Biochimica et Biophysica Acta, 358 (1974) 14–20

BBA 67252

GLUTAMATE DEHYDROGENASE OF *TETRAHYMENA*

ALAN B. HOOPER, K. R. TERRY and KENNETH D. KEMP
Department of Genetics and Cell Biology, University of Minnesota, St. Paul, Minn. 55101 (U.S.A.)
(Received February 13th, 1974)

SUMMARY

Glutamate dehydrogenase [L-glutamate:NAD(P) oxidoreductase (deaminating), EC 1.4.1.3] located in the mitochondria and able to utilize NAD, NADP, NADH or NADPH as substrate, has been purified 67-fold from *Tetrahymena pyriformis*. The activity with the four pyridine nucleotide substrates was catalyzed by a single enzyme as indicated by the constant association of the activites during purification and the coincidence of migration in polyacrylamide gel electrophoresis. Activity with NAD or NADH was stimulated by ADP and inhibited by GTP, Zn^{2+}, α-ketoglutarate, NADPH and NADH. NADH oxidation was inhibited by NH_4Cl or NaCl. Stimulation with ADP was strongly pH-dependent. Activity with NADPH was unaffected by ADP, GTP or Zn^{2+} but was inhibited by NADPH and high concentrations of NH_4Cl or NaCl. The NADH- or NADPH-utilizing activity per mg protein was unaffected by a treatment of cells with chloramphenicol which caused a decrease in the specific activity of succinate dehydrogenase. The specific activity of NADH- and NADPH-utilizing activities increased coordinately by a factor of 2.7 during culture growth.

INTRODUCTION

Glutamate dehydrogenase [L-glutamate:NAD(P) oxidoreductase (deaminating) EC 1.4.1.3] of higher animals is generally able to utilize both NAD(H) and NADP(H) and activity with both substrates is affected by purine nucleotides (often inhibited by GTP and stimulated by ADP [1]). Most bacteria and fungi studied thus far have either an NAD(H)- or NADP(H)-specific glutamate dehydrogenase or two separate enzymes and neither of the two enzymes are affected by purine nucleotides although in some cases an NAD-specific enzyme is affected by adenine and guanine nucleotides [1].

The ciliate protozoan *Tetrahymena pyriformis* is an animal with certain procaryotic characteristics such as the possession of a "bacterial"-type cytochrome *c*-553 [2, 3]. In this work we demonstrate that *Tetrahymena* is an evolutionary intermediate with regard to its glutamate dehydrogenase. *Tetrahymena* is shown to have a single enzyme which utilizes either NAD(H) or NADP(H) in the oxidative deamination of glutamate (or the reductive amination of α-ketoglutarate). The activity with NAD

or NADH is stimulated by ADP and inhibited by GTP or Zn^{2+} whereas the activity with NADPH is unaffected by those factors.

METHODS

Growth of cells

Cells of *Tetrahymena pyriformis* Strain ST (culture obtained from Dr Y. Suyama, University of Pennsylvania, Philadelphia, Pa., U.S.A.) were grown at 28.5 °C to a density of 10^5 cells per ml on 2% proteose-peptone, 0.1% yeast extract, 0.2 μg/ml Sequestrene ferric chelate (Geigy Agricultural Chemicals, Ardsley, N.Y.), sedimented at 3000 × *g* and washed in cold 0.25 M sucrose, 0.15 M KCl, 20 mM Tris, pH 7.4.

Enzyme assay

Glutamate dehydrogenase was routinely assayed spectrophotometrically in a 1-ml volume containing 50 mM potassium phosphate, pH 7.5, 50 mM NH_4Cl, 0.5 mM KCN, 3 mM α-ketoglutarate, and either 80 μM NADH or 200 μM NADPH. In the direction of pyridine nucleotide reduction the reaction mixture consisted of 50 mM potassium phosphate solution, pH 7.5, 12.5 mM glutamate and either 200 μM NAD or 400 μM NADP. When added, ADP was present at 200 μM. These assay conditions gave maximum rates, a linear time course of reaction and enzyme proportionality. NADPH (Type II), NADH (Grade III), NAD (Grade III), NADP (Na salt), ADP (Grade I), GTP (Type II), glutamate and α-ketoglutarate were obtained from Sigma Chemical Co., St. Louis, Mo. Protein was assayed in trichloroacetic acid precipitates by the Lowry procedure as described by Layne [4].

