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# MODIFICATION OF PIG HEART LACTATE DEHYDROGENASE

# A DERIVATIVE POSSESSING ENHANCED THERMAL STABILITY

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## SUMMARY

Pig heart lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) has been modified by reaction with pentanedial to give a water-soluble product. This derivative retains 50% of initial enzymic activity, and its $K_m$ for the reduction of pyruvate is close to that of the native enzyme. Reaction with pentanedial markedly increases the thermal stability of the enzyme at pH 5.0–6.0 and at pH 9.0.

## INTRODUCTION

Insoluble enzyme derivatives often exhibit enhanced stability to inactivation or denaturation[1]. Usually, however, this is at the expense of final yields of enzymic activity and the derivatives themselves often possess markedly altered catalytic parameters. In order to overcome these drawbacks and to use modified enzymes in a wider range of applications than is possible for insoluble enzyme preparations, the possibilities of preparing water-soluble, stabilized enzyme derivatives are being explored.

The stability of proteolytic enzymes to autodigestion in solution has been increased by attachment to high molecular weight polymers[2], but application of this method to non-proteolytic enzymes such as lactate dehydrogenase (L-lactate:NAD oxidoreductase, EC 1.1.1.27) or ribonuclease does not increase their stability to thermal inactivation (Foster, R. L. and Thomson, A. R., unpublished). Pentanedial, a bifunctional reagent, is used extensively as a fixative and for the preparation of insolubilized enzymes[1]. It has been used by us to modify pig heart lactate dehydrogenase and the resulting water-soluble product retains approximately 50% of the activity of the native enzyme. Although the Michaelis constant for reduction of pyruvate is unchanged, the pH profile of its thermal stability is substantially altered.

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## MATERIALS AND METHODS

Pig heart lactate dehydrogenase was obtained from The Boehringer Corporation (London) Ltd. Pentanedial, supplied by the Sigma Chemical Co. as a 50% aqueous solution over barium carbonate, was purified by treatment with activated charcoal followed by distillation. A 5% solution of this dial in 0.1 M phosphate buffer, (pH 6.0) was used to modify the enzyme at 30 °C. Sephadex G-200 was obtained from Pharmacia (G.B.) Ltd, and prepared as directed by the suppliers. Gel chromatography was performed in 50 mM phosphate buffer (pH 7.0). Enzymic activity was assayed according to the procedure of Hakala *et al.*[3]. First estimates of the apparent Michaelis parameters were obtained from Lineweaver–Burk plots, inspection of which indicated no abberations due to substrate inhibition *etc.*, over the pyruvate concentration range ($10^{-5}$–$10^{-3}$ M) used. These values were refined using an iterative procedure based on that of Wilkinson[4].

## RESULTS AND DISCUSSION

The time course of the effect of pentanedial on the activity and stability of lactate dehydrogenase in solution is shown in Fig. 1. During the course of the reaction

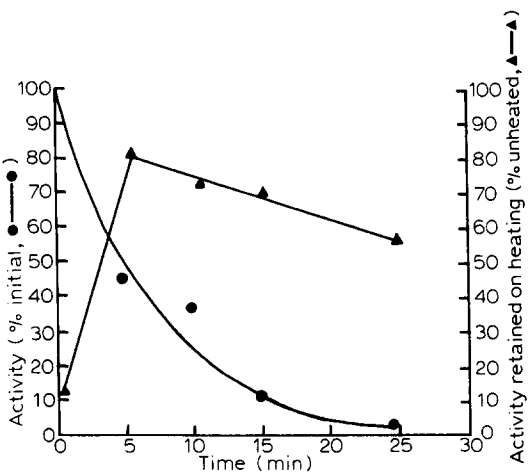


Fig. 1. Effect of pentanedial on the activity and stability of pig heart lactate dehydrogenase. Aliquots were removed at intervals, alkaline sodium borohydride added and the solution centrifuged, dialysed and assayed (●—●). Duplicates were also diluted into 50 mM phosphate buffer (pH 6.0) pre-equilibrated at 70 °C, incubated for 6 min, cooled in ice–water mixtures and then assayed (▲—▲). Both assays were performed in 50 mM phosphate buffer (pH 7.0).

the formation of an insoluble, inactive conjugate was observed which became significant after the first 15 min. The stability of the soluble conjugate reached a maximum after about 5 min exposure to the pentanedial and coincident with approximately 50% retention of activity. Thereafter although the stability of the modified enzyme decreased relatively slowly the soluble activity fell rapidly.

