ALTERNATIVE SUBSTRATES FOR GLUTAMATE DEHYDROGENASES

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SUMMARY

The amide group of glutamine functions as a nitrogen donor in the reactions catalyzed by both the NADP-specific glutamate dehydrogenase of Neurospora crassa and the bovine liver enzyme, but not by the NAD-specific Neurospora enzyme. Asparagine serves as nitrogen source only for the NADP-specific Neurospora dehydrogenase. The optimum for utilization of the amide substrates is at pH 8.4 with both enzymes as contrasted to the value of pH 7.6 to 7.8 obtained with ammonia as the source of nitrogen. The maximal rate of reaction with ammonia is 2.5 times greater than that with glutamine and 10 times greater than that with asparagine at the respective pH optima. Acetamide does not serve as a source of nitrogen for either of these two enzymes.

In recent years, a new group of enzymes, the glutamate synthases (EC 1.4.1.X) have been found to catalyze the transfer of the amide group of glutamine to α-ketoglutarate, resulting in the net formation of 2 molecules of glutamate (1-3). The glutamate synthase of E. coli contains two dissimilar subunits of molecular weights 135,000 and 53,000 (1). The enzyme is an iron-sulfide flavoprotein and also requires NADPH for activity (1). Both the size of the smaller subunit and the specificity of the enzyme for NADPH and α-ketoglutarate suggest possible similarities between this subunit and the glutamate dehydrogenase present in E. coli, which is also specific for NADPH (4). Since many enzymes catalyzing the transfer of the glutamine amide group are also capable of utilizing ammonia as the nitrogen donor (5), we were prompted to investigate the possibility that the glutamate dehydrogenases from

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Neurospora crassa and bovine liver, which normally use ammonia as a nitrogen source, might display an alternative specificity for glutamine. We have found that both bovine liver GDH[1] and the NADP-specific enzyme from Neurospora catalyze the transfer of the amide nitrogen of glutamine to α-ketoglutarate; in addition, the Neurospora enzyme can also utilize asparagine.

MATERIALS AND METHODS

Bovine liver GDH was purchased from Boehringer-Mannheim (GmbH) as a suspension in 50% $(NH_4)_2SO_4$ at pH 7. The NAD- and NADP-dependent GDHs of Neurospora were prepared as described by Veronese et al. (6) and by Blumenthal and Smith (7), respectively. Isophthalic acid and glyoxal were products of Eastman. NADH, NADPH, and α-ketoglutarate were obtained from Calbiochem, L-glutamine and L-asparagine from Sigma, and Nessler's reagent from Harleco Co., Philadelphia, Pa.

The activity of all enzymes used in this study was measured at 30° in a reductive amination assay, unless otherwise specified. For bovine GDH, the assay medium contained 10 mM phosphate, 10 mM Tris, 14 mM α-ketoglutarate, 0.16 mM NADH, and 200 mM glutamine at the desired pH. For the Neurospora GDHs, the medium contained 10 mM phosphate, 93 mM Tris, 7 mM α-ketoglutarate, 200 mM glutamine, and 0.16 mM of the appropriate coenzyme. All reactions were initiated by adding the enzyme.

Enzyme samples were exhaustively dialyzed against 0.05 M Tris-acetate buffer at pH 8.0 prior to assay to remove ammonium sulfate. In the case of GDH(NAD), the dialysate also contained 1.0 mM dithiothreitol to protect the reactive sulfhydryl group (8).

Ammonia concentrations in the test solutions were estimated with Nessler's reagent (9). The absorbance at 460 nm was measured and the ammonia concentration determined by reference to a standard curve.

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[1] The abbreviations used are GDH, glutamate dehydrogenase; GDH(NAD) and GDH(NADP), the NAD- and NADP-dependent glutamate dehydrogenases of Neurospora.

