THERMOSTABLE INHIBITOR(S) OF PYRUVATE DEHYDROGENASE COMPLEX FROM *STREPTOCOCCUS FAECALIS*

Akio YAMAZAKI, Yushi NISHIMURA and Teijiro KAMIHARA
Laboratory of Industrial Biochemistry, Department of Industrial Chemistry, Faculty of Engineering, Kyoto University, Kyoto, Japan

Received 13 December 1976

1. Introduction

Pyruvate dehydrogenase complex (PDH complex) (EC 1.2.4.1) occupies a key position in metabolism between glycolysis and several important metabolic pathways. Consequently, the following regulatory properties of the multienzyme complex have been extensively investigated [1]: (1) product inhibition by acetyl CoA or NADH which is reversed by CoA or NAD^+, (2) feedback regulation by nucleotides, and (3) regulation by covalent modification; inactivation by phosphorylation and reactivation by dephosphorylation. In addition, Wieland [2] has reported inactivation of PDH complex in rat liver mediated by a proteolytic enzyme(s) localized in mitochondrial lysosomes. This inactivation appears to be resulted from disaggregation of the multienzyme complex by limited proteolysis.

The present communication deals with the presence in *Streptococcus faecalis* of a thermostable inhibitor(s) which affects the overall activity of the multienzyme complex.

2. Materials and methods

2.1. *Organisms*

S. faecalis 10C1 (ATCC 11700) and its mutant, H-1-12, isolated as described previously [3] were cultivated in a synthetic medium [4] with supplement of 2 mM sodium acetate.

2.2. *Cell-free extracts*

Cells collected at the end of logarithmic phase of growth were treated with lysozyme prior to a brief sonication [4] to minimize inactivation of PDH complex during sonication. The homogenate was centrifuged at 10 000 × *g* for 40 min. The resulting supernatant was divided into two portions. One was activated with DL-lipoic acid as described by Leach et al. [5] and then dialyzed against three changes of 0.02 M potassium phosphate buffer (pH 7.0) at 4°C for 20–24 h with agitation. The lipoic acid-treated extracts are referred to as 'activated' extracts, in which PDH complex would exist in the form of holoenzyme. The other portion of the supernatant was incubated and dialyzed in the same way as in preparing the 'activated' extracts except that lipoic acid was not added. PDH complex in the lipoic acid-free extracts would contain the dihydrolipoyl transacetylase component in the form of apoenzyme.

2.3. *Enzyme assays*

PDH complex activity was routinely measured according to the method of Leach et al. [5]. The activities of the component enzymes of the complex were assayed by the methods of Reed et al. [6] with the following modifications; (1) for the assay of dihydrolipoyl transacetylase (EC 2.3.1.12), potassium phosphate buffer was used in place of Tris buffer, (2) the reaction of dihydrolipoamide dehydrogenase (EC 1.6.4.3) was followed under anaerobic conditions to eliminate the influence of NADH oxidation.

2.4. *Assay of inhibitor(s)*

The lipoic acid-free extracts obtained as described above were boiled for 5 min and the precipitates formed were removed by centrifugation. The resulting super-

 North-Holland Publishing Company – Amsterdam

natant was dialyzed as above and used as the inhibitor preparation. The activity of inhibitor(s) was determined by measuring the decrease in the activity of PDH complex. One unit of the inhibitor(s) is defined as the amount which causes the reduction of 1 nmol acetyl phosphate formation from pyruvate under the above conditions.

