Structure and Dynamics of a Thermostable Alcohol Dehydrogenase from the Antarctic Psychrophile *Moraxella* sp. TAE123

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**ABSTRACT:** The structure of a recombinant (His-tagged at C-terminus) alcohol dehydrogenase (*MoADH*) from the cold-adapted bacterium *Moraxella* sp. TAE123 has been refined with X-ray diffraction data extending to 1.9 Å resolution. The enzyme assumes a homo-tetrameric structure. Each subunit comprises two distinct structural domains: the catalytic domain (residues 1–150 and 288–340/345) and the nucleotide-binding domain (residues 151–287). There are two Zn ions in each protein subunit. Two additional zinc ions have been found in the crystal structure between symmetry-related subunits. The structure has been compared with those of homologous enzymes from *Geobacillus stearothermophilus* (*GsADH*), *Escherichia coli* (*EcADH*), and *Thermus sp.* ATN1 (*ThADH*) that thrive in environments of diverse temperatures. Unexpectedly, *MoADH* has been found active from 10 to at least 53 °C and unfolds at 89 °C according to circular dichroism spectropolarimetry data. *MoADH* with substrate ethanol exhibits a small value of activation enthalpy $\Delta H^\ddagger$ of 30 kJ mol$^{-1}$. Molecular dynamics simulations for single subunits of the closely homologous enzymes *MoADH* and *GsADH* performed at 280, 310, and 340 K showed enhanced wide-ranging mobility of *MoADH* at high temperatures and generally lower but more distinct and localized mobility for *GsADH*. Principal component analysis of the fluctuations of both ADHs resulted in a prominent open–close transition of the structural domains mainly at 280 K for *MoADH* and 340 K for *GsADH*. In conclusion, *MoADH* is a very thermostable, cold-adapted enzyme and the small value of activation enthalpy allows the enzyme to function adequately at low temperatures.

1. INTRODUCTION

Most of the cold-adapted enzymes that have been characterized to date originate from organisms isolated from Antarctica and the Antarctic seawater. In these low-temperature environments, the rates of chemical reactions responsible for maintaining life are substantially reduced. Nevertheless, organisms native to cold environments achieve metabolic rates sufficient for survival and growth and are comparable to those exhibited by mesophilic organisms at moderate temperatures. These organisms produce enzymes characterized by elevated catalytic activity at low temperatures associated, however, with low thermal stability.$^{1,2}$

Alcohol dehydrogenases (ADHs; EC 1.1.1.1) belong to the oxidoreductase family, a class of enzymes responsible for the catalysis of biological oxidations/reductions. ADHs catalyze the nicotinamide-adenine-dinucleotide (NAD$^+$)/nicotinamide adenine dinucleotide phosphate (NADP$^+$)-dependent interconversion of alcohols to their corresponding aldehydes or ketones and have been identified in all three domains of life: archaea, bacteria, and eukarya.$^{3,4}$ The medium-chain (~370 residues), class I ADHs$^5$ representing most of the ADHs characterized to date share a NAD$^+/$/NADP$^+/^{-}$-binding domain that assumes the Rossmann fold.$^6$ Extensive variability has been observed mainly at the substrate-binding region, leading to diverse specificity, assembly of enzyme subunits, and temperature stability.$^7$

Several crystal structures are available to date including 120 eukaryotic and 90 prokaryotic ADHs.$^8$ The ADH from the psychrophilic bacterium *Moraxella* sp. TAE123 (UniProtKB ID Q8GIX7) with an engineered hexahistidine tag at the carboxy terminus (hereafter *MoADH*) has been purified and crystallized, and the phases have been determined.$^9$ In the present work, the refined structure is presented at 1.9 Å resolution. The enzyme is a Zn-dependent, medium-chain (338 amino acid residues), homo-tetrameric ADH and exhibits a wide range of substrate specificity, oxidizing mainly primary and secondary aliphatic alcohols, utilizing nicotinamide-adenine-dinucleotide (NAD$^+$) as a cosubstrate.$^{10}$ It shows highest reaction rates for ethanol as a substrate and gradually

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decreases its reaction rates as the length and branching of the carbon chain of the alcohol substrates increase. In the latter two referenced works, it has been shown that the enzyme also reduces aldehydes and ketones. In the present work, the crystal structure of MoADH has also been compared with the structures of selected ADHs, which are produced by bacteria thriving in environments of varying temperatures, namely, Escherichia coli (EcADH, UniProtKB ID P39451), Geobacillus stearotherophilus (GsADH, UniProtKB ID P42328) and Thermus sp. ATN1 (ThADH, UniProtKB ID B2ZRE3).

Moreover, circular dichroism (CD) spectropolarimetry and enzyme kinetics of MoADH have been carried out at varying temperatures. Finally, molecular dynamics (MD) simulation results for a single subunit of both MoADH and GsADH are presented in connection with the experimental findings for alcohol dehydrogenase from Moraxella sp. TAE123.

