The Journal of Microbiology, December 2001, p.245-249 Vol. 39, No. 4

MINIREVIEW

Thermostability Mechanisms in Enzymes from *Thermotoga neapolitana* and *Pyrococcus furiosus*

Claire Vieille*, Dinlaka Sriprapundh, Alexei Savchenko, Suil Kang, Harini Krishnamurthy, and J. Gregory Zeikus

Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA

(Received September 17, 200)

Proteins from hyperthermophilic organisms are typically highly thermostable, and they are optimally active at high temperatures (often 90°C and above). Hyperthermophilic enzymes are surprisingly highly similar to their mesophilic homologs: (i) their sequences are typically 40-85% similar; (ii) their three-dimensional structures are superposable; and (iii) they share the same catalytic mechanisms (1, 18, 19, 22). Thus, with the exception of phylogenetic variations, what differentiates hyperthermophilic and mesophilic enzymes is only the temperature ranges in which they are stable and active. When cloned and expressed in mesophilic hosts, hyperthermophilic enzymes usually retain their thermal properties, indicating that these properties are genetically encoded.

In the few cases where the difference in free energy of stabilization between mesophilic and hyperthermophilic homologous enzymes is known, this difference is small, between 5 and 20 kcal/mol. With the free energy of stabilization provided by non-covalent interactions (i.e., hydrophobic interactions, hydrogen bonds, salt bridges, and aromatic interactions) typically ranging between 0.3 and 2.0 kcal/mol, a small number of additional non-covalent interactions are often enough to explain the remarkable stability of hyperthermophilic enzymes. For this reason, identifying the key stabilizing interactions in a hyperthermophilic protein is a difficult task. This task is made even more difficult by the absence of a single mechanism responsible for protein thermostabilization. Stabilization mechanisms vary from one protein to another. Additional salt bridges and extended salt bridge networks seem to be the most common stabilizing mechanism among different proteins and different organisms (19). Due to the high number of hydrogen bonds in proteins, the role of hydrogen bonds in stabilization has not been extensively studied.

* To whom correspondence should be addressed.
(Tel) 1-517-353-4674; (Fax) 1-517-353-9334
(E-mail) vieille@msu.edu

A current working hypothesis is that hyperthermophilic enzymes are more rigid than their mesophilic homologs at mesophilic temperatures, and that this rigidity is a prerequisite for high protein thermostability. This hypothesis is supported by a growing body of experimental data, in particular from hydrogen/deuterium exchange experiments (3, 10, 21). Following from this hypothesis, it has also been proposed that excessive rigidity explains why hyperthermophilic enzymes are often inactive at low temperatures (i.e., around 20°C to 37°C). Denaturants (2, 11, 20), detergents (5, 16), and solvents (4, 12) often activate hyperthermophilic enzymes at suboptimal temperatures. This activation tends to disappear as the temperature gets closer to the enzyme's temperature of maximal activity (2), suggesting that at that temperature, the enzyme is flexible enough in the absence of a denaturant to show full activity. The dynamics of active site residues in relationship to activity has never been directly measured, though, all flexibility studies so far having measured only global enzyme flexibility in relationship to activity. It is also unclear how enzyme rigidity at low temperatures relates with the high activity level shown by a few hyperthermophilic enzymes at mesophilic temperatures. The reason why enzyme rigidity would be a requirement for stability is also not clear: flexibility implies increased conformational entropy of the folded state, and it should, therefore, be favorable to thermodynamic stability (13). More studies on hyperthermophilic enzyme flexibility at various temperatures are needed to get a better understanding of the role of conformational rigidity in protein stability and activity.

In the last few years, our laboratory has been studying enzymes from *Thermotoga neapolitana* and from *Pyrococcus furiosus* to determine which molecular mechanisms make these enzymes thermostable and active at high temperatures. Our study models include *T. neapolitana* adenylate kinase and xylose isomerase, as well as *P. furiosus* α-amylase.

T. neapolitana adenylate kinase

Adenylate kinase (or AK) is an excellent model enzyme for thermostability studies because AK is a small, ubiquitous enzyme that is accessible to NMR, thermodynamic, and molecular dynamics studies, and it is a well-studied enzyme. The AKs from *Escherichia coli* (ECAK, mesophilic) and *Bacillus stearothermophilus* (BSAK, thermophilic) have been characterized. Their thermostability properties and their 3D structures are known.

