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Active-Loop Dynamics within the Michaelis Complex of Lactate Dehydrogenase from *Bacillus stearothermophilus*

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Abstract

Laser-induced temperature-jump relaxation spectroscopy was used to study the active site mobile-loop dynamics found in the binding of the NADH nucleotide cofactor and oxamate substrate mimic to lactate dehydrogenase in *Bacillus stearothermophilus* thermophilic bacteria (bsLDH). The kinetic data can be best described by a model in which NADH can bind only to the open-loop apoenzyme, oxamate can bind only to the bsLDH·NADH binary complex in the open-loop conformation, and oxamate binding is followed by closing of the active site loop preventing oxamate unbinding. The open and closed states of the loop are in dynamic equilibrium and interconvert on the submillisecond time scale. This interconversion strongly accelerates with an increase in temperature because of significant enthalpy barriers. Binding of NADH to bsLDH results in minor changes of the loop dynamics and does not shift the open–closed equilibrium, but binding of the oxamate substrate mimic shifts this equilibrium to the closed state. At high excess oxamate concentrations where all active sites are nearly saturated with the substrate mimic, all active site mobile loops are mainly closed. The observed active-loop dynamics for bsLDH is very similar to that previously observed for pig heart LDH.

Graphical Abstract

An enzymatic reaction involves the diffusion-controlled formation of an encounter complex between the protein and its substrate followed by the appropriate structural and dynamical arrangements, which produce a Michaelis complex capable of product formation. During the formation of the Michaelis complex, the binding pocket is substantially rearranged.

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Specifically, protein flaps or loops often close over the bound ligand; the binding pocket is desolvated, and catalytically important residues are brought into contact with the bound substrate. It is clear from earlier studies that enzymes (proteins) exist in an ensemble of conformations, some of which can competently bind their ligands while others bind poorly or not at all.¹⁻³ It is also found that proteins bind their ligands in a rather complex dynamical pathway.⁴⁻⁹ Moreover, it has been shown directly that conformational changes occur within the ensemble of the enzyme/substrate Michaelis complex(es) on various time scale from femto- to picoseconds through milliseconds and slower.¹⁰ Here we investigate the dynamics of the active site-loop dynamics of *Bacillus stearothermophilus* lactate dehydrogenase (bsLDH) and compare the results with those from mammalian lactate dehydrogenase.

LDH catalyzes the NADH-dependent conversion of pyruvate to lactate. The active site structures of bsLDH and its mammalian counterparts (human or pig heart LDH) are the same. The substrate binding pocket is sequestered inside the protein ~10 Å from the surface.^{11,12} However, computational studies suggest that there are interesting differences in how bsLDH and mammalian LDH accomplish the chemical step of the catalytic mechanism. In human heart LDH, the hydride transfer appears to be directly correlated with the dynamics of the axis residues, which are known as the promoting vibrations. In bsLDH, the hydride transfer is predicted to show a wider range of possibilities that might be correlated with the dynamics of the axis residues, and the promoting vibration is not well-defined.¹³ The computed differences in the enzyme dynamics occur at fewer than tens of picoseconds around the chemical bond breaking/forming event.

In this study, the goal is to determine how slower nanosecond to millisecond conformational fluctuations are used by bsLDH to search the energy landscape for ligand binding pathway(s) to reach catalytically competent conformations. From there, the fast vibrations are predicted to be effective in promoting passage over the transition state.

To preclude the occurrence of enzyme-catalyzed chemistry that would complicate a kinetic analysis, we used a substrate analogue, oxamate, rather than the natural substrate, pyruvate. Oxamate is isoelectric and isosteric to pyruvate and has been shown to have binding kinetics very similar to that of pyruvate. Thus, oxamate is generally a very close mimic of the natural substrate. On the basis of several X-ray crystallographic data, oxamate is placed near the nicotinamide ring of the NADH and the following key active site protein residues: His195, Arg109, and Arg171 (Scheme 1). The C₂=O bond of oxamate forms hydrogen bonds with His195 and Arg109, while C₁OO⁻ forms a salt bridge with Arg171.^{14,15} As the substrate approaches the catalytic site, a catalytically key surface loop (residues 98–110) closes over the ligand, bringing residue Arg109 into hydrogen bonding contact with ligand, water leaves the pocket, and the pocket geometry rearranges to allow for favorable interactions between the cofactor and the ligand, which facilitates on-enzyme catalysis.^{16,17}

We probed the transient events associated with the binding of NADH to bsLDH and of oxamate to the bsLDH·NADH complex, using temperature-jump (*T*-jump) relaxation techniques. *T*-Jump relaxation monitors the re-equilibration of a chemical system following an instantaneous increase in temperature induced by a laser pulse tuned to an infrared water band. The re-equilibration results in changes in the concentration of the species involved,

and the transient changes are characterized using spectroscopic probes. First, we probed the fluorescence emission of NADH in bsLDH to report on the time evolution of the changes within the NADH environment over the microsecond to millisecond time scale. Such analyses should yield data about the dynamics of binding and associated conformational binding. Second, emission of the intrinsic tryptophan in bsLDH is measured to report on conformational changes, such as loop motion. We probed changes in the NADH and the intrinsic tryptophan fluorescence for the bsLDH·NADH binary complex as well as the bsLDH·NADH·oxamate ternary complex. A comprehensive picture of the dynamics of ligand binding and Michaelis complex formation in LDH is obtained from the various structural reports.

MATERIALS AND METHODS

Samples

All required reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) except for NADH and oxamate, which were purchased from Roche Diagnostic Corp. (Indianapolis, IN).

