Evidence for Increased Local Flexibility in Psychrophilic Alcohol Dehydrogenase Relative to Its Thermophilic Homologue†

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ABSTRACT: The psychrophilic alcohol dehydrogenase (psADH) cloned from Antarctic *Moraxella sp.* TAE123 exhibits distinctive catalytic parameters in relation to the homologous thermophilic alcohol dehydrogenase (htADH) from *Bacillus stearothermophilus* LLD-R. Amide hydrogen−deuterium (H/D) exchange studies using Fourier-transformed infrared (FTIR) spectroscopy and mass spectrometry (MS) were conducted to investigate whether the differences are caused by variation in either global or regional protein flexibility. The FTIR H/D exchange study suggested that psADH does not share similar global flexibility with htADH at their physiologically relevant temperatures as has been predicted by the "corresponding state" hypothesis. However, the MS H/D exchange study revealed a more complicated picture concerning the flexibility of the two homologous enzymes. Analysis of the deuteration and exchange rates of protein-derived peptides suggested that only some functionally important regions in psADH that are involved in substrate and cofactor binding exhibit greater flexibility compared to htADH at low temperature (10 °C). These observations strongly suggest that variable conformational flexibility between the two protein forms is a local phenomenon, and that global H/D exchange measurement by FTIR can be misleading and should be used with discretion. These results are supportive of the idea that functionally important local flexibility can be uncoupled from global thermal stability. The structural factors underlying the differences in local protein flexibility and catalysis between htADH and psADH are discussed in conjunction with results from crystallographic and fluorescence spectroscopy studies.

Thermophilic enzymes are characterized by their thermal stability, resistance to denaturant, and reduced catalytic efficiency at low temperature. On the other hand, psychrophilic enzymes are known for their poor thermal stability, vulnerability to denaturant, and high catalytic efficiency in cold environments. There appears to be an inverse correlation between catalytic efficiency and protein stability, as summarized by a hypothesis that links catalytic efficiency and stability to the conformational flexibility of an enzyme (*1*, *2*). It was postulated that thermophilic enzymes gain their thermostability by reducing conformational flexibility and sacrificing catalytic efficiency below physiologically relevant temperatures, and that psychrophilic enzymes maintain their flexibility and catalytic efficiency at the expense of their thermostability (*3*). Another closely related hypothesis is the "corresponding state" hypothesis first proposed by Somero (*1*). The corresponding state hypothesis postulates that psychrophilic enzymes are generally more flexible than their mesophilic and thermophilic homologues at low temperature, and that homologues exhibit comparable flexibilities at their physiologically relevant temperatures.

In recent years, studies of orthologous homologues of lactate dehydrogenases-A from fishes adapted to different temperatures suggested that the evolutionary adaptation of enzymes to temperature is achieved by minor adjustment of flexibility in regions outside of the active site (*4*−*6*). Directed-evolution studies of thermophilic indoleglycerol phosphate synthase, xylose isomerase, and mesophilic *para*-nitrobenzyl esterase showed that it is possible to decouple the catalytic efficiency of an enzyme from its global thermostability, suggesting that only local protein flexibility is critical for catalysis (*7*−*9*). However, direct experimental evidence supporting the temperature-dependent adaptation of either global or local flexibility in enzymes is rare, due to the paucity of experimental methods for probing protein flexibility. Differences in protein flexibility among psychrophilic, mesophilic, and thermophilic proteins have been suggested from the comparisons of thermal factors (*B*-factor) between the crystal structures of homologous protein forms

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(*10*). This comparison suffers from the fact that *B*-factors are affected by other contributions such as resolution, crystal packing, solvent content and experimental temperature. Another approach to probe conformational flexibility is to measure the accessibility of intrinsic tryptophan residue(s), by monitoring the quenching of protein intrinsic fluorescence by small quencher molecules such as acrylamide (*11*, *12*). The limitation of this method is that meaningful comparisons rely on enzymes having the same number of intrinsic fluorophores located at identical positions, a requirement that is not met in most homologous enzymes. Lastly, amide hydrogen−deuterium (H/D)[1] exchange by Fourier-transformed infrared (FTIR) spectroscopy has been employed in recent years to compare the global flexibility of several pairs of homologous proteins (*13*−*15*).

