Accelerated Publications

Dynamics of Protein Ligand Binding on Multiple Time Scales: NADH Binding to Lactate Dehydrogenase†

Hong Deng, Nick Zhadin, and Robert Callender*

Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10461

Received November 15, 2000; Revised Manuscript Received February 8, 2001

ABSTRACT: Although the importance of atomic motion to how proteins function has been conjectured for several decades, the characterization of protein dynamics on multiple time scales is scant. This is because of severe experimental and theoretical difficulties, particularly characterizing the nanosecond to millisecond time scales. Here, we apply advanced laser-induced temperature-jump relaxation spectroscopic techniques to examine the kinetics of NADH binding to lactate dehydrogenase over this time scale. The bimolecular rate process, at about 290 μs, is easily observed as are multiple faster events (with relaxation times of 200 ns, 3.5 μs, and 24 μs), revealing a rich dynamical nature of the binding step. The results show that there are multiple structures of bound enzyme−ligand complexes, some of which are likely to be far from the catalytically productive structure. The results have important implications for interpretations of the binding thermodynamics of ligands to LDH and, by extension, to other proteins. The observed processes likely play a role in the dynamics of the chemistry that is catalyzed by lactate dehydrogenase.

Very little is understood about the dynamical nature of proteins although this may well be of crucial importance in understanding protein function (*1*−*5*). For example, our knowledge of how ligands bind to proteins, the subject of this paper, consists largely either of static structural pictures showing the atomic positions of the protein with and without the bound ligand (*6*) or of thermodynamic parameters characterizing the change in energy taking place when the ligand binds. The time course of how binding occurs and the events that take place from unbound ligand to the protein−ligand complex are uncharacterized because of formidable experimental and theoretical challenges. It is anticipated that binding dynamics will take place on multiple time scales, from picoseconds to milliseconds (and slower). The time scale from nanoseconds to milliseconds is especially inaccessible both experimentally and theoretically. It is very difficult to employ the normal tools used to study chemical kinetics (i.e., stopped-flow techniques), NMR and vibrational spectroscopies (for a variety of reasons), and/or theory to study this time range.

We report here the dynamics of the nucleotide NADH as it binds to lactate dehydrogenase (LDH)[1] using temperature-jump relaxation spectroscopy. The NAD-linked dehydrogenases are a large class of enzymes which employ NADH as coenzyme in the oxidation and reduction of alcohols and aldehydes. Lactate dehydrogenase catalyzes the conversion of NADH and pyruvate to NAD^+ and lactate with a very high efficiency, the catalyzed reaction being approximately 10^{14} times faster than the uncatalyzed one (*7*, *8*). The catalytic pathway for this enzyme is ordered whereby coenzyme binding precedes substrate binding. The enzyme contains two domains: the coenzyme (either NADH or NAD^+) binding domain, which is conserved in the dehydrogenases (the "Rossmann fold"), and a substrate binding domain.

Temperature jumps can provide the "trigger" initiating a reaction, like ligand binding, on very fast time scales, and the structural changes as a function of time can then be probed using a variety of spectroscopies. For example, laser-induced temperature jumps can heat water faster than 50 ps by well over 20 °C (*9*−*13*). In this approach, near-IR light irradiates weak water bands, giving rise to excited water molecules. Since these excited molecules thermalize in about 10 ps, this 10 ps thermalization rate or the excitation pulse width, whichever is slower, sets the time to generate the temperature jump in the irradiated volume and, hence, the resolution of the system (*12*). A system in equilibrium before the temperature jump, such as a solution of free protein, unbound ligand, and protein−ligand complex, now must "relax" to reach a new equilibrium set by the higher temperature. Here, we measure in real time, using a nanosecond spectrometer, the fluorescence from the nicotinamide headgroup of NADH to monitor changes in binding that occur in response to the temperature jump. The emission of NADH is sensitive to the environment of the chromophore (*14*). The observed relaxation times are functions of both forward and backward rate constants. In this way, kinetic

† This work was supported by the National Science Foundation, MCB-9727439, and the Institute of General Medicine, National Institutes of Health, GM35183.

* Corresponding author. Phone: 718-430-3024. Fax: 718-430-8565. E-mail: call@aecom.yu.edu.

[1] Abbreviations: LDH, lactate dehydrogenase; NADH, reduced β-nicotinamide adenine dinucleotide.

10.1021/bi0026268 CCC: $20.00
Published on Web 03/09/2001

events occurring at virtually any time (slower than about 10 ps in principle, tens of nanoseconds in this study) can be measured. Temperature-jump relaxation spectroscopy dates back to the 1960s and has been used in many ligand binding studies with limited success, particularly in probing events that take place faster than the bimolecular rate of diffusion for the encounter of protein and ligand (cf. refs *15* and *16*). Recent technical advances have made such studies much more feasible, and it is now possible to employ these methods on very fast time scales and with a fair amount of structural specificity.

