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Physical and molecular bases of protein thermal stability and cold adaptation

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The molecular bases of thermal and cold stability and adaptation, which allow proteins to remain folded and functional in the temperature ranges in which their host organisms live and grow, are still only partially elucidated. Indeed, both experimental and computational studies fail to yield a fully precise and global physical picture, essentially because all effects are context-dependent and thus quite intricate to unravel. We present a snapshot of the current state of knowledge of this highly complex and challenging issue, whose resolution would enable large-scale rational protein design.

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Introduction

An important challenge in protein science consists in unraveling the mechanisms by which the heat and cold resistance of proteins is modulated in order for their host organism to adapt to extreme environmental conditions, with temperatures that range from about −20°C to over 120°C [1]. The understanding of these mechanisms has important practical applications in the context of the optimization of protein-based biotechnological and biopharmaceutical processes. This involves the rational design of proteins with modified thermal characteristics, and opens the way towards *de novo* design [2].

In the last decades a lot of efforts have been made in this direction (see [3–5] and references therein). The global picture that emerges is that there is no universal, unique, adaptive mechanism but rather an intricate combination of different factors, which frequently differs according to the protein or protein family and is thus highly difficult to disentangle. Moreover, due to the lack of direct and general methods of investigation, the results are often confused and sometimes contradictory, and thus only some general trends are definitely settled.

The present paper reviews what is known regarding the physical mechanisms at the molecular scale that proteins use to remain folded and functional in either hot or cold environments. It is not intended to be a full comprehensive review, but rather a concise point of view of the newest results, debated and contradictory hypotheses, and perspectives for reaching a deeper understanding of the field.

Protein thermal stability

Definitions

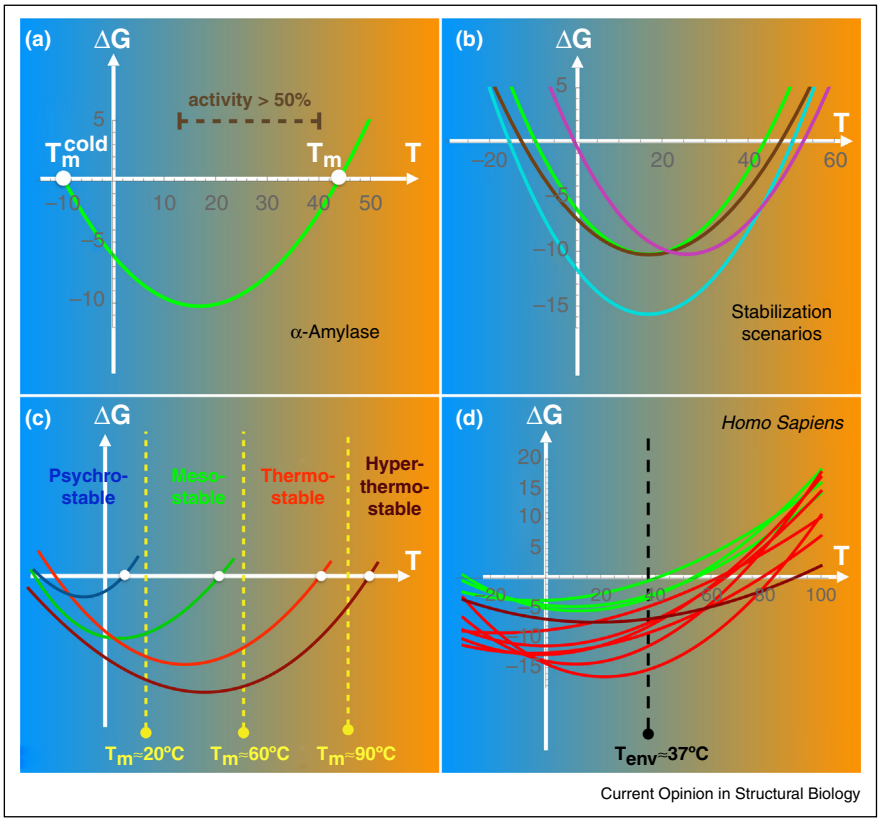
It can be generally assumed that structured proteins occur in two states, the folded and unfolded states, with the former being more populated in the temperature range $T_m^{cold} < T < T_m^{hot}$, with T_m^{cold} and T_m^{hot} the denaturation temperatures where the folding/unfolding transitions occur, whereas the latter is more populated outside this temperature range (Figure 1).

