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MINI-REVIEW

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Toward a molecular understanding of cold activity of enzymes from psychrophiles

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Abstract Despite the fact that a much greater proportion of the earth environment is cold rather than hot, much less is known about psychrophilic, cold-adapted microorganisms compared with thermophiles living at high temperatures. In particular, investigation of the molecular basis of cold-active enzymes from psychrophiles has only recently received concerted research attention, in measure as a result of the EC-funded project COLDZYME. This research effort has been stimulated by the realization that such cold-active enzymes offer novel opportunities for biotechnological exploitation. Only very recently has the first cold-active enzyme, α-amylase, been crystallized, and this success was followed rapidly by others. This effort has facilitated a direct approach to solving the three-dimensional structure of cold-active enzymes to complement the gene homology modeling that had been performed previously. Recently studies have highlighted how different adaptations are used by different enzymes to achieve conformational flexibility at low temperatures, and how such adaptations are not necessarily the opposite of those that confer thermostability to proteins in thermophilic counterparts. This review also highlights initial successes in engineering genetically improved thermal stability in cold-active enzymes to give improved catalysts for low-temperature biotechnology.

Key words Psychrophilic bacteria · Cold adaptation · Cold-active enzymes · Protein structure · Molecular conformation · Biotechnology

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Introduction

Considering that the greater proportion of the planet on which we live is cold, rarely rising above 5°C, and that most of the biota are adapted to grow at temperatures well below what is often considered as normal (i.e., 37°C), it is surprising that we know relatively so much less about the molecular basis of the cold activity of enzymes from psychrophilic, compared with mesophilic or thermophilic, organisms (Russell and Hamamoto 1998). In contrast, the focus of attention has been on the molecular adaptations in membrane lipids (Russell 1989, 1990, 1998; Gounot 1991). Although not diminishing the importance of such adaptive changes for low-temperature growth, this review concentrates on the molecular differences enshrined in the primary sequences and three-dimensional structures of proteins from psychrophiles that enable them to be active at temperatures as low as zero or even below. Recently, there has been a sharp increase in research effort and productivity in this field, with the realization that such cold-active enzymes offer novel opportunities for biotechnological exploitation (Russell 1998). A number of recent reviews have presented the structure of cold-adapted enzymes in fish and psychrophilic bacteria (Feller et al. 1996b; Marshall 1997; Feller and Gerday 1997; Gerday et al. 1997). Therefore, this article, following a general summary of the current status of the field, discusses some examples of cold-adapted bacterial enzymes to illustrate general points and shows how direct structural information is now beginning to be obtained that displays the diversity of protein-adaptive responses: perhaps not surprisingly, those adaptations are not always what one would have predicted from earlier work on thermophiles!

State of the art

The classical approach to elucidating protein structure and for making comparisons between different enzymes was via

Table 1. Psychrophilic bacterial enzymes for which the genes have been cloned and sequenced

Enzyme	Bacterium	Reference
Lactate dehydrogenase	*Bacillus psychrosaccharolyticus*	Vckovski et al. (1990)
Esterase	*Pseudomonas* sp. LS107d2	McKay et al. (1992)
α-Amylase	*Alteromonas haloplanctis*	Feller et al. (1992)
Subtilisin (proteinase)	*Bacillus* sp. TA41	Davail et al. (1994)
Lipase	*Moraxella* sp. TA144	Feller et al. (1991)
	Psychrobacter immobilis	Arpigny et al. (1993)
	Pseudomonas sp. B11-1	Choo et al. (1998)
Triose-phosphate isomerase	*Moraxella* sp. TA137	Rentier-Delrue et al. (1993)
Isocitrate dehydrogenase	*Vibrio* sp. ABE-1	Ishii et al. (1993)
β-Galactosidase	*Arthrobacter* sp. B7	Gutshall et al. (1994)
β-Lactamase	*Psychrobacter immobilis*	Feller et al. (1996b)
3-Isopropylmalate dehydrogenase	*Vibrio* species 15	Wallon et al. (1997)
Alcohol dehydrogenase	*Moraxella* sp. TAE123	Tsigos et al. (1998)
Citrate synthase	*Arthrobacter* sp. DS2-3R	Gerike et al. (1997)
Malate dehydrogenase	*Aquaspirillium arcticum*	Kim et al. (1999)
Elongation factor 2	*Methanococcus burtonii*	Thomas and Cavicchioli (1998)

