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REVIEW ARTICLE

Discussing the roles of proline and glycine from the perspective of cold adaptation in lipases and cellulases

Ahmet Melih Öten[a], Evren Atak[b], Banu Taktak Karaca[c], Sinem Fırtına[d] and Aslı Kutlu[d]

[a]Biology Education Center, Faculty of Science and Technology, Uppsala University, Uppsala, Sweden; [b]Bioinformatics and System Biology, Bioengineering Department, Gebze Technical University, Kocaeli, Turkey; [c]Molecular Biology & Genetics Department, Faculty of Natural Science and Engineering, Atlas University, Istanbul, Turkey; [d]Bioinformatics & Genetics, Faculty of Natural Science and Engineering, İstinye University, Istanbul, Turkey

ABSTRACT

Lipases and cellulases have been extensively used in the field of biotechnology for varied purposes. Mainly, the ongoing researches focus on the improvements of kinetic and enzymatic characteristics of enzymes to meet industrial needs. With the discovery of psychrophilic cellulase and lipase sources, a new era has opened for protein research by allowing the discovery of novel functions together with the description of unique cold adaptation mechanisms to harsh environments. The ability of cold-adapted lipase and cellulases to enable chemical reactions at lower temperatures provides a great opportunity to cut the cost of the finished product by lowering energy and purifying expanses. The advances in the cold-adapted lipase and cellulase enzymes are the cumulative efforts of organic chemists, biophysicists, biotechnologists, and process engineers who greatly contribute to a better understanding of cold-adaptation phenomena from different points of view. In this review, we cover the cold-adaptation aspects of cellulase and lipase enzymes from structural points of view by referring to the roles of Gly and Pro residues. Gly and Pro residues accelerate the cold-adaptation of enzymes by altering the conformational changes in the 3 D structure of proteins The list of microorganisms as a source of cold-adapted cellulases and lipases is given by referring to biotechnological applications. After introducing the thermodynamic background of Gly and Pro residues in the phenomena of cold-adaptation, specific examples are given to emphasise how introducing Gly and Pro into 3 D structure of protein molecules adds value in terms of biotechnological application by contributing to cold-adaptation.

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1. The roles of proline and glycine in protein stability

Protein stability issues

The protein expression adapted to function under extreme conditions of pH, temperature, pressure, or ionic strength has been vital to the survival of organisms. The adapted model as a protein to such variables are a promising resource for commercial biotechnological applications. Temperature is a particularly significant influence on protein adaptation, as a wide variety of biochemical and physiological processes depend upon temperature. Enzymes from species that live in low-temperature environments are less stable than those in organisms living in high-temperature environments. In most cases, decreased stability causes increased conformational flexibility to maintain the stability–flexibility balance in enzymes. Thus, enzyme adaptation to temperature should involve amino acid substitution in either stabilisation (in the case of adaptation to higher temperatures) or destabilisation (in the case of adaptation to lower temperatures) in domains of the catalytic enzyme molecule.

Much of the interest in extremophilic enzymes comes from understanding that cold-adapted enzymes have the potential to be employed in various biotechnological and industrial processes The use of cold-adapted enzymes in reduced-temperature applications enables energy saving by decreasing the reaction temperature without inhibiting enzyme activity. For instance, cellulases and lipases, also known as detergent substitutes, perform the digestion of industrial wastes at low temperatures by allowing for cold wash cycles at 30 °C. (Yuan et al. 2014; Ma et al. 2018). They are two of the most sensitive enzymes to temperature (Fatima et al. 2021). Structural studies of these

CONTACT Aslı Kutlu asli.kutlu@istinye.edu.tr Bioinformatics & Genetics, Faculty of Natural Science and Engineering, İstinye University, 34010 Istanbul, Turkey

enzymes have demonstrated that the functionalities of the enzymes are most responsive to temperature changes, and amino acid substitutions have the potential to lead the functional alterations (Choo et al. 1998; Castiglione et al. 2017). According to these studies, substrate-binding affinity together with the catalytic rate is a key parameter during temperature adaptation. As a result of cold adaptation, the affinity of the enzyme towards its substrate is reduced and it allows for accelerated catalysis (Florczak et al. 2013; Miller 2020). Typically, the functional changes are the result of a single amino acid replacement in an enzyme subunit. The sequence changes occur at surface-exposed regions of the enzyme far from the substrate-binding site, leaving the enzyme's active site structurally unaltered. Such amino acid substitution reduces the stability of cold-adapted forms by modifying intramolecular hydrogen bonding patterns or solvent interactions. For example, the replacement of a Serine (Ser) by a Glycine (Gly) residue in A4-lactate dehydrogenase (A4-LDH) has the potential to increase the conformational flexibility, reducing substrate affinity but accelerating catalysis (Fields and Houseman 2004). The addition of Gly residue in the cold-adaptive model improves the flexibility of the peptide backbone, permitting catalytically required conformational changes. This study enables us to conclude that substrate affinity appears to be a universal target of temperature adaptation in proteins, with more cold-adapted enzymes exhibiting lower substrate affinities (higher K_m values) at a common temperature.

During evolution, cold-adapted proteins sacrificed weak interactions to achieve the structural flexibility required for catalytic activity at low temperatures. As a result, cold-adapted enzymes are involved in a lowered number of salt bridges, altered numbers of proline and glycine residues, reduced cation-pi interactions and increased number of non-polar interactions between the enzyme surface and the solvent. All types of weak interactions have played an important role in proper protein folding and stability. Increased conformational flexibility of cold-adapted proteins produces a more diverse distribution of conformational states, which translates into reduced substrate affinity for enzymes to adapt thermal attributes within the active site (Fields and Houseman 2004). Proline and glycine both had the greatest destabilising effects on model helices as assessed in detergent micelles (Ota et al. 2000; Dong et al. 2012). Proline, a small amino acid, can create persistent kinks within the helix structure, allowing helix flexibility during bending and rotational motions. Glycine contains only hydrogen as its chain. This conformation allows residues more flexibility together with the property of being achiral, and the lack of ability to be involved in intramolecular interactions promotes the destabilisation of helix structures.

Increasing the molecule's rigidity in the partial or overall manner by introducing weak interactions to the enzyme should reduce Km values by decreasing the conformational entropy of the binding site. Moreover, temperature-adaptive increases in rates of enzyme catalysis occur concomitantly with decreases in substrate binding affinity (Chakravarty and Varadarajan 2002; Chakravorty et al. 2017; Sen and Sarkar 2022). Comparing the crystal structure of the cold-adapted enzyme with the crystal structure of its thermophilic counterpart supports the assumption that cold-adapted enzymes have a more flexible three-dimensional (3 D) structure than their thermophilic counterparts. Also, comparing the amino acid compositions of the cold-adapted enzyme and its thermophilic orthologs reveals that the thermophilic ortholog contains a greater percentage of charged residues, whereas the cold-adapted enzyme contains a greater number of hydrophobic amino acids (Imai and Mitaku 2005; Stark et al. 2022).

This review intends to contribute to a better understanding of the structural characteristics of cold-adapted enzymes, particularly lipases and cellulases so that they can be genetically engineered for gaining catalytic functions at or near extreme temperatures. This expanded functioning will be useful in a variety of biotechnological and industrial processes in terms of saving energy.