The *T. neapolitana adk* gene was cloned in our laboratory. Like other long bacterial AK, TNAK is a 25 KDa monomeric enzyme. Most ATP and AMP ligands from ECAK are conserved in TNAK. The four Zn^{2+} liganding cysteines characterized in BSAK (7) are present in TNAK. The purified TNAK was optimally active at 80°C. In contrast to most hyperthermophilic enzymes, which are almost inactive at 30°C, TNAK was as active as ECAK at 30°C. With a melting temperature (T_m) of 99.1°C, TNAK was also much more thermodynamically stable than ECAK (T_m of 51.8°C) and BSAK (T_m of 74.5°C).

As suggested by the TNAKs sequence, atomic emission spectroscopy revealed that TNAK contains one Zn^{2+} per enzyme molecule. EDTA treatments of 1 h at 75°C or 80°C were necessary to deplete TNAK from its Zn^{2+}. As had been observed with BSAK (7), Zn^{2+} was not necessary for TNAK activity. Although both BSAK and TNAK contain the same Zn^{2+} atom, TNAK was significantly more stable than BSAK. This finding indicates that Zn^{2+} alone is not responsible for TNXIs increased stability and that other, unknown, stabilization mechanisms are involved.

We used the FIRST (Floppy Inclusion and Rigid Substructure Topography) software (9) to compare the rigidities of ECAK (i.e., mesophilic), BSAK (i.e., thermophilic), and TNAK (i.e., hyperthermophilic) and to determine whether H-bond networking correlates with stability. FIRST evaluates protein rigidity by modeling covalent forces, H-bonds, and salt bridges as distance constraints, which are then used to decompose the protein into rigid clusters that are linked by flexible regions. The ECAK and BSAK PDB coordinates and the SwissModel coordinates of TNAK were fed into FIRST. The rigid cluster decomposition maps of ECAK (at least eight independent clusters) and BSAK (a single cluster) clearly suggested that BSAK is more rigid than ECAK. BSAK contains approximately seventy more H-bonds than ECAK does. Interestingly, the two enzymes have very similar amino acid compositions, suggesting that the location of the residues in the native enzymes is critical for the difference in the number of H-bonds and for the difference in rigidity between the two enzymes. In addition, higher energy levels are required for similar breakups in the H-bond network in BSAK than in ECAK. These FIRST results on ECAK and BSAK correlate well with the experimental thermostability data. The FIRST results for TNAK (i.e., two rigid clusters and energy levels required for breakups in the H-bond network similar to those for BSAK) suggest that either the TNAK model is not optimal or that H-bonds are not responsible for TNAKs higher thermostability.

T. neapolitana xylose isomerase

Because of their use in the industrial production of high fructose corn syrup, XIs have been extensively studied in terms of structure/function relationships (8). The equilibrium between glucose and fructose favors fructose at higher temperatures, providing an incentive to use thermostable and thermoactive XIs in high fructose corn syrup production. To avoid significant non-enzymatic browning side-reactions occurring at high temperatures and alkaline pHs, the ultimate catalyst should be active and stable at acidic pHs.

Bacterial XIs belong to two groups, classes I and II. XIs activity depends on the presence of two metal cations (taken from Co^{2+}, Mg^{2+}, and Mn^{2+}) in their active sites. The most thermostable XIs known, the *Thermotoga* XIs, are class II enzymes (18). The class II XIs from *E. coli* (mesophilic, ECXI), *Bacillus licheniformis* (BLXI), *Thermoanaerobacterium thermosulfurigenes* (thermophilic, TTXI), and *T. neapolitana* (hyperthermophilic, TNXI) are optimally active at 55°C, 70°C, 80°C, and 95°C, respectively. The reasons for this large temperature range are not clear.

The stability properties of ECXI, BLXI, TTXI, and TNXI were studied to determine if these homologous XIs have similar inactivation patterns. The four enzymes aggregated after inactivation, and no activity could be recovered from the insoluble fraction. Independent of the metal(s) present in their active sites, ECXI, BLXI, and TTXI inactivation courses followed first order kinetics. The three enzymes also showed a single melting transition. BLXI showed a singular, metal-dependent stability behavior. Its T_m increased from 50.3°C (apo-BLXI) to 73.5°C (Co^{2+}-BLXI and Mn^{2+}-BLXI), and the energy of activation for its inactivation increased from 385 kJ/mol (apo-BLXI) to 1,166 kJ/mol (Co^{2+}-BLXI). These results suggest that the first irreversible event in BLXI unfolding occurs when BLXI loses one or both of its metals. The TNXI inactivation course depended on the metal(s) present in its active site. The apo-TNXI or TNXI containing a single type of metal behaved like the other three XIs. In the presence of Mg^{2+} plus Co^{2+}, though, TNXI showed a biphasic inactivation time course and two melting transitions at 99°C and 109°C.