The bacterial thermophilic alcohol dehydrogenase (htADH) cloned from *Bacillus stearothermophilus* strain LLD-R catalyzes the oxidation of alcohol substrates with a large activation energy ($E_a = 21.8$ kcal mol^{-1}) in the temperature range of 4−30 °C, but a reduced activation energy ($E_a =$ 15.2 kcal mol^{-1}) in the temperature range of 30−65 °C (*16*). In contrast, the homologous bacterial psychrophilic alcohol dehydrogenase (psADH) cloned from Antarctic *Moraxella sp.* TAE123 exhibits very different kinetic and activation parameters compared to htADH, including a much smaller E_a (9.8 kcal mol^{-1}) and larger Michaelis−Menten constant (K_m) for substrate (*17*). The differences in kinetic and activation parameters have been attributed to increased global or regional flexibility (disorder) in psADH according to a model that takes the relationship between the hydride-transfer reaction coordinate and protein motions into account (*17*). To provide support for the increased flexibility in psADH, global H/D exchange measurement using FTIR spectroscopy was carried out for psADH, for comparison to htADH and the mesophilic yeast ADH at their physiologically relevant temperatures. In addition, H/D exchange measurements by mass spectrometry was conducted with psADH and htADH at a temperature (10 °C) close to the physiologically relevant temperature of psADH (4 °C) but much lower than that of htADH (70 °C). The analysis of the extent of deuteration and exchange rates for protein-derived peptides provides insight into differences in local protein flexibility. The structural factors underlying the observed differences in H/D exchange between htADH and psADH are discussed in conjunction with the available structural information.

MATERIALS AND METHODS

Materials and Protein Cloning. NAD^+ was purchased from Sigma without further purification. The cloning of htADH and psADH from *B. stearothermophilus* strain LLD-R (NCIMB 12403) and Antarctic *Moraxella sp.* TAE123 is described elsewhere (*18*, *19*).

Global H/D Exchange by FTIR. H/D exchange data for htADH and yeast ADH (YADH) have been reported previously (*15*). H/D exchange measurement with psADH was carried out at 4 °C following the same procedure. For sample preparation, psADH was lyophilized after being exchanged into the working buffer (50 mM KPi, 100 mM KCl, pH 7.0) by dialysis. Measurements were performed on an Applied Systems ReactIR 1000 (ASI) spectrometer with CaF_2 windows (path length: 100 μm). The H/D exchange was initiated by dissolving ca. 2 mg of the lyophilized protein in 100 μL of D_2O. The data collection started after a delay time of 2 min and lasted for 48 h. Parallel activity assay showed that 10−15% of the enzyme activity is lost within the experimental time frame. Deuteron incorporation was readily followed by monitoring the disappearance of the 1550 nm amide II band. The fraction of the nonexchanged amide H's was calculated on the basis of the ratio of the amide II band absorbance at time t and t_0. Relaxation spectra were obtained by plotting the percentage of nonexchanged amide H's vs log (k_0t), where k_0 is the theoretical chemical exchange rate constant at the given temperature (*13*).

H/D Exchange by Mass Spectrometry. H/D exchange measurements and data analysis were carried out following published procedures (*18*, *20*−*22*). Briefly, the exchange reactions were initiated by adding 10 μL of newly thawed protein solution (~0.5 mg/mL, 50 mM KPi, pH 7.4, 250 mM KCl, and 2.5 mM DTT) to 90 μL of D_2O (for htADH) or 90 μL of D_2O containing 100 mM of KCl (for psADH). After the incubation, the exchange reaction was quickly quenched by lowering the pH to 2.0 and temperature to 0 °C, and was followed by rapid pepsin digestion. The resulting peptides were analyzed on a reversed phase capillary column (POROS 20 R1, PerSeptive) coupled to a QStar Pulsar quadrupole time-of-flight (TOF) mass spectrometer with normal electrospray interface (PE Biosystems). The average time from the initiation of digestion to the elution of all peptides is about 22 min. Zero time point controls (the "artifactual in-exchange" control) were performed by adding quenching buffer prior to D_2O. Measured peptide masses were corrected for exchange at $t = 0$, normalized to 100% D_2O, and corrected for back-exchange following published methods (*20*). Typically 12 data points were collected with 7 s and 160 min as the shortest and longest incubation times, respectively. Kinetics of exchange using corrected peptide masses were fitted by nonlinear least squares to the equation $Y = N - Ae^{-k_1t} - Be^{-k_2t} - Ce^{-k_3t}$, where N is the total number of amides exchanging over the observed time course for each individual peptide, and A, B, and C correspond to the number of amides exchanging with rate constants k_1, k_2, and k_3, respectively. The number of nonexchanging amide H's is calculated by subtracting the number of exchanging amides ($A + B + C$) from the total number of backbone amides (N_H) in the peptide, excluding proline residues.