1.1. General characteristics of glycine

The smallest amino acid, Glycine (Gly), is defined with several characteristics such a being a non-essential, non-polar, non-optical and glucogenic amino acid (Berg et al. 2002). It provides flexibility in the secondary structure of proteins and is involved in helix formation. Where it appears in recognition sites on the cell membrane and enzymes it serves as an osmo-protectant and an extracellular signalling molecule that modifies molecular activity via conjugation and Gly extension of hormone precursors (Hall 1998). In the brainstem and spinal cord, Gly is known as the major inhibitory neurotransmitter, and it contributes to various motor and sensory functions (Maciel et al. 2022; Sravya et al. 2022). Gly is working as a co-agonist of the N-methyl D-aspartate subtype of glutamate receptor in the forebrain by promoting the actions of

glutamate. Thus, it serves both inhibitory and excitatory functions within the CNS (central nervous system) (Rousseaux 2008). Gly is naturally synthesised from Serine (Ser), another proteinogenic amino acid naturally produced within cells (Boyle 2005). Herein, the side-chain methylene group of Ser is transferred to tetrahydrofolate, catalysed by Ser transhydroxymethylase enzyme, another pyridoxal-5′-phosphate (PLP) enzyme homologous to aspartate aminotransferase. There is a formation of Schiff base between Ser and PLP, and it labilizes the bond between C_α and C_β atoms of Ser. Then, the side-chain methylene group of Ser is then transferred to tetrahydrofolate. The sulphur atom derived from methionine is used to further the conversion of serine into cysteine for the side-chain oxygen atom (Aurora and Rose 1998).

Because its side chain is composed of only one hydrogen atom, Glycine possesses high conformational flexibility, permitting it to enter spaces others cannot. Generally, a small amount of Gly is incorporated into several proteins. Collagen is one of the exceptions by containing 35% of periodically repeated Gly residues within whole content (Szpak 2011). It is reported that there are individual structural superfamilies such that the large fractions of Gly residues are conserved and played a unique role in the folding patterns of proteins belonging to those superfamilies (Gutfreund 1993; Guo et al. 2003). Due to the small size and minimal steric hindrance within side chain, there is a wide range of conformations that Gly can adopt, and it is a unique property for Gly that are not able to be observed in other naturally occurring amino acids. Gly is also unique in such distinct functional capabilities as side chain backbone binding to phosphates; a conserved Gly substitution will probably bring protein scaffold structure associated with alterations in protein function.

Alamethicin studies specify the role of Gly in protein flexibility. Alamethicin is a voltage-gated channel-forming peptide consisting of a helical 20-amino acid exhibiting segmental flexibility in solution. This flexibility originates along alamethicin's backbone near methyl alanine (MeA)-10 and Gly-11. The presence of MeA at 10^{th} position involves H-bonding with residue at the 14^{th} position. However, the presence of Pro at this position prevents the formation of interhelical H-bonding. For revealing the further impact of Pro at the 14^{th} position on the flexibility of this helical conformation, two alamethicin mutants (only P14A & P14A + G11A) are synthesised. The results provide that the only P14A mutant displays similar C-terminal to C-protein distances. Hence, the only Pro substitution at the 14^{th} position does not alter the flexibility of this helix. For P14A and G11A mutant, there is a significant increase in the averaged distances between C- and N-termini since G-X-X-P motif is responsible to mediate the high-amplitude bending motion existing in the central helical domain of alamethicin in methanol (Jacob et al. 1999). Protein stability is not able to be explained structurally since motion, including bending, angle vibration, and rotation, plays a crucial role in structure and function. For example, the bending motion results in channel formation or any structural changes that alter protein stability dramatically.

1.2. General characteristics of proline

Proline (Pro) is known as the cyclic amino acid with a secondary amino group. It is an α-amino acid unique in that its backbone nitrogen atom is part of its carbon ring structure. Thus, its amino group acts as a secondary rather than a primary amine as being existed in the remaining amino acids. Because of its amide position, the backbone conformation of Pro itself is restricted, which means that available backbone dihedral angles are limited to a small range around $\theta = -65^\circ$, and its side chain is cyclized back to the backbone amide position. This dihedral angle makes Pro the most rigid of the amino acids.

The synthesis of Pro is done from glutamic acid and ornithine, and this reaction is catalysed by P5C (Δ^1-pyrroline-5-carboxylic reductase). During the reaction, Pyruvate carboxylase (PC) is existing as the intermediate of the reaction and is found in equilibrium with its noncyclic isomer glutamic-γ-semialdehyde, an intramolecular imine/cyclization reaction in a reversible manner. Indeed, proline is converted to PC by proline dehydrogenase which further has a role in the oxidation of the glutamic-γ-semialdehyde to glutamic acid. As a product of this reaction, there exists a small amount of ornithine formation from the glutamic-γ-semialdehyde catalysed by aminotransferase (Wang et al. 2013), see Figure 1.

This structural limitation has had considerable consequences, especially in the repeated presence of Pro. Due to bulky N-CH_2 groups, subsequent residues to Pro have restricted conformations, which adversely impacts -helix formation. With the replacement of amide proton by the CH_2 group, Pro is unable to act as a hydrogen bond donor. If Pro presents in the first turn of the helix, it fits well to the backbone conformation by bringing a crucial impact on protein in terms of stabilisation. A less loss in entropy with only one rotatable angle occurs compared to other amino acids

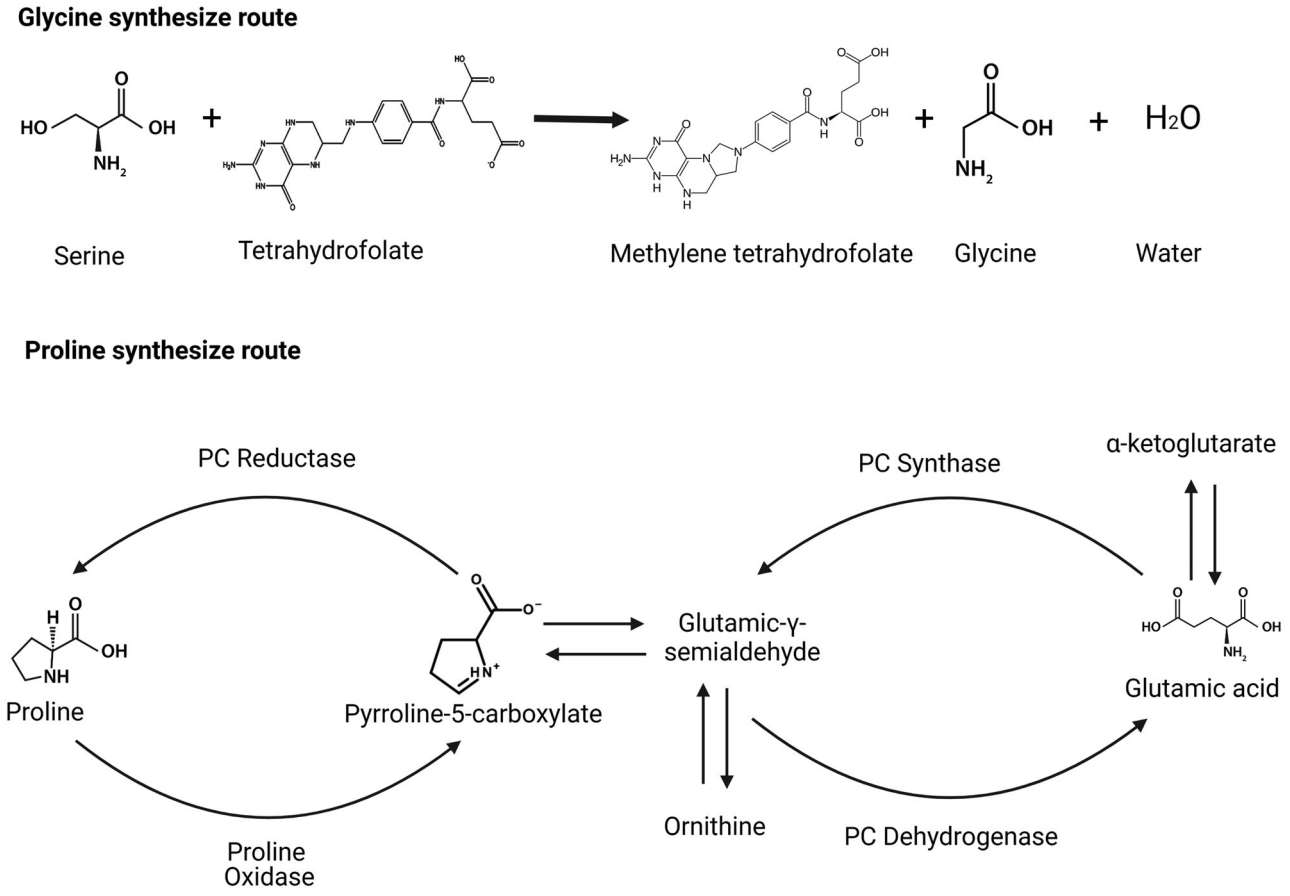

Figure 1. The details of Glycine and Proline synthesis reactions.

in terms of regular structure formation. In addition to that, Pro in the first turn of a helix could block the growth of the helix formation towards the NH2-terminal direction, since its ring produces steric interference with the necessary backbone positioning and also prevents H-bonding both from its own nitrogen that is covalently attached to the ring, and indirectly from at least one neighbouring -NH (Richardson and Richardson 1988). All these features make Pro called the "helix-breaker" (β-sheet breaker).