2. RESULTS

2.1. Enzyme Kinetics and Thermal Unfolding of MoADH. Ethanol oxidation reaction rates have been measured as a function of temperature in the range 10–53 °C (Table S1). The Arrhenius plot based on the latter data (Figure S1) yielded activation enthalpy \( \Delta H^\circ \) of approximately 30 kJ mol\(^{-1}\), which agrees with the value obtained for the original, untagged enzyme of 28.4 kJ mol\(^{-1}\) using data from the publication by Tsigos et al.\(^{10}\) after limitation of the temperature range from 9 to 31 °C to preserve linearity for the respective Arrhenius plot. The original enzyme has been found to reach the peak of its activity at about 37 °C with comparable values of rate constants (kcat) with those of the His-tagged enzyme. Unexpectedly, the His-tagged enzyme remains active up to 53 °C. An analogous value for activation enthalpy (38.2 kJ mol\(^{-1}\)) has been derived for the cold-active FfADH from the psychrotolerant bacterium Flavobacterium frigidimar Gir-1 (UniProtKB ID Q8L3C9) for an even wider temperature range (10–60 °C).\(^{15}\) It is notable that the FfADH shows higher enzymatic activity than MoADH by approximately an order of magnitude. Interestingly, the values of activation enthalpies for the above cold-adapted enzymes are substantially lower than the values 88.8 and 60.7 kJ mol\(^{-1}\) determined for the homologous thermophilic GsADH for two temperature ranges 5–30 and 30–65 °C, respectively.\(^{16,17}\) The enzymatic activation parameters, i.e., activation enthalpy and activation entropy, for MoADH and its related enzymes are summarized in Table 1.

The relatively small value of activation enthalpy of MoADH and FfADH dictates a small dependence of the reaction rates on temperature, which may be the most significant part of the cold adaptation of the Moraxella and Flavobacterium enzymes.

Further, the thermal unfolding of MoADH monitored via CD spectropolarimetry occurs at approximately 89 °C (Figure S2). Moreover, the observed very slow unfolding of MoADH displays a kinetically controlled process because it requires several minutes at each temperature before each measurement. The above results put forward a significant thermal stability of the Moraxella enzyme, which agrees well with the behavior observed for the enzyme from Flavobacterium.\(^{15}\)

2.2. Structure of MoADH. The overall fold of one protomer of MoADH is shown in Figure 1a. The biologically relevant homo-tetrameric structure is presented in Figure 1b. As discussed below in the structure comparison section, the overall fold of the alcohol dehydrogenase structures is remarkably preserved among the class I enzymes. Each subunit comprises two distinct structural domains (Figure 1a): the catalytic domain (residues 1–150 and 288–340/345) and the nucleotide-binding domain (residues 151–287). The only cis-peptide bond observed occurs between residues Pro-55 and Pro-56. Residues Ser-270, Ile-271, and Val-286 assume main-chain disorder in this region. It is also noteworthy that Ser-270 and Ile-271 lie in the interface between protein subunits, while Val-286 lies in the region that connects the two structural domains of the protomers.

2.3. Metal-Binding Site. MoADH like most known medium-chain ADHs binds two Zn\(^{2+}\) ions per subunit. One metal ion is located at the active-site region; thus, it has been termed catalytic. At the catalytic site, the Zn(II) ligand atoms are as follows: Sy-Cys-38, Nε2-His-61, Or1-Glu-62, and Sy-Cys-148 (Figure 2a). The other zinc ion lies at approximately 22 Å from the catalytic one and is bound by four Sy atoms of Cys-92, Cys-95, Cys-98, and Cys-106 (Figure 2b). The presence of the latter Zn(II) ion stabilizes the structure of the small structural subdomain formed by residues Ser-90 to Gln-110. The latter subdomain extends out of the main protein fold and participates in interactions of the subunits in the tetramer. Therefore, the second Zn(II) has been termed structural. Both metal sites are among the basic conserved
The coordination of the metal ions at the catalytic and the structural sites of MoADH is shown in Figure 2 along with the computed, weighted electron density maps. At both sites, the ligand arrangement around Zn$^{2+}$ is nearly tetrahedral. The observed variations at the metal-binding sites are discussed in the following structure comparison section.

The positions of the Zn(II)-binding residues in the sequence of selected ADHs are shown in Figure 3. The geometric parameters of the metal coordination are provided in Table S2. Notably, only in one enzyme subunit (termed B) near the catalytic zinc, there is an ordered water molecule at approximately 2.6 Å from the metal ion. This water is on the opposite side of O$_{e1}$ of Glu-62 and may play the role of a catalytic water molecule. In the other enzyme subunits, no water was found near the catalytic zinc ions as the respective distances with ordered water molecules are greater than 4 Å.

In the MoADH structure, two additional Zn$^{2+}$ have been located, one in the interface between subunit B and the symmetry related to subunit A ($1-y$, $x-y$, $z+2/3$) and the second between subunit D and the symmetry related to subunit C ($y-x$, $-x$, $z+1/3$). These extra metal ions are held in place by residues His-7 and Glu-16 of subunits B or D and His-342 and His-344 of the His-tag epitopes at the carboxy termini of subunits A or C (Figure S3). The latter binding of zinc ions is probably the reason of the order of the terminal residues of chains A and C. In contrast, the six C-terminal residues of subunits B and D are disordered and thus missing from the structure.

2.4. Tertiary Structure and Active-Site Comparisons. The MoADH structure was compared in detail with three selected crystal structures of ADHs produced by other bacteria as described in Section 4. A summary of the superpositions of each structure pair is shown in Table 2. The respective kinetic parameters of the enzymes compared herein are shown in Table S3.

