SGNH-type acetyl xylan esterases (AcXEs) play important roles in marine and terrestrial xylan degradation, which are necessary for removing acetyl side groups from xylan. However, only a few cold-adapted AcXEs have been reported, and the underlying mechanisms for their cold adaptation are still unknown because of the lack of structural information. Here, a cold-adapted AcXE, AIAEase, from the Arctic marine bacterium *Arcticibacterium luteoviscidum* SM1504 was characterized. AIAEase could deacetylate xyloligosaccharides and xylan, which, together with its homologs, indicates a novel SGNH-type carbohydrate esterase family. AIAEase showed the highest activity at 30 °C and retained over 70% activity at 0 °C but had unusual thermostability with a $T_m$ value of 56 °C. To explain the cold adaption mechanism of AIAEase, we next solved its crystal structure. AIAEase has similar noncovalent stabilizing interactions to its mesophilic counterpart at the monomer level and forms stable tetramers in solutions, which may explain its high thermostability. However, a long loop containing the catalytic residues Asp200 and His203 in AIAEase was found to be flexible because of the reduced stabilizing hydrophobic interactions and increased destabilizing asparagine and lysine residues, leading to a highly flexible active site. Structural and enzyme kinetic analyses combined with molecular dynamics simulations at different temperatures revealed that the flexible catalytic loop contributes to the cold adaptation of AIAEase by modulating the distance between the catalytic His203 in this loop and the nucleophilic Ser32. This study reveals a new cold adaption strategy adopted by the thermostable AIAEase, shedding light on the cold adaption mechanisms of AcXEs.
The cold adaptation mechanism of a novel AcXE

reported to be cold adapted (18). AxeA efficiently deacetylates acetylated xylan at low temperatures of 15 to 30 °C (18). However, owing to the lack of structural information, the underlying mechanisms for the cold adaptation of AcXEs are still unknown by far.

Cold-adapted enzymes help their source strains to adapt to extremely cold environments. Compared with mesophilic/thermophilic homologs, cold-adapted enzymes display higher catalytic activity at low temperatures because of their more flexible structures (19, 20). Most cold-adapted enzymes show a global rather than uniform distribution of their flexibility throughout the whole structure, therefore resulting in their low thermostability (21). However, some cold-adapted enzymes are also reported to have unusual thermal stability, such as the vibriolysin E495 from an Arctic sea ice bacterium (22), the phenylalanine hydroxylase from strain SM1504T, which, together with its homologs, is a halotolerant enzyme, whose activity was not influenced by 3.0 M NaCl (Fig. 3A). Among all the characterized GDSL enzymes, AlAXEase is most closely related to the SGNH-type acetyl xylan esterase Axe2 from Geobacillus stearothermophilus (4), with a low sequence identity of 24%, suggesting that AlAXEase is a potential novel SGNH-type CE. To reveal the relationship between AlAXEase and other CEs, a phylogenetic tree was constructed, including AlAXEase and its homologs, Axe2 and its homologs, and characterized enzymes from known SGNH-type CE families 2, 3, 6, 12, and 16 (Fig. 1). The tree showed that AlAXEase and its homologs are clustered as a separate group from all other characterized SGNH-type CEs (Fig. 1). Based on these data, we suggest that AlAXEase and its homologs represent a new SGNH-type CE family.

Multiple sequence alignment showed that AlAXEase contains the four characteristic sequence blocks of SGNH hydrolases, blocks I, II, III, and V (Fig. 2), further supporting that AlAXEase is a SGNH hydrolase. AlAXEase has a catalytic triad possibly formed by Ser32, Asp200, and His203 (Fig. 2). The catalytic Ser32 is located in the conserved GDSxT motif (block I) close to the N terminus, while Asp200 and His203 are located in the conserved DxxHL(P) motif (block V) (Fig. 2). Residues Gly69 and Asn98 were predicted to be involved in the oxyanion hole, which are located in blocks II and III, respectively (Fig. 2).

AlAXEase is a cold-adapted acetyl xylan esterase with unusual thermostability

AlAXEase without the predicted signal peptide was over-expressed in Escherichia coli BL21 (DE3) with the coexpression of the chaperone protein groES-groEL and purified. The recombinant AlAXEase with a calculated molecular mass of 23.5 kDa could hydrolyze p-nitrophenyl acetate (pNPC2), 1-naphthyl acetate, and phenyl acetate but showed no detectable activity against pNP-acylesters with an acyl chain length of more than two carbon atoms (Table 1), suggesting that AlAXEase may have a small substrate-binding pocket. Using pNPC2 as the substrate, AlAXEase exhibited the highest activity at 30 °C and retained more than 70% of the highest activity at 0 °C (Fig. 3A), indicating that it is a cold-adapted enzyme. However, AlAXEase displayed unexpected tolerance to heat treatment, retaining 75% of the highest activity at 50 °C and 45% at 60 °C after 1 h incubation (Fig. 3B). Moreover, AlAXEase had a relatively high $T_m$ value of 56 °C (Fig. 3C). These results indicate that the cold-adapted AlAXEase has unusual thermal stability. The cold-adapted AlAXEase was also resistant to mechanic stirring (Fig. S1). AlAXEase exhibited the highest activity at pH 9.0 and was stable in a range of pH 5.0 to 11.0 (Fig. 3D and Fig. S2). AlAXEase is also a halotolerant enzyme, whose activity was not influenced by 3.0 M NaCl (Fig. 3E). Among all the tested metal ions, only 10 mM of Cu$^{2+}$, Fe$^{2+}$, or Fe$^{3+}$ severely inhibited AlAXEase.

Results

AlAXEase belongs to a novel SGNH-type CE family

A gene encoding a GDSL family protein (GenBank Accession No. WP_111370902) was obtained from the genome sequence of the marine cold-adapted bacterium Aluteiflusbisistations SM1504$^T$ based on gene annotation, which was designated as AlAXEase. AlAXEase is 669 bp in length, encoding a putative lipolytic enzyme of 222 amino acid residues. Based on the SignalP 5.0 prediction, AlAXEase contains an N-terminal signal peptide sequence (14 residues in length).

AlAXEase shows the highest sequence identity (66%) to an uncharacterized GDSL family protein from Entimicia aquatilis (GenBank Accession No. WP_188769581). Among all the characterized GDSL enzymes, AlAXEase is most closely related to the SGNH-type acetyl xylan esterase Axe2 from Geobacillus stearothermophilus (4), with a low sequence identity of 24%, suggesting that AlAXEase is a potential novel SGNH-type CE. To reveal the relationship between AlAXEase and other CEs, a phylogenetic tree was constructed, including AlAXEase and its homologs, Axe2 and its homologs, and characterized enzymes from known SGNH-type CE families 2, 3, 6, 12, and 16 (Fig. 1). The tree showed that AlAXEase and its homologs are clustered as a separate group from all other characterized SGNH-type CEs (Fig. 1). Based on these data, we suggest that AlAXEase and its homologs represent a new SGNH-type CE family.
activity, whereas the other metal ions had no or weak inhibitory effect on AlAXEase activity (Table 2). AlAXEase activity was not influenced by the metal chelator EDTA but severely inhibited by 10 mM PMSF (Table 2), suggesting that AlAXEase is a serine hydrolase.