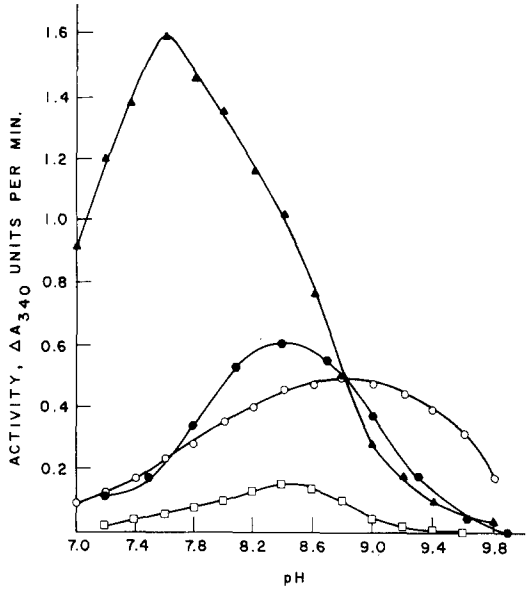

Fig. 1. pH dependence of Neurospora GDH(NADP) for reductive amination using NH_4^+ (▲—▲), glutamine (●—●), or asparagine (□—□) as the source of nitrogen. Substrate solutions are described in "Materials and Methods". For oxidative deamination (○—○) the substrate contained the same buffer, 0.10 M L-glutamate, and 1.0 mM NADP.

RESULTS AND DISCUSSION

The utilization of glutamine as a nitrogen source by Neurospora GDH(NADP) is shown in Fig. 1. At pH values below 7.0, glutamine does not give significant rates of reaction. The optimum is at pH 8.4 in contrast to the value of 7.8 with $(NH_4)_2SO_4$ as the nitrogen source.

The ammonia concentration in all solutions was monitored by reaction of a suitable aliquot with Nessler's reagent (9). In no case did the ammonia concentration exceed 1.0×10^{-5} M, a value at least two orders of magnitude smaller than the known K_m for ammonia (10) and too low to account for the observed rates of reaction. Thus, contamination with ammonia was eliminated as a basis for the observed activity with glutamine.

The possibility that the GDHs were either contaminated with a glutaminase or possessed an inherent glutaminase activity was also investigated. A solution of each enzyme (1.0 mg per ml) in 0.05 M Tris-acetate buffer at pH 8.4

was mixed with an equal volume of 0.1 M glutamine solution and incubated for 18 hours at 30°. No trace of glutamic acid could be detected either by paper electrophoresis at pH 6.5 or by amino acid analysis, and no free ammonia could be detected by the Nessler reaction.

Fig. 2 shows that bovine GDH can use glutamine in place of ammonia as the nitrogen source at pH values as low as 6.4. Inclusion in the reaction mixture of 0.5 mM ADP, an allosteric activator of the enzyme (11), increased the rate of reaction approximately threefold. ADP has about the same qualitative and quantitative effects on bovine GDH when ammonia is used as the source of nitrogen (11). As was observed with Neurospora GDH(NADP) the optimum with glutamine is at pH 8.4 rather than the value of 7.6 observed with ammonia (12).

With the NAD-specific GDH of Neurospora no reaction could be detected with glutamine between pH 7.0 and pH 9.0 (data not shown).

Asparagine as Substrate for the Bovine and Neurospora Enzymes - Fig. 1 shows that asparagine is also used as a nitrogen donor by the Neurospora NADP-dependent enzyme. As with glutamine, there was no detectable reaction