The preparation of bsLDH has previously been described in detail.^{15,18} In brief, the bsLDH gene, obtained from Genomic DNA from *Geobacillus stearothermophilus* ATCC 12980D, was subcloned into the pET3a vector and transformed into C43(DE3) competent *Escherichia coli* cells. The growth conditions of the transformed cells and the protein purification procedures followed a published procedure.¹⁴ Some studies were performed on a mutant bsLDH containing a single Trp residue, G106W, in which the three naturally occurring Trp residues have been mutated to tyrosines.¹⁸

Temperature-Jump Measurements

A custom-built fluorescence *T*-jump instrument was used to measure relaxation kinetics, based on the same principles described previously.¹⁹ Sizable temperature jumps are induced by exposing a volume of water to a pulse of infrared light (1.56 μm wavelength from a D₂ gas Raman shifter pumped by a YAG laser, typically 100–200 mJ/pulse, 1.5 mm diameter spot on the sample). Water absorbs the laser energy, and the temperature of the exposed volume is increased in approximately 7 ns. Typical *T*-jump values ranged from 5 to 10 °C in the study presented here. With a sample cell thickness of 0.5 mm, diffusion of heat out of the interaction volume takes approximately 25 ms. Hence, the apparatus generated a *T*-jump within 10 ns that remained nearly constant until approximately 5 ms. The sample temperature after the jump is monitored over time by probing changes in the water IR absorption at 1460 nm.

The *T*-jump instrument is designed to probe either tryptophan or NADH fluorescence. To probe changes in the fluorescence intensity of the tryptophan, the sample was irradiated by the 300 nm (300.3 and 302.4 nm) emission lines of an Innova 200-25/5 argon ion laser (Coherent, Palo Alto, CA), while the 360 nm (351.1 and 363.8 nm) emission lines of the same laser were used to stimulate NADH fluorescence. To prevent photodamage to the sample, the excitation light was modulated using a shutter that allowed an only 10 ms

exposure for every *T*-jump pulse. Also, the power of the excitation beam was attenuated by a neutral-density filter; the typical intensity was 15–20 μ W. The incident excitation beam is focused onto a 0.05 mm diameter spot on the sample in the center of the beam path of the 1.56 μ m pulse. Fluorescence emission, detected at 50° to the excitation beam, was passed through a narrow band filter (340 ± 12 nm to probe tryptophan fluorescence and 450 ± 20 nm for NADH emission) and monitored using a R4220P photomultiplier tube (Hamamatsu, Bridgewater, NJ). A lab-written program using LabVIEW software (National Instruments, Austin, TX) was used for instrument control and data collection. Data were normalized to the average fluorescence intensity taken before the *T*-jump. Curve fitting was done with Igor Pro version 4.0 (Wavemetrics, Inc., Lake Oswego, OR). The uncertainties in the reported values of relaxation rates were determined from the scatter in values from four or five replicate runs and were approximately $\pm 10\%$ of the measured relaxation rate. Errors in the response amplitudes were analyzed in the same manner and were estimated to be ± 10 –15%.

Samples for *T*-jump measurement were dissolved in 5 mM fructose 1,6-bisphosphate (FBP), 0.1 M triethanolamine (TEA) HCl buffer at pH 6. FBP assures that the protein forms tetrameric uniformity and serves as an allosteric activator of the enzyme.²⁰ The *T*-jump studies typically employed 50 – 80 ± 10 μ N (μ N indicating the active site concentration) bsLDH solutions. Different amounts of NADH and oxamate were added as appropriate to form the “binary” and “ternary” complexes.

RESULTS AND DISCUSSION

Fluorescence *T*-Jump Measurements on bsLDH-NADH Binary Complexes

The fluorescence of NADH is strongly enhanced upon binding to dehydrogenase enzymes, as its quantum yield is increased 2–3-fold,²¹ thus making it a good direct probe of NADH binding. It is also sensitive to the local NADH environment and can report on conformational changes around the active site. Additionally, protein tryptophan fluorescence is partly quenched by bound NADH because of Förster resonance energy transfer and can be used as an independent probe of NADH binding. Depending on the actual value of the tryptophan quantum yield, the Förster radius for this donor–acceptor pair is approximately 15–25 Å,²² and protein tryptophan fluorescence intensity significantly decreases when NADH is bound to the enzyme. Tryptophan fluorescence can also report on changes in the environment of tryptophan residues, i.e., on conformational changes that might be different from those reported by NADH. bsLDH contains three Trp residues (at positions 80, 150, and 203 roughly spread over the protein; cf. ref 18), and this study reports the emission from all three equally depending on their unknown emission efficiency. Additionally, some studies were performed on a mutant bsLDH containing a single Trp residue, G106W, where the three naturally occurring Trp residues have been mutated to tyrosines.¹⁸

NADH and tryptophan fluorescence *T*-jump kinetics were measured within the time span of a few nanoseconds to 15–30 ms for a number of samples with different concentrations of NADH and bsLDH. NADH fluorescence kinetic profiles (Figure 1, top graphs) show an intensity drop from 1 μ s to a few milliseconds. This drop can be attributed to the release of NADH from its complex with the protein, because bound NADH has a fluorescence quantum yield much higher than that of free NADH in solution. Multiexponential curve

fitting of these kinetics resolves three relaxation times in addition to the cooling component, which is ~25 ms and not shown in the figure because a time cutoff of ~10 ms has been imposed. The relaxation times represented, as evaluated from the kinetic profiles, are as follows: a strong midrange component (100–1000 μ s), a weaker slow component with a 1–3 ms relaxation time, and a very weak fast component in the time range of 1–20 μ s. The fast component, which is roughly independent of protein and ligand concentrations, is usually observed in similar systems. It can be partly determined by protein dynamics and may also depend on NADH internal photophysical processes involving the triplet state.

