LACTIC DEHYDROGENASE OF YEAST

II. DIFFERENT FORMS OF THE ENZYME

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SUMMARY

From one to four flavohaemoproteins with different lactic dehydrogenase activity and with two different types of heme spectra (1 and 2) were obtained from yeast extract by fractionation on a NN-diethylaminoethylcellulose column. Type 1 contained 1.0 flavin/heme in its highly active forms, and the spontaneous inactivation of the isolated enzyme was associated with the appearance of fluorescent flavin and dissociation of the flavin. In the completely inactive preparations of type 1 there was no quenched flavin. In contrast, type 2 was isolated from the columns with 0.5 flavin/heme both in its active and inactive forms, and 80 % of the flavin was quenched in an inactive preparation. When isolated neither type 1 nor type 2 changed its spectral properties on spontaneous inactivation. Flavohaemoproteins of type 2 are suggested to be special decomposition products of type 1, formed in crude preparations.

Evidence is presented to show that alteration in the binding of the heme, resulting in altered spectra or in irreducible heme of type 2, does not necessarily result in inactivation. This supports the previous conclusion that the heme can be by-passed in the reduction of cytochrome *c*.

INTRODUCTION

In the first paper of this series the isolation and reaction properties of a highly purified lactic dehydrogenase (LDH) were described[1]. The flavohaemoprotein was eluted as a distinct zone from a NN-diethylaminomethylcellulose (DEAE-cellulose) column. One or two other fractions with LDH activity were frequently obtained. Furthermore, a flavohaemoprotein with no LDH activity was isolated under certain conditions. In this paper some properties of the different flavohaemoproteins are described and compared.

MATERIALS AND METHODS

The following materials and methods were used, in addition to those described previously:

Preparation procedure: In some preparations the extraction temperature was kept at 10° instead of being allowed to rise from 10 to 35° [1]. The two procedures will be called low and high temp. preparations, respectively.

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The flavin coenzyme was dissociated from the protein by the addition of dilute hydrochloric acid to give pH 3.0. Turbidity was usually not formed and fluorescence measurements could be carried out without neutralization.

Cytochrome c *peroxidase* was determined according to ALTSCHUL, ABRAMS AND HOGNESS[2].

Catalase was determined according to VON EULER AND JOSEPHSON[3].

Crystalline chymotrypsin was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, U.S.

Crystalline ribonuclease from pancreas was obtained from L. Light & Co. Ltd., Colnbrook, England.

RESULTS

The preparation applied on the column was the 0.65–0.75 saturated ammonium sulfate precipitate described previously[1] which had been dialyzed against water for 24 h. The soln. contained the following haemoproteins: Lactic dehydrogenase, cytochrome *c* peroxidase, catalase, and cytochrome *c*. The yield of haemoproteins was fairly constant and not very different in low and high temp. preparations. However, the LDH activity of low temp. preparations was on average about half that of high temp. preparations. The yield of LDH was not increased by a longer stirring time or a greater amount of BALLOTINI beads. The absorption band in the SORET region varied from experiment to experiment. A high E_{423}/E_{413} (1.2 or more) indicated high activity.

In 0.2 saturated ammonium sulfate LDH was quite stable from pH 5.0 to 8.0. In several preparations less than 10 % decrease of activity was observed on incubation at 37° for 1 h (pH 7), and the preparation could be kept at 5° for some days. In salt-free solutions, however, LDH decreased some 30 % in 24 h at 5°. The decomposition was associated with increased fluorescence. Furthermore, the formation of ribonucleoprotein particles[4] took place in such solutions, especially in those from low temperature preparations. The solutions contained about 10 % of ribonucleic acid.

The inactivation of the enzyme described above may be partially due to the degrading enzymes of the solution. Thus, on incubation at 37° for 30 min, the biuret reaction decreased some 20 %, and more than 90 % of the ribonucleic acid became dialyzable. Riboflavin was gradually formed from the dissociated flavin nucleotide. The addition of chymotrypsin increased the rate at which LDH was inactivated, whereas pancreatic nuclease had no such effect.

Below pH 5.0 inactivation of LDH occurred and fluorescence appeared quite rapidly. This was accelerated by 0.4 *M* sodium chloride. Above pH 8.0 rapid inactivation took place, again associated with the appearance of flavin fluorescence.