The heat denaturation temperature or melting temperature $T_m^{hot} \equiv T_m$ is commonly taken as the best descriptor of thermal resistance. A protein is considered as more thermally stable than another if its T_m is higher. It is in general biologically active up to this temperature, except if mutations in key sites prevent the enzymatic reaction, ligand binding or conformational change, in a nutshell, the proper functioning of the protein. The separation into psychrostable, mesostable, thermostable, and hyperthermostable proteins is performed on the basis of the T_m.

In contrast, the cold denaturation temperature T_m^{cold} is rarely measured since water usually freezes before this transition is reached. Moreover, even if a protein remains folded at low temperature, it is often inactive, usually due to a lack of flexibility. T_m^{cold} is thus not a good descriptor of cold adaptation, which has instead to be directly related to the activity at low T.

A frequent confusion is made between the thermal stability of a protein and the living or optimal growth temperature of its host organisms (OGT), and the latter is often wrongly taken as thermal stability descriptor. This leads inevitably to some misunderstanding since

Figure 1



Protein stability curves defined by ΔG as a function of the temperature T. **(a)** Experimentally characterized stability curve for the cold-adapted α-amylase from *Alteromonas haloplanktis* (AHA) (Uniprot code P29957, Enzymatic Commission number 3.2.1.1) [6]. The range in which the relative activity is larger than 50% is reported in the figure. **(b)** Different stabilization strategies to increase protein thermal stability with respect to AHA α-amylase (green curve): broadening of the stability curve by increasing the change in heat capacity upon folding (brown), shift of the entire curve towards more stable temperatures by increasing the temperature of maximum stability (magenta) or shift to more negative ΔGs by increasing the folding enthalpy (cyan). **(c)** Example of stability curves of psychrostable, mesostable, thermostable and hyperthermostable proteins that are defined according to their T_m value; note that the thresholds are conventional and are sometimes assigned to different values. **(d)** Experimentally derived stability curves for some proteins belonging to *Homo sapiens*, which hosts proteins with different thermal stability properties: from mesostable proteins with T_m close to the living temperature (37°C) to hyperthermostable proteins with T_m of more than 90°C [7].

the two quantities are only partially correlated: while a thermophilic organism only host thermostable proteins, a mesophilic organism can host both mesostable and thermostable proteins (Figure 1) while psychropholic organisms can in principle host all types of proteins.

Investigation methodologies

Current experimental and computational techniques do not capture a global picture of protein stability and adaptation, and yield a patchwork of results that are sometimes difficult to reconcile. They are summarized in Table 1 with their respective advantages and limitations.

Temperature dependence of the amino acid interactions

A proper analysis of protein thermal stability requires considering that the amino acid interactions are temperature dependent and thus that some are more stabilizing than others at higher or lower temperatures and *vice versa*. We review the interactions whose T-dependence has been discussed in the literature and summarize them schematically in Figure 2.

Hydrophobic effect and amino acid hydrophobicity

The hydrophobic effect constitutes the main driving force of protein folding and results from the tendency of hydrophobic amino acids to cluster together in order to avoid contact with water. Its T-dependence is directly connected to cold denaturation [8,9]. Indeed, whereas the hot denaturation is associated to a thermal increment of the conformational fluctuations, the cold transition is mainly related to the weakening of the hydrophobic effect [10•] that becomes unfavorable below a certain temperature compared to hydrophobe–water interactions.

Table 1

Different methods for getting insight into the molecular-scale origin of stability and adaptation. Abbreviations: 3D: 3-dimensional; H-D exchange: hydrogen–deuterium exchange; NMR: nuclear magnetic resonance; B-factor: X-ray B-factor; OGT: optimal organism growth temperature; MD: molecular dynamics simulations; CNA: constraint network analysis; LM: lattice model

Methods		Information	Drawbacks	References
Statistical analyses of sequences and/or experimental 3D structures of:	• Psychrostable, mesostable, thermostable proteins	Structure-stability relation up to T_m	Lack of data	[10•,11,12,108]
	• Proteins from psychrophilic, mesophilic, thermophilic organisms	Structure-stability relation around OGT	Mixing the notions of stability and OGT	[16,17,25,26,34,36,39–41, 43,49,53,54•,55,60,70•, 73,74,78,80–83,84•,85, 89,90,96–98]
Mutagenesis experiments		Changes in thermal properties and/or activity upon mutations	Context-dependent, difficult to generalize	[13,15,21–23,27,28•,32, 33,35,37,38,42,44–48,50, 51,57,66,67•,68,69,75–77, 86•,87,88,99,107,109–111]
Hydrophobicity measurements		*T*-dependent hydrophobicity	Limited to a subkind of interactions	[14,20•]
Ligand-binding affinity measurements		Ligand-binding thermal and thermodynamic features	Limited to binding properties	[91–95]
Structural and dynamical information from:	• Experimental data (H-D exchange, NMR, B-factor, ...)	Relations between stability, structure and dynamical behavior at different time scales	Mixing different dynamical timescales, force-field biases, computational approximations	[8,9,24,29,31,49,56,61•, 64,65,70•,72,79•]
	• Computational approaches (MD, CNA, LM, ...)			