The formation of secondary structures is also affected by the exceptional conformational rigidity of Pro because of its cyclic pyrrolidine side chain. If the contribution of those residues to the thermal properties of enzymes is known, how their conformational changes are generated is of major interest for understanding the regulatory and recognition properties of proteins. There are several steric constraints on the dihedral angles, φ, and ψ, of a polypeptide backbone that limit its conformational range. Therefore, the secondary structure of any protein is recognised to mainly depend on the combined dihedral angles φ, ψ, and ω relative to the protein backbone. The cyclic structure of Pro and its φ angle value (around −65°) make Pro a structural disruptor amidst regular secondary elements such as α-helix and β-sheets by promoting the initiation and elongation of loop structures (Morris et al. 1992). Herein, it is also important to note that the R-side of Pro is completely aliphatic, and hence it cannot participate in intramolecular interactions which are the actual driving sources for protein folding. The following example demonstrates this feature of Pro. The presence of multiple Pro and/or hydroxyl Pro residues results in the formation of a polyproline helix, which is known as the predominant secondary structure of collagen. Due to the hydroxylation of Pro by prolyl hydroxylase or the addition of electron cancellation substituents, there exists an increase in the conformational stability of collagen in a significant manner (Szpak 2011).

Another study used molecular dynamic (MD) simulation, B-FITTER and framework rigidity-optimised dynamics algorithm (FRODA) to investigate Pro's effect on the rigidity of *Photinus pyralis* luciferase by introducing proline to make these flexible regions more rigid. The study designed two mutants, D476P and H489P, within the most flexible regions and found that H489P's thermostability improved as it

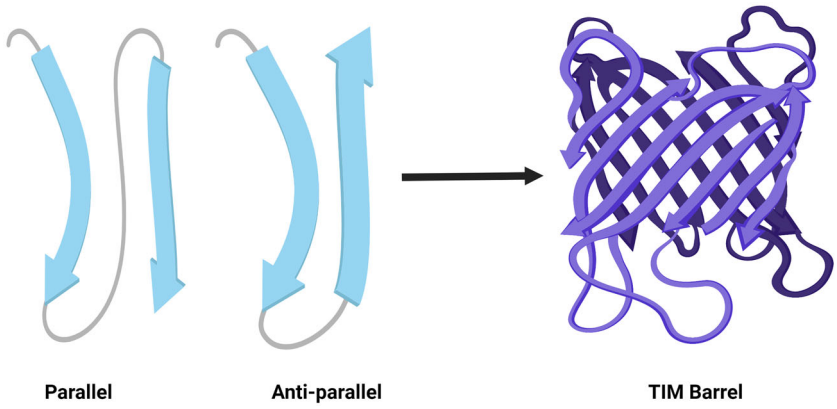

Figure 2. The formation of TIM barrels.

maintained its catalytic efficiency compared to that of WT luciferase. Whereas the overall and local rigidity of the H489P mutant was greatly strengthened, the decreased thermostability of mutant D476P was elucidated at the molecular level. Therefore, the S307P mutant was designed outside the flexible regions as a control. Thermostability analysis demonstrated the control model to have decreased kinetic stability and enhanced thermodynamic stability due to the Ser-Pro replacement (Yu et al. 2015).

1.3. The roles of glycine and proline in the formation of secondary structures

Possessing the highest conformational flexibility (Gly) and rigidity (Pro) are exceptional properties among naturally occurring amino acids. Allowed conformations of proteins are indicated by the Ramachandran diagram. Herein, the sterically allowed values of ψ and φ can be determined by calculating the distances between atoms of tripeptide values of ψ and φ for the central peptide backbone. Mostly in Ramachandran plots, Pro and Gly are known as an outlier due to their restricted ψ and φ angles. Specifically, in Pro, the ring connection to the β-carbon atom causes restriction by allowing fewer degrees of rotation. Contrary to Pro, additional conformation alternatives exist for Gly. Gly, the only residue without a β-carbon atom, is much less sterically hindered than other amino acid residues. These structural features of Pro and Gly are observed as deviations in Ramachandran plots. From the standpoint of thermodynamics, negligible entropy changes are required (ΔS) to initiate and elongate "turns," and the presence of Gly/Pro provides enough entropy changes for secondary structure destabilisation.

Gly is frequently found in locations where a polypeptide backbone makes a sharp turn, causing steric interference with other residues. The presence of Gly and Pro in turn creates one of the most important configurations in protein architecture, e.g. the TIM barrel (α/β barrel). In the single domain of the TIM barrel, there is an alternated pattern of α-helix and β-strand formations; basic βαβ building blocks are repeated eight times to form the TIM barrel (see Figure 2). The inherent stability and function of TIM barrels depend on the connections between αβ and βα loops, in which Gly and Pro are present. In TIM barrels, a solenoid curve closes on itself in a shape known as a toroid (Ewert and Deming 2013). It is believed that the toroid shape evolved in the RNA-protein world from duplication of a $(\beta\text{-}\alpha)_2$ unit, probably by ligation of two identical RNAs. TIM barrels catalyse different reactions (Hegyi and Gerstein 1999; Romero-Rivera et al. 2022) such as the interconversion of dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate (Knowles 1991), the synthesis of orotidine 5′-monophosphate (Miller et al. 2000), and others.

Generally, Gly is located at the N-termini of short αβ but not in βα loops (Huang et al. 2016). In the TIM barrel, βαβα [β1-loop(βα1)-α1-loop(αβ1)-β2-loop(βα2) - α2-loop(αβ2)] repeat unit forms a stable barrel fold, and the N-terminal residues of aβ loops 1 and 2 are Gly, see Figure 3. Gly residue present at the beginning of αβ loops permits them to serve as α-helix breaking signals, preventing the continuation of the α-helix through the loop region. In addition to preventing the continuation of the α-helix, the lack of side-chain Gly could also minimise the existence of steric interferences between residues in the α-helix and loop region. The increased Pro content observed in αβ loops is consistent with the occurrence of increased Pro residues (Watanabe et al. 1997; Bogin et al. 1998; Li et al. 1999). The pyrrolidine ring in Pro is limited in its configurations and restricts the backbone to interact with successive residues. Therefore, it possesses