The crystal structure of MoADH assumes a semiclosed conformation as derived from the results of molecular dynamics analyses (Section 2.5). The rms deviations of MoADH (subunit C) from the open and closed conformations derived from PCA analysis at 340 K are 1.2 Å for 185 C$\alpha$ atoms and 1.7 Å for 264 C$\alpha$ atoms, respectively. The value of
The compared structures show a three-dimensional overlap extending approximately between 75 and 99% of their entire chain-lengths, while the respective sequence identity ranges between 24 and 60%. When the entire tetramers are compared, the corresponding structural overlaps are between 67 and 99% of the chain length, while in the case of the pair EcADH and ThADH, this value decreases to about 50%. This result reflects the slightly different arrangement of the subunits in the latter two enzymes. Figure 3 shows the multiple sequence alignment, which is based on the structural superpositions of the compared proteins. The figure shows the conserved regions, the residues involved in the binding of substrates that have been derived by inference to the homologous structures of the complexes with NAD⁺ (EcADH, ThADH) or trifluoroethanol CF₃CH₂OH (GsADH), and the residues involved in subunit interactions. The distance limit for the latter interactions was set to 4 Å. It is notable that the interactions with the substrates extend over a wide area of the enzymes involving 36 residues. Most of these interactions (24) are located in the dinucleotide-binding domain comprising residues Val-151 to Gly-287 in MoADH. The extent of residue conservation in the interaction region with the substrates is also noteworthy. Only 9 of the 36 residues vary to residues of mostly similar physico-chemical properties in the three, closely related enzymes (MoADH, GsADH, and EcADH). The variations and sequence locations of these residues among the selected enzymes are as follows: Trp to Phe (49), Ile to Leu (173), Val to Ile (194), Ile to Val (196), Asp to Asn and Gly (197), Lys to Gln (200), Ala to Ser and Pro (215), Asn to Val (260), and Ile to Leu (285); the numbers refer to the MoADH sequence. Several more variations are observed for the hyperthermophilic enzyme ThADH (26 of 36), the most prominent being the exchange of the conserved catalytic Zn-ligand Cys-148 in the three closely related ADHs to Asp-152 in ThADH. Moreover, there are several substitutions to bulkier residues as well as an almost equivalent number of substitutions of complementary nature, i.e., large to small residues, among MoADH, EcADH, GsADH, and ThADH.

The above-described variations in the substrate-binding region reflect only in part the corresponding change of electric potential of the enzymes’ surface of the respective active-site regions. This is illustrated in Figure 4 and signifies that more distant residues may also play a role in the development of positive or negative potential in the substrate-binding cleft. Figure 4 displays a nearly uniform distribution of positive and negative potentials among the psychrophilic (MoADH), thermophilic (GsADH), and hyperthermophilic (ThADH) enzymes. On the contrary, the mesophilic enzyme (EcADH) shows an extended surface of positive potential when compared with the other structures. In the same figure, the effect of NAD⁺ binding on the width of the active-site cleft is also clearly visible. In the structures of EcADH and ThADH, where NAD⁺ was cocrystallized with the enzyme, the active-site cleft is much narrower compared to that in MoADH and GsADH, where NAD⁺ was absent and only the analogue of ethanol (CF₃CH₂OH) was present in the structure of GsADH. This movement of the nucleotide-binding domain toward the catalytic domain in ADH structures has been described in detail earlier and agrees with the molecular dynamics results presented below.

The compared ADH structures contain either one cis-peptide bond (MoADH, Pro-55-Pro-56 and GsADH, Leu-55-Pro-56) or no cis linkages at all (EcADH and ThADH). The presence of a small number of cis-peptide bonds in psychrophilic enzymes has been proposed to facilitate the energetics of protein folding at low temperatures.

A last observation referring to the overall structures of the compared ADHs is the remarkably higher average atomic

![Figure 4. (a) MoADH, (b) EcADH, (c) GsADH, and (d) ThADH.](image-url)
temperature factor for the MoADH structure (\(\sim 60 \text{ Å}^2\)) compared to values ranging from approximately 9 to 36 \(\text{ Å}^2\) for the other structures determined at similar resolution limits (1.9–2.4 Å). ThADH has also high atomic temperature factor values (\(\sim 60 \text{ Å}^2\)) most likely because it has been refined at medium resolution (2.74 Å). Molecular packing does not seem to affect atomic displacement parameters because all four compared ADH structures have been derived from crystals that contain more than 50\% (v/v) solvent. The higher temperature factors point generally to higher mobilities of the atoms including those of the active-site region. Increased mobility of MoADH is generally expected in cold-adapted enzymes.2