2.5. *Gel-filtration of inhibitor(s)*

The boiled extracts described above were treated with streptomycin to remove nucleic acids before dialysis. The dialyzed extracts were concentrated in an Amicon (Lexington, Mass. USA) ultrafiltration cell with a UM2 membrane at a constant pressure of nitrogen gas. The preparations obtained were chromatographed on a column of Bio-gel A-0.5 m (1.6 X 60 cm) equilibrated with 20 mM Tris buffer (pH 7.4) containing 1 mM EDTA and 0.1 M NaCl. The column was eluted with the same buffer and fractions of 1 ml were collected. The inhibitor activity of these fractions was assayed as described above. For the determination of the molecular weight of the inhibitor, yeast alcohol dehydrogenase (mol. wt. 148 000), bovine pancreas α-chymotripsinogen A (mol. wt 23 650) and bovine pancreas ribonuclease A (mol. wt 14 000) were used to calibrate the column.

2.6. *Protein determination*

The concentrations of protein in the extracts were estimated according to the method of Lowry et al. [7].

2.7. *Chemicals*

Dihydrolipoamide was prepared from DL-lipoamide according to the method of Reed et al. [8]. All chemicals used were analytical grade.

3. Results and discussion

3.1. *The presence of a thermostable inhibitor(s) of PDH complex*

As illustrated in fig.1A, the activity of PDH complex in cell-free extracts from *S. faecalis* 10C1 was proportional to the concentration of protein up to a range of 4–5 mg/ml when the extracts were activated previously with lipoic acid as described in Materials and methods. However, the enzyme activity decreased markedly upon addition of the lipoic acid-free extracts. The results strongly suggest the existence of an inhibitory factor(s) of PDH complex in the extracts.

As described below, the inhibitory factor(s) was observed to be a thermostable protein(s) and shown to exist also in the 'activated' extracts. However, the

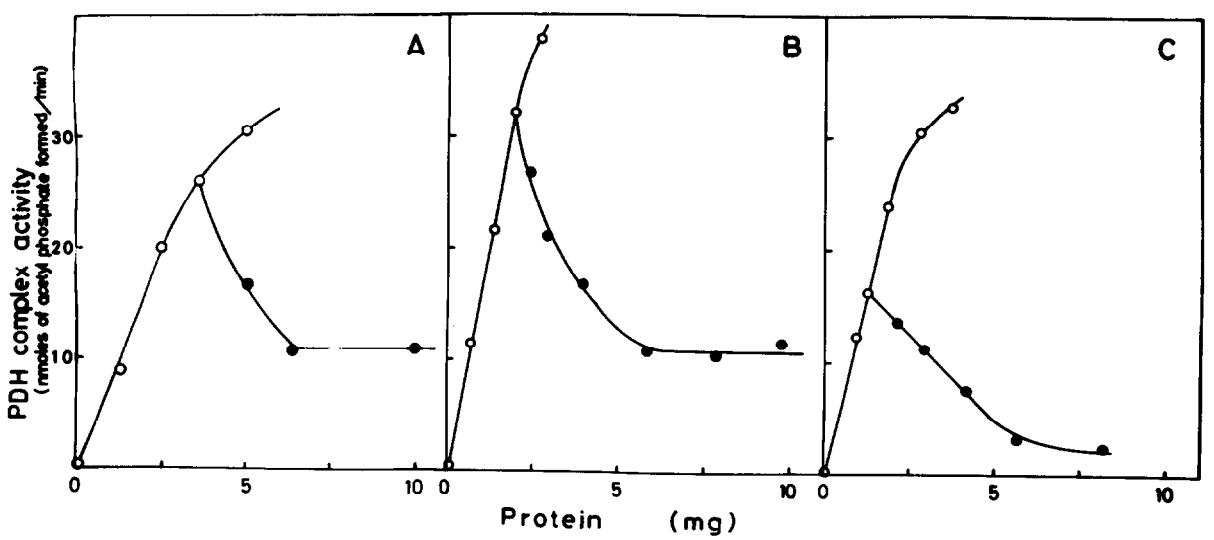

Fig.1. Inhibitory effect of lipoic acid-free extracts from *S. faecalis* on the activity of pyruvate dehydrogenase complex. Open circles refer to the activity of the enzyme as a function of protein concentration (A: parental, B and C: mutant H-1-12). Closed circles indicate change in the enzyme activity upon addition of the lipoic acid-free extracts (A: parental, B: H-1-12, C: H-1-12 (boiled)). Preparation of the cell-free extracts and assay of PDH complex were carried out as described in Materials and methods.

inhibitory effect was not observed in the extracts, presumably due to the higher activity of PDH complex than that of the inhibitor(s).