Table 2. Psychrophilic bacterial enzymes for which crystal structures are known

Enzyme	Bacterium	Reference
α-Amylase	*Alteromonas haloplanctis*	Aghajari et al. (1998)
Citrate synthase	*Arthrobacter* sp. DS2-3R	Russell et al. (1998)
Triose-phosphate isomerase	*Vibrio marinus*	Alvarez et al. (1998)
Malate dehydrogenase	*Aquaspirillium arcticum*	Kim et al. (1999)

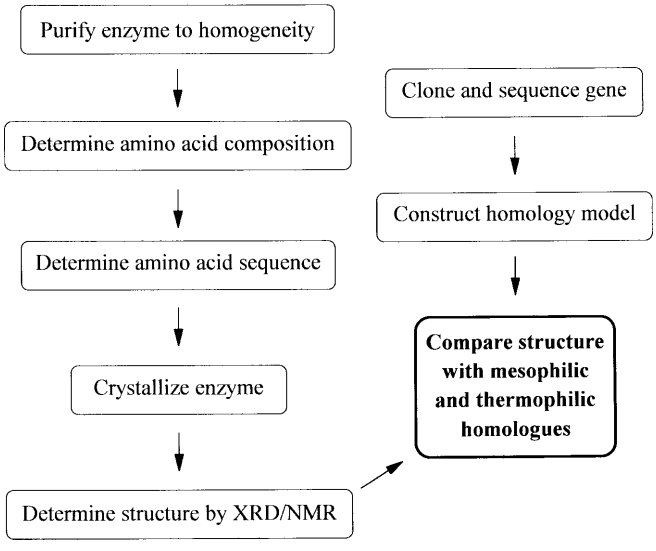

Fig. 1. Approaches toward determining cold-active enzyme structure

protein purification and sequencing; later, this was complemented by the gene cloning approach, as shown in Fig. 1. By the time that much research attention became focused on cold-adapted proteins, invariably the initial approach was to clone and sequence the gene to build homology models by comparison with gene sequences from other (mesophilic and/or thermophilic) homologous proteins. Apart from the obvious fact that the technology was available, the major reason for adopting this approach was that cold-active enzymes were proving difficult to crystallize for direct structural evaluation by XRD and NMR. The number of genes for cold-active proteins that have been cloned and sequenced has already reached double figures (Table 1), and although the number of cold-active enzymes that have been crystallized is much smaller (Table 2), the present pace of discovery is quickening.

The gene–homology–model approach cannot substitute for direct structural determinations of protein crystals, but it is only relatively recently that researchers have succeeded in crystallizing cold-active enzymes from bacteria. The first was an α-amylase by Gerday and Haser and co-workers (Aghajari et al. 1996), who have solved the structure of the native enzyme and its complex with an inhibitor (Aghajari et al. 1998). Another restriction on elucidating the structure of cold-active enzymes from bacteria is the lack of much comparative information on similar enzymes from other organisms. The cold-adapted enzymes in fish from polar waters have been investigated, but the only direct structural data are for trypsin from the Atlantic salmon (Smalås et al. 1994). Therefore, most comparisons are made with the homologous enzyme from mesophilic or thermophilic bacteria (or other organisms). Much of our knowledge is still based on homology models of proteins derived from gene sequences: although homology modeling based on gene sequences is a valid approach to give an indication of structural parameters and, for example, to indicate what useful site-directed mutants might be constructed, it gives only limited information that is based largely on the accuracy of the computer-based predictions of protein folding. This point is evidenced by a comparison of the data obtained from the homology modeling

and direct X-ray structural data of cold-active citrate synthase.

A general fact that has become apparent from such comparisons is that those structural modifications that confer cold activity are not merely the opposite of those which give thermostability: for example, in triose-phosphate isomerase the thermophilic enzyme has modifications to the body of α-helices that stabilize the helix dipole, whereas the psychrophilic enzyme has altered helix-capping residues (Rentier-Delrue et al. 1993). Another feature to become apparent from the homology models is that among the various cold-active enzymes different structural features are used to achieve the same mechanistic goal of maintaining structural flexibility and catalytic activity at low temperatures (Russell and Hamamoto 1997; Feller et al. 1996b; Marshall 1997; Gerday et al. 1997; Russell 1998), which is reminiscent of the different strategies used by thermostable proteins from thermophiles. These structural variations are discussed in more detail below.

