The lactate dehydrogenase

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Enzymes:

Enzymes are protein molecules which catalyst the biological reactions within living systems [23]. They provide a reaction surface to which a substrate can easily bind and a suitable environment for the reaction to occur. They bring the reactants together and orient them in such a way that they attain their transition-state configurations. They weaken the high-energy bonds in the reactants, so that the reaction can occur fast and give a product which is then released [8]. Sometimes, the enzymes may also participate in the mechanism of reaction but remain unchanged at the end of the reaction [6, 18].

An enzyme catalyzed reaction is a reversible reaction which means that the reaction can occur in both the directions. Thus an enzyme can be defined as an agent that speeds up the approach to equilibrium with the starting materials and products being unaffected [8].

One of the most important and basic function of an enzyme is to increase the speed of the reaction they catalyze [6, 18]. This is done by lowering the activation energy, which is the energy difference between the starting material and the transition state, and it is the size of this activation energy that determines the rate of a reaction [8].

“ Most cellular reactions occur about a million times faster than they would in the absence of an enzyme” [6].

Enzymes function with high specificity for only one reactant and catalyze the specific reactions compared to most chemical catalysts which catalyze a wide range of reactions. The enzyme contains an active site which is the

area with an appropriate shape and functional groups to allow the reactant or substrate to bind to the enzyme. The binding can preferably be inhibited by the enzyme with the help of inhibitor. An enzyme also contains a non-protein part called cofactors [8]. The cofactor may be a either

- An organic group called prosthetic group which binds permanently to enzyme [8]
- Positively charged metal ions called activators which bind temporarily to enzyme [8]
- Organic molecules called coenzymes which are derived from protein and bind temporarily to enzyme-substrate complex [8].

Each cell in the living system contains a unique set of some 3000 enzymes and each enzyme is equally important to the cell. Enzymes have greatly been studied by isolating them from cell and make them work in a test tube environment [23].

Lactate dehydrogenase:

“ Lactate dehydrogenase (LDH) is an enzyme present in a wide variety of organisms, including plants and animals”. [23] Its Enzyme Comission number is EC 1. 1. 1. 27 [23] where;

EC 1 = oxidoreductase

EC 1. 1 = acting on the CH-OH group of the donor

EC 1. 1. 1 = With NAD or NADP as acceptor

EC 1. 1. 1. 27 = L-lactate dehydrogenase [23]

Thus, it is an oxidoreductase enzyme that catalyzes the interconversion of pyruvate and lactate accompanied by the interconversion of NADH and NAD+ [2, 5, 7, 12, 14]. The reaction is as following:

During anaerobic glycolysis (limited or no oxygen during intense muscular activity), the enzyme converts pyruvate to lactate [24]. The reverse reaction occurs in liver due to feedback inhibition when the lactate concentration is high [24]. Such a cycle is called Cori cycle which was discovered by Carl Cori and Gerty Cori and the cycle [24] is shown below:

LDH is not specific to any single tissue and is found in many tissues especially the heart, liver, kidney, skeletal muscle, brain, blood cells, and lungs [23].

Kinetic Properties:

Kinetic studies indicate that lactate binds to substrate and co-enzyme in the following order [14]:

The binding order is discussed in detail under the section ‘ LDH Catalytic Cycle’.

LDH Source:

LDH is available in most plants and animals [23]. Commercially available LDH is mainly extracted from the following sources:

- Chicken or turkey (also recombinant and lyophilized)
- Bovine heart (also lyophilized)
- Rabbit muscle (also recombinant)
- Porcine heart and muscle (also lyophilized)

- Human heart and erythrocyte (also lyophilized) [23]

For the laboratory uses, LDH can easily be purified by successive ammonium sulfate precipitations.

LDH Structure:

LDH is present in the form of its isozymes (also called isoenzymes) which are tetramers [12 & 22]. Each tetramer consists of four sub-units or monomers each with mass of 36 kDa giving the tetramer the mass of 144 kDa [12]. Each monomer consists of a peptide chain of 334 amino acids with its own active center [12]. The following figure shows a high-level structure of tetramer (A-1) and detail structure of the active center of one of its monomer (A-2)

It is clear from the above figure (A-1) that the subunits occupy equal positions in a tetramer. In the figure (A-2) shows the peptide backbone, substrate (lactate), the co-enzyme (NAD+) and the three amino acid side chains (Arg-109, Arg-171 and His-195) and peptide loop formed by amino acid residues 98-111 [12]. In the absence of lactate and NAD+, the active centre is open and access to substrate binding site is allowed [12]. In the presence of lactate NAD+ complexes as shown in the figure, the active center is closed by peptide loop and no access is allowed to the binding site [12].