It seems to be probable that the inhibition of PDH complex may occur through that of either lactate dehydrogenase (EC 1.1.1.27) or phosphotransacetylase (EC 2.3.1.8) participating in the dismutation reaction of pyruvate. This possibility could be ruled out since the ratios of these enzymes to the inhibitor(s) in the lipoic acid-free extracts would be the same as those in the 'activated' ones. NADH as well as acetyl CoA is not involved in the inhibitory action of the cell-free extracts since the extracts were exhaustively dialyzed before use. For the same reason, phosphorylation of PDH complex can be ruled out.

As described previously [3], *S. faecalis* possesses three separate enzyme systems for pyruvate dehydrogenation, namely PDH complex, pyruvate formate-lyase and an unidentified enzyme system. Although the enzyme activities can be estimated independently from each other [3], preliminary experiments revealed that the level of PDH complex was affected by the occurrence of the two other enzyme systems (data to be published elsewhere). Hence, a mutant, H-1-12, having only PDH complex for pyruvate dehydrogenation, was employed for further investigation. The activity of PDH complex in this mutant was also inhibited by the addition of lipoic acid-free extracts from its own cells as shown in fig.1B. It would be, therefore, concluded that similar inhibitory factor(s) to that in the parental strain was present in the mutant. Figure 1C shows that the inhibitory factor(s) was thermostable. Boiling of the lipoic acid-free extracts did not affect the inhibitory effect on the activity of PDH complex. The inhibition of PDH complex occurred upon addition of boiled extracts from the parental strain to the 'activated' extracts from H-1-12, or *vice versa*, indicating further the similarity of the inhibitor(s) from both strains. The presence of the inhibitor activity was detected also in the 'activated' extracts by boiling them before use.

3.2. *The properties of the inhibitor(s) of PDH complex*

The activity of the inhibitor(s) was reduced by treatment with a low concentration of trypsin (fig.2). The inhibitory activity was acid-stable, since it was able to be recovered without loss after trichloroacetic acid (3%) treatment.

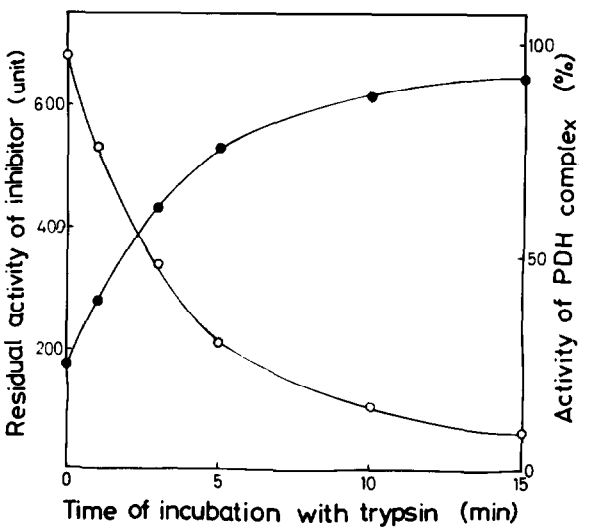

Fig.2. Effect of trypsin on the inhibitor(s) of pyruvate dehydrogenase complex. Boiled extracts (0.78 mg protein) from H-1-12 cells were incubated with bovine pancreatic trypsin at a ratio of 5:1 by weight. At indicated times, tryptic digestion was terminated by the addition of a 4-fold excess by weight of soybean trypsin inhibitor. The inhibitor activity of PDH complex was then determined. Open circles refer to residual activity of the inhibitor(s) after treatment with trypsin. Closed circles indicate activity of PDH complex after incubation with the trypsin-treated inhibitor(s). The enzyme activity in the absence of inhibitor(s) was taken as 100%.