Polyacrylamide gel electrophoresis

Disc electrophoresis was carried out at 4 °C in 7-mm outer diameter glass tubes as described by Davis [5] using 2.4 ml of a 5% separating gel, pH 9.5, and running at 5 mA per tube. A stacking gel was not employed. In the gels protein was detected by Coomassie blue staining [6] and glutamate dehydrogenase activity by enzyme staining with nitro blue tetrazolium as chromogen [7].

RESULTS AND DISCUSSION

Purification of enzyme

Table I summarizes the results of enzyme purification which was carried out at 4 °C, unless otherwise indicated. During the purification procedure NADH and NADPH oxidation was assayed in the presence and absence of ADP. A washed cell pellet weighing 90 g was suspended in 10 vol. of 0.2 M sucrose, 0.18% KCl, 0.01% Triton X-100 solution by one passage of a loose fitting glass–Teflon Potter–Elvehjem homogenizer and the suspension centrifuged at 650 × *g* for 5 min. The resulting pellet of Triton-washed cells was suspended in 4 vol. of 0.5 M sucrose, 0.02 M Tris, 0.05% bovine serum albumin solution, pH 7.4, and homogenized by seven passes with a tight-fitting Potter–Elvehjem homogenizer. The homogenate (Fraction 1) was centrifuged at 48 000 × *g* for 60 min and the resulting 48 000 × *g* particulate fraction (Fraction 2) including the overlying "fluffy layer" partially dried by passing N_2 over the surface of the precipitate for 30 min. The precipitate was then extracted twice by suspension in

TABLE I

PURIFICATION OF GLUTAMATE DEHYDROGENASE OF *TETRAHYMENA*

Fraction	Total activity NADH (− ADP) (units)	Spec. act. NADH (− ADP) (units/mg protein)	Ratio NADH (+ ADP): NADPH	Ratio NADH (+ ADP): NADH (− ADP)
1. Homogenate	6200	1.8	6.7	2.0
2. 48 000 × *g* particulate	6100	2.3	6.6	2.2
3. Extract I of acetone powder	4800	7.8	7.5	1.8
4. Extract II of acetone powder	940	6.2	6.6	1.9
5. Protamine sulfate supernatant	4100	13	8.0	2.1
6. $(NH_4)_2SO_4$ precipitate I	2900	17	9.5	2.0
7. $(NH_4)_2SO_4$ precipitate II	2200	38	5.3	1.9
8. Dialyzed Fraction 7	2700	85	12	1.4
9. DEAE eluate	1000	120	12	2.0

10 vol. of acetone at −10 °C and filtration of the slurry. To form the acetone powder, the filter cake was dried at room temperature until the odor of acetone was replaced by a strong sulphurous odor. The acetone powder was then extracted for 2 h with 40 ml of 0.05 M phosphate solution, pH 7.4, the slurry centrifuged for 10 min at 20 000 × *g*, and the resulting supernatant designated Extract I of the acetone powder (Fraction 3). To form the second extract of the acetone powder (Fraction 4), the precipitate was stirred in 160 ml of 0.05 M phosphate solution, pH 7.4, for 8 h, centrifuged for 60 min at 20 000 × *g*, and the precipitate discarded. The specific activity of Fraction 4 was often approximately equal to that of Fraction 3 so that Fractions 3 and 4 could thus be combined, although that was not done in this instance. Fraction 3 was next adjusted to pH 7.2 with 10% acetic acid and the protein concentration estimated by the absorbance at 280 nm [4]. A volume of aqueous 20 mg/ml protamine sulphate, pH 7.2, was then added to Fraction 3 to a final ratio of protein : protamine sulphate of 50. The slurry was stirred for 2.5 h and centrifuged for 10 min at 20 000 × *g*. The pH of the resulting supernatant (Fraction 5) was adjusted to 7.4 and solid $(NH_4)_2SO_4$ added in seven successive steps to bring the concentration to 35, 42, 52, 59, 66, and 75% saturation. After each addition, the suspension stood for 45 min and was centrifuged for 10 min at 20 000 × *g*. The precipitates sedimenting from solutions which were 52 and 59% saturated with $(NH_4)_2SO_4$ were resuspended in 0.05 M phosphate solution, pH 7.4, and designated Fraction 6. For the second $(NH_4)_2SO_4$ precipitation, solid $(NH_4)_2SO_4$ was added to Fraction 6 until a faint precipitate appeared (approx. 48% saturation). The suspension was allowed to stand for 45 min and centrifuged at 20 000 × *g* for 10 min. The $(NH_4)_2SO_4$ concentration was then increased in successive steps of 4% of saturation and the enzyme activity sedimented from solutions which were approx. 54 and 58% saturated with $(NH_4)_2SO_4$. The second $(NH_4)_2SO_4$ precipitate (Fraction 7) was resuspended in 6 ml of 0.05 M phosphate solution, pH 7.5, and a 5.5-ml volume was dialyzed for 8 h against the same phosphate solution. Dialyzed Fraction 7 was added at a rate of 0.5 ml/min to 1.2 cm × 18 cm chromatography column containing DEAE-cellulose equilibrated with 0.01 M phosphate solution,

