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Mechanism of Thermal Adaptation in the Lactate Dehydrogenases

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Abstract

The mechanism of thermal adaptation of enzyme function at the molecular level is poorly understood but is thought to lie within the structure of the protein or its dynamics. Our previous work on pig heart lactate dehydrogenase (phLDH) has determined very high resolution structures of the active site, via isotope edited IR studies, and has characterized its dynamical nature, via laser-induced temperature jump (T-jump) relaxation spectroscopy on the Michaelis complex. These particular probes are quite powerful at getting at the interplay between structure and dynamics in adaptation. Hence, we extend these studies to the psychrophilic protein cgLDH (*Champsoccephalus gunnari*; 0 °C) and the extreme thermophile tmLDH (*Thermotoga maritima* LDH; 80 °C) for comparison to the mesophile phLDH (38–39 °C). Instead of the native substrate pyruvate, we utilize oxamate as a nonreactive substrate mimic for experimental reasons. Using isotope edited IR spectroscopy, we find small differences in the substrate composition that arise from the detailed bonding patterns of oxamate within the active site of the three proteins; however, we find these differences insufficient to explain the mechanism of thermal adaptation. On the other hand, T-jump studies of reduced β -nicotinamide adenine dinucleotide (NADH) emission reveal that the most important parameter affecting thermal adaptation appears to be enzyme control of the specific kinetics and dynamics of protein motions that lie along the catalytic pathway. The relaxation rate of the motions scale as cgLDH > phLDH > tmLDH in a way that faithfully matches k_{cat} of the three isozymes.

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Supporting Information

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Detailed presentation of the isotope edited IR spectra of the three isozymes as well as kinetic studies (PDF)

Notes

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INTRODUCTION

Biochemical adaptation to biological processes operating at diverse temperatures is well studied but certainly is not well understood. Often, hyperthermophile and psychrophile enzymes employ the same basic active site structural architecture as their mesophilic counterparts. It is thus widely conjectured that evolutionary adaptation of function involves the adaptation of the enzyme's dynamics rather than the arrangement of active site residues and basic arrangement of outlying residues.^{1,2} Adaptation is often particularly viewed as resulting from modulating the Michaelis complex ensemble population characteristics and dynamics. As a well-characterized enzyme, lactate dehydrogenase (LDH) has been a model system for examining such issues.³

The arrangements of first-sphere catalytic residues at the active site of LDH whose physiological operating conditions range from -1.86 °C to over 100 °C are basically identical.^{3,4} Thus, the mechanism of on-enzyme chemistry would appear to be the same. The variation of k_{cat} 's, when measured at the same temperature, is negatively correlated to average body temperature of the species; that is, the catalytic efficiency of low-temperature LDHs is greater than high-temperature LDHs measured at the same temperature. The operating enzyme flux for the pathways in which LDH participates appears to be the same or close to the same for all species at their normal operating temperature.⁵ The rate-limiting step for the on-enzyme chemistry of pyruvate to lactate is not the chemical step but rather is the movement of atoms that occurs when the substrates bind and the system hunts for reactive substates.^{6,7} This shows up in the rather low, even nil, H/D primary isotope effect that all LDHs show.² Hence, k_{cat} 's of LDHs are rate-limited by dynamical considerations, specifically atomic motions on the nanosecond–millisecond time scale.

We have recently thoroughly characterized the dynamics of the Michaelis complex for pig heart lactate dehydrogenase (phLDH) by determining its energy landscape projected along the reaction coordinate.⁸ Upon the binding of substrate, the phLDH-NADH-substrate system undergoes a search through conformational space via a parallel-branched pathway to find a range of reactive conformations over the microsecond-to-millisecond time scale. This detailed body of results on the role of conformational heterogeneity and dynamics in the