General aspects of cold-active enzyme structure and activity

As outlined by Feller et al. (1996b), in comparing cold-active (psychrophilic) enzymes with their mesophilic (or thermophilic) counterparts, we need to be able to explain the following:

- The downward shift in their apparent optimum temperature for activity
- Their higher specific activity (k_{cat}) or physiological efficiency (k_{cat}/K_m) at 0°–30°C
- Their thermolability at moderate temperatures

Despite the fact that proteins are stabilized by the combined effect of a great many, largely noncovalent (and therefore weak), chemical bonds, they have a low energy of stabilization, 30–65 kJ/mole, equivalent to only a few such bonds. Moreover, as discussed by Gerday and co-workers (1997), the stability of the native state is a balance between enthalpy and entropy terms, so that the gain in entropy of unfolding can influence the energy of stabilization. This also means that within a given protein structure there will be regions of differing stability and a relatively unstable region can be sustained by stabilizing forces elsewhere – indeed, it may generate forces that are important in terms of catalytic activity. Because stability and rigidity are closely linked in thermostable proteins, with a decreased amplitude of molecular movement compared to mesophilic counterparts (see Gerday et al. 1997 for references), it might be expected that the reverse is true of psychrophilic proteins. However, taking into account these arguments, a decrease in flexibility may not be reflected in the overall topology of the protein, and its general three-dimensional structure may be very similar to mesophilic (and thermophilic) counterparts. The importance of intramolecular hydrophobic bonding, often largely within the secondary folding structures of α-helices and β-sheets, for the stability of proteins within thermophiles and mesophiles is also reflected in those from psychrophiles in which homologous enzymes have the same groundwork secondary structure despite the requirement for greater conformational flexibility of regions involved in catalytic activitiy and allosteric regulation. It appears that in all proteins, whatever their activity temperature range, it is hydrophobic interactions (together with the accompanying entropically-driven ordering of solvating water) that play a pivotal role in protein stability (Franks 1995). Given these facts, it is hardly surprising that early comparisons of mesophilic protein overall amino acid content and even sequence revealed little about thermophily, and this approach has not been seriously considered for psychrophilic proteins.

The decrease in flexibility of cold-active proteins can arise from changes in intramolecular bonds, interactions with solvent (water and its associated ions), intersubunit interactions, and compactness of core, i.e., size and distribution of cavities.

Hydrogen bonds and electrostatic interactions are formed exothermically, so they are stronger at low temperatures. In contrast, hydrophobic bonds are formed endothermically and will be weaker at low temperatures. These general considerations are reflected in more specific terms by the changes in frequency of particular molecular bonds and amino acid side chains in cold-active proteins from psychrophiles, which include the following:

- More polar and less hydrophobic residues
- Additional glycine residues and low arginine/lysine ratio
- Fewer hydrogen bonds, aromatic interactions, and ion pairs
- Lack of salt bridges
- Additional surface loop(s) with increased polar residues and/or decreased proline content
- Modified α-helix dipole interactions
- Reduced hydrophobic interactions between subunits

No cold-active enzyme displays all these features: each has a suite of changes (relative to mesophilic/thermophilic counterparts) that confer the necessary conformational flexibility to the active site, but at the expense of activity at higher temperatures. Therefore, cold-active enzymes are generally more thermolabile, although there are examples of enzymes from psychrophiles with remarkably wide thermal ranges of activity.

These general points are now illustrated by a more detailed consideration of the molecular adaptations in some selected cold-active enzymes, chosen on the basis that they are the psychrophilic proteins about which we have the best understanding at present.

Lactate dehydrogenase

Lactate dehydrogenase from *Bacillus psychrosaccharolyticus* was the first gene of a cold-active enzyme to be cloned and compared to homologous gene sequences from mesophilic and thermophilic bacilli (Vckovski et al. 1990), chosen in an attempt to minimize genetic (evolutionary)

differences in structure that could confuse the analysis of true thermal effects. The psychrophilic enzyme had already been purified and sequenced (Schlatter et al. 1987), and specific matrix analysis of the enzymes from the different bacilli showed that cold activity was associated with the presence of more polar and charged residues but fewer hydrophobic and ion-pairing residues, changes known to impart increased flexibility to proteins (Jaenicke 1991).

