of internal protein segments, leading to denaturation. The same phenomenon also induces the dissociation of protein complexes, which is the basis of pressure-induced dissociation of protein aggregates, a fashionable tool of biotechnology. On the other hand, in some cases high pressure also promotes intermolecular disulfide bond formation (46).

SUMMARY AND PERSPECTIVES

Some of the most important messages of this review can be summarized as follows:

- water forms an essential part of the internal structure of many proteins constituting proton channels and enabling several forms of efficient catalysis;
- water acts as an adapter in protein-protein, protein-DNA or protein-RNA interfaces;
- water is a highly efficient and necessary "lubricant" of conformational changes in proteins;
- water actively participates in protein folding and its role may be enhanced by molecular chaperones;
- most enzyme reactions require the direct or indirect action of water molecules, in the absence of water protein conformation "freezes" and a "molecular memory" develops;
- Maintenance of "water-supply" is a much more important part of extremophilic adaptation than previously thought.

The paramount importance of water in macromolecular structure has long been recognized. However, recent advances in the field led to numerous exciting open questions, some of which are listed below:

- we still do not know much about the special properties of water molecules buried in hydrophobic protein cavities;
there is much to be explored on the role of water in conformational flexibility of proteins;
- more exciting examples of "frozen" enzymes retaining a molecular memory are anticipated;
- we have to learn much more about kinetically trapped membrane proteins;

- the hypothesis that DNA hydration anomalies may steer DNA-binding proteins to find their binding sites needs to be further established;
- more experiments are needed to learn how "dry" is the "dry molten globule";
- the water percolator model for molecular chaperones needs further experiments to show its validity;
- we are still at the very beginning to study dehydration during RNA folding and the contribution of water molecules to ribozyme action;
- as the experimental methods develop, the old question of cellular water is still holding surprises;
-desiccation is a form of severe stress where the adaptation machinery remanis largely unexplored at the molecular level.

The above examples form a rather incomplete list of exciting questions on the role of water in cellular folding processes, but demonstrate very well that the field, which has been investigated for a long-time, still has many challenges for adventurous scientists.

Acknowledgments – The author would like to thank Dr. László Vígh (Biological Research Centre Szeged, Hungary) for his encouragement, and Dr. James S. Clegg (University of California, Davis and the Bodega Marine Laboratory, USA) for his great help and advices. Work in the author's laboratory was supported by research grants from ICGEB, Hungarian Science Foundation (OTKA-T25206), Hungarian Ministry of Social Welfare (ETT-21/00) and the Volkswagen Foundation (I/73612). P.C. is an International Research Scholar of the Howard Hughes Medical Institute.