At temperatures between 25°C and 100°C, the hydrophobic effect seems to remain essentially constant compared to other interactions, as indicated by the high similarity of the statistical potentials that describe the effective hydrophobic interactions in mesostable and thermostable proteins [11,12] and by mutagenesis experiments [13]. However, its nature changes from entropy-dominated at room temperature to enthalpy-driven above 110°C [15].

An observation that could be viewed as contradictory is that proteins from thermophilic organisms have usually a more hydrophobic core compared to their mesophilic homologues, suggesting a higher stabilization effect of the hydrophobic forces at high T [16,17]. However, it could also be a side effect of, on the one hand, the smaller size of thermophile proteins [18] and, on the other hand, the ratio of hydrophobic to hydrophilic residues which is smaller in small proteins but not sufficiently to counterbalance their smaller volume to surface ratio [19].

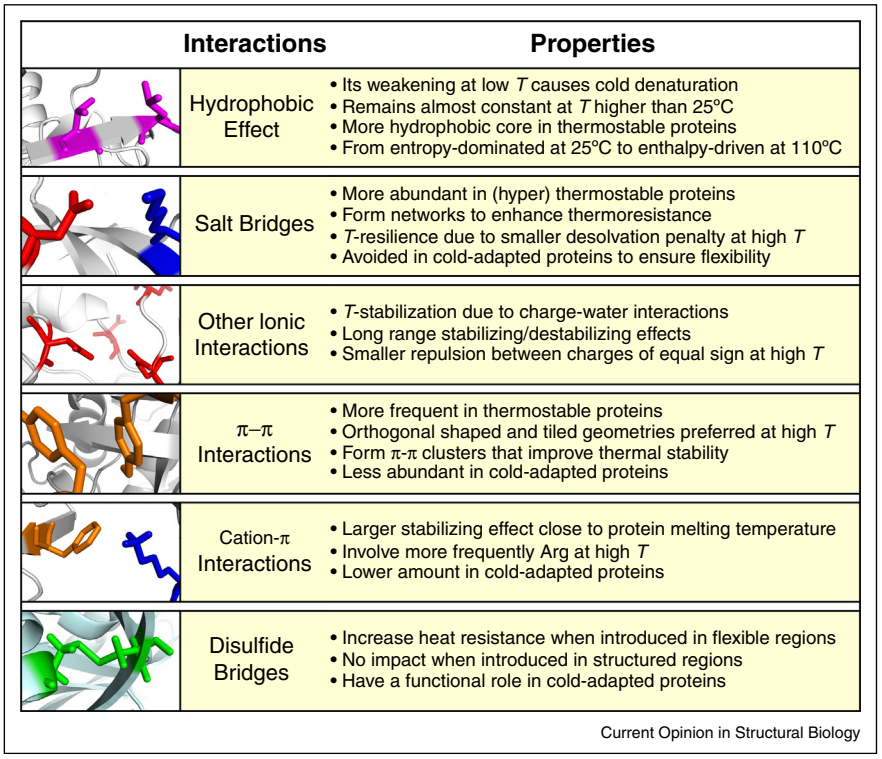
The hydrophobicity is related to the hydrophobic effect, but is defined for both hydrophobic and non-hydrophobic moieties. The *T*-dependence of amino acids' hydrophobicity has been investigated experimentally [20•] and computationally [10•]. At temperatures below 25°C, the hydrophobicity of aromatic and hydrophobic residues weakens, while no significant difference is observed for polar and charged residues [10•]. From 25°C to higher temperature, the hydrophobicity of polar amino acids increases substantially, whereas it is constant for hydrophobic residues (in agreement with the above mentioned results) or increases slightly [10•]. Note that investigations on alkanes rather suggest a mild increase of the hydrophobicity of hydrophobic moieties from 25°C up to a maximum at about 70°C followed by a very slight decrease [14].

Salt bridges

The stabilizing or destabilizing nature of salt bridges at room temperature is still debated. However, it seems established that they contribute to improving the heat resistance of their host proteins and are avoided in cold-adapted proteins. Therefore, salt bridges have been natural targets in protein engineering approaches aiming at increasing thermal stability [15,21].