catalysis of hydride transfer by LDH was possible using isotope edited IR approaches^{9,10} and new instrumentation permitting kinetic studies on the picosecond-to-millisecond time scales.¹⁰ LDH catalyzes the reduction of pyruvate to lactate mediated by the transfer of a hydride from NADH to C-2 of pyruvate as shown in the cartoon of the active site (Figure 1). There are several vibrational modes that report directly on catalysis, but particularly important is the C₂=O group of the bound pyruvate, which is polarized by active site interactions. Clearly, the C₂=O group of the bound pyruvate substrate is an integral component of the reaction coordinate, and the C₂=O stretch monitors the strength of the hydrogen bonding and electrostatic interactions (Figure 1, shown in red). Indeed, it has been shown that the frequency of the C₂=O stretch is a quantitative monitor of propensity toward catalysis.^{11–13} Laser-induced temperature jump spectroscopy, employing isotope edited IR and fluorescence monitors of evolving structure, has yielded the kinetic pathway from ligand binding to catalysis.^{13–16} These studies have led to an unparalleled identification of mechanistically related structure and dynamics within the Michaelis complex of LDH.

The goal of this paper is straightforward. Structure and dynamics are determined on two LDHs at each end of the adaptation temperature range, the psychrophilic protein cgLDH (*Champsocephalus gunnari*; 0 °C) and the extreme thermophile tmLDH (*Thermotoga maritima* LDH; optimal growth 80 °C), for comparison to the well-characterized mesophile, phLDH (see Figure 2 for temperature variation of k_{cat}). The same experimental approaches used for phLDH are employed. Measurement of a high-resolution structure of the substrate bound to the active site is performed via isotope edited IR studies. We follow the emission of bound NADH on the microsecond time scale in response to a rapid change in temperature of the ternary system, LDH·NADH·substrate (i.e., laser-induced T-jump relaxation spectroscopy). It has been found that NADH is an excellent probe of the dynamics of the system by monitoring much of the key motions that take place along the catalytic pathway that finally forms substates poised to perform on-enzyme chemistry, including loop motion and interconversion kinetics among the various substates of the Michaelis complex.^{14,15} Instead of the native substrate, we use oxamate as an unreactive mimic. This is done because it is difficult to isolate the LDH·NADH·pyruvate complex in sufficient concentration for the cgLDH and tmLDH isozymes and because oxamate is a very faithful mimic, both for statics as well as for dynamics,¹⁵ of the native substrate apart from not undergoing on-enzyme chemistry.

MATERIALS AND METHODS

The plasmid of Pig heart LDH (phLDH) with His tag was prepared as described previously.¹² The gene of *Thermotoga maritima* LDH from DSM 3109 (ATCC 43589) was integrated into pET23a. The Antarctic fish *Champsocephalus gunnari* (cgLDH) lactate dehydrogenase gene cloned into pJ express404 vectors was purchased from DNA2.0 Inc. CA. These vectors were freshly transformed into BL21 cells each time for protein expression. For phLDH, Ni column and gel filtration were employed to purify it after cell lysis. For tmLDH, dialysis supernatant after cell lysis was incubated at 80 °C for 30 min before ion exchange and gel filtration. cgLDH was purified with ion exchange and gel filtration. Steady-state kinetic data were measured at room temperature.

Sodium oxamate and NADH were purchased from Roche Scientific. Preparation of $^{13}\text{C}_2$ -oxamate was previously described.¹⁴ All values for K_d were determined in house using standard equilibrium binding assays. k_{cat} values for cgLDH were determined in house using standard steady-state kinetic methods. k_{cat} values for phLDH and tmLDH were adapted from the following refs (phLDH, ref 17; tmLDH, ref 18) on the basis of kinetic measurements performed at room temperature in house.