The process and pathways of ligands binding to proteins are expected to be a complicated process. Even before producing a close encounter complex between the ligand and protein, it is clear that, in some cases, the two molecules can approach each other faster than simple diffusion with the ligand being guided to the protein binding pocket using long-range electrostatic forces (*17*, *18*). In general, once a protein finds its ligand, comparatively short-range forces come into play and a number of events can be imagined. The binding pocket must rearrange to make room for the ligand; the empty pocket must be desolvated; specific ion pairs and/or hydrogen bonds may form between charged and polar groups of the apoprotein and ligand; loops or flaps of protein may close over the ligand. There is experimental evidence and substantial theoretical thought that conformational substates can play an important role in the dynamics of ligand binding, with some conformations being more facile toward binding than others (cf. ref *4*). In such a case, highly nonexponential kinetics are observed as has been found, for example, in the extensive literature concerning small molecule binding to the heme group of myoglobin or hemoglobin.

METHODS

L-Lactate dehydrogenase (L-LDH from pig heart) was purchased as a crystalline suspension in ammonium sulfate solution from Boehringer-Mannheim Co. The enzyme was dialyzed three times against 0.1 M sodium phosphate solution (pH 7.0) at 4 °C. After dialysis, the protein solution was filtered through a 0.45 μm filter and concentrated by centrifugation. NADH (grade I, 100%) was purchased from Boehringer-Mannheim Co. and used without further purification. Concentrations of LDH and NADH were determined using extinction coefficients $\epsilon_{280} = 200\ mM^{-1}\ cm^{-1}$ and $\epsilon_{340} = 6.22\ mM^{-1}\ cm^{-1}$, respectively. Approximately 100 μL solutions were needed to fill a sample cell (0.2 mm path length).

The T-jump experiments were performed by a lab-built apparatus similar in design to that found in ref *19*. Rapid temperature perturbation, i.e., up to a 20 °C jump here, was achieved by irradiating water using IR emission at 1.54 μm with 70 mJ incident energy in a spot of about 1−2 mm diameter. This emission was produced by Raman shifting the fundamental (1.064 μm) of a Quanta-Ray GCR-150 Q-switched Nd:YAG laser (Spectra Physics, Mountain View, CA), using methane gas filled in a 1 m long cell at 450 psi. The pulsed laser was operated at 1 Hz and the emission split into two beams with a combined pulse width of 20 ns full width at half-maximum. These two beams irradiated the sample, one from behind and one from the front to produce a uniformly heated volume. Fluorescent emission was excited by an Innova 200-25/5 argon ion laser (Coherent, Palo Alto, CA) operated at 360 nm. A shutter intercepted the excitation light and was opened only when needed (typically for about 10 ms for each pulse of the heating beam); this permitted a more intense irradiation of the chromophore while still avoiding photodamage. The emission was focused on the sample with a spot of 0.3 mm diameter, which overlapped with the IR spot. Fluorescences filtered through a narrow-band interference filter (450 ± 20 nm) were detected and acquired by a R4220P photomultiplier tube (Hamamatsu, Bridgewater, NJ) at a 50° angle toward the incident excitation beam. All signals were averaged by digitizing oscilloscopes (TDS 754A and TDS 420A, Tektronix Inc., Beaverton, OR).

The experiment was controlled with a computer using LabVIEW Software (National Instruments, Austin, TX). Typically, 1000−2000 averages were recorded in a T-jump experiment. For the studies on LDH solutions, the enzymatic activity of the protein was measured before and after the run, and no degradation was observed. The background signal measured without fluorescence excitation was subtracted from the kinetic data. All data were normalized to the fluorescent intensity before temperature perturbation. Data were processed using IGOR Pro software (WaveMetrics Inc., Lake Oswego, OR).

The temperature jumps used in this study of up to 20−25 °C are large. Cavitations caused by the resulting pressure wave and other less well understood phenomena induced by the temperature jump are sometimes observed as emitted light signals which can give rise to artifacts in the kinetic traces. We have noted these before (*20*). These signals were characterized by runs of NADH alone and solution mixtures of NADH with a protein (protein−tyrosine phosphatase) that does not bind this molecule. In most experiments on these samples, the observed kinetics were featureless (see below), as expected. In some cases, the cavitation signals were observed. In our present apparatus, these signals occurred on random time scales and sometimes with about the same intensity as the signal amplitudes reported below. Typically, the signals were of substantially shorter duration than could be fit by a single exponential and often appeared as random noise; about one out of ten runs yielded a "cavitation" signal with sufficient size and duration to resemble a single exponential relaxation signal. For this reason, several controls were run. First, the results presented below were consistently observed in 10−20 separate runs (depending on the experiment). Additional experiments on LDH and NADH solutions were performed whereby the tryptophan residues were excited at 290 nm and either the Trp emission monitored at 340 nm or emitted light from the nicotinamide headgroup at 450 nm which comes about by energy transfer. The signals from the Trp emission differed from the energy transferred signals while the NADH signals were similar to those observed in the experiments using 360 nm excitation. Finally, the addition of the inhibitor oxamate (*21*) to the mixture of LDH and NADH yielded somewhat different kinetics than that observed for LDH and NADH alone.

