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Fluorescence lifetime analysis and effect of magnesium ions on binding of NADH to human aldehyde dehydrogenase¹

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Abstract

Aldehyde dehydrogenase 1 (ALDH1A1) catalyzes the oxidation of toxic aldehydes to carboxylic acids. Physiologic levels of Mg^{2+} ions decrease ALDH1 activity in part by increasing NADH binding affinity to the enzyme. By using time-resolved fluorescence spectroscopy, we have resolved the fluorescent lifetimes (τ) of free NADH in solution ($\tau = 0.4$ ns) and two enzymebound NADH states ($\tau = 2.0$ ns and $\tau = 7.7$ ns). We used this technique to investigate the effects of Mg^{2+} ions on the ALDH1A1-NADH binding characteristics and enzyme catalysis. From the resolved free and bound NADH fluorescence signatures, the K_D values for both NADH conformations in ALDH1A1 ranged from about 24 μM to 1 μM for Mg^{2+} ion concentrations of 0 μM to 6000 μM , respectively. The rate constants for dissociation of the enzyme-NADH complex ranged from 0.03 s^{-1} (6000 μM Mg^{2+}) to 0.30 s^{-1} (0 μM Mg^{2+}) as determined by addition of excess NAD^+ to prevent re-association of NADH and resolving the real-time NADH fluorescence signal. During the initial reaction of enzyme with NAD^+ and butyraldehyde, there was an immediate rise in the NADH fluorescence, due to the formation of bound NADH complexes, with a constant steady-state rate of production of free NADH. As the Mg^{2+} ion concentration was increased, there was a consistent decrease of the enzyme catalytic turnover from 0.31 s^{-1} (0 μM Mg^{2+}) to 0.050 s^{-1} (6000 μM Mg^{2+}) and a distinct shift in steady-state conformational population from one that favors the ALDH1-NADH complex with the shorter fluorescence lifetime (33% excess) in the absence of magnesium ion to one that favors the ALDH1-NADH complex with the longer fluorescence lifetime (13% excess) at 6000 μM Mg^{2+} . This shift in conformational population at higher Mg^{2+} ion concentrations and to lower enzyme activity may be due to longer residence time of the NADH in the ALDH1 pocket. The results from monitoring enzyme catalysis in the absence of magnesium suggests that the ALDH1-NADH complex with the shorter fluorescence lifetime is the form initially produced, and the complex with the longer fluorescence lifetime is produced through isomerization.

Keywords

Aldehyde dehydrogenase; Fluorescence; NADH

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Conflict of interest

The authors state that there are no conflicts of interest.

1. Introduction

Aldehydes are produced as a consequence of ethanol metabolism and lipid peroxidation that occurs in several diseases. Cells possess enzymes that decrease the levels of these aldehydes, among which the aldehyde dehydrogenases (ALDHs) play a significant role. These enzymes use NAD^+ to oxidize aldehydes to carboxylic acids and produce NADH by an irreversible ordered sequential mechanism involving NAD^+ binding (k_1), thiohemiacetal formation (k_2), hydride transfer (k_3), deacylation (k_4) and NADH dissociation (k_5) [1] (Fig. 1).

Several papers have been published on the effect of Mg^{2+} ions on the specific activity of ALDHs [1–4]. The concentration of free cytosolic Mg^{2+} ions in human erythrocytes has been estimated to be 250 μM [5]. The concentration of Mg^{2+} ions can alter ALDH activity by altering the rate limiting steps of enzyme catalysis, but for ALDH1A1, NADH dissociation has been determined to be the major rate limiting step in the presence, or absence, of Mg^{2+} ions [6]. As Mg^{2+} ions are introduced, ALDH activity decreases due to Mg^{2+} -induced increases in the affinity for the NADH. By decreasing the K_D for NADH, the overall enzyme activity decreases [1].

The interaction of NADH with ALDH1A1 has been previously examined by measuring the fluorescence intensity of the NADH under static conditions using traditional fluorescence techniques [1]. Upon binding to a protein, the fluorescence spectrum of NADH shifts approximately 20 nm toward the blue, and the NADH quantum yield is generally increased fourfold [7]. These types of studies have been used to determine that Mg^{2+} ions increase the affinity of NADH for ALDH1A1.