Nicolet Magna 760 Fourier transform spectrometer was used to obtain Fourier transform infrared (FTIR) spectra. Samples having $^{13}\text{C}_2$ - and $^{12}\text{C}_2$ -oxamate were loaded in two IR cells with CaF_2 windows and 15 μm Teflon spacers at room temperature. Typical concentration of the enzyme was 4 mN (i.e., 4 mM active sites) with stoichiometric amount of NADH and oxamate. A difference spectrum is formed whereby the background IR spectra is nulled leaving positive and negative going IR peaks for any vibrational mode that is frequency shifted by the label. The procedure for isolating isotope edited difference spectra is found in ref 15 and is discussed in the Supplementary Information.

Fluorescence T-jump spectra were obtained on our home-built spectrometer. A detailed setup and procedure could be found in these papers.^{10,16} In the NADH fluorescence measurements, the sample was loaded into quartz cuvettes. The excitation light, with 2 mW incident power in a 10 nm FWHM band centered at a wavelength of 365 nm, is from a Black-LED-365 light source (Prizmatix, Southfield, MI). The excitation beam was focused to 0.7 mm diameter spot in the center of the heated spot of the sample (~1.5 mm diameter). NADH fluorescence passed through a 458 nm narrow-band filter with the bandwidth of 40 nm full width at half-maximum (fwhm) before reaching the detector (Andover, Salem, NH).¹⁹

RESULTS AND DISCUSSION

The goal of this study is to characterize how the specific protein dynamics of an enzyme, specifically the lactate dehydrogenase system, is related to the regulation of activity across a broad range of thermophiles. Here, the structure of a protein is viewed as a vast ensemble of metastable conformational substates that interconvert on multiple time scales, from nanoseconds to minutes. Isotope edited IR spectroscopy is employed to determine those substates that are directly connected to mechanism. Then, it is possible to monitor interconversion kinetics using a variety of spectroscopic techniques. Here, we employ kinetic studies monitoring the emission of bound NADH. This probe is sensitive to protein dynamics as well as to function, since reduction of NADH is involved in the catalytic pathway.

Organization at the Active Site. Isotope Edited FTIR Studies

As mentioned previously, the $\text{C}_2=\text{O}$ stretch of bound pyruvate, or the analogous bond in the mimic oxamate, is a very useful probe of the substates that are key to the reaction coordinate.⁸ The bond is polarized by strong hydrogen bonding with surrounding catalytically key charged residues resulting in marked downward shifts in the stretch frequency. The frequency shifts are strongly correlated to catalytic activity of on-enzyme pyruvate-lactate chemistry. Figure 1S shows the FTIR isotope edited measurements of

oxamate bound to tmLDH·NADH, phLDH·NADH (ref 15), and cgLDH·NADH with ($[^{13}\text{C}_2=\text{O}]\text{oxamate}$ minus $[^{12}\text{C}_2=\text{O}]\text{oxamate}$). In this approach, the IR spectra of the labeled and nonlabeled ternary complexes are taken; the difference is formed and the only resulting modes that show up are those that frequency shift because of the labeling. The $[^{13}\text{C}_2=\text{O}]$ stretch region is shown by positive bands from ca. 1600–1630 cm^{-1} while the $[^{12}\text{C}_2=\text{O}]$ is upshifted about 38 cm^{-1} (as predicted by a simple diatomic oscillator model). Our analysis, by and large, concentrates on the $[^{13}\text{C}_2=\text{O}]$ stretch region since the $[^{12}\text{C}_2=\text{O}]$ stretch can somewhat couple to other oxamate vibrational modes, distorting the mode pattern.²⁰

