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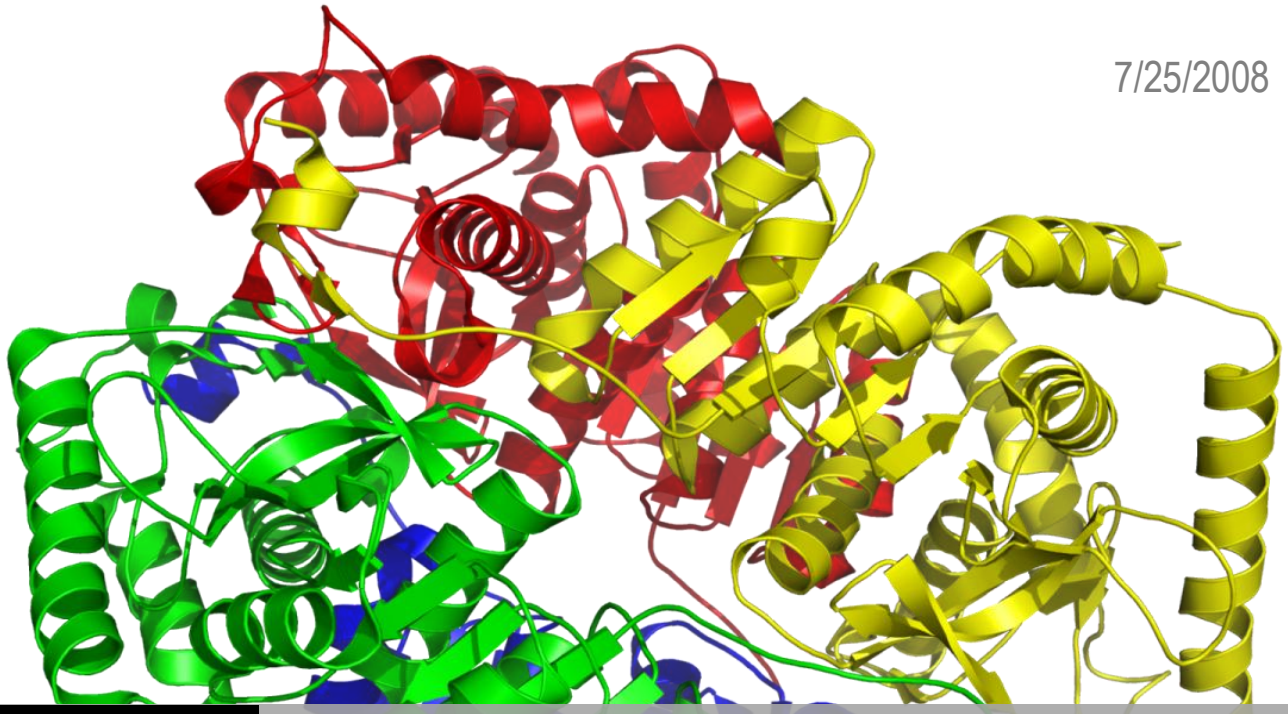

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PURIFICATION, SUBUNIT DETERMINATION, AND KINETICS OF LACTATE DEHYDROGENASE

REPORT BY HEATHER GRAEHL

Introduction: Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate with coenzyme NADH to NAD^+. In muscle tissue, LDH is crucial to maintaining levels of NADH and NAD^+ especially under anaerobic conditions. Glycolysis is the only source of ATP in the absence of oxygen, though it consumes large amounts of NAD^+ to fuel this process. Lactate dehydrogenase serves to replenish this NAD^+ for glycolysis so that it can continue ATP production. LDH exists as a tetramer of several isoforms that catalyze the same reaction but vary slightly in kinetic properties as to better function in the tissue they reside (Experiencing Biochemistry).

We aim to purify LDH from cow shank muscle, determine its subunit composition, and study kinetics of two types of inhibitors. Purification is achieved by homogenization, ammonium sulfate fractionalization, and affinity chromatography though an additional step of ion exchange chromatography could provide useful for separation of LDH-A and LDH-B isoforms. SDS-PAGE and GEC verified LDH as a tetramer and served as diagnostic of purity. The kinetics of purified LDH were then studied to determine its turnover number and the modes of inhibition by oxamate and oxalate.