Tryptophan fluorescence kinetic profiles (bottom graphs in Figure 1) exhibit an intensity rise in the same time range where the main NADH intensity drop is observed. This correlation implies that both kinetic events are dominated by the same process of unbinding of NADH from bsLDH, disrupting energy transfer from tryptophan to bound NADH and enhancing the tryptophan fluorescence. Tryptophan kinetics can be fitted with a single-exponential component with a relaxation time very close to that of the midrange NADH component. Minor evidence of an additional slower component in the millisecond time range was observed for tryptophan kinetics at extreme concentrations and temperatures. To help elucidate the origins of these fast and slow transients, we measured the tryptophan fluorescence *T*-jump traces for the G106W mutant bsLDH·NADH complex (data not shown), which directly measure structural changes associated with the mobile loop.¹⁸ Not surprisingly, the trace yielded a fit to a double exponential with a set of fast and slow transients that have rate constants of ~2000 and ~10000–20000 s^{-1} , respectively. This is significant because the structural changes as reported by the modulation of the NADH signal reflect a change in the loop tryptophan fluorescence. The slow component at ~2000 s^{-1} is likely the bimolecular step associated with the binding/release of NADH. Given the concentrations of free LDH and NADH under the conditions used in this study, the ~2000 s^{-1} transient leads to a second-order rate constant of 150 $mM^{-1} s^{-1}$, which is quite similar to that found for the pig heart protein in previous studies.²³ Moreover, the observed decrease in intensity with time is consistent with the release of NADH from the protein, given that the NADH fluorescence is 4 times higher in the binary complex than in solution. The ~10000–20000 s^{-1} transient represents then a unimolecular structural change within the LDH·NADH complex.

Increasing NADH and enzyme concentrations shift the main features of both NADH and tryptophan kinetics to shorter times. The left graphs of Figure 1 present the kinetic profiles for a sample with stoichiometric amounts of bsLDH and NADH at a low concentration level of 10 μ M, while the right graphs show the kinetics for a sample with an excess of 207 μ M NADH over 50 μ M bsLDH (enzyme subunit concentrations are shown everywhere). Figure 2 displays the effect of an increasing NADH concentration on an 80 μ M sample of bsLDH with regard to the NADH and Trp emission *T*-jump profiles.

The observed rates of tryptophan relaxation and the midrange (major) component of NADH relaxation are plotted in Figure 2 as a function of the total concentration of free NADH and free enzyme, for three after-jump temperatures. Free concentrations were calculated using NADH dissociation constants obtained from steady-state fluorescence data.¹⁸ Two distinct patterns emerge at low (<20 μ M) and high (>100 μ M) concentrations. At low concentrations,

there is a sharp rise in the observed tryptophan relaxation rate and midrange NADH relaxation rate, and at higher concentrations, a much slower, nearly linear rise is observed. Total concentrations of bsLDH active sites in this series varied from 5 to 80 μM , and total NADH concentrations varied from 5 to 670 μM . Within the range of the initial sharp rise, NADH and bsLDH were present in approximately stoichiometric amounts. The slow rise, meanwhile, corresponds to excess NADH concentrations. The observed character of the rate concentration dependence indicates two different binding patterns for the low and high NADH concentrations. This assumption is confirmed by the analysis of kinetic data, which shows that an additional, weak binding type for NADH is required at higher concentrations. The photophysical properties (fluorescence quantum yield and energy transfer efficiency) of weakly bound NADH must be close to those of strongly bound NADH, because for both NADH and tryptophan fluorescence midrate kinetics, the concentration dependence (Figure 2) is essentially the same throughout the concentration range while the fraction of weak binding changes a lot. Indeed, NADH unbinding decreases the NADH fluorescence quantum yield and increases the tryptophan quantum yield because of the disruption of energy transfer. Therefore, the relative change in each quantum yield is approximately the same for both types of NADH binding. Free NADH in solution mainly has a folded conformation, while bound NADH takes on an extended conformation, resulting in a higher fluorescence quantum yield.²¹ Because nearly equal quantum yields are observed for the two NADH binding types, the weakly and strongly bound NADH must both have an extended conformation.

Figure 3 shows the observed rates of the slow NADH relaxation component at 20 and 30 °C. At higher temperatures, this component is much weaker with high fitting errors. The slow component at each temperature is nearly independent of concentration. This suggests that binding of NADH to bsLDH is followed and/or preceded by a unimolecular process that likely involves some conformational rearrangement.

Analysis of the Fluorescence T-Jump Data of bsLDH· NADH Binary Complexes

The observed concentration-dependent kinetic rates shown in Figures 2 and 3 are first analyzed using a minimal reaction model to simulate the NADH binding process: $\text{E} + \text{NADH} \leftrightarrow \text{E1} \cdot \text{NADH} \leftrightarrow \text{E2} \cdot \text{NADH}$. Here, a conformational change occurs after the NADH binds. This problem can be solved analytically to determine two concentration-dependent observable kinetic rate functions in terms of microscopic kinetic constants.⁷ The higher rate function shows minimal concentration dependence at low concentrations and then increases linearly with concentration. The lower rate function shows a linear increase at low concentrations and then gradually becomes saturated when the concentration becomes higher. Furthermore, the slopes of the linear regions in these two functions are the same (cf. Figure 4 in ref 7). Because the observed kinetic rates in Figure 2 show a sharp linear increase in the low-concentration region and a slower linear increase in the higher-concentration region, neither of the two rate functions derived from the minimal model can fit the data accurately in both concentration regions. Our data analysis suggests that when the observed rate shows a linear dependency on the concentration, this rate is typically associated with the bimolecular ligand binding step.