This seminal study formed the benchmark for subsequent comparisons of cold-active proteins with those from mesophiles and thermophiles, but unfortunately it was not followed up by a crystallographic study. Recently, we have been studying the regulation of cold-active L-lactate dehydrogenase (LDH) from some Antarctic psychrophilic bacteria by the allosteric regulator fructose 1,6-diphosphate, which not only activates the enzymes but shifts their apparent optimum temperature for activity upward by 15–20 centigrade degrees and renders them more thermostable on the basis of increased activity half-lives (J. Diez Aguirre and N.J. Russell, unpublished data). By analogy with data on comparable NAD^+-linked LDH from *Bacillus stearothermophilus*, this activation and thermostabilization may be the result of conversion of the enzyme from a dimeric to tetrameric form (Clarke et al. 1986). The gene from the psychrophilic *Bacillus* sp. C2-1 has been cloned, and it will be interesting to compare subsequently the crystal structures of the enzyme with and without its regulator bound to the protein, as this should reveal thermal properties about the active site conformation.

Citrate synthase

The cold-active, thermolabile citrate synthase enzymes have been assayed from a number of psychrophilic and psychrotolerant Antarctic bacteria: they have apparent temperature optima for activity ranging from 17°C to 45°C (Gerike et al. 1997; N.J. Russell, unpublished results), despite the fact that all the bacteria were capable of growing at or close to zero. The citrate synthase gene from *Arthrobacter* sp. DS2-3R was cloned and sequenced, and a homology model derived on the basis of a comparison of its deduced amino acid sequence with the enzymes from the mesophilic bacterium *Mycobacterium smegmatis*, the thermophilic archaeon *Thermoplasma acidophilum*, and the hyperthermophilic archaeon *Pyrococcus furiosus* (Gerike et al. 1997). The enzyme from *Arthrobacter* sp. DS2-3R showed greatest homology with citrate synthases from other Gram-positive bacteria, including *M. smegmatis* (59.9% amino acid sequence identity). Crystal structures were available for the *P. furiosus* and pig enzymes, which were used to identify the secondary structure α-helices, as well as the catalytic residues and the binding sites for CoA-SH and citrate.

The most obvious feature of the cold-active citrate synthase is the presence of an additional surface loop, compared with the mesophilic *M. smegmatis* enzyme. This loop contains nine amino acids of which six are charged; in addition, another surface loop, which is present in both enzymes, has nine substitutions of neutral with charged amino acids, thus further enhancing the surface interaction with solvent and thereby increasing enzyme flexibility. However, overall there are not significantly more charged residues in the enzyme from the psychrophile compared with either the mesophile or the thermophile. The distribution of proline residues within the surface loops of the two enzymes differs, but nothing could be deduced about their significance from the gene homology modeling. Other changes such as increases in the number of isoleucine residues or changes in arginine/(arginine + lysine) ratio, which have been associated with changes in thermostability, are not seen in the *Arthrobacter* sp. DS2-3R enzyme.

This example illustrates well the somewhat limited structural data that can be obtained from gene homology models, particularly when compared directly with those obtained by XRD of protein crystals. The citrate synthase of *Arthrobacter* sp. DS2-3R was expressed in a citrase synthase-deficient strain of *E. coli* and purified to homogeneity (Gerike et al. 1997). Unlike most other cold-active enzymes it proved relatively easy to crystallize, and good crystals were obtained that allowed structural determination to a resolution of 2.09 Å (Gerike et al. 1998; Russell et al. 1998). Because the crystal structure of the *M. smegmatis* enzyme was not available, the *Arthrobacter* sp. DS2-3R enzyme was compared with that from *P. furiosus*, with which it shares 39.5% amino acid sequence identity (Russell et al. 1998). A comparison of the information obtained from the homology model and the crystal structure analysis is given in Table 3. Even though the two comparisons are with citrate synthase from different organisms, it is clear that much less precise information is obtained from the homology modeling exercise.