RESULTS

Fluorescence emission depends on several parameters. An increase in temperature typically quenches emission, so the

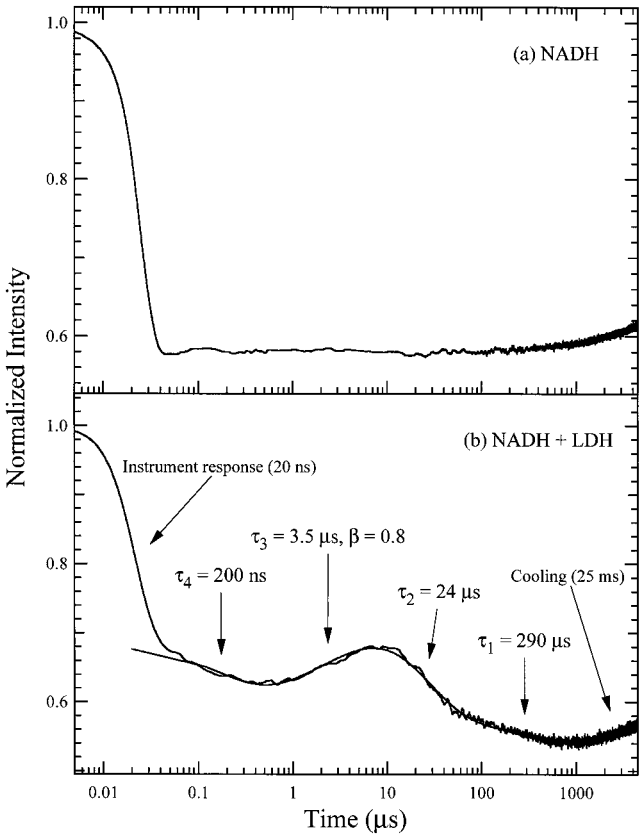


FIGURE 1: Time-resolved fluorescent emission of the nicotinamide ring of NADH from (a) 0.2 mM NADH and (b) binary complex pig heart LDH ([LDH] = [NADH] = 50 μM), following a 20 °C T-jump. The initial temperature of the samples was 12 °C. Exponential fits (solid line) to the transient signals yielded the observed relaxation times shown in panel b. The raw data were smoothed by taking a simple average of a fixed number of time points. Data in time later than 4 μs were smoothed by 1001 points while data before 4 μs were smoothed by 51 points.

change in intensity can be used as a thermometer. Environmental factors around the chromophore also influence emission efficiency. For NADH, the emission of light from the nicotinamide moiety is typically weaker in water than when protein bound. The emission efficiency increases about a factor of 3.75 when NADH binds to LDH.

Figure 1 shows the changes in fluorescence intensity of the nicotinamide moiety at 450 nm as excited by 360 nm in response to a temperature jump from 12 to 32 °C. The time axis is plotted in logarithmic units. Panel a shows the response of NADH in solution. There is an immediate drop in intensity which is due simply to the temperature increase which quenches fluorescence. This drop is unresolved; the observed 20 ns decay time (half-width) is due to the response time of the electronics (ca. 10 ns) and the pump pulse width for this experimental configuration. Hence, there are no observed changes in structure for NADH that are induced by the temperature jump. It is known that intramolecular stacking between the nicotinamide and adenine rings takes place in solution (*22*), and it is expected that destacking resulting from an increased temperature would result in increased emission. The reason this is not observed is probably that the relaxation kinetics of destacking are faster than our experimental resolution. On the long time scale, cooling of the irradiated volume by thermal diffusion is observed and has a lifetime of about 25 ms in this experiment.

Panel b shows the relaxation kinetics of a mixture of NADH and LDH, each having an initial concentration of 50 μN (pig heart LDH contains four independent binding sites per protein, and the units of normality are used to denote the concentration of binding sites). Control runs of LDH alone under otherwise the same conditions yielded emission signals with an amplitude of about 500 times less (likely due to trace amounts of NADH in the apoprotein). At least four relaxation events are observed between the response time of the instrument and sample cooling. These kinetics were fit to a series of "stretched" exponentials of the form $A \exp[-(t/\tau)^{\beta}]$. The parameter β gives an indication of how far from single exponential behavior a particular component in the kinetics is (a β close to one is single exponential). The fit is shown as the solid line in Figure 1b. All but one of the kinetic features yielded good fits to single exponential functions: $\tau_4 = 200$ ns ($\beta_4 = 1.0$); $\tau_3 = 3.5$ μs ($\beta_3 = 0.8$); $\tau_2 = 24$ μs ($\beta_2 = 1.0$); $\tau_1 = 290$ μs ($\beta_1 = 1.0$). A $\beta = 0.8$ indicates just minor departure from single exponential behavior.