The thermal resistance of salt bridge interactions has been firmly demonstrated experimentally using double-mutant cycle techniques [22,23], as well as computationally through the derivation of statistical potentials [11,12] and MD simulations [24]. Other indications come from comparisons of homologous proteins, where the number of single salt bridges and salt-bridge networks tends to increase from psychrophilic to hyperthermophilic organisms [25,26]. These interactions are preferentially located at the protein surface, mediated by arginines that can

Figure 2



Summary of the impact of amino acid interactions on the heat resistance and cold adaptation of proteins.

simultaneously be involved in two salt bridges or H-bonds, and contribute to the structural rigidity that characterize thermostable proteins [27]. Note however that only key nodes (hubs) of salt-bridge networks seem to contribute substantially to thermal stability [28•].

From a thermodynamic point of view, the thermal resistance of salt bridges can probably be attributed to the increase of the hydrophobicity of charged residues with temperature (see previous section), and thus to a smaller desolvation penalty incurred in forming a salt bridge [29]. It is associated to a decrease of the folding heat capacity $\Delta C_p(T)$, resulting in the down-shift and broadening of the protein stability curve (see Figure 1b) [22]. At very high temperatures, above 100°C, where the $\Delta C_p(T)$ can no longer assumed to be constant, the stabilization effect is even more pronounced [15].

In contrast, it was shown that proteins from psychrophilic organisms tend generally to avoid salt bridge formation. The common explanation is that their absence enhances the protein flexibility and thus activity at low temperature [30–32]. Some special kinds of salt bridges seem nevertheless favorable, which form across the hydrophobic core, facilitate water penetration and hence maintain the required flexibility [31].

Other ionic interactions

It has been questioned whether the thermal stabilization due to salt bridge formation is sufficient to explain the observed increment of the number of charged residues at the surface of proteins from thermophilic organisms with respect to their mesophilic or psychrophilic counterparts [25]. This has led to the suggestion that direct interactions between the solvent and charged surface residues provide a stability advantage in hot environments [25]. Other explanations have been proposed, among which the long-range nature of the electrostatic forces, which are strongest when charges form salt bridges but remain non-negligible up to distances of more than 10 Å [33,34], and must be taken into account in the stabilization of salt bridge networks [35]. Also, the strength of the repulsion between identical charges weakens with the temperature, as shown by T-dependent statistical potentials [12]. Another explanation of the abundance of surface charges is their possible role in the destabilization of misfolded or aggregated structures (negative design mechanism) [36].

As a result, targeting surface charges has been proposed as a valuable protein design approach [33,37,38]. However, the success depends on the type of substitution, its environment, and its role in the protein's ion network.

π–π interactions

Interactions between aromatic residues play a fundamental role in protein stabilization, protein-protein interactions and ligand binding [39]. They occur in π–π dimer pairing or extended π–π clusters with variable geometries, which indicates the self-associating property of this interaction [39]. The number of pairwise π–π interactions seems to increase with the growth temperature of the host organism, from psychrophiles to hyperthermophiles [40,41]. Usually, the number of π–π clusters also follows the same trend [40]. The preferred conformations in proteins from thermophilic organisms are the T-shaped orthogonal and tiled geometries, while the near-parallel geometry is less observed. Despite several other observations of the thermostabilizing tendency of these interactions [12,42], a clear understanding is still missing.

Cation–π interactions

Several studies suggest the importance of cation–π interactions in thermal stabilization [12,43]. This characteristic is especially valid for the cation–π interactions involving arginine, whose delocalized charge on the guanidinium group yields dispersion energy contributions in addition to electrostatic ones. Moreover, mutagenesis experiments have shown that while cation–π interactions are at best weakly stabilizing at room temperature and in some cases even destabilizing, they become more stabilizing at elevated temperatures, especially close to the protein melting temperature [44].

In contrast, a reduced number of cation–π interactions are observed in proteins from psychrophilic organisms, which is consistent with the picture that cold adaptation is reached by the weakening of intramolecular interactions to ensure sufficient flexibility [32].

Disulfide bridges

It is difficult to assess the real impact of the temperature on disulfide bridges due to the rarity of cysteines. It seems however established that the disruption of native disulfide bridges in (hyper)thermostable proteins leads to a loss of thermal stability via an increase of conformational entropy of the unfolded state [45–47]. On the other hand, *de novo* engineering of disulfide bonds gives contradictory results that depend crucially on the position where the new interaction is introduced [45,48,49]. Some results suggest that their introduction in flexible regions contribute to enhance the thermal resistance while those introduced in fully structured regions have no impact [48]. Note that the reconciliation of these results could also involve considering whether or not the disulfide bridges are maintained in the unfolded state.