The LDH isozymes are made up of two distinct subunits – M and H, having different amino acid sequences which make them different in terms of catalytic properties [12]. The following figure shows sections from amino acid sequences of M and H genes.

The amino acid sequence of M and H genes [12].

“ The differences in sequence between the M and H subunits are mainly conservative-i. e., both residues are of the same type, e. g. Glycine (G) and alanine (A), or arginine (R) and lysine (K). Non-conservative exchanges are less frequent-e. g., lysine (K) for glutamine (Q), or threonine (T) for glutamic acid (E)” [12]. The H gene is more strongly negatively charged than M gene due to higher number of acidic residues [12, 14]. This can be observed in electrophoresis where LDH-1 containing four H units move faster than other isozymes while the LDH-5 which contains 4 M subunits is the slowest [5, 12 & 14].

LDH Catalytic Cycle:

The figure shows that nicotinamide adenosine dinucleotide (NAD+) acts as a coenzyme for LDH that helps the enzyme to catalyze the dehydrogenation of lactic acid (lactate) to pyruvic acid (pyruvate). NAD+ binds to active site of the enzyme along with lactic acid and acts as an oxidizing agent. It is converted to its reduced form (NADH) at the end of the reaction. On the other hand, NADH acts as a reducing agent and binds to the enzyme and a reverse reaction occurs [8]. The overall effect of LDH is that it catalyzes the transfer of hydride ions from lactic acid to NAD+ or from NADH to Pyruvate. LDH does not affect the equilibrium while catalysing the reaction in either direction [12]. Since LDH catalyses the reaction in both direction, the catalytic process can be represented as a closed loop [12]. Koolman et al. has shown the cycle using six snapshots while describing the steps very short lived making them difficult to detect [12]. The six steps are shown in the following figure. The figure shows the three amino acid residues:

arginine-171, histidine-195 and arginine-109, which play an important role in catalytic cycle. Other amino acid residues also play important role either directly in the one of the steps in the catalytic cycle or indirectly by mediating the binding of the substrate and coenzyme [12]. “ The positively charged guanidinium group of arginine-171 binds the carboxylate group of the substrate by electrostatic interaction. The imidazole group of histidine-195 is involved in acid-base catalysis, and the side chain of arginine-109 is important for the stabilization of the transition state” [12]. The positively charged Arg-171 and Arg-109 do not change their charge during the catalysis while His-195 changes its charge [12]. The peptide loop consisting of amino acid residues 98-111 also plays important role during the catalysis [12]. It closes the active site after the substrate (substrate) binds to the coenzyme (NAD+) in order to exclude the water molecules largely during the electron transfer [12].

LDH catalystic cycle.

The six steps are described as follows.

1. The first step shows the free enzyme. The enzyme is without substrate-coenzyme binding and therefore the active site is open [12]. His-195 is positively charged and is therefore represented as E. H+ [12].
2. In step 2, the coenzyme NADH bounds first [12].
3. Pyruvate binds to the LDH. ” It is important that the carbonyl group of the pyruvate in the enzyme and the active site in the nicotinamide ring of the coenzyme should have a fairly optimal position in relation to each other, and that this orientation should become fixed (proximity

and orientation of the substrates)” [12]. At this point, the active centre is closed by peptide loop [12]

4. This is the transition state where redox reaction occurs [12]. The NADH looses one hydride H- ion which is transferred to the carbonyl carbon [12]. “ The transient-and energetically unfavourable -negative charge on the oxygen that occurs here is stabilized by electrostatic interaction with Arg-109 (stabilization of the transition state)” [12]. The oxygen atom receives a proton released by His-195 [12].
5. Lactate is bound.
6. NAD+ is bound. The peptide loop opens the active centre and lactate is disassociated from the enzyme [12]. “ Finally, the oxidized coenzyme NAD+ is released, and initial state (1) is restored” [12]

The same steps occur in the opposite direction during the oxidation of lactate to pyruvate [12]. The concentration of all the reactants and the pH value determines the direction in which the reaction takes place [12].

LDH Isozymes:

In mammals, LDH is composed of three subunits or genes: H (heart) and M (muscle) [5, 12 & 21]. LDH is a tetramer of H and M subunits which means five different combinations of these two subunits, called isozymes are possible [5, 12 & 21]. The isozymes differ in catalytic, physical and immunological properties [5, 12 & 21] The H subunit is more negatively charged than M subunit which makes the isozyme (HHHH) move faster towards anode than the other isozymes and appear first on the gel [5, 12 & 21]. Therefore these isozymes are named according to their electrophoric

mobility in the gel with the faster being LDH-1 and the slowest LDH-5 [12 & 16].