To reveal the natural substrates of AlAXEase, we also measured the activity of AlAXEase against different kinds of acetylated carbohydrates (Table 1). Similar to the acetyl xylan esterase Axe2 from G. stearothermophilus (Table S1), AlAXEase could deacetylate many acetylated monosaccharides and disaccharides including galactose, glucose, xylose in furanose and pyranose configurations, sucrose, and xylobioside, as well as partially acetylated xylan, with the highest activity toward acetylated glucose and xylopyranose (Table 1), indicating that AlAXEase is a CE. AlAXEase hardly degraded N-acetyl-D-glucosamine (Table 1), suggesting its high specificity for the O-acetyl groups rather than the N-acetyl groups of acetylated carbohydrates. Further kinetic analysis revealed that, among the acetylated monosaccharides, acetylated xylopyranose is the optimal substrate of AlAXEase, to which AlAXEase showed the highest substrate affinity and the highest catalytic efficiency ($k_{cat}/K_m$) (Fig. 3F and Table S2). Moreover, AlAXEase could hydrolyze both acetylated xylobioside and acetyl xylan (Table 1). All these data indicate that AlAXEase is an acetyl xylan esterase.

Analysis of the overall structure and the active site of AlAXEase

To reveal the underlying cold adaption mechanism of AlAXEase, we solved the crystal structure of WT AlAXEase by the molecular replacement method using selenomethionine (SeMet)-AlAXEase structure as the starting model because of the low sequence identities (lower than 24%) shared by AlAXEase and proteins with available structures in the Protein Data Bank.
The crystal of AlAXEase belongs to the P1211 space group, and the structure of AlAXEase was solved at 2.50 Å resolution. The statistics for refinement are summarized in Table 3. Structural data show that each asymmetric unit contains four AlAXEase molecules (Fig. 4A).

Gel filtration analysis showed that AlAXEase tends to form large oligomers in solutions (Fig. 4B), and dynamic light scattering (DLS) analysis indicated that AlAXEase forms stable tetramers in solutions (Fig. 4C).

The overall structure of AlAXEase monomer is similar to those of other SGNH-type AcXEs (Fig. 4D), most closely resembling the structures of an uncharacterized GDSL protein (PDB code 3RJT) from Alicyclobacillus acidocaldarius and Axe2 (PDB code 3W7V) from G. stearothermophilus (15), with the RMSD of 1.34 Å (150 monomer Cα atoms) and 2.63 Å (147 monomer Cα atoms), respectively. Monomeric AlAXEase shows a typical SGNH hydrolase fold, consisting of a central four-stranded parallel sheet flanked by two layers of helices (Fig. 4E). Similar to most AcXEs (1, 14, 15), AlAXEase has a catalytic triad formed by residues Ser32, Asp200, and His203, which are all located on the protein surface (Fig. 4F).

The oxyanion hole is composed of two solvent-exposed residues, Gly69 and Asn98 (Fig. 4F). Both mutations G69A and N98A had a small impact on the Km, but significantly decreased the kcat of AlAXEase (Table 4), consistent with that the oxyanion hole residues are involved in stabilizing the tetrahedral intermediates in the reaction process through their main-chain nitrogen atoms (27). The catalytic residues

Figure 2. Multiple sequence alignment of AlAXEase and reported SGNH-type AcXEs with structures. Using ESPript, secondary structures of AlAXEase are shown above alignment and secondary structures of Axe2 (PDB code 3W7V) under alignment. Helices are indicated by squiggles, β strands by arrows, turns by TT letters, and 310-helices by η letters. Identical amino acid residues are shown in white on a black shadow, and similar residues are in bold black. Stars represent residues belonging to the catalytic triad, and circles represent oxyanion hole residues. The four conserved sequence blocks in SGNH hydrolases are boxed by red dashed lines. The catalytic loop in AlAXEase and the corresponding loops in other SGNH-type AcXEs are boxed by green solid lines.

AcXEs, acetyl xylan esterases; AlAXEase, a cold-adapted AcXE from Arctic marine bacterium Arctobacterium lutei fluvistationis SM1504; PDB, Protein Data Bank.
and the oxyanion hole residues together with their adjacent residues form a shallow substrate-binding pocket of AXEase (Fig. 4F).

In the AXEase tetramer, the interface between chains B and C is the largest, followed by the interface between chains C and D, and the remaining interfaces involving chain A are the least (Fig. 5A). The dimerization interface between chains B and C is mainly stabilized by hydrogen bonds and salt bridges involving eight residues Lys (71, 114), Gly (69, 107, 109), Asp (74, 111), and Thr108 from the interactive monomers (Fig. 5B), and the interface between chains C and D mainly by four hydrophilic residues Asp146, His147, Asn156, and Asn160 (Fig. 5C).

**Structural basis for the high thermostability of AXEase**

Among all the characterized proteins, the sequence and topological structure of AXEase are most closely related to

| Substrate                               | Specific activity (U/mg) |
|-----------------------------------------|--------------------------|
| p-Nitrophenyl acetate                   | 9.10 ± 0.09              |
| p-Nitrophenyl butyrate                  | 1.07 ± 0.01              |
| p-Nitrophenyl caproate                  | 1.10 ± 0.01              |
| p-Nitrophenyl caprylate                 | Undetectable             |
| 1-Naphthyl acetate                      | 2.02 ± 0.03              |
| Phenyl acetate                          | 2.23 ± 0.03              |
| Isopropenyl acetate                     | Undetectable             |
| Menthol                                 | 0.40 ± 0.09              |
| Florenicol                              | 0.07 ± 0.01              |
| Ethyl α,β-unsaturated fatty acid        | 0.10 ± 0.01              |
| Ethyl α,β-unsaturated fatty acid        | 0.10 ± 0.01              |
| Ethyl 4-chloro-3-hydroxybutanoate       | Undetectable             |
| β-1,2,3,5-Tetra-O-acetyl-D-xylofuranose  | 3.02 ± 0.03              |
| β-1,2,3,4-Tetra-O-acetyl-D-xylopyranose  | 3.88 ± 0.03              |
| Sucrose octaacetate                     | 3.41 ± 0.34              |
| 1,2,3,5-Tetra-O-acetyl-D-xylofuranose    | 3.02 ± 0.21              |
| 1,2,3,5-Tetra-O-acetyl-D-xylopyranose    | 3.88 ± 0.14              |
| Benzyl β-D-xyloside pentaacetate        | 0.38 ± 0.03              |
| Xylan (partially acetylated)            | 0.29 ± 0.03              |
| N-acetyl-D-glucosamine                  | Undetectable             |

