TRANSIENT DISSOCIATION OF LACTATE DEHYDROGENASE RESULTING IN INCREASED REACTIVITY TOWARDS IODOACETAMIDE

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1. Introduction

Under ordinary laboratory conditions, no hybridization takes place between LDH* tetramers composed of different polypeptide chains even in high dilution and/or after prolonged incubation, whereas hybridization is easily obtained by a short exposure to extreme conditions of pH, ionic strength, temperature, pressure, to high concentrations of urea or guanidine hydrochloride, or by freezing and thawing [1–4]. This indicates that under normal laboratory conditions there is no dissociation of LDH tetramers.

This communication shows that by freezing and thawing, a transient, inactive and dissociated state of LDH is obtained. In this transient state LDH shows a markedly increased sensitivity towards irreversible inactivation by iodoacetamide. In the absence of freezing, inactivation of LDH by iodoacetamide occurs at a very slow rate which is not, or only slightly increased by lowering the protein concentration. This supports the view that in the absence of freezing, the tetramers do not dissociate.

2. Materials and methods

LDH-H_4 was prepared from pig heart muscle, and LDH-M_4 from pig skeletal muscle [5, 6]. Iodoacetamide (A.G.Fluka) was recrystallized from carbon tetrachloride. All other reagents were analytical grade commercial preparations.

* Lactic dehydrogenase.

The pH values were measured at room temperature, protein concentration was determined from the absorbance at 280 nm, and LDH activity was measured with pyruvate and NADH as substrates [5, 6]. All freeze-thaw treatments were carried out by freezing 1-ml aliquots in test tubes, immersed for 2 hr in an ethylene glycol-water bath thermostatically controlled at $-18 \pm 1°C$. For thawing, water (bath(s) at room temperature and/or 0°C were used.

3. Results and discussion

3.1. *Transient dissociation of LDH by freezing and thawing*

When a 1 mg/ml solution of LDH-H_4 is frozen and thawed quickly, and enzyme activity is determined from samples withdrawn successively between 2 to 50 min following the commencement of thawing [fig. 1], full reactivation can be observed, which starts from a very low initial activity. Reactivation is arrested by dilution. This concentration-dependence of reactivation indicates that the transition of an inactive dissociated state to an active associated state of the protein is involved. This idea is further supported by the findings shown in fig. 2. When decreasing concentrations of the enzyme protein were subjected to standard freeze-thaw treatment, the extent of final reactivation decreased to less than 10 percent (LDH-H_4), and to about 30 percent (LDH-M_4), respectively (fig. 2). If reassociation is the limiting step in the reactivation process, this would account for a lower rate of reactivation, but not for a decrease in the final extent of

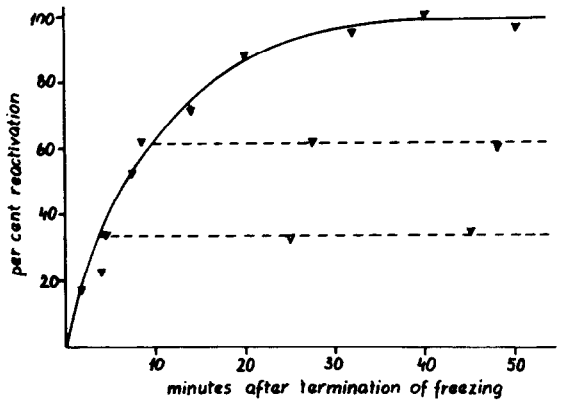

Fig. 1. Reversible inactivation of 1 mg/ml LDH-H_4 by freezing and thawing in 0.1 M sodium phosphate buffer, pH 7.0. Following the completion of thawing, the solution was kept at 0°C. 0.02 ml aliquots were with drawn at various times as indicated on the abscissa, and diluted 50-fold before the determination of enzyme activity. LDH activity was determined from the diluted samples immediately (solid line) and after various times of further incubation in the diluted state (broken lines).