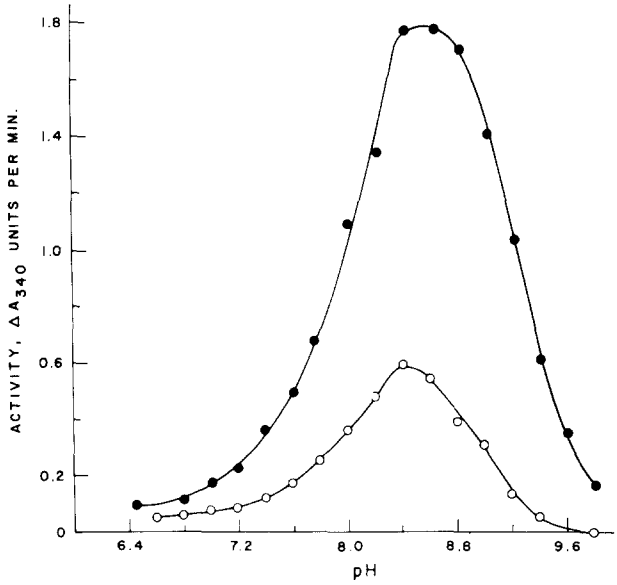

Fig. 2. pH dependence for use of glutamine by bovine GDH in the presence (●—●) or absence (○—○) or 0.5 mM ADP. The substrates are described in "Materials and Methods".

below pH 7.0 and the optimum is at pH 8.4. Asparaginase activity was shown to be absent in the enzyme by experiments similar to those described for glutamine.

Asparagine was not used as a substrate by bovine GDH over the pH range 6.9 to 9.3 and at concentrations of asparagine from 1.0 to 100 mM in the presence and absence of 0.5 mM ADP.

Acetamide in the concentration range 2.0 to 200 mM was not a substrate for either the bovine or the Neurospora enzymes at pH 7.5 or 8.5.

The bovine and Neurospora (NADP) GDHs display a number of similarities. For both enzymes, the optima for reductive amination are at pH 7.6-7.8 with ammonia and at pH 8.4 for the amide substrates. For bovine GDH the optimum for oxidative deamination has been shown to be about pH 8.8 (12); as shown in Fig. 1 this is also the case for Neurospora GDH(NADP). In addition, at the respective pH optima, the maximum velocity of reductive amination with NH_4^+ is approximately 2.6 times that obtainable with glutamine for Neurospora GDH; in the case of the bovine enzyme the reaction is about 2.2 times faster with NH_4^+.

Both isophthalate (13) and glyoxal (14) are inhibitors of bovine GDH, competitive with substrate, and with K_i values of 5.6×10^{-4} M (13) and 2.0×10^{-3} M (14), respectively. We have found that both these compounds are likewise competitive inhibitors of GDH(NADP) with K_i values of 3.6×10^{-4} M for isophthalate and 3.4×10^{-3} M for glyoxal, similar to the values previously obtained for bovine GDH.

Since the pH optima for reductive amination differ by approximately 0.6 pH unit depending on the source of nitrogen, contaminating ammonia is not the nitrogen source in the reactions involving either glutamine or asparagine, in agreement with the failure to detect free ammonia or liberation of ammonia by an amidase activity. Our data suggest, therefore, that direct transfer of the amide group from glutamine (or asparagine) to α-ketoglutarate takes place, and that there is no liberation of ammonia. Alternatively, the amides might be hydrolyzed but with the enzyme retaining bound ammonia at the site of amination.

Of the three enzymes tested, only the NAD-specific enzyme from Neurospora did not utilize glutamine under conditions giving significant rates of reaction with the other two GDHs. This lack of activity with glutamine may be explained by the fact that GDH(NAD) is thought to be primarily involved in the oxidative deamination of glutamate *in vivo* (15). Furthermore, it is noteworthy that among this group of enzymes two, GDH(NADP) and bovine GDH, show considerable sequence homology (16,17), whereas no such homology has been detected thus far for GDH(NAD)[2] which is a tetramer of molecular weight 464,000 (6) in contrast to the smaller hexameric enzymes of bovine liver and Neurospora (18).

Our studies have shown that the structurally homologous GDHs of vertebrates and the Neurospora GDH(NADP) are similar with respect to alternative substrates, competitive inhibitors, and pH dependence. In addition, the fact that the amide group of glutamine can serve as a nitrogen source for these enzymes raises a number of interesting questions concerning possible structural and evolutionary relationships among the small subunit of glutamate synthase (1), the vertebrate GDHs, GDH(NADP) of Neurospora, and the GDH(NADP) of *E. coli*.

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