To test other reaction models, we used the kinetic simulation procedure based on the biochemical reaction modeling program, GEPASI,^{24,25} as was previously implemented in our earlier studies.^{19,26} Briefly, the following algorithm was used. The prejump equilibrium concentrations of all the reaction components required by the tested model are first obtained from the GEPASI run, using some initial guesses for the prejump rate constants. In the next GEPASI run, using the obtained prejump equilibrium concentrations and guessed after-jump rate constants, the time dependence is obtained for each transient concentration on the approach to the new equilibrium after the *T*-jump. The simulated NADH fluorescence kinetic profile is calculated using previously determined values for relative quantum yields and some reasonable guess values for the less reliably known yields. The rate constants are adjusted to obtain the best fit to the experimental kinetic profile. Rates derived from the exponential fits of the experimental and simulated kinetic profiles should approximate one another. The resulting optimal rate constants are extrapolated to find the projected values for the next pair of pre- and postjump temperatures, and the process is repeated for all available temperature pairs. Then the whole sequence is repeated iteratively. In the early iterations, the quantum yields are also adjusted within their experimental uncertainties, as needed, to obtain the most consistent fit to the experimental kinetics.

The results for several reaction systems with different concentrations of NADH and bsLDH were preliminarily analyzed this way in the initial attempts to establish a consistent reaction model. The final detailed analysis was performed for two reaction systems: a nearly stoichiometric 50 μ M bsLDH/48 μ M NADH system and a 50 μ M bsLDH/207 μ M NADH system with excess NADH. First, it was found that the kinetics for low-concentration stoichiometric systems could be satisfactorily described by the simple two-step reaction model $E + \text{NADH} \leftrightarrow E1\cdot\text{NADH} \leftrightarrow E2\cdot\text{NADH}$ in which a conformational change follows NADH binding, and unbinding is not possible after this change. Another two-step model in which a conformational change in the enzyme would precede NADH binding and binding would be possible to only one enzyme conformation was less representative of the data. For the systems with a large excess of NADH, kinetic reconstruction was possible only with two NADH binding steps (strong binding and weak binding), plus a conformational change after the strong NADH binding step. After this rearrangement, the weak NADH binding would not be possible. However, the reaction rate constants required for the kinetic reconstructions with this model were considerably different for low- and high-concentration systems. This discrepancy was eliminated by the addition of a conformational change in the apoenzyme that makes strong NADH binding possible. The best fit, with nearly perfect reconstruction of all the kinetic profiles (using GEPASI) for both low- and high-concentration systems, corresponds to approximately the same equilibrium constants for the two conformational changes before and after strong NADH binding. This suggests that all three conformational changes have the same nature. The X-ray crystallography data,^{27,28} from Protein Data Bank entries 1LDN and 2LDB, show that the NADH binding pocket in bsLDH is partly covered by the active site loop (amino acid residues 98–110) when it is in the closed conformation. Therefore, closing of this loop can block NADH from entering its binding pocket and block the substrate from binding to the active site. Apparently, the conformational changes required by the model are the opening and closing motions of the active site mobile loop, and NADH binding is possible to only the open-loop conformation.

The minimum model for NADH binding reaction (Scheme 2) includes four steps: (1) opening the active site loop in apo-bsLDH, (2) strong NADH binding with formation of a binary complex between open-loop bsLDH and NADH (bsLDH^{OPEN}·NADH), (3) closing of the active site loop in the binary complex, and (4) weak binding of an additional NADH molecule to the binary complex in the open-loop conformation.

The microscopic rate constants resulting from the kinetic reconstructions, along with other derived parameters, are listed in Table 1. The errors for the absolute values of rate constants were estimated as their variances in the last iterations (30–50%); however, the forward and back rates vary mainly in parallel, and their relative changes for different temperatures and forward/back ratios have smaller errors. This evaluation of errors is rough, but a more rigorous estimate is not currently possible.

The fraction of open-loop state that determines binding competence for the apoenzyme [$C^{\text{apo}} = k_1/(k_1 + k_{-1})$] and binding or unbinding competence for the binary complex [$C^{\text{bin}} = k_{-3}/(k_3 + k_{-3})$] significantly drops with an increase in temperature (Table 1). While its value of 0.5–0.6 at 20 °C corresponds to nearly equal populations of competent and noncompetent conformations, the value is 2 times lower at 40°C, signifying a strong domination of noncompetent states. The pattern of temperature dependence of NADH binding competence, with an equilibrium shift to the closed-loop noncompetent state with an increase in temperature, is similar to what was previously observed for pig heart LDH (phLDH).^{19,26}

In an experiment in which the concentration of bsLDH was held constant at 80 μM (Figure 4 and Table 2), exponential fits of the experimental profiles possibly reveal that as we increase the NADH concentration in the binary complex, we observe a different kinetic event. A close inspection of the rate kinetics indicates that at low NADH concentrations of 30–300 μM, the transient (the observed relaxation rate, $k_{1_binary_trp}$) derived from the tryptophan *T*-jump trace approximates the fast transient ($k_{1_binary_NADH}$) derived from the fit of the NADH *T*-jump trace. However, at a NADH concentration of 400 μM, that no longer is the case; the transient ($k_{1_binary_trp}$) from the tryptophan trace matches that of the slower transient ($k_{2_binary_NADH}$) derived from the NADH trace. It is even worse at 500 μM NADH, as $k_{1_binary_trp}$ no longer matches any of the transients derived from the NADH *T*-jump kinetics. The change in the kinetic profile of the tryptophan *T*-jump seems to be gradual as it remains fairly constant at 30–50 μM NADH and starts to increase to 1 order of magnitude larger at 300 μM. It is fairly interesting that the two limiting values approximate the transients derived from the biexponential fit of the tryptophan *T*-jump trace for the single-loop-containing Trp mutant, G106W bsLDH. Perhaps at low NADH concentrations, the tryptophan *T*-jump trace prominently detects the NADH unbinding event. Then at higher NADH concentrations where unbinding is expected to be negligible as the system is fairly saturated with NADH, the conformational change, which is loop motion, is evident.

