LACTIC DEHYDROGENASE OF YEAST

III. A COMPARATIVE STUDY OF THE KINETIC PROPERTIES AND THE STABILITY OF TWO ISOLATED FORMS OF THE ENZYME

AGNAR P. NYGAARD

Johan Throne Holst's Institut for Ernaeringsforsking, Blindern, Oslo (Norway)

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SUMMARY

The kinetic properties and the stability of two isolated fractions of yeast lactic dehydrogenase (LDH II and III) have been compared. LDH II oxidized L(+) and LDH III oxidized D(—) lactic acid.

LDH II reduced cytochrome b_2. LDH III contained a heme which was irreducible by LDH III as well as by LDH II.

Crude and purified LDH II were completely inactivated by incubation at 55–57° for 5 min and by low concentrations of *p*-chloromercuribenzoate (10^{-4} *M* or less). LDH III lost the ability to reduce ferricyanide and 2,6-dichlorophenol indophenol under the same conditions, but the reduction of cytochrome *c* was unaffected.

The reduction of cytochrome *c* by LDH II was not influenced by variations in the salt concentration. LDH III was either inhibited by the addition of small amounts of several salts, or its affinity for cytochrome *c* was greatly decreased.

The affinities for ferricyanide and 2,6-dichlorophenol indophenol appeared to be similar for both forms of the enzyme. The reactions with these dyes were unaffected by great changes in the salt concentration.

LDH II and III had rather flat pH optima with cytochrome *c* and with ferricyanide as acceptors. The regions of optimum pH were somewhat different for the two forms of the enzyme.

It is argued that the two forms of LDH are no artefacts; they must occur already in the yeast cell.

INTRODUCTION

In the previous papers of this series[1,2,3] the isolation of several forms of yeast lactic dehydrogenase (LHD) was described. These forms could be separated on a NN-diethylaminoethylcellulose exhanger and therefore differed in the negative charge available to the exchanger. Furthermore, the spectral properties, the flavin/heme ratio, and the activity were different in the various forms. In the present paper the kinetic properties and the stability of two forms of LDH will be described and compared.

MATERIALS AND METHODS

The following materials were used in addition to those described previously[2,3].

D(+) *lactic acid* (Ca. salt) was purchased from Mann Research Laboratories, N.Y. The specific rotation in molybdate solution was + 36.3°.

L(+) *lactic acid* was obtained from Mann Research Laboratories (40 % solution) and from California Foundation for Biochemical Research, Los Angeles (Ca. salt. $[\alpha]_D = -3.84$; 6.65 % in water at 15°).

Trypsin (twice crystallized) was purchased from Nutritional Biochemicals Corporation, Clevland, Ohio.

Desoxyribonuclease was obtained from L. Light & Co. Ltd., Colnbrook, England.

Relative activities

The ratio's between the turnover numbers with cytochrome *c* ($2 \cdot 10^{-5}$ *M*), ferricyanide ($5 \cdot 10^{-4}$ *M*), and 2,6-dichlorophenol indophenol ($6 \cdot 10^{-5}$ *M* = $1.2 \cdot 10^{-4}$ *N*) will be called "relative activities" except where otherwise stated. The reactions were carried out at 23° in phosphate buffer, μ 0.01, in the presence of *M*/1000 Versene.

The reaction with ferricyanide was measured at 420 mμ where the extinction coefficient was $1.0 \cdot 10^3$ M^{-1} cm^{-2}.

NOMENCLATURE

The first step in the purification procedure from yeast extract was the precipitation of the enzyme with acetone[2]. This precipitate, dissolved in water, had a pH of 6.3 and will be called "crude LDH".

A number of fractions of LDH were eluted from the column[1,2,3] and pooled in three mixtures of eluates, indicated by I, II and III, according to the order of elution.

LDH I

The variants of the enzyme which were eluted between pH 7 and 6 with 0.04 *M* phosphate (group I of eluates) will be called LDH I. They contained about 0.5 flavin per heme and showed spectral properties deviating from those of the crystalline enzyme of APPLEBY AND MORTON[5].

LDH II

The variants of the enzyme which were eluted at pH 5.3 with 0.04 *M* phosphate and zero to 0.08 *M* NaCl (group II of eluates) will be called LDH II.

LDH II contained one flavin per heme and the purest preparation obtained had an equivalent weight of 100,000. The spectral properties were similar to those of the crystalline enzyme[5].

LDH III

LDH III was eluted at pH 5.3 with 0.04 *M* phosphate and 0.4 *M* NaCl[1,4] (group III of eluates).