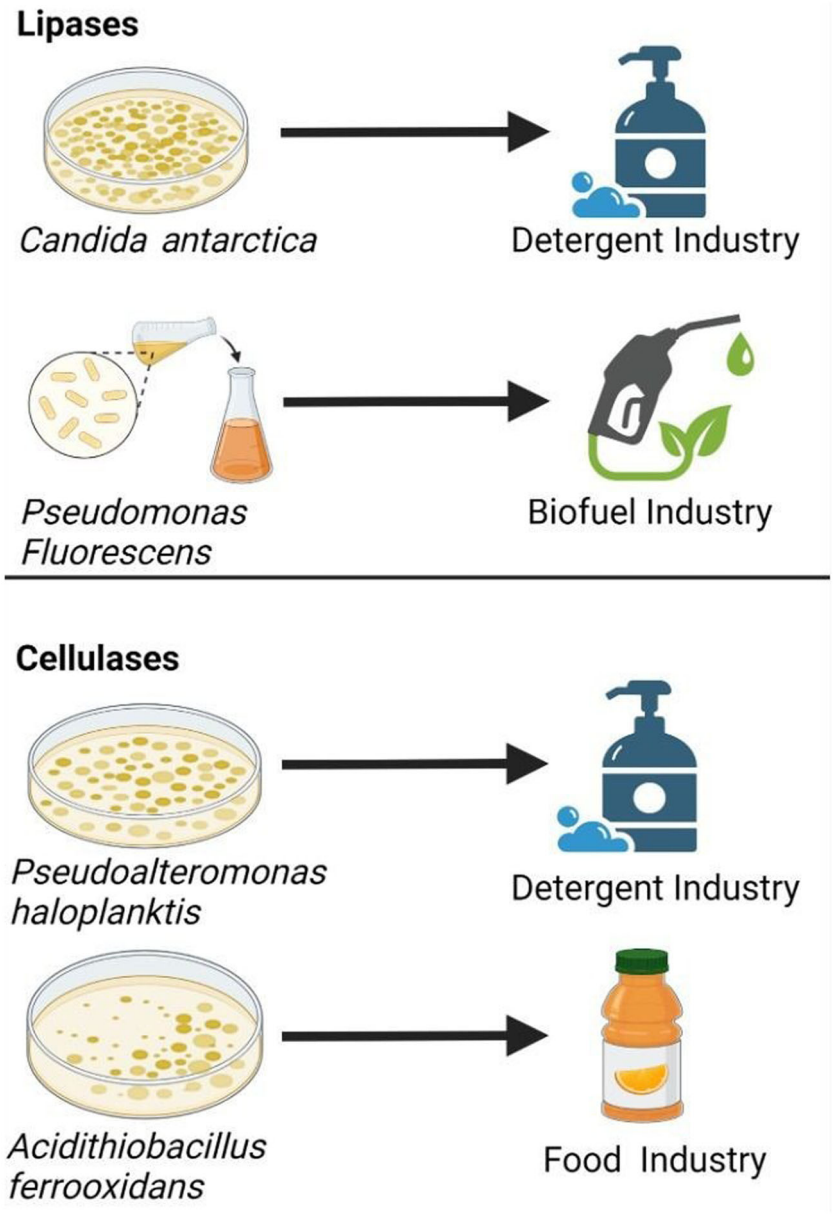

Figure 3. Examples of biotechnological applications carried out by cold-adapted lipases and cellulases.

the lowest conformational entropy by leading to a decrease in the entropy of the unfolded state (Matthews et al. 1987). As in line with thermodynamical fact, the substitution of non-Pro and non-Gly residues increases the stability of the folded state (Kimura et al. 1992; Hardy et al. 1993; Stites et al. 1994; Hegyi and Gerstein 1999; Takano et al. 2001; Trevino et al. 2007) and supports the preferential distribution of Gly and Pro residues in αβ loops (Kadumuri and Vadrevu 2018).

The several motifs presented at the end of peptide α-helices are known as the main contributors to the local conformation of polypeptide chains. The α-helix cap is one of the examples of these motifs by consisting of polar residues at the N-termini, where the α-helices form H-bonds with exposed amide protons (Aurora and Rose 1998), and these capping sequences are found in 5 out of 8 helices in TIM barrels. A second motif, the α-helix stop signal, consists of Gly and Pro residues at either end of α-helices, which are thought to prevent helical secondary structure propagation into the adjacent sequence (Gunasekaran et al. 1998). The presence of two Gly together with a single Pro residue in the TIM barrel falls into this category. It has been demonstrated that the presence of any mutation in each of two Gly and Pro residues results in a less than 10-fold reduction *in vivo* activity (Silverman et al. 2001). This highlights the relevance of Gly and Pro conservation in terms of thermodynamic stability, which is critical for protein activity.

There are also several examples in which Gly and Pro residues contribute to the destabilisation of β-sheet formation in the TIM barrels. Here, Gly and Pro residues are presumably able to block β-strand extension of terminal regions. It is stated that the presence of any mutation in four Gly residues located at C-terminals of TIM barrel β-strands reduces *in vivo* activity by more than 10-fold (Silverman et al. 2001). However, any mutation in the single Pro located at the C-terminal end of β-strand 6 has no measurable impact. All structural Pro residues in the TIM barrel seem capable of substitution. Gly β-stops appear to be more important than Pro in terms of secondary structure punctuation motifs in TIM barrel (Silverman et al. 2001).

1.4. Homology modelling problems regarding glycine and proline

Identifying protein structure is essential to developing a detailed functional understanding of proteins. Methods for determining the structure of proteins can be roughly classified into three categories: those based on various microscopy techniques, those based on scattering/diffraction and spectroscopy (such as X-ray and nuclear magnetic resonance (NMR) techniques), and those based on computational modelling. Experimental methods such as X-ray crystallography, NMR spectroscopy or Cryo-EM are used to identify protein structures existing under different conditions such as membrane proteins, intrinsically disordered proteins, and so on (Muhammed and Aki-Yalcin 2019). Each of these experimental approaches has been associated with several restrictions, e.g. size limitations for NMR, solvent limitations for X-ray spectroscopy, the required numbers of skilled technical people for solving encountered problems, the other case-specific problems as well. The advances in the field of sequencing and recombinant DNA technologies speed up the discovery of new sequences, and there exists a gap between a number of discovered sequences and revealed 3 D protein structures. Herein, the integration of computational power into the field of structural biology helps more to predict 3 D structures of protein molecules, which are essential to explain their functions. Mainly, two different strategies are employed to perform 3 D structure prediction of protein molecules; (1) template-based and (2) template-free approaches.

Comparing these two approaches, several pros and cons arise such that template-based approaches use the evolutionary information between query protein sequences and others existing in databases, and it has a significant contribution to the prediction process but template-free approaches are not. If the sequences are not similar or homolog enough, the template-free approach works better to perform 3 D structure prediction since the utilised force-fields behind template-free approaches are accurate enough to mimic the actual intramolecular interactions within protein molecules. If the query protein sequences share a certain level of similarity or homology with others existing in the database, 3 D structure prediction would be more cost-effective compared to alternative approaches. The algorithms behind the 3 D structure prediction tools are varied from one to another. Since it is not possible to consider all parameters describing the actual physiological state of protein molecules, several parameters are "included" or "excluded" while constructing the prediction algorithms (Xiang 2006). So, the existing prediction tools are employed in a case-specific manner (Kinch et al. 2021; Sisakht et al. 2022).

Especially, in the template-based approach, the sequence similarity and/or other empirical information about the query are significant to build the predicted protein model. Even Gly and Pro are reported as structural breakers; there is still a lack of complete understanding of their roles, and especially how both should be taken into consideration during 3 D structure prediction (Morris et al. 1992). The intramolecular interactions are primary factors for 3 D architecture, and various amino acid combinations can promote similar local interactions. Therefore, it is important to analyse how Gly and Pro interact intra-molecularly. In addition to the widespread characteristics of amino acids and their potential contributions to 3 D structure prediction, environmental factors play a significant role. Secondary structures appear to be more stable if they have a concept of neighbouring segments. For -helix and -sheet forms, the breakers in loop segments, notably Gly and Pro, have different levels of hydrophobicity and helical periodicity than these two types. Due to this, predicting secondary structures becomes more challenging when evaluating environmental effects (Zhang 2008). There may be a local or overall contribution to thermodynamic stability from Gly and Pro's existence, however, this is either ignored or underweighted in the development of prediction algorithms.