It is expected that one of the relaxation events is due to the bimolecular association of ligand and protein. The kinetics of this reaction can be fit to the form $k_{obs} = k_1([NADH] + [LDH]) + k$,where k_1 is the bimolecular association rate and k is an "apparent" dissociation rate constant which is actually composed of the dissociation rate constant, k_{-1}, of the first encounter complex and other rate constants if there are conformational rearrangements that take place after formation of the encounter complex. A series of runs were performed, varying the initial concentrations of LDH and NADH. Independent titration studies were performed using NADH fluorescence to monitor concentration, and the dissociation constant was determined ($K_d = 6.8$ μM) in a manner previously described (*23*, *24*) at the final temperature reached in the T-jump experiments (32 °C). Using this value and the initial concentrations, the amounts of [NADH] and [LDH] were determined (this procedure must be considered approximate in view of the existence of multiple bound structures; see below). The $\tau_1 = 290$ μs rate observed in Figure 1b was sensitive to concentration while the three faster relaxation events observed in Figure 1b were not. This concentration dependence is plotted in Figure 2 (dots). A least-squares fit (solid line) yielded $k_1 = 9.5 \times 10^7$ M^{-1} s^{-1} and $k = 416$ s^{-1}. These rate constants are in line with previous measurements (*23*); it can be noted that previous studies employed smaller concentrations to bring measured times out to the millisecond time domain. The three new observed faster steps, apparent single exponential kinetics at 200 ns and 24 μs and the multiple exponential process centered at about 3.5 μs, have never before been observed. Previous results have shown that the apparent dissociation rate constant observed in relaxation studies is the same rate observed in stopped-flow studies (*23*, *25*), suggesting there are no protein conformational steps slower than k.

The equations describing relaxation spectroscopy are well developed (*15*, *16*, *26*, *27*). Each observed relaxation time corresponds to a change in concentration from one chemical species to another with a forward and backward rate constant. Thus, the minimum kinetic scheme consistent with the

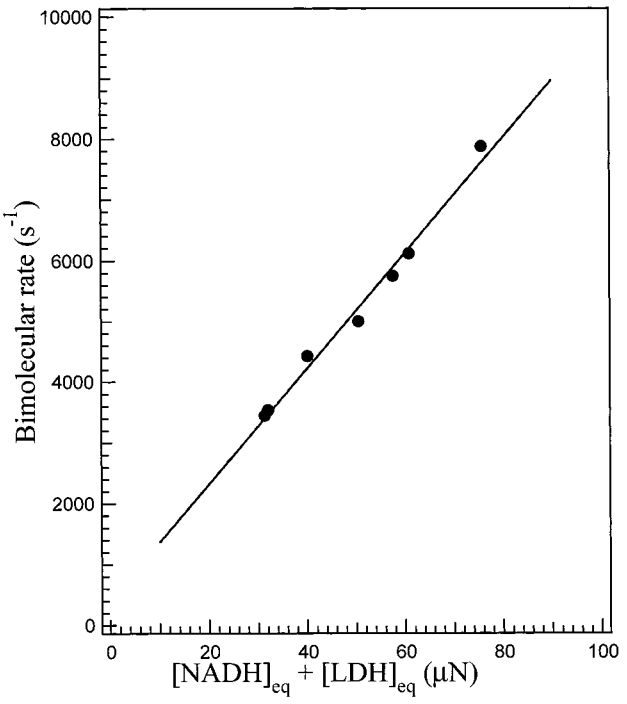
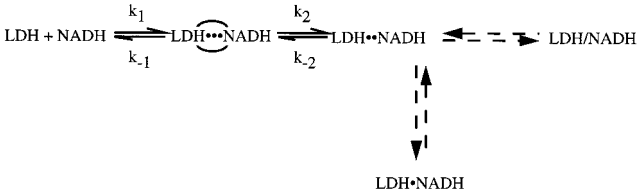


FIGURE 2: Plot of the bimolecular rate, $1/\tau_1 = k_{obs}^1$, as a function of the sum of free LDH and NADH concentration at 32 °C. The solid line is a nonlinear fit of the data to $k_1([NADH] + [LDH]) + k$, which yields $k_1 = 9.5 \times 10^7$ $M^{-1}\cdot s^{-1}$ and $k = 416$ s^{-1}. A dissociation constant of 6.8 μM was used to calculate the unbound LDH and NADH concentrations in solution.