The crystal structure showed that the overall structures of the psychrophilic and thermophilic enzymes are similar: both are dimers with comparable α-helical arrangements in the large and small subunits. Structural comparisons can be considered in relation to the active site, the surface loop regions, the subunit interfaces, and the solvent-exposed surface. Changes in these structural features appear to combine in achieving an increased catalytic rate at low temperatures.

The active site of the psychrophilic enzyme has a larger entrance that is more accessible due to the shortening of a loop region on one side and the creation of more space by the substitution of a bulky arginine reside with alanine. The overall strongly basic nature of the active site is retained, but the surface surrounding the entrance is more negatively charged, which could help to force approaching substrates into the pocket. The catalytic triad of an aspartate and two histidine residues is conserved, but the number of binding sites for CoA-SH are less and are located on a single monomer, probably contributing to the lower affinity of the cold-active enzyme for this substrate.

Several enzymes from psychrophiles are known to have additional or longer surface loops containing a preponderance of charged residues, which has been proposed as a mechanism for increasing flexibility and therefore cold ac-

Table 3. A comparison of the distinctive structural features of cold-active citrate synthase from psychrophilic *Arthrobacter* sp. DS2-3R from a gene homology model and the protein crystal structure[a]

Structural feature	Homology model	Protein crystal structure
Loop regions	Extended hydrophilic loop	Several loops longer
	Increased charge	Increased charge
	Altered distribution of prolines	More prolines
Surface	More charge (?)	More exposure of hydrophobic residues to solvating water
Active site	No information	Larger entrance/shortened loop
		Favorable charge distribution
		Fewer large residues
Subunit interfaces	No information	Smaller area
		Fewer ion pairs/reduced network
		No isoleucine clusters

[a] Information compiled from Gerike et al. (1997) and Russell et al. (1998); the gene homology model is based on comparison with the mesophile *Mycobacterium smegmatis*, whereas the protein crystal structure is compared with that from the thermophile *Pyrococcus furiosus*

tivity (Davail et al. 1994; Feller et al. 1996b). The citrate synthase of *Arthrobacter* DS2-3R has two longer and one shorter surface loops compared with the *P. furiosus* enzyme, affecting mainly the small subunit; the largest addition is particularly flexible but is not involved in crystal contacts. Whether these changes really do stimulate catalytic efficiency at low temperatures by making the small subunit more flexible remains to be determined, but this hypothesis is supported by the fact that its average B-chain factor is higher in the cold-active enzyme (Russell et al. 1998).

The small–large subunit interface of both enzymes is formed by four antiparallel pairs of α-helices, plus contacts between residues in the C- and N-termini. However, in the psychrophilic enzyme there is a reduction in the extent of the ion-pair network, with fewer intersubunit ion pairs and a lack of isoleucine clusters, which are recognized features of thermostable enzymes from hyperthermophiles. The C-terminus of the cold-active enzyme is six residues shorter, leading to loss of a key intersubunit ion pair and reducing the extent of the subunit interface. Overall the surface of the cold-active citrate synthase is more hydrophobic, which destabilizes the enzyme by means of entropy-driven ordering of the surrounding solvent water molecules, further contributing to low-temperature activity.

Thus, in summary, there are multiple and complex interacting structural adaptations in the cold-active enzyme, which are believed to contribute toward its cold activity. Further understanding of their individual contributions will come from investigations of site-directed mutants and measurements of molecular dynamics.

α-Amylase

The α-amylase secreted by the Antarctic bacterium *Alteromonas haloplanctis* was the first cold-active enzyme to be crystallized (Aghajari et al. 1996), and its native structure has been compared by X-ray diffraction at 2.0 Å resolution with the pancreatic α-amylase of the pig (Aghajari et al. 1998), with which it shares 66% amino acid sequence identity – a value greater, in fact, than with any other microbial α-amylase. This comparison confirmed that the bacterial enzyme has the classical three-domain structure of other α-amylases, comprising an $(\alpha/\beta)_8$ barrel in the major domain A, a small β-pleated domain B, and a globular domain C made up of eight β-strands, a structural arrangement that had been deduced previously using the gene homology modeling approach (Feller et al. 1994).

