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The Enzyme Kinetics of NADH Dehydrogenase After the Addition of the Inhibitory Molecules, EDTA and Mg^{2+}

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The oxidation of NADH to NAD+ is done by NADH dehydrogenase, a component of the electron transport chain in *Escherichia coli.* It has been demonstrated that when *E. coli* K12 cells were lysed by the lysozyme lysis method, NADH dehydrogenase activity was inhibited. On the contrary, when cells were lysed by French press, NADH dehydrogenase activity was not inhibited. In our investigation, some common components used in cell lysis were examined for their effect on purified NADH dehydrogenase activity. Attempts to partially purify the enzyme to eliminate contaminating factors were unsuccessful and potential causes were examined. It was found that individually, EDTA and $MgCl_2$ both inhibited the activity of NADH dehydrogenase. However, the pattern in the kinetics of inhibition of EDTA and $MgCl_2$ were different and $MgCl_2$ appeared to be a stronger inhibitor of the enzyme at lower concentrations and limiting substrate levels.

Previous studies used crude extracts of clarified supernatants. These extracts were potentially a problem because interference of NADH dehydrogenase by components of the raw lysate could have been significant. As well, although NADH dehydrogenase activity can still be measured from the lysate of cells broken by the French press method, this measured activity may not be optimal and therefore, investigating the lysozyme lysis method is critical. We aimed to obtain a purer form of NADH dehydrogenase to avoid such interference. This was attempted with the use of the Diethylaminoethyl (DEAE)-cellulose ion exchange chromatography column. Ion exchange chromatography is based on the fact that proteins are ionically charged, and will interact with any material in solution that has opposite charge. The protein with stronger charge affinity will bind longer than ones with weak affinity and therefore changing the ionic strength of the buffer will elute the bound materials at differing concentrations.

Using this pure form of the NADH dehydrogenase, we can investigate the kinetics and inhibitory characteristics of the enzyme by EDTA and Mg^{2+} and provide their comparison that will lead to future experiments to further optimize the extraction of the enzyme from intact cells using the lysozyme lysis method. This method involves using EDTA and $MgCl_2$ to aid in the disruption of cell membranes by lysozyme. Gram-negative cells are incubated with lysozyme and are slowly broken by agitating the mixture over a period of time until it becomes viscous, indicating cell breakage and DNA release. In comparison, French press uses mechanical energy to lyse cells, where bacterial cells are subject to a shift from a high-pressure environment to a normal atmospheric pressure environment. However, in addition to the stresses induced on the cells in the French press protocol, high pressures generate heat that may result in inactivated and denatured proteins. As well, NADH dehydrogenase is an enzyme embedded in the cellular membrane and improper operations of the French press may result in large chunks of membranes remaining unbroken, containing the enzyme, that will end up in the pellet when centrifuging out the cellular debris.

Inhibition of the interaction of key co-factor ions with NADH dehydrogenase by the chelating capability of EDTA, accounts for the major caveat of the lysozyme lysis method. Therefore, with the results from our experiment, a future design of a modified lysozyme lysis method can be determined such that the inhibition of the activity of NADH dehydrogenase is minimal.

MATERIALS AND METHODS

Bacterial Cell Culture and Growth: *E. coli* K12 B23 was grown in a standard LB medium (10g Tryptone, 5g yeast extract and 10g NaCl per liter of water) to an early-late log phase (5 hrs). Harvest was done by spinning the cells at 5,000 rpm for 10 minutes. The pellet was resuspended in 25 ml TS50G (50mM Tris Hcl pH 7.9, 50 mM Nacl, 10% glycerol) to prepare for cell breakage.

Cell Breakage: The resuspended cell pellet was passed through the Y110L Microfluidizer (Duong Lab, Univeristy of British Columbia) at 15,000 psi until the suspension became less viscous (~ 4 passes).The lysate was then centrifuged at 10,000 rpm for 10 minutes to remove cellular debris, the supernatant was collected for solubization.

Membrane Solubilization: The supernatant after cell breakage was treated with a final concentration of 1% Triton X-100. The solution was incubated at 4˚C for 1 hr under constant rotation. The solution was centrifuged at 48,800 x g for 1 hr to remove any unsolubilized membrane. One hundred µM DTT was added to the supernatant and kept for further purification/enzyme assays.