2.4.1. Metal-Binding-Site Comparisons. Table S2 provides the geometric parameters of the metal coordination in all four compared ADH structures. The tetrahedral arrangement of the ligands around Zn(II) is generally preserved. The structural zinc ions are bound invariably by four S\(_\text{r}\) atoms of cysteines whose relative position in the protein sequence is also conserved (Figure 3). This is expected for a region that contributes to the stability of the biologically relevant tetrameric structure, i.e., the small structural subdomain comprising residues Ser-90 to Gln-110 (MoADH numbering) whose integrity relies on the presence of a tightly bound Zn(II). On the contrary, the catalytic zinc ions exhibit variation of the fourth (weak) ligand, namely, O\(_e\)1 of Glu-62 in MoADH (distance to zinc ion \(\sim 2.7 \text{ Å}\)), which is substituted by oxygen of an ordered water molecule in EcADH and ThADH, whereas in GsADH, the fourth ligand is the oxygen atom of the substrate alcohol. The exchange of ligands could be attributed to the fact that EcADH and ThADH structures have been determined as the enzyme complexes with NAD\(^+\) and the active-site architecture rearranges, as mentioned above, allowing thus the replacement of O\(_e\)1 by the oxygen of water. The other metal ligands, namely, Cys-38, His-61, and Cys-148 (MoADH numbering) are preserved except in the hyperthermophilic ThADH, whereby Cys-148 is replaced by Asp-152. Additional subtle variations in the catalytic zinc site are observed for the angles between the metal and the fourth ligand (water) in the structures of EcADH and ThADH. This again could be due to the rearrangement of the active site upon binding of the cosubstrate dinucleotide.

2.4.2. Quaternary Structure Comparisons. Figure 3 shows underlined the residues engaged in the subunit interactions of the enzymes’ quaternary structures. More detailed information is provided in Table S4, which displays the type and number of interactions among the protomers (subunits) in the biologically relevant homo-tetrameric unit. The increase of the number of ionic interactions is prominent from the psychrophilic MoADH (12) toward the mesophilic EcADH (28) and the two thermophilic, GsADH (30) and ThADH (23), enzymes. The same trend also holds true for the intramolecular ionic interactions (not shown), providing further evidence to the earlier observation regarding the means of the cold adaptation of enzymes.21

On the other hand, the interchain hydrogen bonds are more than twice as many in EcADH and ThADH compared to MoADH, while they are approximately the same in MoADH’s closest relative GsADH. The number of hydrophobic interactions increases from the psychrophilic to the other enzymes although it is again approximately the same in GsADH. Finally, there is a small increase in the interchain aromatic interactions of the thermophilic enzymes.

As regards the accessible surface areas of each tetramer, MoADH buries the least of its surface area (16.5\%) to form its tetramer with a well-defined cavity of 3165 Å\(^3\) as shown in Table S5. The other related ADHs bury more of their surface (22–27\%) as expected from the more extensive inter-subunit interactions mentioned above, yielding either well-defined cavities of relatively small volume (EcADH), or cavities accompanied by channels, leading to the surface (GsADH, ThADH) thus enclosing much larger volumes. The latter observation on the variation of the quaternary structure of MoADH may also play a role in the cold adaptation of the alcohol dehydrogenase from *Moraxella*.

2.5. Molecular Dynamics simulations. To get an in-depth look at the dynamics of the two closely related alcohol dehydrogenases MoADH and GsADH at all-atom resolution, we have run all-atom classical molecular dynamics (MD) trajectories. The dynamics are run at three temperatures (280, 310, and 340 K) for 150–400 ns as described in Section 4.5. The results focus on two distinct and important features of the two dehydrogenases, namely, their thermostability and the characteristics of their substrate-binding cavity. These results supplement the knowledge obtained from the presented crystal structure of MoADH and give further insight into the dynamical behavior and adaptation of the *Moraxella* enzyme at low temperatures. The Zn\(^{2+}\) ligands of both MoADH and GsADH display for the last 100 ns of the simulations (at 310 K) rms deviations of about 0.07 and 0.30 nm for the catalytic and the structural zinc ions, respectively.

2.5.1. ADH Thermostability between Species. To investigate the influence of the temperature on the dynamical behavior (thermostability) of the two closely related alcohol dehydrogenases MoADH and GsADH, the root-mean-square fluctuations (RMSF) of the Ca atoms of each ADH monomer are evaluated for the first 3–11 eigenvectors, which sum to \(\sim 60\%\) of the total protein motion, as derived from a principal component analysis (PCA) or essential motion analysis22 on each trajectory. The PCA suggests that MoADH exerts on average considerably noisier "essential" motions compared to GsADH. The RMSF values are mapped on the respective crystal structures of MoADH and GsADH and are shown in Figure 5. MoADH displays more fluctuations than GsADH as the temperature is increased from 280 to 310 and 340 K. In stark contrast, GsADH seems more stable at higher temperatures and displays local flexibility. Selected residues that are identified to show different flexibility between the two ADH species are labeled in Figure 5. It is readily observed that the majority of the common conformational changes between the two ADH molecules are located toward the base of the substrate-binding cavity.