Scheme 1: Pictorial Representation for the Binding of NADH to LDH Showing the Formation of an Initial Diffusional Encounter Complex and Subsequent Formation of Multiple Bound Complexes Which Are in Equilibrium[a]



[a] The dashed arrows emphasize that both sequential and parallel kinetic paths are possible.

observation of four relaxation times involves eight rate constants and five species. Both parallel and sequential schemes are possible as are mixed parallel and sequential pathways. Only one of the relaxation times is dependent on concentration, and so this event involves the formation of a diffusional encounter species from free NADH and LDH. We depict this encounter complex as

Then, a simple picture of the species involved and their interconversions are shown in Scheme 1. Here, LDH•• NADH, LDH•NADH, and LDH/NADH represent monomolecular binary protein−ligand complexes, and the scheme emphasizes the possibility of both parallel and sequential interconversions. The dashed arrows suggest the possibility of more interconverting bound complexes than are actually observed.

The T-jump changes the equilibrium distribution toward unbound ligand and free protein (ligand dissociation) so that the amplitudes of the relaxation kinetics are determined by the changes in concentration of the species as a result of the temperature jump, going right to left in Scheme 1, and the emission amplitudes of the nicotinamide moiety for each species. Kinetic steps may not be observed if the emission amplitude is not sensitive to the structural change or if a fast process follows a slower process during ligand dissociation (since the relatively slow process is rate limiting). The structural changes that are represented in the kinetic results may be due to a substantial change in relative positioning of the nicotinamide headgroup with respect to the protein (and final protein binding pocket) or may be due to a more subtle protein conformational change that affects the emission quantum yield of the nicotinamide ring. The bimolecular rate reaction is easily disentangled in the kinetics because it is the slowest step and because the observed rate is dependent on concentration: $1/\tau_1 = k_{obs}^1 = k_1([NADH] + [LDH]) + k$. The apparent dissociation rate constant, k, and the three faster relaxation events observed on the microsecond time scale are then functions of the other rate constants (k_1, k_2, k_{-2}, etc.). If $k_{-1} \ll k_2$, $k_{-2} \gg k_3$, k_{-3}, etc., then it can be calculated that $k \approx k_{-1}k_{-2}/(k_2 + k_{-2})$. The apparent dissociation constant, k_{-1}/k_1, $= 4.4$ μM. This is somewhat smaller than the measured value of $K_d = 6.8$ μM, which is expected if $k_{-2} \approx k_2$.

It is now clear why previous studies were generally unsuccessful in observing kinetic steps apart from the bimolecular association. Most of the signal observed in Figure 1 comes from the change in temperature of the sample due to the T-jump. The incremental emission signals arising from the temperature-induced concentration change in going from the binary LDH/NADH complex to free LDH and NADH are relatively small, and the corresponding emission changes in amplitudes are small and are only revealed by the large temperature change made possible with the laser-induced T-jump and the high signal-to-noise ratio which can be obtained only with very bright light sources (here laser). The total expected concentration-dependent emission change can be estimated from an approximate calculation of the concentration changes, taking into account that the nicotinamide emission quantum yield changes when free NADH complexes with LDH. The concentration of free enzyme and NADH at 12 °C is 9 μM for the 50 μM initial conditions (the conditions for data in Figure 1b) using a K_d of 1.9 μM, and this is increased to 15 μM at 32 °C ($K_d = 6.8$ μM). A simple calculation, using these figures and weighting the emission quantum yield for bound versus free NADH (a factor of 3.75/1, respectively, from ref *24*), suggests about a 10% change in emission intensity (or 0.10 in the units of the *y*-axis of Figure 1b). This concentration-induced emission change would be fully expressed at the time of the bimolecular rate relaxation event (near 500 μs) since it is at this point that enzyme and coenzyme form their encounter complex. Hence, about 20% of the observed signal changes arises from the effects of a concentration change while about 80% is due to the increase in temperature.

That significant signals of transient intermediates are observed in the kinetics shows that substantial populations of structurally different bound states exist at equilibrium. Signal size in T-jump kinetics is a function of the change in concentration induced by the temperature change weighted by the change in emission quantum yield. The values of the

emission quantum yields are not known, and the specific values of the various rate constants are also not known. Therefore, it is not now possible to calculate exact populations. However, the amount of enzyme−coenzyme that exists in intermediates between the final binary complex and free state can be estimated, crudely, from the amplitudes in the relaxation kinetics relative to the expected 12% change between LDH/NADH and free NADH. The relative changes in emission amplitudes are on the order of 5% so that the concentration of the intermediates is several micromolar (about half of the 6 μM change in bound to free NADH induced by the T-jump).

DISCUSSION

An important result from this study is that the experimental approach taken here, laser-induced temperature-jump spectroscopy on fast time scales, reveals much about the dynamical nature of proteins on the nanosecond to millisecond time scale that has generally been inaccessible both theoretically and experimentally. It is also evident that there is a very rich dynamical nature to proteins on these time scales. This is certainly anticipated theoretically and also from studies of those few systems where pulsed laser irradiation of a strategic chromophore initiates ligand binding reactions on very fast time scales (for example, studies of the binding of small ligands to protein heme groups).