RESULTS

Crude preparations of LDH

The properties of crude LDH varied from preparation to preparation: (1) The relative activities have been observed to vary from 0.2:1:1 to 0.8:1:1; (2) the con-

centration of cytochrome *c* at which half-maximal activity was attained (in phosphate μ 0.08, pH 7.1) varied from $0.4 \cdot 10^{-5}$ *M* to $2 \cdot 10^{-5}$ *M*; and (3) the fraction of lactic dehydrogenase activity which remained after incubation at 55–57° for 5 min varied from 5–20 %. The same fraction of the enzyme remained after incubation with high concentrations of *p*-chloromercuribenzoate. This fraction (heat-stable and pCMB-stable) will be called stable LDH. The properties of stable LDH were different from those of the original preparation. In the first place, the ratio of the reduction of cytochrome *c* to the reduction of the dyes was higher for the stable enzyme. The relative activities varied from 12:1:1 to 7:1:1. Secondly, the reaction of the stable enzyme with cytochrome *c* was strongly affected by changes in the salt concentration, whereas that of the original preparation was for the most part uninfluenced by the same conditions. In some preparations the activity of the stable enzyme decreased by about 80 % when the ionic strength of the phosphate buffer was increased from 0.01 to 0.08 (pH 7.1), in others the affinity of the enzyme for cytochrome *c* was greatly decreased.

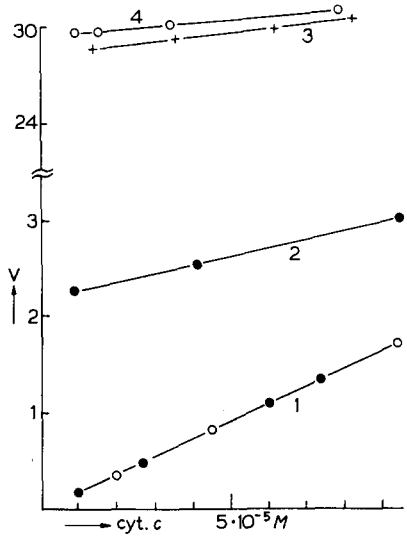

Fig. 1. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (DL, 0.02 *M*) by a crude preparation of LDH (curves 3 and 4) and by the heat-stable (curves 1 and 2) and the pCMB-stable (curve 1, marked O—O—O) enzyme contained in this preparation (see, the text). All solutions were at pH 7.1 and contained *M*/1000 Versene. Curve 1: phosphate μ 0.08; curve 2: phosphate μ 0.01; curve 3: phosphate μ 0.08; curve 4: phosphate μ 0.01.

Fig. 1 illustrates the reaction properties of a crude preparation which contained 10 % stable enzyme. The relative activities of the original preparation was 0.8:1:1 compared with 12:1:1 for the stable enzyme. It is seen from the figure that both the original preparation and the stable enzyme followed zero-order kinetics with cytochrome *c* as acceptor in the region 10^{-5}–10^{-4} *M* when the phosphate concentration was very low (μ 0.01). When the buffer concentration was increased to μ 0.08, however, the reaction properties of the original enzyme were practically unaltered, whereas the reaction of the stable enzyme changed from zero to first-order kinetics.

Incubation of the crude preparation at 37° for 30 min did not change the amount of stable enzyme.

A comparison of the properties of isolated LDH II and III

Yield, composition, and purity. In preparations which contained only small amounts of LDH I[3], LDH II constituted the main part of the LDH activity of crude preparations. The composition and purity of this form of the enzyme have been described previously[2,3].

When the DEAE-column was eluted by a gradual increase in the NaCl concentration from 0.08 to 0.4 *M*, an inactive haemoprotein was eluted with 0.4 *M* NaCl, which contained one mole of cytochrome b_2/100,000 g of protein. The haemoprotein was not associated with flavin. Between this fraction and LDH II cytochrome *c* peroxidase was eluted as a brown zone with one heme/200,000 g of protein. In order to obtain active LDH III, the salt concentration was increased stepwise from 0.08 to 0.4 *M* NaCl. In this way, preparations which contained 0.2 flavin/heme/500,000 g of protein were obtained. The heme was of type 2[3] and irreducible by LDH III as well as by LDH II. The amount of LDH III obtained corresponded roughly to the amount of stable enzyme in the crude preparation.

The stability. LDH II was completely inactivated by incubation at 55–57° for 3 min (pH 7.1) and by 10^{-4} *M* pCMB (0°, pH 7.1). The reduction of ferricyanide and 2,6-dichlorophenol indophenol by LDH III was inhibited under the same conditions; the cytochrome *c* reduction, however, was unaffected. Thus, the relative activities of LDH III could be changed to 1:0:0 by heat or pCMB. The relative activities of LDH II could not be altered in this way, and they remained roughly the same also during inactivation due to ageing and freezing (see Table I).