REFERENCES

1. Arumugam, S., Pascal, S., North, C.L., Hu, W., Lee, K.-C., Cotton, M., Ketchem, R.R., Xu, F., Brenneman, M., Kovacs, F., Tian, F., Wang, A., Huo, S. and Cross, T.A., Conformational trapping in a membrane environment: a regulatory mechanism for protein activity? *Proc. Natl. Acad. Sci. USA* 1996, **93**: 5872-5876.
2. Auffinger, P. and Westhof, E., Hydration of RNA base pairs. *J. Biomol. Struct. Dyn.* 1998, **16**: 693-707.
3. Barron, L.D., Hecht, L. and Wilson, G., The lubricant of life: a proposal that solvent water promotes extremely fast conformational fluctuations in mobile heteropolypeptide structure. *Biochemistry* 1997, **36**: 13143-13147.
4. Becker, M., Lerum, V., Dickson, S., Nelson, N.C. and Matsuda, E., The double helix is dehydrated: evidence from the hydrolysis of acridinium ester-labeled probes. *Biochemistry* 1999, **38**: 5603-5611.
5. Bett, K.E. and Cappi, J.B., Effects of pressure on the viscosity of water. *Nature* 1965, **207**: 620-621.
6. Billeter, M., Guntert, P., Luginbuhl, P. and Wuthrich, K., Hydration and DNA recognition by homeodomains. *Cell* 1996, **85**: 1057-1065.
7. Bukau, B. and Horwich, A.L., The Hsp70 and Hsp60 chaperone machines. *Cell* 1998, **92**: 351-366.
8. Bryngelson, J.D., Onuchic, J.N., Socci, N.D. and Wolynes, P.G., Funnels, pathways, and the energy landscape of protein folding: a synthesis. *Proteins* 1995, **21**: 167-195.
9. Caflish, A. and Karplus, M., Molecular dynamics simulation of protein denaturation: solvation of the hydrophobic cores and secondary structure of barnase. *Proc. Natl. Acad. Sci. USA* 1994, **91**: 1746-1750.
10. Careri, G., Gratton, E., Yang, P.-H. and Rupley, J.A., Correlation of IR spectroscopic, heat capacity, diamagnetic susceptibility and enzymatic measurements on lysozyme powder. *Nature* 1980, **284**: 572-573.
11. Clegg, J.S., Properties and metabolism of the aqueous cytoplasm and its boundaries. *Am. J. Physiol.* 1984, **246**: R133-R151.
12. Colombo, M.F., Rau, D.C. and Parsegian, V.A., Protein solvation in allosteric regulation: a water effect on hemoglobin. *Science* 1992, **256**: 655-659.
13. Covell, D.G. and Wallqvist, A., Analysis of protein-protein interactions and the effects of amino acid mutations on their energetics. The importance of water molecules in the binding epitope. *J. Mol. Biol.* 1997, **269**: 281-297.
14. Crowe, J.H. and Clegg, J.S. (eds.), *Anhydrobiosis*. Dowden, Hutchinson and Ross, 1973.
15. Crowe, J.H., Hoekstra, F.A. and Crowe, L.M., Anhydrobiosis. *Annu. Rev. Physiol.* 1992, **54**: 579-599.
16. Cunningham, E.L., Jaswal, S.S., Sohl, J.L. and Agard, D.A., Kinetic stability as a mechanism for protease longevity. *Proc. Natl. Acad. Sci. USA* 1999, **96**: 11008-11014.
17. Csermely, P., Proteins, RNAs and chaperones in enzyme evolution: a folding perspective. *Trends Biochem. Sci.* 1997, **22**: 147-149.
18. Csermely, P., The "chaperone-percolator" model: a possible molecular mechanism of Anfinsen-cage type chaperone action. *BioEssays* 1999, **21**: 959-965.
19. Denisov, V.P., Johnsson, B.-H. and Halle, B., Hydration of denatured and molten globule proteins. *Nat. Struct. Biol.* 1999, **6**: 253-260.
20. Dill, K.A., Bromberg, S., Yue, K., Fiebig, K.M., Yee, D.P., Thomas, P.D. and Chan, H.S., Principles of protein folding – A perspective from simple exact models. *Prot. Science* 1995, **4**: 561-602.
21. Dobson, C.M., Evans, P.A. and Radford, S.E., Understanding how proteins fold: the lyzozyme story so far. *Trends Biochem. Sci.* 1994, **19**: 31-37.
22. Edsall, J.T. and McKenzie, H.A., Water and proteins. II. The location and dynamics of water in protein systems and its relation to their stability and properties. *Adv. Biophys.* 1983, **16**: 53-183.
23. Elcock, A.H., The stability of salt bridges at high temperatures: implications for hyperthermophilic proteins. *J. Mol. Biol.* 1998, **284**: 489-502.
24. Ernst, J.A., Clubb, R.T., Zhou, H.-X., Gronenborn, A.M. and Clore, G.M., Demonstration of positionally disordered water within a protein hydrophobic cavity by NMR. *Science* 1995, **267**: 1813-1817.
25. Fedorov, B.B. and Fedorov, B.A., The effect of fixed water molecules on fractal properties of globular protein surfaces. *Biofizika* 1993, **38**: 611-618.
26. Fischer, S. and Verma, C.S., Binding of buried structural water increases the flexibility of proteins. *Proc. Natl. Acad. Sci. USA* 1999, **96**: 9613-9615.
27. Garay-Arroyo, A., Colmenero-Flores, J.M., Garciarrubio, A. and Covarrubias, A.A., Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. *J. Biol. Chem.* 2000, **275**: 5668-5674.m
28. Gerstein, M. and Chothia, C., Packing at the protein-water interface. *Proc. Natl. Acad. Sci. USA* 1996, **93**: 10167-10172.
29. Glasel, J.A., ^{17}O nuclear magnetic relaxation in a protein-water system, *Nature* 1968, **218**: 953-955.
30. Hartl, F.-U., Molecular chaperones in cellular protein folding. *Nature* 1996, **381**: 571-580.
31. Hoeltzli, S.D. and Frieden, C., Stopped-flow NMR spectroscopy: real-time unfolding studies of 6-^{19}F-tryptophan-labelled