The binding of NADH to LDH involves several steps that are faster than the diffusional encounter rate between ligand and protein, which at the concentrations employed here occurs around 290 μs. That is, after the protein and ligand first come together to produce an encounter complex, a number of bound species can be formed. Our results reveal three distinct "steps" faster than diffusional encounter with relaxation times of 200 ns, 3.5 μs, and 24 μs, which suggests that there are at least three bound enzyme−ligand structures. There may be more bound structures since the present experiment only observes fluorescence from the nicotinamide headgroup of NADH and so misses binding kinetics associated with other moieties of the coenzyme. Moreover, relatively faster kinetic steps will be unobserved if they precede a relatively slower step assuming the binding pathway is sequential.

The view of ligand binding that results from the present study is quite different than is suggested in static crystal structures where the ligand is either in solution or bound to the protein in a single conformation. The results reveal that the "bound state" actually consists of substantial populations of several structures in a dynamic equilibrium. Some of these structures may lie quite far from the structure defined in crystallographic studies (see below). This observation is in agreement with a recent NMR study that suggests that the adenine moiety is bound to LDH in a way suggested by the crystal structure while the nicotinamide moiety retains considerable conformational flexibility (*28*). Moreover, the C4−H vibrational stretch mode of the nicotinamide group of NADH, which is sensitive to the planarity of the ring (the ring can adopt chair/boat forms) and other factors, is multiply peaked and heterogeneously broadened in the binary complex of LDH with NADH, clearly showing the existence of multiple bound structures of the nicotinamide moiety (*29*). This has two important consequences. In the first place, it wreaks havoc with the common notion that the binding constant between a ligand and a protein can be understood in a two-state approximation as a simple equilibrium between free and bound structures. Hence, conclusions from calculations of the thermodynamics of binding of NADH to LDH in terms of the two-state approximation and/or binding studies using, for example, mutant proteins that incorporate features of the nicotinamide binding pocket and/or NADH analogues may need revisions to incorporate multiple bound species. The second consequence following from multiple populated bound structures has to do with the dynamical nature of enzymatic catalysis, which has recently become a matter of intense speculation (*30*). With regard to LDH, the hydride transfer step from NADH to substrate in the reaction catalyzed by this protein can occur only when the two molecules are quite close. The rate of forming a productive encounter complex is ultimately governed by dynamics, and the kinetic processes observed here must be involved in this rate since some of the LDH−NADH binary complexes almost certainly have an improperly placed NADH.

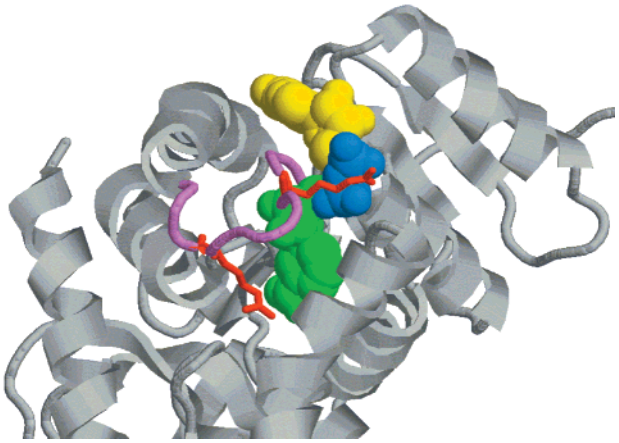


FIGURE 3: Graph of spacial arrangements of NADH bound to LDH determined from crystallographic studies (*31*; Protein Data Bank ID number 9LDB). One monomer of the enzyme (partially blocked) is shown as cartoon in silver. The three different moieties of NADH are displayed in different colors: adenosine in yellow, pyrophosphate in sky blue, and nicotinamide plus the nicotinamde ribose in green. Two arginines, Arg-101 and Arg-109, are presented in red while the loop 98−112 is displayed in violet.

The kinetics do not tell us anything directly about the structural changes that take place except that the environment of the nicotinamide headgroup changes in such a way as to produce the observed relaxation rates and amplitudes. To make structural assignments will require using other spectroscopies sensitive to different structures types (like IR), monitoring other fluorophores (like the indole ring of tryptophans), use of fluorescing NADH analogues, introduction of fluorophores into the structure of the protein and the use of FRET studies, and/or studies in conjunction with mutant proteins so that an association with a protein structural modification can be made with modified kinetics and amplitudes. It seems possible to us that NMR spectroscopy may be useful also when the results are analyzed in terms of the kinetic times and amplitudes observed in the relaxation measurements of the sort presented here.

