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Thermodynamic and Structural Adaptation Differences between the Mesophilic and Psychrophilic Lactate Dehydrogenases

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Abstract

The thermodynamics of substrate binding and enzymatic activity of a glycolytic enzyme, lactate dehydrogenase (LDH), from both porcine heart, phLDH (*Sus scrofa*; a mesophile), and mackerel icefish, cgLDH (*Chamapscephalus gunnari*; a psychrophile), were investigated. Using a novel and quite sensitive fluorescence assay that can distinguish protein conformational changes close to and distal from the substrate binding pocket, a reversible global protein structural transition preceding the high-temperature transition (denaturation) was surprisingly found to coincide with a marked change in enzymatic activity for both LDHs. A similar reversible structural transition of the active site structure was observed for phLDH but not for cgLDH. An observed lower substrate binding affinity for cgLDH compared to that for phLDH was accompanied by a larger contribution of entropy to ΔG , which reflects a higher functional plasticity of the psychrophilic cgLDH compared to that of the mesophilic phLDH. The natural osmolyte, trimethylamine *N*-oxide (TMAO), increases stability and shifts all structural transitions to higher temperatures for both orthologs while simultaneously reducing catalytic activity. The presence of TMAO causes cgLDH to adopt catalytic parameters like those of phLDH in the absence of the osmolyte. Our results are most naturally understood within a model of enzyme dynamics whereby different conformations of the enzyme that have varied catalytic parameters (i.e., binding and catalytic proclivity) and whose population profiles are temperature-dependent and influenced by osmolytes interconvert among themselves. Our results also show that adaptation can be achieved by means other than gene mutations and complements the synchronic evolution of the cellular milieu.

Graphical abstract

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Notes

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Supporting Information

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A more detailed presentation of various emission studies (PDF)

One of the main challenges in investigating the adaptation of enzymes is understanding the evolutionary mechanisms involved with adapting to a wide range of environmental conditions, particularly to the operating temperature of a specific organism. Low-temperature-adapted enzymes tend to have decreased affinity for their substrate (K_m) and markedly increased chemical reaction rates (k_{cat}) compared to those of their higher-temperature counterparts at the same temperature. A common notion is that these issues are related to enzyme “flexibility” in some sense. In particular, a higher rate of reaction for one isozyme compared to another at the same temperature is usually associated with higher structural flexibility and inversely related to protein stability or substrate affinity.^{1–5}

The glycolytic enzyme lactate dehydrogenase (LDH) serves as an excellent model system for studying the thermal adaptation of enzymes.^{1,5} LDHs catalyze the conversion of pyruvate and NADH to lactate and NAD⁺, which is accompanied by hydride and proton transfer. The structural and kinetic dynamics of a variety of LDH isoforms^{6–8} have been characterized, and the crystal structures for LDHs from mesophilic, psychrophilic, and thermophilic organisms have been determined, though at different resolution, and can be used for comparison.^{9–11} It was shown by infrared (IR) spectroscopy¹² and X-ray analysis^{9–11} that the geometry and the electrostatic fields of the active sites of most (if not all) LDHs are virtually identical, showing no differences between isoforms isolated from prokaryotic and eukaryotic or mesophilic and thermophilic organisms despite the considerable lack of homology in the amino acids that are peripheral to the active site. The thermodynamic parameters of activation for enzymes adapted to different temperatures are largely dissimilar^{4,13} and can be attributed to minor changes in the LDH primary structure.^{9,11} These findings demonstrate that the adaptation differences in k_{cat} and/or K_m among the various LDHs are likely due to effects of global conformational changes of the protein rather than structural changes close to the active site. Thus, the detailed bonding patterns of the substrate within the active site of temperature-adapted LDH orthologs are insufficient to explain the mechanism of thermal adaptation.

These observations assume that the mechanism of thermal adaptation for enzymes lies within their dynamics.¹⁴ Steady-state¹⁵ and kinetic^{6,8} studies have shown that LDH, as an apoenzyme, the LDH/NADH binary complex, or the LDH/NADH/substrate ternary complex occupies a range of interconverting conformations either competent or non-competent for binding ligands. As we have shown in some LDHs,^{8,16} a substantial amount of the protein undergoes a remarkable major transient conformational change (partially unfolded) for its

substrate (pyruvate or lactate) to reach the binding pocket.^{16,17} This suggests that the formation of ternary complexes competent for chemistry is preceded by the formation of looser ternary structures. Moreover, several experimental^{18–20} and theoretical²¹ analyses come to a similar conclusion that the ternary complex, LDH/NADH/substrate, exists as a set of interconverting conformations with a range of specific proclivities toward on enzyme catalysis.

