SHORT COMMUNICATION

Some Properties of the NAD-specific Glutamate Dehydrogenase from *Crithidia fasciculata*

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The NAD-specific glutamate dehydrogenase from *Crithidia fasciculata* has been purified 135-fold. Its molecular weight was about 380000. The apparent K_m values for 2-oxo-glutarate, NADH and NH_4Cl were 3, 0.17 and 47 mM, respectively, at pH 7.8, and for L-glutamate and NAD, 10 and 1.4 mM, respectively, at pH 8.6. The enzyme was strongly inhibited by ATP and GTP, and by thiol-binding reagents, such as 5,5'-dithiobis(2-nitrobenzoate) and *p*-chloromercuribenzoate. Several organic acids, such as pyruvic and L-malic acids, were also inhibitory.

INTRODUCTION

Glutamate dehydrogenase (GDH) is a key enzyme for the metabolism of amino acids in most organisms studied so far. Higher animals have one enzyme, which is able to act with either NAD or NADP as the coenzyme [NAD(P)-GDH, EC 1.4.1.3], whereas plants, fungi and most bacteria have GDHs specific for either NAD or NADP [NAD-GDH, EC 1.4.1.2; NADP-GDH, EC 1.4.1.4] (Smith *et al.*, 1975). Several lower eukaryotes, including *Neurospora crassa* and *Saccharomyces cerevisiae*, have both coenzyme-specific enzymes; in such cases the NAD-GDH usually fulfils a degradative role, whereas the NADP-GDH is thought to be biosynthetic (Smith *et al.*, 1975).

Relatively little is known about these enzymes in protozoa. *Tetrahymena pyriformis* seems to have only one enzyme, which is coenzyme-unspecific (Hooper *et al.*, 1974). On the other hand, *Plasmodium chabaudi* (Walter *et al.*, 1974) and *Trypanosoma cruzi* (Cazzulo *et al.*, 1977; Juan *et al.*, 1978) have a NADP-GDH, with properties quite distinct from those of the enzyme present in their respective mammalian hosts.

We report in this paper the presence, partial purification and some properties of a NAD-GDH in the insect trypanosomatid *Crithidia fasciculata*; the organism, under our growth conditions, also had a much lower NADP-GDH activity.

METHODS

Maintenance and growth of C. fasciculata. The anopheles isolate (ATCC 11745) was kindly supplied by Dr S. Hutner of the Haskins Laboratories, Pace University, New York, U.S.A. The strain was maintained on agar slants of complex medium (Bacchi *et al.*, 1974), and grown in the same medium without agar; 150 ml cultures were grown in 500 ml flasks shaken in a New Brunswick G-76 Gyrotory Shaker at 28 °C.

Partial purification of the enzyme. When a culture reached a cell density of about 1×10^8 organisms ml^{-1} (early stationary phase; usually 40 to 48 h), the cells were harvested by centrifugation (1000 *g*, 10 min, 4 °C) and then washed three times in 0.25 M-sucrose/5 mM-KCl solution (BS) by centrifugation as above. The final pellet was mixed with glass powder (2.5 g per g wet wt of cells) and the cells were broken by grinding

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in a mortar (5 min, 4 °C); under these conditions over 90 % of the flagellates were broken, as judged from microscopic observation. The resulting paste was suspended in BS (125 mg wet wt ml⁻¹) and centrifuged (800 g, 3 min, 4 °C) to remove the glass powder. The homogenate was then centrifuged (14000 g, 10 min, 4 °C). The pellet was discarded, and the supernatant cell-free extract was brought to 50 % saturation with solid (NH₄)₂SO₄. The precipitate was removed by centrifugation (14000 g, 15 min, 4 °C) and dissolved in 0.8 ml 50 mM-Tris/HCl buffer, pH 7.6, containing 1 mM-EDTA and 0.4 M-KCl. This enzyme solution was loaded on to a column (22 × 1.2 cm) of Sephadex G-200 and eluted with similar Tris buffer (7.2 ml h⁻¹, 10 °C). Fractions (1 ml) were collected and assayed for protein and NAD-GDH activity. The active fractions were pooled, glycerol was added to a final concentration of 20 % (v/v) and then the solution was dialysed (3 h, 10 °C) against 200 vol. 20 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-EDTA and 20 % (v/v) glycerol. The dialysed material was then loaded on to a column (9 × 1 cm) of DEAE-cellulose equilibrated with similar phosphate buffer and stepwise elution was carried out with increasing concentrations (30, 60, 90 mM) of KCl in the same buffer (9 ml h⁻¹, 10 °C); the enzyme was eluted at 0.1 M-KCl. The active DEAE-cellulose fractions were pooled and dialysed (3 h, 10 °C) against 200 vol. 10 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-EDTA and 20 % (v/v) glycerol, and then applied to the top of a small column (0.7 × 1.0 cm) of hydroxylapatite equilibrated with similar phosphate buffer. The column was eluted stepwise with buffer solutions containing increasing concentrations (10, 30 mM) of potassium phosphate (6 ml h⁻¹, 10 °C); the peak of enzyme activity was eluted at 60 mM-potassium phosphate. The fractions with the higher specific activities were pooled and used in subsequent experiments. In a representative preparation, NAD-GDH was purified about 135-fold to a specific activity of 87 μmol NADH oxidized min⁻¹ (mg protein)⁻¹; the yield was low (about 2 %), probably because of the lability of the enzyme after the Sephadex G-200 step. The addition of 20 % glycerol to the buffer solutions afforded sufficient stabilization to allow us to perform the subsequent purification steps.

