POSSIBLE ISOZYME STRUCTURES IN MALATE DEHYDROGENASE FROM BACILLUS SUBTILIS

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Received February 23, 1970

It was reported that NAD dependent malate dehydrogenase (MDH) from crude extracts of Bacillus subtilis, as well as the crystalized enzyme, showed a single band of activity in polyacrylamide gel electrophoresis (Yoshida, 1965). Murphey et al. (1967) and Murphey & Kaplan (1967) have found the same homogeneity of MDH activity in starch gel electrophoresis, but they have reported a heterogeneity of the subunits isolated from MDH tetrametric molecules with respect to their molecular weights.

The present report illustrates the followings: i) MDH in crude extracts from Bacillus subtilis exhibits two isozymes which are different with respect to electrophoretic running, substrate afinity and sensitivity to heat treatment and to 4-chloromercuri benzoate. ii) The existence of two MDH isozymes in Bacillus subtilis, appears to account for the unexplained heterogeneity of the MDH subunits.

Material and Method

Bacillus subtilis WT strain kindly provided by Anagnostopoulos [+)] was used. The origin of the WT strain was explained by Barat et al. (1965).

Fresh culture of bacteria was inoculated in concentration of 10^7 cells/ml in Penassay Broth (Difco Antibiotic Medium No 3) and was incubated with aeration at 37^0 for 16 hours. The cells were harvested by centrifugation and washed once in phosphate buffer

(o. o1 M Na_2HPO_4, o. oo2 M KH_2PO_4. pH 7. 5). The pellet was resuspended in the same buffer (1 g wet pellet in 5 ml), lysozyme (Mann, Res. Lab. N. Y) 2oo/µg/ml was added and the suspension was incubated at 37°C for 1 hour. In the clearer, high viscous suspension, desoxyribonuclease (NBC-Cleveland) 1/µg/ml and ribonuclease (NBC-Cleveland) 10/µg/ml were added to remove the viscosity. After 1O min at 37°C the product was centrifuged with refrigeration (+2°C) at 12, ooo g for 1 hour. The clear yellowish supernatant was used as crude cell free extract.

To test the heat inactivation, aliquots from crude extract were heated at 55°C, 60°C and 70°C for 30 minutes. The sensibility to chloromercuri benzoate was tested by adding 10^{-3} M Sodium 4-chloromercuri benzoate (Fluka AG, Buchs SG) in the same extract. From each aliquot (treated or untreated) samples of 40 µl in electrophoretic analysis were immediately used.

Disc electrophoresis in polyacrylamide gel was carried out as described by Ornstein (1964) and Davis (1964). Soft and separation gels were 3 and 7 % acrylamide respectively. For the separation gel the pH was 9. o and for the soft gel was 6. 8. The buffer was glycine-tris (hydroxymethyl) aminomethane (Merck-Darmstadt-Germany) pH 9. 3. The electrophoretic running was carried out in a cell constructed in this laboratory after that of Langvad (1968 a and b). The running time was 3-4 hours at a current of 5 mA (source Karl Zeiss Jena, Germany).

The MDH were stained by p-nitrotetrazolium blue (Mann. Res. Laboratories N. Y.) using a modified staining mixture (Markert and Apella, 1961) as following: tris (o. o5 M) -HCl buffer pH 7. 5-15 ml, sodium L-malate (N. B. C. Cleveland Ohio) (o. 3 M) pH 7. 5-6 ml, nitrotetrazolium blue (2 mg/ml) 7. o ml, phenasine methosulphate (2 mg/ml) o. 3o ml, NAD (N. B. C. Cleveland Ohio) 2o mg, $MgSO_4$. 6 H_2O (1M) o. 1 ml. The gels were incubated in this mixture at 37°C for 60 min and at 24°C for 30 min. The gel columns were then fixed and stored in 7. 5 % acetic acid (Langvad a, b)

The quantitative measurements of the MDH activities were

carried out by densitometry in a Karl Zeiss type ERI_{10} microdensitometer (Karl Zeiss, Jena, Germany).

Results

In the crude extracts of Bacillus subtilis four bands of MDH activity were found. With respect to their electrophoretic velocity there were two fast bands and two slow bands (fig. 1.1.). The most anodal (thefastest) band had 82-85 per cent from the total MDH activity (fig. 1.1.).