DEAE-Cellulose Ion Exchange Purification of Enzyme: Tris buffer (10mM Tris, pH 8, 1 litre) and TN10 buffer (10mM Tris, pH 8.0 and 1M NaCl) was used to prepare a series of different buffers

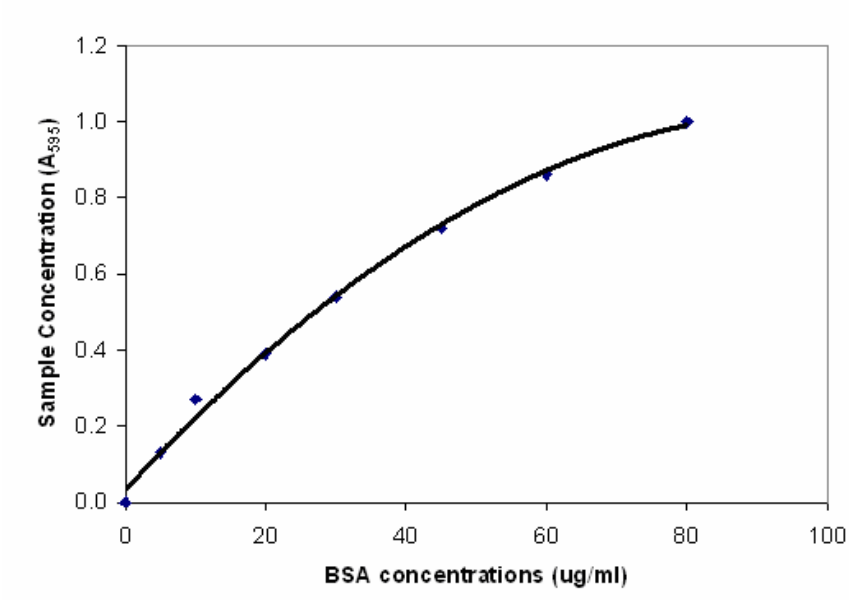

FIG. 1 Bradford standard curve with known concentration of BSA.

with different ionic strengths: TN1, TN2, TN4, TN6, TN8. Samples were loaded in Vivapure minispin DH membrane spin columns (Cat# VS-IX01DH24, Vivascience Sartorius Group Inc., Edgewood, NY, USA). All centrifugations were at 5000 x g for 2 minutes. Filtrate was pooled and saved for Bradford and enzyme assays. The column was then subsequently washed with increasing concentrations of TN buffers. TN samples were saved for Bradford and enzyme assays (8). Bradford Assay: Protein was measured with the Bradford dye reagent, Coomasie Blue G-250 biding assay with chicken egg albumin as the standard (8).

NADH dehydrogenase Activity Assay: The assay for NADH dehydrogenase was based on the observation that NADH absorbs light at 340nm but NAD does not. This means that a tube containing NADH and NAD dehydrogenase should show a decrease in absorbance which is inversely proportional to the amount of enzyme converting the NADH to NAD. The spectrophotometer was blanked with 1.35 ml of 50 mM Tris-HCl buffer, pH 7.4 and 1.5 ml of dH_2O. One hundred µl of 5 mg/ml NADH (Sigma) was added to bring the absorbance (A_{340}) to 0.6. To each reaction tube, 100 µl of sample was added. The absorbance was continuously monitored in intervals of five minutes using the SpecX software (Vernier Inc, Seattle) (8).

RESULTS/DISCUSSION

The aerobic respiratory chain of *Escherichia coli* is composed of a number of membrane-bound, multi-subunit enzymes, including nicotinamide adenine dinucleotide (NADH) dehydrogenase (type I dehydrogenase). This enzyme is an oxidoreductase that oxidizes NADH to NAD^+ and reduces ubiquinone-8 to ubiquinol-8 within the cytoplasmic membrane (1). Previous studies have shown that inactivation of this enzyme is caused by the lysozyme lysis method (8) and more specifically, the EDTA and Mg^{2+} required in this method (5). Further, the only lysis method shown to conserve enzyme activity is via the French press (5,8) and therefore, we used this technique in order to examine the extent of inactivation of EDTA, Mg^{2+}, and polymixin B post lysis.

