INTERACTION OF LACTATE DEHYDROGENASE ISOENZYMES WITH LIGANDS

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Isoenzymes of bovine lactate dehydrogenase (H_4, H_3M, and H_2M_2) were prepared by affinity chromatography on a 5'-AMP-Sepharose 4B column. The interaction of isoenzymes with two ligands, coenzyme NADH and the competitive inhibitor Cibacron Blue F3GA was followed by means of kinetic measurements and by affinity electrophoresis. The Michaelis constants of NADH were compared with the inhibition constants of Cibacron Blue and dissociation constants of enzyme–inhibitor complexes. It was found that the M subunit of lactate dehydrogenase exhibits always higher affinity both to NADH and Cibacron Blue in comparison to the H subunit. This finding corresponds to the physiological role of lactate dehydrogenase isoenzymes.

Animal lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase EC 1.1.1.27) is a tetrameric protein composed of subunits of two types called H (heart) and M (muscle), according to their main localization in the organism[1]. There exist five different lactate dehydrogenase isoenzymes H_4, H_3M, H_2M_2, HM_3, and M_4, that play different physiological roles; in aerobic tissues, e.g. heart or brain, they catalyze mainly oxidation of lactate with the simultaneous formation of NADH, in anaerobic tissues, e.g. muscles, the enzyme catalyzes the reduction of pyruvate and at the same time, the reoxidation of NADH to NAD^+. From the physiological point of view the affinity of lactate dehydrogenase subunits to coenzyme should, therefore, be different.

We have studied the affinity of lactate dehydrogenase isoenzymes to the coenzyme and to their inhibitor, triazine dye Cibacron Blue F3GA, that binds to the same binding site of the apoenzyme[2].

EXPERIMENTAL

Material

The crude lactate dehydrogenase preparation was obtained by 60% saturation of the water extract of bovine heart muscle with ammonium sulphate. Nicotinamide adenine dinucleotide reduced disodium salt (NADH) (Reanal, Budapest), Blue Dextran and 5'-AMP-Sepharose 4B (Pharmacia, Uppsala), Cibacron Blue F3GA (Ciba-Geigy, Switzerland). Other chemicals used were of analytical grade of purity.

Methods

Lactate dehydrogenase isoenzymes were isolated by means of affinity chromatography on AMP-Sepharose column according to Brodelius and Mosbach[3]. The isoenzymes were eluted from the column by a concave concentration gradient of NADH (0—0·5 mmol/l in 250 ml). The active fractions were pooled, deslated by dialysis and lyophilized.

The activity of lactate dehydrogenase was followed spectrophotometrically at the wave length 340 nm at a temperature of 30°C by measuring the rate of NADH oxidation. The reaction mixture contained 0·3 mol/l phosphate buffer pH 7·5, 0·6 mmol/l sodium pyruvate and 0·2 mmol/l NADH. The reaction was started by adding the enzyme to the reaction mixture. In kinetic experiments 0·2 mol/l Tris-glycine buffer pH 8·3 was used.

Affinity electrophoresis. Polyacrylamide gel electrophoresis was performed according to Davies[4] in discontinuous alkaline buffers. Affinity gels were prepared by adding a solution of Blue Dextran to the polymerization mixture. The enzyme samples in 20% glycerol were applied to the tube; electrophoresis proceeded for 2 hours at a current density of 4 mA per tube. Gels were stained specifically for lactate dehydrogenase activity[5]. The dissociation constants of enzyme–Cibacron Blue complexes (K_d) were determined graphically using the equation $1/(d_0 - d) = (K_d/d_0)(1/c_i) + 1/d_0$, where d_0 represents the mobility of enzyme in control gel, d represents that in the affinity gel, and c_i the concentration of the immobilized ligand[6].

The concentration of Cibacron Blue was determined spectrophotometrically at a wave length of 610 nm using molar absorption coeficient 13·6 . 10^3 l/mol cm (ref.[2]).

RESULTS AND DISCUSSION

The isoenzymes of bovine heart lactate dehydrogenase were prepared by a procedure involving extraction of the heart muscle, ammonium sulphate precipitation of the extract to 60% saturation and, finally, biospecific chromatography of the sulphate fraction on a 5'-AMP-Sepharose 4B column. Individual isoenzymes were eluted from the column by the increasing concentration of NADH. Three highly purified isoenzymes, namely H_4, H_3M, and H_2M_2, were prepared. The affinity chromatography and electrophoretic analyses of individual fractions are documented by Fig. 1.

Both, Michaelis constants for NADH (K_m) and inhibition constants for Cibacron Blue (K_i) were determined by kinetic measurements for lactate dehydrogenase isoenzymes. It was found that K_m values decrease with an increase in the content of the subunit M in the isoenzyme. Cibacron Blue inhibited all isoenzymes competitively to NADH (Fig. 2) and the value of K_i correlated well with values of K_m for NADH (Table I). It could be concluded that the inhibitor Cibacron Blue binds to the coenzyme binding site and it is also clear that affinity of the M subunit, both to NADH and Cibacron Blue, is higher than that of the H subunit.

