COMPLEX FORMATION BETWEEN NADase AND PROTEIN INHIBITOR FROM *MYCOBACTERIUM BUTYRICUM*

N.OGASAWARA, N.SUZUKI, M.YOSHINO and Y.KOTAKE
Department of Biochemistry, School of Medicine, Nagoya University, Nagoya, Japan

Received 9 January 1970

1. Introduction

The importance of naturally occurring protein inhibitors in the control of enzyme activity for regulation of cellular metabolism has been suggested [1] and suitable protein inhibitors have been discovered for a number of enzymes.

The existence of an inhibitor for NADase in cell-free extracts of *Mycobacterium butyricum* was first described by Kern and Natale [2]. This paper is concerned with evidence for the formation of a complex between NADase and its protein inhibitor obtained from *M. butyricum*.

2. Materials and methods

M. butyricum was grown as described by Kern and Natale [2]. NADase was purified from a sonic extract and assayed as described recently [3]. The inhibitor was purified by calcium phosphate gel, ammonium sulfate fractionation, DEAE-cellulose chromatography, Sephadex G-75 gel filtration and hydroxylapatite. A 1,000-fold increase in the specific activity of the inhibitor was achieved. A unit of enzyme is defined as that amount cleaving 1 μmole of NAD per min at 37°C. A unit of inhibitor activity is defined as that amount needed to inhibit 1 unit of enzyme activity. To test the inhibitor activity, the enzyme and inhibitor were preincubated for 20 min before the addition of substrate. To assay a NADase-inhibitor complex, samples were heated for 2 min in boiling water and the resultant NADase activity was measured.

3. Results and discussion

Complex formation between NADase and its protein inhibitor was shown by means of gel filtration on Sephadex G-100. In experiments A and B, respectively, free NADase (4 units) and inhibitor (8 units) were filtered separately on the same column (1.5 × 100 cm) to determine their positions in the effluent (fig. 1). In experiment C, NADase (2 units) was mixed with excess amounts of inhibitor (8 units) and passed through a column identical to that in experiments A and B. In this experiment, the inhibitor peak appeared in the expected position for inhibitor and the NADase peak was missing. However, large amounts of NADase activity were released by heating fractions in boiling water and a new peak appeared in chromatogram. The position of this peak was as expected for an additional compound between NADase and inhibitor.

To clarify the stoichiometry of NADase-inhibitor reaction, the molecular weights of NADase, inhibitor and the complex were determined as 39,000, 26,000 and 60,000, respectively, from the positions on Sephadex G-100 gel filtration [4]. The data indicated that 1 mole of enzyme was bound to 1 mole of inhibitor. The same molecular weight was obtained for the complex irrespective of whether it was prepared with an excess of NADase or an excess of inhibitor, indicating that complexes of higher order were not readily formed. These results indicated that the inhibitor acts through the formation of a stable complex with NADase.

Complex formation was also analyzed by electrophoresis on a cellulose acetate membrane. In these

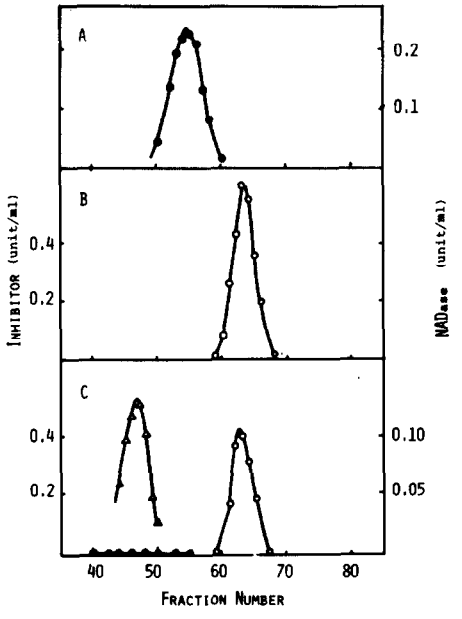

Fig. 1. Sephadex G-100 chromatography of NADase, inhibitor and the mixture containing two proteins. The samples of NADase (A) and protein inhibitor (B) were chromatographed on the column (1.5 X 100) of Sephadex G-100, previously equilibrated with 0.1 M potassium phosphate buffer, pH 7.5 and fractions of 2.5 ml were collected. In experiment C, NADPase was mixed with excess amounts of inhibitor and passed through the column. The fractions obtained were analyzed for NADase activity (–●–), inhibitor activity (–○–) and for the presence of the NADase-inhibitor complex, which was measured on samples of the fractions after heating for 2 min in boiling water (–△–).

experiments, NADase, inhibitor and a mixture of both proteins were added to a series of electrophoresis strips and analyzed in the same electrophoresis run. As seen in fig. 2, the enzyme-inhibitor complex differs in mobility from the native enzyme and the inhibitor; the complex migrates to cathode faster than native NADase, and slower than inhibitor.

The formation of a complex between NADase and inhibitor was also shown by sucrose gradient centrifugation [5]. 0.2 ml Samples were layered onto 4.4 ml of a 3 to 15% linear gradient of sucrose containing 0.05 M potassium phosphate buffer, pH 7.5 and centrifuged for 24 hr at 37,500 rpm in a Hitachi RPS 40 rotor at 8°C. After centrifugation, fractions of approximately 0.2 ml were collected from the bottom

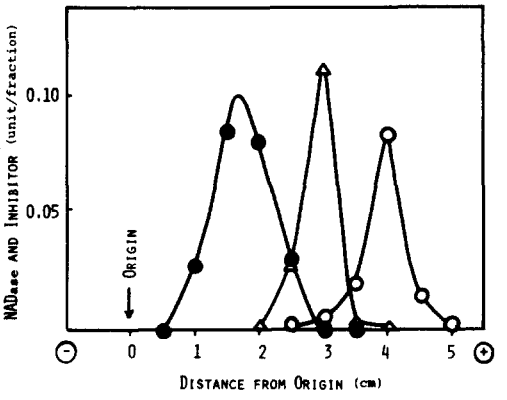

Fig. 2. Electrophoretic analysis of NADase, inhibitor and their complex. Samples approximately 2 μl volume were applied to a series of cellulose acetate strips and electrophoresis was applied at pH 8.6 (Veronel buffer) for 4 hr at 0.5 mA per cm width. After electrophoresis, membranes were cut into sections which were eluted with 0.1 M potassium phosphate buffer, pH 7.5 and assayed for NADase (–●–), inhibitor (–○–) and the complex (–△–) activities.

and analyzed for NADase, inhibitor and the complex. When centrifuged separately, the NADase activity showed an S value of 3.1 and the inhibitor activity a value of 2.3. When NADase and inhibitor were preincubated and centrifuged together under the same conditions, the NADase activity of the complex appeared with an S value of about 3.6.

The results of these experiments suggest that the mechanism of action of the specific protein inhibiting NADase involves a direct interaction between the enzyme and the inhibitor protein. This interaction results in the formation of one type of stable complex only, regardless of the relative proportions of the reacting proteins.

References

[1] M.N.Swartz, N.O.Kaplan and M.E.Frech, Science 123 (1956) 50.
[2] M.Kern and R.Natale, J. Biol. Chem. 231 (1958) 41.
[3] N.Suzuki, J. Vitaminol. (Kyoto) 14 (1968) 121.
[4] D.Andrews, Biochem. J. 91 (1964) 222.
[5] R.G.Martin and B.N.Ames, J. Biol. Chem. 236 (1961) 1372.