The behavior of the inhibitor preparation on a Bio-gel 0.5 m agarose column is shown in fig.3. Two distinct peaks of inhibitory activity were observed, although the inhibitor in fraction 86–95 should be the major component. This main inhibitor was found to have a molecular weight of 17 000 as determined by the column chromatography on Bio-gel A-0.5 m. These results strongly suggest that the inhibitor(s) of PDH complex found in *S. faecalis* is a thermostable protein(s).

As shown in fig.4 and 5, the three component enzymes of PDH complex exhibited no loss in activity when examined individually with the lipoic acid-free extracts. This would mean that the inhibitor(s) causes a certain disaggregation of the multienzyme complex as found for the inactivation of PDH complex by extracts from rat mitochondria [2] and for the inactivation of 2-oxoglutarate dehydrogenase complex by extracts from bovine kidney mitochondria [9]. How-

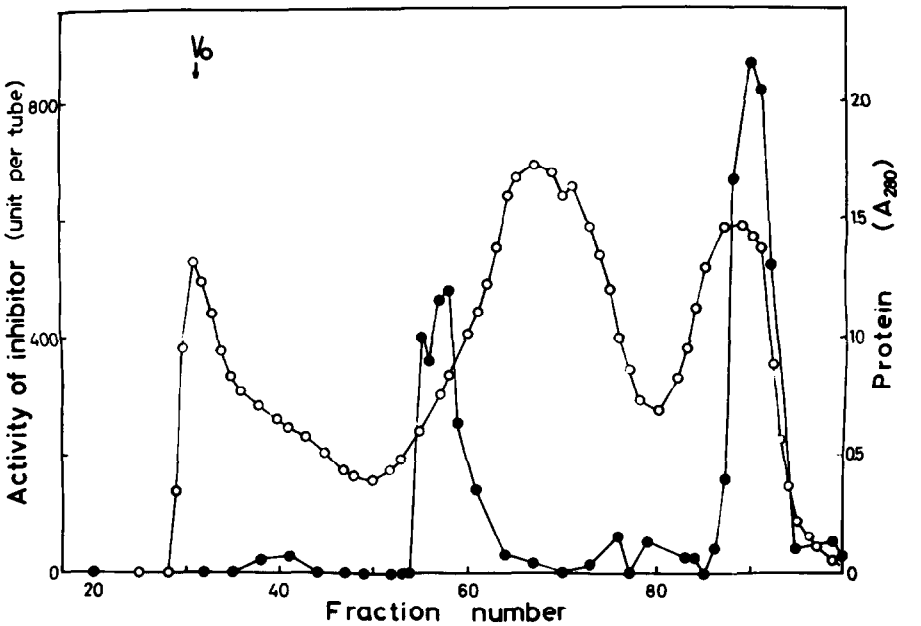

Fig.3. Bio-gel A-0.5 m chromatography of the inhibitor preparation. Boiled extracts from H-1-12 cells were treated with streptomycin and concentrated as described in Materials and methods. The inhibitor preparation (0.8 ml) was applied to a column of Bio-gel A-0.5 m (1.6 × 60 cm) equilibrated with 20 mM Tris buffer (pH 7.4) containing 1 mM EDTA and 0.1 M NaCl. One-milliliter fractions were collected. The column was monitored for absorbance at 280 nm (open circles) and for inhibitor activity (closed circles).