Not many differences in TTXI and TNXIs structures can explain their different stabilities, with the exception of a few additional prolines in TNXI than in TTXI. We noted, though, a direct correlation between the number of non-conserved salt bridges and XI thermostability. Substituting Pro for another residue can increase protein sta-

bility, provided that proline does not create volume interference, and does not destroy stabilizing non-covalent interactions. Two additional prolines in TNXI (P58 and P62) are found in a large loop that participates in subunit-subunit interactions. The corresponding residues in TTXI (Q58 and A62) were substituted to prolines. Mutation Q58P extended TTXIs half-life at 85°C by 43%. The stabilization provided by this mutation was attributed to a reduction of TTXI unfolding entropy (17). Mutation A62P significantly destabilized TTXI through an unfavorable Van der Waals contact between P62's pyrrolidine ring and K61's sidechain that probably led to destabilizing local conformational changes (17).

We have in the past increased TTXI catalytic efficiency on glucose by engineering the enzyme's substrate binding site (14, 15). We introduced the same mutations (i.e., W138F, V185T, and W138F/V185T) into TNXI and characterized the catalytic and stability properties of the mutant enzymes (17). In contrast to the effect of the mutations on TTXI activity, mutation V185T was the best at increasing TNXI catalytic efficiency on glucose. The only significant structural difference between TTXI and TNXI active sites is a 1.23Å shift in the position of metal II. This metal shift might explain why mutations had different effects on TTXI and TNXI activities on glucose. The most stable and active XI we have developed by site-directed mutagenesis is the TNXI V185T mutant. We have since obtained (by random mutagenesis) a TNXI derivative that is five times more active at 60°C and three times more active on glucose at pH 5.2 than TNXI (Fig. 1).

P. furiosus α-amylase

The hyperthermophile *P. furiosus* produces an extracellular α-amylase that belongs to glycosyl hydrolases family 13 (6). *P. furiosus* α-amylase (PFA) is more thermostable than its bacterial (i.e., *B. licheniformis* α-amylase) and archaeal (i.e., *Pyrococcus* KOD1 α-amylase) homologues. Uncommon for a hyperthermophilic enzyme, PFA contains five cysteines (C152, C153, C165, C387, and C430). To test the role of these cysteines in PFA stability, these residues were mutated one by one and in sequence to produce eight mutant enzymes. Mutation C165S (CCSCC) dramatically destabilized PFA, showing that C165 is essential for stability. In contrast, the quadruple mutant enzyme C152S/C153S/C387S/C430A (or SSCSA) was as thermostable as PFA. Atomic emission spectrometry showed the presence of at least two Ca^{2+} and one Zn^{2+} atoms in PFA. Mutants CCSCC and SSSSA contained less Zn^{2+}, and an EDTA treatment at 20°C removed most Zn^{2+} from these two mutants. Only EDTA treatments at 90°C and above could remove the Zn^{2+} from wild-type PFA and

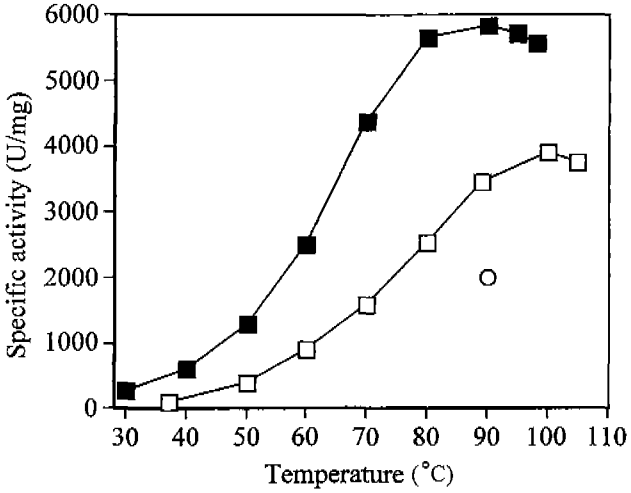

Fig. 2. Effect of temperature on the activities of G-PFA, PFA, and BLA. Closed squares: G-PFA; open squares: PFA; open circle: BLA specific activity at 90°C.