pH 7.5. A small amount of inactive material absorbing at 280 nm was eluted with 90 ml of 10 mM phosphate solution and a linear gradient was then employed between 100 ml of 10 mM phosphate, pH 7.5, and 100 ml of 0.2 M phosphate, pH 7.5. Material with 280 nm absorption eluted in a major peak between 60 and 90 mM phosphate with a shoulder of 20% of the peak height extending to 0.2 M phosphate. The enzyme activity was concentrated in the major peak of material absorbing at 280 nm. The active enzyme fractions were combined to give the DEAE eluate (Fraction 9). The specific activity of Fraction 9 was 67 times Fraction 1 with a recovery of 17%.

Because the enzyme lost activity rapidly in dilute solution, active fractions were routinely stored in the presence of 1 mg/ml bovine serum albumin at −10 °C. Fraction 9 was especially unstable. The NADPH activity was more labile to storage, dilution or heating than the NADH activity. The NADH and NADPH activities were inactivated 15% and 40%, respectively, by heating for 10 min at 60 °C and almost completely inactivated at 70 °C.

As shown in Table I, at all steps during purification of the enzyme, the ratio of ADP-stimulated NADH activity : NADPH activity was approximately constant, increasing gradually from 6.7 to 12. Within any one of the nine purification steps such as $(NH_4)_2SO_4$ precipitation or chromatography on DEAE-cellulose, all fractions had the same ratio of NADH : NADPH activity. It thus seems likely that the increase in ratio during purification was due to preferential inactivation of NADPH activity rather than the separation of an NADPH activity from the NADH activity.

Enzyme activity was located in 0.35 M sucrose following centrifugation of a sample of Fraction 7 for 2 h at 100 000 × *g* in a 0.0–2.0-M discontinuous sucrose gradient with steps of 0.2 M sucrose.

Changes with growth conditions

Glutamate dehydrogenase was localized in the mitochondrial fraction of *Tetrahymena* extracts [8, 9]. Inhibition of in vivo mitochondrial protein synthesis with chloramphenicol caused a 50% decrease in the activity per mg protein of succinate dehydrogenase but no change in NADH-dependent glutamate dehydrogenase or malate dehydrogenase activity per mg protein in the mitochondrial fraction [8, 10]. We now report that the ratio of NADH : NADPH-dependent activity also remained constant during chloramphenicol treatment. During growth of cultures of *Tetrahymena* from $4.6 \cdot 10^3$ to $1 \cdot 10^5$ cells per ml the activity of glutamate dehydrogenase per mg cellular protein increased by a factor of 2.7, whereas the ratio of NADH : NADPH-dependent enzyme activity remained constant (7.5–7.0).

Gel electrophoresis

To further substantiate the apparent unity of the NADH- and NADPH-utilizing enzyme, fractions were analyzed by polyacrylamide gel electrophoresis as shown in Fig. 1. Protein staining of gels following electrophoresis of the purified Fraction 9 and a fraction containing the supernatant resulting from centrifugation of purified mitochondria after freezing and thawing three times (soluble mitochondrial proteins), showed that many fewer bands occurred in Fraction 9. When either the mitochondrial supernatant (Gel F) or Fraction 9 (Gel G) were stained for glutamate dehydrogenase activity the pattern closely resembled the protein-stained Fraction 9 (Gel I). The appearance of multiple bands may reflect polymerization of the enzyme