In cold-adapted enzymes, the observed disulfide bridges are often likely to have a functional role instead of being related to thermal stability, for example, by providing the required conformation in the active site regions [50,51].

Interestingly, a series of disulfide bridge prediction methods have been developed to automatically suggest where to insert such interactions to improve stability (see [52] and references therein).

Interaction networks

It becomes increasingly clear that the whole network of interactions that make up the proteins must be considered to understand the thermal and cold adaptation mechanisms, as illustrated in Figure 3. Indeed, these are related not only to the type of interactions but also to their specific role in the network which they are part of.

In some cases, the network hubs play a pivotal role to confer a higher thermoresistance [40,53,54•,55], while in others peripheral nodes seem important in this respect [54•]. In yet other cases, the stronger heat resistance of thermostable proteins can be attributed to differences in size and geometry of the interaction networks and to the network resilience to temperature [34,56].

Considering full interaction networks makes the elucidation of the molecular bases of thermal stability more complex, but is essential to capture emergent features such as the cooperativity and anti-cooperativity of the interactions and their role in the adaptation mechanisms. Interaction network analyses could also suggest new approaches in protein engineering in view of designing proteins with modified thermal properties [57].

Thermal effects of structure and dynamics

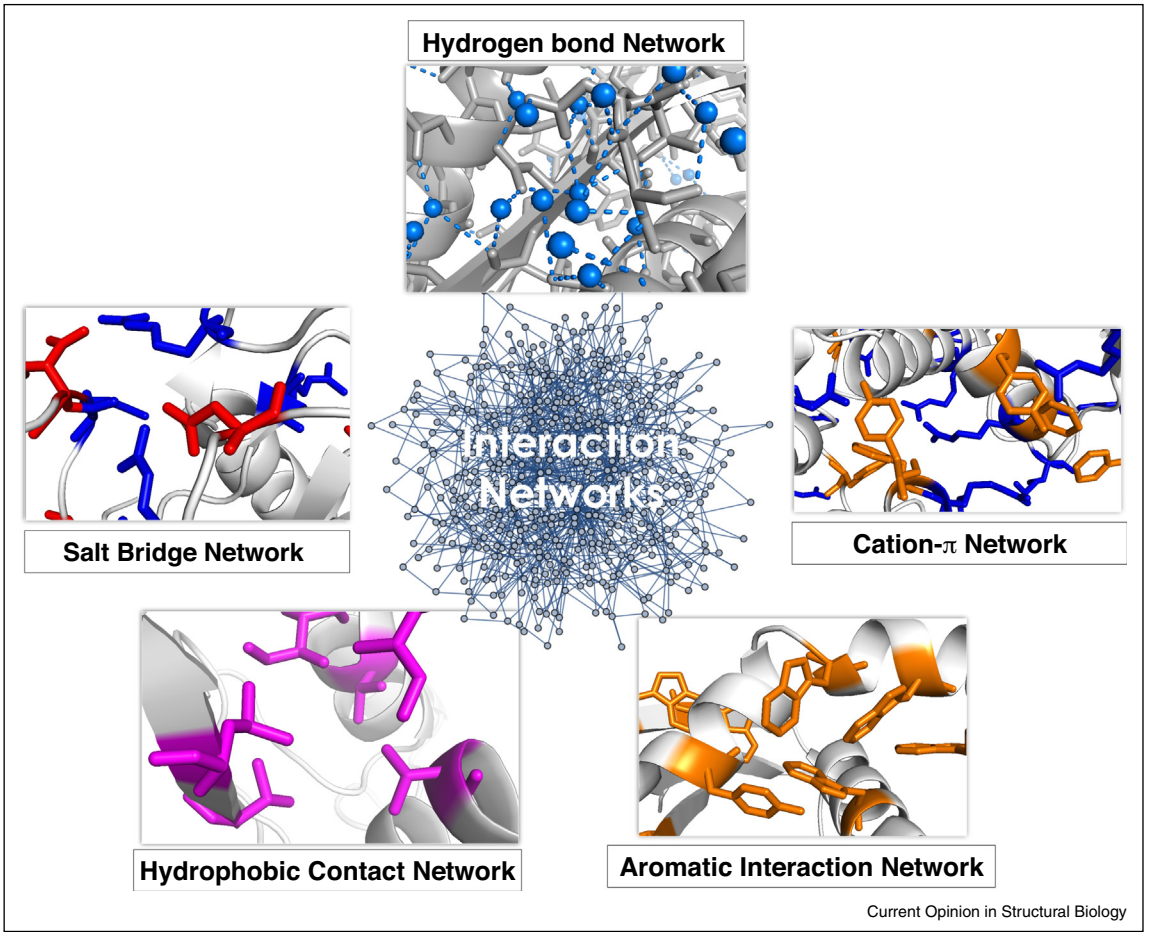
The global structural and dynamical features that improve thermal resistance or cold adaptation are here discussed and illustrated in Figure 4. Note, however, that these different characteristics are often interdependent and also related to the amino acid interactions, and that it is impossible to completely separate their effects.

