Vol. 80, No. 1, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS

January 13, 1978 Pages 276-282

APPLICATION OF AN IMMUNOASSAY TO THE STUDY OF YEAST MALATE DEHYDROGENASE INACTIVATION

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Received November 21, 1977

SUMMARY

In derepressed yeast cells the cytoplasmic malate dehydrogenase activity disappears after addition of glucose to the culture medium. Using specific antisera, it seemed possible to isolate an inactive enzyme protein if the inactivation resulted from an allosteric inhibition or from a chemical modification. The present studies show that after the inactivation an inactive enzyme protein is immunologically not detectable. Together with the irreversibility of the inactivation in vivo and in vitro this result supports a proteolytic mechanism of enzyme inactivation.

In the yeast Saccharomyces cerevisiae two enzymes of gluconeogenesis (fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase (1, 2, 3, 4) and two enzymes of the glyoxylate pathway (isocitrate lyase (9) and cytoplasmic malate dehydrogenase (6, 7, 8, 9) are inactivated if glucose is added to the culture medium. Allosteric control, chemical modification and specific proteolytic degradation have been discussed as possible mechanisms (5, 7, 12). With previous in vivo (8) and in vitro (10, 11) experiments it has not as yet been possible to establish which of the proposed mechanisms is responsible for this phenomenon. Therefore immunological techniques were applied to this problem. Such experiments were possible now since pure malate dehydrogenase isoenzymes are available (12).

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0006-291X/78/0801-0276$01.00/0

METHODS AND MATERIALS

The experiments in this paper were performed with the yeast strain Saccharomyces cerevisiae M_1 (8). The growth conditions, the derepression and inactivation of cytoplasmic malate dehydrogenase, the enzyme assay and the protein determination have been described in a previous paper (10). The preparation of pure cytoplasmic and mitochondrial malate dehydrogenase, the chemical and physical characterisation of these enzymes and the preparation of antisera have recently been published (12). The immunological experiments described here were performed by addition of various amounts of these antisera to pure malate dehydrogenase preparations or to crude cell extracts. After 30 min of agglutination at 25°C and incubation at 4°C over night the enzymatic activity could be pelleted by centrifugation for 5 min at 16 000 x g.

RESULTS

Antiserum specificity: The specificity of the antisera was tested by the diffusion technique of Ouchterlony (not shown) (13) and by titration experiments (Figure 1). Both methods showed that antisera from 9 rabbits to cytoplasmic malate dehydrogenase were specific for this isoenzyme. In contrast the antisera to mitochondrial malate dehydrogenase (5 rabbits) precipitated both isoenzymes. Neither antiserum reacted with pig heart mitochondrial malate dehydrogenase (12).

Immunoassay for an inactive cytoplasmic malate dehydrogenase protein: The inactivation kinetics of cytoplasmic malate dehydrogenase have been shown in previous papers (7, 8, 10). In our experiments derepressed cells were suspended in 0.1 M phosphate buffer pH 6.5 and 1 % solid glucose was added. Omission of a nitrogen source prevented cell growth during inactivation (11). By removing cell samples just before and 20, 40, and 70 min after glucose addition, crude extracts were obtained which contained decreasing amounts of cytoplasmic malate dehydrogenase activity and which were expected to contain increasing amounts of the proposed inactive enzyme. After adjustment of these extracts to the same protein concentration (3 mg/ml)

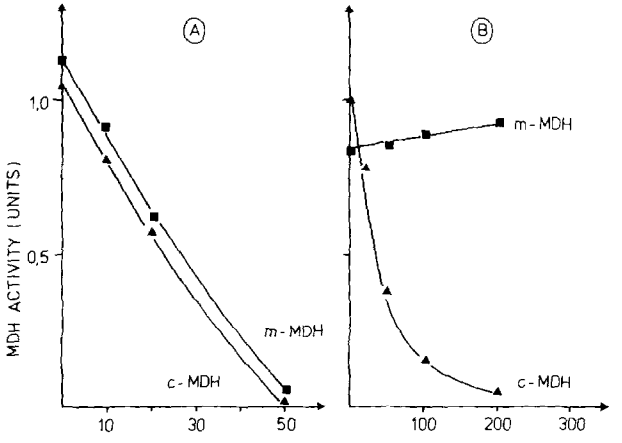

Figure 1. Specificity of the antisera.