Assay methods. Protein was estimated throughout the purification procedure by the Coomassie Blue procedure (Spector, 1978), using bovine serum albumin as standard.

NAD-GDH was assayed by measuring the change in absorbance at 340 nm concomitant with the oxidation of NADH, or the reduction of NAD⁺, at 30 °C. Unless stated otherwise, the final reaction mixtures for reductive amination of 2-oxoglutarate contained (μmol ml⁻¹): Tris/HCl buffer, pH 7.6, 50; NH₄Cl, 90; NADH (or NADPH, when the NADP-GDH was assayed), 0.12; 2-oxoglutarate, 3; plus 0.02 ml of enzyme preparation. The reaction mixtures for oxidative deamination of L-glutamate contained (μmol ml⁻¹): Tris/HCl buffer, pH 8.6, 50; L-glutamate, 20; NAD⁺, 1; plus 0.1 ml of enzyme preparation. In both cases, the reaction was started by adding the enzyme and activity was measured after 0.5 min.

Determination of molecular weight. The approximate molecular weight of the NAD-GDH was determined by gel filtration through Sephadex G-200 (Andrews, 1965). The experimental conditions were similar to those used in the Sephadex step of the purification method, except that a 30 × 1.8 cm column was used. The proteins used as markers were citrate synthase (EC 4.1.3.7; Sigma; 0.02 mg; mol. wt 100000), lactate dehydrogenase (EC 1.1.1.27; Sigma; 0.05 mg; mol. wt 140000), pyruvate kinase (EC 2.7.1.40; Sigma; 0.1 mg; mol. wt 240000) and apoferritin (Sigma; 2 mg; mol. wt 480000). The corresponding elution volumes were 31.4, 28, 22.8 and 19.2 ml; the elution volume of the NAD-GDH [13 mg of the 0 to 50 % (NH₄)₂SO₄ fraction] was 20.4 ml. The void volume of the column was 17.2 ml, as determined with Blue Dextran 2000 (Pharmacia). Apoferritin was assayed in a separate experiment by measuring the absorbance at 280 nm; lactate dehydrogenase was assayed as described by Andrews (1965), citrate synthase as described by Srere (1969) and pyruvate kinase as described by Bücher & Pfeleiderer (1955).

RESULTS AND DISCUSSION

Extracts of the insect trypanosomatid *C. fasciculata* contained two GDH activities, one NAD-linked and one NADP-linked; in a representative experiment, their specific activities were 0.65 μmol NADH and 0.046 μmol NADPH oxidized min⁻¹ (mg protein)⁻¹, respectively. The two GDH activities corresponded to different proteins, since (i) the NAD-GDH/NADP-GDH ratio increased from 14.1 in the cell-free extract, to 191 in the 0 to 50 % (NH₄)₂SO₄ fraction, and to 415 in the Sephadex G-200 eluate; (ii) the two enzyme activities were partially separated by Sephadex G-200, with NADP-GDH having the lower molecular weight.

The molecular weight of the NAD-GDH, determined by gel filtration through Sephadex G-200 (Andrews, 1965), was about 380000. This is lower than that of the NAD-GDH from *N. crassa*, which was about 480000, but higher than the values reported for other GDHs (Smith *et al.*, 1975).