Upon completion of the Bradford assay, we have measured the protein concentration of the lysate after solubilization and centrifugation to be 12 mg/ml. The high level of proteins within the lysate indicated that the cell breakage mechanism was sufficient to release the proteins from the cells.

Partial purification of NADH dehydrogenase with DEAE cellulose ion exchange column was unsuccessful. In order to avoid loss of NADH dehydrogenase recovery from the lysate, the removal of cellular debris was incubated with Triton X-100. Triton X-100 is a non-ionic detergent that has the ability to solubilize membranes, and therefore, release any membrane coated NADH dehydrogenase in the solution (5). The intent was to partially purify NADH dehydrogenase in order to perform inhibition assays on a purer form of the enzyme containing higher activity. The purification of NADH dehydrogenase by means of DEAE-cellulose ion exchange chromatography was not successful in our investigation. Sufficient enzyme activity was only observed in the raw cell lysate and the flow through of our samples (Fig.2). This means that either our protein was not eluted by our series of TN solutions, or that our protein did not bind to the column at all. A previous study used DTT in the purification process (3). High concentrations of DTT may have been a problem in previous studies since it reduces disulfide bonds, causing the protein to lose its three-dimensional structure. Because of this reason, we left the dithiothreitol (DTT) out of our protocol.

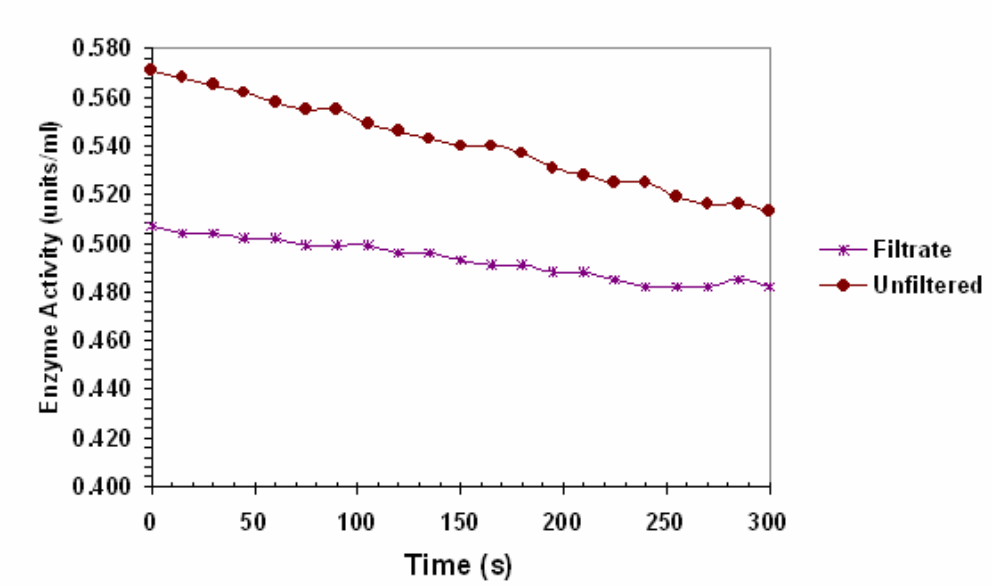

FIG. 2 The observed activity of NADH dehydrogenase after and before treatment on a DEAE ion exchange filter.

However, DTT is commonly used to reduce disulfide bonds quantitatively and maintain monothiols in the reduced state (2). At low concentrations, DTT stabilizes enzymes and other proteins which possess free sulfhydryl groups and has been shown to restore activity lost by oxidation of these groups *in vitro*. It seems that without DTT, our NADH dehydrogenase may have in fact denatured and lost its activity. We should have used a lower concentration of DTT than used in previous studies, instead of leaving it out of the protocol completely. Also, it is possible that the pH we used (pH = 7.64) was too low, so our protein was did not possess enough negative charge to bind to the column. Therefore, NADH dehydrogenase activity was only observed in the flow through, and in the original raw cell lysate. However, this is unlikely, since the previous study by Dancey *et al.* (2) had binding occur at a pH of 7.5. It is possible that the material did not elute at the tested salt concentrations, or that the NaCl was inhibitory at the level that the elution occurred, causing an absence of enzyme activity in the filtrate. Spin column membranes tend to bind more strongly than particulate columns, so it might take higher salt concentrations to elute the material out of spin columns.The activity of NADH dehydrogenase does appear to be affected by the addition of DTT (Fig. 3). There is a significant decrease in enzyme activity when DTT was omitted. Therefore, omitting DTT from our purification protocol may have resulted in the aggregation of available NADH dehydrogenase, leading to a lower measured activity.