- LDH1: This is composed of four H subunits (HHHH). Mostly found in heart.
- LDH2: This is composed of three H subunits and one M subunit (HHHM). LDH2, LDH3 and LDH4 are found in all tissues.
- LDH3: This is composed of two H and two M subunits (HHMM).
- LDH4: This is composed of one H and three M subunits (HMMM).
- LDH5: This is composed of four M subunits (MMMM) and is found in skeletal muscle in all species and the liver in horses and small animals.

LDH Isoenzymes

The isozymes have different Km values for pyruvate and therefore are better suited for different environments inside the body. A pure tetramer of M subunit i. e. M4, has lower Km for pyruvate and is concentrated in skeleton muscles which are anaerobic [5 & 21]. Therefore M4 promotes glycolysis by catalyzing the production of lactate from pyruvate quickly and efficiently [14]. On the other hand pure tetramer of H subunit i. e. H4, has greater Km for pyruvate and is more concentrated in heart muscles which is aerobic [14 & 21]. Since heart is not anaerobic, it is not necessary to turn the pyruvate into lactate [5, 14]. Therefore the enzymatic activity of H subunit is restrained by pyruvate and other compounds in the heart structurally similar to the pyruvate [14]. This pyruvate in heart can then be used in glycolysis to generate ATP for the heart muscle [14].

As said before, H subunit predominates the heart while the M subunit predominates the muscle[13]. As an example, the rat heart contains 78% of

H subunit while the legs have only 11% of this subunit and instead have greater number of M subunit.

In 1962, some researchers investigated the banding patterns of LDH isozymes in different animals and found that same isozymes of different animals have different electrophoretic mobility [5]. The result of their study is shown below:

In this case, the sera were all started at the center line, and bands would migrate either to the positive or negative electrode. They noticed that HHHH migrated the most strongly to the positive electrode and MMMM moved to the negative electrode [5].

LDH isozymes separation techniques:

There are a number of techniques used to separate and assay the LDH isozymes. One of the most effective techniques is the separation using cellulose acetate as separation medium [5]. Gel based electrophoresis separation techniques are also used [5]. Gels used for separation in such techniques include agar gels, starch and polyacrylamide gel [5]. Isoelectric focusing in acrylamide has also been used [5]. However, beside their usefulness for LDH isozyme separation, electrophoresis techniques have several disadvantages [5]. The following table shows the distribution of LDH enzymes in human tissues and serum using different separation techniques.

“ Different distributions are given by different electrophoretic media in the same tissue, and studies on the same tissue by different workers using the same medium often reveals disagreement” [5]. Artefactual bands on the gel appearing in the absence of substrate are considered to be causing this [5].

These bands are caused by due to the reaction between alcohol dehydrogenase and traces of ethanol present in the reagent [5]. This has also been attributed to the action of LDH on traces of hydroxy-acids found in gel media [5]. " Other sources of concern include the wide range of reaction conditions used by various authors for carrying out the visualisation procedure and which in many instances are far from optimal" [5]. Furthermore, the electrophoretic techniques are not best suited for a large throughput in routine laboratories. To overcome the issues relating to these techniques, other techniques were developed to utilize the chemical properties of isoenzymes instead of electrophoretic properties [5]. For this purpose, chemical and physical differences between the pure tetrameric forms, LD1 and LDs were successfully exploited [5 & 12]. Initial techniques employed chloroform and acetone to inactivate all fractions except LD1 but required centrifugation to remove denatured protein prior to carrying out the assay which posed an inconvenience [5]. Oxalate and Oxamate were also utilized which inhibit LD1 more than LD5 with Oxamate inhibiting human heart extract (LD1) by 50% and human liver extract (LD5) by only 20% [5]. Neither inhibitor is sufficiently specific to provide clear-cut diagnostic discrimination [5]. On the other hand, urea is seen as more powerful reagent inhibiting LD5 more powerfully than LD1 [5].

LDH Applications

LDH is an important enzyme and has found various applications in clinical biochemistry, pharmaceuticals, nanotoxicology etc. Some of the applications of LDH are as following:

LDH in clinical biochemistry:

LDH can serve as a great marker for various diseases and cell problems [2, 5, 12 & 20]. LDH resides inside the cell and never gets into the blood in a healthy cell. When a cell damages LDH finds its way into the blood. This elevates the level of LDH in the blood which can be measured and considered as a general indicator of the existence and severity of acute or chronic tissue damage [5 & 20]. LDH level in blood can also be measured regularly to monitor the treatment progress. LDH level can be measured either as total LDH or LDH isozyme. Total LDH level is a general marker of the tissue damage and does not give any indication of the cause or location of tissue damage [5, 12 & 20]. Since LDH isozymes are present in various tissues at different levels, their elevated levels are helpful in identifying the exact nature or cause of the tissue damage and location of the damaged tissue and organ [5, 12 & 20]. " For example, the liver and skeletal muscles mainly produce M subunits of lactate dehydrogenase (M for muscle), while the brain and cardiac muscle mainly express H subunits (H for heart). In consequence, each organ has a characteristic isozyme pattern. Following cardiac infarction, for example, there is a strong increase in the amount of LDH-1 in the blood, while the concentration of LDH-5 hardly changes" [12]. Lactate dehydrogenase isoenzyme 1 (LD-1) is used as a tumor marker of germ cell tumors [4].