Separation on DEAE-cellulose

Lactic dehydrogenase, catalase and cytochrome *c* peroxidase, but not cytochrome *c*, were absorbed on the column. In the most active high-temp. preparations practically all flavohaemoprotein was eluted in one zone. This was the highly purified LDH described in the previous paper[1]. From many preparations, however, 2, 3 or even 4 fractions with partially quenched (protein-bound) flavin were obtained. The fact that fluorescent flavin was present in all fractions indicates that the appearance of

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fluorescence does not necessarily mean complete dissociation of the flavin from the protein. The flavoprotein fractions were always associated with a heme. Two different spectra were obtained, types 1 and 2, illustrated in Figs. 1 and 2. Types 1 and 2 had the same absorption maxima, but different extinction coefficients. In both types the α bands were located at 557 mμ and the SORET band in the reduced and oxidized states were located at 423 mμ and 413 mμ, respectively. In type 2, however, there was no difference in the heights of the reduced and oxidized SORET band, whereas a great difference was noted for type 1. The extinction coefficient of the reduced band at 423 mμ was about $100 \cdot 10^3\ M^{-1}$ cm^2 for type 2 and $200 \cdot 10^3\ M^{-1}$ cm^2 for type 1. The peaks of the reduced bands were much sharper in type 1 than in type 2. E_{423}/E_{413} was used as a measure of the sharpness of the band. The values were 2.1 and 1.2 for type 1 and 2, respectively. However, intermediate values were obtained in a few cases. From the difference in the spectrum between types 1 and 2 (in the oxidized and reduced states) it appears unlikely that type 2 is a mixture of type 1 and an unknown haemoprotein.

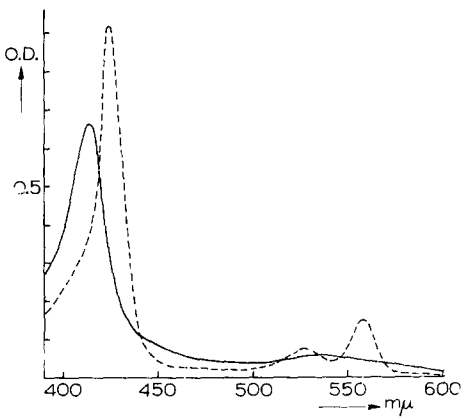

Fig. 1. The spectral properties of flavohaemoproteins of type 1. ——— in the oxidized form; – – – – in the reduced form.

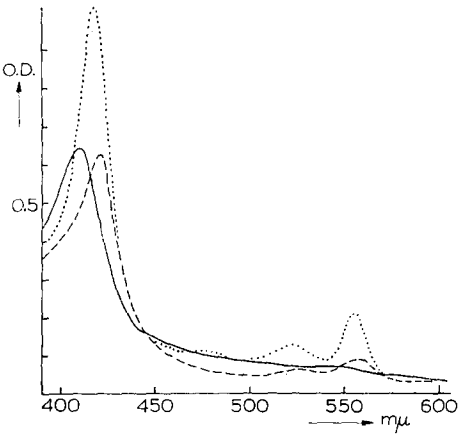

Fig. 2. The spectral properties of flavohaemoproteins of type 2. ——— in the oxidized form; – – – – in the reduced form; ····· the pyridine haemochromogen band.

One of the fractions obtained was usually inactive, whereas the others had different LDH activity. In general, the more affinity for the column, the higher the activity. The least active preparations contained the fractions most readily eluted. Furthermore, the least active preparations lost more activity on the column than the most active preparations. In one case 80 % loss was observed.

In high temp. preparations one highly active fraction of type 1 and one less active (or inactive) of type 2 were frequently isolated. The amount of type 1 decreased and that of type 2 increased with decreasing activity. When a high temp. preparation was applied on the column at pH 8.5 (0.04 M Na_2HPO_4), where some alkaline degradation was likely to take place, 3 fractions of type 1, with different turnover numbers (TN) and flavin contents, were obtained. Furthermore, one inactive fraction of an intermediate type with no flavin was isolated. The results are shown in Table I. The ability to reduce the heme decreased with decreasing activity. All fractions were distinctly separated except 3 and 4, which overlapped slightly.

