BIOCHEMICAL DEFICIENCY ASSOCIATED WITH ad3 MUTATIONS IN SACCHAROMYCES CEREVISIAE

II. SEPARATION OF TWO FORMS OF METHYLENETETRAHYDROFOLATE DEHYDROGENASE

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Received February 3, 1970

SUMMARY : Two forms of methylenetetrahydrofolate dehydrogenase, separable by TEAE-cellulose chromatography, are present in wild type extracts of S.cerevisiae, one of which is absent in ad3 mutant extracts.

We showed in the precedent communication that the ad3 mutation is associated with a decrease in the activity of three enzymes related to tetrahydrofolate metabolism. In the present report we studied one enzyme particularly, methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5). The existence of this enzyme was first demonstrated in pigeon liver by Jaenicke (5) and has been studied after partial purification from liver (3, 7), bakers' yeast (8), thymus (11), Escherichia coli (6), Salmonella typhimurium (2) and Streptococcus faecium var. durans A_k (1).

Searching for some differences among crude extracts from ad3 and wild type strains as far as this enzyme is concerned, we were able to show that the apparent K_m for NADP is higher in the mutant than in the wild type. This difference is due to two forms of methylenetetrahydrofolate dehydrogenase present in wild type extracts one of which is absent in ad3 mutants.

MATERIAL AND METHODS

Yeast strains, growth conditions and the preparation of extracts have been described in the preceding paper.

Enzyme assay : Two different assays were carried out, the first based on the spectrophotometric measurement of the reduction of NADP and

the second based on the formation of N^5-N^{10} methenyltetrahydrofolate.

1. The incubation mixture contained : 50 mM potassium phosphate buffer pH 7.5 ; 6.6 mM 2-mercaptoethanol ; 0.2 mM dl-L-tetrahydrofolic acid ; 0.6 mM NADP ; 0.59 mM formaldehyde ; enzyme and water in a total volume of 1.2 ml. The reference cell contained all the reactants except formaldehyde and enzyme. NADP was added to start the reaction and the absorbance change at 340 nm was recorded over a 1 min interval with a Beckman DU spectrophotometer, the cell being maintained at 37°C.

2. The incubation mixture contained all the compounds described above. The reaction was carried out at 37°C for 10 min and 0.12 ml of 1N HCl was added. After 10 min standing the absorbance at 355 nm was measured. A zero time value, was obtained with a mixture acidified directly after the addition of NADP. The reference cell contained all the reactants except NADP.

Chemicals were obtained from the following sources : d,l-L tetrahydrofolic acid from Sigma Chemical Co. or prepared as described by Hatefi et al. (4). TEAE-cellulose (0.495 meq/gm) from Bio-Rad. Laboratories.

RESULTS

K_m in crude extracts.

Fig. 1A shows a Lineweaver-Burk plot for NADP in the crude extracts from ad3 and wild type strains. The K_m values for the two extracts are very different. Five different enzyme preparations have given an average value of K_m = 46×10^{-5} M in the case of ad3 and 7.0×10^{-5}M in the case of wild type. This latter value is of the same order of magnitude as the one found in bakers yeast previously (3×10^{-5}M) (8).

With regard to tetrahydrofolate, the Lineweaver-Burk plot for the two extracts are shown in Fig. 1B and no differences are found. The value we observe (4.5×10^{-5}M) is similar to the one reported previously for the pigeon enzyme (2.7×10^{-5}M) (3).

When NADP was replaced by NAD, FAD, FMN or pyridoxal phosphate no reaction was observed either in the wild type or in the ad3 mutant, in agreement with the result obtained in bakers yeast (8).

TEAE-cellulose chromatography :

The crude extract containing ca. 58 mg of protein in 2.5 ml was applied directly to a TEAE-cellulose comumn (1.5 x 20 cm) freshly equilibrated with 0.01 M potassium phosphate buffer pH 7.5. Elution was car-