Gly, Pro, and the amphiphilicity index are all factors that can be used to accurately predict secondary structure breakers. To calculate the amphiphilicity index, one needs to know how much energy is required to transport the hydrophobic stem group from one surface to another, depending on the

attainable surface (Imai and Mitaku 2005). It varies according to the length and composition of the peptide chain. For instance, the polar side chains are carried at the first five residues while the weakly polar ones are carried at the last two residues. In terms of the amphiphilicity index, A-index and A′-index are used to denote strongly polar and weakly polar residues, respectively. These three evaluated properties result in similar structural features in proteins called secondary structure breakers: the peak amphiphilicity index is located between the aqueous phase and nonpolar regions and the segments on both sides of the amphiphilic segment are nonpolar parts of the protein, breaking the secondary structure. Yet; the experimental studies reveal that the clusters of amphiphilic residues acting as structural breakers are only 12% of all loop segments (Uchikoga et al. 2005). These data imply that Gly and Pro are more important than other amino acids when predicting structural breaks in protein 3 D architecture. There are three steps involved in analysis of the presence of secondary structure breakers during protein 3 D structure prediction. First, the potential breakers are numbered based on the existing peaks of the primary features; next, they are categorised based on their relative position in the secondary structure's termini. Thirdly, primary component analysis is utilised to compare a regional average of environmental factors and to establish a differentiation function for the secondary structure. Based on the cumulative results of this analysis, the decisions on secondary structure breakers are determined. Nonetheless, one difficulty remains unsolved: the actual position of the secondary structure breaks in the remaining parts of the tertiary structure. Though 66% of loop regions include the predicted breakers with up to 90% accuracy, the mechanism of secondary structure breakage in the remaining loop regions remains unclear. There is a plausible explanation for it either the secondary structure breakage is facilitated by the entire tertiary structure or perhaps an alternative method is used to predict the secondary structure. Furthermore, these potential explanations highlight the importance of noting Gly and Pro positions relative to their neighbours and including additional parameters into algorithms predicting the 3 D structure of proteins (Imai and Mitaku 2005). The study of protein structure is undergoing a revolution. In structural bioinformatics accurately modelling proteins has been one of the greatest challenges. There is a critical need for accurate computational approaches to address this challenge and allow large-scale structural bioinformatics applications. AlphaFold was recognised as a solution to the protein folding problem in 2020, a biannual challenge for research groups to verify the accuracy of their predictions against actual experimental evidence. Then, AlphaFold evolved as a DeepMind-developed artificial intelligence system that produces accurate predictions of a protein's structure based on its amino acid sequence (Jumper et al. 2021). Since only a small fraction of the known protein sequences are structurally determined, the need for a modelling method that can resolve structures with atomic accuracy is crucial. Now, AlphaFold is at the centre of the search for modelling proteins, and its already resolved most of the human proteins. This big step made by AlphaFold will speed up especially hypothesis-driven research in structural bioinformatics, but it also helped as a pioneer in understanding the importance of homology modelling by attracting attention to the field. With these recent developments, it is believed that for every known protein sequence, there will be either an experimentally determined or a high-quality homology modelled protein structure. Research in biotechnology related to drug discovery, molecular diagnostics and biological metabolism, will be accelerated as a result of this. Despite the lack of research on cold adaptive lipases or cellulases recently, scientists will have access to an increasing number of biotechnologically important cellulases and lipases. The research will start to be done with atomic accuracy and with more detailed protein models. In 2021, AlphaFold2 (AF2), a programme used to predict protein and peptide models, was combined with glycine (Ko and Lee 2021). In general, the predicted structures have very low RMSD scores; hence, they qualify as c high-quality homology models. İmportantly, more than fifty percent of the predicted models are close to native. This work revealed the potential for future studies into the role of glycine and proline as structural breakers. in the future. This also applies to the study of other biological systems, including cold adaptation. Moreover, the models predicted with high accuracy suggest that AF2 can be used to forecast and generate biotechnologically important cellulases and lipases without any prior information. This is vital since the majority of these key enzymes are active under extreme conditions and have been referred to as extremophiles previously in our review. As a result, AlphaFold ushered in a new era in homology modelling, and it is difficult to say yet what impact this new era will have on the other studies mentioned in this review. Hopefully, the breaking effect of proline and glycine on the protein structure and the cold adaptation mechanism will be studied in more detail. If these

developments occur, hypothesis-driven research in structural bioinformatics will become even more important. Thus, it will be easier to study enzymes that are active in extreme environments such as extremophiles, and the biotechnology industry will make greater use of these and comparable organisms.

2. Cold adaptation in lipases and cellulases

2.1. Cold-adapted enzymes

Previously, it has been mentioned that organisms have experienced changing life conditions that have necessitated adaptation during the evolutionary process. Psychrophilic microorganisms provide the first example, able to live at low temperatures between −20 and 10 °C and unable to grow at temperatures higher than 15 °C. To thrive at low temperatures, psychrophilic organisms possess enzymes that have a high specific activity at low temperatures for carrying out vital reactions and are collectively termed cold-adapted or cold-active enzymes. Psychrophilic enzymes display different features compared to their mesophilic and thermophilic counterparts A mesophilic organism grows best in moderate temperatures, with an optimum growth range of 20–45 °C (Willey et al. 2011). A thermophilic organism thrives at relatively high temperatures, between 41 °C and 122 °C (Madigan et al. 2006). Various secondary structures, structural features and molecular interactions have emerged among enzymes coming from those different sources to satisfy their functional requirements.

Mostly in protein engineering studies, we encounter the term 'thermal-stabilisation' referring to enzymatic activity. The common strategy is to improve the overall packing of whole protein structure via H-bonds, reducing the length of loop structures to positively contribute to overall packing, introducing the covalent interactions, and so on (Santiago et al. 2016). However, the functional requirements of all protein-engineering studies are not the same, and somehow there is a need to perform reverse engineering strategies to make protein molecules as cold active. Herein, the engineering strategies would be relied on comparing the mesophilic and thermophilic counterparts of psychrophilic enzymes to gain insight into how protein dynamics compensate for the negative effect of low temperature while avoiding catastrophic cold-induced unfolding events that impede proper function.

These distinct features of psychrophilic enzymes depend on the number of Pro residues in their primary sequences. Many psychrophilic enzymes tend to possess reduced Pro content compared to their mesophilic and thermophilic counterparts (Feller and Gerday 2003; Siddiqui and Cavicchioli 2006; Feller 2010). The reduction of loop Pro residues enhances the chain flexibility between secondary structures. Pro isomerisation is a rate-limiting step for the folding process of many proteins and reduced Pro in psychrophilic enzymes facilitates structural adaptation by lowering the activation energy. Thus, it is possible to alter the local mobility of protein structure by reducing Pro numbers. Indeed, the less organised conformational state of the protein improves local mobility, increasing control of enzyme aggregation (Berlemont et al. 2009). A comparison of psychrophilic enzymes and their mesophilic counterparts indicates that longer external loops with reduced Pro content result in less compact and stable proteins. As a result of this 3 D architecture, the catalytic site of psychrophilic enzymes together with surrounding residues displays improved structural flexibility and mobility (Feller and Gerday 2003; Feller 2007), enhancing the accessibility of substrates to the active site and possibly reducing catalytic energy costs (Feller 2010; 2013). Finally, high-resolution models of psychrophilic enzymes show an improved number and size of cavities on the surface of cold-adapted proteins compared to their mesophilic counterparts (Margolles et al. 2012). If we consider the fact that apparent cavities retain a high number of hydrophilic groups at the binding locations of a greater number of water molecules, it results in increasing enzyme flexibility by enhancing internal solvation (Margolles et al. 2012).

Arginine (Arg) and Lysine (Lys) contents of primary protein sequences are considered another distinct feature between psychrophilic and mesophilic enzymes. A reduced ratio of Arg to Lys exists in psychrophilic enzymes compared to mesophilic enzymes. This low ratio is associated with reduced hydrogen bonding and salt bridge formation (De Maayer et al. 2014). This lower ratio would increase conformational flexibility due to a lower number of intramolecular interactions, especially on the protein surface, conferring enhanced conformational flexibility (Kavitha 2016). Additionally, the reduced number of arginine residues diminishes the number of ion pairs and aromatic interactions compared to mesophilic enzymes, and hence it results in the lowered number of associated ions as cofactors (Feller 2013). Alternatively, the replacement of basic residues with Glutamine (Glu) or Asparagine (Asp) improves the conformational flexibility of the protein by altering the distribution of charged residues on its surface. Engineering applications employ these

Table 1. The types of CLPs come from different sources.