However, real-time measurement of NADH fluorescence lifetimes (τ) can provide additional details not revealed by traditional fluorescence measurements. Free NADH has a relatively short lifetime (τ) of near 0.4 ns, and the lifetime increases upon binding to proteins [7]. A fluorescence-lifetime analysis allows for the differentiation of bound and unbound forms of NADH, thus giving the potential to actively monitor the enzymatic reaction during enzyme catalysis in greater detail as compared to traditional fluorescence techniques.

In these current studies, we tested the hypothesis that NADH exists in multiple fluorescent states during enzyme catalysis and that these states are affected by the presence of aldehydes and Mg^{2+} ions. By employing time-resolved fluorescence techniques, we have found that NADH has multiple fluorescence lifetimes in the presence of enzyme with (or without) magnesium ion and aldehyde. During enzymatic reactions in the presence of ALDH1A1, NAD^+ , aldehyde, and Mg^{2+} , we resolved the fluorescence signature of two NADH-ALDH1 complexes from the free NADH signal.

2. Materials and Methods

2.1. Chemicals

β -Nicotinamide adenine dinucleotide, disodium salt, reduced form (NADH) was purchased from Acros (Pittsburgh, PA). SigmaUltra grade β -nicotinamide adenine dinucleotide (NAD^+), butyraldehyde, and Chelex 100 sodium form resin were purchased from Sigma-Aldrich (St. Louis, MO). Magnesium chloride, electrophoresis grade EDTA (free acid), enzyme grade HEPES, and glycogen (beef liver) were purchased from Fisher Scientific (Pittsburgh, PA). Dithiothreitol (DTT) was purchased from Research Products International Corp. (Mount Prospect, IL). The EDTA and HEPES buffer solutions were adjusted from their acidic forms to pH 7.4 using sodium hydroxide.

2.2. Preparation of recombinant human ALDH1

Recombinant human ALDH1 containing an N-terminal 6X histidine tag was prepared as previously described using Rosetta 2 (DE3) *E. coli* cells (Novagen, EMD BioSciences, San Diego, CA) [8]. Following nickel agarose affinity chromatography, purified ALDH1 was dialyzed at 4 °C versus two changes of buffer (1:1000) consisting of 40 mM HEPES buffer, pH 7.4, treated with Chelex 100, containing 1 mM DTT to preserve enzyme activity. Purity of protein at just above 50 kDa was over 95% as judged by SDS-PAGE analysis. The tetrameric protein concentration was determined based on the Bradford colorimetric assay with bovine serum albumin as the standard [1].

2.3. Time-resolved fluorescence instrument

Time-domain fluorescence data were collected with a custom instrument, developed by Dakota Technologies, Inc. (Fargo, ND), that has been described previously [9]. The Cary Eclipse fluorometer (Varian, Inc.) has been enhanced with the addition of a pulsed laser excitation source, a fast rise-time photomultiplier tube (PMT), and a transient digitizer. The output of the modified excitation source, a JDS Uniphase NV-10410-DA1 Microchip NanoLaser, was delivered with vertical polarization to the standard cuvette using a periscope arrangement consisting of two dichroic mirrors, used to select the 355 nm output. A Quantum Northwest TLC 50 temperature-controlled sample holder was used to maintain temperature control, spin a stirbar in the cuvette, and deliver argon gas over the cuvette surfaces at lower temperatures. All fluorescence measurements were conducted while the solutions were stirred with a 7 mm micro stir bar and the temperature maintained at 25 °C. To eliminate anisotropy effects, the time domain fluorescence was detected at magic angle (54.7°) polarization. The emission collection optics of the fluorometer was left untouched but a Hamamatsu R7400U-20 PMT with 0.7 ns risetime was installed for the lifetime work. The R7400-20 PMT was slid into place at the monochromator exit slit or moved to the side for conventional steady-state data collection. The PMT current was processed using a fast digitizer from Fluorescence Innovations (1 GS/s sampling rate, 150 MHz bandwidth, 10-bit ADC, and internal trigger) interfaced with a computer through the USB port.