Figure 3 shows the FTIR isotope edited measurements of oxamate bound to tmLDH·NADH, phLDH·NADH, and cgLDH·NADH in the $^{13}\text{C}_2=\text{O}$ stretch region, all taken at the same temperature, 20 °C. Specifically, two major $^{13}\text{C}_2=\text{O}$ bands are located near 1606.4 and 1622.8 cm^{-1} for phLDH as reported previously.¹⁵ It is concluded that the Michaelis complex exists in two major populated conformations: a mimic of a more competent conformation as represented by the lower frequency species (1606 cm^{-1} ; less downward shift means that the active site H-bonds are weaker) and a less competent form represented by a higher stretch frequency (1623 cm^{-1}). Also, the architecture of more competent conformation is such that the reduced nicotinamide ring is puckered in such a way that the pro-R hydrogen on C₄ of the reduced nicotinamide ring of NADH adopts a pseudoaxial geometry. This conformation is poised for catalysis since its ring structure is consistent with the transition-state structure of the hydride transfer reaction. The less competent conformation adopts a planar NADH ring, a less reactive form.¹⁵ We do not have any correlations between chemical activity and the band position for oxamate since this is an unreactive mimic. For pyruvate, the relationship is exponential;¹¹ a 35 cm^{-1} frequency shift yields an enhancement in catalytic rate of about 10^6 . Hence, small changes in the peak position of the $\text{C}_2=\text{O}$ stretch indicate significant changes in the catalytic rate. Laser-induced temperature jump kinetic studies monitoring the response at the IR peaks showed that the two conformations do not interconvert directly among each other but rather via a separate conformation (called an encounter complex since such a species is required in the kinetic pathway of binding ligand) that is relatively sparsely populated.^{14,15,20} The encounter complex is not observed directly in the spectra of Figure 3 because of its relatively small population and perhaps relatively broad C=O stretch. In studies of the phLDH·NADH·pyruvate complex, a small IR band representative of the encounter complex species is in fact observed.^{11,13}

The three proteins show qualitatively similar spectra, quite consistent with crystallographic studies showing that the active sites of the three proteins are basically identical and that substrate (mimic) occupies the active site with the same first sphere positioning. The resolution here is, however, substantially more accurate, about 0.2 Å for X-ray compared to better than 0.01 Å for the vibrational results. This enhanced resolution shows clear quantitative differences in the IR spectra presenting evidence of protein oxamate binding pattern differences within the active site among the three enzymes. Given the sensitivity of the $\text{C}_2=\text{O}$ stretch to catalytic rate, the changes in the IR spectrum can be compared to the relative catalytic rates of the three isozymes at 20 °C (Figure 2). Relative to the phLDH spectrum, the population of the cgLDH protein is mostly located in the single low frequency conformer (ca. 1606.4 cm^{-1}) compared to two populated substates. There may be a

relatively poorly populated conformation located at an even larger redshift at 1595 cm^{-1} . It is clear that the folding energy of the cgLDH isozyme brings about a shift in the structure of the protein populating conformations that are relatively more active than that found in phLDH, entirely consistent with the shift in k_{cat} from 245 s^{-1} in phLDH to 582 s^{-1} in cgLDH (at $22\text{ }^{\circ}\text{C}$). Conversely, the spectra of the tmLDH isozyme is shifted toward higher frequencies (the $[\text{C}_2=\text{O}^{13}]$ stretch bands lie at 1609.3 and 1624.8 cm^{-1} , upshift by ca. $2\text{--}3\text{ cm}^{-1}$). This is also well correlated to the shift in k_{cat} from 245 s^{-1} in phLDH to 6.5 s^{-1} in tmLDH (at $22\text{ }^{\circ}\text{C}$).