Fluorescence Experiments on bsLDH-NADH-Oxamate Ternary Complexes

Binding of a substrate to dehydrogenase enzymes requires preliminary binding of the NADH cofactor.^{29–31} Without a cofactor, the binding of substrate or its mimic, oxamate, to apo-LDH is very weak and does not noticeably occur under our conditions. We do not consider this further. We also do not consider the level of unbinding of NADH from a ternary

bsLDH·NADH·oxamate complex that is known to be negligibly low under conditions close to ours.³²

Bound oxamate strongly quenches the fluorescence of bound NADH. Therefore, binding of oxamate to the bsLDH·NADH binary complex, much like NADH binding, can be monitored directly by measuring changes in NADH fluorescence intensity. NADH fluorescence *T*-jump kinetics for a ternary sample with stoichiometric amounts of bsLDH, NADH, and oxamate (50 μ M of each) are shown in the top graph of Figure 5, and green curves on the same graph show kinetics for the corresponding binary sample without oxamate. In these kinetic events, the dominating features are (a) unbinding of NADH from bsLDH resulting in the intensity drop for both systems in approximately the same submillisecond time range and (b) oxamate unbinding that produces an intensity rise around 1 ms.

At high oxamate concentrations (Figure 5, bottom panel), the NADH emission kinetics at 20 °C exhibit a minor drop of fluorescence intensity in submilliseconds. At higher after-jump temperatures, however, there is a large increase in intensity throughout the whole microsecond to millisecond time range. In the multiexponential fits of NADH emission kinetics for ternary samples, in addition to the known cooling component (~ 25 ms, which is only partially shown in the figure), three exponential components can be resolved: fast ($20000\text{--}30000\text{ s}^{-1}$), mid ($1500\text{--}5000\text{ s}^{-1}$), and slow ($250\text{--}2000\text{ s}^{-1}$). The dependence of the free species concentration on the observed relaxation rates (Figure 6) displays complicated patterns that imply a multistep reaction mechanism.

The traces in Figure 5 represent measurements involving a “ternary complex” of bsLDH, NADH, and oxamate taken at low oxamate concentrations and final temperatures. Exponential profiles were used to fit the kinetic data to yield three time constants within the observational time range of 1 μ s to 5 ms. In a less complete earlier study exploring binding of oxamate to phLDH,^{5,7,8} using a combination of stopped flow-based and laser *T*-jump spectroscopies, results suggested a reaction that starts with a bimolecular step followed by unimolecular transformations within the phLDH·NADH·oxamate complex. After the association of the LDH·NADH complex with oxamate and formation of a bimolecular encounter complex, the observed kinetics show key hydrogen bond formation between the ligand and His195 ($\sim 500\text{ }\mu$ s), followed by the closure of the surface loop (~ 2 ms). Using these results as a guide in interpreting the data presented here, the slowest observed transient (k_1 in Table 3) can be attributed to a unimolecular transformation involving loop motion while the fastest (k_3), which scales linearly with the oxamate concentration (see below), is primarily the bimolecular on rate between LDH/NADH and oxamate for forming an encounter complex (see Scheme 3). A third weakly observed transient, which is more distinct in complexes with a high oxamate concentration and a low final temperature (not observed in the phLDH system⁷), with a relaxation rate, k_2 , of $\sim 1500\text{ s}^{-1}$ and an amplitude of opposite sign was first thought to involve the formation of the binary complex (see Figure 1). The simplest kinetic model that could account for the *T*-jump NADH emission studies for oxamate complexes is shown in Scheme 3.

Linearization of the kinetic equations describing the model in Scheme 3 yields the following provided the rearrangement after oxamate binding is by far the slowest step:^{33,34}

$$k_1 = k_r + \frac{k_f}{1 + \frac{K_{EC}}{[\text{LDH}/\text{NADH}] + \frac{[\text{oxamate}]}{K_{bin} + 1}}} \quad (1)$$

$$k_2 = k_{on_binary} + \frac{k_{off_binary}}{1 + \frac{[\text{oxamate}]}{[\text{LDH}/\text{NADH}] + \frac{K_{EC} K_{loop}}{K_{loop} + 1}}} \quad (2)$$

$$k_3 = k_{off_ternary} + k_{on_ternary}([\text{LDH}/\text{NADH}] + [\text{oxamate}]) \quad (3)$$

where $K_{bin} = k_{off_binary}/k_{on_binary}$, $K_{EC} = k_{off_ternary}/k_{on_ternary}$, and $K_{loop} = k_r/k_f$. They represent the (linearized) analytical solutions for each of the observed transients.

Kinetic simulations using GEPASI and initial guesses based on values observed for phLDH⁷ helped associate k_2 with concomitant concentration changes of the binary complex. In phLDH, k_2 was not observed because the rate at which the LDH/NADH concentration changes occurs in the millisecond time regime, which is much slower than the rate of ternary complex formation. Another distinction between phLDH and bsLDH systems is that the change in the LDH·NADH·oxamate complex concentration is 1 order of magnitude slower in bsLDH (Table 2) than in phLDH.⁷ Furthermore, while the rates for phLDH decrease with an increase in temperature, the opposite was observed for the bsLDH system. This behavior may be consistent with the thermophile bsLDH having an optimal temperature higher than that of the pig heart protein.³⁵

While the structural similarities for phLDH and bsLDH in the active site are apparent, the structural differences remote from the active sites are also obvious. For example, two cofactor fructose-bisphosphate molecules are required to bind two bsLDH dimers to form a tetramer, and this feature is believed to be a critical component for the rigidity of bsLDH to resist heat-induced denaturation. Our previous and current *T*-jump studies on phLDH and bsLDH binary and ternary complexes revealed that the more rigid bsLDH structure results in several interesting differences in the ligand binding dynamics in these two isozymes as discussed below.