The instrumental response function of the detection system was collected under the same condition as the sample using a scattering solution of dissolved glycogen. The fluorescence data were analyzed in a customized spreadsheet using an iterative procedure based on the Marquardt algorithm. Data were presented as the sum of the exponential components

$$I(t) = \sum_i \alpha_i e^{(-t/\tau_i)}$$

where α_i are pre-exponential amplitudes and τ_i are the lifetimes of the fluorescence decay components. Accuracy of fits was characterized by the reduced χ^2 statistic and a plot of weighted residuals.

2.4. Wavelength-time matrix and kinetic data modes

The capability of the detection system to rapidly generate fluorescence decay curves with high signal-to-noise was used to collect multidimensional wavelength–time matrices (WTMs) and kinetic data [9]. In these NADH studies, decay curves over a 100 ns time range were collected with a 5000 shot average at each wavelength as the emission monochromator was advanced from 420 nm to 545 nm in steps of 5 nm. Integration of the area under the decay curve converts the WTM to a conventional fluorescence spectral representation. Fluorescence spectra were not corrected for grating and PMT efficiencies so the emission

maximum of free NADH was observed at 480 nm instead of 460 nm [7]. The emission monochromator wavelength was held at 480 nm for the kinetic studies.

2.5. Calibration of NADH solutions

All NADH solutions were freshly prepared and were calibrated using a CHEMUSB4-UV-VIS Ocean Optics spectrophotometer to measure the absorbance at 340 nm through sequential addition of the stock solution to 40 mM chelexed HEPES buffer (pH 7.4) over a concentration range of 0–30 μM . WTMs containing NADH fluorescence spectral and temporal information were also collected after each sequential addition to determine a fluorescence response function. In order to obtain consistent wave profiles, the fluorescence peak intensities were maintained near 50 mV by adjusting the high voltage of the PMT.

2.6. Relative NADH fluorescence efficiencies

The fluorescence efficiencies of ALDH1-NADH binary complexes were determined through sequential addition of MgCl_2 stock solution to a solution of NADH (2 μM) and ALDH1 (0.50 μM tetramer) in 40 mM chelexed HEPES buffer (pH 7.4) to cover a concentration range of 0–6 mM. WTMs were collected after each sequential addition of magnesium to determine a fluorescence lifetime and intensity of each contributing NADH-ALDH1 fluorophore. Fluorescence data were resolved into short-lived (free NADH) and long-lived (protein-bound NADH) components. Due to the limited sampling rate of our system, the fluorescence decay of free NADH fit easily to a single exponential lifetime of 0.4 ns instead of the bi-exponential lifetimes near 350 and 760 ps reported with other systems [10,11]. The relative fluorescence efficiencies were determined based on the established fluorescence response function of free NADH and the initial concentration of NADH in solution prior to the addition of enzyme. Adjusting the high voltage of the PMT was not required.

2.7. Determination of NADH dissociation constant (K_D)

The dissociation constant of the ALDH1-NADH binary complex was determined through sequential addition of NADH to ALDH1 and various concentrations of MgCl_2 in 40 mM chelexed HEPES buffer (pH 7.4). As with the NADH calibrations, in these NADH binding experiments the NADH concentration range was 0–30 μM while the WTMs were collected. Over the course of nine experiments, the MgCl_2 concentrations ranged from 0 to 6 mM while the ALDH1 tetrameric concentration remained at 0.50 μM . As a control, an experiment involving 40 mM EDTA was conducted and compared against the 0 mM MgCl_2 results. The resulting bound NADH fluorescence intensities as a function of total NADH concentration data were fit by nonlinear regression using the one-site binding (hyperbola) equation in Prism 5 software (GraphPad) to determine the K_D and B_{max} values, where the B_{max} value for each curve is the asymptotic limit of the hyperbola that provides the number or concentration of binding sites available.