However, the nature of the substates as reported by the mechanistically sensitive IR spectra among the three proteins is similar; the differences appear to be much too small to account for the temperature adaptation across these thermophiles. Some preliminary conclusions are available about the relationship between the binding patterns revealed by the IR spectrum patterns and the effects of catalytic rates. The quantitative relationship between the k_{cat} 's of specific conformations and $\text{C}_2=\text{O}$ stretch is worked out for the substrate pyruvate, and the spectra of Figure 3 are for the substrate mimic, oxamate. Oxamate is an excellent mimic, however, and we make the qualitative assumption that spectral features contained in Figure 3 will carry over to pyruvate (as demonstrated by previous studies of phLDH). The concentration of the unreactive component for phLDH, as represented by the intensity of the 1622.8 cm^{-1} peak, is about 30% of the total population. Hence, its absence in the cgLDH with all the intensity in a reactive population at 1606.4 cm^{-1} suggests that 30% of the factor of 1.8 increased reactivity of cgLDH over phLDH is due to the change in packing forces between the two active sites. The 1595 cm^{-1} species for cgLDH, if real, would be a reactive substate. However, its population is insufficient to account for the relative value of k_{cat} for cgLDH versus the other two isozymes. The shift of 2.9 cm^{-1} in the $\text{C}_2=\text{O}$ stretches between tmLDH and phLDH (the $1606.4\text{--}1609.3\text{ cm}^{-1}$ in Figure 3) is equivalent to a factor of 2.9 in relative k_{cat} .¹¹ This can be compared to the measured change in k_{cat} of a factor of 35. Hence, we observed some shifts in the substate populations that are generally in line with the relative k_{cat} values but that are too small to explain the differences in the temperature profile of the three thermophiles.

Figure 2S shows the temperature dependence of the FTIR isotope edited difference spectra for the three isozymes over quite a broad range. In general, there is very little temperature dependence, quite insufficient to account for the temperature dependence in k_{cat} values. For example, the main oxamate $^{13}\text{C}_2=\text{O}$ stretch frequencies near 1605 cm^{-1} in these three LDH isozymes do not show significant change with temperature change. This is in agreement with the general idea that the activity temperature profile among LDH thermophiles is regulated by the individual dynamical nature of the proteins; that is, the flux from substrate to product on the enzyme is a dynamical process; the system generally hunts through a restricted set of conformations with a restricted set of kinetic pathways among the conformations with any specific Michaelis conformation undergoing chemistry stochastically.⁸ Given that the actual chemical event is generally faster than the interconversion kinetics, values of k_{cat} 's are more determined by the interconversion dynamics.

Dynamical Features of the Populated Conformations. Laser-Induced T-Jump Studies

To give us an idea of interconversion kinetics and thermodynamics, we performed laser-induced T-jump studies monitoring the response of NADH optical emission to the sudden change in temperature. Previously, we performed several in-depth studies on the kinetics of interconversion of the conformational substates of phLDH·NADH-oxamate Michaelis complex mimic^{14,15,21,22} as well as the phLDH·NADH-pyruvate Michaelis complex.^{12,13,16,23} Our studies included stopped-flow and laser-induced T-jump approaches to launch ligand binding and unimolecular conformational changes, employing several types of probes: UV-vis and IR absorbance and NADH and Trp emission.^{8,10} The dynamics of both systems, pyruvate and oxamate, are similar. Several relaxation events are observed: a binding event as ligand binds to LHD·NADH and generally two (oxamate system) or more (pyruvate system) unimolecular events occurring within the ternary complex. The unimolecular and bimolecular events are disentangled by performing studies as a function of free ligand (e.g., ref 14). In brief, conformations that tightly bind substrate of the Michaelis complex (broadly defined here as the protein-cofactor-substrate ternary complex) are formed from conformations that bind substrate loosely.

It appears that the transformation from loosely bound to tightly bound substrate involves fairly large-scale motions such as the closure of the key surface loop (Figure 1), motions of internal loops, and smaller more localized motions. The tightly bound substates interconvert on time scales faster than $1/k_{\text{cat}}$. The substates do not interconvert directly but via a weakly populated substate whereby oxamate or pyruvate are loosely bound. The observed kinetic lifetimes range from 10 μs to 10 ms. Such a broad range requires several different types of spectrometers: laser-induced T-jump techniques, which in our hands have a resolution of ca. 20 ns and extend to ca. 1–5 ms, and also conventional stopped-flow techniques, with a resolution of ca. 1 ms. Structural probes of evolving structure have included IR absorbance (including monitoring of the important $\text{C}_2=\text{O}$ stretch) as well as fluorescence emission from the NADH nicotinamide headgroup and NADH optical absorption.^{8,10}

