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Review

Review: Protein function at thermal extremes: balancing stability and flexibility ☆

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Abstract

No organism can survive across the entire temperature range found in the biosphere, and a given species can rarely support active metabolism across more than a few tens of °C. Nevertheless, life can be maintained at surprisingly extreme temperatures, from below −50 to over 110°C. That proteins, which are assembled with the same 20 amino acids in all species, can function well at both extremes of this range illustrates the plasticity available in the construction of these macromolecules. In studying proteins from extremophiles, researchers have found no new amino acids, covalent modifications or structural motifs that explain the ability of these molecules to function in such harsh environments. Rather, subtle redistributions of the same intramolecular interactions required for protein stabilization at moderate temperatures are sufficient to maintain structural integrity at hot or cold extremes. The key to protein function, whether in polar seas or hot springs, is the maintenance of an appropriate balance between molecular stability on the one hand and structural flexibility on the other. Stability is needed to ensure the appropriate geometry for ligand binding, as well as to avoid denaturation, while flexibility is necessary to allow catalysis at a metabolically appropriate rate. Comparisons of homologous proteins from organisms spanning a wide range of thermal habitats show that adaptive mutations, as well as stabilizing solutes, maintain a balance between these two attributes, regardless of the temperature at which the protein functions.

Keywords: Compatible solutes; Conformational microstates; Extremophile; Flexibility; Habitat temperature; Protein stability; Psychrophile; Thermal adaptation; Thermophile

1. Introduction

The beautiful and informative models of proteins that are generated using X-ray crystallography and nuclear magnetic resonance techniques have led to a much clearer understanding of the intricacies of protein structure, as well as the modes by which intramolecular interactions among amino acids lead from a one-dimensional amino acid sequence to secondary and tertiary structures. Unfortunately, these images also give the impression that enzymes in solution are static structures, rigidly waiting for ligands to approach and bind. That this is not the case has been amply shown by studies using a variety of techniques. Indeed, an enzyme active in solution is most accurately represented as a statistical distribution of microstates with conformations differentiated by varying levels of local unfolding (Bai et al.,

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1995; Wooll et al., 2000). It follows that enzyme molecules occupying different conformational microstates will show different affinities for substrate (Ma et al., 1999), which may explain experiments finding molecular sub-populations with discrete substrate affinities in a 'homogeneous' enzyme preparation (Xue and Yeung, 1995). However, as long as the temperature of the solution stays within a range defined by the stability of a given enzyme, the population of molecules will occupy a distribution of conformations that can be considered the native state.

This ability to move easily among closely related conformations is often the basis of enzyme functionality. Many catalytic proteins undergo conformational shifts, such that different structural components reorient themselves during the catalytic cycle (Creighton, 1993). This may be to form the appropriate geometry for binding substrate or cofactors, to bring chemically reactive species together after binding, to create a 'catalytic vacuole' with the appropriate physicochemical characteristics separate from the surrounding medium, or to release products after chemical catalysis has occurred. Thus, the molecular flexibility that is important for adaptation to temperature affects the mobility of secondary structural components of protein molecules, and operates on μs–ms scales rather than on the ps-scale, which corresponds to the thermal fluctuations of individual atoms within the protein structure (Zaccai, 2000). Because the functionally important conformational motions of enzymes require thermal energy from the surrounding medium, the flexibility of any enzyme molecule is temperature-dependent. If the environment is too cold, the enzyme may move so slowly that catalysis no longer occurs at a metabolically useful rate; if too warm, the structure may be so loose that substrate and cofactor can no longer bind. At extremes of temperature, complete denaturation will be the fate of protein molecules.

Nevertheless, if the structures of orthologs are examined (orthologous homologs, enzymes in different species that derive from a common ancestral form) in disparate taxa living across a broad range of temperatures, the gross three-dimensional structure is usually remarkably similar (Auerbach et al., 1998). In other words, the adaptive modifications necessary to carry out catalysis at different temperatures do not include wholesale rearrangements of secondary structural components or the development of new structural motifs. Instead, subtle changes in intramolecular interactions, and equally importantly, protein–solvent interactions, are enough to modify flexibility and stability sufficiently across the entire range of temperatures experienced in the biosphere. Thus, the initial structural solution to a particular catalytic task in distant ancestors is usually maintained with little gross modification in descendants that go on to occupy a variety of thermal niches.

In this paper, I discuss the evidence from a number of studies of orthologs, showing that stability and flexibility co-vary with habitat temperature. I then describe the modifications in primary structure, that is, amino acid composition, that are believed to alter the intrinsic stability of enzymes, and thus to allow function at extremes of heat and cold. I provide a number of examples of enzymes from extremophiles that show modifications in structure that lead to optimal stability and flexibility in environments humans would view as harsh. And finally, I discuss the role of the solvent medium, and especially the addition of protein-stabilizing solutes, on function at environmental extremes.

2. Covariance of flexibility and stability in enzymes

Although enzyme molecules comprised of hundreds of amino acids can have many thousands of stabilizing intramolecular interactions — salt bridges between oppositely charged side chains, hydrogen bonds, and hydrophobic and van der Waals interactions — *net* stabilization of these macromolecules usually corresponds to the energy of only a few hydrogen bonds (Jaenicke, 1991, 2000). This is because the destabilizing forces acting upon protein three-dimensional structures in solution, both unfavorable interactions among residues, but also the increase in entropy on unfolding, nearly balance the stabilizing forces. Notably, researchers assume that this is a necessary precondition for enzyme function, given the trade-offs between stability and catalytic flexibility described above (Zavodszky et al., 1998). The interactions between thermal energy and the stabilizing and destabilizing forces acting on a protein molecule in solution are complex, but to a first approximation, increases in tempera-

ture will favor destabilization of protein structure, and a sufficient input of heat energy will ultimately lead to protein denaturation. Such consequences are obviously detrimental to growth and survival, as denaturation of a small proportion of the cell's cytosolic protein population will reduce metabolic scope while increasing demands on the cell's protein-degradative or recovery pathways. Denaturation of larger percentages of cytosolic proteins will lead to death through protein aggregation and metabolic collapse. (This is not meant to imply that heat-death is mediated only by protein denaturation; the effect of heat on other macromolecular systems, especially lipid-based membranes, will be highly detrimental as well.)