Rigidity and flexibility

A wide series of experimental and computational investigations have demonstrated that protein thermal stability is related to the rigidity/flexibility properties of the protein structure (see [58,59] and references therein). Thermostable proteins are indeed generally characterized by an enhanced global conformational rigidity with respect to their mesostable homologues, whereas cold-adapted proteins usually present a higher flexibility.

The general validity of this relation has been frequently discussed, since both heat and cold adaptations require an intricate correlation between local flexibility and rigidity, the former being important for activity while the latter for correct folding and stability [58–60,61•]. Moreover, enzymes that show a high flexibility associated with an

Figure 3



Schematic illustration of interaction networks that contribute to the temperature adaptation of proteins.

unexpectedly high stability and *vice versa* have been recently characterized [62,63].

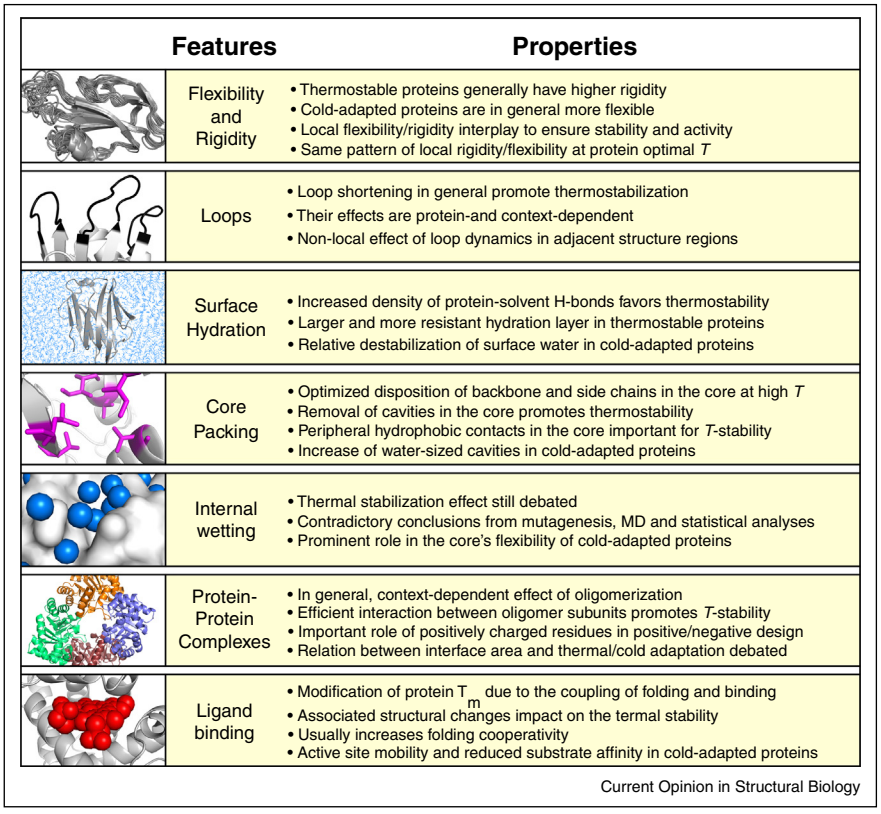
Despite the fact that the study of the rigidity/flexibility distribution throughout the protein structure and its relation to the thermal stability remain a debated field of research, more and more experimental (H/D, NMR) and computational (MD, CNA) results seem to converge towards the validity of the 'corresponding state hypothesis' affirming that enzymes display the same pattern of local rigidity and flexibility at their respective optimal temperatures [64,65].

Loops

Loops are intimately related to the flexibility/rigidity patterns of proteins and, as a direct consequence, impact on their thermal stability properties. Usually loop shortening results in thermal stabilization, but the magnitude of this effect is still debated, probably because it is protein-dependent and context-dependent. Thermodynamically, the loss of enthalpy due to the shortening is overcome by a gain in conformational entropy of the folded state and/or by an entropy loss in the unfolded state [66,67•].