For the binding of oxamate to LDH·NADH binary complex, the bimolecular step (step 1 in the model) can be observed in the time range of tens of microseconds in both isozymes. However, in phLDH, the oxamate molecule can reach its final position in this step, as shown by the observation that the NADH fluorescence is already quenched in this time range.⁷ In bsLDH, the NADH fluorescence quench by oxamate is no more than approximately one-third in this step, and additional enzyme conformational changes on the slower time scale are required to bind oxamate to its final position to complete the quench (Figure 5).

For the binding of NADH to phLDH, the observed concentration-dependent rate for the bimolecular step (the concentration-dependent rate) is in time range of hundreds of microseconds and shows a well-defined linear relationship, and a single binding site is sufficient to describe the binding process.²³ Interestingly, in both phLDH and bsLDH, concentration-independent rates faster than the rate associated with the binding are observed. However, concentration-independent rates slower than the rate associated with binding are observed only in bsLDH (Figure 1).²³ One possible explanation is that in phLDH, the faster motions, including unfolding of NADH and active site local residue rearrangements to accommodate the extended conformation of NADH, are in sync with the bimolecular step and no additional slower environmental changes to the nicotinamide are required to anchor NADH to its final position. This is possible because of a special feature of the *T*-jump experiment: the faster kinetic processes can be observed regardless of whether they occur before, after, or at the same time as the slower processes. On the other hand, the slower additional protein conformational changes are required in bsLDH, as in the case of binding of oxamate to the LDH·NADH complex.

The analysis described above is adequate for samples with low oxamate concentrations where the contribution from the binary complex equilibrium may be significant but not for samples with high oxamate concentrations where the ternary bsLDH·NADH·oxamate species dominates the reaction mixture. At high oxamate concentrations, a different kinetic scheme could be in place. Indeed, in a recently published paper, we proposed the plausible scheme depicted in Scheme 4.¹⁸

In this kinetic scheme, which is in agreement with various results,^{7,8,15} the encounter complex, presumed to have an “open”-loop conformation, leads to two other conformations of the Michaelis complex, labeled inactive and active, which are probably of “closed”-loop conformations with varying loop structure and an atomic arrangement in the active site. The two closed conformations do not appear to directly interconvert but do so via the encounter complex. The observed increase in the magnitude of emission with an increasing oxamate concentration and a lower final *T*-jump temperature (Figure 7) supports this contention. Again, the higher the oxamate concentration, the smaller the expected contribution from the binary complex equilibrium. Because the “bump” in the ~100 μs region of the kinetic profile becomes prominent at a higher oxamate concentration (green traces in Figure 7), this kinetic event cannot be due to the binding of NADH to LDH. In Figure 7, there is no hint of the “bump” at a lower oxamate concentration (blue traces); more evident is a decrease in the signal in the ~100 μs region reminiscent of a similar decrease in intensity observed in the kinetic traces for the “binary complex” (Figures 1 and 4). Although the changes in the concentration of the binary complex are very small, its effect on the final *T*-jump trace is magnified as the NADH emission quantum yield of this species is 12 times that of the ternary complex. The “bump” in the *T*-jump traces (Figure 7) occurs only at high oxamate concentrations and low temperatures when the rate of ternary complex formation is faster than the rate of formation of the binary complex.

CONCLUSION

T-Jump studies on phLDH (previous work) and bsLDH (this work) binary and ternary complexes revealed that the more rigid bsLDH structure results in several interesting differences in the ligand binding dynamics in these two isozymes. The kinetic data can be best described by a model in which NADH can bind only to the open-loop apoenzyme, oxamate binding is possible only to the bsLDH·NADH binary complex in the open-loop conformation, and oxamate binding is followed by closing of the active site loop preventing oxamate unbinding. The loop open and closed states are in dynamic equilibrium and interconvert on the submillisecond time scale and faster, strongly accelerating with an increasing temperature due to significant enthalpy barriers. Binding of NADH to bsLDH results in minor changes of the loop dynamics and does not shift the open–closed equilibrium, but oxamate substrate mimic binding shifts this equilibrium to the closed state. At high excess oxamate concentrations when all active sites are nearly saturated with the substrate mimic, all active site mobile loops are mainly closed.

The closing of an active site mobile loop that follows substrate binding and brings the atomic groups required for the catalysis into proper contacts is a ubiquitous pattern of key importance in modern enzymology and is addressed by different experimental and molecular modeling approaches.^{3,9,26,36–41} The loop dynamics for bsLDH initiated by substrate binding was found to be in the submillisecond time range^{38,42,43} and is assumed to be rate-limiting. Interestingly, this work suggests that the key surface loop of LDH does not undergo a rigid body motion upon closure. Very typically, these studies show at least two motions for the loop, one on the $\sim 300\text{ s}^{-1}$ scale and another 5 times or so faster (see the G103W data in Table 2). Also, motions of the surface loop appear to be coupled to motions of internal loops.⁴⁴ Our laser-induced *T*-jump studies of binding of the substrate or substrate mimic to phLDH and the number of other proteins^{3,9,26,39–41,45} showed similar temporal characteristics of active site-loop dynamics.

However, quite little is known about the loop motion in free enzymes and their complexes with cofactors. In this study, we applied the laser-induced *T*-jump method to investigate the active site-loop dynamics in all binding states of bsLDH on the way to the Michaelis complex. The reaction model that describes the reaction and the microscopic kinetic parameters for individual reaction steps were determined from the experimental kinetics using computer simulation. We found that the time scale of active site-loop (residues 98–110) motion is quite similar for both the apoprotein and the binary complex (Table 1) and in turn is quite similar to that found in the ternary complex; all proceed with an $\sim 300\text{ s}^{-1}$ motion. We found that the conformation of the active site loop (residues 98–110) affects both NADH cofactor and oxamate substrate mimic binding and that binding is possible only when the loop is open.