It is clearly advantageous to develop proteins that will be stable in the temperature range that the organism experiences. We can predict, then, that the global stability of proteins will correlate with habitat temperature. A number of studies examining this hypothesis have shown that, indeed, when orthologs from a broad range of species are compared, the temperatures at which the molecules denature often correspond to the organisms' thermal niches. Davies et al. (1994) showed that the denaturation temperatures of myosins of a group of food fishes correlated well with habitat, whether measurements were carried out in situ (in whole muscle) or on isolated myosins. A study using organisms from a broader range of thermal environments (Maes et al., 1999), including both psychrophiles and hyperthermophiles, showed that triosephosphate isomerase thermal stability could also be related to habitat temperature. An especially interesting example used vertebrate eye-lens crystallins, which contain a number of proteins, including several cytosolic enzymes. In this study, McFall-Ngai and Horwitz (1990) used circular dichroism (CD) spectroscopy to show that habitat temperature of a variety of species, from extremely cold-adapted Antarctic fishes to warm-adapted desert lizards, correlates well with the temperature of unfolding (T_m) of the crystallins (Fig. 1). Thus, it would appear that some aspect of the structure in each of these proteins has been selected to provide stability in relation to the amount of thermal energy available in the environment.

Interestingly, the temperatures at which the eye-lens proteins denature are much higher than those experienced by the species in their habitats, and are well above the lethal temperatures for all the species. Furthermore, the slope of the regression between environmental temperature and T_m is much lower than unity (Fig. 1), indicating that complete compensation for increasing habitat

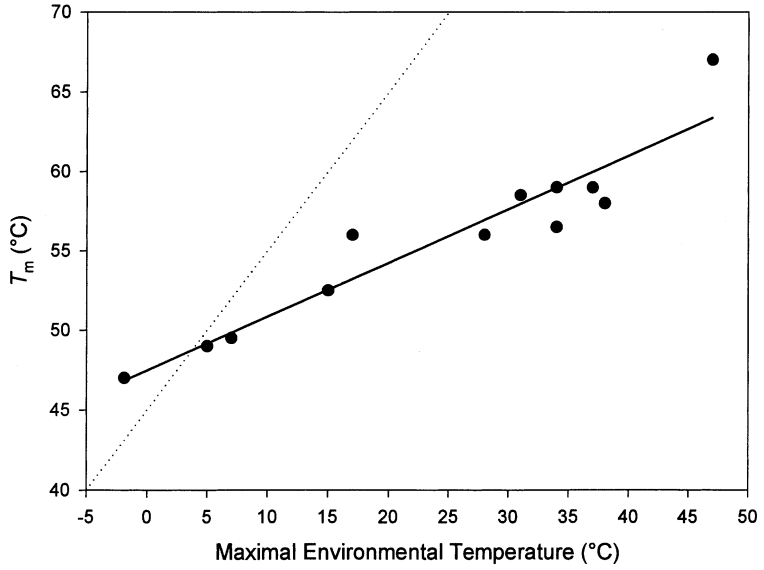

Fig. 1. The denaturation temperature (T_m; temperature at which 50% of the secondary structure has been lost, measured by CD spectroscopy) of eye lens crystallins from a variety of vertebrate species adapted to a range of temperatures, correlated to habitat temperature. The solid line represents the least-squares fit to the data ($r^2 = 0.90$), the dotted line is unity. Data from McFall-Ngai and Horwitz (1990).

temperature is not found, and presumably is unnecessary. Thus, denaturation temperature per se cannot be the attribute that is under selection in these protein molecules. Instead, it is apparent that global stability is an experimentally tractable correlate of functionally important changes in stability that must occur at temperatures lower than those necessary for denaturation. In other words, the denaturation temperatures measured by CD spectroscopy and other whole-molecule methodologies likely are indirect indicators of dissociations that occur among intramolecular secondary structures and domains at lower, more physiologically meaningful temperatures (Zavodszky et al., 1998). It is these intramolecular destabilizations that have an impact on enzyme function (Ma et al., 1999), and thus are the targets of natural selection.

Molecular stability is only one part of the relationship between enzyme function and temperature — if enzyme molecules could perform their tasks while remaining completely rigid, there would be no need to fine-tune stability to environmental temperatures. Instead, the necessity for enzymes to maintain a certain level of flexibility ultimately dictates the levels of stability that are allowable for a given thermal niche. An analysis of the kinetic parameters that describe enzyme function, and their response to temperature change, provides a clear illustration of how delicate the balance is among temperature, protein stability, and optimal function. One of the most basic descriptors of enzyme function is k_{cat}, or turnover number, that is, the rate at which one enzyme active site converts substrate to product. As the k_{cat} of an enzyme is measured at ever-increasing temperatures, the rate of the catalyzed reaction increases exponentially, until an enzyme-specific break-point is reached; at higher temperatures, k_{cat} slows and finally plummets to zero. This behavior is taken as an indication of localized, and ultimately global, denaturation of the enzyme molecule. Thus, as in the case of enzyme stability per se discussed above, the catalytic function of any enzyme is highly temperature-dependent, even in the temperature range where molecular denaturation is not a danger. Increased thermal energy in the medium will increase the number of enzyme molecules that have the necessary energy to undergo catalytic conformational changes, and this increase in the proportion of catalytically active molecules will be measured as an increase in catalytic rate, or k_{cat}.

The importance of k_{cat} to enzyme function can be illustrated by comparing k_{cat} values measured for an enzyme, muscle-type lactate dehydrogenase (A_4-LDH), from organisms occupying a range of thermal habitats (Fields and Somero, 1998). As can be seen in Fig. 2, when k_{cat} is measured at a constant experimental tempera-