Table 1: The substrate specificity of AXEase

| Compound                        | Relative/residual activity (%) |
|---------------------------------|---------------------------------|
|                                 | 1 mM                           | 10 mM                          |
| K+                              | 1.13 ± 0.19                    | 1.21 ± 0.22                    |
| Li+                             | 1.05 ± 0.22                    | 1.06 ± 0.22                    |
| Ba2+                            | 1.12 ± 0.24                    | 1.10 ± 0.22                    |
| Ca2+                            | 1.21 ± 0.27                    | 1.15 ± 0.19                    |
| Mg2+                            | 1.08 ± 0.19                    | 1.12 ± 0.11                    |
| Mn2+                            | Undetectable                   | Undetectable                   |
| Ni2+                            | 1.05 ± 0.19                    | 1.03 ± 0.19                    |
| Zn2+                            | 1.05 ± 0.12                    | 0.91 ± 0.12                    |
| Fe2+                            | 1.11 ± 0.14                    | 1.07 ± 0.14                    |
| Fe3+                            | 1.11 ± 0.14                    | 1.07 ± 0.14                    |
| EDTA                             | 1.04 ± 0.15                    | 0.99 ± 0.15                    |
| PMSF                             | 0.77 ± 0.11                    | 0.68 ± 0.12                    |

Table 2: Effects of metal ions and potential inhibitors on AXEase activity

* Undetectable.

Effect of metal ions and potential inhibitors on AXEase activity

**Figure 3. Biochemical characterization of AXEase.** A, the effect of the temperature on the activity (solid line) and stability (dashed line) of AXEase. B, the effect of the temperature on the stability of AXEase. The enzyme was incubated at 40 °C, 50 °C, and 60 °C for different time intervals, and the residual activity was measured at pH 8.0 and 30 °C. C, thermal unfolding of AXEase and its mutant monitored by CD. The CD was monitored at 222 nm. The inset shows the first derivative of the CD signal versus temperature. D, the effect of pH on the activity (solid line) and stability (dashed line) of AXEase. For stability, the enzyme was incubated in buffers ranging from pH 2.0 to 12.0 at 0 °C for 1 h, and the residual activity was measured at pH 8.0 and 30 °C. E, the effect of NaCl on the activity (solid line) and stability (dashed line) of AXEase. For stability, the enzyme was incubated at 0 °C for 1 h in buffers containing NaCl ranging from 0 to 4.8 M, and the residual activity was measured at pH 8.0 and 30 °C. F, kinetic parameters of AXEase against different acetylated mono/oligosaccharides. Enzyme kinetic assays of AXEase were carried out at pH 9.0 (20 mM Hepes) using 1,2,3,4-tetra-O-acetyl-D-xylopyranose, 1,2,3,5-tetra-O-acetyl-D-xylofuranose, β-D-glucose pentaacetate, and β-D-galactose pentaacetate at concentrations from 0.5 to 20 mM, respectively. The Km and kcat/Km values of AXEase against 1,2,3,4-tetra-O-acetyl-D-xylopyranose are considered to be 100%. In panels A, B, D, E, and F, the graphs show data from triplicate experiments (mean ± SD). AXEase, a cold-adapted AcXE from Arctic marine bacterium Arcticibacterium luteifluviustis strain SM1504T.
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Table 3
Data collection and refinement statistics of WT AlAXEase and SeMet-AlAXEase

| Parameters                        | AlAXEase            | SeMet-AlAXEase |
|-----------------------------------|---------------------|----------------|
| Space group                       | P12₁                | P12₁           |
| Unit cell dimensions              |                     |                |
| a, b, c (Å)                       | 76.87, 80.61, 82.06 | 72.22, 79.04, 81.80 |
| α, β, γ (°)                       | 90, 103.132, 90     | 90, 104.28, 90 |
| Wavelength (Å)                    | 0.979               | 0.979          |
| Resolution range (Å)              | 50.00–2.50 (2.54–2.50) | 50.00–2.30 (2.34–2.30) |
| Redundancy                        | 3.4 (3.5)           | 3.0 (2.3)      |
| Completeness (%)                  | 98.8                | 91.6           |
| Rmerge                            | 0.137 (0.306)       | 0.153 (0.437)  |
| I/σ(I)                            | 8.25 (2.67)         | 8.94 (1.27)    |
| Resolution range (Å)              | 42.07–2.51 (2.60–2.51) | 42.07–2.30 (2.34–2.30) |
| Rwork (%)                         | 17.92 (20.02)       |                |
| Rfree (%)                         | 23.54 (26.95)       |                |
| B-factor (Å²)                     | 28.09               |                |
| Macromolecules                    | 29.64               |                |
| Solvent                           | 29.64               |                |
| RMSD from ideal geometry          |                     |                |
| Bond lengths (Å)                  | 0.01                |                |
| Bond angles (°)                   | 1.02                |                |
| Ramachandran plot (%)             | 93.91               |                |
| Favored (%)                       | 93.91               |                |
| Allowed (%)                       | 6.09                |                |

* Numbers in parentheses refer to data in the highest resolution shell.

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Figure 4. Overall structural analysis of AlAXEase. A, overall structure of tetrameric AlAXEase in one asymmetric unit. B, gel filtration analysis of AlAXEase. Aldolase (158 kDa), protein E40 (137 kDa) (48), and protein DdDP (110 kDa) (49) were used as protein size markers. The theoretical molecular weight of monomeric AlAXEase without signal peptide is 23.5 kDa. C, DLS analysis of AlAXEase. D, superimposition of AlAXEase and other SGNH-type enzymes. AlAXEase is colored in green, the uncharacterized GDSL protein (PDB code 3RJT) from Alicyclobacillus acidocaldarius in cyan, CtCes3 (PDB code 2VPT) in magenta, and Axe2 (PDB code 3W7V) in yellow. E, overall structure of monomeric AlAXEase. The monomer has four β-sheets and eight α-helices. The catalytic triad residues (Ser32, Asp200, and His203) and the oxyanion hole residues (Gly69 and Asn98) are shown as sticks. The catalytic loop is colored in blue. F, surface view of monomeric AlAXEase. Active site residues Ser32, Gly69, Asn98, and His203 are colored in red, yellow, green, and magenta, respectively, and the catalytic loop in blue. AlAXEase, a cold-adapted AcXE from Arctic marine bacterium Arcticibacterium luteovisitatis SM1504; DLS, dynamic light scattering.

those of Axe2 albeit with a low similarity of 24% (Figs. 1 and 4). Axe2 is a mesophilic enzyme with the highest activity between 50 °C and 60 °C and a Tm value of 72 °C (4). The most common determinants for increased thermostability of hyperthermophilic proteins are more noncovalent stabilizing interactions (21, 28, 29). At the monomer level, AlAXEase has
similar numbers of hydrogen bonds and ionic interactions as Axe2 (Table 5), suggesting that AlAXEase has a high overall stability, thus leading to the high thermostability of AlAXEase.