TABLE I

ABSOLUTE AND RELATIVE ACTIVITIES* OF FRESHLY PREPARED AND OF PARTIALLY INACTIVATED LDH II AND III

Treatment	*Turnover numbers*			*Relative activities*
	Cytochrome c	*Ferricyanide*	*2,6-Dichlorophenol indophenol*	
II Freshly prepared	6,300	10,000	6,000	1.0:1.7:1.0
II Aged at 6° for 70 h	3,300	5,500	3,000	1.1:1.8:1.0
II Frozen, thawed	2,000	2,900	2,000	1.0:1.5:1.0
II Stored at −10° for 3 weeks	120	240	120	1.0:2.0:1.0
II Trypsin-digested	180	400	—	1.0:2.2 —
III Freshly prepared	6,000	210	180	15:1.2:1.0
III Trypsin-digested	420**	40	40	10:1:1
III Venom-digested	480**	40	40	10:1:1

* The assays were carried out in phosphate μ 0.1 + *M*/1000 Versene, pH 7.1 LDH III was determined with 10^{-4} *M* cytochrome *c*.

** The rate was proportional to the cytochrome *c* concentration at least up to 10^{-4} *M* cytochrome *c*, just as for the original enzyme.

The reduction of cytochrome *c* by LDH III was inhibited within one minute at 100°. In $3 \cdot 10^{-3}$ *M* pCMB 20 % of the activity was destroyed within 5 h at 0° (pH 7.1).

The reaction with cytochrome c. Neither LDH II nor LDH III had constant kinetic properties. The concentration of cytochrome *c* at which half-maximal activity was attained varied from preparation to preparation. The sensitivity to salts was, however, widely different for LDH II and III. The cytochrome *c* dependency curve for LDH II and its insensitivity to salts was similar to that of the special crude preparation from which it was obtained, and the cytochrome *c* dependency curve for LDH III and its sensitivity to salt was similar to the stable enzyme of the crude preparation.

In general, LDH II followed zero-order kinetics down to a concentration of 10^{-5} *M* cytochrome *c*, and the rate was the same in phosphate μ 0.01, 0.08, and 0.08 + 0.04 *M* NaCl. LDH III, on the other hand, was either strongly inhibited by salts, or the affinity for cytochrome *c* was strongly decreased by changes in the salt concentration. Fig. 2 shows the reaction properties of LDH III in three different solutions. In phosphate, μ 0.01, the enzyme followed zero-order kinetics at least down to 10^{-5} *M*; in phosphate, μ 0.08, the reaction was of first order up to a concentration of 10^{-4} *M* cytochrome *c*. Fig. 3 illustrates the fact that the same salt dependency exists at pH 7.9. NaCl, Na_2SO_4, and NaAc all had very strong effects. The effect of glycylglycine was much smaller, and this was therefore a good buffer to use for the determination of pH dependency.

The pH-dependency curve of LDH II and III with cytochrome *c* as acceptor is shown in Fig. 4. Both enzymes exhibited relatively flat pH optima, and the curves were similar in the acidic range. In the alkaline range, however, the activity of LDH II decreased rapidly above pH 8.6, whereas the activity of LDH III decreased above pH 7.6 or 7.9, depending upon the salt concentration.

LDH II has previously[2] been shown to be stimulated to varying degrees by

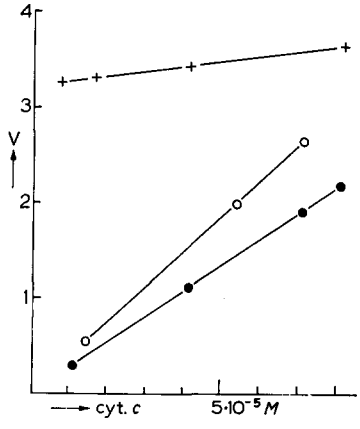

Fig. 2. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (D, 0.02 *M*) by a preparation of LDH III obtained from the crude preparation described in Fig. 1. All solutions were at pH 7.1 and contained *M*/1000 Versene. ●—●—● phosphate μ 0.08 + 0.04 *M* NaCl; O—O—O phosphate μ 0.08; +—+—+ phosphate μ 0.01.

