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KINETIC PROPERTIES OF LACTATE DEHYDROGENASE FROM TROUT MUSCLE

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INTRODUCTION

The kinetics of pyruvate reduction by LDH isozymes in the presence of NADH is said to be an ordered bisubstrate reaction characterized by Michaelian concentration dependence (Borgmann et al., 1975). If the reaction is ordered, no cooperativity can occur between the two substrates (NADH and pyruvate) (Neet, 1995). Experiments with fish LDH showed, however, that this enzyme might display two types of saturation profile, hyperbolic and sigmoidal, depending on the pH in the reaction medium. At pH 9, for example, the initial velocity of pyruvate reduction by epaxial muscle LDH from goldfish showed a sigmoidal dependence upon substrate concentration, whereas at pH 8 the kinetics were of the hyperbolic type (Hochachka, 1965). Our experiments showed, in addition, that LDH from trout muscle (*Onchorhynchus mykiss)* could display both types of saturation profile at one and the same pH, but in the presence of different concentrations of the LDH preparation. Rainbow trout (*Onchorhyncus mykiss*) were obtained in October from the Goč Fishpond of the School of Forestry, University of Belgrade, Serbia. White skeletal muscle was immediately excised and kept in liquid nitrogen until use. After thawing, muscle samples were deprived of skin and spine, cut into small pieces, and homogenized in 5 volumes (w/v) of Tris-EDTA buffer. The homogenate was centrifuged for 10 min at 10 000×g. Partial purification with ammonium sulfate was performed according to Place and Powers, 1984. After centrifugation, solid ammonium sulfate was added to the supernatant to give 30% saturation at 0°C. The suspension was stirred for 1 hour and centrifuged for 30 min at 10 000×g. The pellet was discarded, and the supernatant brought to 70% saturation. The suspension was then stirred again for 1 h at 0°C, and centrifuged as already described. This time, the supernatant was discarded and the pellet redissolved in Tris-EDTA buffer and dialyzed overnight at 4°C against four 1liter replacements of the same buffer. The concentration of protein, determined by the method of Lowry (Lowry et al., 1951), amounted to 5.3 mg/ml of the final enzyme suspension.

LDH kinetic studies were carried out by measuring the change in optical density at 340 nm in a Shimadzu UV-160 spectrophotometer supplied with a thermostatically controlled cell holder and using a stopped-flow accessory (Hi-Tech SFA-11). The enzyme preparation, containing an appropriate concentration of NADH, was held in one syringe while a second syringe contained the chosen concentration of pyruvate. Absorbance changes were recorded at 3 or 5 s intervals during 1 or 2 min. The velocities were calculated by linear regression analysis of the initial linear segments of the curves. All measurements were performed at 15°C.

At least triplicate assays were conducted at 5-12 substrate concentrations, ranging from 0.0125 to 2