Some investigations point towards a minimal effect on the protein thermal stability of the deletion of specific surface loops as well as of the insertion of proline residues to rigidify them [68]. In other analyses on the contrary, a substantial increase of the protein thermoresistance is observed in proteins in which the loops have been shortened [66,67•], or in mesostable proteins in which loops from hyperthermostable homologues were transplanted [69]. The *T*-dependence of loop dynamics has been investigated by computational methods [70•], indicating that loops related to protein activity are more flexible at low temperature, while loops involved in stability show an opposite dynamical behavior. Note that the structures adjacent to flexible loops generally show larger fluctuations compared to the other parts of the

Figure 4



Summary of the effect of structural and dynamical features on the heat resistance and cold adaptation of proteins.

structure, which suggests a non-local effect of the loop dynamics [70•].

Surface hydration

The role of protein surface hydration and its dynamics in promoting thermal stability has still to be fully understood (see [71] and references therein) even though different investigations point out its relevant role.

Hydration in thermostable proteins seems usually characterized by an increased density of solvent-protein hydrogen bonds, a larger shell size and a stronger shell resistance to the temperature compared to mesostable proteins. The formation of a highly connected network of water-water hydrogen bond interactions coupled to the protein surface could prevent the thermal destabilization by avoiding the penetration of water molecules [56,72,73]. This effect is amplified in thermostable proteins since they have a reduced number of apolar residues at the surface counterbalanced by an increase in charged residues.

In cold-adapted proteins, which have instead more exposed non-polar residues on the surface, the water structure around the surface is destabilized compared to bulk water [74], which leads to an increase of the overall conformational flexibility of the protein.

Core packing

Multiple investigations show that the core packing efficiency seems related, albeit not crucially, to protein thermal stability. Indeed, thermostable proteins seem to have removed the cavities in their core regions and optimized the disposition of their backbone and side chains to adapt to high temperatures [54•,75–78]. Less trivial and still debated is the understanding of the physiochemical origin of this improved core packing: some investigations identified as pivotal factor the hydrophobic effect, the van der Waals interactions [75] or the gain in water-entropy upon folding [76]. Interestingly, it has been suggested that while the hubs of core residue clusters contribute equally to the packing efficiency in mesostable and thermostable proteins, the core peripheral residues play instead a fundamental role in thermostable proteins to improve this efficiency and thus thermoresistance [54•].

Proteins from psychrophilic organisms have larger internal cavities that can accommodate more buried water molecules compared to those of their mesophilic

homologues, and that are characterized by a higher polarity [74]. As a consequence, they are usually characterized by a weaker, less packed and more flexible hydrophobic core.

Internal wetting

Cavity hydration is expected to impact on the thermal characteristics of proteins, but the results are anything but clear-cut. Experimental mutagenesis experiments aimed at modifying the internal cavity characteristics are not conclusive regarding the nature of the internal wetting: this seems to act as a stabilizing factor in some proteins but not in others [79•]. Recent MD simulations that estimate the hydration free energy in a set of proteins from mesohophilic and thermophilic homologues support the important role played by the internal wetting in the modulation of the thermal resistance: in thermophile proteins, the hydration of the cavities could contribute more substantially to the overall stability than in their mesophile counterparts [79•]. In contrast, a large scale study of protein 3D structures indicates that thermophile proteins have less buried water than their mesophile homologues [80], thus suggesting a less important role of the internal wetting.

These two apparent contradictory results can be reconciled by considering that thermostable proteins have on the average less cavities and that, when they nevertheless have, these are more hydrated. Further investigations are needed to get a definite picture.

In proteins from psychrophilic organisms, the observed increase of the water-sized cavities could indicate a prominent role of the internal solvation in the increase of the core flexibility characterizing their heat and cold labile structures [74].

Protein–protein and protein–ligand complexes in thermal and cold adaptation

Proteins often form complexes to accomplish their biological functions, and this modifies their thermal characteristics, as summarized in Figure 4.

Protein–protein interactions

The temperature adaptation of a protein complex is achieved by the combination of different mechanisms that tend to stabilize the protein–protein interfaces and the structure of the complex, while reducing the affinity for non-native interfaces and other aberrant assemblies.

A large comparison of modeled structures of protein complexes in different host organisms [81] show that a prominent role is played by the positively charged residues, whose amount correlates with the OGT. This enrichment is suggested to prevent the assembly of misfolded chains. Moreover the charged residues in the interface are likely to strengthen the binding energy of the complexes through electrostatic interactions. The hydrophobic contact area is significantly increased in proteins from thermophilic and hyperthermophilic organisms and reduced in psychrophilic ones [82,83]. This trend could be related to the temperature dependence of the hydrophobic surface solvation energy (see 'Hydrophobic effect and amino acid hydrophobicity' section).