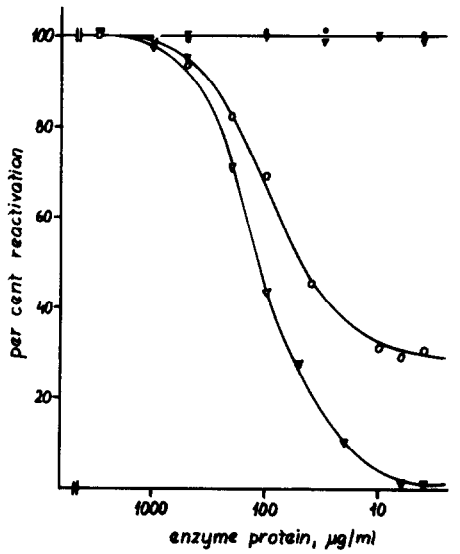

Fig. 2. Maximal reactivation following freezing of LDH-H_4 (△, ▲) and LDH-M_4 (○, ●) at decreasing protein concentrations. The enzymes were dissolved in 0.1 M sodium phosphate buffer, pH 7.0 (△, ○), and in 0.2 M tris-HCl, pH 7.5 (▲, ●), respectively.

reactivation (fig. 2). However, since it is known that LDH in the dissociated state is easily denatured [7], we suggest that subunit denaturation and reassociation are parallel reactions, denaturation becoming more and more predominant as the rate of reassociation is lowered by reducing the protein concentration. Similarly the difference between the behaviour of the LDH-s (fig. 2) can be explained by the assumption that the relative rate of reassociation of the M-chains is higher.

Above a threshold protein concentration of about 1 mg/ml, any further transient structural change accomparying dissociation was fully reversible (figs. 1 and 2). Moreover, re-formation of the "active tetrameric structure" was obtained at a low temperature (≤ 0°C) and with a half-life of about 5–10 min. For these reasons, chemical reactivity of the products of transient dissociation can be studied by the freeze-thaw procedure.

No enzyme activity was lost, even at very low protein concentrations, by freezing and thawing LDH in pH 7.5 tris-HCl buffer instead of the standard sodium phosphate (fig. 2). Neither did freezing in tris buffer lead to a transient inactivation as shown in fig. 1. It follows that LDH does not dissociate upon freezing in tris buffer. This conclusion is in full accordance with the finding [6] that hybridization is obtained by freezing and thawing in sodium phosphate, but not in tris buffer [2, 8], and also with the following results obtained in the presence of iodoacetaminde (fig. 3).

3.2. *Inactivation by iodoacetamide in the transient dissociated state*

Fig. 3 shows that practically full inactivation is obtained by freezing and thawing of 1 mg/ml LDH in sodium phosphate buffer, in the presence of less than 0.01 M iodoacetamide. In contrast, in unfrozen controls incubated at 0°C, or when freezing and thawing was carried out in tris-HCl buffer instead of sodium phosphate, no loss of activity was obtained even with 0.1 M iodoacetamide (fig. 3).

Results obtained by adding iodoacetamide to previously frozen LDH at various intervals following the termination of freezing are shown in fig. 4. The reaction between LDH and iodoacetamide takes place upon thawing during the period of time when the protein exists in the transient dissociated state (cf. fig. 1). We therefore suggest that freezing-induced inactivation of LDH by iodoacetamide is due to the marked increase in sensitivity of the enzyme protein in the short-lived dissociated state.