Fluorescence Spectroscopy. Fluorescence spectra were taken at 10 °C on a FluoroMax-3 fluorometer with excitation wavelength set at 295 nm to selectively excite tryptophan residues. Buffer (50 mM KPi, pH 7.4, 100 mM KCl) and protein solution were filtered and degassed shortly before measurement.

Homology Modeling. The crystal structures of htADH and *Escherichia coli* ADH have been determined recently (*23*, *24*). Using the structures of htADH and *E. coli* ADH as templates, a homology model of psADH was constructed and energy-minimized using Swiss-Model and Insight II (*25*).

[1] Abbreviations: htADH, thermophilic alcohol dehydrogenase; psADH, psychrophilic alcohol dehydrogenase; MS H/D exchange, hydrogen−deuterium exchange by mass spectrometry; FTIR H/D exchange, hydrogen−deuterium exchange by Fourier-transformed infrared spectroscopy; LC−MS/MS, liquid chromatography coupled tandem mass spectrometry.

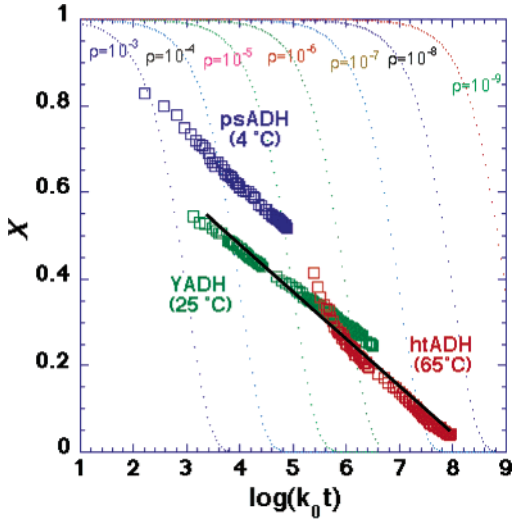

FIGURE 1: H/D exchange probability distribution as presented in the form of relaxation spectra for psADH (4 °C), YADH (25 °C), and htADH (65 °C) as measured by FTIR spectroscopy. *X* is the fraction of unexchanged peptide hydrogens, *t* is time in seconds, and k_0 is the calculated chemical exchange rate constant at the relevant temperature. The dotted lines represent exchange curves for hypothetical polypeptides characterized by a ρ value, which is defined as the probability of finding a particular hydrogen exposed to the solvent (*13*). Coincidence of the curves of YADH and htADH as indicated by the solid straight line has previously been reported to suggest similar flexibility (*15*).

RESULTS

Global H/D Exchange. By monitoring the disappearance of the amide N−H band at 1550 nm, FTIR spectroscopy offers a convenient tool for studying protein H/D exchange. Data are presented for psADH in the traditional form of a relaxation spectrum, with the fraction of unexchanged hydrogens plotted against $\ln(k_0t)$ (Figure 1). In this representation, k_0 is the calculated intrinsic exchange rate constant at a given temperature and *t* is the time of exchange. According to the corresponding state hypothesis, global H/D exchange profiles for homologous proteins that share similar flexibility at their functional temperatures should fall approximately on the same line (*13*). As can be seen in Figure 1, the exchange profiles for htADH (65 °C) and YADH (25 °C) appear to coincide, but the profile for psADH is shifted to lower ρ values and does not coincide with the other two. This indicates that psADH at 4 °C does not share a similar global flexibility with htADH and YADH at their physiological temperatures, with psADH being more rigid overall than YADH and htADH at their physiological temperatures.