Here, we concentrate on NADH emission spectroscopy. NADH emission is unusual in LDH complexes in that it is highly quenched in ternary complexes. The relative emissions of NADH fluorescence with excitation at 340 nm for three structures of NADH in solution, in LDH·NADH binary complex, and in LDH·NADH-substrate are 1.0:2.4:0.45 (peak emission).¹⁴ The analogous values for the cgLDH and tmLDH systems are 1.0:1.7:0.22 and 1.0:1.7:0.31, respectively (data not shown). The wide variation is responsible for the sensitivity of NADH emission in kinetic spectrometers. Importantly, NADH monitors much of the key motions that take place along the catalytic pathway including loop motion and interconversion kinetics among the various substates of the Michaelis complex.^{14,15}

Figure 4 shows the time dependence of emission from the nicotinamide group of the NADH cofactor at room temperature (final temperature) of the tmLDH·NADH plus oxamate system following a 10 °C T-jump for several concentrations of oxamate. These data are very similar to that found for the phLDH system. Because the binding of oxamate to LDH·NADH binary complexes is exothermic, a sudden rise in temperature induces the ligand release, a bimolecular step. This in turn brings about unimolecular transitions involving a change in

concentrations of the various species and concomitant NADH emission even if there is no enthalpic difference between the substates. However, there is usually also an enthalpic difference, and this also leads to interconversion among the substates. The data can be fit to two exponentials, and their analysis is provided in the Supporting Information. One of the observed relaxation rates scales with the concentration of free binary complex plus free ligand, while the other does not (see Figure 3S). From the relationship showing a linear dependence on oxamate concentration under pseudo-first-order conditions, the ligand on-rate, k_{on} , is readily calculated (as the slope¹⁴). The step independent of concentration is a unimolecular event of the ternary, LDH·NADH·ligand, complex. There is also a second unimolecular event that is too slow to be observed in the T-jump relaxation spectrometer but is easily observed in stopped-flow studies following the binding of oxamate to the binary complex, LDH·NADH (data not shown). The analogous slow step in the mesophiles is generally assigned to loop closure with concomitant quenching of NADH emission and is often taken as rate-limiting in k_{cat} (although the actual rate-limiting steps are more complicated¹³). It seems safe to assign the slow step found in tmLDH as loop closure given the close correspondence of the rate to k_{cat} (see Table 1). Figure 5 and Figure 5S show the analogous data for cgLDH.

The kinetic results are summarized in Table 1. There are three observed relaxation rates for all three enzyme systems: (1) a ligand binding step (which gives rise to a linear dependence on oxamate concentration under pseudo-first-order conditions and which yields a value for the apparent binding constant of oxamate with LDH·NADH, k_{on} ; see Supplementary Information), (2) a slow relaxation step (associated with loop closure of the key surface loop), and (3) a fast step likely involving the motions of loops internal to the proteins. These data along with the k_{cat} 's and the K_{d} 's of NADH with LDH and oxamate with LDH·NADH are summarized in Table 1.

CONCLUSIONS

The parameter k_{cat} describes the catalytic flux in the conversion of substrate to product in standard Michaelis–Menten kinetics formulation. While accurate in describing the overall kinetics, it yields little in the way of atomic mechanism, partly because Michaelis–Menten describes many different types of kinetic pathways. For the lactate dehydrogenases, it is well-known that the rate-limiting step in catalysis involves protein motion. For example, the primary H/D isotope effect on k_{cat} is generally very small. In our hands, k_{cat} measured under single turnover conditions yields a primary isotope KIE of 1.6 for phLDH. Previously, in quite detailed studies of pig heart LDH (and others), we have shown that the actual kinetic pathway describing the catalytic flux is complicated.^{8,12,13} The ground state of the Michaelis complex (here, the LDH·NADH·pyruvate ternary complex) consists of an ensemble of conformations. Populating these conformations and then conversion to product involves multiple pathways and widely varying time scales. Thus, there are three aspects of the Michaelis complex that must be determined in order to describe such a system: the distribution of the substate ensemble, the interconversion dynamics (pathway and kinetics), and the relative propensity toward catalysis of each substate within the ensemble.