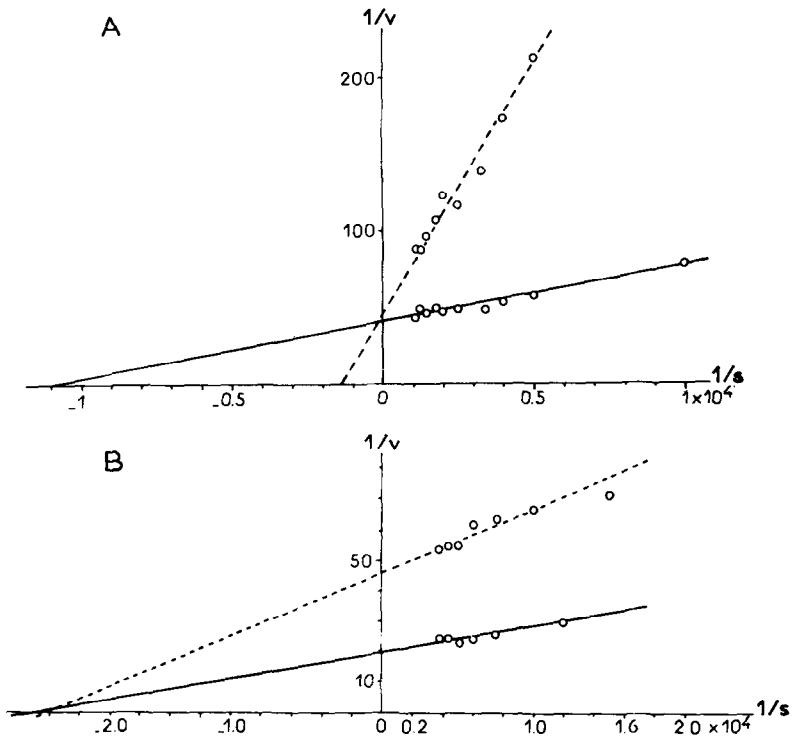

Fig. 1 : Lineweaver-Burk plot of initial velocity vs substrate concentration

full line : ωt dashed line : ad3-10

A : standard assay conditions with constant 0.2 mM d,l-L-THF and variable NADP concentrations.

B : standard assay conditions with constant 0.6 mM NADP and variable d,l-L-THF concentrations.

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ried out by a first gradient : 100 ml of 0.01 M potassium phosphate pH 7.5 in the mixing vessel and 100 ml of 0.1 M phosphate buffer in the reservoir. A second gradient was then applied : 100 ml of 0.1 M phosphate buffer in the mixing vessel and 100 ml of 0.5 M phosphate buffer in the reservoir, 2 ml fractions were collected at a flow rate of 24 ml per hour, and tested for activity.

The elution pattern obtained assaying for the formation of 5.10-methenyltetrahydrofolate is shown in Fig. 2A for wild type and in Fig. 2B for ad3 (The first sharp peak is due to a slight overload of the column, which can be easily avoided). The same pattern (Fig. 3A and 3B) is obtained when fractions are assayed for the reduction of NADP and the gradient is changed to a linear one, with 200 ml of 0.01 M phosphate buffer in the

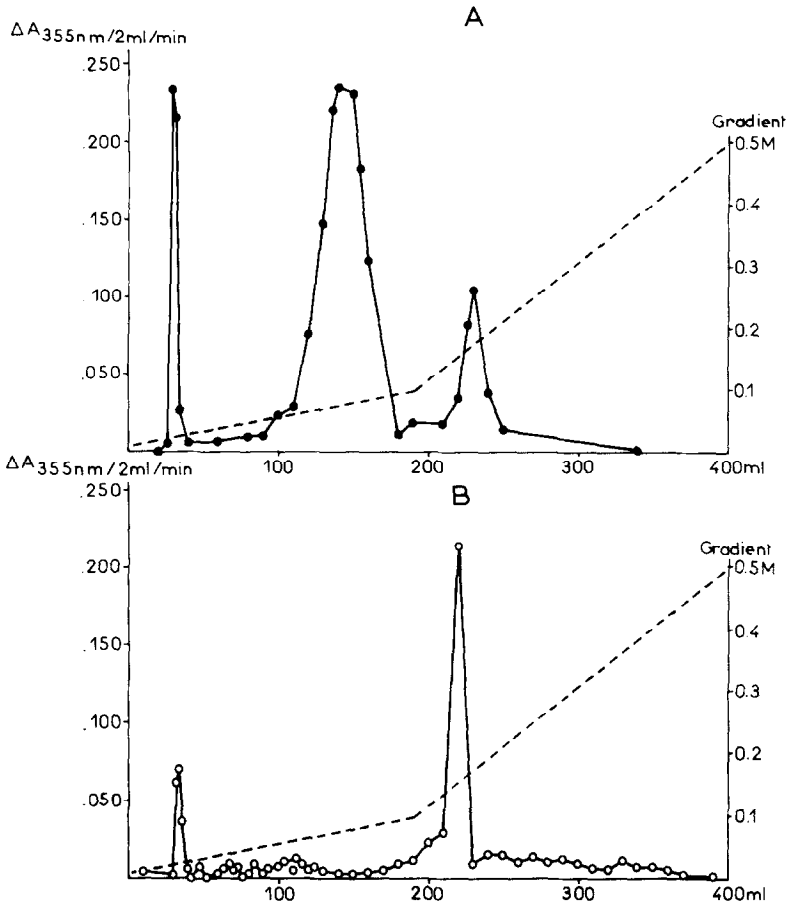

Fig. 2 : Elution pattern of 5,10-methylene-THF dehydrogenase from TEAE-cellulose column. The determination of enzyme activities was based on the formation 5,10-methenyl-THF.
A : wild type strain B : ad3 mutant

mixing vessel and 200 ml of 0.25 M phosphate buffer in the reservoir, and 4 ml fractions collected.