Materials and Methods: Experimental procedures are outlined in Experiencing Biochemistry lab manual and Course Reader Companion with a few notable modifications. During homogenization to obtain the crude extract, cheese cloth was used instead of wool glass as a filtering device. When running the affinity column, approximately 1.5ml of AMP-agarose stationary phase was used instead of the outlined 1.0ml. Also load and rinse fraction assays were not performed at all for enzyme assays and not in duplicate for protein assays due to limited time and their insignificance in a properly run affinity column.

Table of Responsibilities

	Heather	Ivan	Jessica	Albert	Brian	Everyone
LDH Lab 1	Assay Team		Homogenization and ammonium sulfate precipitation team			cleanup
LDH Lab 2	Column Team		Assay Team			cleanup
LDH Lab 3	enzyme assays	protein assays	enzyme assays	protein assays	GEC and protein assays	cleanup
LDH Lab 4	prepared sample mixtures	prepared gel	prepared samples and loaded gel	prepared gel	prepared gel	ASSAYS! Cleanup
LDH Lab 5	Dilutions, enzyme assays	substrate mixtures	Dilutions, enzyme assays	Substrate mixtures	Substrate mixtures, gel drying	cleanup

Results/Discussion

a) **Crude Extract**: Cow shank muscle was butchered to eliminate excess fat and homogenized in a blender with phosphate buffer in order to lyse cells, releasing LDH into a slurry of tissue components. Centrifugation separated membranes, nuclei, and other large cellular components to a pellet leaving a supernatant of crude product. Controlling temperature was a major consideration after homogenization since not only did this step release LDH from the cell, but it also released proteases that can now interact to degrade the LDH. Keeping samples on ice, pre-cooling the phosphate buffer, and avoiding excess kinetic energy through conservative blending were methods to minimize activity of these proteases. Excessive blending also increases LDH's exposure to air that can cause oxidation of the enzyme, or it can cause surface denaturation in which LDH folds differently due to the atmosphere interacting with LDH instead of its native aqueous environment. To improve yield, alternative methods of cell lysis to homogenization could be investigated to avoid surface denaturation, using inert gas could avoid atmospheric oxidation, and protease inhibitors could combat unwanted protease activity.

The presence of LDH was confirmed via enzyme assays through monitoring a rate of decrease of 340nm absorbance corresponding to the conversion of NADH (A340nm) to NAD^+. The assay provided initial reaction rates and through Beer's Law, the total enzyme was calculated to 7000±100units (Table 1).

b) **40% ammonium sulfate:** Highly soluble ammonium sulfate salt is added to the crude extract as to outcompete unwanted impurities for water solvation. As increasing amounts of ammonium sulfate are added, the weakly soluble proteins, membranes, and fats are outcompeted for hydration and precipitate from solution. Centrifugation yields a pellet of these impurities and a 40% supernatant containing LDH. The addition of ammonium sulfate must be done slowly with stirring as to avoid microenvironments of high concentration that would prematurely precipitate LDH into the 40% pellet and decrease yield.

To monitor progress throughout purification steps, both enzyme and protein assays are utilized to confirm the sample is becoming more concentrated with LDH and significant loss has not occurred. Protein assays are a way to determine protein concentration, but these proteins are not specific to LDH. The protein concentration is obtained through use of Coomassie Blue dye, which at low pH, binds to hydrophobic pockets of proteins to form a dye-protein complex that absorbs at 595nm. Sample serial dilutions are assayed until a reliable range at 595nm with Coomassie blue is met and then interpolated on a standard curve of known BSA concentrations with Coomassie blue. When combining both enzyme and protein assay results, an assessment can be made if the enzyme to protein ratio or specific activity has increased with each purification step.