2.8. Determination of NADH dissociation rate constants

NADH was displaced from the binary ALDH1-NADH complex by addition of an excess of NAD^+ (1 mM) to a solution of 0.5 μM ALDH1 (tetrameric), 2.0 μM NADH, and 0–6 mM MgCl_2 in 40 mM chelexed HEPES buffer, pH 7.4. The PMT high voltage was adjusted to maximize the initial fluorescence signal at 50 mV. Fluorescence waveforms were collected with the emission monochromator fixed at 480 nm and the fast digitizer set at varying shot averages (145 to 5000). The relative contributions from the individual NADH fluorophores were resolved in each waveform collected to obtain the decrease in complex NADH fluorescence intensity. To determine the NADH dissociation rate constant (k_5), the change in fluorescence intensity for the three fluorophores (free and two NADH complexes) as a function of time was modeled using DynaFit software (by BioKin) [12].

2.9. ALDH1 activity

Enzyme activity experiments were conducted with the emission monochromator fixed at 480 nm and the PMT high voltage fixed at 700 V. Solutions of ALDH1 (0.50 μ M tetrameric), NAD⁺ (1 mM), and MgCl₂ (0–6 mM) in 40 mM chelexed HEPES buffer (pH 7.4) were stirred until thoroughly mixed. After data collection was initiated and a starting fluorescence baseline was established (for 3–5 seconds), 10 μ L of butyraldehyde (50 μ M cuvette concentration) was injected into the cuvette using a pipette to initiate the reaction. The fluorescence waveforms were collected until the total fluorescence intensity reached 50 mV. Once the collected waveforms were resolved into the individual contributing NADH fluorophores, the dehydrogenase activity was determined based upon the initial rate of free NADH production. The 0 mM Mg²⁺ experiment was again compared to an experiment including 40 mM EDTA as a control. Typically a small amount of fluorescent contaminant was carried over with the purified protein that led to initial fluorescence signals before the butyraldehyde was introduced.

3. Results

In applying time-resolved fluorescence to solutions containing NADH and ALDH1 enzyme in HEPES buffer, three main fluorescent contributors were apparent. The free, unbound NADH in solution had a single exponential fluorescence decay of 0.4 ns and a bound NADH fluorophore with a single exponential fluorescence decay of 2.0 ns, and a bound NADH fluorophore with a single exponential fluorescence decay of 7.7 ns. Similar to the results observed with ALDH2 [9], as increasing amounts of Mg²⁺ ion were introduced, the fluorescence lifetime of these three contributors did not change significantly, nor did any additional fluorescent contributors arise.

As the concentration of Mg²⁺ ion in the NADH-ALDH1 system was increased, the fluorescence intensity of free NADH decreased while the fluorescence intensity of both bound NADH fluorophores increased. The contribution from each of the NADH fluorophores over a range of Mg²⁺ ion concentrations was measured to determine the fluorescence efficiency of each fluorophore. The relative fluorescence efficiency of the bound NADH fluorophores was 2.1 and 1.5 for the bound NADH fluorophores that have fluorescence lifetimes of 2.0 and 7.7 ns, respectively, relative to the fluorescence efficiency of free NADH (set at 1.0). The molar concentration of each fluorophore at each Mg²⁺ ion concentration was determined (Table 1). Even at this low concentration of total NADH, increasing the magnesium ion concentration up to 2000 μ M caused the bound NADH concentration to reach 1.0 μ M, which represents half of the binding sites available with 0.5 μ M tetrameric ALDH1.

At higher NADH concentrations, such as those necessary to determine NADH dissociation constants, a similar half site NADH saturation was reached (Table 2). As the concentration of Mg²⁺ ion was increased, the NADH dissociation constant for both bound NADH complexes decreased significantly in a similar manner. Resolving the fluorescence signature of the bound NADH fluorophore with the 2.0 ns fluorescent signature from the large free NADH signal was more difficult than resolving the 7.7 ns signature and this was reflected in the standard deviation of the K_D and B_{max} values.