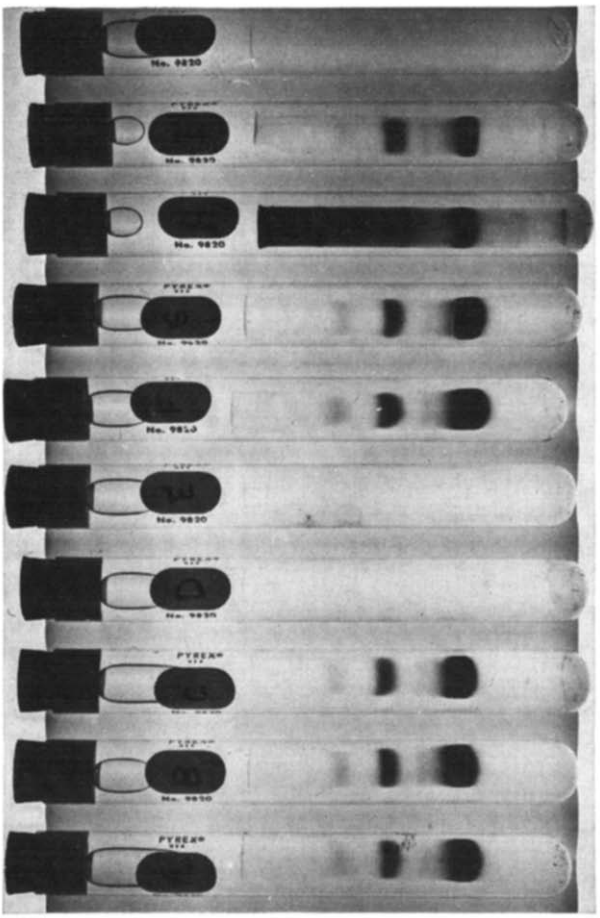

Fig. 1. Polyacrylamide gel electrophoresis of extracts of *Tetrahymena*. A, Fraction 9, enzyme stain, NAD as substrate; B, Fraction 9, enzyme stain, NADP as substrate; C, Fraction 9, enzyme stain, NADP plus NAD as substrate; D, Fraction 9, enzyme stain, NAD as substrate, glutamate omitted; E, Fraction 9, enzyme stain, pyridine nucleotide omitted; F, supernatant resulting from centrifugation of purified mitochondria* after freezing and thawing 3 times, enzyme stain, NAD as substrate; G, Fraction 9, enzyme stain, NAD as substrate (same as gel A); H, supernatant from frozen–thawed mitochondria, protein stain; I, Fraction 9, protein stain; J, blank, enzyme stain, NAD as substrate. (A–J, top to bottom).

[11]. The band pattern of Fraction 9 was the same when enzyme-stained utilizing NAD, NADP or a mixture of both pyridine nucleotides as would be the case if both activities were associated with the same enzyme.

To summarize, the behavior of the NADH- and NADPH-utilizing glutamate dehydrogenase activities during purification and polyacrylamide gel electrophoresis indicated that the two activities were catalyzed by a single enzyme or by two enzymes which were tightly bound to each other.

* The purified mitochondrial preparation consisted of material from Fraction I (Table I) which did not sediment at 1000 × *g* but sedimented at 3000 × *g* and formed a band at the interface between 1.7 M and 1.5 M sucrose when centrifuged at 100 000 × *g* for 1 h.

Preliminary characterization of enzyme

The ratio of maximum measured velocities per mg protein for the four reactions (ADP absent) NADH : NADPH : NAD : NADP was approx. 30 : 10 : 3 : 1. This enzyme was thus able to utilize both NADH and NADPH at significant rates in common with other animal enzymes [1] but shared with the enzyme from *Micrococcus aerogenes* [12] a low rate with NADP.

The concentrations of substrates resulting in half the maximum observed rate of activity were: 25 μM NADH, 0.5 mM α-ketoglutarate, 20 mM NH_4Cl; 0.13 mM NADPH, 0.2 mM α-ketoglutarate, 15 mM NH_4Cl; 60 μM NAD, 1.5 μM glutamate. Because of the low activity, values were not obtained for oxidative deamination utilizing NADP. With NAD as substrate, the enzyme did not oxidize D-glutamate, L-alanine, L-aspartate, L-norvaline, L-valine, β-alanine, or L-methionine at significant rates. Reduction of NAD was 80% inhibited by 6 mM α-ketoglutarate, 0.8 mM NADPH or 80 μM NADH. Oxidation of NADH was 40% inhibited by 0.25 M NH_4Cl and 10% inhibited by 0.25 mM NADH or 10 mM α-ketoglutarate. Oxidation of NADPH was 75% inhibited by 0.7 mM NADPH, 0.25 M NH_4Cl. Both NADPH and NADH oxidation were inhibited by 0.2 M NaCl indicating that inhibition by NH_4Cl was a non-specific effect. Sensitivity to inhibition by NADH or NADPH is a common property of glutamate dehydrogenases from several animal and bacterial sources [1, 13].