In this study, the IR spectrum of the $C_2=O$ group of the mimic substrate oxamate within the Michaelis complex is an accurate measure of the distribution of the substrate ensemble projected along the catalytic pathway. This is because the downshift in the $C_2=O$ stretch is a direct measure of the distance between the oxygen of $C_2=O$ and the active site polarizing residues and is also a monitor, indirectly, of the packing of NADH against oxamate at the active site.^{12,15,24} Here, we have determined key aspects of the dynamics within the Michaelis complex via time-resolved fluorescence studies monitoring the emission of NADH. NADH has been shown to be a good probe, if a partial one, of many of the protein motions that accompany the system from substrate binding to the catalytic event.

We find in this study that all three isozymes, cgLDH, phLDH, and tmLDH, form a substrate population that can be labeled as conformations that are relatively reactive or unreactive as give by the IR profile of the $C_2=O$ stretch of bound oxamate, the mimic for the substrate pyruvate. As discussed earlier, the profile differences are in agreement with the enzyme activity trends of cgLDH > phLDH > tmLDH. However, the specific differences in structure are small and insufficient to explain the differences in activities of the three enzymes. In addition, there is virtually no change in the substrate distribution as a function of temperature; the temperature dependence of the $C_2=O$ stretch for each protein is very small. There is one surprising feature of the first-sphere binding patterns of substrate with its protein environment at the active site. From the shifts in the IR spectra of the $C_2=O$ stretch, the substrate is held more tightly as cgLDH > phLDH > tmLDH while the protein's folding stability (Gibb's free energy) goes as the reverse, cgLDH < phLDH < tmLDH (Figure 2 shows a loss of folded protein concomitant with a loss in k_{cat} as the temperature increases).

By performing T-jump studies of NADH emission to monitor the key motions that place along the catalytic pathway, we have demonstrated that the most important parameter affecting thermal adaptation seems to be enzyme control of specific kinetics and dynamics. The relaxation times of the motions scale as cgLDH > phLDH > tmLDH (at a fixed temperature, Table 1) in a way that faithfully matches k_{cat} of the three isozymes. It has been noted previously that the active site arrangement and interactions are universally conserved across the megafamily that are the lactate dehydrogenases in agreement with the results found here.^{2,3} Coquelle et al.³ find that the adaptation of LDHs from hot to cold correlates, by and large, with a decrease in thermal stability. Thermal stability in the LDHs seems to come about, at least in part, from a small number of nonconserved residues.³ The mesophile to hyperthermophile is correlated with an increased number of ionic residues in mobile regions of the protein. In the case of cold adaptation, examples are found which involve an increase in flexibility locally rather than in a global change. For example, an increase in glycine residues, which increase flexibility compared to a proline residue that they might replace, is located in the loop regions of the psychrophilic enzyme. These studies are inferences between sequence comparisons looking for non-conserved residues and correlating these with the thermal dependence of k_{cat} and the overall stability of a particular LDH.

Given many of these inferences from sequence comparisons coupled with biochemical and structural data, these specific suggestions and others can be probed very directly with our methods, really for the first time. Further, it would be premature to say if regulation of the

dynamics of a protein, via perhaps subtle changes in structure, is a general feature of adaptation across many proteins. This remains to be investigated. However, we are in a position to develop quantitative, atomic level, theories about adaptation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