As figure 4 indicates, EDTA has a significant inhibitory effect on NADH dehydrogenase even at a low concentration of 0.6mM. At 0.6mM, the EDTA has suppressed roughly 40% of the original activity. Further increases of EDTA concentration to 6.3 mM and 12.6 mM did not have a proportionate increase in suppression. There is an optimal Ca^{2+} requirement for maximal NADPH dehydrogenase enzyme activity in the plant *Helianthus tuberosus*, where even minimal amounts of EDTA will chelate available Ca^{2+} (7). The evolutionary conservation of the NADH dehydrogenase enzyme suggests that the same may be true for bacterial species. It is interesting to note that in the bacteria *Neurospora crassa*, the mitochondrial NADH dehydrogenase shows an internal site that specifically binds Ca^{2+} (6) This fact, in concert with suggested Ca^{2+} regulation of plant NADPH dehydrogenases provides a strong argument that Ca^{2+} concentrations will significantly effect the activity of NADH dehydrogenase that we isolated from *E. coli*. At low substrate levels there seems to be an EDTA concentration dependent effect on the enzyme activity. When there is 0.6 mM of EDTA with 0.07 mg/ml of NADH in the assay solution, there is a 67% increase in enzyme activity compared to the activity of NADH dehydrogenase when incubated with 12.6 mM of EDTA. However, this difference in inhibition is not as well visualized once we have higher substrate concentrations (0.16mg/ml, 0.23 mg/ml). This observation may be caused by substrate saturation at 0.23 mg/ml of NADH. When the enzyme is saturated with substrate, the relative degree of difference of inhibition between the different concentrations of EDTA added into the assay would not be as great as when there is less substrate in the solution.

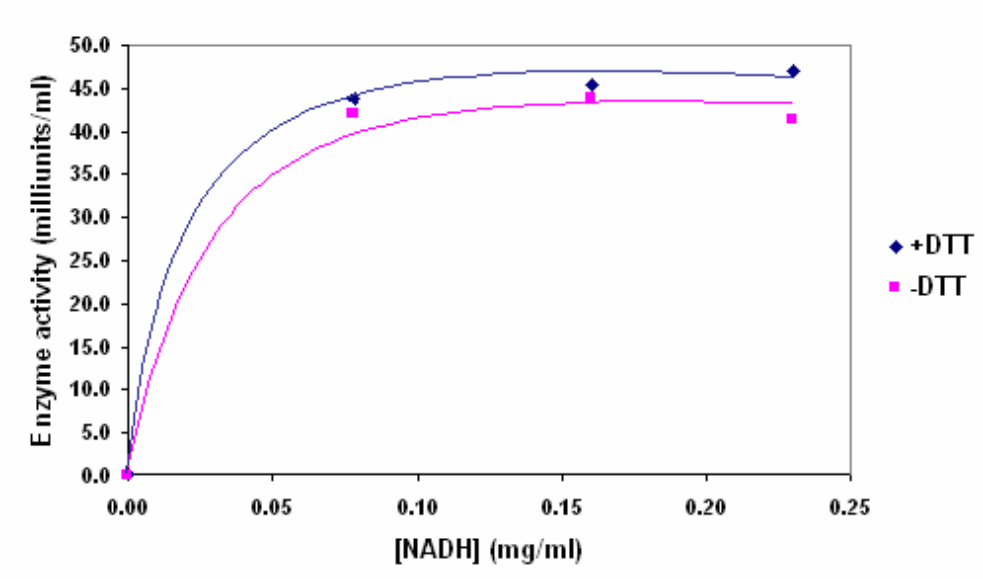

FIG. 3 The effects of DTT on the enzyme activities of NADH dehydrogenase expressed over various concentrations of NADH substrate.