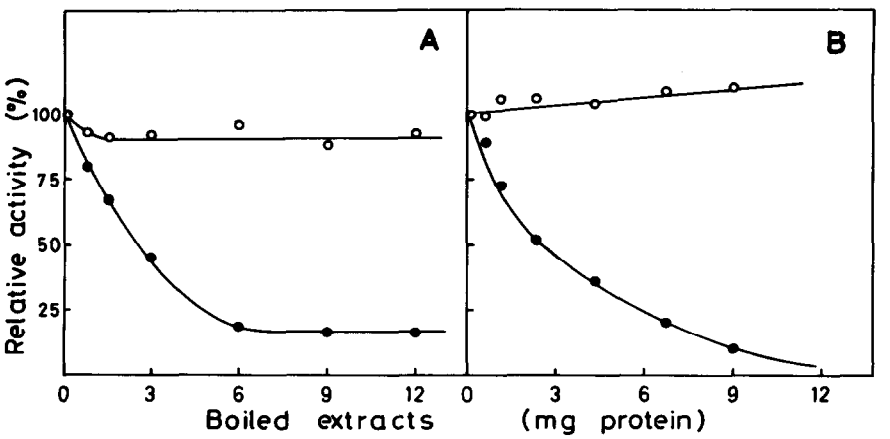

Fig.4. Effect of boiled extracts from H-1-12 cells on the activities of pyruvate dehydrogenase (A) and dihydrolipoyl transacetylase (B). In each case, open circles represent change in the enzyme activity as a function of the amount of boiled extracts added. The change in the activity of PDH complex under the same conditions is also represented as control (closed circles). The enzymes were assayed as described in Materials and methods.

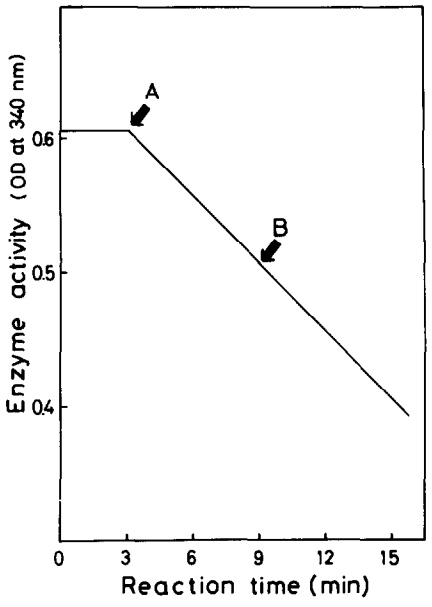

Fig.5. Effect of boiled extracts from H-1-12 cells on the time course reaction of dihydrolipoamide dehydrogenase. Arrows A and B represent the addition of the substrate, DL-lipoamide and that of the boiled extracts (8.8 mg protein), respectively. The amount of the boiled extracts was enough to inhibit the activity of PDH complex. The enzyme activity was determined as described in Materials and methods.

ever, the enzyme inactivation reported in this literature has been considered caused by limited proteolysis and hence the mechanism would be quite different from the PDH complex inhibition in *S. faecalis*.

Acknowledgements

The authors wish to express their sincere gratitude to Professor Saburo Fukui for his continuous encouragement during the course of this investigation. Thanks are also due to Dr Jun-itsu Saito, Fujisawa Pharmaceutical Co., for supplying DL-lipoamide.

References

[1] Reed, L. J. (1974) Acc. Chem. Res. 7, 40–46.
[2] Wieland, O. H. (1975) FEBS Lett. 52, 44–47.
[3] Yamazaki, A., Watanabe, K., Nishimura, Y. and Kamihara, T. (1976) FEBS Lett. 64, 364–368.
[4] Kamihara, T. (1969) Arch. Biochem. Biophys. 133, 137–143.
[5] Leach, F. R., Yasunobu, K. and Reed, L. J. (1955) Biochim. Biophys. Acta 18, 297–298.
[6] Reed, L. J. and Williams, C. R. (1966) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O. eds) Vol. 9, 247–265, Academic Press, N.Y.
[7] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[8] Reed, L. J., Koike, M., Levitch, M. E. and Leach, F. R. (1958) J. Biol. Chem. 232, 143–158.
[9] Linn, T. C. (1974) Arch. Biochem. Biophys. 161, 505–514.