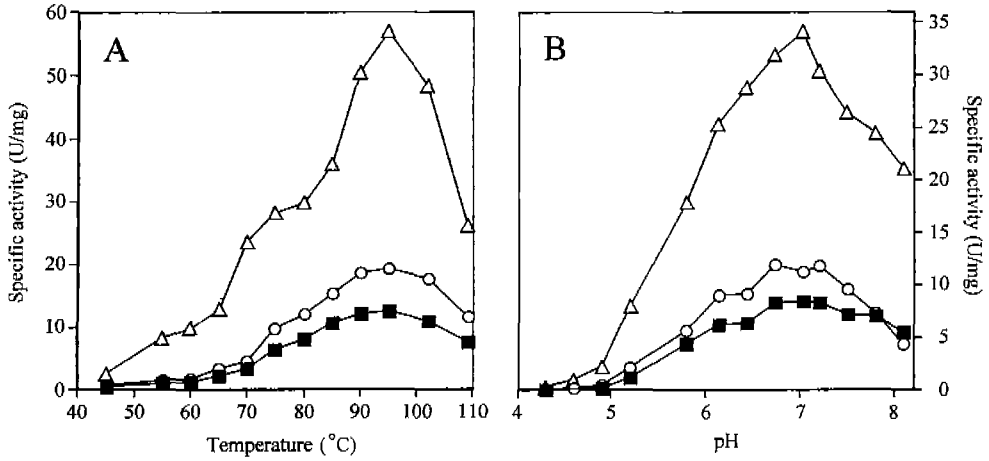

Fig. 1. Effects of temperature (A) and pH (B) on the activity of TNXI and its mutant derivatives on glucose. Closed squares: TNXI; open circles: TNXI V185T mutant; open triangle: TNXI directed evolution mutant 3A2.

from mutant SSCSA. These results suggest that C165 is a Zn^{2+} ligand, and that Zn^{2+} is required for PFA thermostability. PFA contains at least two Ca^{2+} per monomer, but these ions are not removed by EDTA at temperatures below 75°C. PFA was inactivated by EDTA after 30 min at 95°C. PFA's full activity was restored by adding Ca^{2+}, suggesting that Ca^{2+}, but not Zn^{2+}, is required for PFA activity. PFA stability was Ca^{2+}-dependent only at temperatures above 95°C.

PFA easily aggregates at concentrations as low as 1 mg/ml. To improve PFA's solubility, we expressed it in the yeast *Pichia pastoris*. In this system, PFA was secreted into the culture supernatant as a glycosylated protein (or G-PFA). We expected glycosylation to mask some of PFA's hydrophobic surfaces. The purified G-PFA had a half-life of 10 h at 90°C, remained soluble at 2.5 mg/ml, and it was 2.9 times more active than BLA at 90°C. Surprisingly, G-PFA was also 1.7 times more active than the non-glycosylated PFA at 90°C.

Conclusions

In only three enzyme families, we identified at least four different stabilization mechanisms: (i) metal binding, (ii) additional prolines; (iii) additional H-bonds; and (iv) additional salt-bridges. These results concur with other thermostability studies showing that no single molecular mechanism is responsible for protein thermostabilization. Our results also show that a hyperthermophilic enzyme can be as active as a mesophilic one at 30°C (i.e., TNAK) and that improved catalysts can be derived from hyperthermophilic enzymes (i.e., TNXI and PFA).