To constant amounts of pure cytoplasmic malate dehydrogenase or mitochondrial malate dehydrogenase increasing amounts of antiserum to mitochondrial malate dehydrogenase (A) and cytoplasmic malate dehydrogenase (B) were added. After centrifugation the malate dehydrogenase activity of the supernatants was measured.

all extracts were mixed with a constant amount of antiserum to mitochondrial malate dehydrogenase. The malate dehydrogenase activity precipitated in this step stems from mitochondrial and cytoplasmic malate dehydrogenase. It is given in column 2 of table 1. To the supernatants of these samples 1 unit pure cytoplasmic malate dehydrogenase was added. The activity precipitated in this second step is given in column 3 of the table. From the total activity precipitated (column 4) it is obvious that in extracts containing less cytoplasmic malate dehydrogenase activity less antiserum was absorbed in the first titration. The fact that a similar total activity was precipitated in all the samples indicates, that inactive cytoplasmic malate dehydrogenase protein does not bind antibodies. Because of its broader specificity antiserum to mitochondrial malate dehydrogenase was used in the experiment described here. Almost identical results were obtained with antiserum to cytoplasmic malate dehydrogenase.

Table 1. Complex formation between m- MDH antiserum and in vivo inactivated cytoplasmic malate dehydrogenase.

Crude extracts from	Initial activity (c- and m-MDH) in units / ml	Activity precipitated after addition of 50 µl antiserum	Activity precipitated after addition of 1 U pure c- MDH to aliquots of the supernatants*	Total activity precipitated in both titrations
Derepressed cells	1.13	0.85	0.30	1.15
Cells , 20 min after glucose addition	0.87	0.70	0.41	1.11
Cells, 40 min after glucose addition	0.68	0.60	0.47	1.07
Cells , 70 min after glucose addition	0.40	0.34	0.70	1.04

* all values corrected by a factor which results from the amount of antiserum present in an aliquot of supernatant.

Control experiments: The cytoplasmic malate dehydrogenase is easily labeled with ^{14}C-leucine when glucose grown cells are incubated on a medium containing acetate as the only carbon source. Titration experiments with crude extracts from such labeled cells showed that the amount of radioactivity precipitated was always proportional to the precipitated malate dehydrogenase activity. Additional control experiments were performed with cytoplasmic malate dehydrogenase which was chemically modified by glyceraldehyde-3-phosphate. This metabolite has been shown to inactivate the enzyme irreversibly in vitro and it has been discussed as a physiological effector of malate dehydrogenase inactivation (10). However, if active cytoplasmic malate dehydrogenase was added to samples which already contained glyceraldehyde-3-phosphate inactivated cytoplasmic malate dehydrogenase and increasing amounts of antiserum the resulting titration curve (Figure 2) indicates that the modified enzyme binds normal amounts of antiserum. The same result was obtained with heat

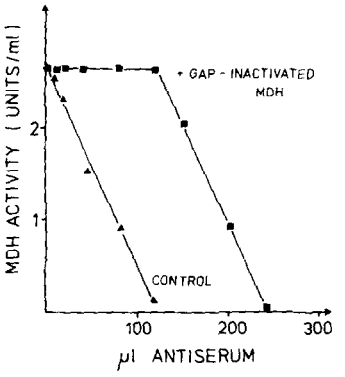

Figure 2. Titration of glyceraldehyde 3-phosphate inactivated cytoplasmic malate dehydrogenase with antiserum.