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In low temp. preparations type 2 was predominant with one active and one inactive fraction. However, small amounts of a highly active fraction of type 1 were also obtained.

In the following, some frequently isolated flavohaemoproteins of types 1 and 2 are described in the order in which they were eluted from the column. The approximate pH of the eluting agent, 0.04 *M* phosphate, is given.

TABLE I

FRACTION OF LACTIC DEHYDROGENASE AND CYTOCHROME b_2, OBTAINED FROM A DEAE-CELLULOSE COLUMN*

Eluate number	*Turnover number*	E_{423}/E_{413} *reduced with*		*Flavin/Heme*
		lactate	*dithionite*	
1	0	0.6	1.6	0
2	400	1.5	1.9	0.1
3	1600	1.7	2.0	1.0
4	4000	2.1	2.1	1.0

* The sample was applied on the column at pH 8.5 (see text).

Type 2, eluted at pH 7.1, contained 0.4 flavin/heme, and about half of the fluorescence was quenched. The fraction had no lactic dehydrogenase, catalase or cytochrome *c* reductase activity. Large amounts of this flavohaemoprotein were obtained from low temp. preparations which had lost activity on storage.

Type 2, eluted at pH 6.5, contained 0.5 flavin/heme, and 50–80 % of the fluorescence was quenched. TN varied from zero to 3000 (or 6000/mole flavin). Inactive preparations with 80 % quenched flavin have been isolated. In one preparation with TN 1800, the position of the SORET peak was observed to change from 423 mμ to 413 mμ in the course of 24 h at 5° with no concomitant decrease of activity. Thus, the heme may become irreducible by lactate without a consequent decrease of activity. This type of inactivation has been observed also for a special preparation of type 1[1].

The fraction has been isolated both from high and low temp. preparations. Catalase was eluted about simultaneously, but not in quantities sufficient to influence the spectral properties.

Type 1, eluted at pH 5.3, contained 1.0 flavin/heme, and a maximal TN of 6000 was recorded. The enzyme was very unstable, and there was a direct relationship between inactivation and the appearance of fluorescence. In this respect the preparation was similar to crystalline LDH[5]. The inactivated preparation had the spectral properties of the active enzyme (type 1).

Type 1, eluted at pH 5.3 with 0.04 M *sodium chloride*, contained 1.0 flavin/heme, with TN as high as 15,000. This enzyme was described previously[1]. On storage and inactivation the enzyme behaved as the previous fraction.

DISCUSSION

Type 2 is likely to be a decomposition product of type 1, for type 2 increased and type 1 decreased on inactivation of high temp. preparations. It is puzzling, however,

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that the greatest amount of type 2 was isolated from low temp. preparations, where the least decomposition would be expected to take place. Possibly, the low temp. preparations contain the highest concn. of the decomposing agent. The decomposition of type 1 to type 2, which must occur in crude preparations or on the column, is different from the inactivation directly observed for the isolated LDH of type 1. This inactivation was associated with the appearance of fluorescence and no spectral changes. From the resulting products the mechanism of the decomposition of type 1 to type 2 can be reconstructed. Initially, half of the flavin is dissociated and the binding of the heme is altered so that half or more of the activity is lost and the spectral properties are changed. Further destruction can take place without dissociation of the remaining flavin (or increase in fluorescence).

Alteration of the binding of the heme does not necessarily cause inactivation. This is evident from the facts that type 2 had been isolated with a high TN (6000/flavin) and that the heme was observed to become irreducible by lactate without decrease in activity.

ACKNOWLEDGEMENT

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DENATURATION OR DISSOCIATION OF DEOXYRIBONUCLEIC ACID IN DILUTE SOLUTION

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SUMMARY

Ultracentrifugal analysis of dilute solutions of deoxyribonucleic acid at low ionic strength shows that the sedimentation boundary is composed of two molecular species at pH values lower than 5. The proportion of each component varies from 100 to 0 % in the pH range from 5 to 4.

Combination of the sedimentation and viscometric data at pH 7 and 4 indicates that one component is constituted of native molecules and the other one of denatured material.

The sedimentation pattern proves also that the denaturation process is "all-or-none" in these conditions.

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