From psychrophiles to mesophiles to thermophiles, a clear trend can be observed that shows an increase in the number of ionic attractions on the protein surface (23, 30). Compared with Axe2, AlAXEase has a more positively charged interface near its active site (Fig. 6A), fewer stabilizing prolines, and more thermally labile residues asparagine and lysine on its surface (Table 5 and Fig. 6, B and C), which may result in the lower thermostability of AlAXEase than Axe2.

In addition, oligomerization also contributes to the thermal stability of proteins (31, 32). Axe2 forms a ‘doughnut-shaped’ homo-octamer with two staggered tetrameric rings both in the crystal and in solution, and the oligomerization of Axe2 is mainly stabilized by a cluster of hydrogen bonds and π-stacking interactions involving residues near the active sites of all eight monomers (15). Similar to Axe2, AlAXEase also forms large oligomers. AlAXEase forms tetramers both in the crystal and solution (Fig. 4), which may play a role in maintaining the structural stability and thermostability of AlAXEase. Different from Axe2 octamers, AlAXEase tetramers are mainly maintained by residues far away from their active sites (Fig. 5). Moreover, the smaller oligomerization interfaces of AlAXEase than those of Axe2 suggest that AlAXEase tetramers are less compact than Axe2 octamers, which may also contribute to the lower thermostability of AlAXEase.

AlAXEase has a long and flexible catalytic loop around its active site

At the monomer level, the largest structural difference between AlAXEase and Axe2 is that the loop containing the catalystic triad

| Enzyme | Temperature (°C) | $V_{\text{max}}$ (μM/min/mg) | $K_m$ (mM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_m$ (mM$^{-1}$ s$^{-1}$) |
|--------|-----------------|----------------|-------------|----------------|-------------------------------|
| WT     | 10              | 5.2 ± 0.17    | 3.4 ± 0.08  | 2.0 ± 0.07     | 0.60 (83%)                     |
| WT     | 20              | 7.2 ± 0.01    | 4.5 ± 0.35  | 2.8 ± 0.01     | 0.63 (88%)                     |
| WT     | 30              | 9.2 ± 0.46    | 5.0 ± 0.33  | 3.6 ± 0.18     | 0.72 (100%)                    |
| WT     | 40              | 4.6 ± 0.67    | 5.6 ± 0.57  | 1.8 ± 0.26     | 0.33 (46%)                     |
| WT     | 50              | 1.0 ± 0.06    | 6.1 ± 0.16  | 0.41 ± 0.02    | 0.07 (10%)                     |
| S32A   | 30              | –             | –           | –              | –                             |
| G69A   | 30              | 3.1 ± 0.04    | 5.3 ± 0.30  | 1.2 ± 0.01     | 0.23 (31%)                     |
| N98A   | 30              | 0.05 ± 0.01   | 6.0 ± 0.36  | 0.02 ± 0.01    | 0.01 (1.4%)                    |
| D200A  | 30              | 0.05 ± 0.02   | 5.0 ± 0.16  | 0.02 ± 0.01    | 0.01 (1.4%)                    |
| H203A  | 30              | –             | –           | –              | –                             |
| E190A  | 10              | 4.4 ± 0.37    | 3.6 ± 0.25  | 1.7 ± 0.14     | 0.45 (63%)                     |
| E190A  | 20              | 5.7 ± 0.95    | 5.6 ± 0.21  | 2.2 ± 0.37     | 0.49 (68%)                     |
| E190A  | 30              | 2.0 ± 0.06    | 6.0 ± 0.16  | 0.77 ± 0.02    | 0.13 (18%)                     |
| E190A  | 40              | 0.48 ± 0.10   | 6.7 ± 0.13  | 0.19 ± 0.04    | 0.03 (4.2%)                    |

*Undetectable.

Figure 5. Oligomerization of AlAXEase. A, surface view of tetrameric AlAXEase. The four chains of AlAXEase are shown in different colors, and the catalytic triad and the residue Glu190 in the catalytic loop of each chain are highlighted in red and blue, respectively. B, the hydrogen-bond network between chains B and C. Residues in chain B are shown in cyan, and residues in chain C in magenta. For both chains, catalytic triad residues are shown in ball-and-stick representation. C, the hydrogen-bond network between chains C and D. Residues in chain C are shown in magenta and residues in chain D in yellow. For both chains, catalytic triad residues are shown in ball-and-stick representation. AlAXEase, a cold-adapted AcXE from Arctic marine bacterium Arcticibacterium lutefluidstationis SM1504$^\text{A}$.

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Table 5
Structure and sequence comparison of AlAXEase and Axe2

| Sequence/structural information | AlAXEase | Axe2 |
|--------------------------------|-----------|------|
| $T_m$ (°C)                     | 56        | 72   |
| $T_{m}$ (°C)                   | 30        | 50–60|
| Recombinant protein sequence length | 208 | 219 |
| Hydrophobic residues (%)       | 38.5      | 45.7 |
| Polar residues (%)             | 31.7      | 27.9 |
| Charged residues (%)           | 29.8      | 26.5 |
| Net charges (Arg + Lys - Asp - Glu) | -3 | -3 |
| No. of Gly/Met/Pro             | 16/5/4    | 19/7/9|
| No. of Asn/Gln                | 16/8      | 6/7  |
| Arg/(Arg + Lys)                | 0.296     | 0.577|
| Sequence identity to AlAXEase  | 100%      | 24%  |
| PDB entry                     | This study | 3W7V |
| Resolution (Å)                | 2.50      | 1.85 |
| No. of residues per monomer in the crystal structure | 199 ± 1 | 219 ± 0 |
| RMSD (Å) (no. of residues)    | 2.374 ± 0.04 | 2.297 ± 0.04 |
| No. of hydrogen bonds per residue in monomer | 0.398 ± 0.04 | 0.416 ± 0.05 |
| No. of side-chain to side-chain hydrogen bonds per residue | 0.550 ± 0.02 | 0.506 ± 0.03 |
| No. of side-chain to main-chain hydrogen bonds per residue | 1.426 ± 0.02 | 1.574 ± 0.03 |
| No. of main-chain to main-chain hydrogen bonds per residue | 12 ± 1.6 | 14 ± 2.8 |
| No. of ion pairs per monomer at 4 Å | 22.5 ± 0.6 | 26 ± 2.8 |
| No. of ion pairs per monomer at 6 Å | 0.060 ± 0.01 | 0.064 ± 0.01 |

The catalytic loop contributes to the cold-adapted characteristics of AlAXEase by modulating the distance between the catalytic residues Ser32 and His203

To further investigate the role of the catalytic loop in the cold adaptation of AlAXEase, site-directed mutagenesis on the residue Glu190 in the catalytic loop with the highest B factor was performed. Compared with WT AlAXEase, mutant E190A had a lower optimum temperature ($T_{opt}$) of 20 °C (Figs. 3A and 8B). At 10 °C, mutant E190A retained 92% of its maximal catalytic efficiency ($k_{cat}/K_m$), higher than that of the WT (83%) (Table 4), suggesting that mutant E190A is more active than the WT at low temperatures. Mutant E190A also had a lower thermostability, quite unstable at temperatures above 20 °C (Fig. 8, B and C). These data suggest that mutant E190A is more cold-adapted than WT AlAXEase.