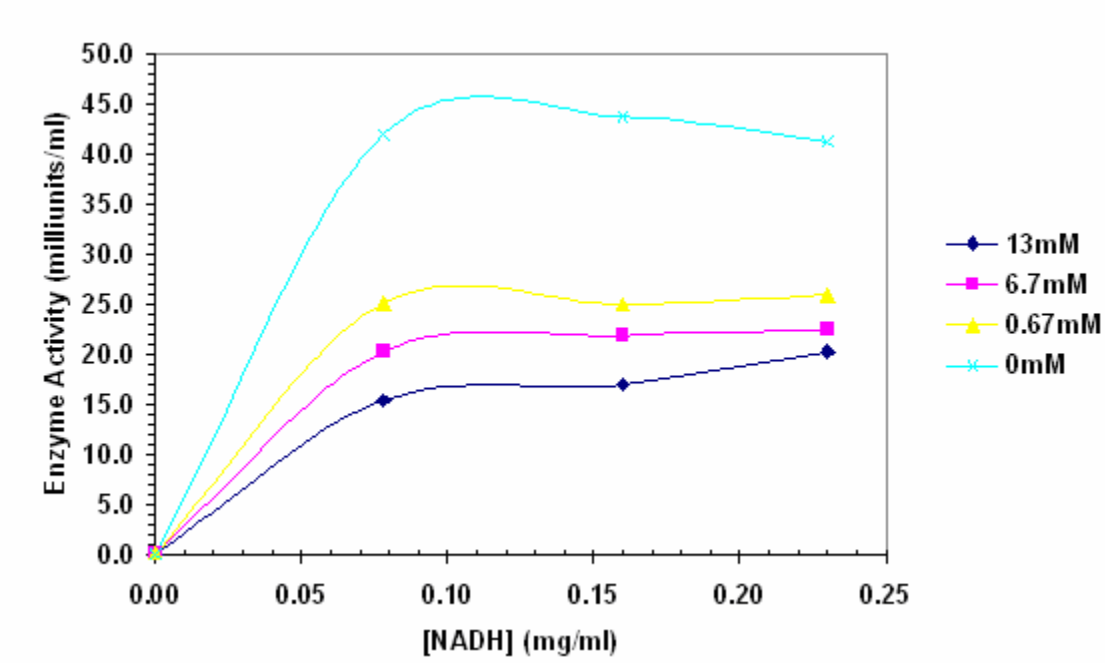

FIG. 4 The effects of different concentration of EDTA on the enzyme activities of NADH dehydrogenase expressed over various concentrations of NADH substrate. EDTA significantly inhibits the activity of NADH dehydrogenase by chelating available Ca^{2+} ions.

When the concentration of EDTA increases, we have an increased Km (Fig. 5). Furthermore, as the EDTA concentration increases, there is a decrease in the V_{max} (the maximum enzyme activity) of the enzyme. The Km indicates the amount of substrate required for ½ of the maximal enzyme activity. This observation is valid because the inhibitory characteristics of EDTA would result in a higher amount of substrate needed for the enzyme to reach its Km. Consequently; the maximum activity of the enzyme is also reduced.