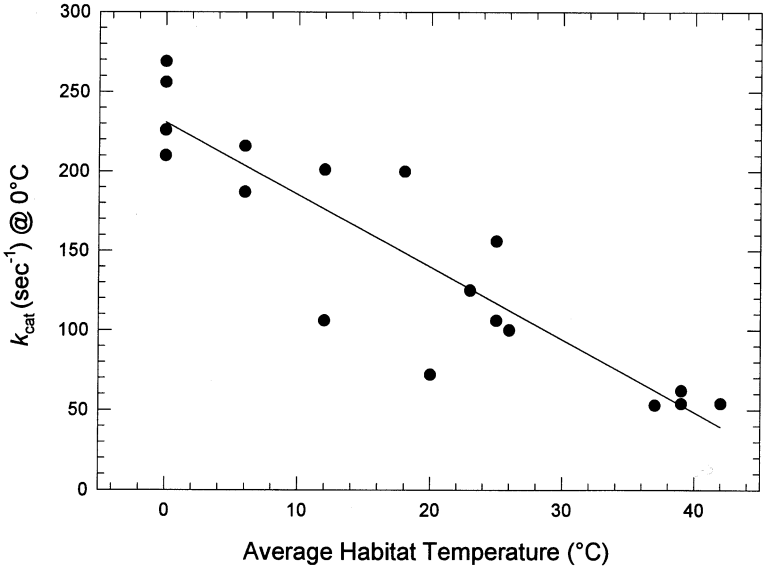

Fig. 2. Correlation ($r^2 = 0.81$) between average habitat temperature and A_4-LDH k_{cat} values measured at 0°C. Data from Fields and Somero (1998) and references therein.

ture, A_4-LDHs from warm-adapted species convert substrate to product more slowly than do the orthologs from more cold-adapted species. This shows the increasing intrinsic stabilities of orthologs as species become adapted to warmer temperatures. For the most warm-adapted orthologs, a much larger proportion of the enzyme population at 0°C is below the activation energy threshold, and is therefore unable to catalyze the reaction, than is the case for the cold-adapted forms. Because this is due to stability differences intrinsic to the various enzymes, the same relationship is true at any level of thermal energy, i.e. at any temperature, until the more flexible forms begin to denature at high temperatures.

Another parameter of enzyme function, the apparent Michaelis constant, or K_m, measures the binding affinity of enzyme for substrate and can also be used as an indirect measure of the inherent flexibility of an enzyme molecule. The ability of an enzyme to bind substrate and cofactor depends on the maintenance of an appropriate geometry, such that the complementary surfaces of ligand and enzyme can interact closely. Indeed, it is the specificity of these interactions that determines the ability of enzymes to efficiently catalyze reactions that otherwise would be energetically unfavorable. Thus, if the appropriate geometry of the binding surface of the protein is perturbed, this can be measured as an increase in K_m (Fields and Somero, 1998). One source for such perturbation is excess flexibility in the enzyme molecule, as can occur when inputs of thermal energy become excessive. Again using A_4-LDH as an illustrative example, Fig. 3a shows that for any ortholog, an increase in assay temperature leads to an increase in K_m for the substrate pyruvate (K_m^{PYR}), meaning a decrease in enzyme–substrate affinity. This is consistent with an increase in molecular flexibility leading to a disruption of binding site geometry. Furthermore, if K_m^{PYR} values of orthologs are examined at one measurement temperature, it is apparent that more warm-adapted orthologs have lower K_m^{PYR} values than cold-adapted forms. This suggests that the greater inherent stability of warm-adapted enzymes, as discussed above, leads to the maintenance of appropriate binding geometries to higher temperatures. Finally, however, if we examine K_m^{PYR} values within the physiological temperature ranges of the various species studied (Fig. 3b), in a manner analogous to the comparison of k_{cat} values above, we see that K_m^{PYR} values are conserved within a narrow range, regardless of habitat temperature. Thus, both K_m and k_{cat} are enzyme parameters that are protected by natural selection, and to the extent that they depend on enzyme flexibility, it is changes in this structural parameter that will be the raw material that natural selection acts upon to modulate enzyme function.

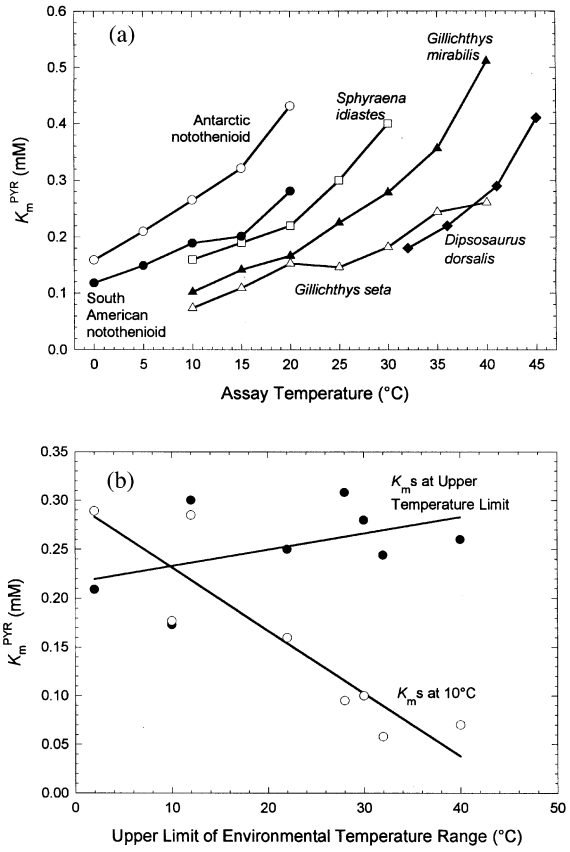

Fig. 3. (a) Apparent A_4-LDH Michaelis constants for the substrate pyruvate (K_m^{PYR}) from vertebrates adapted to different habitat temperatures. The more warm-adapted orthologs have lower K_m^{PYR} values at any assay temperature. (b) K_m^{PYR} values for A_4-LDH orthologs measured at 10°C vs. habitat temperature (open symbols; $r^2 = 0.83$), and K_m^{PYR} values for the same orthologs measured at the upper habitat temperature limit of each species vs. habitat temperature (closed symbols; $r^2 = 0.23$). Data from Fields and Somero (1998).