The effect of the total interface area on the thermal stability of the complexes is currently debated. Some recent analyses find a positive correlation between the level of expression of the protein complex and the interface size and this relation is stronger in thermophilic than in mesophilic organisms [84•]. This has been suggested to be related to the evolutionary pressure for keeping the thermostability of the complex while avoiding detrimental subunit interactions [84].

Oligomerization mechanisms

The formation of higher-order oligomeric structures is a possible strategy for protein thermal adaptation [85]. Indeed, protein–protein contacts usually ensure a more efficient packing of the oligomeric structure compared to the monomeric one, thereby increasing the heat tolerance of the protein. Note also that the folding kinetics of higher-order oligomers is often a multistate scheme, which can lead to the kinetic stabilization of some proteins.

It is intriguing to note that not only mutations at the interface can occur in promoting the higher oligomerization state and thermal stability but also mutations in the core far from the interface. In the latter case, protein subunits undergo conformational changes that promote the oligomer formation to compensate the possible loss of stability of the individual subunits [86•,87,88].

The oligomerization mechanism is a strategy that can be used differently by different proteins and/or protein families. For example, psychrostable proteins have usually lower oligomerization forms with respect to their mesostable homologues. Sometimes, however, the oligomerization strategy can be used inversely, promoting the flexibility and the activity of the proteins at low temperatures, while reducing the stability [89].

Protein–ligand binding

Ligand and metal binding usually affect the protein thermal stability by modifying the melting temperature, the folding heat capacity and the folding enthalpy. These modifications are specific to each protein–ligand pair, and are due to the coupling between the folding and binding equilibrium processes [91]. The binding mechanisms are relatively well understood even though their complexity, which arises from the fact that binding occurs not only in folded structures but also in partially or fully unfolded ones [92], has still to be fully deciphered.

The thermal stability changes are also a consequence of the structural changes associated to the binding [93]. Indeed, these frequently induce an increase of the folding cooperativity and a more efficient protein packing, which in turn generally lead to a higher thermostability [94].

Cold-adapted proteins require an increased conformational mobility of the active site region with respect to their homologues from mesophilic and thermophilic organisms to maintain the catalytic activity. This implies that a higher number of protein conformational states must be able to bind the ligands, among which some have low binding affinity. As a consequence, the average affinity for the ligands is usually rather low compared to that of mesophilic and thermophilic homologues [95].

Conclusion

Summary of the adaptation strategies to cold and heat

It is very difficult to pinpoint the features that specifically ensure cold or heat adaptation, since they mix stability requirements with function, solubility, aggregation propensities and other environmental parameters. This is particularly true for proteins from psychrophilic organisms, in which maintaining a stable folded structure at low temperature is usually less problematic than keeping a sufficient flexibility in the functional regions. For proteins from (hyper)thermophilic organisms, the main issue is sometimes to avoid alternative, non-functional or aggregated, structures rather than to increase the T_m.

Moreover, it has become manifest that these mechanisms cannot be fully rationalized on the basis of the sum of individual interactions, but require considering feature networks. This adds a level of complexity to the problem, but can be expected to reconcile some contradictory results described in this paper: what is true in some protein environments is false in others. Clearly, more systematic and quantitative measurements as well as computational analyses are required to fully elucidate the molecular bases of the thermal stability and activity properties of proteins.

Figures 2 and 4 contain a summary of the characteristics that appear — in the present state of knowledge — to contribute to heat and cold adaptation, and of the features whose effects are still debated.

Future perspectives

Despite all the efforts devoted to the understanding of the heat and cold adaptation mechanisms, the picture remains incomplete. Some (partially) unexplored tracks that would be valuable to explore are listed hereunder.

- **Proteome-wide analyses of protein structures** could give more precise information and deeper understanding of the structural strategies used by proteins to adapt to different thermal conditions [96,97].
- **The role of evolution** in the thermal adaptation process has still to be deciphered. Interesting evolutionary hypotheses that differ according to the organism family and its living environment have been proposed [36,98–100], but need to be checked and enriched.
- **Chaperones** that assist protein (re)folding are known to adapt their functions to the OGT [101] and to be essential for the cell to respond to temperature fluctuations [102,103].
- ***In vivo* crowding** has been shown to modulate the thermal and thermodynamic stabilities but needs to be further investigated [104–106]).
- **Membrane proteins** have characteristics that differ profoundly from those of globular proteins and their specific thermal properties have not yet been analyzed in detail [107].
- **Improved prediction methods** of thermal stability or of its variation upon mutations, with faster and more accurate outcomes, is of utmost importance in biotechnological and biopharmaceutical applications [108–111].

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