The temperature dependences of the flexibility of the Ca atoms of MoADH and GsADH are comparatively presented in Figure 6a,b. The latter results allow an estimation of the conformational variability between the two ADHs. In Figure 6a, the differences are shown in the RMSF values between MoADH and GsADH. On average, there exist more negative peaks that are associated with increased MoADH flexibility at any temperature. The squares of the generalized order parameters \(S^2\), per residual backbone N–H bond vector, were evaluated in line with earlier work.23 The order parameters were scaled by a 0.89 factor, and their temperature dependence \(A\) was also calculated.23 The \(\Lambda\) values indicate the temperature dependence of the effective potential for the fluctuations of the N–H bond vectors. Large \(\Lambda\) values are
associated with higher disorders as temperature increases. In the proposed model, an average $\Lambda$ of 1.93 for MoADH and 1.81 for GsADH suggest that the MoADH structure is slightly more sensitive to temperature. Based on the evaluated slope ($\sim 0.0095$) of the linear dependence between $\Lambda$ and the melting temperature ($T_m$) previously proposed for members of the bacterial ribonuclease H (RNase H), a rather small difference in $T_m$ values of $\sim 13^\circ \text{C}$ is obtained between the two analyzed ADHs. In the case of interacting protomers, such as in the tetrameric ADHs, additional stability could be introduced, and the above proposed value may be different.

2.5.2. Substrate-Binding Cavity. The substrate-binding cavity consists of two connecting subdomains, defined herein by the following residues, subdomain-1: $37-40, 43, 48-51, 61-62, 86-88, 146-149, 151-152, 285-287, 323-331$ and subdomain-2: $151-152, 171-176, 193-200, 214-216, 237-240, 242-245, 260-262, 283-287$. The solvent (water) accessible surface area (SASA) of the cavity was evaluated throughout the MD trajectories for each ADH species and temperature. The results are shown in Figure 7a. We observe opposite trends in the SASA for MoADH and GsADH. However, it is interesting to note that around the assumed physiological temperatures of function for the two ADHs (280 K for MoADH and 340 K for GsADH) the values of SASA are comparable with a mean value at around 60 nm$^2$. To elucidate the fine details of the above trends in SASA, we have evaluated the number of hydrogen bonds between the residues within the substrate-binding cavity and adjacent water molecules in a probability density scheme (Figure 7b). The cutoff angle between hydrogen–donor–acceptor was set at 30°, while the hydrogen bond cutoff radius, at 0.35 nm.

As a general trend, for the H-bonding network profile, increase in the temperature leads to a lower number of H-bonds between the protein and water molecules within the cavity. It is remarkable that this trend is enhanced in the GsADH species but it is not prominent in MoADH. For GsADH, the latter fact indicates that at higher temperatures there could be a higher in/out flux of water molecules, leading to cavity variation between a partially collapsed and a fully hydrated conformation that has a larger effect on the SASA.
Throughout the MD trajectories and based on the PCA reported herein, we have observed a distinct open–close motion of the substrate-binding cavity, as depicted for GsADH in Figure 8a. The determined crystal structures of MoADH and GsADH overlap in an ideal way with the average structure of GsADH within the 340 K trajectory. The catalytic site is designated as a shaded area. (b) Distance of residues Lys-200 and Glu-329 of MoADH and Lys-200 and Asn-329 of GsADH at the gate of the substrate-binding cavity is consistent with the transition between the open and closed ADH states. (c) Open–closed transition of the ADHs in the frequency domain and (d) along with the distances between the residues defining the gates over the probed temperature range.

Figure 7. (a) Solvent (water) accessible surface area (SASA) of the substrate-binding cavity. (b) Number of hydrogen bonds between residues within the substrate-binding cavity and adjacent water molecules.

Figure 8. (a) Open–closed conformations calculated from the extreme structures for the first eigenvector of GsADH within the 340 K trajectory. The catalytic site is designated as a shaded area. (b) Distance of residues Lys-200 and Glu-329 of MoADH and Lys-200 and Asn-329 of GsADH at the gate of the substrate-binding cavity is consistent with the transition between the open and closed ADH states. (c) Open–closed transition of the ADHs in the frequency domain and (d) along with the distances between the residues defining the gates over the probed temperature range.
residue to return to the α-helical conformation in repeating cycles.

3. DISCUSSION AND CONCLUSIONS

The structure (Figure 1) of MoADH determined herein from psychrophilic Maraxella sp. TAE123 in combination with the homologous ADH structures derived from mesophilic (E. coli), thermophilic (G. stearothermophilus), and hyperthermophilic (Thermus sp. ATN1) bacteria provided interesting results as regards the features that underlie cold adaptation. These results diverge from those of previous studies on other enzymes from psychrophiles.225 The enzyme kinetics results (Table S1 and Figure S1) along with the thermal protein unfolding studies probed via CD spectroscopy (Figure S2) signify a thermostable MoADH. The enzyme is active in a wide range of temperature (10–53 °C), and its catalytic rates exhibit a very small temperature dependence (ΔH° 30 kJ mol⁻¹).