However, knowledge of the final structure of the binary complex and other binding attributes can help to make initial guesses into tying structure to kinetics. Figure 3 shows the atomic arrangement as of NADH bound to pig heart LDH

as determined from crystallographic studies (*31*). The NADH nucleotide binding domains in the NAD-linked dehydrogenases have been shown to be quite similar, consisting of six parallel strands of pleated sheets packed against four helices (*32*; the so-called Rossmann fold motif). In these structures, NADH binds in an extended conformation. There are several specific features of the LDH−NADH complex which are to be noted. The adenosine moiety lies in a generally hydrophobic crevice lined by several valines and glycines with hydrogen bond interactions between protein and the adenosine ribose. A substantial portion of the adenosine moiety is solvent exposed in the LDH/NADH binary complex. In the competent ternary complex of LDH, consisting of protein, NADH, and substrate, the mobile loop, approximately residues 98−110, moves about 13 Å and folds over the pyrophosphate group inserting Arg-101 into contact with pyrophosphate and also inserting the catalytically crucial Arg-109 in contact with substrate. The loop has been drawn in the open position in Figure 3 since (1) it is found open in the binary complex crystallographic structure (*31*), (2) spectroscopic results on bound NADH show considerable conformational flexibility for the nicotinamide moiety in the binary (but not ternary) complex (*29*), and (3) the kinetics of loop closure in ternary complexes are relatively slow (*33*, *34*) and so cannot be responsible for the nanosecond−microsecond kinetics that are observed. The nicotinamide ribose and the nicotinamide ring itself are buried (*35*). Several interactions orient the nicotinamide group in its binding pocket. The most important of these include the several hydrogen bond interactions with the C═O and $-NH_2$ moieties of the nicotinamide carboxamide group which serve to orient the ring so that the C4 hydrogen of the *re* face of NADH points toward bound substrate in the ternary complex (cf. ref *21*). It has been estimated that the difference in energy between this conformation and the one rotated 180° about the glycosidic bond (which brings the carboxamide group into a hydrophobic environment) is more that 10 kcal/mol (*21*). In addition, these calculations suggest that the nicotinamide ring is not free to rotate sterically from an anti to a syn conformation as there is a substantial energy barrier between the two geometries.

The bimolecular kinetics, formation of

from NADH and LDH (Scheme 1), likely involves some sort of recognition center that LDH has for NADH apart from the full binding pocket. The changes in the NADH emission intensity are fairly small, and there has not been enough time in the ca. 290 μs for the entire binding process to take place since faster relaxation processes are clearly observed. This recognition center almost certainly involves the adenine binding pocket. This protein region lies at the surface of the protein as outlined above, and NADH fragments that include the adenosine moiety (AMP, etc.) and aromatic compounds bind quite well to LDH. Overall, the adenine binding pocket is recognized as a general purpose binding pocket for aromatic molecules (*36*). In addition, as mentioned above, the NMR study suggests that the adenine moiety is properly bound to its protein pocket while the nicotinamide moiety is not (*28*). This first binding step does involve some protein interaction with the nicotinamide ring since its emission is modulated. We suspect that the nicotinamide binding in the encounter complex is rather nonspecific. The minor decrease in emission observed in the T-jump in the ca. 290 μs time range results from unbinding so that the emission of the nicotinamide ring is a bit quenched by its association with LDH in the bimolecular association. This would be consistent with a slightly more hydrophobic environment on the enzyme than in solution.

On the basis of the time scale, the steps faster than the bimolecular association may then be due to motions whereby, with the adenine moiety anchored, the nicotinamide group works its way into the binding pocket (perhaps the slower 24 μs kinetics) and, arriving closer to its binding pocket, searches and makes its correct contacts that orient the nicotinamide group properly for the later catalysis steps (the ca. 200 ns and 3.5 μs kinetics). Alternatively, there may well be subpopulations of bound states where the adenosine moiety is very loosely anchored, or not anchored, and the nicotinamide group forms strong contacts with protein. With regard to the fastest step, a ca. 200 ns time implies a kinetic barrier of about 9 kcal/mol using a 10^{13} preexponential factor. This is consistent with molecular dynamics calculations that yield a substantial energy barrier between the *syn* and *anti* configuration of the nicotinamide ring in the LDH binding pocket (*21*). The 3.5 μs step most likely involves conformational substates since it shows multiple exponential behavior. That is, the nicotinamide moiety apparently binds to several conformations of the protein at this step. The kinetics of all these steps have to be influenced as well by the dynamical nature of the interactions between LDH and NADH along the entire NADH molecule. The time-dependent binding interactions occurring at NADH's pyrophosphate group will likely influence the kinetics of the interactions between the nicotinamide headgroup and apoprotein, for example, since the two moieties are connected through covalent bonds. Internal motions ("breathing" motions, for example) within the apoprotein itself may also be important in determining characteristic kinetics. That there are substantial group motions within the protein itself, apart from motions of the mobile loop, are shown by T-jump experiments on LDH itself, monitoring the emission of the indole rings of Trp residues (LDH contains six Trp residues per monomer, none close to the binding pocket), which indicate substantial structural changes in the environments of one or more of these groups on the 40 and 150 ns time scales (data not shown).