The catalytic parameters k_{cat} and K_m are highly informative regarding relative levels of flexibility in enzyme orthologs, but they remain indirect measures of this molecular attribute. By using physical techniques, we can develop a more direct view of how differently-adapted orthologs

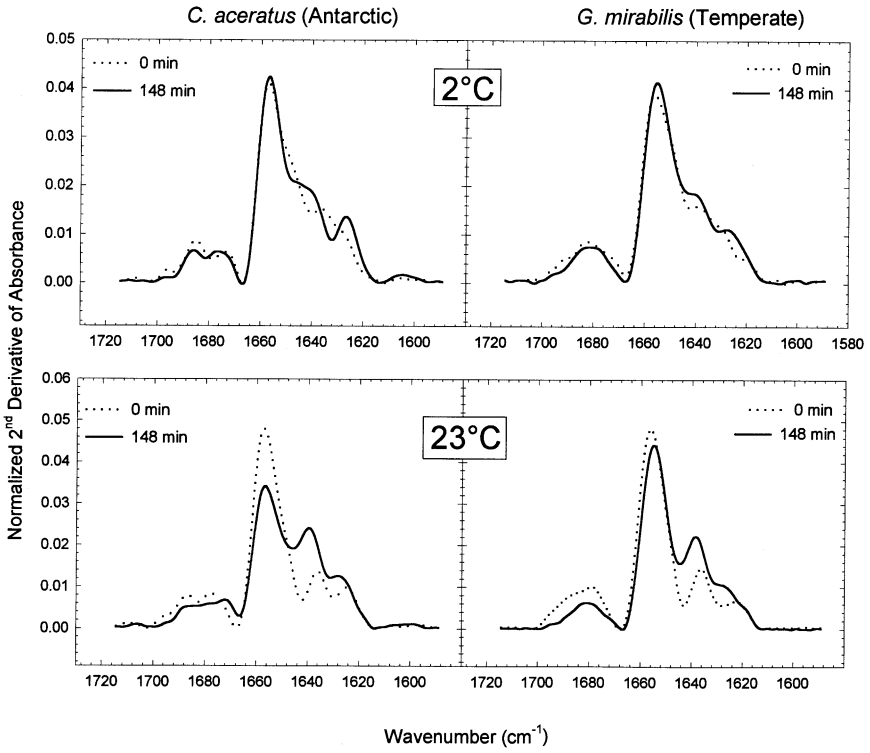

Fig. 4. Hydrogen/deuterium exchange experiments monitored by Fourier-transform infrared spectroscopy, measuring the rate of localized unfolding of A_4-LDH orthologs of two teleost fishes, the Antarctic *Chaenocephalus aceratus* and the warm-temperate *Gillichthys mirabilis.* In each panel, the dotted line corresponds to the absorbance of each ortholog immediately after transfer to D_2O. The solid line shows the absorbance after 148 min in D_2O. Peaks in the spectra correspond to specific secondary structures (for example, the peak $\sim 1655\ cm^{-1}$ is due to absorbance by α-helices), and changes in peak height and breadth correspond to the amount of hydrogen–deuterium exchange that has taken place in those structures. The only 148-min spectrum that shows a significant difference from its corresponding 0-min spectrum is that of *C. aceratus* A_4-LDH at 23°C, indicating that at this temperature the Antarctic ortholog is considerably more flexible than it is at 2°C, and is more flexible than the temperate ortholog is at either temperature.

'breathe' in solution at different temperatures. One of the most useful techniques for this type of study is hydrogen–deuterium exchange, as monitored by Fourier-transform infrared spectroscopy (H/D-FTIR). Briefly, in this technique, purified enzyme is transferred into deuterated water, and the exposed hydrogen atoms on the protein molecule rapidly exchange with the deuterium atoms in the D_2O. Monitoring changes in the infrared absorbance of the protein, which shifts in stereotypical ways as deuterons replace protons, can quantify the rate of this exchange. Exchange is extremely rapid on the surface of the protein, but for those hydrogen atoms buried in the protein interior, exchange is dependent on the rate at which molecular 'breathing' transiently exposes them to the solution. Thus, the technique ultimately measures the rate at which enzyme molecules expose interior residues, which is a direct quantification of molecular flexibility.

Fig. 4 shows the results of an experiment in which A_4-LDH orthologs from thermally divergent species were compared using H/D-FTIR. *Chaenocephalus aceratus* is an Antarctic notothenioid fish, and it lives in a habitat where temperatures continuously remain between 0 and −1.86°C. *Gillichthys mirabilis* is a temperate goby fish, which experiences temperatures between 10 and 35°C in its estuarine habitat. When the A_4-LDHs of these two species were compared at 2°C (Fig. 4, upper panel), there was no difference in the rate at which interior hydrogens exchanged with deuterium atoms. Thus, at this low temperature, it appears that there was little difference between the orthologs in their 'breathing', or inherent flexibility. In contrast, when the same

experiment was performed at 23°C (Fig. 4, lower panel), the rate of H/D exchange was much higher in the Antarctic ortholog than in the temperate one. In other words, the internal hydrogens of the more cold-adapted form were exposed to the deuterated solvent much more quickly, revealing a more flexible structure than the warm-adapted ortholog. This direct evidence of correlation between differences in molecular flexibility and habitat temperature provides a valuable confirmation of the relationship suggested by the k_{cat} and K_m data presented above — that flexibility of enzyme molecules varies inversely with habitat temperature.

In summary, the conformational mobility inherent in enzyme catalysis requires a specific level of stability and of flexibility in order to achieve optimal function. Each of these parameters is temperature-sensitive, and thus natural selection has acted to maintain each at comparable levels in orthologs adapted to varying thermal habitats. It is not clear that the same structural modifications lead to changes in both parameters, and in fact recent laboratory work has shown that k_{cat} can be modified without changes in global stability (Giver et al., 1998; Haney et al., 1999a; Miyazaki et al., 2000). Nonetheless, studies using differently adapted orthologs, as well as site-directed mutagenesis, have begun to suggest what types of structural changes must occur in order to have selectively advantageous impacts on stability, flexibility, and ultimately, function.

3. Changes in amino acid composition leading to thermal adaptation

The role that amino acid substitutions play in altering the stability and flexibility of enzymes during adaptation to temperature has been the subject of much study in recent years. The ever more rapid production of gene sequences, and now entire genomes, has allowed comparison of enzymes from mesophiles and extremophiles in order to deduce the importance of various types of amino acid substitution, usually through reliance on statistical tests regarding the prevalence of particular amino acids or amino acid groups (e.g. polar, charged, hydrophobic) in one group vs. the other. For example, Haney et al. (1999b) compared the genome of one hyperthermophilic archaeon, *Methanococcus jannaschii* (85°C growth temperature), with sequences from its mesophilic congeners *M. voltae*, *M. vannielii* and *M. maripaludis*, allowing the direct sequence alignment of 115 proteins. The researchers then performed an exhaustive pairwise comparison of all 20 amino acids, examining over 7000 amino acid replacements, and ultimately found 26 pairs that showed significant changes between the mesophiles and the thermophile. In the direction mesophile → thermophile, these changes can be categorized into four distinct groups (Haney et al., 1999b): (1) a decrease in uncharged polar residues, mainly in favor of non-polar amino acids that participate in hydrophobic interactions (see below). These replacements may also minimize problems related to deamidation of asparagine and glutamine by serine and threonine at high temperatures (Tomazic and Klibanov, 1988); (2) an increase in charged residues, which may be involved in larger numbers of stabilizing ion pairs and networks, although location of ion pairs within molecular structures also appears to be important in determining stability (Xiao and Honig, 1999); (3) an increase in residue hydrophobicity, which is expected to stabilize warm-adapted proteins as the hydrophobic effect becomes stronger with increasing temperature; and (4) increased residue volume, which may enhance stability in the thermophilic proteins by more efficient packing and reduced rotational entropy of the unfolded form.