Local H/D Exchange. Peptides generated by pepsin digestion were identified by LC−MS/MS experiments and found to encompass 95% (htADH) and 90% (psADH) of the polypeptide sequences (Figure 2). H/D exchange was carried out using cofactor-free enzymes under identical conditions, except that 100 mM KCl was present with psADH to prevent the enzyme from losing its quaternary structure. The difference in salt concentration in the exchange of psADH is not expected to compromise the results, as several studies have shown that salt has a negligible effect on H/D exchange rate (*13*, *26*). Independent activity assays confirmed that both enzymes maintain over 95% of their activities after incubation at 10 °C for 180 min under the exchange conditions.

For each individual peptide, the number of incorporated deuterons (N_D) at a given experimental time point is the difference between the weighted average mass of the peptide before and after H/D exchange corrected for back-exchange (*20*). Only the exchange of backbone amide hydrogens is monitored because deuterons incorporated into side chains completely back-exchange with H_2O during HPLC (*20*). The plots of N_D vs time were fitted with a three-exponential model to give the number of exchanging H's as well as the exchange rate constants k_1, k_2, and k_3 for each peptide. On the basis of their exchange rates, the amide H's are categorized into four groups: fast-exchanging H's ($k_{ex} > 1$ min^{-1}), intermediate-exchanging H's (1 min^{-1} > k_{ex} > 0.1 min^{-1}), slow-exchanging H's (0.1 min^{-1} > k_{ex} > 0.001 min^{-1}), and nonexchanging H's. The total numbers of fast, intermediate, and slow-exchanging H's for the whole protein are obtained by summing over all peptides. Unexpectedly, the overall extent of deuteration is almost identical for htADH (42%) and psADH (41%). However, there are 22% fast-exchanging H's, 11% intermediate-exchanging H's, and 9% slow-exchanging H's for htADH, in contrast to 14% fast-exchanging H's, 4% intermediate-exchanging H's, and 23% slow-exchanging H's for psADH. The excess in slow-exchanging H's for psADH is evidenced by the fact that the exchange for most of the psADH peptides does not reach a plateau within 160 min. (See data in Supporting Information.)

The variation in deuteration is illustrated by comparing the H/D exchange of "homologous peptides", which are generated when pepsin cleaves the polypeptides of htADH and psADH at identical or very similar positions. There are five pairs of homologous peptides: T3/P3, T4/P4, T7/P6, T8/P8, T10/P9, and T15/P14 (see Figure 2). Some of the homologous peptides differ in composition by a few residues. We also "bundled" consecutive peptides to give five pairs of bundled homologous peptides that include (T1-T2)/(P1-P2), (T5-T6)/P5, (T11-T12)/(P11-P12), and (T18-T19-T20)/(P16-P17-P18). Among the 10 pair of single or bundled homologous peptides, which cover ~75% of the polypeptide sequences, only one pair (T1-T2)/(P1-P2) shows almost identical deuteration progression curves (data not shown). Among the remaining nine pairs, four exhibit substantially lower deuteration in psADH than the corresponding peptides in htADH (Figure 3). An examination of the numbers of exchanging H's for these peptides shows that the differences in deuteration are mainly because of decreases in the numbers of fast- and intermediate-exchanging H's for psADH peptides. (See Figure S3 in Supporting Information.) By contrast, the remaining five peptides in psADH show increased deuteration compared with the corresponding peptides in htADH (Figure 4). Note that although the deuteration level of P14 appears to be lower than that of T15 at the end of 160 min (Figure 4e), the number of exchanging H's obtained from model fitting suggests that P14 has more exchangeable H's than T15, indicating that P14 has some slow-exchanging amide H's that have not exchanged within the experimental time frame. The fast exchange rates and low deuteration for the htADH peptides, and the slow exchange rates and high deuteration for the psADH peptides, give rise to the crossover between each set of curves in Figure 4.

FIGURE 2: Alignment of htADH and psADH sequences. The overlaying and underlying bars represent the peptides generated by pepsin digestion, where T-peptides are from the thermophilic htADH and P-peptides from the psychrophilic psADH.