To further understand the relationship between enzyme dynamics and thermal adaptation, we conducted extensive fluorescence studies to establish thermodynamic parameters for the reaction between the substrate mimic oxamate and lactate dehydrogenase from mesophilic porcine heart (phLDH, *Sus scrofa*) and psychrophilic mackerel icefish (cgLDH, *Chamapsocephalus gunnari*). We employed the inhibitor oxamate because its binding kinetics are essentially identical to those of the substrate pyruvate and the complex does not undergo catalysis, which greatly complicates data analysis.^{22,23} It is important to distinguish the difference between global and local structural changes of the enzyme in relation to its function. For example, ligand binding and release by a protein induces a wide range of structural changes, varying from small movements of loops or side chains in the binding pocket to large-scale domain hinge-bending and shear motions as well as partial unfolding that facilitates the capture and release of a ligand.^{7,16} To this aim, we developed a novel three-in-one excitation assay that simultaneously monitors changes to the global protein structure, structural changes near the active site, and aggregation of the enzyme in response to an increase in temperature and an increase in concentration of the osmolyte trimethylamine *N*-oxide (TMAO). We believe this assay to be particularly useful for our study as both phLDH and cgLDH are tetramers that contain six tryptophan residues per monomer at the same positions (150, 190, 203, 225, 248, and 323 for phLDH and 149, 189, 202, 224, 247, and 322 for cgLDH), which allows us to simultaneously monitor the fluorescence and fluorescence resonance energy transfer (FRET) of these residues in response to changes in solution conditions.

Herein, we demonstrate a reversible structural transition for both orthologs at “physiological” temperatures preceding the irreversible structural transition that is accompanied by aggregation. The observed differences in the stability and temperature transitions preceding the denaturing of LDH support the hypothesis for the existence of a reversible equilibrium between different structural forms of the enzyme, which differ in function (i.e., k_{cat}), preceding its irreversible denaturation. The natural osmolyte, TMAO, increases stability and shifts all structural transitions to the higher temperatures for both orthologs and simultaneously reduces their catalytic activity. The presence of TMAO causes cgLDH to adopt a structural transition leading to an activation energy for the catalytic reaction similar to those for phLDH in the absence of the osmolyte. We also measured the relative entropy–enthalpy contribution to the free energy of the substrate binding to provide a quantitative basis for the discussion of the energetic contribution to the evolution of the enzyme adaptation to different thermal conditions.

EXPERIMENTAL PROCEDURES

Proteins and Other Reagents

Porcine heart LDH (phLDH) was purchased from Sigma-Aldrich (catalog no. L725, lot no. SLBM5803V). The mackerel icefish *C. gunnari* lactate dehydrogenase (cgLDH) gene cloned into pJexpress404 vectors was purchased from DNA2.0 Inc. These vectors were freshly transformed into BL21 cells each time for protein expression. cgLDH was purified with primarily ion exchange and gel filtration as needed. Both phLDH and cgLDH were stored in a suspension of approximately 70–80% ammonium sulfate at 4 °C. Amicon Ultra-4, 30K molecular weight cutoff centrifugal filters (Merck Millipore) were used for desalting and buffer exchange of phLDH and cgLDH prior to performing assays.

Fluorescence and Absorption Spectroscopy

All experiments were performed in buffer containing 100 mM sodium phosphate buffer in the absence or presence of the natural osmolyte TMAO at pH 7.2 over a range of temperatures (20–85 °C). Absorption measurements were taken with a NanoDrop 2000 ultraviolet–visible (UV–vis) spectrophotometer and a Cary 100 Bio UV–vis spectrophotometer to collect k_{cat} data. Fluorescence measurements were taken with a Jobin Yvon (Edison, NJ) Fluoromax-4 spectrofluorometer. The intensity of the Raman scattering band of water was used as the internal standard of fluorometer sensitivity. Intrinsic tryptophan and NADH fluorescence was used as a measure of the structural changes of the binary and ternary LDH complexes (Figure S1). Oxamate binding quenches tryptophan and NADH emission in the ternary Michaelis complex (Figure S2). Protein emission ($\lambda_{\text{max}} \sim 348$ nm), NADH emission ($\lambda_{\text{max}} \sim 442$ nm), and NADH excitation ($\lambda_{\text{max}} \sim 280$ and 340 nm) can be used to measure the fluorescence of LDH (Figure S2). We used the “280 nm” and “340 nm” peaks in the excitation spectrum as a measure of FRET and active site structure changes, respectively (Figure 1). The “280 nm” peak in the NADH excitation spectrum arises from FRET of the six Trp residues similarly placed in both proteins studied here (at positions 150, 190, 203, 225, 248, and 323 for phLDH and positions 149, 189, 202, 224, 247, and 322 for cgLDH), while the “340 nm” peak is direct absorption of the light by NADH. We used emission at 470 nm to register NADH excitation, shifted from the emission maximum at 442 nm (Figure S1B) to avoid overlap with the protein emission (Figure S1A).

Light Scattering and “Three-in-One” Excitation Assay

Elastic light scattering (ELS) collected at an angle 90° to the incident illumination as a control for protein aggregation was also recorded using the Fluoromax-4 spectrofluorometer. Although the same wavelength for incident and scattering light is usually used in the ELS experiments, in our light scattering experiments we used the second order of the monochromator grating with the experimental excitation and emission setup of 240 and 470 nm, respectively. When a parallel beam of monochromatic light is incident on a grating, the relationship between the wavelength of the light (λ) and the angle of diffraction (Θ) is $\lambda = (d/n) \times \sin \Theta$, where d is the distance between grating lines of the monochromator diffraction grating and n is the order number. The light is diffracted from the grating in directions corresponding to $n = -2, -1, 0, 1, 2, 3$, etc., and dispersed so that each wavelength satisfies the diffraction grating equation. The first and second order of the monochromator diffraction

grating are overlapping, and we used the second order of the monochromator grating for registration of the light scattering.