Table II shows the effect of ADP, GTP and Zn^{2+} on glutamate dehydrogenase of *Tetrahymena*. Essentially the same result was obtained with fractions at any stage

TABLE II

EFFECT OF NUCLEOTIDES AND Zn^{2+} ON ACTIVITY OF *TETRAHYMENA* GLUTAMATE DEHYDROGENASE

Enzyme activity of the DEAE eluate was assayed as described in Methods.

Substrate	Activity (% control)		
	ADP (100 μM)	GTP (100 μM)	Zn (20 μM)
NADH	350	73	31
NADPH	100	104	90

of the purification. Although NADPH activity was unaffected by ADP, GTP or Zn^{2+}, activity with NADH (or NAD) was stimulated in the presence of 0.1 mM ADP and inhibited by 0.1 mM GTP or 20 mM Zn^{2+}.

Stimulation of the enzyme by ADP had the following kinetic properties: (a) the apparent maximum observable velocity of enzyme activity was increased (2–3-fold) with NH_3, NAD, NADH, glutamate or α-ketoglutarate as substrates; (b) the concentration of NH_3 and glutamate giving half the maximum observed rate of activity was decreased (2–4-fold) in the presence of ADP whereas the corresponding concentrations of NADH, NAD and α-ketoglutarate appeared to be changed very little in the presence of ADP; (c) the pH for maximum enzyme activity was higher in the presence of ADP (pH 7.5 vs pH 8.5) and thus the extent of stimulation by ADP was greater at higher pH values. Variation with pH of the effect of activators or inhibitors has been

observed for glutamate dehydrogenases from other sources such as bovine or *Blastocladiella* [1, 14].

Glutamate dehydrogenase from *Tetrahymena* appears to be the evolutionarily most advanced enzyme found to date among microorganisms. It shares with the enzyme from dogfish, frog, chicken and bovine a dual pyridine nucleotide specificity and NAD-dependent activity which is affected by purine nucleotides [1]. In common with the frog enzyme [15] and in contrast to other animal enzymes, the NADP activity of *Tetrahymena* is unaffected by certain purine nucleotides. Assuming that the NADH-dependent and NADPH-dependent glutamate dehydrogenase activities are catalyzed by a single enzyme which presumably consists of a number of subunits, this enzyme may have as an evolutionary precurser either (a) an unregulated enzyme with dual substrate specificity or (b) a regulated NAD-utilizing enzyme and a separate unregulated NADP-utilizing enzyme. *Thiobacillus novellus* has the latter glutamate dehydrogenase composition [16].

The kinetic and regulatory properties of glutamate dehydrogenases vary greatly between species presumably in keeping with each individual metabolic niche. We add a protozoan enzyme to the list of enzymes studied. Because it is the most primitive enzyme observed thus far with dual pyridine nucleotide specificity but regulation of activity with only NAD and NADH, more detailed studies of its kinetic and regulatory properties should prove interesting.

REFERENCES

1 Goldin, B. R. and Frieden, C. (1971) in Current Topics in Cellular Regulation (Horeker, B. L. and Stadtman, E. R., eds), Vol. 4, pp. 77–117, Academic Press, New York
2 Yamanaka, T., Nagata, Y. and Okunuki, K. (1968) J. Biochem. 63, 753–760
3 Hill, D. L. (1970) The Biochemistry and Physiology of Tetrahymena, Academic Press, New York
4 Layne, E. (1967) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. III, pp. 448–450, Academic Press, New York
5 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
6 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
7 Shaw, C. R. and Prasad, R. (1970) Biochem. Genetics 4, 297-320
8 Mason, T. L. (1971) Ph.D. Thesis, University of Minnesota
9 Porter, P., Blum, J. J. and Elrod, H. (1972) J. Protozool. 19, 375–378
10 Mason, T. L., Hooper, A. and Cunningham, W. (1970) J. Cell. Biol. 47, 130a
11 Josephs, R. and Borisy, G. (1972) J. Mol. Biol. 65, 127–155
12 Kew, O. M. and Woolfolk, C. A. (1970) Biochem, Biophys. Res. Commun. 39, 1126–1133
13 Hooper, A. B., Hanson, J. and Bell, R. (1967) J. Biol. Chem. 242, 288–296
14 LeJohn, H. B. (1968) J. Biol. Chem. 243, 5126–5131
15 Wiggert, B. O. and Cohen, P. P. (1966) J. Biol. Chem. 241, 210–216
16 LeJohn, H. B. and McCrea, B. (1968) J. Bacteriol. 95, 87–94