Then, structural analyses and MD simulations of WT AlAXEase and its mutant E190A at different temperatures were carried out to further probe the molecular mechanism for the cold adaptation of AlAXEase (Figs. 9 and 10 and Fig. S3). At all simulated temperatures, no significant differences were observed in the RMSD values of the backbone atoms of both enzymes (Fig. 10A), suggesting that the introduction of the E190A mutation in the catalytic loop has little impact on the overall structures of AlAXEase monomers under different temperatures. However, the fluorescence peak position of AlAXEase began to change with a blue shift at 60 °C, and that of mutant E190A at 20 °C (Fig. 9A), indicating that the tertiary structure of the mutant is less rigid and less stable than that of the WT against high temperatures. Moreover, different from the WT (with a $T_m$ value of 56 °C), mutant E190A presented two thermal transitions (Fig. 3C), one at ~30 °C and the other at ~60 °C, suggesting that some regions of the enzyme unfold first at a low temperature, followed by the unfolding of the

catalytic residues Asp200 and His203 (18 residues in length) in AlAXEase is much longer than the corresponding one in Axe2 (8 residues in length) (Fig. 7A). The catalytic loop of AlAXEase is also the longest one among all the characterized AcXEs with solved structures (Fig. 2). Based on the B factor analysis, the flexible regions in AlAXEase and Axe2 are similar, except that the active site of AlAXEase is more flexible, especially the long catalytic loop (Fig. 6C). In AlAXEase, the catalytic loop is mainly stabilized by forming hydrogen bonds with two residues (Glu143 and Asp146) in the loop between β4 and α6 and hydrophobic interactions involving four residues (Ile196, Leu197, Val202, and Leu204) in the catalytic loop and eight hydrophobic residues in the other regions of AlAXEase (Fig. 7, B and D). For Axe2, similar hydrogen bonds and hydrophobic interactions are found to stabilize its short catalytic loop (Fig. 7, C and E). However, AlAXEase has less hydrophobic interactions (a 12-member cluster) around the catalytic loop than Axe2 (a 16-member cluster) (Fig. 7F). Moreover, no interaction is present to maintain the structure of the region $192^{KDRG}_{195}$ in the catalytic loop of AlAXEase (Fig. 7, B and D), and this region and its upstream residues are rich in destabilizing asparagine and lysine residues (Fig. 6B). In addition, the catalytic loop in Axe2 also forms intermolecular hydrogen bonds between interactive monomers (15), which, however, are absent from AlAXEase. All these differences make the catalytic loop of AlAXEase more flexible than that in Axe2, which would improve the flexibility of the catalytic center and lead to the high activity of AlAXEase at low temperatures. When the catalytic loop of AlAXEase was shortened (mutants Δ2 and Δ3 in Fig. 8A) or substituted by the short catalytic loop of Axe2 (mutants L1 and L2 in Fig. 8A), all the mutants were inactive (Fig. 8A), indicating that the length and flexibility of the catalytic loop is important for maintaining the catalytic activity of AlAXEase.
remaining regions at relatively high temperature. These data suggest that the introduced mutation E190A may cause an increased flexibility in local rather than overall structure of AlAXEase to enhance its cold adaptation.

Root mean square fluctuation values often reflect the fluctuation of individual residues during the MD simulation process (33). As shown in Figure 10B, both AlAXEase and its mutant contain three major unstable regions, including (1) the loop between β2 and α3 and the initial proportion of α3 (residues 67–76), (2) the latter part of α4 and the loop between α4 and α5 (residues 103–111), and (3) the region near the active site involving the catalytic loop. The latter part of the loop between β4 and α6 (residues 143–152) is also unstable in AlAXEase but stable in the mutant. Except for the unstable regions near the active site, all other unstable regions are located in the oligomerization interfaces of AlAXEase (Fig. 5), suggesting that heat treatment may influence the oligomerization of protein. Notably, at 45 °C, AlAXEase lost most of the enzymatic activity (Fig. 3A) but still retained tetramers (Fig. 9B), demonstrating that the cold-adapted characteristics of AlAXEase come from the flexibility of its monomeric rather than oligomeric structure. Different from the WT, a part of the tetramers of mutant E190A were depolymerized to monomers at its T_{opt} of 20 °C (Fig. 9B), suggesting that the introduced mutation E190A makes AlAXEase tetramers tend to depolymerize to decrease its thermostability.

MD simulations also showed that the regions around the active sites of both AlAXEase and mutant E190A become flexible at a high temperature (Fig. 10B). Compared with the small unstable part of the catalytic loop (residues 201 and 202) in AlAXEase, mutant E190A possessed a larger unstable region around the active site including the α7 and the following long catalytic loop (residues 180–194, 201, and 202) (Fig. 10B). It has been found that high flexibility, particularly around the active site, is usually associated with low substrate affinity in cold-adapted enzymes (21, 24). Similarly, compared with Axe2, AlAXEase and mutant E190A showed increased K_{m} values, and the K_{m} values of mutant E190A were higher than those of AlAXEase (Table 4 and Table S3), further indicating a flexible active site in AlAXEase and a more flexible active site in the
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Figure 7. Analysis of the interactions between the catalytic loop and other regions in AlAXEase and Axe2. A, the superimposition of AlAXEase (green) and Axe2 (cyan). Catalytic loop in AlAXEase is colored in magenta, and the counterpart in Axe2 in yellow. For both AlAXEase and Axe2, the catalytic triad residues and the oxyanion hole residues are shown as sticks. B, the hydrogen-bond network between the catalytic loop (magenta) and other regions (green) in AlAXEase. Key residues involved in these interactions are shown as sticks. C, the hydrogen-bond network between the catalytic loop (yellow) and other regions (cyan) in Axe2. Key residues involved in these interactions are shown as sticks. D, the hydrophobic interactions between the catalytic loop (magenta) and other regions (green) in AlAXEase. Key hydrophobic residues are shown as sticks. E, the hydrophobic interactions between the catalytic loop (yellow) and other regions (cyan) in Axe2. Key hydrophobic residues are shown as sticks. F, the superimposition of hydrophobic residues in AlAXEase and Axe2 involved in the hydrophobic interactions between the catalytic loop (magenta for AlAXEase and yellow for Axe2) and other regions (green for AlAXEase and cyan for Axe2). AlAXEase, a cold-adapted AcXE from Arctic marine bacterium Arcticibacterium lutefluivistisationis SM1504.