In fact, the setup of 240 and 470 nm (excitation and emission monochromators, respectively) was used to measure light scattering of the aggregated protein solution (Figure S3). Control experiments, including those with 350 and 350 nm, and 240 and 470 nm, wavelength combinations of the incident and scattered light, were conducted to determine a set of experimental parameters suitable for the monitoring of light scattering. Thus, all three properties [light scattering, fluorescence resonance energy transfer (FRET), and NADH fluorescence] could be measured simultaneously via excitation at 240 nm (light scattering), 280 nm (FRET), and 340 nm (NADH fluorescence) using a fixed emission at 470 nm (Figure 1).

Equilibrium Binding of Oxamate to the Binary LDH/NADH Complex

The binding isotherms were obtained by titration of the binary LDH/NADH complex at a constant concentration of 4 μM with increasing concentrations of oxamate. Because the concentration of the binary complex is comparable to or relatively higher than the binding affinity of the complex, the simple assumption that $[\text{ligand}]_{\text{total}} \approx [\text{ligand}]_{\text{free}}$ cannot be used in the analysis of the titrations of this study. In this case, the equation for the binding isotherms should contain concentrations of all reactants.²⁴ The fractional saturation of the LDH/NADH monomer binding site, $\bar{Y} = (P_{\text{obs}} - P_{\text{min}})/(P_{\text{max}} - P_{\text{min}})$, is T/B_{tot} , where P_{obs} , P_{min} , and P_{max} are the observed, minimal, and maximal values, respectively, of the measured parameter [intensities at 280 or 340 nm (Figure 1)] and T and B_{tot} are equal to the concentrations of the ternary and binary complexes, respectively. The binding equation described by Khrapunov and Brenowitz²⁴ was used for fitting the experimental data:

$$P_{\text{obs}} = P_{\text{min}} + (P_{\text{max}} - P_{\text{min}}) \left[(K_d + B_{\text{tot}} + \text{Ox}_{\text{tot}}) - \frac{\sqrt{(K_d + B_{\text{tot}} + \text{Ox}_{\text{tot}})^2 - 4B_{\text{tot}}\text{Ox}_{\text{tot}}}}{2B_{\text{tot}}} \right] \quad (1)$$

where K_d is the equilibrium dissociation constant and B_{tot} and Ox_{tot} are the binary complex and oxamate concentrations, respectively.

Estimation of the Thermodynamic Parameters of the Michaelis Complex

The association constant, K_a , of binding of oxamate to the binary LDH/NADH complex was measured from 20 to 35 $^{\circ}\text{C}$, and the Gibbs free energy ($G = -RT \ln K_a$) was determined as a function of temperature. The isotherms were measured and fitted as described above. To determine the enthalpy (H) for formation of the ternary complex, LDH/NADH/oxamate, the data were analyzed by a van't Hoff plot, $\ln(K_a)$ versus $1/T$. A meaningful estimate of H can be derived from the slope ($-H/R$), according to the equation

$$\ln K_a = -\Delta H/RT + \Delta S/R \quad (2)$$

where R is the gas constant and T is the temperature in kelvin. However, eq 2 cannot give more than a rough idea of entropy, S , from the y -intercept (S/R) because this requires knowledge of the ordinate intercept by extrapolation of the experimental data to infinite temperature.^{25,26} Thus, we estimated the entropy of oxamate binding from the Gibbs equation ($G = H - TS$) using independent measurements of G and H as described above.

For Arrhenius analysis of the kinetic data, the linear form of the Eyring–Polanyi equation was used:

$$\ln \frac{k_{\text{cat}}}{T} - \frac{-\Delta H^\ddagger}{R} \frac{1}{T} + \ln \frac{k_B}{h} + \frac{\Delta S^\ddagger}{R} \quad (3)$$

where k_{cat} is the reaction rate constant, T is the absolute temperature, $H^\ddagger$ is the enthalpy of activation, R is the gas constant, k_B is equal to the Boltzmann constant, h is equal to Planck's constant, and $S^\ddagger$ is the entropy of activation. Graphing $\ln(k_{\text{cat}}/T)$ versus $1/T$ yields a straight line with a slope from which the enthalpy of activation can be derived. The y -intercept from which the apparent entropy of activation is derived is uncertain because of extrapolation of the experimental data to infinite temperature.

Calculation of the Midpoint of the Temperature Denaturation Transition

$T_{1/2}$ was obtained by fitting to the light scattering data to the sigmoidal Boltzmann equation

$$f_N = \frac{1}{1 + \exp[(T - T_{1/2})/sT]} \quad (4)$$

where f_N is the molar fraction of the native state and equal to $(P_{\text{obs}} - P_1)/(P_2 - P_1)$, where P_{obs} , P_1 , and P_2 are the observed, initial, and final values of the measured parameters, respectively, T is the temperature, $T_{1/2}$ is the midpoint of the transition, and sT is the slope of the transition at $T_{1/2}$.