Lactate dehydrogenase, crosslinked by reaction with pentanedial for 5 min, was used for further study. This derivative was found to behave similarly to native

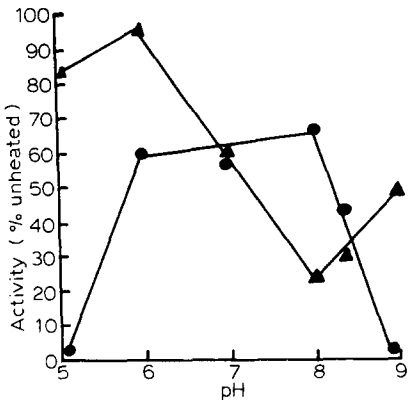


Fig. 2. Effect of pentanedial modification of lactate dehydrogenase on its pH dependent thermal stability. Samples were diluted into appropriate buffer, incubated at 70 °C for 5 min and then assayed as described in Fig. 1. Lactate dehydrogenase, ●—●; modified lactate dehydrogenase, ▲—▲.

lactate dehydrogenase on gel filtration. The pH dependences of the thermal inactivation of this and native lactate dehydrogenase are shown in Fig. 2. Between pH 5.0 and 6.0 and at pH 9.0, there is considerable stabilization, but between these extremes, pronounced stabilization at pH 6.0 is followed by progressive sensitization of the soluble conjugate between pH 7.0 and 8.0. Such behaviour is markedly different

TABLE I

APPARENT MICHAELIS CONSTANTS FOR THE REDUCTION OF PYRUVATE BY NATIVE AND PENTANEDIAL-MODIFIED PIG HEART LACTATE DEHYDROGENASE AT 30 °C, 50 mM PHOSPHATE (pH 7.0)

| *Fraction* | $K_{pyr}(\mu M)$ | *S.E.* ($\mu M$) |
|---|---|---|
| Native enzyme | 35.8 | 4.9 |
| Modified enzyme | 46.2 | 3.8 |

from that of the native enzyme, the stability of which remains constant throughout this intermediate range. Although modification of lactate dehydrogenase by pentanedial alters its pH–stability profile there is no statistically significant effect on the apparent Michaelis parameter for the reduction of pyruvate ($K_{pyr}$) (Table I). At low pyruvate concentrations and with [NADH] $\gg K_{NADH}$ the reaction rate data fit the equation of Hakala *et al.*[3]:

$$\frac{V}{v} = 1 + \frac{K_{pyr}}{[\text{pyruvate}]} + \frac{K_{NADH}}{[\text{NADH}]} + \frac{K_{pyr \cdot NADH}}{[\text{pyruvate}]\,[\text{NADH}]}$$

where $v$ is the observed initial reaction velocity and $V$ is the maximal reaction velocity at high concentrations of pyruvate and NADH, relative to their apparent Michaelis constants $K_{pyr}$, and $K_{NADH}$, respectively; $K_{pyr \cdot NADH}$ is a complex constant, (see Alberty[6]). The values of $K_{pyr}$ for both native and modified lactate dehydrogenase are slightly high, but this does not affect any comparisons made.

Insoluble, immobilized enzyme derivatives exhibiting enhanced stability to

inactivation have been studied extensively[1]. By comparison, soluble enzyme conjugates, other than those for proteases[2], have received scant attention. Amylase has been attached to several polysaccharides and marked increases in its thermal stability have been reported[7]. In addition a higher proportion of enzyme activity was retained in these conjugates than in previously reported insoluble amylase preparations. Lactate dehydrogenase has been coupled directly to activated dextrans giving soluble conjugates containing 30% protein and 70% of the initial activity (Foster, R. L. and Thomson, A. R., unpublished). Unfortunately, the thermal stability of the enzyme was reduced significantly. These results are in contradistinction to insoluble polysaccharide–lactate dehydrogenase conjugates which retain less than 1% of their initial enzymic activity after coupling but exhibit greater stability to high temperatures[8]. Soluble or insoluble conjugates of lactate dehydrogenase can be prepared by reaction with pentanedial, however the dialdehyde rapidly inactivates the enzyme on prolonged reaction. This loss in activity which may result from direct reaction at the active site, or from steric hindrance or conformational changes elsewhere in the enzyme caused by more extensive reaction of the bifunctional reagent, is not paralleled by any further increase in the stability of the remaining enzymically active molecules. The stability of the modified lactate dehydrogenase is more dependant on pH between pH 5.0 and pH 9.0 than that of the native enzyme. This presumably partly reflects changes in the microenvironment of the protein and partly the probable presence of intersubunit crosslinks. Both of these will cause the pH dependent mechanisms of inactivation for native and modified enzyme to be different.

Alterations in the protein conformation cannot be discounted as contributing to the stabilization of lactate dehydrogenase reported here, although large effects are not transmitted to the active sites which exhibit kinetic characteristics similar to those of the native enzyme.

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