Organism	Environment	References
Halomonas sp. BRI 8	Antarctic habitat	Jadhav et al. 2013
Moritella sp. 2-5-10-1	Antarctic habitat	Wang et al. 2013
Pseudoalteromonas sp.	Antarctic marine	Lo Giudice et al. 2006
Pseudoalteromonas h. TAC125	Antarctic marine	de Pascale et al. 2008
Pseudomonas sp. AMS8	Antarctic soil	Ali et al. 2013
Psychrobacter sp. Ant300	Antarctic habitat	Kulakovaa et al. 2004
Psychrobacter sp. 7195	Antarctic habitat	Zhang et al. 2007
Psychrobacter sp. TA144	Antarctic marine	De Santi et al. 2010
Candida antarctica	Antarctic habitat	Siddiqui et al. 2005
Geomyces sp. P7	Antarctic habitat	Florczak et al. 2013
Pseudomonas antarctica sp.	Antarctic habitat	Reddy et al. 2004
Vibrio Sp.	Antarctic habitat	Lo Giudice et al. 2006
Pseudomonas sp. B11-1	Alaskan soil	Choo et al. 1998
Acinetobacter sp. strain no. 6	Siberian tundra soil	Suzuki et al. 2001
Stenotrophomonas maltophilia	Oil soil	Li et al. 2013
Psychrobacter sp.	Antarctic	Shuo-shuo et al. 2011
Shewanella frigidimarina NCIMB 400	Antarctic marine	Parra et al. 2015
Acinetobacter sp. XMZ-26	Glacier soil	Zheng et al. 2016
Psychrobacter cryohalolentis K5	Siberian cryopeg	Novototskaya-Vlasova et al. 2013
Penicillium expansum	Antarctic habitat	Mohammed et al. 2013

principles to improve enzymatic and kinetic properties and maximise their enzyme use in cold environments. The next section discusses cold-adapted lipases and cellulases that are integral to biotechnological applications, whose examples are displayed in Figure 3.

2.2. Cold-adapted lipases

Lipases (triacylglycerol lipases EC 3.1.1.3) constitute the third most important category of enzymes, next to carbohydrases and proteases. In general, thermostable enzymes preferred for industrial applications are obtained from mesophilic and thermophilic organisms, but some thermostable enzymes are also found in psychrophiles. Among all known sources of lipases, special interest is given to microbial ones that are relatively easier to handle and more stable in terms of catalysing a diverse range of chemical reactions (Veno et al. 2019). Cold-adapted lipases (CLPs) demonstrate high specific activity in the temperature range of 0–30 °C towards substrates, and their origins are mostly psychrophilic organisms living in polar regions such as deep water and marine sediments of the oceans and the glaciers of mountains (Struvay and Feller 2012). If CLPs are effectively used and well-integrated into biotechnological applications, they are the most vulnerable products of molecular adaptation through evolutionary processes. To understand the critical effect of thermal adaptation on an enzyme's continued activity in the cold, consider that the metabolic and growth rates of psychrophilic and psychrotolerant species near the freezing point of water exceed those of mesophilic organisms at the same temperature. CLPs are active under low water conditions due to superior flexibility, stability and activity compared to their mesophilic and thermophilic counterparts, which are characterised by higher rigidity, low production costs, wide variety, stability in the presence of organic solvents, specificity in action, mild reaction condition and low energy consumption. Table 1 lists microorganisms that are cold-adapted lipase sources and their living conditions.

Lipases have been used in many different applications in the fields of biotechnology, including the detergent industry, medical and pharmaceutical applications, fine chemical synthesis, and others. Since lipases are able to catalyse reactions at oil/water interfaces as well as organic solvents, they have integral to chemical reactions such as hydrolysis, esterification, and transesterification. This makes them more attractive for further research potentials in the biotechnology industry. In Table 2, the biotechnological applications of CLPs are listed.

A set of evolutionary genomic mutations has led to changes in the lipid composition of cell membranes and CLP structures by ensuring the efficiency of biochemical reactions essential to the life cycle even at lowered temperatures. With these mutations, CLP's enzymatic activity has been optimised by reducing the free energy barrier of the transition state in which substrates bind to the catalytic site. Due to the presence of genomic mutations, structural reorganisation has happened within lipase molecules through intramolecular forces. This newly existing organisation in the active site and whole molecule involve a reduction in the number and strength of weak interactions or the disappearance of stability factors, resulting in improved dynamics of active site residues in the cold (Struvay and Feller 2012). Being active in cold refers that there is resistance in the molecular integrity of lipase molecules towards cold that is not destructive.

Table 2. CLPs are used in different applications.

Organism	Application	Purpose	References
Candida Antarctica	Detergent industry	Detergent additive	Uppenberg et al. 1994
Microbacterium phyllosphaerae	Detergent industry	Detergent additive	Joseph et al. 2013
Bacillus sphaericus	Detergent industry	Detergent additive	Joseph et al. 2013
Pseudomonas fragi	Detergent industry	Detergent additive	Tutino et al. 2009
Bacillus pumilus ArcL5	Detergent industry	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	Wi et al. 2014
Pseudoalteromonas haloplanktis TAC125	Detergent industry	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	de Pascale et al. 2008
Colwellia psychrerythraea 34H	Detergent industry	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	Do et al. 2013
Polaromonas vacuolata	Detergent industry	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	Irgens et al. 1996
Shewanella frigidimarina	Detergent industry	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	Parra et al. 2015
Moritella sp. 2-5-10-1	Detergent industry	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	Yang et al. 2008
Vibrio sp.	Detergent industry	Detergent additives used at low temperatures and biocatalysts for the biotransformation of heat-labile compounds	Lo Giudice et al. 2006
Candida antarctica	Medical and pharmaceutical Applications	Synthesis of optically active amines	Smidt et al. 1996
Pseudomonas sp.	Medical and pharmaceutical Applications	Synthesis of optically active amines	Smidt et al. 1996
Geotrichum sp. F0401B	Medical and pharmaceutical Applications	Synthesis of aryl aliphatic glycolipids	Otto et al. 2000
Candida antarctica	Medical and pharmaceutical Applications	Formation of citronellol laurate	Ganapati et al. 2005
Candida antarctica	Medical and pharmaceutical Applications	Synthesis of single isomer chiral drugs	Gotor-Fernandez et al. 2006
Candida antarctica	Medical and pharmaceutical Applications	Synthesis of nitrogenated compounds	Gotor-Fernandez et al. 2006
Microbacterium phyllosphaerae	Medical and pharmaceutical Applications	Synthesis of citronellol laurate	Joseph et al. 2008
Candida antarctica	Medical and pharmaceutical Applications	Cosmetics	Joseph et al. 2008
Candida antarctica	Fine chemical synthesis	Asymmetric synthesis of amino acids/amino esters	de Maria et al. 2005
Candida antarctica	Fine chemical synthesis	Ester synthesis	Zhang et al. 2003
Candida antarctica	Fine chemical synthesis	Formation of butyl lactate	Pirozzi et al. 2004
Candida antarctica	Fine chemical synthesis	Enantioselective esterification of *(R)-ketoprofen*	Ong et al. 2006
Candida antarctica	Fine chemical synthesis	Organic synthesis of chiral intermediates	Gotor-Fernandez et al. 2006
Candida antarctica	Fine chemical synthesis	Flavour modification, optically active esters	Joseph et al. 2008
Acinetobacter sp.	Environmental applications	Bioremediation and bioaugumentation	Suzuki et al. 2001
Pseudomonas fluorescens P38	Food industry	Formation of butyl caprylate as a flavour compound	Tan et al. 1996
Acinetobacter sp.	Environmental applications	Bioremediation, degradation and removal of xenobiotics and toxic compounds	Joseph et al. 2008
Pseudomonas fluorescens	Biofuels and energy production	Biodiesel production by trans-esterification of oils and alcohols	Tutino et al. 2009

Several computer-based proteome analyses performed with mesophiles and psychrophiles support evidence that psychrophiles possess more Gly than mesophiles at rates of 7.1%–6.7%, respectively, accelerating their adaptation to cold environments (Struvay and Feller 2012).