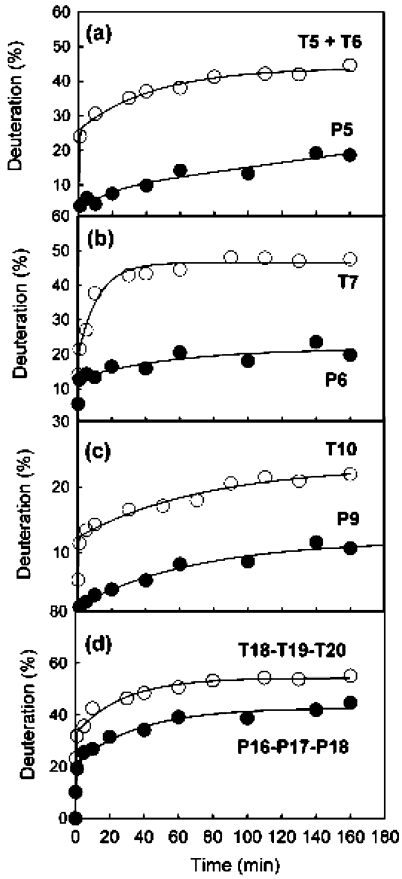

FIGURE 3: Comparison of hydrogen−deuterium exchange time-course plots for homologous peptides (htADH peptides (○), psADH peptides (●)). The four peptides of psADH (P5, P6, P9, and P16-P17-P18) show lower deuteration relative to their homologous peptides in htADH (T5-T6, T7, T10, and T18-T19-T20).

Fluorescence Spectroscopy. psADH has four intrinsic tryptophan residues, and htADH only has three. The fluorescence λ_{max} for psADH (345 nm) is unexpectedly red shifted by 9 nm relative to those of htADH, yeast and horse liver ADHs (336 nm). (See data in Supporting Information.) This red-shifted λ_{max} suggests that either all the tryptophan residues in psADH are more exposed to solvent or the extra tryptophan residue is solvent exposed and dominates the fluorescence spectrum.

DISCUSSION

Kinetic and thermodynamic activation parameters for psADH and htADH obtained from steady-state kinetic measurements have been reported recently (*17*). htADH and psADH exhibit comparable catalytic turnover rates at 10 °C, in contrast to the trend that psychrophilic enzymes generally show greater turnover numbers (k_{cat}) at low temperature than their thermophilic homologues (*27*). However, the Michaelis−Menten constant (K_m (benzyl alcohol)) for psADH (11.8 mM) is significantly greater than that of htADH (3.6 mM), consistent with the observations that cold-adapted enzymes usually have greater K_m for substrates (*5*). Activation parameters derived from temperature-dependent kinetic measurements revealed significant differences in both the enthalpy of activation ($\Delta H^{\ddagger}$) and entropy of activation ($\Delta S^{\ddagger}$). psADH exhibits a smaller enthalpy of activation ($\Delta\Delta H^{\ddagger} = -12.0$ kcal mol^{-1}), and a negative entropy of activation (-24.2 cal mol^{-1} K^{-1}) relative to a positive value of 18.4 cal mol^{-1} K^{-1} for htADH ($\Delta\Delta S^{\ddagger} = -42.6$ eu). These differences in catalytic parameters have been attributed to a significantly increased disorder or flexibility in the ground state of psADH relative to htADH, which entropically reduces the probability of the psADH−NAD$^+$−substrate ternary complex achieving the optimal configuration for hydride transfer (*17*). The H/D exchange results presented here allow a spatial resolution of changes in protein flexibility and suggest that the enhancement of protein flexibility in psADH occurs in a local rather than global fashion.

Global H/D Exchange and Its Limitation. H/D exchange by FTIR spectroscopy detects hydrogens that exchange at intermediate and slow rates due to a delay time of ca. 2 min and long exchange time (48 h). This method has been used