The effect Mg²⁺ ions have on the rate constants for NADH dissociation (k_5) was examined by adding an excess of NAD⁺ to a solution containing the binary enzyme-NADH complex with increasing concentrations of Mg²⁺, monitoring the disappearance of the fluorescent signature of the binary complex, and using kinetic modeling software to resolve the dissociation and association rate constants [12]. Four different kinetic mechanisms of NADH release were examined (Fig. 2), (1) binary complex isomerization followed by

NADH release from the fluorophore with the shorter fluorescence lifetime (2.0 ns), (2) binary complex isomerization followed by NADH release from the fluorophore with the longer fluorescence lifetime, (3) binary complex isomerization followed by NADH release from both fluorophores, and (4) independent release of NADH from both fluorophores, and isomerization. From the fluorescence results collected and modeled, there was no clear mechanism for NADH release but the independent release results were consistently slightly better than the other kinetic mechanisms and are also shown in Table 2. Similar to the previous study with ALDH2 [9], our modeling results show that the rate constant for NADH association remains around $0.020 \mu\text{M}^{-1}\text{s}^{-1}$.

We applied time resolved fluorescence of ALDH1-NADH interactions to monitor these same interactions during enzymatic catalysis, in which enzyme, aldehyde, NAD^+ , and Mg^{2+} were present (Fig. 3). From the analysis it is clear that during catalysis the acyl-ALDH1-NADH complexes have no distinct fluorescence lifetimes beyond the two already present in the ALDH1-NADH systems. Also, as the Mg^{2+} ion concentration was increased, we observed no new fluorescent signatures from abortive products as observed previously with the ALDH2 system [9].

By monitoring the production of free NADH, we noted that, similar to our previous results with ALDH2, there was an immediate rise in the NADH fluorescence, due to the formation of bound NADH complexes, with a constant steady-state rate of production of free NADH. As the Mg^{2+} ion concentration was increased, we observed a consistent decrease of the enzyme catalytic turnover from 0.31 s^{-1} ($0 \mu\text{M Mg}^{2+}$) to 0.050 s^{-1} ($6000 \mu\text{M Mg}^{2+}$) and a distinct shift in steady state conformational concentrations from one that favors the ALDH1-NADH complex with the shorter fluorescence lifetime (33% excess) in the absence of magnesium ion to one that favors the ALDH1-NADH complex with the longer fluorescence lifetime (13% excess) at $6000 \mu\text{M Mg}^{2+}$ ion (Fig. 4). This shift in steady state conformational concentrations at higher Mg^{2+} concentrations could be due to longer residence time of the NADH in the ALDH1 pocket.

In the absence of magnesium ion, there appears to be a significantly larger concentration of the ALDH1-NADH complex with the shorter fluorescence lifetime before it reaches its steady state concentration (Fig. 3A). This observation suggests that the ALDH1-NADH complex with the shorter fluorescence lifetime (2.0 ns) is initially produced in the reaction and the complex with the longer fluorescence lifetime (7.7 ns) is produced through isomerization of the initial form. At higher temperatures, this pre-steady state behavior was even more pronounced and was even present at low Mg^{2+} concentrations (2 and $6 \mu\text{M}$).

4. Discussion

The dissociation constant for NADH with ALDH1 has been determined previously as an inhibition constant (K_{iq}) for NADH acting as a competitive inhibitor against NAD^+ . From this approach, a K_{iq} value of $52 \mu\text{M}$ was reported in the absence of metal, and a value of $19 \mu\text{M}$ was reported in the presence of 5 mM Mg^{2+} [1]. Later, a K_{iq} value of $45 \mu\text{M}$ was reported in the absence of metal, and a value of $25 \mu\text{M}$ was reported in the presence of $250 \mu\text{M Mg}^{2+}$ in similar experiments [13]. With our approach, the determined K_{D} values for both ALDH1-NADH complexes were very similar to each other but significantly lower than the previously reported values. This observed deviation could be due to a difference in methodology in that our approach is a thermodynamics-based approach to measure of NADH binding, whereas the other is kinetics-based.