As discussed earlier, low numbers of Pro and Arg residues and high numbers of Gly residues are associated with reduced salt-bridge formation in CLPs compared to mesophilic enzymes, especially on protein surfaces. Several studies conducted with psychrophilic enzymes show that non-polar residues on protein surfaces increase the solvent-accessible area (Aghajari et al. 1998; Russell et al. 1998; Georlette et al. 2003). Upon the reorganisation of water molecules around exposed hydrophobic side chains, the entropy-driven destabilisation factor is created. According to the calculations of electrostatic potential, it is revealed that there is an excess negative charge at the surface of the protein, and hence it results in lowered pH value of cold-adaptive enzyme with respect to mesophilic or thermophilic counterparts. This lowered pH value is related to the enhanced interactions of an enzyme(s) through solvent molecules (Feller et al. 1999). Due to the presence of this situation, there is a reduction in the balance of charges and salt bridges existing in the protein surface. The typical correlation between surface ion pairs and temperature adaptation is explained such that there is an increased pattern of number in electrostatic interactions from psychrophiles to mesophiles, thermophiles, and hyperthermophiles, with hyperthermophiles showing arginine-mediated multiple ion pairs and interconnected salt bridge networks (Yip et al 1995; Vetriani et al. 1998; Struvay and Feller 2012; Liu et al. 2022). Due to the presence of these electrostatic interactions, there exists improved protein dynamics or the "breathing" of the external shell of cold-active enzymes (Feller 2013). In terms of structural features, aromatic-aromatic interactions could be also pointed out to explain the mechanism of cold adaptations. It has been concluded that CLPs have a smaller number of aromatic-aromatic interactions when compared with their mesophilic counterparts.

Another example is the *Bacillus subtilis* lipase A by demonstrating the roles a single Gly plays when substituted at different positions on the protein's structure (Kumar et al. 2014). This study concludes that four out of five Gly substitutions (F17G, A132G, M134G, M137G, G155D) improve enzymatic activity at low temperature by displaying a seven-fold enhancement in activity at 10 °C compared with WT lipase. These experimental results have been also validated through molecular dynamics (MD) simulations along 100 ns, and it is described that loop flexibility increases with a single Gly replacement in a catalytic residue (Kumar et al. 2014). Gly replacement around the active site increases the local mobility of enzyme that also turns into E_a (activation energy) together with the increased ΔRMSF along 100 ns, and hence it facilitates enzymatic activity even at lower temperatures. As in line with expectations, the mutant lipases have lower melting temperatures (T_m^{app}) than their wild-type counterpart.

Besides the side-directed mutagenesis studies performed close to the active site, there are several studies aiming to enhance lid mobility in overall lipase structure. For lipases, the function of lid structures is so specified, and the increased local mobility around lid structure is a crucial item for adaptation along the evolutionary process. The function of lid structure is defined as hydrolysis reactions facilitated in the water/oil interface due to the aggregation of lipid molecules on this lid region. The main role of the lid is to cover the active site so that the interfacial activation proceeds in a controlled manner upon the close contact between the substrates. By introducing a mutation to the lid region of CLPs from *P. fragi*, the possible contribution of the lid region is questioned to cold adaptation, and the answer is yes by reporting the improved selectivity and thermal stability in the cold environment (Kavitha 2016). Here again, they report that mutated cold-active lipase possesses high plasticity, being responsible for lowered activation energy by conferring structural adaptation. The whole story of cold adaptation has been summarised such that there exists the optimal equilibrium between enzyme stability, conformational flexibility, plasticity, and catalytic efficiency, and the reorganisation of intramolecular interactions via mutation ensures the existence of this optimal equilibrium. In the following Figure 4, 3D structure of CALB lipase is displayed by indicating the positions of Gly and Pro residues.

2.3. Cold-adapted cellulases

Cellulases are a major group of industrial enzymes, involved in reactions that include cotton processing, paper recycling, detergent formulation, and agricultural biotechnology. Cellulases are secreted by microbes, bacteria, fungi, plants, etc, and their primary role is to catabolize biomass into simple units that could be further utilised for varied purposes. Especially, the tremendous growth around the world results in the existence of the highest need for energy, and it results in the search for alternative energy sources instead of fossil fuels, contributing to global warming and to other environmental challenges. Among renewable energy resources (hydroelectric, solar, wind, biomass and hydrogen), biomass is most attractive due to its availability and economic feasibility together

with the presence of already optimised chemical reactions to convert it to renewable energy (Yuan et al. 2014; Paixão et al. 2021). Herein, the roles of cellulases gain so important since they play a major role in breaking down the cellulose components of biomass into useful smaller units. Depending on their modes of action and substrate specificity, cellulases are classified as endoglucanase, cellobiohydrolase, and β-glucosidase (Zheng et al. 2016). Endoglucanases (EC 3.2.1.4) randomly hydrolyse amorphous parts of solid cellulose molecules, generating new ends; cellobiohydrolases (EC 3.2.1.91) attack the ends of cellulose molecules to release soluble cellobiose or glucoses; and β-glucosidases (EC 3.2.1.21) hydrolyse cellobiose to glucose to prevent inhibition by cellobiose (Zhang et al. 2007). So, their synergistic combination effectively converts natural lignocellulose to crystalline cellulose and then glucose, and hence positively contributes to the clean energy cycle.

Among different types of sources, microbial cellulases have been attracting more due to their being economically feasible and easily handled. Cellulase enzymes obtained from bacteria living in cold and in alkaline environments would be great candidates for producing stable enzymes for use in extreme conditions. The utilisation of cellulases coming from these extreme conditions could contribute to the conservation of energy due to carry out chemical reactions at low temperatures while retaining overall enzymatic efficiency. Table 3 presents studied cold-adapted enzymes and their sources.

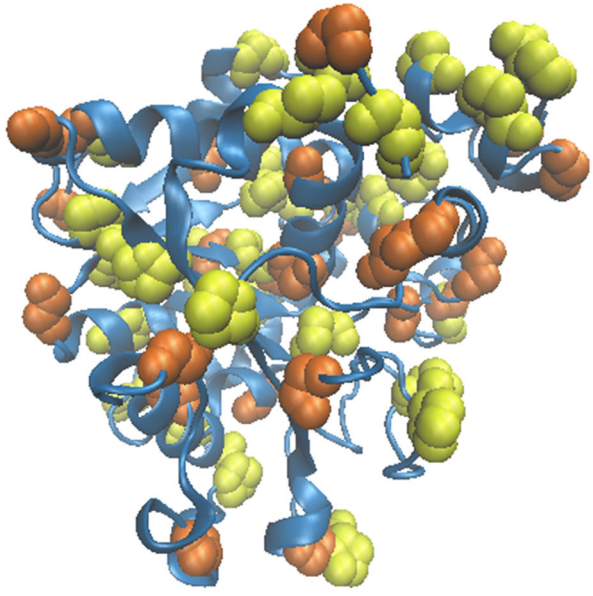

Figure 4. The positions of Gly and Pro residues in cold-adapted lipase CALB (pdb: 1tca).

For the discovery of cold-adapted cellulases, the metagenomic approach, a combination of computer-based and wet lab-based studies, is also used to explore the potential of nature as a source. Here, the host and bacterial diversity have been revealed by indicating functionality without the cultivation of microbes. After the identification of the microbial population, novel strategies are employed to discover more about the wealth of resources (Wang et al. 2013), and hence the isolated cellulase enzymes from extreme environments are effectively used for industrial applications as given in Table 4.