Escherichia coli dihydrofolate reductase. *Proc. Natl. Acad. Sci. USA* 1995, **92**: 9318-9322.
32. Israelachvili, J. and Wennerström, H., Role of hydration and water structure in biological and colloidal interactions. *Nature* 1996, **379**: 219-225.
33. Jaenicke, R., Stability and stabilization of globular proteins in solution. *J. Biotechnol.* 2000, **79**: 193-203.
34. Jeffery, J., Enzymes: chemistry and biochemistry. In: *Interface between Chemistry and Biochemistry*, Jolles, P. and Jornvall, H. (eds.), Birkhauser Verlag, Basel, 1995, pp. 79-88.
35. Jesior, J.-C., Hydrophilic framework in proteins? *J. Prot. Chem.* 2000, **19**: 93-103.
36. Kandori, H., Iwata, T., Hendriks, J., Maeda, A. and Hellingwerl, K.J., Water structural changes involved in the activation process of photoactive yellow protein. *Biochemistry* 2000, **39**: 7902-7909.
37. Kiefhaber, T., Labhardt, A.M. and Baldwin, R.L., Direct NMR evidence for an intermediate preceding the rate-limiting step in the unfolding of ribonuclease A. *Nature* 1995, **375**: 513-515.
38. Klibanov, A.M., What is remembered and why? *Nature* 1995, **374**: 596.
39. Kuntz, I.D. and Kauzmann, W., Hydration of proteins and polypeptides. *Adv. Prot. Chem.* 1974, **28**: 239-345.
40. Kuwajima, K., The molten globule state as a clue for understanding the folding and cooperativity of globular-protein structure. *Proteins* 1989, **6**: 87-103.
41. Leikin, S., Parsegian, V.A., Yang, W.-H. and Walrafen, G.E., Raman spectral evidence for hydration forces between collagen triple helices. *Proc. Natl. Acad. Sci. USA* 1997, **94**: 11312-11317.
42. Leporc, S., Mauffret, O., El Antri, S., Convert, O., Lescot, E., Tevanian, G. and Fermandjian, S., An NMR study of d(CTACTGCTTTAG). d(CTAAAGCAGTAG) showing hydration water molecules in the minor groove of a TpA step. *J. Biomol. Struct. Dyn.* **16**: 639-649.
43. Löffler, G., Schreiber, H. and Steinhauser, O., Calculation of the dielectric properties of a protein and its solvent: theory and a case study. *J. Mol. Biol.* 1997, **270**: 520-534.
44. Marjan, M., Kurik, M., Kikineshy, A., Watson, L.M. and Szász, A., Two-structure model of liquid water. *Model. Simul. Mater. Sci. Eng.* 1999, **7**: 321-331.
45. Mayer-Jung, C., Moras, D. and Timsit, Y., Hydration and recognition of methylated CpG steps in DNA. *EMBO J.* **17**: 2709-2718.
46. Mentré, P. and Hui Bon Hoa, G., The effects of high hydrostatic pressures on living cells: a consequence of the properties of macromolecules and macromolecule-associated water. *Int. Rev. Cytol.* 2001, **201**: 1-84.
47. Mozhaev, V.V., Heremans, K., Frank, J., Masson, P. and Balny, C., High pressure effects on protein structure and function. *Proteins* 1996, **24**: 81-91.
48. Narlikar, G.J. and Herschlag, D., Isolation of a local tertiary folding transition in the context of a globally folded RNA. *Nat. Struct. Biol.* 1996, **3**: 701-710.
49. Otting, G., Liepinsh, E. and Wüthrich, K., Protein hydration in aqueous solution. *Science* 1991, **254**: 974-980.
50. Pappenberger, G., Saudan, C., Becker, M., Merbach, A. and Kiefhaber, T., Denaturant-induced movement of the transition state of protein folding revealed by high-pressure stopped-flow measurements. *Proc. Natl. Acad. Sci. USA* 2000, **97**: 17-22.
51. Parak, F., Correlation of protein dynamics with water mobility: Mössbauer spectroscopy and microwave absorption methods. *Meth. Enzymol.* 1986, **127**: 196-206.
52. Pertsemlidis, A., Soper, A.K., Sorenson, J.M. and Head-Gordon, T., Evidence for microscopic, long-range hydration forces for a hydrophobic amino acid. *Proc. Natl. Acad. Sci. USA* 1999, **96**: 481-486.
53. Pethig, R., Protein-water interactions determined by dielectric methods. *Annu. Rev. Phys. Chem.* 1992, **43**: 177-205.