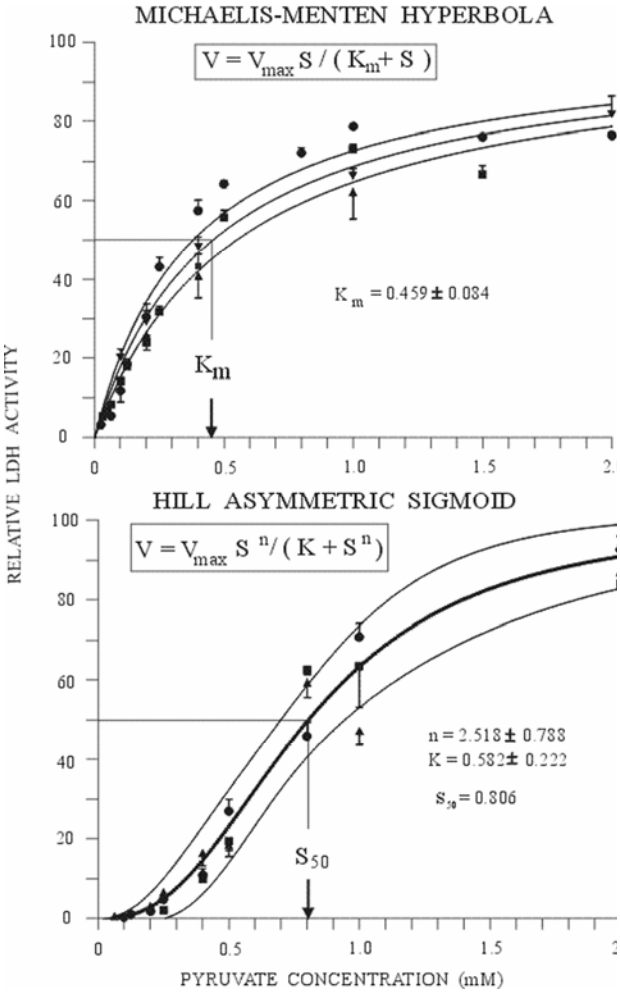

Fig. 1. Saturation curves of trout white muscle LDH (means ± SE, n = 3). Lower panel: Sigmoidal curve obtained in experiments with relatively concentrated enzyme preparations. Circles and squares: 40- and 60-fold dilutions, respectively, tested at pH 7.7; triangles: a 60-fold dilution tested at pH 7.5. - Upper panel: Hyperbolic saturation curve observed in experiments with highly diluted enzyme preparations. Circles, squares and triangles: 200-, 400-, and 600-fold dilutions, respectively, tested at pH 7.5. Data fitted with the framed equations. Dotted lines: 99.9% confidence limits.

mM. Five different dilutions of the enzyme preparation were tested for LDH activity (40-, 60-, 200-, 400- and 800-fold). The preparation was diluted with Tris-EDTA buffer (pH 7.72 at 15°C) or with 0.2 M phosphate buffer (pH 7.5). The concentration of NADH was adjusted to amount to 0.5 mM, 0.25 mM, and 0.125 mM when assaying, respectively, low (40- and 60-fold), medium (200- and 400-fold) and high (800-fold) dilutions of LDH. Initial velocities were plotted against pyruvate concentration and the curves (sigmoids or hyperbolae) were obtained by appropriate fitting procedures (equations in Fig. 1). Fitted curves were used for the determination of the S_{50} and K_m values (Fig. 1). With concentrated preparations (40- and 60-fold dilutions), pyruvate reduction proceeded rapidly: at substrate concentrations close to saturation (i.e. 1 mM pyruvate) and the linear segment lasted not more than 5 to 10 s. With a lower substrate concentration (a subsaturation concentration of 0.2 mM) pyruvate reduction was still fast, the linear segment lasting only about 15 s. In both cases (saturating and subsaturating substrate concentration), a lag period was conspicuous at the beginning of the reaction.

Further dilutions of the preparation slowed down the reaction considerably. The initial linear segment lasted much longer, and the lag period was missing, enabling a greater accuracy of rate determination. With the 800-fold dilution, the linear segments lasted up to 30 and 60 s in the case of 1 and 0.2 mM pyruvate, respectively. When sigmoids were obtained, the ratio of substrate concentration required for 90% and 10% of V_{max} was only 5 or 8 (Fig. 1, lower diagram). When testing the 200-, 400- and 800-fold diluted LDH preparation, the kinetics obeyed the hyperbolic relationship of reaction velocity to substrate concentration (Fig. 1, upper diagram), and the ratio of substrate concentration required for 90% and 10% of V_{max} was 81.

The concentration of LDH ensuring Michaelis-Menten-type kinetics, and thus optimal for studies of thermal modulation of affinity, was found to be the one giving an absorbance change of 0.1 to 0.2 per minute at 15°C, in the presence of 0.2 mM pyruvate. It was obtained by an 800-fold dilution of the partially purified enzyme preparation.

During the last thirty years considerable information has been accumulated on enzymes capable of undergoing a conversion between oligomer and