The 40% ammonium sulfate step had a slight decrease in protein amount from 220±20mg/ml to 170±20mg/ml and relatively no change in enzyme activity from 7000±100units/ml to 7100±200units/ml (Table 1). This is consistent with the theory of salting out since 0% to 40% saturated solution will keep most to all of LDH in solution while precipitating fats, membranes, and less soluble proteins. Though this step doesn't increase specific activity significantly from 32±3units/mg to 43±5units/mg (table 1), it is crucial since affinity column chromatography would be crippled by the presence of bulky fats. Furthermore, specific activity is only a calculation based on protein removal which doesn't give any quantitative consideration to removal to non-protein contaminants like the fats and membranes removed in this step. Yield was 103±4% (table 1) which is suspiciously high but can be excused within delta values. Protein and enzyme assays were repeated from new reaction mixtures in duplicate to confirm this high yield was not in error.

c) **60% Pellet :** The 60% ammonium sulfate step has the same theory as the previous step in which ammonium sulfate competes for hydration which precipitates out less soluble components; however, in this step LDH is targeted for precipitation rather than impurities. The resulting 60% pellet theoretically contains most of the original LDH which is re-suspended in about tenfold less volume to create a more pure and concentrated sample. The purification factor of 1.3±0.2 is not particularly impressive considering such a

large loss of enzyme and yield of 67±6% (table 1), but this step is still required since it separates out LDH from salts and nucleic acids. If the salts were not removed they would interfere with subsequent steps such SDS-PAGE causing significant gel smiling, and if nucleic acids were not removed they could absorb at 280nm during GEC causing additional or overlapping peaks. This step was expected to have no more than 33% loss and with a yield of 67±7% (table 1) the experimental enzyme loss was considerable. This can be accounted for by unequal splitting of two pellets in which the assayed pellet caused an underestimate of enzyme activity. This could be tested by performing an enzyme assay on the second 60% pellet sample to see if it has a significantly higher enzyme activity than the first pellet. Another source of loss could be attributed to degradation of enzyme since it was stored for several days between assaying the 40% supernatant and 60% pellet. The loss of enzyme cannot be blamed on not obtaining high enough saturation to precipitate all the LDH since enzyme assays on the 60% supernatant showed insignificant amounts of enzyme present. Ideally, all assays would be performed in the same lab period to avoid possibility of enzyme degradation during prolonged storage as well as splitting of pellets could be performed in a more precise manner and checked via enzyme assays.

d) **Affinity Column:** The affinity column serves as a very effective purification step that manipulates LDH's affinity toward specific ligands for purification. The 60% cut is loaded onto a column containing AMP-agarose beads. The AMP-agarose beads temporally bind and release LDH as well as other AMP-binding proteins, slowing their descent down the column while other proteins pass through the mobile phase with high velocity. Phosphate buffer is loaded to ensure all non-AMP binding proteins have completely passed through. A NADH-pyruvate adduct is used to elute LDH very effectively because it contains both substrates that LDH binds which brings LDH into the mobile phase for elution while other AMP-binding proteins remain bound to the AMP-agarose beads.

Fractions were collected and initially tested for enzyme presence qualitatively by tetrazolium spot plate color change. An elution profile was constructed using protein and enzyme assays of fractions (figure 2).

Protein assays were performed on all fractions while enzyme assays were only performed for elute fractions; however, overload was observed in which tetrazolium spot plate detected some enzyme activity in fractions load 3 and rinse 4. Since enzyme assays were not performed on these overload fractions, the spot plate results can be qualitatively compared to similar degree of color change to elute fractions 13 and 14 which did have enzyme assays. Though this method is has high error, the amount of overload enzyme is so low that it is less than the delta for peak fraction 11 and thus this qualitative method is acceptable to determine enzyme amount of overload fractions.