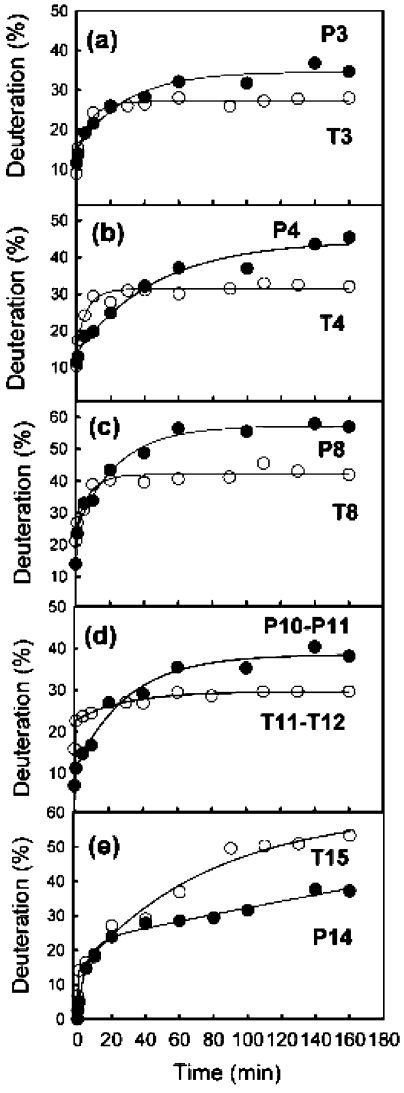

FIGURE 4: Comparison of hydrogen−deuterium exchange time-course plots for homologous peptides (htADH peptides (○), psADH peptides (●)). The five peptides of psADH (P3, P4, P8, P10-P11, and P14) show higher deuteration relative to their homologous peptides in htADH (T3, T4, T8, T11-T12, and T15).

to study the flexibility of several pairs of thermophilic and mesophilic enzymes such as 3-isopropylmalate dehydrogenases from *E. coli* and *T. thermophilus* (*13*), glyceraldehyde phosphate dehydrogenases from *T. maritima* and rabbit (*28*), and alcohol dehyrogenases from *B. stearothermophilus* (htADH) and yeast (*15*). These studies showed that the exchange profiles of the thermophilic and mesophilic homologues near their physiological temperatures are positioned close to each other. The authors suggested that the proximity of the exchange profiles support the corresponding state hypothesis, which predicts that orthologous psychrophilic, mesophilic, and thermophilic proteins will share similar flexibility at their physiological temperatures (*1*, *13*). Following the same logic, the shift of the psADH profile in Figure 1 from those of htADH and YADH would suggest that psADH is less flexible than htADH and YADH at their corresponding physiological temperatures. Decreased global H/D exchange was also observed for the cold-adapted 3-isopropylmalate dehydrogenase relative to its mesophilic and thermophilic homologues (*14*). In light of the present MS H/D exchange study, it appears that proteins should not be treated as homogeneous objects with uniform H/D exchange rates throughout the peptide backbone. The "corresponding state" hypothesis may only be appropriate if considered in the context of specific functional regions of proteins, i.e., because variations in protein flexibility may be expected to be local, averaged global H/D exchange measurements using FTIR spectroscopy may be misleading.

Local H/D Exchange. Although some aspects of the H/D exchange mechanism are still under debate, most current models explain the exchange of a native protein with D_2O by invoking transient structural fluctuations that allow the access of the catalyst OD^- (*29*−*32*). H/D exchange processes can be described by a "two-state" model as illustrated by eq 1.

$$E_{closed}(H) \underset{k_{close}}{\overset{k_{open}}{\rightleftharpoons}} E_{open}(H) \xrightarrow{k_{ex}} E(D) \qquad (1)$$

Under native conditions, the closing step is much faster than the exchange step ($k_{close} \gg k_{ex}$), corresponding to the so-called EX2 limit. In this case, $k_{H/D}$ is a composite rate constant and can be expressed as the product of k_{ex} and K_{eq}, i.e. $k_{H/D} \approx K_{eq}k_{ex} = (k_{open}/k_{close})k_{ex}$. Under unfolding conditions, the opening step becomes rate-limiting ($k_{close} \ll k_{ex}$) and exchange is in the EX1 limit whereby $k_{H/D} \approx k_{open}$. In the EX1 limit, bimodal isotope patterns are anticipated in the mass spectra of peptides (*20*, *33*), which were not observed in this study.

Under native conditions as for the current case, opening/closing is achieved either by fast local fluctuations or by slow fluctuations involving collective motions. Fast fluctuations are usually associated with rapidly exchanging amide hydrogens located on flexible surface loops, and collective fluctuations are often associated with more protected amide hydrogens that reside inside the protein core or are involved in strong hydrogen-bonding (*34*). Given the high sequence homology between htADH and psADH, the intrinsic exchange rate constant k_{ex} should be similar for the homologous peptides. This was confirmed by the calculation of the average k_{ex} for all the homologous peptides based on the methods described by Bai et al. (*26*). According to the equation $k_{H/D} \approx K_{eq}k_{ex}$, when variation in k_{ex} is small, exchange rates are governed by the equilibrium constant K_{eq}, or the free energy gap (ΔG) between states E_{closed} and E_{open}. On the basis of these considerations, it is logical to reason that a peptide located in a rigid protein environment that lacks large fluctuations may show low deuteration but fast H/D exchange rates (small ΔG and large K_{eq}), while the same peptide may show higher deuteration but slower exchange rates (large ΔG and small K_{eq}) as the surrounding protein scaffold becomes more mobile.