54. Persson, M., Hammarström, P., Lindgren, M., Jonsson, B.-H., Svensson, M. and Carlsson, U., EPR mapping of interactions between spin-labeled variants of human carbonic anhydrase II and GroEL: evidence for increased flexibility of the hydrophobic core by the interaction. *Biochemistry* 1999, **38**: 432-441.
55. Ptitsyn, O.B., How does protein synthesis give rise to the 3D-structure? *FEBS Lett.* 1991, **285**: 176-181.
56. Quiocho, F.A., Wilson, D.K. and Vyas, N.K., Substrate specificity and affinity of a protein modulated by bound water molecules. *Nature* 1989, **340**: 404-407.
57. Rand, R.P., Raising water to new heights. *Science* 1992, **256**: 618.
58. Rashin, A.A., Iofin, M. and Honig, B., Internal cavities and buried waters in globular proteins. *Biochemistry* 1986, **25**: 3619-3625.
59. Record, M.T. Jr., Courtenay, E.S., Carley, S. and Guttman, H.J., Biophysical compensation mechanisms buffering *E. coli* protein-nucleic acid interactions against changing environments. *Trends Biochem. Sci.* 1998, **23**: 190-194.
60. Riepe, A., Beier, H. and Gross, H.J., Enhancement of RNA self-cleavage by micellar catalysis. *FEBS Lett.* 1999, **457**: 193-199.
61. Robinson, C.R. and Sligar, S.G., Changes in solvation during DNA binding and cleavage are critical to altered specificity of the EcoRI endonuclease. *Proc. Natl. Acad. Sci. USA* 1998, **95**: 2186-2191.
62. Rupley, J.A., Gratton, E. and Careri, G., Water and globular proteins. *Trends Biochem. Sci.* 1983, **8**: 18-22.
63. Rye, H.S., Burston, S.G., Fenton, W.A., Beechem, J.M., Xu, Z., Sigler, P.B. and Horwich, A.L., Distinct actions of *cis* and *trans* ATP within the double ring of the chaperonin GroEL. *Nature* 1997, **388**: 792-798.
64. Sclavi, B., Sullivan, M., Chance, M.R., Brenowitz, M. and Woodson, S.A., RNA folding at millisecond intervals by synchrotron hydroxyl radical footprinting. *Science* 1998, **279**: 1940-1943.
65. Shaknovich, E.I. and Finkelstein, A.V., Theory of cooperative transitions in protein molecules. *Biopolymers* 1989, **28**: 1667-1680.
66. Sidorova, N.Y. and Rau, D.C., Removing water from an EcoRI-noncognate DNA complex with osmotic stress. *J. Biomol. Struct. Dyn.* 1999, **17**: 19-31.
67. Sorenson, J.M. and Head-Gordon, T., The importance of hydration for the kinetics and thermodynamics of protein folding: simplified lattice models. *Fold. Des.* 1998, **3**: 523-534.
68. Sóti, C. and Csermely, P., Molecular chaperones and the aging process. *Biogerontology*, 2000, **1**: 225-233.
69. Sunnerhagen, M., Denisov, V.P., Venu, K., Bonvin, A.M., Carey, J., Halle, B. and Otting, G., Water molecules in DNA recognition. I: Hydration lifetimes of trp operator DNA in solution measured by NMR spectroscopy. *J. Mol. Biol.* 1998, **282**: 847-858.
70. Thirumalai, D. and Guo, Z., Nucleation mechanism for protein folding and theoretical predictions for hydrogen-exchange labeling experiments. *Biopolymers* 1994, **35**: 137-140.
71. Timasheff, S.N., The control of protein stability and association by weak interactions with water. *Annu. Rev. Biophys. Biomol. Struct.* 1993, **22**: 67-97.
72. Vossen, K.M., Wolz, R., Daugherty, M.A. and Fried, M.G., Role of macromolecular hydration in the binding of the *Escherichia coli* cyclic AMP receptor to DNA. *Biochemistry* 1997, **36**: 11640-11647.
73. Waldegger, S., Barth, P., Raber, G. and Lang, F., Cloning and characterization of a putative human serine/threonine protein kinase transcriptionally modified during anisotonic and isotonic alterations of cell volume. *Proc. Natl. Acad. Sci. USA* 1997, **94**: 4440-4445.
74. Wiggins, P.M., Role of water in some biological processes. *Microbiol. Rev.* 1990, **54**: 432-449.
75. Woda, J., Schneider, B., Patel, K., Mistry, K. and Berman, H.M., An analysis of the relationship between hydration and protein-DNA