References

1. Bauer, M.W. and R.M. Kelly. 1998. The family 1 β-glucosidases from *Pyrococcus furiosus* and *Agrobacterium faecalis* share a common catalytic mechanism. *Biochemistry* 37, 17170-17178.
2. Beaucamp, N., A. Hofmann, B. Kellerer, and R. Jaenicke. 1997. Dissection of the gene of the bifunctional PGK-TIM fusion protein from the hyperthermophilic bacterium *Thermotoga maritima*: design and characterization of the separate triosephosphate isomerase. *Protein Sci.* 6, 2159-2165.
3. Bönisch, H., J. Backmann, T. Kath, D. Naumann, and G. Schäfer. 1996. Adenylate kinase from *Sulfolobus acidocaldarius*: expression in *Escherichia coli* and characterization by Fourier transform infrared spectroscopy. *Arch. Biochem. Biophys.* 333, 75-84.
4. D'Auria, S., R. Nucci, M. Rossi, E. Bertoli, F. Tanfani, I. Gryczynski, H. Malak, and J.R. Lakowicz. 1999. β-Glycosidase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: structure and activity in the presence of alcohols. *J. Biochem.* 126, 545-552.
5. De Montigny, C. and J. Sygusch. 1996. Functional characterization of an extreme thermophilic class II fructose-1,6-bisphosphate aldolase. *Eur. J. Biochem.* 241, 243-248.
6. Dong, G., C. Vieille, A. Savchenko, and J.G. Zeikus. 1997. Cloning, sequencing, and expression of the gene encoding extracellular α-amylase from *Pyrococcus furiosus* and biochemical characterization of the recombinant enzyme. *Appl. Environ. Microbiol.* 63, 3569-3576.
7. Glaser, P., E. Presecan, M. Delepierre, W.K. Surewicz, H.H. Mantsch, O. Barzu, and A. M. Gilles. 1992. Zinc, a novel structural element found in the family of bacterial adenylate kinases. *Biochemistry* 31, 3038-3043.
8. Hartley, B.S., N. Hanlon, R.J. Jackson, and M. Rangarajan. 2000. Glucose isomerase: insights into protein engineering for increased thermostability. *Biochim. Biophys. Acta* 1543, 294-335.
9. Jacobs, D.J., L.A. Kuhn, and M.F. Thorpe. 1999. Flexible and rigid regions in proteins, p. 357-384. *In* M.F. Thorpe and P. Duxbury (ed.), Rigidity Theory and Applications. Kluwer Academic/Plenum Publishers.
10. Jaenicke, R. and G. Böhm. 1998. The stability of proteins in extreme environments. *Curr. Opin. Struct. Biol.* 8, 738-748.
11. Kozianowski, G., F. Canganella, F.A. Rainey, H. Hippe, and G. Antranikian. 1997. Purification and characterization of thermostable pectate-lyases from a newly isolated thermophilic bacterium, *Thermoanaerobacter italicus* sp. nov. *Extremophiles* 1, 171-182.
12. Kujo, C. and T. Oshima. 1998. Enzymological characteristics of the hyperthermostable NAD-dependent glutamate dehydrogenase from the archaeon *Pyrobaculum islandicum* and effects of denaturants and organic solvents. *Appl. Environ. Microbiol.* 64, 2152-2157.
13. Lazaridis, T., I. Lee, and M. Karplus. 1997. Dynamics and unfolding pathways of a hyperthermophilic and a mesophilic rubredoxin. *Protein Sci.* 6, 2589-2605.
14. Meng, M., M. Bagdasarian, and J.G. Zeikus. 1993. The role of active-site aromatic and polar residues in catalysis and substrate discrimination by xylose isomerase. *Proc. Natl. Acad. Sci. USA* 90, 8459-8463.
15. Meng, M., C. Lee, M. Bagdasarian, and J.G. Zeikus. 1991. Switching substrate preference of thermophilic xylose isomerase from D-xylose to D-glucose by redesigning the substrate binding pocket. *Proc. Natl. Acad. Sci. USA* 88, 4015-4019.
16. Sako, Y., P. C. Croocker, and Y. Ishida. 1997. An extremely heat-stable extracellular proteinase (aeropyrolysin) from the hyperthermophilic archaeon *Aeropyrum pernix* K1. *FEBS Lett.* 415, 329-334.
17. Sriprapundh, D., C. Vieille, and J.G. Zeikus. 2000. Molecular determinants of xylose isomerase thermal stability and activity: analysis by site-directed mutagenesis. *Protein Engin.* 13, 259-265.
18. Vieille, C., J.M. Hess, R.M. Kelly, and J.G. Zeikus. 1995. *xylA* cloning and sequencing and biochemical characterization of xylose isomerase from *Thermotoga neapolitana*. *Appl. Environ. Microbiol.* 61, 1867-1875.
19. Vieille, C. and J.G. Zeikus. 2001. Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol. Mol. Biol. Rev.* 65, 1-43.
20. Wilquet, V., J.A. Gaspar, M. van de Lande, M. Van de Casteele, C. Legrain, E.M. Meiering, and N. Glansdorff. 1998. Purification and characterization of recombinant *Thermotoga maritima*

dihydrofolate reductase. *Eur. J. Biochem.* 255, 628-637.
21. Závodszky, P., J. Kardos, Á. Svingor, and G. A. Petsko. 1998. Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. *Proc. Natl. Acad. Sci. USA* 95, 7406-7411.
22. Zwickl, P., S. Fabry, C. Bogedain, A. Haas, and R. Hensel. 1990. Glyceraldehyde-3-phosphate dehydrogenase from the hyperthermophilic archaebacterium *Pyrococcus woesei*: characterization of the enzyme, cloning and sequencing of the gene, and expression in *Escherichia coli*. *J. Bacteriol.* 172, 4329-4338.