RESULTS

Thermodynamics of the Formation of the Michaelis Complex in the Temperature Range Preceding Denaturation

We have studied the affinity of oxamate for two LDH orthologs in the temperature range preceding denaturation. Binding of the substrate to the LDH/NADH binary complex strongly quenches the emission of bound NADH, likely because of interactions with the polar groups of the substrate or its mimic, oxamate, with NADH.^{27–29} We observed the change in intensity of the 280 and 340 nm peaks (Figure 1) to determine isotherms, the association constant, and the free energy for the binding of oxamate to the binary LDH/NADH complex (see Experimental Procedures). van't Hoff analysis (eq 2) in the temperature range of 20–35 °C reveals the association enthalpy of the ternary complexes

containing phLDH and cgLDH, and the association entropy was calculated using the Gibbs equation (see Experimental Procedures) (Figure 2 and Table 1). The results with TMAO will be discussed later together with Figure 4.

The affinity at 20 °C is higher for phLDH ($K_d \sim 6 \mu\text{M}$) than for cgLDH ($K_d \sim 94.8 \mu\text{M}$) upon excitation at 340 nm. The affinity changes are reversible; the affinity at 20 °C returns to its initial value after both orthologs were heated to 35 °C. The binding of oxamate is enthalpically driven (ΔH is negative) and entropically disfavored (ΔS is negative). Variations of the entropy component upon oxamate binding are caused not only by the conformation of the active site itself but also by the flexibility of the whole protein structure, including portions distal from the active site.³⁰ The more flexible the structure of the protein, the more negative the entropy component because binding of the substrate restricts the number of conformations for both the substrate and the protein. In our case, this restriction is more profound for the more flexible protein, cgLDH, as expected for the cold-adapted enzyme.^{31,32}

As shown in Figure 2A, determining the affinity for the phLDH/NADH binary complex for oxamate does not depend on the excitation wavelength used to monitor NADH emission but does for cgLDH. Thus, temperature-dependent changes in the affinity of substrate binding coincide for the active site and global protein structure in the case of phLDH but are asynchronous for cgLDH. Thermodynamic signatures of ternary LDH complexes, with the Gibbs free energy of binding (ΔG), enthalpy (ΔH), and entropy (ΔS), are different for phLDH and cgLDH and dependent on the excitation wavelength in the case of cgLDH (Figure 2B). The difference in ΔG between phLDH and cgLDH is almost the same when measured via excitation at 280 and 340 nm (7.0 and 5.8 kcal/mol for phLDH and 7.0 and 5.4 kcal/mol for cgLDH, respectively) (Table 1). At the same time, the contribution of ΔH and especially ΔS to free energy differs for phLDH and cgLDH. These differences in ΔS are especially large for cgLDH upon excitation at 280 and 340 nm. The contribution of entropy to the free energy is practically the same for phLDH with fluorescence excited at 280 and 340 nm (10.2 and 10.4 cal mol⁻¹ K⁻¹, respectively) but differs by a factor of 2 for cgLDH (15.0 and 8.6 cal mol⁻¹ K⁻¹, respectively).

Thus, the loss of entropy upon substrate binding is largest in the case of cgLDH when NADH fluorescence is excited through FRET with excitation at 280 nm (Figure 2B and Table 1). These data show the high flexibility of the global protein structure of cgLDH compared to that of phLDH. In general, the results shown in Figure 2 and Table 1 confirm the idea that the stability of the catalytic site and global protein domains can evolve differently upon thermo-adaptation.^{1,33}

Structural Changes and Temperature Stability of the Ternary Complexes of phLDH and cgLDH

Taking into consideration the affinity of oxamate for the binary LDH/NADH complex (Table 1), we have conducted our studies of the ternary complex at high (saturated) concentrations of the components. A wide temperature range has been used, including those in which reversible (Figure 2) and irreversible (accompanied by aggregation) structural changes of LDH occurred. The excitation spectra at different temperatures for both orthologs are shown

in Figure S4. According to our “three-in-one” excitation assay (see Experimental Procedures), we used intensities at 280, 340, and 240 nm as a measure of the conformational changes of the protein global structure, enzyme active site, and aggregation of the ternary complex, respectively (Figure 3). The “340 nm” peak of the cgLDH spectrum (Figures 1 and 3B, empty circles) decreases with an increase in temperature following the expected temperature quenching of the NADH fluorescence. At the same time, the change in the “280 nm” peak (filled circles, looks like a solid line) is more complicated.

The fluorescence intensity of cgLDH with excitation at 280 nm increases until 50 °C, demonstrating the structural transition with a T_m of 36.1 °C (Figure 3B and Table 2), and then decreases with further heating. Aggregation of the cgLDH ternary complex started at >50 °C (Figure 3C) when the low-temperature (predenaturation) structural transition was completed.