Figure 8. Analyses of the thermodependence of activity and thermostability of mutant E190A. A, enzymatic activities of the mutants of AlAXEase. Mutant L1 with mutation to replace residues L188NKNPENKDRGILTR199 in the catalytic loop of AlAXEase with KTLYPAALAW187 of Axe2, mutant L2 with mutation to replace residues L181KTLYPAALAW187 of AlAXEase with L181KTLYPAA187 of Axe2, mutant Δ2 with mutation to delete residues Asn188 and Pro189 of AlAXEase, and mutant Δ3 with mutation to delete residues Asn188, Pro189, and Glu190 of AlAXEase. The activities of WT AlAXEase and mutant E190A were measured under their respective optimum temperatures. For all other mutants, no enzymatic activity was detected at temperatures ranging from 0 to 60 °C. B, the effect of temperature on the activity (solid line) and stability (dashed line) of mutant E190A. For stability, the enzyme was incubated from 0 to 60 °C for 1 h, and the residual activity was measured under optimal conditions. C, the effect of the temperature on the stability of mutant E190A. The enzyme was incubated at 20 °C and 30 °C for different time intervals, and the residual activity was measured under optimal conditions. In panels A–C, the graphs show data from triplicate experiments (mean ± SD). AlAXEase, a cold-adapted AcXE from Arctic marine bacterium Arcticibacterium lutefluivistisationis SM1504.
mutant. Different from the reversible active site of AlAXEase, the active site of mutant E190A was irreversibly disrupted at a high temperature and thus led to the distortion of the α7 helix upstream of the catalytic loop to a random coil structure (Fig. 10B), indicating that the introduced mutation E190A in the catalytic loop makes the region around the active site more susceptible to thermal denaturation than other regions of AlAXEase as shown by CD (Fig. 3C).

During the MD simulation, we measured the distances between the key residues in the active site to further assess the effect of heat treatment on the active site of AlAXEase. At all simulated temperatures, the distance variations between the key residues in the active site were kept at a very small range in AlAXEase and mutant E190A except for the distance between the two catalytic residues, Ser32 and His203 (Table 6). For both enzymes, the distances between Ser32 and His203 at 400 K were significantly enlarged compared with those at 280 K, and these distance variations were irreversible when the proteins were cooled from 400 K to 280 K (Table 6). The enlargement of the distance between Ser32 and His203 resulted in the reduction in both activity and substrate affinity of both enzymes at temperatures higher than their respective $T_{opt}$ as indicated in Table 4. Notably, at 280 K, the distance between Ser32 and His203 in mutant E190A (6.5 ± 1.0 Å) is greater than that in the WT (4.3 ± 0.4 Å) (Table 6). Moreover, mutant E190A lost its catalytic activity and substrate-binding ability at a temperature (40 °C) lower than that for the WT (50 °C) (Table 4). All these results suggest that the flexible catalytic loop contributes to the cold-adapted characteristics (high catalytic activity and high substrate affinity at low temperatures) of AlAXEase by modulating the distance between the catalytic His203 in this loop and the nucleophilic Ser32, and that the introduced mutation E190A causes a further increase of flexibility in the catalytic loop of AlAXEase, leading to an improvement of its cold adaptation.

Discussion

AcXEs play important roles in both marine and terrestrial xylan degradation and recycling (1). AcXEs, dominated by SGNH-type enzymes, are distributed in nine CE families in the CAZy database (3) in addition to the recently discovered Axe2 family (4, 5). Compared with the extensive study on terrestrial mesophilic/thermophilic AcXEs, study on marine cold-adapted AcXEs is still scarce. Until now, owing to the lack of
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Structural information, the cold adaptation mechanisms for SGNH-type AcXEs are still unknown. In this study, a novel cold-adapted AcXE, \( \text{AlAXEase} \), was characterized from the Arctic marine bacterium \( \text{A. lutei} \) \( \text{fluviationis} \) SM1504\( ^{T} \). AlAXEase shares low sequence identities (≤24%) with characterized AcXEs, and phylogenetic analysis suggests that AlAXEase and its homologs represent a new SGNH-type CE family. AlAXEase had the highest activity at 30°C and displayed high catalytic activity at 0 to 20°C, showing its cold-adapted character. However, different from other cold-adapted enzymes that are generally thermolabile (34–36), AlAXEase has unusual thermostability, with a relatively high \( T_m \) value of 56°C and stable at temperatures up to 50°C, suggesting that the cold adaption strategy adopted by AlAXEase is different from other thermolabile cold-adapted enzymes.

Most cold-adapted enzymes are highly flexible in their overall structures, leading to their high catalytic activity at low temperatures but low thermostability (21). However, a few cold-adapted enzymes are also reported to be locally flexible without compromising the global stability of proteins (23, 24, 37–39). Biochemical and structural analyses suggested that AlAXEase has high overall stability but is flexible in the loop containing the catalytic residues Asp200 and His203 because of the reduced stabilizing hydrophobic interactions and increased destabilizing residues asparagine and lysine (Figs. 6 and 7). Further structural and enzyme kinetic analyses of WT AlAXEase and its mutant E190A combined with MD simulations at different temperatures revealed that, the flexible catalytic loop contributes to the cold-adapted characteristics of AlAXEase by modulating the distance between the catalytic His203 in this loop and the nucleophilic Ser32. The cold-adapted enzymes CpPAH and DplIDH are also reported to be locally flexible around their active sites because of the disrupted hydrogen-bonding abilities for the cofactor BH4 (23) and the increase in destabilizing residues such as methionine and charged amino acids (24), respectively. Both CpPAH and DplIDH have flexible active sites through increasing flexibilities in noncatalytic residues in their catalytic cavities, which contribute to their cold-adapted characteristics (23, 24).
However, different from *CpPAH*, *DpIDH*, and other cold-adapted enzymes (23, 24, 40), the flexible active site of *AlAXEase* comes from the increased flexibilities in the catalytic residues Asp200 and His203 rather than noncatalytic residues. Therefore, the cold adaption mechanism of *AlAXEase* is different from those of other reported cold-adapted enzymes. The flexible active site contributes to the cold adaptation of *AlAXEase* by modulating the distance between the catalytic residues His203 and Ser32. These data indicate that optimization of the flexiblity of the catalytic residues is also a strategy for cold adaptation of enzymes.