Fig. 1. Inhibition of the NAD-specific glutamate dehydrogenase from *Crithidia fasciculata* by adenine and guanine nucleotides. The enzyme activity was determined in the direction of reductive amination of 2-oxoglutarate as described in Methods, except that the buffer used was 50 mM-Tris/acetate, pH 7.8, and AMP (Δ), ADP (□), ATP (○), GDP (■) or GTP (●) was added at the concentrations indicated. The activity of the control was 12.5 nmol NADH oxidized min⁻¹.

The optimum pH, determined in the presence of 50 mM-Tris/acetate buffers, was about 7.8 for the reductive amination of 2-oxoglutarate, and about 8.6 for the oxidative deamination of L-glutamate. Differences of about 1 pH unit between the optima have been reported for most of the other GDHs that have been studied (Smith *et al.*, 1975; Meredith *et al.*, 1978).

The apparent K_m values for 2-oxoglutarate, NADH, NH_4Cl , NAD and L-glutamate, determined at the respective pH optima, were 3, 0.17, 47, 1.4 and 10 mM, respectively. The concentrations of the fixed substrate(s) were those stated in Methods. All the double-reciprocal plots were linear. These apparent K_m values are of the same order as those reported for the NAD-GDHs from *Fusarium oxysporum* and *N. crassa* (Smith *et al.*, 1975), with the exception of the apparent K_m for NAD, which was about 10-fold higher in the case of the *C. fasciculata* enzyme. The apparent $V_{\max}$ calculated from the plot for 2-oxoglutarate as variable substrate of the reductive amination reaction was about 70-fold greater than the apparent $V_{\max}$ for L-glutamate oxidation, calculated from the plot for L-glutamate.

As shown in Fig. 1, the NAD-GDH from *C. fasciculata*, assayed in the direction of reductive amination, was strongly inhibited by ATP and GTP (I_{50} , concentration of inhibitor for 50% inhibition, about 0.07 mM), less inhibited by ADP and GDP (I_{50} about 0.3 mM) and little affected by AMP (25% inhibition at 1 mM). These effects may have a physiological significance similar to that of the inhibition by GTP and the activation by ADP shown by the mammalian GDH (Smith *et al.*, 1975), reflecting regulation by the energy charge of the cell (Atkinson, 1969). Most coenzyme-specific GDHs seem not to be affected by purine nucleotides, with the exception of the NAD-GDHs from *N. crassa*, inhibited by GMP (Smith *et al.*, 1975), and from *Chlorella sorokiniana*, inhibited by ADP (Meredith *et al.*, 1978).

Several mono- and dicarboxylic acids of metabolic significance were tested for their effect on the activity of the NAD-GDH from *C. fasciculata*, assayed as described in the legend to Fig. 1. The enzyme was inhibited by pyruvate and L-malate (I_{50} about 4.5 and 7.5 mM, respectively), less affected by phosphoenolpyruvate and L-aspartate (38 and 35% inhibition, respectively, at 10 mM) and very little affected by succinate (10% inhibition at 10 mM). L-Malate and succinate also inhibited the NAD(P)-GDH of *Rhodopseudomonas sphaeroides*, but L-aspartate was an activator of the latter enzyme (Engelhardt & Klemme, 1978).

The GDHs obtained from different sources differ considerably in their response to thiol-binding reagents; thus, the mammalian enzyme is little affected, whereas the NAD-

GDH from *N. crassa* (Smith *et al.*, 1975) and the NADP-GDH from *T. cruzi* (Juan *et al.*, 1979) are strongly, although not completely, inhibited by a variety of thiol-binding reagents. The NAD-GDH from *C. fasciculata*, assayed as described in the legend to Fig. 1, behaved similarly, being strongly inhibited by 5,5'-dithiobis(2-nitrobenzoate) (I_{50} about 0.07 mM, 100% inhibition at 0.2 mM) and by *p*-chloromercuribenzoate, which reached a maximal inhibition of about 65 % at 0.2 mM, with an I_{50} of about 0.05 mM. The enzyme was less inhibited by *o*-iodobenzoate and *N*-ethylmaleimide; the maximal inhibitions attained at 2 mM were 43 and 35 %, respectively. Iodoacetate and iodoacetamide were considerably less effective; the inhibitions attained at 7.5 mM were 11 and 32 %, respectively.

Our results indicate that *C. fasciculata* is similar to other eukaryotes, such as *N. crassa* and *S. cerevisiae*, in possessing two different, coenzyme specific GDHs, which differ from the coenzyme-unspecific enzyme from mammals. The effects of nucleotides and some intermediates of glycolysis and the Krebs cycle on the activity of the NAD-GDH suggest that this enzyme may be a control point for amino acid metabolism in this flagellate.

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