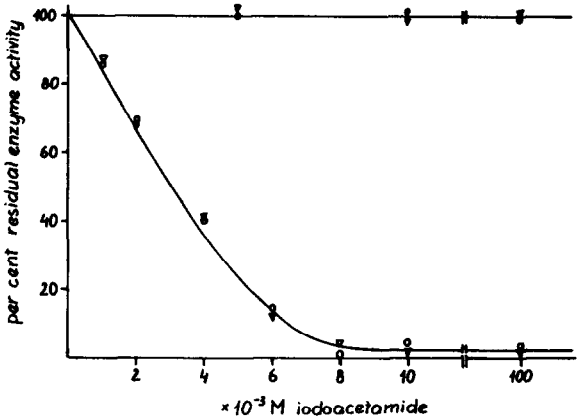

Fig. 3. Irreversible inactivation of 1 mg/ml LDH by freezing and thawing in the presence of increasing concentrations of iodoacetamide. LDH-H_4 (△, ▲) and LDH-M_4 (○, ●) were dissolved in 0.1 M sodium phosphate, pH 7.0 (△, ○), and in 0.2 M tris-HCl, pH 7.5 (▲, ●), respectively. Residual activity was determined 30–60 min after thawing.

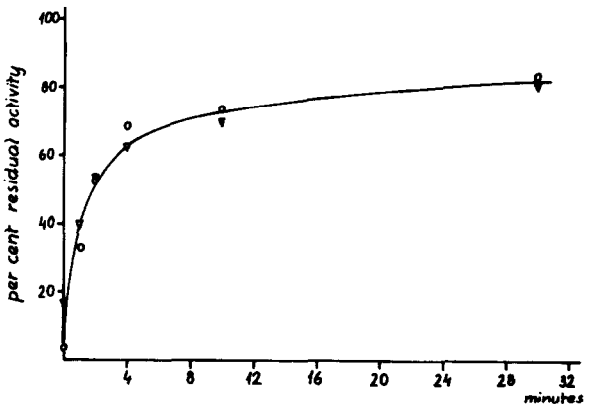

Fig. 4. Irreversible inactivation of LDH by the addition of iodoacetamide to previously frozen enzyme. 1 ml aliquots of a 2 mg/ml solution of LDH-H_4 (△) and LDH-M_4 (○) were frozen in 0.1 M sodium phosphate, pH 7.0. At 0–30 min after termination of freezing, a 1 ml aliquot of a 0.2 M iodoacetamide solution in the same buffer was added. Enzyme activity was determined after further incubation for 30–60 min at 0°C.

3.3. *Inactivation by iodoacetamide in the stable tetrameric state*

It is an intriguing question whether the slow inactivation of the tetrameric form of LDH by iodoacetamide occurs through an undetectably low equilibrium concentration of a dissociated form of the enzyme. If the dissociated form were much more sensitive towards iodoacetamide, the rate of inactivation should be markedly increased by lowering protein concentration. However, we found that the apparent first-order rate constant of inactivation of the two LDH-s by iodoacetamide was not markedly affected by a dilution of protein more than 300-fold. A 0°C the time required for 50 percent inactivation of LDH in the presence of 0.1 M iodoacetamide in 0.1 M sodium phosphate buffer, pH 7.0, was lowered from 77 ± 8 hr to 69 ± 7 hr (LDH-M_4), and was constant at 70 ± 7 hr (LDH-H_4), respectively, by lowering the concentration of protein from 1 mg/ml to 0.003 mg/ml. This further supports the idea that, under these conditions, the reversible dissociation of LDH tetramers is a prohibited structural transition (see Introduction).

4. Concluding remarks

We conclude that LDH shows a markedly increased sensitivity towards irreversible inactivation by iodoacetamide in a transient, inactive and dissociated state which is formed upon freezing and thawing. Further studies are necessary to decide whether or not the amino acid side chains which react in the dissociated state are directly involved in the association of tetramers. Some years ago, Gold and Segal [9] claimed to have labelled specifically the essential SH-group in the active center of LDH by freezing and thawing the enzyme in the presence of *N*-(4-dimethylamino-3,5-dinitrophenyl)-maleimide. It is certainly possible that the freezing-induced dissociation of LDH tetramers is coupled to marked modifications of the tertiary structure of the polypeptide chains, and that these modifications also involve a steric change in the active center. However, we should like to point out that the associating surfaces must be masked by dissociation, and that transient dissociation may prove to be a means of specifically labelling the amino acid side chains of the associating surfaces.

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