A second study (Chakravarty and Varadarajan, 2000) used the same concept as Haney et al. (1999b), but a different technique to confirm and extend the findings described above. These authors compared the amino acid composition of soluble proteins (without alignment of orthologs) from eight thermophiles and 12 mesophiles in order to find correlations between amino acid replacement and habitat temperature. This study confirmed the ubiquity of polar uncharged → non-polar substitutions in species occurring at higher temperatures, as well as the increase in charged residues among these thermophiles. Chakravarty and Varadarajan also confirmed the findings of Thompson and Eisenberg (1999), concluding that soluble thermophilic proteins are smaller than mesophilic ones, with an average of 268 ± 38 vs. 310 ± 16 residues. Chakravarty and Varadarajan argue that smaller protein size leads to lower heat capacity, and thus to greater stability at high temperatures, while Thompson and Eisenberg suggest that shortened exposed loops

in thermophilic proteins lead to a smaller difference in entropy between the unfolded and the folded states, thereby stabilizing the folded state. Finally, Chakravarty and Varadarajan predicted secondary structure of the proteins based on the primary structural information available, and found that β-sheets are more likely to be found in thermophiles, but that α-helices are not. However, they discovered that α-helices of thermophiles are more likely to have stabilizing ionic interactions, and to have stronger charge-dipoles (i.e. charged residues positioned to enhance the natural dipole of the helix), which can also stabilize structure (Hol et al., 1978).

At the opposite extreme of temperature, the same general changes in amino acid composition have been found to apply in enzymes from organisms adapted to life at constant cold temperatures. The enhanced flexibility necessary for sustaining rates of catalysis in a range similar to that of mesophilic enzymes functioning at moderate temperatures necessitates a reduction in protein stability, and this is achieved by reversing many of the processes described above for proteins from thermophiles. Although there are fewer sequences and structures of psychrophilic enzymes available than there are for thermophilic forms, a number of patterns have become apparent (Feller et al., 1997): (1) fewer ionic interactions in psychrophilic proteins likely lead to less structural stability; (2) intramolecular hydrogen bonds are less numerous in psychrophilic proteins, again suggesting a less stable structure; (3) a reduction in the hydrophobicity index of psychrophilic proteins is apparent, which would reduce the stabilizing effect of hydrophobic interactions. However, it should be noted that the hydrophobic effect itself is weakened by low temperatures, and the loss of hydrophobic residues may simply be an artifact of their reduced utility in intramolecular stabilization; (4) increased numbers of polar or charged groups (i.e. increased hydrophilicity) on protein surfaces in psychrophiles, which may lead to destabilization through enhanced interactions with solvent (see below); and (5) in counterpoint to the study of Chakravarty and Varadarajan described above, it appears that psychrophilic enzymes are likely to have more unfavorable α-helical charge–dipole interactions (i.e. charged residues positioned to disrupt the helix dipole), thus destabilizing these secondary structures (Rentier-Delrue et al., 1993; Feller and Gerday, 1997). An additional strategy that may be employed by psychrophilic proteins involves the enhancement of the entropy of the peptide backbone, by reducing the number of rigid proline residues while increasing the number of unconstrained glycines. However, the reverse strategy may (Haney et al., 1999b) or may not occur (Chakravarty and Varadarajan, 2000) in thermophiles.

Although the studies described above provide a number of different routes by which enzyme structure can be modified in order to maintain optimal function at extreme temperatures, one potential type of modification has not been found. The residues and geometry of the active site are conserved across broad taxonomic groups and from one extreme of temperature to the other. It appears that substrate binding is so specific that any alteration in residues involved in catalysis almost invariably leads to a reduction in catalytic efficiency, if not outright loss of function. Thus, the locations of the residue substitutions described above, both for psychrophiles and thermophiles, must occur at some distance from substrate binding sites, and must alter function by changing the relative mobility of structures, rather than attributes of the binding surfaces.

These studies have provided valuable information regarding the importance of certain types of amino acid substitutions in adaptation to extreme temperatures. However, they have not been able to provide information regarding where in enzyme molecules changes occur. It is unlikely that a given amino acid substitution, for example serine to alanine, would have the same effect on enzyme stability if it occurred on the solvent-exposed surface rather than the hydrophobic core, or a rigid α-helix rather than an unstructured loop, or the geometrically constrained active site rather than a location far from any ligand-binding structures. Thus, although this particular replacement is the most favored (i.e. highest probability of directional bias) in the *Methanococcus* mesophile → thermophile study of Haney et al. (1999b), it remains unclear how the substitution exerts its effect in individual enzymes, and why the same change occurs in the thermophile → mesophile direction in 23% of the substitutions involving the two residues. Obviously, there are times when this substitution is more important to temperature adaptation than others, and it can be surmised that much of this change in relative

effect must be due to the position of the residue in the protein molecule.

4. Localization of amino acid substitutions important for thermal adaptation

The discussion above illustrated the breadth of the general understanding that has come about regarding the relationship between changes in habitat temperature and modifications in amino acid composition. Correlations between the types of amino acids favored by extremophiles and the putative roles the amino acids play in stabilization (or destabilization) have also been strengthened by the ever-increasing sample sizes available for comparison between species adapted to different habitats. Nevertheless, in order to understand how individual enzymes become adapted to function at thermal extremes, it is necessary to know the details regarding how each substitution stabilizes or destabilizes the enzyme molecule of interest, or, equivalently, how each substituted residue interacts with the other amino acids in the protein and with the surrounding solvent. To obtain such detailed information, the three-dimensional structure of the protein must be known, usually from X-ray crystallography, but occasionally developed through homology modeling of closely related proteins whose structures have been determined. In addition, it is best to compare orthologs differentiated by relatively few substitutions, in order to most efficiently separate truly temperature-adaptive substitutions from those that are simply phylogenetic noise — drift owing to the distance between the mesophile and the extremophile under study. This is usually accomplished by comparing closely related species, in which the signal of adaptive change is not masked by large numbers of neutral mutations.