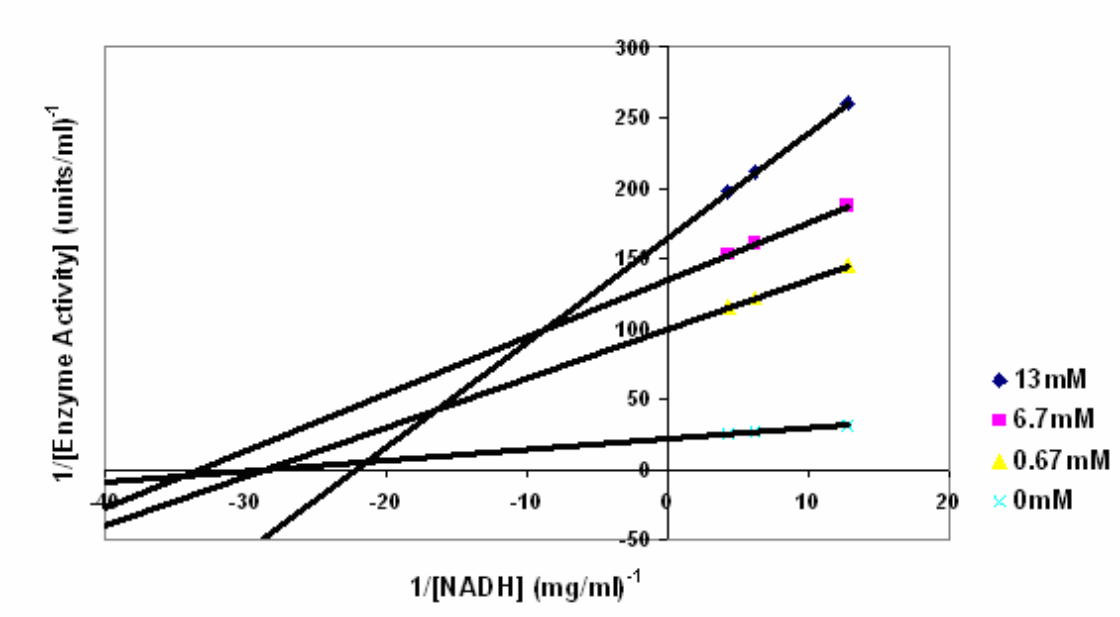

FIG. 5 Lineweaver-Burk plots of 1/enzyme activity and 1/[S] to determine the theoretical maximum rate (V_{Max}) and Km values for the enzyme activity of NADH dehydrogenase teated with different concentrations of EDTA.

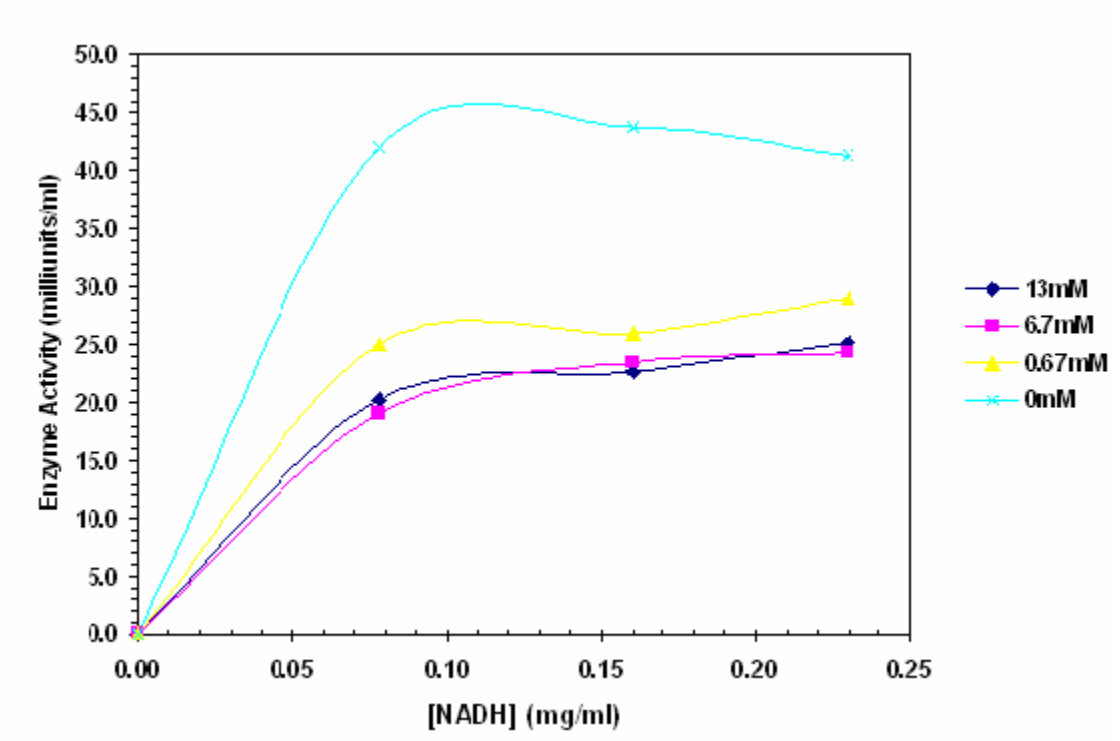

FIG. 6 The effects of different concentration of Mg^{2+} on the enzyme activities of NADH dehydrogenase expressed over various concentrations of NADH substrate.