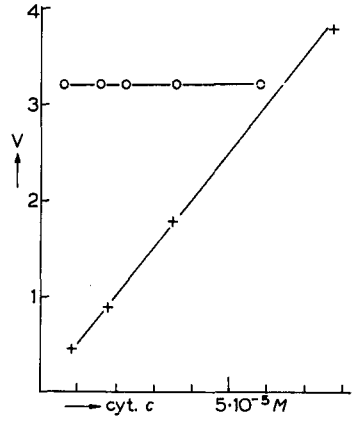

Fig. 3. The effect of the concentration of cytochrome *c* on the rate of oxidation of lactate (DL, 0.02 *M*) by a preparation of LDH III (the same as in Fig. 2). Both solutions were at pH 7.9. O—O glycylglycine 0.005 *M*; +—+ glycylglycine 0.005 *M* + 0.04 *M* NaCl.

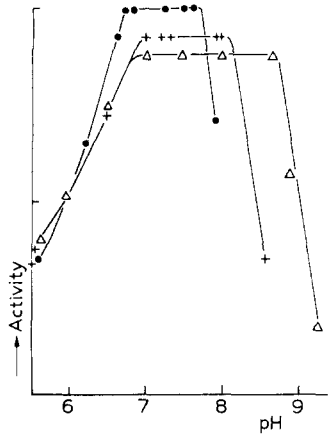

Fig. 4. The rate of the reduction of cytochrome *c* ($2 \cdot 10^{-5}$ *M*) by LDH II and III as a function of pH (DL lactate, 0.02 *M*). ●—● LDH III in 0.002 *M* phosphate + 0.002 *M* glycylglycine; +—+ LDH III in phosphate μ 0.08 + 0.002 *M* glycylglycine + *M*/1000 Versene; △—△ LDH II in phosphate μ 0.08 + *M*/1000 Versene, below pH 7.7; in glycylglycine, 0.05 *M*, from pH 7.7 to 8.6; in glycine, 0.05 *M*, from pH 8.6 to 9.7. Change of buffer at pH 8.6 and 7.7 did not affect the activity.

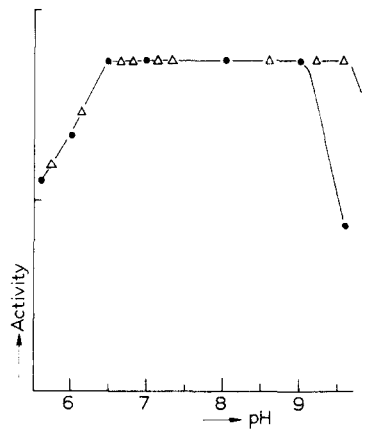

Fig. 5. The rate of the reduction of ferricyanide ($5 \cdot 10^{-5}$ *M*) by LDH II and III as a function of pH (DL lactate 0.02 *M*). ●—● LDH II; △—△ LDH III. Buffers: phosphate μ 0.08 at pH 7.7 and below; glycylglycine, 0.05 *M*, from pH 7.6 to 8.6; glycine, 0.05 *M*, from pH 8.6 to 9.7. Change of buffer at pH 8.6 and 7.7 did not affect the activity.

phenazin methosulfate. This compound had no effect on the preparations of LDH III thus far investigated.

The reaction with ferricyanide and 2,6-dichlorophenol. The reaction of LDH II and III with ferricyanide as acceptor was of zero order at least down to a concentration of $2 \cdot 10^{-5}$ *M*, and the reaction with 2,6-dichlorophenol indophenol attained half-maximal velocity around $3 \cdot 10^{-5}$ *N*. Thus, the reaction of the two forms with the dyes were practically of zero order at the concentrations used for the determination of relative activities.

Both enzymes were unaffected by great variations in the salt concentration. Thus, no change in the activity of LDH III with ferricyanide was observed when 0.8 *M* NaCl was added at pH 7.1.

The effect of pH on the activity of LDH II and III with ferricyanide as acceptor is shown in Fig. 5. The curves were similar except for the drop in rate on the alkaline side. In contrast to the reaction with cytochrome *c*, the activity of LDH III extended further than that of LDH II. The drop in the rate of LDH III on the alkaline side was due to enzyme inactivation.

With 2,6-dichlorophenol indophenol as acceptor the pH optimum of LDH II and III was at pH 6.5 or below. The optical properties of this dye changed in the acidic range.

Absolute and relative activities. The most active preparation of LDH II now obtained had relative activities 1.0:1.0:1.0 and turnover numbers 15,000 min^{-1} (per mole of flavin or heme) with cytochrome *c*. Both the absolute and the relative activities varied greatly[2,3]. In crude preparations which contained only small amounts of LDH I, the relative activities of LDH II were similar to those of the original preparation.