Fraction 11 was selected as the peak fraction with highest specific activity of 490±40units/mg (table II). Yield after affinity column was 60±7% which indicates very little loss since previous step was at 67±6% yield (table 1). This was by far the most effective purification step with a purification factor of 8.5±0.9 though previous purification steps were still necessary as they provided a way to remove components that would clog the column. Though overload did not contribute much to enzyme loss, it could be prevented by further diluting the 60% cut prior to loading and using a larger column with more AMP-agarose beads.

e) **SDS-PAGE:** Now that the enzyme has gone through several purification steps, SDS-PAGE is employed to evaluate progress of purification as well as determine subunit molecular weight of LDH. SDS-PAGE is an electrophoresis technique that separates proteins based on size by use of a porous acrylamide gel and electric field. An aliquot of purified LDH is denatured so that LDH exists as its individual subunits instead of a tetramer. Since different proteins have varying charges, negatively charged SDS is employed due to its ability to bind to proteins at a constant one SDS per two amino acids. This negates the initial charge a protein might have held since all proteins will have a similar charge to mass ratio with SDS bound. Since velocity is dependent on this charge to mass ratio, which is made relatively constant with SDS, the proteins will separate by size.

There is an exponential relationship between distance traveled through the gel and molecular weight. By running a set of known subunit Mr proteins, a linear trend can be observed when plotting logMr versus

distance (figure 4). The column purified LDH is interpolated to this graph in the linear range to obtain subunit molecular weight. Two different isoforms might be present as 36598Da LDH-A or 36724 Da LDH-B (Swiss-Prot/TrEMBL database). Both isoenzymes are very similar size, but since LDH-B heart isoform is more negatively charged than the LDH-A muscle isoform, it would travel slightly further. Lane 5 has a very faint single band while lane six is twice as concentrated and a second overlapping lower band can be observed (figure 3). This could be due to the existence of LDH-B in much lower concentration than LDH-A so that the LDH-B lower band only shows at higher concentration. Interpolating the LDH-B band would not be very useful since the isoform separation due to charge difference and not size, so it is not reliable to distinguish their molecular weights this way. Gel stacking was already employed, so to further enhance resolution a larger percent acrylamide resolving gel, longer running time, and standards closer in molecular weight to LDH could be used. Other methods such as ion exchange chromatography, HPLC, or PAGE (without SDS) could be used better distinguish LDH-A and LDH-B forms.

The observed interpolated molecular weight of 37931 Da (table III) is slightly higher than the 36kb isoenzyme published values. One explanation is that the published values are unprocessed precursors while purified LDH will have glycosylation and other post transcriptional modifications, though this won't likely significantly increase the molecular weight. Another explanation is that the LogMr versus Distance (figure 4) relationship is truly exponential so the linear relationship is prone to error, especially as standards become further from the molecular weight of the interpolated LDH. The drawn best fit line including BSA results in 37931 Da while neglecting this largest 66kb protein adjusts the interpolation of LDH subunit molecular weight to 36083 Da (table III) which is closer to published values.

In addition to determining subunit molecular weight, SDS-PAGE was also run with previous purification steps to determine if LDH is pure since specific activity only provides a gage of relative increases in purity. The column purified LDH appears pure as it shows a single band that can be matched in all previous purification samples (figure 3). Crude extract is not possible to read though luckily this is the least important

lane. Oddly the 60% pellet has several additional high molecular weight weak bands that the previous 40% supernatant step did not show, though this can be explained since the 60% pellet is suspended in a tenfold smaller volume making these components more concentrated and weakly visible in 60% and not in the 40% lane (figure 3).

f) **GEC:** Similarly to SDS-PAGE, a set of standards are used to create a log Mr plot to interpolate a molecular weight of LDH, though GEC aims to determine a native molecular weight instead. GEC uses a different mode of size separation in which porous beads target to separate intermediate molecular weight. A linear range exists where these intermediate size standard proteins elute, so LDH was interpolated to obtain a native molecular weight of 159294 Da (Table IV). Ferritin was removed in constructing a linear range since it has a molecular weight of 770,000 Da which is far outside the resolution capability of 5-250kD and artificially increases the molecular weight determination to 200kD, which is nowhere close to published values of 140kD (Holbrook et al., 1975).