Mg^{2+} addition to NADH dehydrogenase resulted in an similar inhibition to that by EDTA. There appears to be little difference in the inhibition of NADH dehydrogenase by the three different concentrations of $MgCl_2$ (0.7 mM, 6.7 mM, and 13 mM) that were implemented in the experimental protocol. Even at the lowest concentration, the presence of Mg^{2+} ions decreased the observed activity of NADH dehydrogenase by 40%. The lack of significant

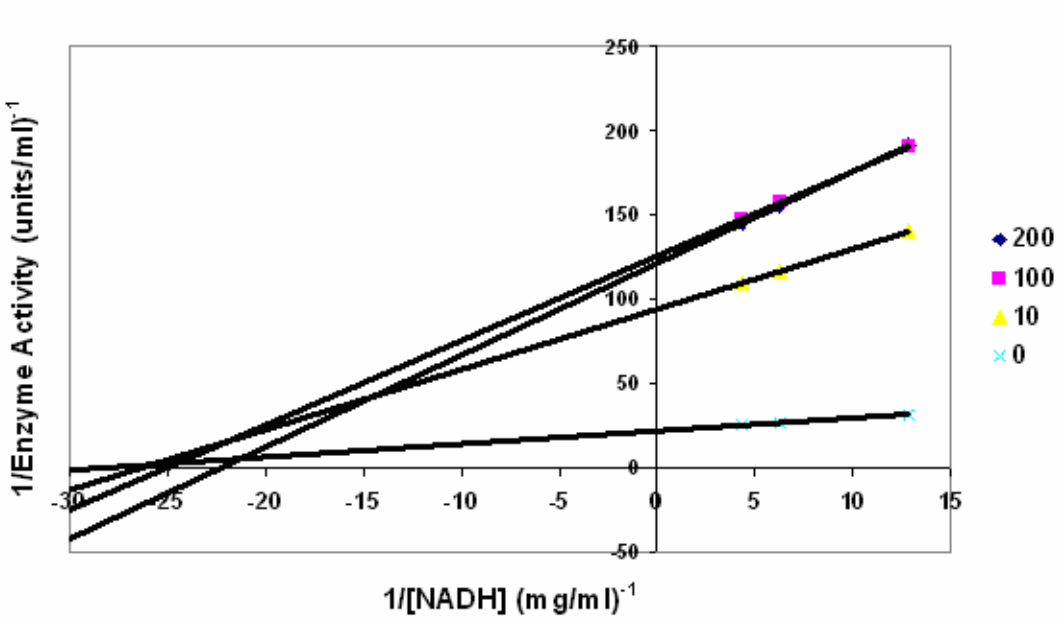

FIG. 7 Lineweaver-Burk plots of 1/enzyme activity and 1/[S] to determine the theoretical maximum rate (V_{Max}) and Km values for the enzyme activity of NADH dehydrogenase teated with different concentrations of Mg^{2+}.

differences in inhibitory activities between the different concentrations of $MgCl^{2+}$ used and at different substrate concentrations could mean that we were already at saturation of both the inhibitor and substrate levels (NADH substrate quantities used in the Mg^{2+} experiments were the same as the experiments with EDTA). Therefore, any further increase in substrate or inhibitor would not have made much difference in our data plot.

It is seen in literature that Mg^{2+} can act as an inhibitor to certain enzymes that are analogous to NADH dehydrogenase. Mg^{2+} has been postulated as a ligand of glutamate dehydrogenase that regulates its activity. However, the level of inhibitory effect of Mg^{2+} on glutamate dehydrogenase is regulated by ADP/ATP ratios. ADP is an allosteric activator of glutamate dehydrogenase. In the absence of ADP, Mg^{2+} is an inhibitor of the enzyme. (4). Therefore, by a similar mechanism, Mg^{2+} can potentially inactivate NADH dehydrogenase at the concentrations that we used.

Since Mg^{2+} had similar inhibition patterns as EDTA, the Lineweaver-Burk plot for Mg^{2+} resembles that for EDTA; when the concentration of Mg^{2+} increased, the V_{max} of the enzyme decreased while the Km increased. These results were expected of inhibitory compounds.