The first-order kinetic constant of a preparation of LDH III in phosphate μ 0.08,

pH 7.1, has primarily been estimated at $1 \cdot 10^6\ M^{-1}\ \text{sec}^{-1}$. The constant was determined on the basis of flavin content. The relative activities of LDH III varied from preparation to preparation. The relative reduction of cytochrome *c* was much higher than for LDH II, and the relative activities were similar to those of the heat- and pCMB-stable enzyme of the crude preparation.

The effect of decomposing enzymes. Attempts were made to alter the kinetic properties of LDH II and III by means of degrading enzymes. Trypsin inactivated both LDH II and III (see Table I), but the relative activities remained the same during inactivation, and the partially inactivated LDH III followed first-order kinetics with cytochrome *c* (from 10^{-5} to $10^{-4}\ M$) in phosphate μ 0.08, pH 7.1, just as did the original enzyme. The same was true for a preparation of LDH III that had been 80 % inactivated by the venom of *Crotalus terrificus terrificus*.

Although LDH II as well as LDH III appeared to be associated with polynucleotides[1,4,6], neither ribonuclease nor desoxyribonuclease affected the kinetic properties of the two forms of the enzyme.

Stereospecificity. The stereospecificity of LDH II and III has been investigated in phosphate μ 0.01, pH 7.1, and with $2 \cdot 10^{-5}\ M$ cytochrome *c* as acceptor LDH II was found to be specific for L(+) and LDH III for D(—) lactic acid.

DISCUSSION

The data obtained show clearly that both L(+) and D(—) lactic dehydrogenase are present in yeast extracts. The fact that neither LDH II nor LDH III had constant kinetic properties indicates that decomposition may take place during the extraction procedure. This may account for the fact that LDH with differing reaction properties has been obtained by other authors as well[7,8,9].

If only one form of the enzyme is present in the cell, LDH II must have changed its stereospecificity and decomposed to yield LDH III, or vice versa. This seems improbable, however. LDH II has been observed to decompose to yield an enzyme with irreducible heme[2,3]. However, LDH III is a much more stable enzyme than is LDH II and for that reason is not likely to be a decomposition product of LDH II. On the other hand, LDH III does not have the ability to reduce cytochrome b_2 and for that reason is not likely to form LDH II. Therefore, more than one form of LDH may be present in the cell. In this connection it is interesting that respiratory enzymes in yeast are inducible.

The LDH-preparation of BOERI AND TOSI[10] was inhibited by salts. Salts either inhibited LDH III or decreased the affinity of cytochrome *c* for the enzyme. The nature of the last effect could be to prevent the formation of a definite complex between LDH-lactate and cytochrome *c*. This would result in first-order kinetics.

The following sequence of reactions could take place:

1. E + lactate $\rightleftharpoons$ E. lactate
2. E. lactate + cytochrome $c \cdots \rightarrow$ E + pyruvate + cytochrome $c \cdot \cdot$

For LDH II the reduction of cytochrome *c* as well as the reduction of the dyes are sensitive to pCMB. For LDH III, however, exposed sulfhydryl groups appear to be essential for the reduction of the dyes only.

In conclusion, the salt effects as well as the effect of pCMB indicate that one

difference between LDH II and III is in the reaction with cytochrome *c*, where two different attachment sites may be operating.

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THE ACTION OF MAMMALIAN LIVER ENZYME PREPARATIONS ON ASPARAGINE AND ASPARAGINE DERIVATIVES

N. DE GROOT AND N. LICHTENSTEIN

Department of Biological Chemistry, The Hebrew University, Jerusalem (Israel)

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SUMMARY

α-N-alkyl derivatives of DL-asparagine were not hydrolyzed by rat-liver asparaginase or guinea pig-liver asparaginase. They markedly inhibited the action of these enzymes on L-asparagine. In all cases tested the inhibition was found to be competitive. Rat and guinea pig liver asparaginase did not hydrolyze N-(β-DL-aspartyl)-alkylamines and were inhibited by these compounds only slightly, if at all. The asparagine-α-keto acid transamination-deamidation system did not act on both kinds of asparagine derivatives and was not inhibited by them. Rat-liver preparations which exhibited asparaginase activity also catalyzed β-aspartyl transfer from asparagine to hydroxylamine. The specificity of the transfer reaction towards both kinds of asparagine derivatives was the same as in the case of asparaginase.

INTRODUCTION

Asparaginase is widely distributed in animal and plant tissues, and in microorganisms[1]. A comprehensive study of the specificity of asparaginase activity was carried out by