One example of such a study used A_4-LDH orthologs from nine species of Antarctic notothenioid fishes (Fields and Somero, 1998), which are adapted to cold, constant temperatures of ~ -1°C. The amino acid sequences of LDH-A were derived from *ldh-a* cDNA, and a 331-amino-acid-long consensus sequence was produced. This consensus was aligned with a consensus sequence from six temperate teleost LDH-As, and 10 non-conservative differences in the amino acid sequence were noted (Fig. 5a). Interestingly, the substitutions were not distributed randomly across the primary structure of the molecule; instead, potentially functionally important differences were grouped together. By combining this information with the three-dimensional structure of vertebrate A_4-LDH available from the literature (Abad-Zapatero et al., 1987), these substitutions could be placed in the context of secondary and tertiary structural interactions (Fig. 5b). In addition, the conformational changes that occur during catalysis have been described in detail for LDH (Dunn et al., 1991; Gerstein and Chothia, 1991), so these potentially adaptive substitutions could be associated with structures that require mobility for catalysis, and therefore can be altered in order to modify catalytic rate — the raw material upon which natural selection must operate.

Given the high level of flexibility inherent in the Antarctic orthologs implied by their high k_{cat} and K_m values (Fields and Somero, 1998), specific substitutions were targeted as likely to increase the mobility of catalytically important structures in the Antarctic orthologs with respect to the temperate forms. These included two extra glycine residues, which, by increasing the rotational entropy of the peptide backbone, have been argued to increase flexibility (Matthews et al., 1987). These two substitutions are 10 residues apart, and are located in an area of the molecule (loop βH–α1G, Fig. 5b) that may control the mobility of one side of the catalytic vacuole (helix α1G–2G).

A second substitution common to the Antarctic notothenioid LDH-As is a proline → alanine at the N-terminal of helix αH, which also borders the catalytic vacuole. Because proline is extremely restricting on peptide backbone movement, due to its unique pyrrolidine ring (Creighton, 1993), the loss of proline in the cold-adapted forms might lead to greater mobility in helix αH. Because both helix α1G–2G and helix αH have been described as 'major movers' during the A_4-LDH catalytic cycle (Gerstein and Chothia, 1991), the presence of destabilizing substitutions bordering these structures in the Antarctic forms provides a plausible mechanism for cold-adaptation within this enzyme.

However, such correlations between potentially destabilizing substitutions and cold-adaptation remain enticing hypotheses until direct proof of the importance of the structural alterations can be developed experimentally. This has been carried

out in a number of studies, which have used site-directed mutagenesis to modify extremophilic enzymes, replacing potentially adaptive residues with those found in the same position in me-

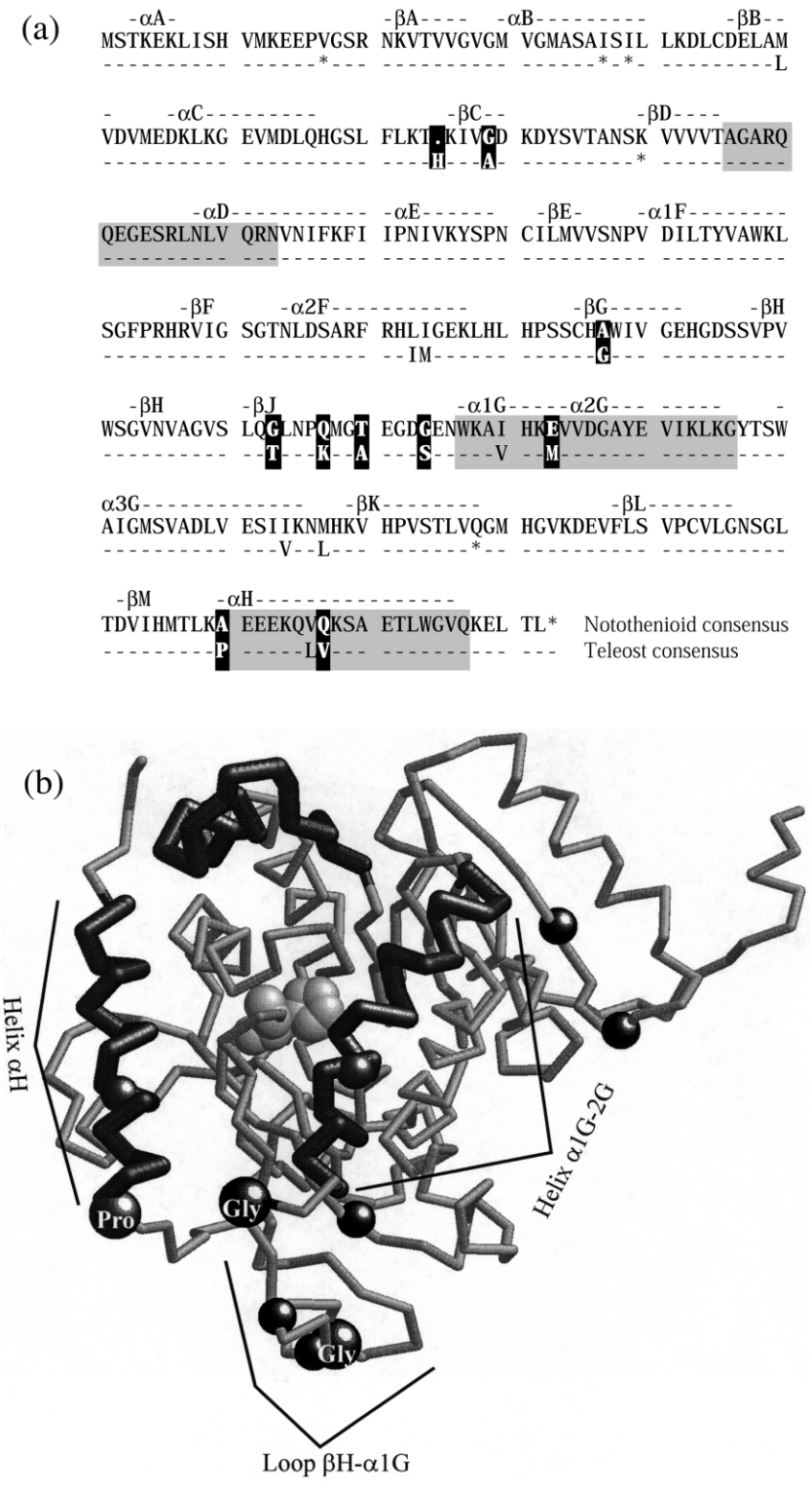

Fig. 5.

sophilic orthologs. Interestingly, when we compare the mechanisms by which specific amino acid substitutions alter the stability of study enzymes, no particular method appears to predominate. This can be illustrated by succinctly describing some recent studies employing the site-directed mutagenesis technique.