Table 4. Physiological efficiency (k_{cat}/K_m) of mesophilic and psychrophilic subtilisins

Assay temperature (°C)	5	15	25	35	45
Mesophile	3.0	2.8	3.5	5.4	5.9
Psychrophile (wild type)	1.2	3.0	3.1	3.6	4.8
Psychrophile (mutant[a])	4.5	7.7	11.1	12.0	10.4

This table is derived from data given in Table 2 of Feller et al. (1996b)
[a] See text for explanation

The apparent temperature optimum of the psychrophilic α-amylase is approximately 30°C lower than that of the (mesophilic) pig enzyme, and at 4° and 25°C its catalytic efficiency (k_{cat}/K_m) is 6.6 and 3.7 times greater. The enzymes have the same catalytic triad of two aspartyl and one glutamyl residues in the active site, although the orientation of the side chain of one of the aspartyl residues is the opposite of that in the pig enzyme (Aghajari et al. 1998). In addition, several aromatic residues at the active site are conserved, and function in substrate recognition and binding. Both enzymes require a chloride ion for activity, the anion acting as an allosteric activator; however, in contrast to the porcine enzyme, the thermostability of the cold-active bacterial enzyme is not increased by the chloride ion, which is bound with tenfold less affinity (Feller et al. 1996a). Overall, the 24 residues most intimately involved in forming the active site cleft are strictly conserved in the cold-active α-amylase compared with the porcine enzyme (Aghajari et al. 1998). The question remains: "Why is the psychrophilic enzyme more catalytically efficient at low temperatures?" The twin approaches of gene homology modeling and crys-

tal structure determination reveal a number of features that favor more conformational flexibility of the cold-active enzyme:

- Reduced number of salt bridges
- One less disulfide bond
- Fewer arginine-mediated hydrogen bonds
- Fewer proline residues in surface loops
- Reduced hydrophobicity of the protein core

Triose-phosphate isomerase

The triose-phosphate isomerase (TIM) from the psychrophilic bacterium *Vibrio marinus* has been purified and crystallized as complexes with sulphate or 2-phosphoglycolate (a substrate analogue) and the structures at a resolution of 2.7 Å, described by Alvarez et al. (1998). Like other TIM enzymes, the cold-active enzyme from *V. marinus* does not require cofactors or metal ions; it is not regulated allosterically, although it is dimeric, and reaction rates are controlled by diffusion. The enzyme normally interconverts the glycolytic intermediates dihydroxyacetaone phosphate and D-glyceraldehyde-3-phosphate. The mechanism involves the binding of the phosphate moiety of the substrate to loop 8, which comprises a so-called 3/10 helix whose backbone —NH groups hydrogen bond to the phosphate oxygens, holding the substrate in place for catalysis to occur.

Comparison of the crystal structure of the *V. marinus* enzyme with that from mesophilic and thermophilic bacteria, and from other organisms, revealed that a difference was the replacement of the normally conserved serine-238 in the phosphate-binding helix by an alanine. The *V. marinus* enzyme has a high catalytic efficiency (2.2×10^5) but is thermolabile ($t_{1/2}$ = 10 min) at 25°C (Alvarez et al. 1998). The key role of this residue change in both catalytic efficiency and thermolability was demonstrated by constructing a site-directed mutant in which the serine-238 was restored to an alanine. The mutant had a 3.7-fold-lower catalytic efficiency and a 2.7-fold-greater thermostability than the wild-type enzyme. Such serine–alanine exchanges in other proteins usually do not generate such big differences in structural stability. However, in the case of the cold-active TIM, the change generates two additional (stabilizing) hydrogen bonds because of the unique positioning of the βC-atom of alanine-238 within the protein interior. Loop 8 is rather rigid in all TIM enzymes, so there is little overall structural difference between the cold-active enzyme and its homologue in the mesophilic *E. coli*. This example illustrates the subtlety of the changes that may be responsible for both thermolability and catalytic efficiency at low temperatures.

Malate dehydrogenase

The crystal structures of the cold-active malate dehydrogenase (MDH) from the psychrophile *Aquaspirillium arcticum* has recently been described as the apo-enzyme, as a binary complex with NADH, and as a ternary complex with NAD^+ plus oxaloacetate (Kim et al. 1999). The structures were compared with MDH from the mesophilic *E. coli* and the thermophilic *Thermus aquaticus*. The enzyme displays the usual property of high catalytic efficiency at low temperatures, and even at 37°C it is still 1.25 times more efficient than the enzyme from *E. coli*.