Our NADH binding and displacement studies showed that Mg^{2+} ions restricted the release of NADH from ALDH1. Our fluorescence results demonstrated that both ALDH1-NADH

complexes showed about a 10-fold decrease in the rate of NADH dissociation from ALDH1 as the Mg^{2+} ion concentration was increased from 0 to 6 mM. Previous work conducted under similar conditions also reported a 10-fold decrease in the NADH dissociation rate constant as the Mg^{2+} ion concentration was increased from 0 to 2 mM Mg^{2+} [1]. Whereas this previous study focused on the loss of fluorescence enhancement as the NADH was displaced from ALDH1, our approach focuses on discriminating the individual ALDH-NADH fluorescent signatures from the significant background fluorescence of the free NADH in solution. Remarkably, the fold-differences derived from both experimental methods were quite similar. This 10-fold difference in the dissociation rate constant seems more consistent with the 10-fold change in NADH dissociation constants obtained by time resolved fluorescence than values obtained by competitive inhibition constants.

Similar to our previous results [9], the fluorescence lifetime of bound NADH fluorophores remained constant in the presence and absence of Mg^{2+} ion. Unlike the previous work with ALDH2, the ALDH1 system does not appear to have an abortive product pathway that leads to a different fluorescent signature at significant levels of Mg^{2+} and aldehyde. With NADH dissociation already being the rate limiting step for ALDH1 without Mg^{2+} ion added, this apparent lack of aldehyde-ALDH1-NADH adduct formation was unexpected.

Given the previous NMR/fluorescence work [14] and other crystallographic studies [15], this consistency in dual fluorescence lifetimes is not surprising, but the continued presence of both fluorophores with increasing Mg^{2+} ion is unexpected. Fluorescence anisotropy and NMR results suggest that the reduced nicotinamide ring retains significant mobility after NADH formation, but this mobility becomes more restricted in the presence of magnesium [14]. These studies also indicate that the nicotinamide ring becomes reoriented when Mg^{2+} ions are added. One possible explanation for this lack of change in fluorescence lifetime of the NADH-ALDH1 complexes is that these changes to the nicotinamide ring with the addition of Mg^{2+} ions are not significant compared to the extent of elongation of the NADH while bound to enzyme. NADH bound to protein in an elongated fashion, which prevents contact between adenine and the fluorescent reduced nicotinamide group, increases the quantum/fluorescence efficiency and the fluorescence lifetime [7].

One key aspect of the mechanism of the ALDH family, including GAPN and lactaldehyde dehydrogenase [16,17], is the requirement for the NAD to contract from a conformation that is necessary for efficient hydride transfer to a conformation that allows Glu-268 to activate the deacylating water molecule. This required coenzyme isomerization step that occurs between hydride transfer (k_3) and deacylation (k_4) is most likely not the process leading to the two different NADH-ALDH1 complexes that are responsible for the two different fluorescence lifetimes observed. Since all three fluorophores (0.4 ns, 2.0 ns, and 7.7 ns) are present when NADH is added to ALDH1 in the absence and presence of Mg^{2+} ion (Table 1) and not just during enzyme catalysis, the origin of the two bound NADH fluorophores is most likely from a two-step process for NADH dissociation (k_5).

A clear mechanism for NADH release was not resolved, however the enzymatic catalysis result obtained in the absence of magnesium ion suggests the fluorophore with the shorter lifetime is formed first and then isomerization leads to the fluorophore with the longer fluorescence lifetime. The kinetic mechanism involving binary complex isomerization followed by NADH release from the fluorophore with the longer fluorescence lifetime (7.7 ns) gave similar results to the other three mechanism proposed.

In conclusion, this work demonstrates that as Mg^{2+} ion is introduced in the ALDH1 system, the release of NADH from the enzyme is restricted and leads to a buildup of two bound NADH conformations with distinct fluorescence lifetimes. Our results show that (1) under

static conditions the equilibrium concentration of these two ALDH1-NADH complexes remains nearly equal, (2) the NADH dissociation constants and the apparent NADH dissociation rate constants appear to be nearly equal. The only significant difference between the ratios of the NADH complexes is when enzyme catalysis occurs. As the Mg^{2+} ion concentration increases, the concentration of both steady state complexes approach equality.