It is clear that methylenetetrahydrofolate dehydrogenase in the wild type is eluted in two peaks : peak I at ca. 0.06 M potassium phosphate, the peak II at ca. 0.16 M. Peak I is totally absent in the elution pattern from the ad3 mutant, while peak II is preserved unchanged in its position. Enzyme activity present in peak II represents ca. 20 % of the total activity recovered from the column in the case of the wild type and more than 90 % of the total activity recovered in the case of the mutant. The specific activity of peak II in the mutant is at least as high, if not higher, than the corresponding one in the wild type.

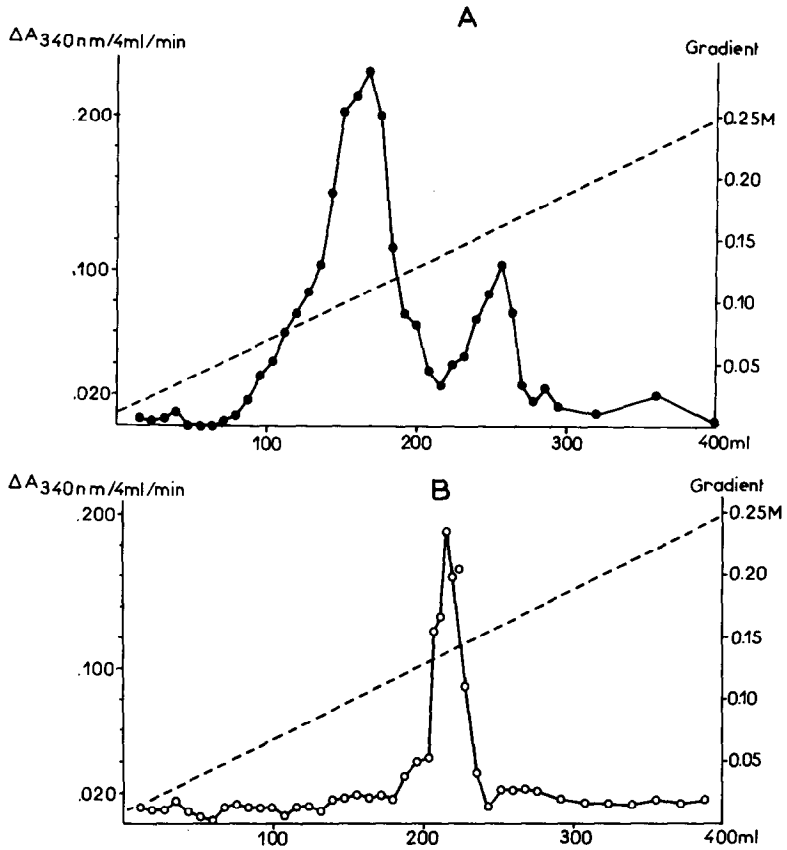

Fig. 3 : Elution pattern of 5,10-methylene-THF dehydrogenase from TEAE-cellulose column. The determination of enzyme activities was based on the formation NADPH.
A : wild type strain B : ad3 mutant

K_m values with regard to NADP have been determined for the peaks in both the wild type and ad3 strains. It was found that peak I has a $K_m = 6 \times 10^{-5}$ M (average of 3 determinations) very similar to that found in the crude extract. This is understandable because peak I represents the most abundant form of methylenetetrahydrofolate dehydrogenase in the wild type. Therefore the measurement of activity in crude extracts corresponds to the titration of activity of form I of the dehydrogenase. The K_m of peak II from ad3 strain has given a value of 56×10^{-5} M (average of 2 determinations), almost identical to the one measured in the crude extract of this strain, which is in agreement with the observation that the II is the only one present in this strain. The K_m of peak II for the wild type has given a value of 16×10^{-5} M (average of 3 determinations). This value

is certainly different from the K_m of form I, but more experiments are needed to establish whether it is significantly different, or not, from the K_m of peak II of the ad3 mutant.

DISCUSSION

We have shown that the wild type of S. cerevisiae synthetises two molecular forms of the methylenetetrahydrofolate dehydrogenase which can be easily separated by TEAE-cellulose chromatography and differ in the K_m for NADP but do not differ in the K_m for dl-L-THF, ad3 mutation causes complete disappearence of the first form, which is the predominant one in the wild type and leaves apparently intact the second form. This explains in a satisfactory way the diminution of the overall activity of the dehydrogenase observed in crude extracts of the mutant. Two directions are now open for futher studies, which are necessary to explain the pleiotropy of ad3 mutations. a) characterisation of the molecular properties of form II of the methylenetetrahydrofolate dehydrogenase and of its physiological role. Of special interest will be a comparison of the properties of form I and II in the wild type and of forms II in the mutant and wild strains ; b) characterisation of the molecular properties (multiple forms and possible aggregates) of enzymes catalysing adjacent steps in the tetrahydrofolate metabolism i.e. methenyltetrahydrofolate cyclohydrolase and formyltetrahydrofolate synthetase, the overall activity of which is strongly diminished albeit present in ad3 mutant.

ACKNOWLEDGEMENTS

We are grateful to Pr. Slonimski P. for discussion and suggestions
This work was supported by a grant from D.G.R.S.T. Biolog. Molec. N° 66.00.168

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