In summary, the following conclusions can be deduced as regards the low-temperature adaptation of MoADH. First, the observed small value of activation enthalpy (ΔH°, 30 kJ mol⁻¹) may well be the most significant molecular adaptation at low temperatures by MoADH. Second, another important physicochemical parameter for the cold-adapted MoADH appears to be the limited ionic interactions between the subunits in the tetramer.5 There are about half salt-bridges in MoADH compared to the mesophilic or thermophilic homologues (Table S4). The same is true although to a smaller extent for the intrachain protein interactions. Ionic interactions are considered to play an important role in stabilizing thermophilic proteins26 but are not apparently required for the cold-adapted enzymes. Moreover, the H-bonds are also limited (less than half) among the subunits in MoADH compared to the corresponding values for the mesophilic EcADH and hyperthermophilic ThADH. On the contrary, the numbers of H-bonds among the subunits of MoADH and GsADH are approximately equal. It is worth stating that the respective numbers of intramolecular H-bonds in all four enzymes are also approximately equal. The above increased interactions in the mesophilic and thermophilic enzymes result also into a tighter binding of their subunits to yield a more stable tetramer. The latter is also supported by the observed values of the buried surface area in the respective oligomeric structures (Table S5). Third, the substitution of few (9/36) residues in the substrate-binding area of the three closely related enzymes, namely, MoADH, EcADH, and GsADH, may also affect the accessibility and electrostatic potential of the active site of these ADHs. The effect on the surface potential is quite pronounced in EcADH but only minor in the GsADH structure (Figure 4). Moreover, active site analysis of the four compared structures did not result in any significant increase of the sidechain volume for the thermophilic enzymes.

Finally, the conclusions of the molecular dynamics simulations of MoADH and GsADH provided further insight into some interesting dynamical properties of the binding cavity and correlate with the above experimental results. First, MoADH shows higher mobilities than its thermophilic counterpart GsADH (Figure 6a) at all three temperatures investigated (280, 310, and 340 K). Second, both enzymes exhibit increased local flexibility in certain areas of their structures, which vary as a function of temperature (Figure 5). Third, the dynamics of the substrate-binding cavity of MoADH point to open-close transitions occurring in two events (10 and 60 μs⁻¹) at 280 K (Figure 8c). The latter transition is quite reduced at 340 K. In contrast, for GsADH, this transition is hindered at 280 K, but it occurs predominantly in one event (10 μs⁻¹) at 340 K, with comparatively higher intensity. On average, the gate at the substrate-binding cavity appears more open (larger distance between gate residues) in GsADH compared to MoADH (Figures 7 and 8). To what extent the differences in the gate/base residues contribute to the open-close transition of the substrate-binding cavity remains thus far unclear. The elucidation of the fine details of such a transition requires enhanced sampling and free-energy calculations.27 These will be addressed in future work, while the study presented herein focuses mainly on the thermostability of the two ADHs and the differences between them at all-atom resolution and variant temperatures.

4. EXPERIMENTAL SECTION AND COMPUTATIONAL METHODS

4.1. Thermal Protein Unfolding via CD Spectropolarimetry. The CD spectra were collected with a J-810 spectropolarimeter (JASCO, Inc.) in a quartz cuvette with a 1 mm optical length and a protein concentration of approx. 0.67 g L⁻¹ in 20 mM Tris–H₂SO₄, pH 8.0, and 20 mM Na₂SO₄. The temperature was controlled by a Peltier system. Data points were taken manually at wavelength 217 nm from 81 to 98 °C. The sample was equilibrated for 20 min at each temperature. The signal was recorded in units of mean residue ellipticity (mdeg). The value of ellipticity at 81 °C was similar to those at lower temperatures (10–80 °C).

4.2. Enzyme Assays. The standard assay mixture contained 70 nM enzyme, 20 mM Tris–HCl, pH 7.4, 10 mM CaCl₂ and 90 mM imidazole in total volume of 200 μL. For the determination of the kinetic constants for NAD⁺ as the variable substrate, a series of 0, 0.1, 0.5, 1.1, 2.0, and 4.0 mM NAD⁺ were added and the reaction was started by injection of 35 mM ethanol. Experiments have been carried out with approximately 5° intervals at temperatures ranging between 10 and 53 °C. The latter value is the maximum temperature attained by the employed instrument. A FLUOStar Galaxy microplate reader spectrometer (MTX Lab Systems, Inc.) was used with Costar 3596 96-well plates (Cole-Parmer, Co.) in absorption mode at 340 nm wavelength. Every absorption measurement including the blanks was performed three times, and the values were averaged.

4.3. Structure Determination and Refinement. The protein purification, crystallization, diffraction data collection, and processing statistics have been presented in detail earlier.9 The initial model of the structure was obtained by applying the single-wavelength anomalous diffraction (SAD) method. The structure was solved in the space group P₃₂₁. All diffraction data (F > 0) extending to 1.9 Å resolution were used in refining the structure of the biologically relevant unit comprising four protein subunits, each of which contains 346 residues. The latter include the engineered His-tag epitope sequence LEHHHHHH at the protein’s carboxy terminus. The initial structure was refined with REFMACS29 as implemented in the CCP4 suite of programs.29 Interventions of manual inspection and search for ordered solvent and fitting employed Xfit.30 Table 3 summarizes the refinement statistics.

The geometry of the final model was analyzed by PROCHECK,31 and MolProbity32 was used for rotamer and clash score calculations. The figures of the structures were
produced by the molecular graphics system PyMol (www.pymol.org).