We also found that binding of the second NADH molecule to the bsLDH·NADH binary complex occurs at high excess NADH concentrations and is also only possible for the open-loop complex. This dimerization of NADH at the bsLDH binding pocket can affect the loop dynamics and needs to be taken into account in LDH studies if high NADH concentrations ($>0.1\text{ mM}$) are to be used to ensure enzyme saturation with the cofactor.

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Figure 1.

Laser-induced *T*-jump kinetics for two samples: 10 μM bsLDH with 10 μM NADH (left) and 50 μM bsLDH with 207 μM NADH (right). The temperatures after jump are indicated on the bottom graph.

Figure 2.

Dependence of the NADH midrange kinetic rate and tryptophan kinetic rate on the sum of equilibrium concentrations of the free enzyme and ligand in the bsLDH-NADH binary system. The error bars represent fitting errors. Straight lines are linear fits for the first four lowest concentration points, and for the rest of the points.

Figure 3.

Dependence of the NADH slow kinetic rate on the sum of equilibrium concentrations of the free enzyme and ligand in the bsLDH·NADH binary system. The error bars represent fitting errors.

Figure 4.

Kinetic response of the fluorescence signal shown for the emission of the NADH as excited at 360 nm and probed at 450 nm (top) and tryptophan emission excited at 290 nm and probed at 340 nm (bottom), subjected to an $\sim 10^\circ\text{C}$ T -jump final T -jump temperature, T_f , of 30°C for a variety of wild-type bsLDH·NADH solutions. All samples were dissolved in 0.1 M triethanolamine buffer (pH 6.0), and each trace represents an average of 1500–3000 T -jumps. Fits using double exponentials to the 1 μs to 5 ms region of the NADH kinetic profile yielded relaxation rates of approximately 1000–3000 and 10000–25000 s^{-1} that vary with NADH concentration. A single-exponential fit of the Trp T -jump traces, on the other hand, yielded relaxation rates of approximately 1000–3000 s^{-1} that again varied with NADH concentration.

Figure 5.

NADH emission T -jump kinetics of bsLDH with NADH and oxamate at different concentrations. The temperatures after jump are indicated in the bottom graph. The kinetic response of the fluorescence signal is shown for the emission of the NADH as excited at 360 nm and probed at 450 nm, subjected to an ~ 10 °C T -jump for a variety of wild-type bsLDH/NADH/oxamate solutions and final T -jump temperature, T_f : 80 μ M LDH, 80 μ M NADH, and 600 μ M oxamate for $T_f = 20$ °C; 80 μ M LDH, 80 μ M NADH, and 1800 μ M oxamate for $T_f = 20$ °C; and 80 μ M LDH, 80 μ M NADH, and 1800 μ M oxamate for $T_f = 40$ °C. All samples were dissolved in 0.1 M triethanolamine buffer (pH 6.0), and each trace represents an average of 1500–3000 T -jumps. Fits (—) using triple exponentials to the 1 μ s to 5 ms region of the kinetic profile yielded relaxation rates of approximately 250–2000, 1500–5000, and 20000–30000 s^{-1} .

Figure 6. Observed NADH kinetic rates as a function of the sum of equilibrium concentrations of the free binary bsLDH·NADH complex and oxamate ligand. Error bars represent fitting errors.

Figure 7.

Kinetic response of the fluorescence signal for the emission of the NADH as excited at 360 nm and probed at 450 nm, subjected to an ~ 10 °C T -jump, for a variety of wild-type bsLDH-NADH solutions. Temperature-jump relaxation kinetics were measured at various final T -jump temperatures, T_f , 20 °C (top), 30 °C (middle), and 40 °C (bottom). All samples contain 80 mM bsLDH and 80 mM NADH with varying amounts of oxamate and were dissolved in 0.1 M triethanolamine buffer (pH 6.0), and each kinetic trace represents an average of 1500–3000 T -jumps. Fits using triple exponentials to the 1 μ s to 5 ms region of the NADH kinetic profile yielded relaxation rates of approximately 100–500, 1000–3000, and 10000–25000 s^{-1} that vary with oxamate concentration and T_f (see also Table 3).

Scheme 1. Active Site Contacts of Pyruvate and NADH Bound to LDH with Key Residues As Determined by X-ray Crystallography^a

^aThe reaction catalyzed by LDH involves the direct transfer of a hydride ion from C4 of the reduced nicotinamide group of NADH to C2 of pyruvate accompanied by the protonation of pyruvate's keto oxygen, the proton being supplied by His195.^{16,17} It is known that either electrostatic stabilization of the transition state in the pyruvate–lactate interconversion, which contains a highly polarized carbonyl moiety, $^+C-O^-$, or destabilization of the $>C=O$ ground state (or a combination) is responsible for approximately half of the rate enhancement caused by LDH. The other half of the rate enhancement can be attributed to bringing the cofactor and substrate close together in a proper orientation and “activating” the cofactor toward catalysis.

Scheme 2.

A Kinetic Model for the Binding of NADH to bsLDH That Is Adequate for Explaining T -Jump NADH Emission Studies

Scheme 3. A Kinetic Model for the Binding of Oxamate to bsLDH Adequate for Explain *T*-Jump NADH Emission Studies^a

^aEC stands for encounter complex. Additional unimolecular interconversions within the LDH·NADH and LDH·NADH·oxamate complexes are indicated by *T*-jump Trp emission and IR absorption studies, respectively, described below.

Scheme 4^a

^aThe encounter complex, presumed to have an “open”-loop conformation, leads to two other conformations of the Michaelis complex, labeled inactive and active, which are probably of “closed”-loop conformations with varying loop structures and atomic arrangements in the active site. The two closed conformations do not appear to directly interconvert but do so via the encounter complex. Because oxamate is a non-reactive species, the arrow labeled “chemical step” purely reflects the presumed relative reactivity between the observed conformations (cf. refs 5 and 15).