For EDTA, the Km values were almost identical for the control (no inhibitor), 0.7 mM and 6.7 mM but 13 mM showed a higher Km value. For Mg^{2+}, 0.7 mM and the control had similar Km values while 6.7 and 13 mM had similar, but higher values. This difference in pattern indicates that Mg^{2+} is a stronger inhibitor than EDTA at lower concentrations. While both of the inhibitors will decrease the activity of NADH dehydrogenase significantly (both suppresses enzyme activity to about 40% of the control, uninhibited enzyme), at limiting substrate concentrations, Mg^{2+} will inhibit the enzyme more efficiently than EDTA. The inhibitor constants, KI, for EDTA and Mg^{2+} were of values 3.1 and 3.5. The similarity in KI values indicate that The slightly higher KI value for Mg^{2+} suggests that Mg^{2+} is a stronger inhibitory reagent for NADH dehydrogenase.

FUTURE EXPERIMENTS

In the future, since we postulated that Ca^{2+} may be a component required for the activity of NADH dehydrogenase, additional experiments should assess whether it is the Mg^{2+} competing with, or the EDTA chelating the available Ca^{2+} that results in a lowered activity. Another parameter that should be assessed is whether the Cu^{2+} binding site found in *E. coli* NADH dehydrogenase II (9) contributes to the activity of the enzyme. An interesting question to ask is whether it is the chelation of Cu^{2+} or Ca^{2+} by EDTA that is responsible for the decreased enzyme activity measured in lysates with the addition of EDTA. These experiments could be done by the addition of the individual components in different combinations into a purified extract of *E. coli* lysate and measuring the activity of NADH dehydrogenase.

Furthermore, experiments could be done where we try to determine if there are any co-factors that could activate NADH dehydrogenase. It has been discovered that for glutamate dehydrogenase, ADP could serve as a co-factor that would in turn transform the Mg^{2+} that was previously inhibitory to the enzyme to an enzyme activator, increasing glutamate dehydrogenase's activity (5). Since we have postulated that the mode of action by Mg^{2+} on NADH dehydrogenase could be analogous to the divalent cation's inhibitory effect on glutamate dehydrogenase, a co-factor that can activate glutamate dehydrogenase could potentially increase NADH dehydrogenase's activity.

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REFERENCES

1. **Calhoun, M.W., G. Newton, and R.B. Gennis.** 1991. Physical Map Locations of Genes Encoding Components of the Aerobic Respiratory Chain of *Escherichia coli*. J. Bacteriol. 173:1569-1570.
2. **Cleland, W.W.** 1964. Dithiothreitol, A New Protective Reagent for SH Groups. Biochem. 3: 480-2.
3. **Dancey, G., Levine, A., and Shapiro, B.** 1976. The NADH dehydrogenase of the respiratory chain of Escherichia coli. J. Biol. Chem. 251: 5911-5920.
4. **Fahien L.A., Teller J.K., Macdonald M.J., and Fahien C.M.** 1990. Regulation of glutamate dehydrogenase by Mg^{2+} and magnification of leucine activation by Mg^{2+}. Mol. Pharmacol. 37: 943-9.
5. **Madden, M., Song, C., Tse, K., and Wong, A.** 2004. The Inhibitory Effect of EDTA and Mg^{2+} on the Activity of NADH Dehydrogenase in Lysozyme Lysis. J. Exp. Microb. Immun. 5:8-15.
6. **Melo, Ana M.P. *et al.*** 1999. Primary structure and characterisation of a 64 kDa NADH dehydrogenase from the inner membrane of *Neurospora crassa* mitochondria. Biochimica et Biophysica Acta 1412: 282-287.
7. **Møller, I. M., Johnston, S. P., and Palmer, J. M**. 1981. A specific role for Ca2+ in the oxidation of exogenous NADH by Jerusalem-artichoke (Helianthus tuberosus) mitochondria. J. Biol. Chem. 194:487–495.
8. **Ramey, W.D.** 2002. Experiment C2: Effect of the method of cell breakage on the distribution patterns of enzyme activity observed after ultracentrifugation, p. 1-10. *In* Microbiology 421: Source experiments for experimental projects. Department of Microbiology and Immunology, the University of British Columbia, Canada.
9. **Rapisarda, Vivianna A., *et al.*** 2002. Evidence for Cu(I)-thiolate ligation and prediction of a putative copper-binding site in the *Escherichia coli* NADH dehydrogenase-2. Arch. Biochem. Biophys 405: 87-94.