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References

1. Ho KK, Alli-Hassani A, Hurley TD, Weiner H. Differential effects of Mg^{2+} ions on the individual kinetic steps of human cytosolic and mitochondrial aldehyde dehydrogenases. *Biochemistry*. 2005; 44:8022–8029. [PubMed: 15924421]
2. Dickinson FM, Hart GJ. Effects of Mg^{2+} , Ca^{2+} , and Mn^{2+} on sheep liver cytoplasmic aldehyde dehydrogenase. *Biochem. J.* 1982; 205:443–448. [PubMed: 7138510]
3. Bennett AF, Buckley PD, Blackwell LF. Inhibition of the dehydrogenase activity of sheep liver cytoplasmic aldehyde dehydrogenase by magnesium ions. *Biochemistry*. 1983; 22:776–784. [PubMed: 6838823]
4. Vallari RC, Pietruszko R. Interaction of Mg^{2+} with human liver aldehyde dehydrogenase. II. Mechanism and site interaction. *J. Biol. Chem.* 1984; 259:4927–4933. [PubMed: 6715329]
5. Levy LA, Murphy E, Raju B, London RE. Measurement of cytosolic free magnesium ion concentration by ^{19}F NMR. *Biochemistry*. 1988; 27:4041–4048. [PubMed: 3415971]
6. Blackwell LF, Motion RL, MacGibbon AK, Hardman MJ, Buckley PD. Evidence that the slow conformation change controlling NADH release from the enzyme is rate limiting during the oxidation of propionaldehyde by aldehyde dehydrogenase. *Biochem. J.* 1987; 242:803–808. [PubMed: 3593277]
7. Lakowicz, JR. Principles of Fluorescence Spectroscopy, second ed. New York: Kluwer Academic/Plenum Publishers; 1999.
8. Leiphon LJ, Picklo MJ. Sr., Inhibition of aldehyde detoxification in CNS mitochondria by fungicides. *Neurotoxicology*. 2007; 28:143–149. [PubMed: 17010440]
9. Gonnella TP, Leedahl TS, Karlstad JP, Picklo MJ. NADH fluorescence lifetime analysis of the effect of magnesium ions on ALDH2. *Chem. Biol. Interact.* 2011; 191:147–152. [PubMed: 21276780]
10. Vishwasrao HD, Heikal AA, Kasischke KA, Webb WW. Conformational dependence of intracellular NADH on metabolic state revealed by associated fluorescence anisotropy. *J. Biol. Chem.* 2005; 280:25119–25126. [PubMed: 15863500]
11. Yu Q, Heikal AA. Two-photon autofluorescence dynamics imaging reveals sensitivity of intracellular NADH concentration and conformation to cell physiology at the singlecell level. *J. Photochem. Photobiol. B.* 2009; 95:46–57. [PubMed: 19179090]
12. Kuzmic P. Program DYNAFIT for the Analysis of Enzyme Kinetic Data: Application to HIV Proteinase. *Anal. Biochem.* 1996; 237:260–273. [PubMed: 8660575]
13. Ho KK, Hurley TD, Weiner H. Selective Alteration of the Rate-Limiting Step in Cytosolic Aldehyde Dehydrogenase through Random Mutagenesis. *Biochemistry*. 2006; 45:9445–9453. [PubMed: 16878979]
14. Hammen PK, Allali-Hassani A, Hallenga AK, Hurley TD, Weiner H. Multiple conformations of NAD and NADH when bound to human cytosolic and mitochondrial aldehyde dehydrogenase. *Biochemistry*. 2002; 41:7156–7168. [PubMed: 12033950]
15. Perez-Miller SJ, Hurley TD. Coenzyme isomerization is integral to catalysis in aldehyde dehydrogenase. *Biochemistry*. 2003; 42:7100–7109. [PubMed: 12795606]

16. Talfournier F, Pailot A, Stinès-Chaumeil C, Branlant G. Stabilization and conformational isomerization of the cofactor during the catalysis in hydrolytic ALDHs. *Chem. Biol. Interact.* 2009; 178:79–83. [PubMed: 19028478]
17. Costanzo LD, Gomez GA, Christianson DW. Crystal Structure of Lactaldehyde Dehydrogenase from *Escherichia coli* and Inferences Regarding Substrate and Cofactor Specificity. *J. Mol. Biol.* 2007; 366:481–493. [PubMed: 17173928]

Fig. 1.
Schematic of the ALDH1 catalytic mechanism.