The same story for cold adaptation of cellulases is true as being discussed for lipase above. There is an example from literature about the cold adaptation of cellulases such that a high Gly/Pro ratio (1.07) has been reported in XynGR40 as a source of cold adaptation. The study conducted with GaExg55 has revealed that performing multiple alignments with mesophilic exo-1,3-b-glucanases of *S. cerevisiae* (1H4P), *Candida albicans* (1EQP), and *Candida albicans* (1EQP) (2PB1) explains the cold adaptation mechanism of GaExg55. According to the multiple alignment results, there is a conservation of the catalytic residues across all exo-1,3-b-glucanases examined. Also, it is revealed that there is a variety of novel replacements for the hallmark psychrophilic proline and glycine residues, and these substitutions are classified as either flexibility substitutions or stability substitutions in which the property of cold adaption in GaExg55 is achieved by the combination of structural features. The role of proline's pyrrolidine ring is to impose severe constraints on the conformation of the preceding residue. Proline increases the stability and local stiffness of proteins by decreasing the configurational entropy of unfolding and restricting the main chain's flexibility.

Table 3. The types of cold-adapted cellulase come from different sources.

Organisms	Environment	References
Eisenia foetida	Sapporo, Japan	Ueda et al. 2014
Pseudomonas stutzeri	Antarctic soil	Berlemont et al. 2009
Verticillium sp.	Antarctic soil	Wang et al. 2013
Pseudoalteromonas haloplanktis	Antarctica	Violot et al. 2005
Arthrobacter sp. TAD20	Antarctic sea sediments	Lonhienne et al. 2001
Psychrophiles	Deep-sea sediments	Gerday et al. 1997

Table 4. Cold-adapted cellulase is used in different applications.

Organisms	Application	Purpose	References
Psychrophiles	Textile industry	Biopolishing process	Gerday et al. 1997
Psychrophiles	Textile industry	Stone-washing processes	Gerday et al. 1997
Eisenia foetida	Biofuels and energy production	Conversion of cellulose to ethanol	Ueda et al. 2014
Pseudoalteromonas haloplanktis	Detergent industry	Additive in detergent industry	Violot et al. 2005
Acidithiobacillus caldus	Food and beverages	Removal of hemicellulosic material from feed	Navarro et al. 2013
Acidithiobacillus ferrooxidans	Food and beverages	Feed component	Navarro et al. 2013
Extremophilic microorganisms	Detergent industry	Wash of cotton fabrics	Sarmiento et al. 2013
Extremophilic microorganisms	Textile industry	Bio-finishing combined with dyeing of cellulosic fabrics	Sarmiento et al. 2013
Extremophilic microorganisms	Food and beverages	Hydrolysis of hemicellulose and cellulose to lower molecular weight polymers in brewing	Sarmiento et al. 2013
Extremophilic microorganisms	Pulp and paper	Modify cellulose and hemicellulose components of virgin and recycled pulps	Sarmiento et al. 2013

Through the substitutions of hydrophobic residues into GaExg55, there is a critical decrease in GaExg55's flexibility. However, in mesophilic exo-1,3-b-glucanases, proline residues in certain places in the psychrophilic GaExg55 were substituted by other residues in equivalent positions. This examination supports the notion that structural components engaged in the catalytic cycle of psychrophilic enzymes are more flexible, whereas non-catalytic regions may display stiffness comparable to or even greater than that of their mesophilic counterparts (Mohammadi et al. 2014). In the following Figure 5, 3D structure of cold-adapted cellulase CEL5G is displayed by indicating the positions of Gly and Pro residues.

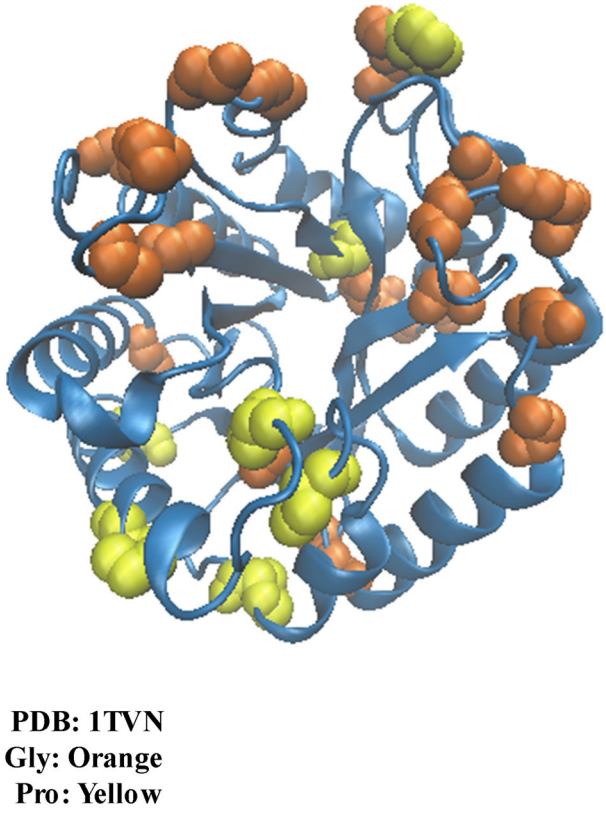

Figure 5. The positions of Gly and Pro residues in cold-adapted cellulase CEL5G (pdb: 1tvn).

3. Future perspectives

Biotechnology fields have used lipases and cellulase intensively for different purposes. Until now, scientific efforts have been devoted to improving their kinetic and enzymatic properties to meet industrial demands. Through the discovery of psychrophilic organisms, a new era has opened for protein science in the discovery of new structures and functions, and novel cold adaptation mechanisms to extreme conditions are described. Both cellulases and lipases are attractive enzymes for biotechnology, and their cold adaptation has opened new application areas.

For instance, lipases are playing a special role in the synthesis of fine chemicals, mostly considered value-added, by displaying unusual enzymatic activities at low temperatures (Shuo-shuo et al. 2011; Guo et al. 2021). Functionality at decreased temperature leads to slower reaction rates via new intramolecular interactions, and we have observed altered enzyme-substrate interactions, increased solvent viscosities and changes in the solubility of proteins or other associated macromolecules (Rousseaux 2008). These changes make it possible to synthesis of chemicals, which are difficult to synthesise but economically so valuable (Yuan et al. 2014) Another important concern to be included in the reaction design is the stabilisation of synthesised product(s), and lower reaction temperatures can be advantageous, especially in the case of chiral intermediates and frail compounds (Vetriani et al. 1998; Trevino et al. 2007). In addition to facilitating chemical reactions at lower temperatures, cold-adapted enzymes reduce the costs of the final product

by reducing energy expenditures or purification costs. Any improvements to cold-adapted lipase and cellulase enzymes will affect organic chemists, biophysicists, biotechnologists, and process engineers (Tan 1996), whose demands are dominating the future of research studies. We believe that understanding enzymes' cold adaptation mechanisms will accelerate the application of synthetic design principles to the field of protein engineering, accelerating the introduction of engineered proteins to our lives. Protein engineering research takes three primary approaches: guided evolution, rational design, and de novo design. Once the three-dimensional structure and mechanisms of proteins are well understood, rational design becomes an efficient approach for protein engineering. In comparison, directed evolution does not require a lot of information or a three-dimensional structure of the desired protein; rather, random selection and mutagenesis identify enzymes with desirable characteristics. De novo design methods customise synthetic proteins by utilising three-dimensional structures and folding principles of natural proteins. All these methods can be used for cold-adapted enzymes.

Ethics statement

No biological or human material were used in this study.

Informed consent

This manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. It is also confirmed that the order of authors listed in the manuscript has been approved by all of us.

Author contributions

This manuscript has been read and approved by all named authors. A.M.Ö, E.A, and A.K. designed the manuscript, A.K, A.M.Ö.,E.A, and B.T.K wrote the manuscript. S.F. provided a critical reading to manuscript.

Disclosure statement

The authors have no conflicts of interest to declare that are relevant to the content of this article.

ORCID

Ahmet Melih Öten http://orcid.org/0000-0002-3899-8836
Evren Atak http://orcid.org/0000-0001-9310-9205
Banu Taktak Karaca http://orcid.org/0000-0002-9657-4679
Sinem Fırtına http://orcid.org/0000-0002-3370-8545
Aslı Kutlu http://orcid.org/0000-0002-9169-388X

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