interactions. *Biophys. J.* **75**: 2170-2177.
76. Wolfenden, R., Influences of solvent water on the transition state affinity of enzymes, protein folding and the composition of the genetic code. *Mol. Biol. Biochem. Biophys.* 1980, **32**: 43-61.
77. Xu, T. and Cross, T.A., Water: foldase activity in catalyzing polypeptide conformational rearrangements. *Proc. Natl. Acad. Sci. USA* 1999, **96**: 9057-9061.
78. Yu, B., Blaber, M., Gronenborn, A.M., Clore, G.M. and Caspar, D.L.D., Disordered water within a hydrophobic protein cavity visualized by X-ray crystallography. *Proc. Natl. Acad. Sci. USA* 1999, **96**: 103-108.
79. Zanotti, J.-M., Bellissent-Funel, M.-C. and Parello, J., Hydration-coupled dynamics in proteins studied by neutron scattering and NMR: the case of the typical EF-hand calcium binding parvalbumin. *Biophys. J.* 1999, **76**: 2390-2411.
80. Zimmerman, S.B. and Trach, S.O., Estimation of macromolecule concentrations and excluded volume effects for the cytoplasm of *Escherichia coli*. *J. Mol. Biol.* 1991, **222**: 599-620.

Questions:

Ref. 14 : Dowden, Hutchinson and Ross is the publishing house? place? + pp?

Fig. 1: Ref Guo and Thirumalai is not in the list; We should add the complete ref. in the legend.