REFERENCES

1. McCammon, J. A., and Harvey, S. C. (1987) *Dynamics of Proteins and Nucleic Acids*, Cambridge University Press, Cambridge.
2. Karplus, M., and McCammon, J. A. (1983) *Annu. Rev. Biochem. 53*, 263−300.
3. McCammon, A. (1998) *Curr. Opin. Struct. Biol. 8*, 245−249.
4. Frauenfelder, H., Sligar, S. G., and Wolynes, P. G. (1991) *Science 254*, 1598−1603.
5. Zhou, H.-X., Wlodek, S. T., and McCammon, J. A. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 9280−9283.
6. Gerstein, M., Lesk, A. M., and Chothia, C. (1994) *Biochemistry 33*, 6741−6749.
7. Holbrook, J. J., Liljas, A., Steindel, S. J., and Rossmann, M. G. (1975) in *The Enzymes* (Boyer, P. D., Ed.) pp 191−293, Academic Press, New York.

8. Burgner, J. W., and Ray, W. J. (1984) *Biochemistry 23*, 3636−3648.
9. Pimentel, G. (1964) *Appl. Phys. Lett. 5*, 231.
10. Holzwarth, J. F., Schmidt, A., Wolff, H., and Volk, R. (1977) *J. Phys. Chem. 81*, 2300−2301.
11. Turner, D. H., Flynn, G. W., Sutin, N., and Beitz, J. V. (1972) *J. Am. Chem. Soc. 94*, 1554−1559.
12. Callender, R., Gilmanshin, R., Dyer, R. B., and Woodruff, W. (1994) in *Physics World*, pp 41−45.
13. Dyer, R. B., Gai, F., Woodruff, W., Gilmanshin, R., and Callender, R. H. (1998) *Acc. Chem. Res. 31*, 709−716.
14. Lakowicz, J. R. (1999) *Principles of Fluorescence Spectroscopy*, 2nd ed., Kluwer Academic/Plenum Publishers, New York.
15. Hammes, G. G., and Schimmel, P. R. (1970) in *The Enzymes* (Boyer, P. D., Ed.) pp 1−584, Academic Press, New York.
16. Cantor, C. R., and Schimmel, P. R. (1980) *Biophysical Chemistry, Vol. 2*, W. H. Freeman and Co., San Francisco.
17. Schreiber, G., and Fersht, A. R. (1996) *Nat. Struct. Biol. 3*, 427−431.
18. Gabdoulline, R. R., and Wade, R. C. (1997) *Biophys. J. 72*, 1917−1929.
19. Gulotta, M., Gilmanshin, R., Callender, R. H., and Dyer, R. B. (2001) *Biochemistry* (in press).
20. Gilmanshin, R., Williams, S., Callender, R. H., Woodruff, W., and Dyer, R. B. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 3709−3713.
21. van Beek, J., Callender, R., and Gunner, M. (1997) *Biophys. J. 72*, 619−626.
22. Oppenheimer, N. J. (1987) in *Coenzymes and Cofactors* (Dolphin, D., Avramovic, O., and Poulson, R., Eds.) pp 185−230, John Wiley & Sons, New York.
23. Heck, H. D. A. (1969) *J. Biol. Chem. 244*, 4375−4381.
24. Fromm, H. J. (1963) *J. Biol. Chem. 238*, 2938−2944.
25. Heck, H. D. A., McMurray, C. H., and Gutfreund, H. (1968) *Biochem. J. 108*, 793.
26. Bernasconi, C. F. (1976) *Relaxation Kinetics*, Academic Press, New York.
27. Fersht, A. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, Freeman and Co., New York.
28. Vincent, S. J. F., Zwahlen, C., Post, C. B., Burgner, J. W., and Bodenhausen, G. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 4383−4388.
29. Deng, H., Zheng, J., Sloan, D., Burgner, J., and Callender, R. (1992) *Biochemistry 31*, 5085−5092.
30. Bruice, T. C., and Benkovic, S. J. (2000) *Biochemistry 39*, 6267−6274.
31. Dunn, C. R., Wilks, H. M., Halsall, D. J., Atkinson, T., Clarke, A. R., Muirhead, H., and Holbrook, J. J. (1991) *Philos. Trans. R. Soc. London, Ser. B 332*, 177−185.
32. Branden, C., and Tooze, J. (1991) *Introduction to Protein Structure*, Garland Publishing, New York.
33. Parker, D. M., Jeckel, D., and Holbrook, J. J. (1982) *Biochem. J. 201*, 465−471.
34. Clarke, A. R., Waldman, A. D. B., Hart, K. W., and Holbrook, J. J. (1985) *Biochim. Biophys. Acta S29*, 397−407.
35. White, J. L., Hackert, M. L., Buehner, M., Adams, M. J., Ford, G. C., Lentz, P. J., Smiley, I. E., Steindel, S. J., and Rossmann, M. G. (1976) *J. Mol. Biol. 102*, 759−779.
36. Subramanian, S., and Ross, P. D. (1978) *Biochemistry 17*, 2193−2197.

BI0026268