The marine strain SM1504<sup>T</sup> where *AlAXEase* comes from was reported to be cold adapted, growing at temperatures between 4 °C and 30 °C (optimum of 20 °C) (25). The cold adaptation of *AlAXEase* is consistent with the growth characteristics of strain SM1504<sup>T</sup>, suggesting that its structural and biochemical properties are optimized to low temperatures. Genomic analysis showed that this strain contains some genes encoding potential xylanases, arabinofuranosidases, and other xylan-degrading enzymes (26). *AlAXEase* could hydrolyze many kinds of acetylated monosaccharides and disaccharides as well as xylan, with acetylated xylopyranose as the optimal substrate, suggesting that *AlAXEase* is likely involved in xylan/xylooligosaccharide degradation together with other xylan-degrading enzymes to provide carbon source and energy for its source strain. Moreover, the cold-adapted characteristics of *AlAXEase* with unusual thermostability may also help its source strain SM1504<sup>T</sup> adapt to the cold polar environment.

**Experimental procedures**

**Gene cloning and mutagenesis**

Based on blasting analysis, a gene *AlAXEase* encoding a GDSL family lipolytic protein (GenBank Accession No. WP_111370902) was identified from the genome sequence of marine bacterium *A. lutefluiuvistatis*on SM1504<sup>T</sup>. *AlAXEase* without the signal peptide sequence was amplified from the genomic DNA of strain SM1504<sup>T</sup>, and the amplified fragment was ligated into the vector pET22b. All of the site-directed mutations and the truncated mutations in *AlAXEase* were introduced with the QuikChange mutagenesis kit (41) using plasmid pET22b-*AlAXEase* as the template. All recombinant plasmids were verified by sequencing.

**Protein expression and purification**

WT *AlAXEase* protein and all mutants were expressed in *E. coli* BL21 (DE3) with the coexpression of the chaperone groES-groEL. The cells were cultured at 37 °C to an absorbance at 600 nm of 0.6 to 1.0 and then induced by the addition of 1 mM IPTG and 0.5 mg/ml L-arabinose at 20 °C for 16 h. Cells were collected and disrupted by a JN-02C French press (JNBIO) in 50 mM Tris HCl buffer (pH 8.0) containing 100 mM NaCl and 5 mM imidazole. After centrifugation at 15,000g for 1 h at 4 °C, the recombinant proteins were first purified by Ni affinity chromatography (Qiagen) and then by ion-exchange chromatography on a SOURCE 15Q column (GE healthcare). The eluted enzyme fractions were further purified by gel filtration chromatography on a Superdex 200 column (GE healthcare) with 10 mM Tris HCl buffer (pH 8.0) containing 100 mM NaCl. The target protein was collected, and the protein concentration was determined by Pierce BCA Protein Assay Kit (Thermo Scientific).

**Enzyme activity assay**

Esterase activity was measured as described (42). The standard reaction system (1 ml) contained 50 mM Tris HCl buffer (pH 8.0), 0.02 ml of 10 mM pNP-acylesters (Sigma), and 0.02 ml enzyme with an appropriate concentration. After incubation at 30 °C for 5 min, the reaction was terminated by the addition of 0.1 ml 20% SDS (w/v). The absorbance of the reaction mixture at 405 nm was measured using a SpectraMax Plus384 microplate spectrophotometer (Molecular Devices). One unit of enzyme (U) is defined as the amount of enzyme required to liberate 1 μmol p-nitrophenol per minute.

The CE activity of *AlAXEase* was determined by detecting the release of acetic acid using synthetic substrates 1-naphthyl acetate, phenyl acetate, isopropenyl acetate, menthyl acetate, chlorfenicol, ethyl 2-chlorobenzoate, and ethyl 4-chloro-3-hydroxybutanoate as well as acetylated carbohydrates β-D-galactose pentaacetate, β-D-glucose pentaacetate, sucrose octaacetate, 1,2,3,5-tetra-O-acetyl-D-xylofuranose, 1,2,3,4-tetra-O-acetyl-D-xylopyranose, benzyl β-D-xylobioside

**Table 6**

The distances between key residues in the active sites of WT *AlAXEase* and its mutant E190A based on MD simulations

| Enzyme                | Crystal/MD simulation | S32 (Ca) - G69 (N) | S32 (Ca) - N98 (N) | S32 (OG) - H203 (NE2) | D200 (OD1) - H203 (ND1) | D200 (OD2) - H203 (ND1) |
|-----------------------|-----------------------|--------------------|--------------------|------------------------|-------------------------|-------------------------|
| WT                    | Crystal*              | 5.0 ± 0.1          | 9.1 ± 0.1          | 3.6 ± 0.2              | 2.6 ± 0.3                | 3.5 ± 0.2                |
| 280 K                 | 4.7 ± 0.2             | 8.6 ± 0.6          | 4.3 ± 0.4          | 4.3 ± 1.4              | 4.8 ± 1.7                |                         |
| 400 K                 | 5.7 ± 1.2             | 7.0 ± 0.5          | 10.5 ± 2.4         | 5.5 ± 2.2              | 5.5 ± 2.2                |                         |
| 400 K back to 280 K   | 4.3 ± 0.3             | 6.8 ± 0.3          | 11.1 ± 1.4         | 4.3 ± 1.5              | 4.9 ± 1.3                |                         |
| E190A                 | Crystal               | -<sup>c</sup>      | -<sup>c</sup>      | -<sup>c</sup>          | -<sup>c</sup>            | -<sup>c</sup>            |
| 280 K                 | 6.2 ± 0.3             | 10.1 ± 0.3         | 6.5 ± 1.0          | 3.3 ± 0.3              | 2.9 ± 0.2                |                         |
| 400 K                 | 7.2 ± 1.5             | 7.4 ± 0.9          | 9.3 ± 2.1          | 4.4 ± 2.0              | 4.4 ± 2.0                |                         |
| 400 K back to 280 K   | 7.7 ± 0.7             | 7.5 ± 0.4          | 8.2 ± 1.1          | 3.0 ± 0.5              | 3.3 ± 0.6                |                         |

Abbreviation: SeMet, selenomethionine.

* The corresponding atom/group of a given residue used for distance calculation is shown in parentheses.

<sup>c</sup> The distances were calculated based on the active sites of four chains in the crystal structure of WT *AlAXEase*.