Dividing GEC native molecular weight by SDS-PAGE subunit molecular weight gives a value of 4.4 which corresponds to number of subunits in LDH. Dividing published 140kD (Holbrook et al., 1975) and 36kb (Swiss-Prot/TrEMBL database) gives a value of 3.9 which establishes LDH as a tetramer. An additional modification to the GEC best fit line (figure 5) could improve comparability to published values through neglecting hemoglobin standard which would give a native Mr of 143244 Da and number of subunits to 4.0. Hemoglobin cannot be rejected based on being out of resolution, though it does not fit well on the best fit line. Little was known about the condition standard proteins and they were not handled directly, though it is possible the hemoglobin was degraded to smaller Mr causing time to elute to increase.

g) **Kinetics:** With a modification to enzyme assays performed in purification steps, the kinetics of LDH and two inhibitors were investigated. Purified LDH is assayed with varying pyruvate concentrations with and without inhibitors. Inhibitors oxamate and oxalate are assayed at high and low concentrations to determine their identity and mode of inhibition.

Inhibitor A's double reciprocal plot identifies it as competitive inhibitor oxamate due to 1/Vmax remaining relatively equal in the presence and absence of inhibitor (figure 7). Oxamate binds to LDH preventing formation of ES complex which lowers the apparent Km. At theoretical infinite substrate concentrations, the inhibitor would be outcompeted to produce the same Vmax value. Since Km is defined as substrate concentrations at ½ Vmax, the Km change is only apparent represented by α. K_I of oxamate calculated using 0.2mM and 0.04mM inhibitor concentrations and alpha to average a K_I disassociation constant of $1.49x10^{-2}$ (table V).

Inhibitor B's double reciprocal plot shows only uncompetitive nature even though this inhibitor must be oxalate which is mixed inhibition (figure 8). Uncompetitive inhibition binds to enzyme-substrate complex making this complex more stable decreasing Vmax and thus an apparent Km decrease. The slope (Vmax/Km) remains constant at 0.003 with both low and high oxalate concentrations, indicating strong uncompetitive inhibition. In order to observe the true mixed inhibition by oxalate, lower than 0.04mM oxalate could be used to demonstrate its more competitive nature. The K_I' of oxalate is solved to be 0.154 (table V). Oxalate does not bind to ES complex as strong as oxamate binds to E based on K_I and K_I' values, though these cannot be compared directly since they act on different sites. LDH binding affinity of E+S to ES appears greater in oxalate than oxamate by observation of oxalate's lower Km values which is logical since oxalate stabilizes the ES complex reducing the k_{-1}. On the other hand oxalate has lower observed Vmax than oxamate, which can be explained because available E_{total} is lowered as oxalate binds and creates a stable ESI complex. In oxamate, increasing substrate can outcompete the inhibitor so that Vmax is unaffected, though with oxalate increasing substrate does not hinder the formation of an ESI complex, thus Vmax is lower in oxalate (Table V). K_2 or K_{cat} of LDH was determined to be 29400 catalytic events per enzyme site per second (Table V).

h) **Purity Assessment:** The affinity column elution profile (figure 2) has one defined elution peak, but this doesn't necessarily mean it is pure since it could be possible for another component to bind to the NAD-

pyruvate adduct. GEC shows one peak corresponding to LDH elution (figure 5), though a similar native weight protein in low concentration would not be detectable. SDS-PAGE is most useful when assessing purity because it gives suggestion that both LDH-A and LDH-B forms are present. Lane 5 is purified LDH from elute fraction 11 and shows a faint single band. Lane 6 is twice as concentrated and a second lower band appears due to low concentration LDH-B (figure 3). It is known that LDH-B has a lower Km or higher binding affinity, so LDH-B would also bind to the elution adduct stronger than LDH-A. Thus elution fraction 10 likely has more LDH-B while later fractions like 12 and 13 likely have more LDH-A. This hypothesis can be confirmed by performing a PAGE without SDS on all elute fractions to compare strength of bands. To obtain complete purity of LDH-A without any LDH-B, ion exchange chromatography could be performed as a final purification step to separate the two based on their charge differences.

References

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