Fig. 2.

Four mechanisms for NADH release tested using kinetic modeling software.

Fig. 3.

The effect of Mg^{2+} ion concentration on enzyme activity. The fluorescence waveforms collected during enzymatic catalysis are used to determine the changes in concentration of free and bound forms of NADH. The reaction mixture contained $0.50 \mu\text{M}$ ALDH1 (tetrameric), 1 mM NAD^+ , $50 \mu\text{M}$ butyraldehyde, with (A) no Mg^{2+} and (B) $2000 \mu\text{M}$ Mg^{2+} . * E and F denote the enzyme conformations responsible for the bound NADH fluorescence lifetimes 2.0 ns and 7.7 ns , respectively.

Fig. 4. Time-resolved fluorescence analysis of ALDH1 activity. The effect of Mg²⁺ ion concentration on the steady state concentration of ALDH1-NADH complexes compared against the measured enzyme activity.

Table 1

Effect of Mg^{2+} on the Equilibrium Concentrations of Free NADH and ALDH1-NADH complexes in 40 mM chelexed HEPES buffer (pH 7.4). ¹

[Mg ²⁺] (μM)	Free NADH Conc. (μM)	Bound ² NADH Conc. (μM)	Bound ³ NADH Conc. (μM)
0	1.9	0.1	0.1
2	1.8	0.1	0.1
6	1.7	0.1	0.1
20	1.6	0.1	0.2
60	1.5	0.2	0.3
200	1.3	0.3	0.4
600	1.2	0.4	0.4
2000	1.0	0.5	0.5
6000	0.9	0.5	0.5

¹ Molar concentrations are were determined based on relative fluorescence efficiencies of $\Phi_{\text{free}} = 1.0$, $\Phi_{2.0} = 2.1$, and $\Phi_{7.7} = 1.5$, respectively.

² NADH-ALDH binary complex with a 2.0 ns fluorescence lifetime.

³ NADH-ALDH binary complex with a 7.7 ns fluorescence lifetime.

Effect of Mg²⁺ on the NADH dissociation constants, dissociation and association rates, NADH saturation maxima for ALDH1

Table 2

Bound NADH ($\tau_f=2.0\text{ns}$)					Bound NADH ($\tau_f=7.7\text{ns}$)				
[Mg ²⁺] (μM)	K_D (μM)	B_{max} (μM)	k_5 (s ⁻¹)	k_{-5}^a ($\mu\text{M}^{-1}\text{s}^{-1}$)	K_D (μM)	B_{max} (μM)	k_5 (s ⁻¹)	k_{-5}^a ($\mu\text{M}^{-1}\text{s}^{-1}$)	k_{cat} (s ⁻¹)
0	13[8]	0.3[0.2]	-	-	24[7]	0.3[0.2]	-	-	0.31
2	17[14]	0.3[0.3]	0.21	0.012	19[5]	0.3[0.2]	0.31	0.017	0.26
6	8[2]	0.4[0.1]	0.22	0.027	8[2]	0.3[0.1]	0.29	0.036	0.19
20	10[3]	0.5[0.1]	0.14	0.014	5[2]	0.3[0.1]	0.10	0.021	0.14
60	9[3]	0.6[0.1]	0.073	0.008	9[4]	0.4[0.1]	0.079	0.009	-
200	4.0[0.1]	0.6[<0.1]	0.050	0.012	4[1]	0.4[<0.1]	0.043	0.011	0.072
600	2.5[0.8]	0.6[<0.1]	0.036	0.015	2.3[0.5]	0.4[<0.1]	0.019	0.0083	-
2000	1.5[0.3]	0.6[<0.1]	0.029	0.019	1.2[0.2]	0.4[<0.1]	0.014	0.011	0.052
6000	1.4[0.4]	0.7[0.1]	0.034	0.024	0.5[0.7]	0.3[<0.1]	0.011	0.021	0.050

The k_5 and k_{-5} value could not be kinetically modeled in the absence of magnesium ion.

[] indicate the standard deviation based on three experiments where <0.1 indicates that the standard deviation is below the limit of quantification.

^a $k_{-5} = k_5/K_D$.