<sup>c</sup> Undetectable.
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pentaacetate, partially acetylated xylan, and N-acetyl-D-glucosamine. 1-Naphthyl acetate, phenyl acetate, β-D-galactose pentaacetate, β-D-glucose pentaacetate, sucrose octaacetate, and N-acetyl-D-glucosamine were purchased from Sigma. Menthol acetate, flornofenil, ethyl 2-chlorobenzoate, ethyl 4-chloro-3-hydroxybutanoate, and 1,2,3,4-tetra-O-acetyl-D-xylopyranose were purchased from Aladdin. 1,2,3,5-Tetra-O-acetyl-D-xyloluranose and benzyl β-D-xylobioside pentaacetate were purchased from Zzstandard, and the partially acetylated xylan from Megazyme. The standard assay system contained 0.01 ml of 20 mM substrate dissolved in 50 mM Tris-HCl buffer (pH 9.0) containing 40% (v/v) isopropyl alcohol, and 0.01 ml enzyme with appropriate concentration. The reaction took place for 1 h at 30 °C. The release of acetic acid was determined with an Acetic Acid (ACS Analyser Format) Assay Kit (Megazyme, Ireland) according to the manufacturer’s instructions. One unit of enzyme activity (U) was defined as the amount of enzyme required to release 1 μmol of acetic acid per minute.

Biochemical characterization of AIAESe and its mutants

By using pNPC2 as the substrate, the biochemical characteristics of AIAESe and its mutants were studied. The T_{opt} for AIAESe activity was measured in the temperature range of 0 to 60 °C at pH 8.0. For thermostability assay, the enzyme was incubated at 40 °C, 50 °C, and 60 °C for different periods, and then, the residual activity was measured at 30 °C. The optimum pH of AIAESe was determined at 30 °C in the Britton–Robinson buffers ranging from pH 2.0 to pH 12.0. For pH stability assay, the enzyme was incubated in buffers with a pH range of 2.0 to 12.0 at 0 °C for 1 h, and then, the residual activity was measured at pH 8.0 and 30 °C. The effect of NaCl on AIAESe activity was determined at NaCl concentrations ranging from 0 to 4.8 M. For salt tolerance assay, the enzyme was incubated at 0 °C for 1 h in buffers containing NaCl ranging from 0 to 4.8 M before the residual activity was measured at 30 °C. The effects of metal ions and potential inhibitors on AIAESe activity were examined by the addition of various chemical agents to the reaction mixture.

Enzyme kinetic assays of AIAESe and its mutants were carried out at pH 9.0 (20 mM Hepes) using 1,2,3,4-tetra-O-acetyl-D-xylopyranose at concentrations from 0.5 to 20 mM. Kinetic parameters were calculated by nonlinear regression fit directly to the Michaelis–Menten equation using the Origin9.0 software.

Crystallization, data collection, and structure determination

Crystals suitable for X-ray diffraction were obtained using the hanging-drop vapor-diffusion method. WT AIAESe crystals grew at 18 °C in the buffer containing 0.1 M succinic acid and 15% (w/v) PEG 3350 for 1 week. Selenomethionine-AIAESe crystals grew at 18 °C in the buffer containing 0.2 M potassium thiocyanate, 0.1 M Bis-Tris propane (pH 7.5), and 20% (w/v) PEG 3350 for 1 week. X-ray diffraction data were collected on the BL17U1 beam line at Shanghai Synchrotron Radiation Facility using Area Detector Systems Corporation Quantum 315r. The initial diffraction data sets were processed by the HKL3000 program (43). AIAESe structure was determined by molecular replacement using the SeMet-AIAESe structure as the starting model. The refinement of AIAESe structure was performed using Coot (44) and Phenix (45). All structure figures were processed using PyMOL.

DLS and CD spectroscopy

The DLS experiments of AIAESe protein and its mutants were carried out using DynaPro NanoStar (Wyatt Technology). The protein concentration was 1 mg/ml (10 mM Tris HCl buffer, pH 8.0, 100 mM NaCl). Data analysis was performed with the Dynamics 7.1.0 software.

CD spectra of WT AIAESe and its mutants were recorded at 25 °C on a J-1000 spectropolarimeter (JASCO). All the spectra were collected from 200 to 250 nm at a scanning rate of 200 nm/min with a path length of 0.1 cm. The protein concentration was 0.1 mg/ml. The thermal unfolding curves were recorded using the spectropolarimeter equipped with a CTU-100 temperature control unit (JASCO). The signal was recorded at 222 nm with a bandwidth of 1 nm. The temperature was monitored using an internal sensor, and the heating rate was 1 °C per min. A 0.1-cm path length cell was used. The protein concentration was 0.2 mg/ml.

Fluorescence measurements

Steady-state fluorescence measurements were performed using an FP-6500 spectrofluorometer (JASCO) equipped with a JULABO computer-controlled thermostat. The excitation wavelength was set at 280 nm and the emission wavelengths at 300 to 500 nm, respectively. Both excitation and emission bandwidths were 5 nm. Cuvettes with a 1-cm path length were used. Proteins were at a concentration of ~0.06 mg/ml in 50 mM Tris HCl buffer (pH 8.0). Fluorescence spectra of AIAESe and its mutants after incubation at different temperatures for 1 h were recorded, respectively.

MD simulations

The MD simulations of WT AIAESe and its mutant E190A were conducted by using software package GROMACS 2019.6 (46), with the force field Amber99sb-ildn (47) adopted. The enzyme structure was first placed into the center of a virtual cubic box with side length of 7.57 nm for WT and 7.36 nm for E190A and then solvated with 12,613 and 11,875 TIP3P water molecules for WT and E190A, respectively. Sodium ions were added to the virtual water box as counter ions to neutralize the negative charge of the entire system (5 Na⁺ for WT and 4 Na⁺ for E190A). Energy minimization of the system was conducted using the steepest descent algorithm for 10,000 steps, followed by a 1-ns equilibration simulation with harmonic position restraints on the heavy atoms of protein to equilibrate the solvent molecules around the protein at the desired temperature. Subsequently, the simulation was performed for 200 ns at the target temperature without any position restraints. All simulations were performed under the NPT ensemble with periodic boundary conditions and a time step of 2 fs. The system was kept at a certain temperature using the v-rescale method, as well as the pressure was kept at 1 bar.
using the Parrinello–Rahman method. The temperature of the simulation was set to 280 K and 400 K. The final frame of the simulation performed under 400 K was used as the initial conformation to conduct another simulation under 280 K. According to the plot of the RMSD, trajectories that reached the equilibrium state (100 ns–200 ns) were used for analysis. The dynamics changes of the root mean square fluctuation values and the secondary structure against time were analyzed by using the built-in tools of GROMACS.

Data availability

The atomic coordinates and structure factors of AlAXEase have been deposited in the PDB with accession code 7DDY.

Supporting information—This article contains supporting information.

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Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: AcXE, acetyl xylose esterases; AlAXEase, a cold-adapted AcXE from Arctic marine bacterium Arcicibacterium luthefluvlustrationis SM15041; CEs, carbohydrate esterases; CpPAH, phenylalanine hydroxylase from Colwellia psychrerythraea 34H; DLS, dynamic light scattering; DiPDH, isocitrte dehydrogenase from Desulfitotalea psychrophila; PDB, Protein Data Bank; pNPD, p-nitrophenyl acetate; SeMet, selenomethionine; T_{opt}, optimum temperature.

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