Four peptides in psADH show decreased deuteration relative to their corresponding homologous peptides in htADH (Figure 3 and Figure 5a). Considering the high sequence and structure similarity among the two protein forms and *E. coli* ADH, the decreases in deuteration for these psADH peptides are unlikely to arise from the differences in secondary and tertiary structures. Because the decreases in deuteration are mainly caused by the decreases in the number of fast- and intermediate-exchanging H's, rather than the number of slow-exchanging hydrogens, the differences in deuteration are likely caused by differences in fast local

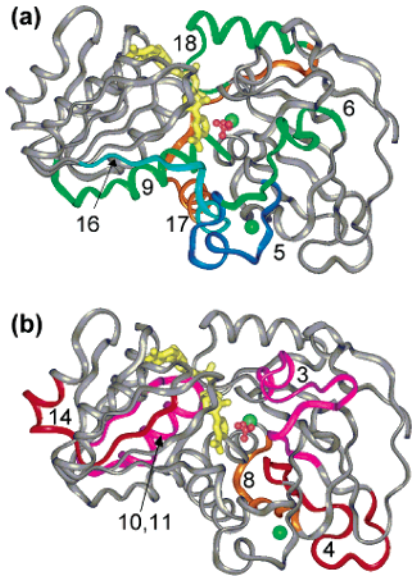

FIGURE 5: Structural representation of the psychrophilic ADH subunit model. The cofactor-binding domain is shown on the left, and the substrate-binding domain is on the right. The zinc ions (green) are shown in CPK representation, and the "modeled-in" NAD^+ (yellow) and substrate analogue trifluoroethanol (pink) are shown in ball-and-stick representation. (a) The peptides that display lower deuteration in comparison with their corresponding homologous peptides in htADH are colored and labeled. (b). The peptides that display higher deuteration in comparison with their corresponding homologous peptides in htADH are colored and labeled.

fluctuations with small amplitude. This interpretation is in accordance with the suggestion that adaptation of thermophilic proteins for a high-temperature environment has resulted in a restriction of large deviations from the native state and a corresponding increase in smaller scale fluctuations (*35*).

Most interestingly, five peptides in psADH exhibit higher deuteration but slower exchange rates compared to their htADH counterparts, with three of these residing in the catalytic domain (P3, P4, and P8), and the other two (P10−P11 and P14) in the cofactor binding domain (Figure 5b). An important feature of the plots in Figure 4 is that the curves for the htADH and psADH peptides cross over each other within the first 30 min. This crossover reflects the fact that T3, T4, T8, and (T11-T12) are dominated by fast, and intermediate-exchanging H's with lower deuteration, and that P3, P4, P8, and (P10-P11) are dominated by slow exchange rates but with higher deuteration. Slow-exchanging H's are often associated with large collective structural fluctuations because of the small K_{eq} resulting from a large free energy difference between the open and closed states. Increased deuteration and the dominance of slow-exchanging H's for psADH peptides compared to htADH peptides suggests that P3, P4, P8, P10, and P11 are located in environments capable of large-scale conformational fluctuations.

These conclusions are consistent with the results from a temperature-dependent H/D exchange study of htADH (*18*). It was found that 10 peptides, including T3, T4, and T8, were in a nonexchanging rigid environment at reduced temperatures (<30 °C), due to the lack of cooperative motions or conformational flexibility. At elevated temperatures, slow-exchanging hydrogens started to emerge, as a result of the activation of large structural fluctuations. The involvement of P3, P4, and P8 in substrate binding and P11 and P12 in cofactor binding implies that the observed differences are closely related to function and, thus, that under evolutionary pressure, enzymes may adjust conformational flexibility within regions important to catalysis. This is supportive of the view proposed by Somero and coworkers based on the systematic studies of orthologous homologues of lactate dehydrogenase-A from fishes adapted to different temperatures (*4*−*6*), and the view that catalytic efficiency can be altered by adjusting local, rather than global, flexibility on the basis of directed evolution and mutagenesis studies (*7*−*9*, *36*).