Since LDH is present in many tissues, it is very useful in diagnosing tissue damages and various diseases [5 & 20]. There are a wide variety of conditions in which LDH level is elevated. Some of these conditions include myocardial and pulmonary infarction, megaloblasic anemia, extensive

carcinomatois, severe shock and hypoxia, granulocytic or acute anemia, hemolytic anemia, infectious mononucleosis, progressive muscular dystrophy, hepatitis, cirrhosis, obstructive jaundice, and in delirium tremens [5 & 20].

LDH obtained from Lactobacillus strains, has been employed for the stereospecific reduction of pyruvate to D-lactate and phenylpyruvate to D-phenyllactate [9].

LDH in Industries:

LDH has recently been found to be very useful in industrial production of lactate which is widely used in industrial applications such as chemical production. Colombi et al. and Branduardi et al. showed the effectiveness of LDH in terms of efficiency and economics to produce large amounts of lactate [1 & 3]. Conventionally, lactate is produced from lactic acid bacteria in which LDH converts pyruvate to lactate [3]. But this method is less economic due to expensive purification procedures required and the lactate produced as a result is less acid-tolerant [3]. An alternative and improved method uses yeasts to produce the lactate by fermentation process [1 & 3]. Colombi et al. and Branduardi et al showed how LDH can be used to produce lactate from yeast [1 & 3]. They integrated LDH gene from Lactobacillus Plantarum into genome of metabolically engineered yeast Saccharomyces cerevisiae. “ The LDH gene expression in a budding yeast cell introduces a novel and alternative pathway for the NAD + regeneration, allowing a direct reduction of the intracellular pyruvate to lactate, leading to a simultaneous accumulation of lactate and ethanol” [1].

Due to its efficiency, extreme stability to thermal denaturation and low cost, LDH has also found its uses in pharmaceautical industry to produce chirally pure ?-lactate which is used to produce a large variety of pharmaceuticals such as antihypertensives, semi-synthetic pencillins etc. It also has medicinal uses and is used as a diagnostic aid in phenylketonuria [10]. However, LDH requires stoichiometric amounts of NADH which is a very expensive cofactor. Therefore, NADH has to be recycled to make the process cost-effective. For this purpose, the dehydrogenase of choice is " formate dehydrogenase" (FDH), since it is more stable and has a relatively high activity. Also, the reducing agent " formate" is cheap and the oxidized product " CO2" can be removed easily from the reaction and thus NADH is reduced completely [10 & 19]. Furthermore, LDH also requires an allosteric activator " fructose 1, 6-bisphospate" (FBP) for its maximal activity and has limited substrate specificity [10]. Karaguler et al. employed protein engineering to improve the properties of LDH and to overcome the disadvantages it shows in large-scale production of chirally pure lactate [10]. They used a DNA shuffling approach to produce a mutant shuffled bsLDH, whose substrate specificity is now switched from pyruvate/lactate to malate/oxaloacetate and it no longer requires FBP for its activity [10].

Another application of LDH is its use as a catalyst in organic synthesis. Simon et al. found L-LDH very useful in organic synthesis due to the fact that it can convert a wide range of 2-oxoacids into S-2-hydroxy acids [19]. They also attempted to make a comparison between usefulness of L-LDH and D-LDH for use in organic synthesis [19]. They observed that L-LDH was

comparatively more useful in this regard as compared to D-LDH since the later shows narrower substrate specificity [19].

Lactate dehydrogenase assay is used for cell proliferation test in a culture in order to measure and study the increased number of cells as a result of cell division and cell growth [17].

LDH in Nanotoxicology:

LDH assay is also widely used in nanotoxicology for the screening of in vitro cytotoxicity resulting in cell damage. Nanotoxicity leads to cell membrane damage. Once the cell membrane is damaged, the cytoplasmic enzymes, including LDH, are released. From the amount of LDH released, the extent of cell toxicity can be measured. A fluogenic indicator such as resazurin can be used to measure this LDH release from the damaged cells. " Released LDH transfers lactate to pyruvate with co-reaction of NAD+ to NADH transition, and then the oxidation reaction of NADH to NAD+ transfers the non fluorescent resazurin to red fluorescent resorufin. Viable cells, however, produce negligible fluorescent signal with LDH assay" [25]. Thus the fluorescent signal of only the damaged cells can be measured which can be used in detecting the extent of cytotoxicity [25].

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