4.4. Structure Comparisons. Homologous structures of alcohol dehydrogenases from bacteria living in environments of diverse temperatures (mesophilic, thermophilic, and hyperthermophilic) were selected from the deposited ADH structures in the Protein Data Bank and compared with the MoADH structure. The biological origins of the compared ADHs with the structure presented herein are as follows: *E. coli* (EcADH, PDB ID 4GKY),12 *G. stearothermophilus* (GsADH, PDB ID 1RJW),13 and *Thermus* sp. ATN1 (ThADH, PDB ID 4CPD).14 The structures of EcADH and ThADH were determined as complexes with NAD*. Nevertheless, the structural comparisons have been made possible because the crystal structures of the apoenzymes (MoADH and GaADH) assume a semiclosed conformation. The latter observation has been derived from the molecular dynamics simulations presented in Section 2.5. Moreover, to date, there are no other closely related apo-ADH structures to MoADH from *E. coli* or from any other hyperthermophilic bacterium. The next closest homologue from *E. coli* whose structure has been determined in the apoenzyme form is the alcohol dehydrogenase-like protein yaHK (PDB ID 1UUF) with about 30% sequence identity and rms deviation 1.4 Å of 284 Ca atoms from MoADH.

Both individual subunits and the homo-tetrameric structures were compared by three-dimensional superpositions using SwissPDBViewer (http://spdbv.vital-it.ch/).35 The sequence analyses and multiple alignments were carried out via the ProtParam tool and ClustalW as implemented in the ExPaSy suite.34 The computations of the type and number of interactions among the enzyme subunits were carried out by the PIC server (http://pic.mbu.iisc.ernet.in/).35 Computations of the protein accessible surfaces were carried out by PISA (http://www.ebi.ac.uk/pdbe/pisa/).36 The computations of the cavities were carried out by POCASA (altair.sci.hokudai.ac.jp/g6/service/pocasa/).37 Finally, the calculations of the electrostatic potentials of the enzymes were carried out by the PBEQ Solver module38 at the CHARMM-GUI server (http://www.charmm-gui.org/)39

4.5. Molecular Dynamics Computational Protocol. 4.5.1. Model Setup. The crystal structures of MoADH (PDB code 4Z6K, chain C) and GaADH (PDB code 1RJW, chain B) were used as initial coordinates to build the models, due to improved resolution of the respective chains. Only one monomer per ADH was probed herein, for computational efficiency. For consistency, the residues 339–345 were removed from the crystal structure of MoADH, whereas the substrate (trifluoroethanol) was also removed from the crystal structure of GaADH. The Glu-62/catalytic-Zn coordination was also restored in the latter upon changing a side-chain torsion angle of Glu-62, to match the one in MoADH. No co-enzyme or other alcohol substrates were included in the MD simulations. The protonation states of titratable residues were simulated at neutral pH, thus all Glu and Asp residues were left deprotonated, in accordance also with the PDB 2PQR (propka 3.0 method) predictions.30 For both MoADH and GaADH, the cysteinyl residues 92, 95, 98, and 106, ligands of the structural Zn, were simulated at the sulfide protonation state. The cysteinyl ligands of catalytic Zn (Cys-38 and Cys-148) were also treated as sulfides. Cys-259 (MoADH) and Cys-35, Cys-124, and Cys-257 (GaADH) were treated at the protonated states, and His-7, His-76, His-94, and His-232 (MoADH) and His-76, His-108, and His-232 residues (GaADH) were protonated only at the Nε site. The rest of His residues were protonated only at the Nδ site, to maintain the hydrogen bonding network within the crystal structures. All crystallographic water molecules are retained within each crystal structure. Two samples (MoADH and GaADH) were thus, prepared in a consistent way. The all-atom models, as defined previously, were embedded in a triclinic box of around 8.5 nm × 7.6 nm × 9.4 nm in the x, y, and z dimensions, respectively. Around 18 000 SPC/E water molecules31 were used to hydrate each protein. Ion (K+ and Cl−) concentration at 120 mM was added to mimic the physiological salt concentration, in addition to a K+ surplus to neutralize the protein charges in each sample, resulting in models of around 60 800 atoms. The Amber03 protein force field was used for the residues and ions.

4.5.2. Equilibration—Production Setup. The equilibration—relaxation for the all-atom systems is employed based on published protocols.32 This contains a steepest descent energy minimization with a tolerance of 0.5 kJ mol−1 for 1000 steps and a sequence of isothermal (nVT), isothermal–isobaric (nPT) runs with the gradual relaxation of the constraints on protein heavy atoms (from 104 in steps 1−2 to 106 kJ mol−1 nm−2 in step 4) and Ca atoms (from 105 in step 5 to 102 in step 6, 10 in step 7, 1 in step 8, and 0 kJ mol−1 nm−2 in step 9) for around 30 ns, with a time steps of 1.0 fs (steps 1−4) and 2.0 fs (steps 5−9). The details are as follows: (step 1) constant-density and -temperature (nVT) Brownian dynamics (BD) at 100 K for 50 ps that employs the Berendsen
thermostat, with a temperature coupling constant at 1.0 fs\textsuperscript{44} (steps 2–3) two short constant-density (nVT) and constant-pressure (nPT) runs for 100 ps each, with a weak coupling Berendsen thermostat and barostat\textsuperscript{43} at 100 K employing time coupling constants of 0.1 ps for the temperature and isotropic 50.0 ps coupling for the pressure with a compressibility of \(4.6 \times 10^{-5}\). (step 4) heating from 100 to 250 K in a constant-density ensemble (nVT) for 3 ns employing the v-rescale thermostat,\textsuperscript{45} with a time coupling constant of 0.1 ps; (step 5) heating from 250 to 280, 310, or 340 K in a constant-pressure ensemble (nPT) for 2 ns, employing the v-rescale thermostat\textsuperscript{46} and Berendsen barostat,\textsuperscript{43} with time coupling constants of 0.1 ps for the temperature and 2.0 ps for the pressure, removing also all but the Ca-atom protein position restraints; (step 6) equilibration at 280, 310, or 340 K (0.1 ps temperature coupling constant) for 5 ns (nPT, 1 atm, 2.0 ps coupling constant for pressure); (steps 7–8) equilibration at 280, 310, or 340 K (0.5 ps temperature coupling constant) for 5 ns (nPT, 1 atm, 2.0 ps coupling constant for pressure); and (step 9) equilibration at 280, 310, or 340 K (0.5 ps temperature coupling constant) for 10 ns (nPT, 1 atm, 2.0 ps coupling constant for pressure). The barostats—thermostats employed for steps 6–9 were the same as in the production trajectories that follow.