Structural Basis for Increased Flexibility psADH. The comparison of a psADH homology model with the crystal structures of htADH and *E. coli* ADH shows that the main chains of the three bacterial enzymes are almost superimposable, which is not surprising given the high sequence homology among the three protein forms (60% sequence identity and 76% sequence similarity between htADH and psADH). Comparison between the model of psADH and the structure of htADH reveals the absence of prolines in psADH at several key positions connecting helices and sheets. Proline is known to restrict conformational freedom and stiffen local structure, and, thus, the loss of these prolines is likely to increase local flexibility in psADH. Structural analysis also shows that psADH carries fewer bulky hydrophobic residues than htADH, particularly in the substrate-binding domain. The lack of bulky hydrophobic residues can cause poor protein packing and make the protein more soft or flexible. The deficiency in proline and bulky hydrophobic residues is probably the leading factor behind the increased local flexibility in psADH.

The red-shifted fluorescence λ_{max} for psADH, suggesting increased solvent exposure of Trp residue(s), may be one of the indicators of loose protein packing and increased protein flexibility. Increased exposure of hydrophobic residues to solvent is not unprecedented and has been seen in the crystal structures of other cold-adapted enzymes such as xylanse from *Pseudoalteromonas haloplanktis* and citrate synthase from Antarctic bacterial strain DS2-3R (*37*, *38*). Three of the four Trp's in psADH reside in the same location as their counterparts in htADH, including two on the peptides (P3 and P4) that show increased flexibility. Although there is a small possibility that the difference in λ_{max} is solely caused by the extra Trp in psADH, it is highly unlikely that the fluorescence spectrum would be dominated by a single Trp residue. It appears more reasonable to attribute the red-shifted fluorescence λ_{max} in psADH to contributions from all four Trp residues.

CONCLUSIONS

The H/D exchange results presented here provide evidence for enhanced local flexibility in psychrophilic ADH compared to its thermophilic homologue. The MS H/D exchange results suggest that only certain regions within psADH exhibit greater flexibility compared to htADH at reduced temperature. Although the picture is incomplete due to the missing peptides and the lack of comparable homologous peptides for some regions (P7, P13, and P15 do not have corresponding homologous peptides in htADH), the available comparison provides direct support for differences in local

flexibility between the two homologous ADHs. Most interestingly, the regions that exhibit enhanced flexibility in psADH are involved in substrate and cofactor binding and, thus, are likely to have functional implications. These results support the view that adjustments in local flexibility are likely to be a strategy used by psychrophilic enzymes for cold adaptation, and are consistent with the observation that psADH exhibits a very large reduction in the entropy of activation ($\Delta\Delta S^{\ddagger} = -42.6$ cal mol^{-1} K^{-1}), together with a smaller enthalpy of activation ($\Delta\Delta H^{\ddagger} = -12.0$ kcal mol^{-1}). Despite the reduction in its $\Delta H^{\ddagger}$, psADH catalyzes its hydride transfer only 1.6-fold faster than htADH at 5 °C (*17*), indicating the degree to which its reaction is under the control of an unfavorable activation entropy. With hydride transfer as the rate-determining step for both enzymes, these results can be rationalized by the recognition that the hydrogen-transfer coordinate is tightly coupled to surrounding protein heavy-atom motions (*17*, *39*, *40*), and that adjustments in protein dynamical properties can have a large impact on the hydrogen-transfer parameters: In the case of psADH, an "over-compensation" in active site flexibility may be the origin of the relatively small catalytic advantage, relative to htADH, at low temperature. It will be very interesting to see if rigidification of the flexible peptides in psADH (Figure 5b), via site specific mutagenesis, produces smaller values for $\Delta S^{\ddagger}$ and corresponding increases in k_{cat}.

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SUPPORTING INFORMATION AVAILABLE

Hydrogen−deuterium exchange time course plots, tables of least-squares fitted exchange parameters, and deuteration percentage of homologous peptides for htADH and psADH peptides. Normalized fluorescence spectra of htADH and psADH. This material is available free of charge via the Internet at http://pubs.acs.org.

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