Unfolding of the Michaelis complex mimic phLDH/NADH/oxamate ternary complex is accompanied by different and more profound changes compared to that of cgLDH (Figure 3A). NADH emission excited at either 340 nm (empty circles) or 280 nm (filled circles, looks like a solid line) does not follow the simple picture expected for the temperature quenching of fluorescence and increases until the temperature reaches 65–70 °C with a half-transition of 51.7 °C (Table 2). Aggregation of the complex began at >60 °C (Figure 3C) when the predenaturation structural transition was nearly complete.

Fluorescence changes for both LDHs in the temperature range preceding denaturation can occur for different reasons: oxamate dissociation (the binary LDH/NADH complex shows NADH emission higher than that of the LDH/NADH/oxamate complex), temperature quenching, and conformational changes in the Michaelis complex. In turn, conformational changes can be associated with the active site or the global protein structure. Oxamate dissociation is unlikely to cause the increase in the fluorescence in the predenaturation temperature range for several reasons. First, as follows from Figure 2, hypothetical dissociation of oxamate does not exceed 5% for phLDH or 15% for cgLDH in the temperature region of the transitions shown in Figure 3. Second, NADH emission upon oxamate dissociation should be increased in a manner independent of the excitation wavelength (Figure 1), but NADH emission for cgLDH varies in the opposite way upon excitation at 280 or 340 nm (Figure 3B). Moreover, the affinity for oxamate is approximately 1 order of magnitude lower for the cgLDH binary complex ($K_d \sim 53.1 \mu\text{M}$) than for phLDH ($K_d \sim 6.0 \mu\text{M}$) (Table 1). For these reasons, oxamate dissociation and consequently the relative increase in NADH emission should be much higher for cgLDH than for phLDH, yet this is not observed experimentally. Fluorescence is considered as a temperature-dependent exponential quenching process^{34,35} as shown for *N*-acetyl-L-tryptophan ethyl ester (AcTrpEE) modeling the fully accessible protein tryptophan residues (Figure 3A,B, filled triangles). However, the tryptophan fluorescence of phLDH and cgLDH changes in the opposite direction compared to that of AcTrpEE.

We have built a quenching theoretical curve of the Michaelis complexes that assumes only oxamate dissociation and temperature quenching of the tryptophan fluorescence are observed, and there are no structural changes in the complex (red line, Figure 3A,B). The

theoretical curves do not follow the observed fluorescence change for both LDHs when NADH emission is excited at 280 nm. Thus, the low-temperature emission change (excitation at 280 nm) for both LDHs (Figure 3A,B) is probably due to the structural transition of the Michaelis complex in the temperature range preceding denaturation with half-transition points of 51.7 ± 0.4 and 36.1 ± 0.5 °C for phLDH and cgLDH, respectively (Table 2). Interestingly, the low-temperature (predenaturation) transitions reach completion at quasi-optimal temperatures of less than 50–60 °C (Figure 3A,B), where the activities for both LDHs reach their maxima.¹⁴ The observed concerted changes for the NADH emission excited at 280 and 340 nm for the phLDH tertiary complex and the lack of such a transition for the cgLDH tertiary complex upon excitation at 340 nm suggest that changes in the conformation of the active site and the global protein structure of the enzyme can occur in concert or independently.

Osmolyte Control of the Protein Dynamics

Numerous studies have shown that the natural osmolyte TMAO stabilizes protein structure and affects folding rates of proteins.^{36–38} TMAO strongly affects the rates and thermodynamics of specific events along the LDH-catalyzed reaction: binding of substrates, loop closure, and the chemical event. Qualitatively, these results can be understood as an osmolyte-induced change in the energy landscape of the LDH complexes, shifting the conformational nature of functional substates within the protein ensemble.^{1,39} We investigated the behavior of phLDH and cgLDH in the presence of 1.5 M TMAO to better understand how the osmolyte affects the determined structural transitions and aggregation of the LDH ternary complex shown in Figure 3 (Figure 4 and Tables 1 and 2). The high-temperature (denaturation) transition accompanied by aggregation is higher by ~10 °C for the Michaelis complex for both orthologs in the presence of TMAO compared to that without the osmolyte. The increasing melting temperature and level of aggregation at higher temperatures of the studied LDHs are consistent with the general stabilization of proteins in the presence of TMAO.^{38,40} The fluorescence increase observed for phLDH in the absence of TMAO is shifted to the higher temperature in the presence of TMAO using a wavelength of either 280 or 340 nm for excitation (Figures 3A and 4A). The full predenaturation transition is not observed for phLDH because the fluorescence increase for phLDH practically coincides with aggregation of the ternary complex (Figure 4A,B).

More striking differences are observed for the ternary complex containing cgLDH in the presence of TMAO (Figures 3B and 4A). The low-temperature transition (excitation at 280 nm) is shifted to 43 °C compared to 36 °C in the absence of TMAO (Table 2) and reaches completion before 60 °C, before cgLDH aggregation starts (Figure 4). Moreover, cgLDH fluorescence measured with excitation at both 280 and 340 nm reveals a clear structural transition (Figure 4A) in the presence of TMAO that is absent with excitation at for cgLDH in the absence of TMAO (Figure 3B). Thus, the structure of the cgLDH ternary complex in the presence of TMAO resembles that of the phLDH ternary complex in the absence of TMAO (compare Figures 3A and 4A).