For example, in the DNA-binding protein HU from the thermophilic bacterium *Bacillus stearothermophilus*, Kawamura et al. (1998) showed that five different residue substitutions relative to the homolog from the mesophile *B. subtilis* play a role in increasing thermostability. These act via two different mechanisms: four of the five stabilize two α-helices, either directly through their enhanced helix-forming propensities (serine → alanine and asparagine → glutamate), through the formation of a stabilizing salt bridge (asparagine → glutamate and asparagine → lysine), or through the stabilization of a helix–turn–helix motif (glutamate → glycine). The second mechanism of stabilization in this mesophile → thermophile comparison appears to be more efficient packing of the hydrophobic core (serine → alanine and isoleucine → valine).

In another study using *B. stearothermophilus*, triosephosphate isomerase in the thermophile was compared with its ortholog in the mesophilic *Thermotoga maritima* (Alvarez et al., 1999). Here, the replacement of a glycine by a lysine stabilizes the position of nearby active-site residues, thereby enhancing function at high temperatures, without altering global stability.

A third example of the value of site-directed mutagenesis for teasing out the effects of different substitutions is a study carried out on cold-shock proteins, comparing the *B. caldolyticus* (thermophile) with the *B. subtilis* ortholog (Perl et al., 2000). In this case, stabilization occurs because of the loss of an unfavorable electrostatic interaction between two glutamate residues, when one is substituted for a leucine. Furthermore, stabilization in the thermophilic homolog is also enhanced by the addition of an arginine on the surface of the molecule, replacing a glutamate in the mesophilic form; this stabilization seems to occur via long-range electrostatic interactions, instead of local ion pairing.

A final study deserves mention (Narinx et al., 1997), because it focuses on psychrophilic rather than thermophilic *Bacillus*. Moreover, the mode by which the psychrophilic homolog of subtilisin is destabilized illustrates a further mechanism for altering protein stability. In addition to a number of other stabilizing structural modifications, the replacement of a threonine by an aspartate in the psychrophilic subtilisin increased the affinity of the enzyme for the allosteric ligand Ca^{2+}. When bound, the ionic ligand presumably acts as a non-covalent scaffold maintaining tertiary structure, and increases the thermostability of the mutant to a level equivalent to mesophilic subtilisin.

One striking aspect of these studies is that each finds a different mechanism by which thermophilic proteins are stabilized, or psychrophilic proteins are destabilized. Ion-pair formation, increased α-helical propensity, more efficient hydrophobic packing (see also Auerbach et al., 1998), changes in electrostatic potential, and enhanced ligand affinity have all been discovered when individual stabilizing substitutions were scrutinized. Other studies have shown the importance of additional proline residues (in some places in the *Clostridium beijerinckii* alcohol dehydrogenase molecule, but not others; Bogin et al., 1998), enhanced interactions of aromatic sidechains (in *Thermomonospora fusca* xylanase; Georis et al., 2000), and stronger subunit hydrophobic interactions within multimeric proteins (such as citrate synthase from *Thermoplasma acidophilum*, Erduran and Kocabiyik, 1998, or LDH from *Thermotoga maritima*, Auerbach et al., 1998).

Thus, although studies of amino acid preva-

Fig. 5. (a) Consensus sequence of LDH-A cDNAs from nine Antarctic notothenioid species, aligned with a consensus sequence from six temperate teleost LDH-As (Fields and Somero, 1998). The top row indicates secondary structural components, the second row provides the notothenioid consensus, and the third row shows the non-notothenioid teleost consensus. Dashes represent identical residues, asterisks represent residues that were evenly divided among the non-notothenioid LDH-As (none represented a non-conservative substitution). Non-conservative substitutions are highlighted in black, secondary structures that are 'major movers' during catalysis (Gerstein and Chothia, 1991) are highlighted in gray. (b) Three-dimensional model of one monomer of A_4-LDH, based on the dogfish A_4-LDH structure of Abad-Zapatero et al. (1987) (PDB code 1LDM). Dark gray structures are 'major movers' as shown in Fig. 5a; black spheres are non-conservative substitutions between notothenioids and non-notothenioids. Substitutions that may impact flexibility of mobile structures are labeled. The active site is indicated by a space-filled histidine residue (light gray).

lence in combined sequences or entire genomes of mesophiles vs. thermophiles may provide a broad picture of what changes are possible, or even likely, in adaptation to a given environment, we still do not have the ability to predict the changes that are important for an individual enzyme of interest. This is true even if an alignment of the mesophilic and extremophilic orthologs is available — in order to be sure that the adaptively important substitutions are unambiguously identified, site-directed mutagenesis must be employed. One hope for the future is that, as mutagenesis studies become more common, patterns will emerge relating amino acid prevalence in extremophiles to particular locations in enzymes — α-helices, loops, inter-subunit contact areas, etc. This may allow a further level of predictive ability regarding the role of individual substitutions, but will probably not do away with the need for ultimate confirmation via site-directed mutagenesis.