Compared with the *T. aquaticus* enzyme, the oxaloacetate-binding site in the MDH from *A. arcticum* enzyme is conserved apart from the substitution of alanine-227 with glycine. This structure could provide additional flexibility in the substrate-binding site because the backbone of glycine has more conformational freedom than that of any other amino acid. Comparing the B-factors for the whole molecule revealed that the cold-active enzyme is more conformationally flexible, including the regions close to the binding sites for the substrates NADH and oxaloacetate. This result is comparable to that obtained with citrate synthase by Russell et al. (1998), as discussed earlier. A possibly greater contribution to the greater catalytic efficiency of the MDH at low temperature is the substitution of glutamine-228 with lysine, which increases the positive charge in the region of the oxaloacetate-binding site, possibly drawing in the substrate more efficiently. Comparable changes around the NADH-binding site also alter the charge distribution favorably.

The greater thermolability of the cold-active MDH appears to arise from the fact that it has only about half the number of intersubunit ion pairs compared to the enzyme from the thermophile. Other potential contributory factors, such as intrasubunit ion pairs or the number and distribution of glycine and proline residues, are essentially the same in the enzymes from the psychrophile and thermophile. The importance of intersubunit interactions is consistent with the finding that thermostability of enzymes from thermophiles is often linked to increased interactions between subunits.

Closing comments

Besides the enzymes listed in Table 2, several others have recently been crystallized, including a Zn^+ metalloproteinase (C. Gerday and R. Haser, personal communication) and glutamate dehydrogenase (L. Camardella and D. Rice, personal communication). The direct structural data from X-ray diffraction of four enzymes already have provided information that both corroborates and builds upon that derived from models based on gene sequences. For example, the crystal structure of citrate synthase reveals that there are indeed weaker subunit interactions but also that there is increased surface exposure of hydrophobic residues and a modified structure to the entrance of the active site, neither of which could be predicted from the gene homology model (Gerike et al. 1997; Russell et al. 1998). This experimental approach is being supported by site-directed mutagenesis studies, and Narinx et al. (1997)

have produced mutants of subtilisin with improved catalytic efficiency, not only at low but also at moderate temperatures. This result has particular significance for the exploitation of cold-active enzymes in novel biotechnological applications (Russell 1998). Taguchi et al. (1998) have used "evolutionary engineering" to double the activity of a cold-active proteinase at 10°C by using sequential random mutagenesis and a screening program. However, a much better understanding of the structural determinants of cold activity is necessary before such engineering can be done in a rational manner, and simple assumptions based on knowledge of thermostable enzymes may be misleading. For example, the cold-active citrate synthase of *Arthrobacter* sp. DS2-3R is, unexpectedly, less flexible than the thermostable counterpart of *P. furiosus*. However, there is a larger inequality between the flexibility of the large and small subunits in the psychrophilic enzyme, which is not the result of crystal contacts, and it has been postulated that this might help to drive substrate-triggered conformational changes, thereby enhancing enzyme activitiy at low temperatures (Russell et al. 1998).

This observation calls into question the general statement that, compared with their mesophilic and thermophilic counterparts, psychrophilic enzymes are intrinsically more flexible. Molecular dynamics simulation of psychrophilic and mesophilic trypsins revealed little difference in their conformational flexibilities (Heimstad et al. 1995). Such molecular dynamics measurements are necessary because even when crystal structures are available it may not be possible to determine what structural features are responsible for cold activity. Moreover, there may be apparently conflicting structural indicators. For example, in the *Arthrobacter* citrate synthase there are additional proline residues in two internal α-helices and fewer prolines in surface loops, compared with the *Pyrococcus* enzyme, both changes that would increase overall flexibility. In contrast, the distribution of glycine residues in the two enzymes indicates the opposite effect.

Hence, the title of this review, "*Toward* a molecular understanding.…" The recent upsurge of interest in cold-active enzymes, the coordination of European research in this field, and the rapid increase in number of publications has resulted in a much improved understanding of cold activity. However, much remains to be done, and no doubt more surprises await researchers to show how Nature has kept pace with a cooling world in the evolution of enzymes capable of working efficiently at the low temperatures that cover most of our planet today.

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