Temperature-Dependent Activity Profile of the Lactate Dehydrogenases

The structural transitions that exist in the physiological temperature range and are shown above are expected to influence the function (k_{cat}) of the enzyme. It was shown previously that there are multiple structures of bound enzyme–ligand complexes, some of which are likely to be far from the catalytically productive structure.^{18,19} We studied the kinetics of the two LDHs under different conditions, presumably changing the set of the subtle protein conformations involved in the enzyme function.

Enzyme activities (k_{cat}) for phLDH and cgLDH were determined at 20 °C in the absence and presence of 1.5 M TMAO; increasing the concentration of TMAO decreases activity for both orthologs (Figure S5). An Eyring plot, $\ln(k_{\text{cat}}/T)$ as a function of $1/T$, in a temperature range of 10–50 °C is shown in Figure 5A. The activation enthalpy, $H^\ddagger$, which corresponds to the activation energy (E_a), was evaluated using eq 3. The value of E_a increases in the following order: cgLDH ~ phLDH (high T) < cgLDH (TMAO) $\ll$ phLDH (low T) (Figure 5B). The decrease in E_a for phLDH at high temperatures where the protein structure is more flexible, compared to that of cgLDH, follows the results of the thermodynamic analysis (Figure 2) and confirms the conclusion that flexibility of the global protein structure is important for modulating the substrate–LDH affinity and resulting enzyme function. The change in activation energy follows the change in the low-temperature conformational transition of the Michaelis complex formed by cgLDH (Table 2). At the same time, the stability of the Michaelis complexes reflected by the denaturation transition (Table 2) is changed: cgLDH (55 °C) < cgLDH (TMAO) (66.5 °C) ~ phLDH (66.7 °C). The ability of TMAO to stabilize proteins against denaturing stresses originates from the unfavorable interaction of the osmolyte with the peptide backbone^{41–43} mainly through a decrease in protein flexibility and significant rigidification of the protein backbone.⁴⁴ Thus, the increase in the rigidity of the protein conformation by TMAO shifts the stability and activity of cgLDH toward those of phLDH in the absence of TMAO. All these observations strongly suggest that the changes in the structural transition preceding denaturation shown for two LDHs in the presence of TMAO (Figures 3 and 4 and Table 2) are physiologically significant.

DISCUSSION

There is substantial qualitative understanding of how lactate dehydrogenases have adapted.^{1,9,11,14} In general, these enzymes are produced by many species with physiological operating temperatures that span from 0 to 100 °C and have very similar protein structures across a wide array of orthologs.^{9–11} The active sites are nearly identical with regard to both the type of residues present and the resulting field strengths generated by the charged groups within the active site.^{2,12} Taken together, thermal adaptation has resulted in the LDHs having approximately the same values for k_{cat} and K_m for all species at their respective physiological operating temperature.¹ Given this remarkable observation about these important catalytic parameters and the high degree of homology of the LDH active site structure among species, it has been surmised previously that adaptation is not brought about by a regulation of the active site structure or by the transition-state structure but rather lies in changes in the dynamical nature of the LDH family.⁵ Our own studies of the dynamics and

structure of a set of LDH thermophiles¹⁴ agree with this hypothesis, showing that transition-state structure is essentially unchanged and that low-temperature adapted LDHs are more “flexible” whereas high-temperature adapted LDHs are more “rigid”.

It has been well shown that proteins exist as an ensemble of interconverting conformational substates, some having more important mechanistic implications than others, that differ in their k_{cat} and K_{m} values and can be tuned to adapt to a specific temperature.¹ We have explicitly shown this for lactate dehydrogenase with the reported values of k_{cat} and K_{m} representing an average for all conformational substates.^{14,17–19}

The results presented here agree with those of our previous studies and extend some quantitative understanding to the matter at hand. The LDH substrate mimic oxamate at 20 °C binds substantially more loosely to cgLDH than phLDH and tightens up considerably as cgLDH's operating temperature is approached (Figure 2). Comparing the van't Hoff dependence of substrate binding for the two orthologs (Figure 2 and Table 1), one can conclude, first, that all changes are reversible and therefore physiologically significant and, second, that the changes for the active site (excitation at 340 nm) and global protein structure (excitation at 280 nm) coincide for phLDH and do not for cgLDH (particularly for H and S ; the G values are probably more similar because of the tendency toward entropy–enthalpy compensation). We anticipate that there may be differences in the thermodynamic binding parameters, G , H , and S , depending on whether the excitation employed to study the binding lies at 280 or 340 nm. As explained above, studies employing 340 nm report on changes happening close to the active site while those employing 280 nm additionally monitor more global protein structure, i.e., including portions distal from the active site. This result shows that the mechanism of adaption affects different portions of the protein structure differently from mesophile to psychrophile.