Dissociation constants of complexes lactate dehydrogenase–Cibacron Blue were determined also by the method of affinity electrophoresis. For this experiment, a mixture of isoenzymes could be used because specific detection of enzyme activity on electrophoretic gels was employed. It was found that the values of K_d depended

on the concentration of the enzyme applied to the gel, therefore the values of K_d were determined for different amount of protein and extrapolated to zero concentration of protein (Table II).

TABLE I

Kinetic evaluation of the interaction of lactate dehydrogenase isoenzymes with ligands

Isoenzyme of lactate dehydrogenase	NADH K_m, μmol/l	Cibacron Blue K_i, μmol/l
H_4	19·0	7·1
H_3M	16·0	5·3
H_2M_2	10·0	3·8

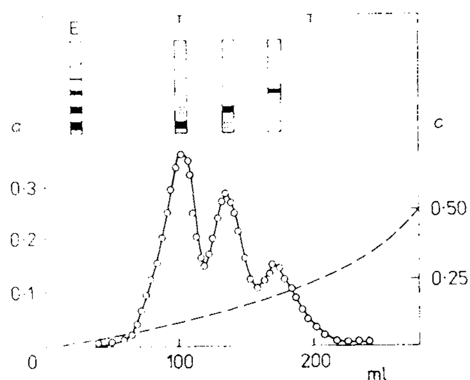

FIG. 1

Affinity chromatography of bovine heart lactate dehydrogenase on AMP-Sepharose 4B column and electrophoretic analysis of pooled fractions. *a* activity of enzymes, mU/ml; *c* concentration of NADH, mmol/l; E sulphate fraction of lactate dehydrogenase

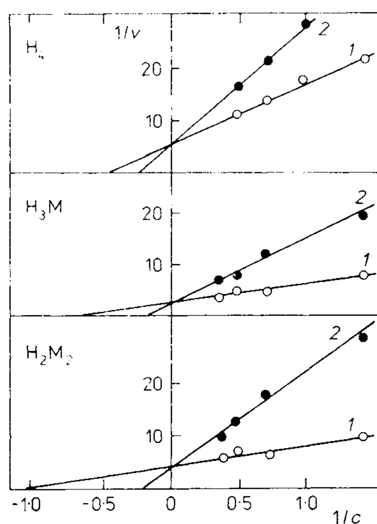

FIG. 2

Inhibition of lactate dehydrogenase isoenzymes by Cibacron Blue F3GA. *1* control reaction, *2* reaction inhibited by 1·2 . 10^{-5} mol/l Cibacron Blue; abscissa: reciprocal values of NADH concentrations 10^5 l/mol; ordinate: reciprocal values of reaction rate 1/mU

The results of both, the kinetic method and affinity electrophoresis, confirmed that M subunit of bovine lactate dehydrogenase has a higher affinity to the coenzyme – NADH, and to the competitive inhibitor Cibacron Blue than H subunit. This finding is in good agreement with their physiological role. In this respect, our results agree well with those of Thompson and Stellwagen[2], but they differ from the data published elsewhere[7,8] (Table III).

TABLE II

The effect of the protein concentration on the value of the dissociation constant of the complex lactate dehydrogenase isoenzyme–Cibacron Blue (K_d) determined by affinity electrophoresis

Concentration of protein mg/ml	K_d, µmol/l		
	H_4	H_3M	H_2M_2
4·0	2·3	1·2	0·74
2·0	—	1·1	0·71
1·0	1·4	0·9	0·50
0·2	0·8	0·6	0·34
[a]	0·8	0·5	0·36

[a] Extrapolated to zero concentration.

TABLE III

Differences in the values characterizing the interaction strength of lactate dehydrogenase isoenzymes with ligands

Interaction constant µmol/l	Ligand	Isoenzyme			Ref.
		H_4	H_2M_2	M_4	
K_d	NADH	0·38	—	2·20	7
K_d	NADH	0·38	—	1·10	8
K_d	NADH	7·50	—	5·00	2
K_i	Cibacron Blue	2·50	—	0·35	2
K_m	NADH	19·00	6·30	—	[a]
K_d	Cibacron Blue	0·80	0·27	—	[a]
K_i	Cibacron Blue	7·10	3·80	—	[a]

[a] This paper.

We may conclude that there are certain differences in data published up to now on the strength of the interaction of lactate dehydrogenase apoenzymes with the coenzyme or the triazine dye Cibacron Blue, which binds to the same site as the coenzyme. The reason may be the application of different methods by different authors. Our results characterizing the affinity of the apoenzyme to ligands obtained by two different methods differ from each other. One reason may be the different forms of ligands – free in kinetic measurements and bound to dextran in the electrophoresis. It should be also admitted that the dissociation constant K_d obtained by affinity electrophoresis and the inhibition constant K_i determined kinetically characterize different events. In spite of this, the affinity of the M subunit to ligands was, according to our observation, always higher than that of the H subunit.

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