Table 1

Rate Constants Obtained from *T*-Jump Kinetic Reconstructions for the Binary Enzyme/Cofactor System As Fitted to Scheme 2, and Parameters Derived from Them^a

		20 °C	30 °C	40 °C
apoenzyme	loop opening k_1 (s ⁻¹)	300 ± 90	790 ± 240	2000 ± 600
	loop closing k_{-1} (s ⁻¹)	260 ± 80	1200 ± 400	5300 ± 1600
	open/closed k_1/k_{-1}	1.1 ± 0.3	0.7 ± 0.2	0.4 ± 0.1
	open-loop fraction C^{apo}	0.54 ± 0.17	0.40 ± 0.12	0.27 ± 0.08
strong binding	NADH-on k_2 (μM ⁻¹ s ⁻¹)	250 ± 80	330 ± 100	430 ± 130
	NADH-off k_{-2} (s ⁻¹)	330 ± 100	1100 ± 400	3500 ± 1000
	k_{-2}/k_2 (μM)	1.3 ± 0.7	3.4 ± 1.7	8 ± 4
binary complex	loop closing k_3 (s ⁻¹)	270 ± 140	2000 ± 1000	12000 ± 6000
	loop opening k_{-3} (s ⁻¹)	440 ± 220	1500 ± 800	5200 ± 2600
	open/closed k_{-3}/k_3	1.6 ± 0.8	0.8 ± 0.4	0.4 ± 0.2
	open-loop fraction C^{bin}	0.62 ± 0.25	0.43 ± 0.20	0.30 ± 0.12
weak binding	NADH-on k_4 (μM ⁻¹ s ⁻¹)	22 ± 7	34 ± 10	34 ± 10
	NADH-off k_{-4} (s ⁻¹)	2700 ± 800	5600 ± 1700	7000 ± 2000
	k_{-4}/k_4 (μM)	120 ± 60	160 ± 80	210 ± 110

^aErrors for the rate constants were evaluated from their variations in the last reconstruction iterations. The rate absolute values have errors that are larger than their ratios. All measurements were taken at pH 6.0.

Table 2

Observed Relaxation Rates for a 10–20 °C *T*-Jump for the Binary Complexes of 50 μ M bsLDH with Varying NADH Concentrations^a

[NADH] (μ M)	NADH fluorescence <i>T</i> -jump trace		tryptophan fluorescence <i>T</i> -jump trace	
	k_{1_binary} (s^{-1})	k_{2_binary} (s^{-1})	k_{1_binary} (s^{-1})	k_{2_binary} (s^{-1})
30	1239 $\pm$ 58, 76%	14200 $\pm$ 1600, 24%	1663 $\pm$ 30	
50	1196 $\pm$ 40, 76%	14100 $\pm$ 1200, 24%	1856 $\pm$ 33	
300	1320 $\pm$ 110, 62%	29200 $\pm$ 3200, 38%	8240 $\pm$ 340	
400	1.670 $\pm$ 210, 60%	13100 $\pm$ 1700, 40%	11600 $\pm$ 900	
500	5000, 62%	100000 $\pm$ 40000, 38%	18300 $\pm$ 1300	
G106W	not measured	not measured	1960 $\pm$ 250, 60%	11400 $\pm$ 2500, 40%

^aMost binary mixtures contain 50 μ M LDH and varying concentrations of NADH in TEA buffer (pH 6.0). The only exception was for G106W, for which a 150 μ N LDH/150 μ M NADH mixture was prepared in TEA buffer (pH 7.2). Moreover, the final temperature for G106W was 25 °C instead of 20 °C. The percent contribution of each component to the amplitude follows each relaxation rate.

Table 3

Observed NADH Relaxation Rates for a 10° T-Jump Measurement of Ternary Complexes Containing 80 μM LDH, 80 μM NADH, and Various Oxamate Concentrations, Monitored at Different Final T-Jump Temperatures^a

[oxamate] (μM)	20 °C			30 °C			40 °C		
	k_1 (s ⁻¹) (loop motion)	k_2 (s ⁻¹)	k_3 (s ⁻¹) (bimolecular on rate)	k_1 (s ⁻¹)	k_2 (s ⁻¹)	k_3 (s ⁻¹)	k_1 (s ⁻¹)	k_2 (s ⁻¹)	k_3 (s ⁻¹)
80	125 ± 28	1870 ± 90, decreasing amplitude	28000 ± 2400, decreasing amplitude	589 ± 15	3970 ± 220, decreasing amplitude	55000 ± 6000, decreasing amplitude	1294 ± 30	9000 ± 3000, decreasing amplitude	36000 ± 15000, decreasing amplitude
600	270 ± 40	1700 ± 700, decreasing amplitude	11000 ± 3000	520 ± 160	1000 ± 300, decreasing amplitude	33000 ± 6000	1011 ± 19		18800 ± 1900
1200	80 ± 100	1700 ± 900, decreasing amplitude	13000 ± 9000	280 ± 50	2100 ± 1400, decreasing amplitude	23000 ± 7000	660 ± 170		73200 ± 500
1800	140 ± 40	1800 ± 1000, decreasing amplitude	18000 ± 13000	299 ± 35	1400 ± 1000, decreasing amplitude	55000 ± 7000	640 ± 120	1200 ± 2600, decreasing amplitude	75000 ± 5000

^a All ternary mixtures are in TEA buffer at pH 6.0. The slowest observed transient (k_1 in Table 3) can be attributed to a unimolecular transformation involving loop motion, while the fastest (k_3), which scales linearly with oxamate concentration (see the text), is primarily the bimolecular on rate between LDH/NADH and oxamate for forming an encounter complex (see Scheme 3). k_2 is associated with concomitant concentration changes of the binary complex (see eq 2).