The change in substrate binding G is determined by the total entropy change with parameters related to either protein–water interactions or protein conformational entropy. It has been shown generally that the configuration portion of protein entropy that consists of the conformational and vibration components is essentially higher than its hydration portion.^{45–47} These fluctuations among the protein substates can contribute significantly to the entropy of proteins.^{48,49} With regard to the present determinations of K_{m} , the topology of the active site is virtually the same for cgLDH and phLDH as well as the binding of oxamate at the active site. Any residual hydration effects will cancel out in the difference in the entropy component of K_{m} . Moreover, it is known that the differing enthalpy and entropy values of the variants of the same enzyme originate from changes in the internal structure of the protein.⁵⁰ It has also been shown that the most probable basis for the differing entropy and enthalpy values among varying cold-adapted LDHs lies in localized increases in conformational flexibility and is not the result of hydration effects.⁵¹ Thus, the change in the contribution of entropy to the free energy of substrate binding for cgLDH shown in Figure 2 most likely originates from the protein conformational entropy and high conformational flexibility of cgLDH. The enzymes from warm-adapted species are more rigid and have lower conformational entropy and higher energy barriers, causing them to denature at higher temperatures, as shown for phLDH and cgLDH (Table 2).

The observation of structural transitions that occur before the denaturation of LDH sheds new light on the relationship between protein structure and function. As shown in Figure 3, the temperature-dependent reversible conformational transition for phLDH occurs before the start of the irreversible transition (denaturation). Starting at approximately 35–40 °C, the increase in NADH emission with temperature can be caused by only an increasing population of conformations within the phLDH/NADH/oxamate Michaelis complex that have larger NADH emission coefficients. These shifting conformational changes observed before denaturation are quite subtle and can be observed only by our highly sensitive three-in-one excitation assay as there are no gross changes involving secondary structure.^{8,9} This temperature-dependent structural change is concomitant with changes in the k_{cat} of phLDH (Figure 5). This is a remarkable result that demonstrates that protein activity and stability are affected by subtle yet important conformational changes preceding denaturation.

In this study, we hypothesized that we could direct a cold-adapted enzyme in part to adopt the substrate affinity, structural stability, and catalytic activity of a warm-adapted ortholog by modifying environmental conditions using the natural osmolyte TMAO. The ability of TMAO to stabilize proteins against denaturation originates from significant rigidification of the peptide backbone through unfavorable interactions with the osmolyte.^{37,38,40–44,52} We have shown previously that TMAO induces a change in the energy landscape of the LDH complexes, shifting the conformational nature of functional substates within the protein ensemble.³⁹ The results of our experiments in the presence of TMAO confirm these expectations (Figures 3–5 and Tables 1 and 2). In the absence of TMAO, the structural transition preceding the irreversible aggregation is evident for both LDHs when the fluorescence of NADH is excited through FRET from tryptophan residues (Figure 3A,B). A similar transition is observed for phLDH but not for cgLDH when direct excitation of NADH fluorescence is used (○, Figure 3A,B). As shown, the affinity and melting temperature are lower (Tables 1 and 2) while the activity is higher (Figure 5) for psychrophilic cgLDH than for mesophilic phLDH. These trends are seen to continue (Figure S5) with the addition of the stabilizing osmolyte TMAO. In the presence of TMAO, both LDHs are more stable as shown by the approximately 10 °C increases in the aggregation and denaturation temperatures (Table 2).

The enthalpy of activation (activation energy) of cgLDH in the presence of TMAO increases in the direction of that for phLDH in the absence of TMAO (Figure 5B). As a result, the structure and activation energy of cgLDH in the presence of TMAO resemble those of phLDH. In fact, the addition of the osmolyte comes close to turning the low-temperature-adapted (psychrophilic) LDH into a warm-temperature-adapted (mesophilic) LDH (Figure 5). The loss of conformational entropy and the increase in conformational flexibility for psychrophiles presume that the thermal adaptation of proteins is closely related to the evolution of species under different thermal conditions. Also, the different “weight” of the entropy term in the value of ΔG provides a mechanism of conservation of the Michaelis–Menten constant (K_m) for all LDH orthologs at normal body temperatures.^{1,53} Thus, it seems possible that evolution of the thermal adaptation of enzyme structure may be complemented by the evolution of the cellular milieu.¹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

LDH	lactate dehydrogenase
TMAO	trimethylamine <i>N</i> -oxide
NADH	nicotinamide adenine dinucleotide (reduced form)

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Figure 1.

Excitation spectrum (emission at 470 nm) of the ternary (phLDH/NADH/oxamate) complex with a change in oxamate concentration. Reaction conditions: buffer of 100 mM sodium phosphate (pH 7.2), concentrations of the components, 4 μ M phLDH, 5 μ M NADH, and from 0 to 1.5 mM oxamate (from top to bottom, respectively).

Figure 2.

van't Hoff analysis of the reversible binding of oxamate to the binary NADH complex with phLDH and cgLDH in temperature range of 20–35 °C. (A) The data are colored black (phLDH) and blue (cgLDH) and calculated from excitation spectra (Figure 1), NADH emission at 470 nm, and excitation at 280 nm (filled circles) or 340 nm (empty circles). Red triangles depict the reverse data at 20 °C after heating to 35 °C. K_a is the association constant, and the temperature scale is shown in reciprocal kelvin. The buffer was 100 mM sodium phosphate (pH 7.2). (B) Thermodynamic parameters estimated from the data shown in panel A. H is the enthalpy in kilocalories per mole. S is the entropy in calories per mole per kelvin. G is the free energy in kilocalories per mole. The error bars reflect the standard deviations of the data.