Increasing amounts of mitochondrial malate dehydrogenase antiserum were added to samples containing 2.7 units of pure cytoplasmic malate dehydrogenase which had been inactivated by addition of 10 mM D, L-glyceraldehyde-3-phosphate. After precipitation and centrifugation 2.7 U of active cytoplasmic malate dehydrogenase were added to the supernatants. The samples were again centrifuged and the resulting supernatants were assayed for malate dehydrogenase(■). Samples without glyceraldehyde-3-phosphate inactivated enzyme served as controls(▲) .

inactivated cytoplasmic malate dehydrogenase (not shown). These results argue against a physiological role of glyceraldehyde-3-phosphate in cytoplasmic malate dehydrogenase inactivation and they show that the physiological reaction effects a more marked structural alteration than does chemical modification or heat denaturation.

DISCUSSION

In order to explain the glucose dependent inactivation of cytoplasmic malate dehydrogenase three molecular mechanisms have been proposed: Allosteric control, covalent modification and specific proteolytic degradation (7). The irreversibility of the reaction (8) is a strong argument against allosteric control or a chemical modi-

fication. These regulatory processes are usually reversible under appropriate culture conditions (14). Aside from the sensitivity of cytoplasmic malate dehydrogenase to proteolytic attack (15, 16) there is as yet no experimental evidence for a proteolytic model.
Purification and characterisation (12) of the two malate dehydrogenase isoenzymes has made possible the immunological studies described here. The experiments showed that an inactive enzyme protein could not be detected either by the specific antiserum to cytoplasmic malate dehydrogenase or by the antiserum to mitochondrial malate dehydrogenase which reacts with both isoenzymes. Since enzyme which was either heat inactivated or inactivated in vitro by glyceraldehyde-3-phosphate forms an antigen-antibody complex as well as the active enzyme molecule, a more drastic alteration of the enzyme protein must occur during the in vivo reaction. This finding presents strong evidence that yeast cytoplasmic malate dehydrogenase "inactivation" is a selective enzyme degradation.

ACKNOWLEDGEMENTS

This work was supported by the "Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 76" and by the "Fonds der chemischen Industrie".

REFERENCES

1. Gancedo, C. (1971) J. Bacteriol., 107, 401 - 405
2. Molano, J. & Gancedo, C. (1974) Eur. J. Biochem. 44, 213 - 217
3. Haarasilta, S. & Oura, E. (1974) Eur. J. Biochem. 52, 1 - 7
4. Gancedo, C. & Schwerzmann, K. (1976) Arch. Microbiol. 109, 221 - 225
5. Witt, I., Kronau, R. & Holzer, H. (1966) Biochim. Biophys. Acta 118, 522 - 537
6. Witt, I., Kronau, R. & Holzer, H. (1966) Biochim. Biophys. Acta 128, 63 - 73
7. Ferguson, J.J., Boll, M. & Holzer, H. (1967) Eur. J. Biochem. 1, 21 - 25
8. Duntze, W., Neumann, D. & Holzer, H. (1968) Eur. J. Biochem. 3, 326 - 331

9. Duntze, W., Neumann, D., Gancedo, J.M., Atzpodien, W. & Holzer, H. (1969) Eur. J. Biochem. 10, 83 - 89
10. Neeff, J. & Mecke, D. (1977) Arch. Microbiol. 115, 55 - 60
11. Neeff, J. (1974) Dissertation, Biol. Fakultät d. Universität Freiburg i.Br.
12. Hägele, E., Neeff, J. and Mecke, D. (1977), Eur. J. Biochem., in press.
13. Ouchterlony, Ö. (1949) Acta Patholog. Microbiol. Scand. 26, 507 - 511
14. Wieland, O., Helmreich, E., Holzer, H., eds. (1971) Second International Symposion on Metabolic Interconversion of Enzymes, Springer-Verlag, Berlin, Heidelberg, New York
15. Neeff, J., Hasilik, A. & Mecke, D. (1974) Abstract of the 9th FEBS-Meeting, Budapest p. 90 s.2.s.5 published by the Hungarian Biochemical society.
16. Jusic, M., Hinze, H. & Holzer, H. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 735 - 740