LDH catalyzes the interconversion of $\text{NADH} + \text{pyruvate} + \text{H}^+$ with $\text{NAD}^+ + \text{lactate}$ (cf. refs 25 and 26). Binding is strictly ordered with cofactor binding preceding substrate. It is widely believed that hydride and proton transfer are concerted. Calculations show multiple routes for proton and hydride transfer occurring in traversal of the transition state within the time frame of a single bond vibration (ca. 5 fs).²⁷ Shown is a schematic of the LDH active site showing the residues stabilizing the substrate pyruvate and the proximity of the cofactor, NADH. The catalytically key surface loop (residues 98–110) closes over the active site, bringing residue Arg109 in hydrogen bond contact with the ligand, and water leaves the pocket. Creation of the pocket is accompanied by the motions of mobile areas within the protein, rearranging the pocket geometry to allow for favorable interactions between the cofactor and the ligand that facilitate on-enzyme catalysis.¹⁹ Of particular interest to this work are the hydrogen bonds formed between Arg-109 and His-195 to the C2 carbonyl of pyruvate (emphasized in red). These bonds dictate the polarity of the carbonyl when pyruvate is bound. Figure taken from ref 13.

Figure 2. k_{cat} temperature dependence of tmLDH,¹⁸ phLDH,¹⁷ and cgLDH (our results). The values at 22 $^{\circ}C$ are 6.6, 245, and 582 s^{-1} , respectively.

Figure 3.

Isotope edited FTIR spectra in the region of the $^{13}\text{C}_2=\text{O}$ stretch of oxamate within the LDH·NADH-oxamate complex for the extreme thermophile *Thermotoga maritima* (tmLDH), mesophile pig heart (phLDH), and *Charmpsoccephalus gunnari* (cgLDH) lactate dehydrogenases. Results are taken at 20 °C. It is uncertain if the 1595 cm^{-1} peak in the chLDH spectrum is a $^{13}\text{C}_2=\text{O}$ stretch since we are unable to verify a similarly positioned mode in the $^{12}\text{C}_2=\text{O}$ spectrum.

Figure 4.

T-jump NADH emission study of tmLDH·NADH plus oxamate for various oxamate concentrations. Excitation at 365 nm, emission at 458 or 460 nm. Initial concentrations for tmLDH·NADH-oxamate are 50/50/50, 50/50/300, 80/80/600, and 80/80/1200 μM . 100 mM phosphate with 3 mM FBP buffer, pH = 7, 22–24 $^{\circ}\text{C}$.

Figure 5.

T-jump NADH emission study of cgLDH-NADH plus oxamate for various oxamate concentrations. Excitation at 365 nm, emission at 458 or 460 nm. Initial concentrations: [NADH] = 80 μ M, [cgLDH] = 80 μ M, [oxamate] as listed in figure. Buffer: 100 mM sodium phosphate, pH = 7, 22–24 $^{\circ}$ C.

Table 1

Observed Relaxation Rates and Kinetic Parameters^a

species	k_{cat} (s^{-1}) (22 °C)	slow relaxation s^{-1} (22–24 °C)	fast relaxation s^{-1} (22–24 °C)	k_{on} ($\text{M}^{-1} \text{s}^{-1}$)	K_{d} (μM) (20 °C)
c _g LDH	582 s^{-1}	3300	28600	N/A	0.3 (NADH) 100 (oxamate)
enthalpy	5.9		10–11		
entropy	–26.7		~0		
phLDH ^b	245 s^{-1}	400	4000	2.1×10^7	0.85 (NADH) 14 (oxamate)
enthalpy	16.3		1.8		
entropy	7.5				
tmLDH	6.6 s^{-1}	4.4 ^c	606	1.5×10^7	22.6 (NADH) 37 (oxamate)
enthalpy	10.2		10.2		
entropy	–20.0				

^a All kinetic rates are given in s^{-1} . The activation enthalpy and entropy are calculated by the Eyring equation, and the units are kcal/mol and cal/mol/ K, respectively.

^b Data from ref 14.

^c Stopped-flow experiment monitoring NADH emission. Data not shown.