Figure 3.

Temperature-dependent changes in (A and B) fluorescence and (C) light scattering of the ternary complex: NADH (40 μ M)/LDH (40 μ M)/oxamate (1 mM) with (A) phLDH and (B) cgLDH. NADH emission was measured at 470 nm with excitation at 280 nm (filled circles, looks like the top solid line) or 340 nm (empty circles). Triangles show the temperature quenching of *N*-acetyl-L-tryptophan ethyl ester (AcTrpEE). The red line is a quenching theoretical curve of the complex assuming only oxamate dissociation and concomitant quenching of the tryptophan emission are observed (see the text). (C) Light scattering was measured at 240 nm. The y-axis is for phLDH (left) and cgLDH (right). The buffer is 100 mM sodium phosphate (pH 7.2).

Figure 4.

Influence of the neutral osmolyte trimethylamine *N*-oxide (TMAO) on the temperature-dependent changes in (A) NADH emission (470 nm) and (B) light scattering (240 nm) of the NADH (40 μ M)/LDH (40 μ M)/oxamate (1 mM) ternary complex with phLDH (circles) and cgLDH (triangles). In panel A, excitation was at 280 nm (filled symbols) and 340 nm (empty symbols). The y-axis is for phLDH (left) and cgLDH (right). The buffer was 100 mM sodium phosphate (pH 7.2) containing 1.5 M TMAO.

Figure 5.

(A) Eyring plot (see eq 3 in Experimental Procedures) of the relationship between the k_{cat} of pyruvate hydrogenation and temperature for phLDH (without TMAO) and cgLDH with or without 1.5 M TMAO. The data for phLDH are split into two parts, ph_lowT (10–35 °C) and ph_highT (35–47.5 °C). Note the abrupt change in the catalytic function near 35 °C. (B) Activation energy (enthalpy) of the reaction in kilocalories per mole. All measurements were performed in triplicate with average values reported. The buffer was 100 mM sodium phosphate (pH 7.2) with or without 1.5 M TMAO.

Thermodynamic Parameters of the Formation of the Michaelis Complex (LDH/NADH/oxamate) at 20 °C [100 mM sodium phosphate (pH 7.2)] for phLDH and cgLDH in the Absence and Presence of 1.5 M Trimethylamine *N*-Oxide (TMAO)

Table 1

	excitation at 280 nm (FRET)						excitation at 340 nm (NADH quenching)					
	buffer			buffer with 1.5 M TMAO			buffer			buffer with 1.5 M TMAO		
	phLDH	cgLDH	phLDH	cgLDH	phLDH	cgLDH	phLDH	cgLDH	phLDH	cgLDH	phLDH	cgLDH
K_d (μ M)	6.0 $\pm$ 0.3	53.1 $\pm$ 3.2	ND ^a	11.1 $\pm$ 1.0	6.0 $\pm$ 0.28	94.8 $\pm$ 6.9	ND ^a	24.0 $\pm$ 3.3	ND ^a	24.0 $\pm$ 3.3	ND ^a	24.0 $\pm$ 3.3
G (kcal/mol)	-7.0 $\pm$ 0.4	-5.8 $\pm$ 0.3	ND ^a	-6.7 $\pm$ 0.8	-7.0 $\pm$ 0.4	-5.4 $\pm$ 0.4	ND ^a	-6.2 $\pm$ 0.9	ND ^a	-6.2 $\pm$ 0.9	ND ^a	-6.2 $\pm$ 0.9
H (kcal/mol)	-10.2 $\pm$ 0.3	-10.1 $\pm$ 0.6	ND ^a	17.0 $\pm$ 1.4	-10.1 $\pm$ 0.6	-7.9 $\pm$ 0.4	ND ^a	-14.4 $\pm$ 1.2	ND ^a	-14.4 $\pm$ 1.2	ND ^a	-14.4 $\pm$ 1.2
S (cal mol ⁻¹ K ⁻¹)	-10.2 $\pm$ 0.4	-15.0 $\pm$ 2.1	ND ^a	35.3 $\pm$ 4.6	-10.4 $\pm$ 2.0	-8.6 $\pm$ 0.7	ND ^a	-27.0 $\pm$ 3.9	ND ^a	-27.0 $\pm$ 3.9	ND ^a	-27.0 $\pm$ 3.9

^aNot determined. The results with TMAO will be discussed below together with Figure 4

Table 2

Structural Thermal Transitions of the Michaelis Complex (LDH/NADH/Oxamate) for phLDH and cgLDH in the Absence and Presence of 1.5 M Trimethylamine *N*-Oxide (TMAO)

	<u>low-temperature (predenaturation) transition, T (°C)</u>		<u>high-temperature (denaturation) transition, T (°C)</u>	
	buffer	buffer with 1.5 M TMAO	buffer	buffer with 1.5 M TMAO
phLDH	51.7 ± 0.4	NA ^a	66.7 ± 0.1	75.1 ± 0.6
cgLDH	36.1 ± 0.5	43.0 ± 0.8	55.0 ± 0.1	66.5 ± 0.1

^aNot applicable.