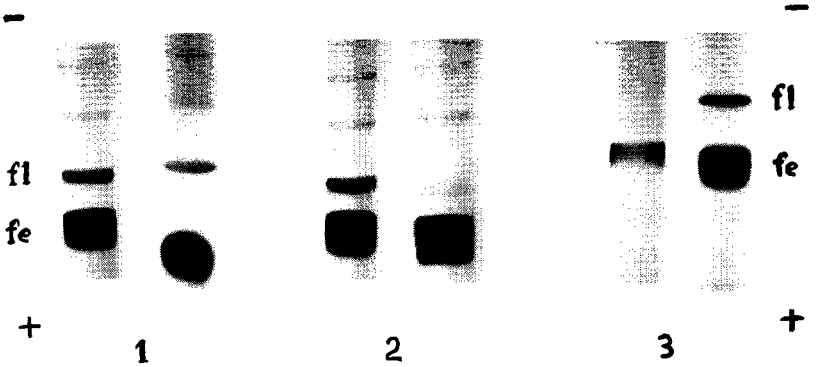

Fig. 1 Different electrophoretic patterns of MDH. The explanations are in the text.

For control, gel columns (in which the electrophoresis of crude extracts were carried out) in a substrate free staining mixture were incubated. This incubation resulted in a poor staining of the two slow bands. The presence of substrate (L-malate) increased their intensity of staining. The fast bands were not stained at all in the control staining mixture.

The two fast bands (fig. 1.1.) were very distinct from one another, exhibiting a distance of running of o.3-o.8 cm. Moreover, between these bands there was a marked difference about the time required for staining: whereas the faster one appeared earlier (fig. 1.3) in the first 10 min. of incubation [fast early (fe) band] the other appeared at 40-50 min of incubation [fast late (fl) band] (fig. 1.3.)

The heat treatment of extracts at 60°C for 30 min. resulted in a more marked inactivation of the fl band (fig. 1, 2) If the fe band was inactivated in a proportion of 10-15 per cent the fl band in a percentage of more than 90 was inactivated. The fe band was completly inactivated at 70°C for 30 min. The 4-chloromercuri benzoate treatment resulted in a complete inactivation of fl band, whereas the fe band was not inactived at all.

Discussion

MDH from crude extracts of Bacillus subtilis was electrophoretically heterogenous, exhibiting a zymogram of two bands of specific activity: fast early (fe) band and fast late (fl) band. The entire pattern, at a longer incubation time in the staining mixture could be visualized.

The two slow bands which appeared later during the incubation period, were not considered MDH isozymes since they gave the nitrotetrazolium blue conversion, in a control staining mixture free of substrate. This suggested that they were nitroblue tetrazolium oxydoreductases. Their more intensive staining in presence of malate was explained by the small MDH residues remained at the level of these reductases, in the running columns.

The difference in the electrophoretic running of the two fe and fl bands of MDH activity, indicates the existence of two MDH isozymes in the crude homogenates of Bacillus subtilis. Moreover, the difference in the substrate afinity (infered from the inequality between the incubation time periods for staining of the two bands), as well as the different sensitivities to heat inactivation and 4-chloromercuri benzoate inactivation, support the concept of the two isozymes pattern for MDH of Bacillus subtilis.

Whereas the single band pattern was in marked contrast with the existence of two types of subunits in MDH tetramers (Murphey et al. 1967), the existence of two isozymes for this enzyme is in agreement with the molecular weight heterogeneity of the subunits

isolated by Murphey et al (1967) from MDH of Bacillus subtilis.

However the two MDH isozymes appear to be encoded by a single gene, because the both isozymes were missing in the MDH defective mutants obtained in this laboratory (Antohi et al. 1970). It would be possible to suggest that MDH gene would encode for the subunit of 31,500 Daltons (Murphey et al. 1967), which can partly be deleted into subunits of 15,000 Daltons as in the case of hexokinases (Kaji 1965, Gazith et al. 1968). In the resulted subunit pool, in which the subunit of 15,OOO Daltons is a minor component (Murphey et al. 1967), two tetrameric forms would be established: the major fe MDH isozyme and the minor fl MDH isozyme.

Acknowledgements

We are indebted to Dr. Langvad (Fibiger Laboratory Lyngby Denmark) for his theretical counselling in disc electrophoresis and to Mrs. Adriana Costake for her technical assistance.

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