5. Extrinsic stabilizers

Much of the preceding discussion has focused on modifications to enzyme stability arising from changes in primary structure; i.e. intrinsic stabilization. Another route by which to achieve changes in stability, and one that allows greater phenotypic plasticity, is through the use of low molecular-weight stabilizing solutes, often termed 'compatible solutes.' Organisms that rely on such extrinsic stabilizers to attain and retain appropriate levels of enzyme stability at temperature extremes enjoy a two-fold advantage over those that, via random mutation, have modified the protein primary structure. First, they are able to occupy broader thermal niches by modifying the concentrations of thermostabilizing solutes in response to transient changes in temperature. That is, they can exploit thermal habitats that would be either overly stabilizing or destabilizing to the proteins of an organism that did not have access to varying levels of stabilizing solutes. Second, in order to colonize a newly available thermal habitat, they need not structurally modify every thermally sensitive protein in their repertoire — a process that might take many thousands of generations. Instead, the same levels of stabilizing solutes would have comparable effects on all proteins, because the mechanism by which these compounds stabilize is generally independent of specific physicochemical attributes of the proteins being stabilized. Instead, compatible solutes tend to act non-specifically by minimizing the surface area of proteins, thus favoring the folded over the unfolded state (Timasheff, 1992).

Because of the valuable biotechnological applications of solutes that stabilize proteins without negatively impacting their function, much research has been carried out on the different species of stabilizers that are found in various extremophiles. Hensel and Konig (1988) showed that 2,3-diphosphoglycerate accumulates in some methanogenic bacteria when growth temperatures are increased, and coupled their finding with data showing that this compound acts as an enzyme stabilizer in vitro. This provided strong evidence that stabilizing solutes can have an adaptive value to thermophilic organisms in vivo.

Upon the introduction of heat stress, the changes in concentration and composition of thermostabilizing compounds can be striking. Martins et al. (1996) showed that the entire stock of solutes in the bacterium *Thermotoga neapolitana* increased approximately seven-fold as growth temperature was increased from 65 to 88°C. Furthermore, protein-stabilizing *myo*-inositol-phosphate derivatives increased from less than 0.1 to nearly 1.5 μmol/mg across this temperature range. The same family of stabilizers has also been found in archaeal species (Ciulla et al., 1994; Martins et al., 1997). Other stabilizing solutes that have been shown to increase in certain species at high growth temperatures include α-mannosylglycerate (*Rhodothermus obamensis*; Silva et al., 1999), diglycerol phosphate (*Archaeoglobus fulgidus*; Martins et al., 1997), and inorganic ions (Coolbear et al., 1992; Hansen et al., 1999; Mueller et al., 2000).

Just as stabilizing solutes appear advantageous for some organisms that live at high temperatures and experience even higher temperatures transiently, it would seem that some psychrophilic organisms would take advantage of destabilizing solutes to lend increased flexibility to proteins. Indeed, there seems to be no physical reason why such solutes should be avoided during adaptation or acclimation to cold temperatures. Nevertheless, the evidence for such a role by destabilizing solutes is slight. In a study comparing elongation factor-2 from an Antarctic and a thermophilic methanogen, Thomas and Cavicchioli (2000)

found that the psychrophilic form possessed maximal activity at a temperature well above the optimal growth temperature of the organism, *Methanococcoides burtonii*. From this, they argued the possibility of as-yet unidentified cytosolic factors that might increase protein flexibility, thus boosting activity at physiologically meaningful temperatures. The presence of such factors remains speculative, however, and further research is required to confirm the presence of destabilizing solutes in psychrophiles.

Conversely, it is possible that the proteins of some psychrophilic organisms would require *stabilization* by solutes at low temperatures. This is because the hydrophobic effect, one of the main intrinsic stabilizers of protein structure, is progressively weakened as temperature decreases. In essence, the hydrophobic effect depends on the entropically favorable loss of water structure as hydrophobic structures are removed from the solvent — which organizes cage-like structures to accommodate them — and are buried together in the protein interior. As temperature decreases and the bulk water becomes more structured, the entropy increase upon the burial of hydrophobic residues is reduced, and the entropically driven hydrophobic stabilization of proteins is weakened. At the same time, the thermal energy necessary to melt the solvent cages around the hydrophobic functional groups is reduced, pushing the system equilibrium toward the unfolded state. Thus, proteins reach a maximum stability, as measured by ΔG of stabilization, at some intermediate temperature, and at either colder or warmer temperatures stability is reduced. Thus cold-denaturation becomes a theoretical possibility for proteins (Privalov and Gill, 1989), and stabilization may be required for adequate function at low temperatures. This aspect of solute–protein interactions has been little studied, and no evidence for stabilizing solutes in psychrophilic organisms has yet been presented.

A final mechanism of protein stabilization via extrinsic modulators involves stress proteins, which act to protect proteins from denaturation and aggregation at both hot (heat shock) and cold (cold shock) extremes. Their occurrence in organisms is nearly ubiquitous, indicating the importance of the roles of these proteins in the regulation of cellular processes at suboptimal temperatures. A description of the complexities of the regulation and function of the stress proteins is beyond the scope of this paper, but the interested reader is directed to a recent review that focuses on the comparative aspects of stress proteins in different species (Feder and Hofmann, 1999).

6. Summary

It has become axiomatic that enzyme function depends on a careful balance between structural stability and flexibility (Jaenicke, 1991; Somero, 1995). Temperature affects both of these attributes, and so proteins adapted to work at one temperature are inherently unable to maintain function at temperatures far removed from this optimum. This indeed may be one of the most important factors in determining the geographical distributions of ectotherms. Nevertheless, protein structure can be modified to optimize function, such that homologs with similar primary structures, and almost identical tertiary structures, can be found across the entire temperature range experienced in the biosphere. In fact, it could be argued that the range of temperatures in which proteins can conceivably maintain a native conformation might define the temperature limits of life.

Thermophilic and psychrophilic enzymes do not employ any ‘special tricks’ to maintain an appropriate balance between flexibility and stability in their respective extremes — the same stabilizing factors can be found in both. Unfortunately, this underlying symmetry does not help us make a priori predictions regarding the ability of a *particular* amino acid modification to lead to an expected change in stability. Studies comparing large numbers of homologs from closely related mesophiles and extremophiles have provided data regarding which amino acid substitutions are favored in adaptation to extreme temperatures. Furthermore, studies relying on site-directed mutagenesis of individual enzymes, informed by comparisons of sequences from closely related homologs, have allowed us to see the breadth of possible strategies available to natural selection in designing proteins optimized for specific habitats. Nevertheless, the system by which a one-dimensional string of amino acids is transformed into a working, ‘breathing’ enzyme is so complex that we do not, except in the most simple cases, have the ability to predict how a change in pri-

mary structure will impact protein stability, flexibility, and ultimately, function.

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