For the production all-atom classical molecular dynamics (MD), Newton’s equations of motion are integrated with a time step of 2.0 fs at three different temperatures (280, 310, and 340 K). All production simulations are run with the leapfrog integrator in GROMACS 5.1.5\textsuperscript{46} for 150 ns each. They were performed at the constant-pressure nPT ensemble, with isotropic coupling (compressibility at \(4.5 \times 10^{-5}\)) employing the v-rescale thermostat\textsuperscript{45} (280, 310, or 340 K, temperature coupling constant 0.5) and the Parrinello– Rahman barostat\textsuperscript{47,48} (1 atm, pressure coupling constant 2.0). Details of parameters can be found in earlier work.\textsuperscript{43} The first 50 ns were considered for further equilibration from each independent trajectory per sample and were disregarded in the analysis. Van der Waals interactions were smoothly switched to zero between 1.0 and 1.2 nm with the VERLET cutoff scheme. Electrostatic interactions were truncated at 1.2 nm (short-range), and long-range contributions were computed within the PME approximation.\textsuperscript{49} All bond lengths were constrained employing the LINCS algorithm.\textsuperscript{50}

It was noticed that, throughout the MD trajectory of GsADH at 340 K, a water molecule was coordinated on the catalytic Zn site, whereas the MoADH trajectory at 340 K gave such a coordination only transiently. For the other temperatures (280 and 310 K), no water coordination was observed within the simulation time window (150 ns) in any protein. Therefore, we chose to extend the sampling at 340 K in a second independent trajectory of 150 ns per ADH. For these second independent trajectories, initial coordinates for GsADH were chosen from the 310 K trajectory, while for MoADH, they were chosen from the 340 K trajectory at the transient coordination state. Reassignment of velocities in a Maxwell distribution and de novo equilibration was performed for these initial structures, prior to the production. For these second trajectories, on GsADH, a water molecule was again coordinated at the catalytic Zn site (at around 60 ns), whereas for MoADH, the trajectory sampled exclusively the water coordinated on the catalytic Zn site. Results for both GsADH and MoADH reported at 340 K herein are averaged over the two independent trajectories (after 25 ns for the second sampling). The initial MoADH trajectory at 340 K was also extended up to 400 ns. In the latter, a stable water coordination was sampled on the catalytic Zn site, at around 270 ns and beyond. Thus, there must be a barrier associated with the water coordination to the catalytic Zn site, so the different catalytic Zn coordination states are only sampled at the highest (340 K) temperature. However, this barrier along with the associated minimum populations is beyond the scope of this study, and they will be addressed in a future work.

The produced root-mean-square (RMSF) values, per eigenvector, are weighted based on the respective eigenvalues or contributions to the total protein motion and averaged. To reproduce almost 60% of the total protein motion, less PCA eigenvectors are needed for GsADH (3 at 280 K, 7 at 310 K, and 3 at 340 K), while more are needed for the MoADH models (11 at 280 K, 6 at 310 K, and 5 at 340 K).

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01210.

MoADH rate constants for the oxidation of ethanol with increasing temperatures (Table S1), Arrhenius plot of MoADH kinetic data (Figure S1), thermal denaturation curve of MoADH based on circular dichroism data (Figure S2), interface Zn-binding site (Figure S3), superposition of protomers of MoADH and GsADH (Figure S4), comparison of Zn(II)-site geometries of selected alcohol dehydrogenases (Table S2), kinetic parameters of the compared alcohol dehydrogenases (Table S3), intermolecular contacts between the subunits of tetrameric structures of alcohol dehydrogenases (Table S4), and buried surfaces and cavities among subunits of related ADH structures (Table S5) (PDF)

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Notes
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ABBREVIATIONS
MoADH, alcohol dehydrogenase from Moraxella sp. TAE123; FjADH, alcohol dehydrogenase from Flavobacterium frigidimaritis KUC-1; EzADH, alcohol dehydrogenase from Escherichia coli; GsADH, alcohol dehydrogenase from Geobacillus stearothermophilus; ThADH, alcohol dehydrogenase from Thermus sp. ATN1; B-factor, atomic displacement parameter; temperature factor; CD, circular dichroism spectropolarimetry; MD, molecular dynamics; PDB, Protein Data Bank; PCA, principal component analysis; RMSF, root-mean-square fluctuations; SASA, solvent (water) accessible surface area

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