monomer, with a concomitant change in enzyme activity (Traut, 1994). Sigmoid curves relating initial velocity to substrate concentration are diagnostic of such enzymes, rather than the rectangular hyperbolae typical of the Michaelis-Menten relationship. The sigmoid curve is interpreted as indicating that the binding of the first substrate enhances the binding of the second substrate to another site via a different mechanism (Traut, 1994). Although lactate dehydrogenase is a tetramer and could therefore be subject to the tetramer-oligomer transition, a considerable number of studies revealed that the kinetics of LDH catalysis was of the Michaelis-Menten type and that the possibility of cooperativity or allosteric regulation should be excluded. It is known, however, that some LDH isozymes are allosterically regulated. In aerobic tissues, such as heart muscle and other red muscles, the predominating isozyme form (LDH-H) has a low K_m (high affinity) for pyruvate, but reduces it to lactate at a relatively low rate and becomes inhibited allosterically by high pyruvate levels. On the other hand, the other isozyme (LDH-M), located in anaerobic tissues (skeletal muscle and liver), has a higher K_m (lower affinity) for pyruvate, but reduces pyruvate at a relatively high rate and is not inhibited by the substrate (Voet and Voet, 1995).

Studies of the kinetics of pyruvate reduction by purified LDH isozymes from rat kidney (LDH-M and LDH-H) demonstrated that LDH kinetics differed markedly at highly diluted and at physiological concentrations of the enzyme (Wuntch et al., 1970). Substrate inhibition, prominent in assays with low LDH-H concentration, was absent at physiological enzyme concentrations, even in the presence of a very high substrate concentration (20mM pyruvate). The possibility was discussed that, in comparison to the kinetic properties found at highly diluted concentrations, the kinetics at the much higher enzyme concentrations that occur in vivo might be quite different. The type of the kinetics in the latter case was not specified, although the results were suggestive of the Michaelis-Menten kinetics. These results are at variance with our own findings which showed that a Michaelis-Menten rectangular hyperbola occurred only when applying high dilutions of the partially purified enzyme, while sigmoidal behavior was regularly observed with concentrated LDH preparations. The differences may reside in the experimental methods applied, particularly as a result of the different degrees of enzyme purification. The preparations of the cited authors were highly purified (Wuntch et al., 1970), while our preparations were purified only partially, imitating thus closer physiological conditions in tissues. Studying LDH from goldfish epaxial muscle (aerobic tissue) and plotting initial reaction velocities against pyruvate concentration, a sigmoid-type kinetics was observed at pH 9, irrespective of the extent of purification (5- or 40-fold) (Hochachka, 1965). At pH 8, however, the assays yielded a hyperbola. On the other hand, when using liver preparations (anaerobic tissue), the hyperbolic shape of the curves was present irrespective of pH, from pH 7 to pH 10, and the effect of a pH increase was similar to that of competitive inhibitors. K_m increased with pH, while maximal velocities (V_{max}) remained the same. This suggested the possibility that the second LDH isozyme might be regulated by a mechanism similar to competitive inhibition. An analogy was suggested with aspartate transcarbamylase, an allosteric tetramer enzyme. In the presence of NADH, according to this analogy, LDH from goldfish epaxial muscle would have a low reactivity towards the first pyruvate molecule encountered. Once this first molecule is bound, the reactivity toward the second pyruvate molecule would be increased and so forth, until all sites were occupied (Hochachka, 1965). The same author also found, working with LDH preparations from goldfish muscle and liver and from rat heart and kidney, that after irradiation with large doses of ionizing radiation of dry enzyme preparations, the remaining catalytic activity corresponded to a target mass close to one subunit of the tetramer molecule (Hochachka, 1965). This implied independent catalytic activities of the subunits.

In our experiments the observed transition from sigmoidal to hyperbolic behavior was certainly not the result of a change in pH, since hyperbolae

were observed at pH 7.5 and sigmoids both at pH 7.5 and pH 7.7. The transition was undoubtedly the consequence of a change in the dilution of the preparation. We can speculate that in concentrated preparations subunits of the LDH tetramer are closely associated, so that interactions analogous to positive cooperativity can proceed. Binding of the first pyruvate molecule would then loosen the tetrameric association, thus increasing the reactivity of the remaining binding sites. In the case of highly diluted preparations, on the other hand, the tetrameric structure would be unrestrained, each subunit would therefore be capable of undergoing an equally efficient catalysis, and no cooperativity would be possible. When considering the effect of different dilutions, the fact must also be taken into consideration that dilutions of the test-preparations do not change only the concentration of the enzyme, but also that of the accompanying compounds present in the cytosol and capable of modifying enzyme activity. Finally, the possibility should not be neglected of an artifact due eventually to an aggregation of enzyme molecules in concentrated preparations.

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