Rapid Papers

(Pages 711–718)

Reaction of Formiminoglutamate with Liver Glutamate Dehydrogenase

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(Received 22 November 1977)

1. Kinetic aspects of the reaction between crystalline bovine liver glutamate dehydrogenase and formiminoglutamate were investigated to establish the conditions under which the latter may interfere with the assay of glutamate by using glutamate dehydrogenase and to explain why formiminoglutamate accumulates *in vivo* after histidine loading, although it can react with glutamate dehydrogenase. The K_m and V_{max} values were compared with those of the enzyme reacting with glutamate. At pH 7.4 K_m for formiminoglutamate was much higher and V_{max} much lower than the values for glutamate. 2. The equilibrium constant at pH 7.0 was $0.017 \mu M$ with formiminoglutamate, i.e. about one two-hundredths that with glutamate. 3. *In vivo* the interaction between glutamate dehydrogenase and formiminoglutamate is minimal even when the concentration of the latter in the liver is greatly raised, as in cobalamine or folate deficiency after histidine loading. 4. At pH 9.3, i.e. under the conditions for the assay of glutamate by glutamate dehydrogenase, formiminoglutamate reacts readily with the enzyme.

We reported (Krebs *et al.*, 1976) that crystalline bovine liver glutamate dehydrogenase readily reacts with formiminoglutamate under the conditions used for the quantitative determination of glutamate. The comment in the previous paper was limited to the relevance of this observation to the use of glutamate dehydrogenase for determination of glutamate. The present paper reports details of the interaction between glutamate dehydrogenase and formiminoglutamate. The object of the work was to define the conditions under which formiminoglutamate reacts with glutamate dehydrogenase *in vivo* or may interfere with the assay of glutamate by glutamate dehydrogenase, which is carried out at pH 9.3.

Materials and Methods

Bovine liver glutamate dehydrogenase (EC 1.4.1.3) in 50% (v/v) glycerol was obtained from Boehringer Corp. (London), Lewes, E. Sussex, U.K. Glutamate decarboxylase (EC 4.1.1.15) was an acetone-dried powder from *Escherichia coli*, supplied by BDH, Poole, Dorset, U.K. Formiminoglutamate was obtained as the hemi-barium salt from the same company. This was found to be contaminated with about 1.5% glutamate. Barium was removed by precipitation with the calculated amount of Na_2SO_4 , and glutamate was removed by treatment of the stock solution (about 0.2M) with 1 mg of glutamate decarboxylase/ml. Glutamate and formiminoglutamate

were measured as previously described (Krebs *et al.*, 1976). Formamidine acetate was obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K.; per 100 μmol of formamidine a contamination with 9.5 μmol of ammonia was found.

Results and Discussion

K_m and V_{max} of glutamate dehydrogenase for formiminoglutamate

The initial rates of the reaction between glutamate dehydrogenase and glutamate or formiminoglutamate were measured at pH 7.4 under the following conditions: each cuvette contained 1 ml of 0.2M-sodium phosphate buffer, pH 7.4, 0.2 ml of 75.5 mM-NAD⁺ or 0.2 ml of 53.5 mM-NADP⁺, 0.02 ml of 0.1 M-ADP, various amounts of glutamate or formiminoglutamate, and water to give a final volume of 2 ml. With NAD⁺ the initial glutamate concentrations were 0.166, 0.20, 0.25, 0.285, 0.33 and 0.40 mM. The initial formiminoglutamate concentrations were 2.0, 2.5, 3.4 and 6.8 mM. With NADP⁺ the initial glutamate concentrations were 3.00, 2.00, 1.50, 1.00, 0.70 and 0.50 mM and the initial formiminoglutamate concentrations were 4.00, 3.00, 2.50, 2.00 and 1.50 mM. The ionic strength was 0.26. The values for K_m and V_{max} under these conditions are shown in Table 1. The K_m values for formiminoglutamate are about 3 times those for glutamate, and the V_{max} value for formiminoglutamate was about one-fortieth with NAD⁺, but only one-third with NADP⁺. These findings, it should be emphasized, apply to the test conditions. They may vary with the coenzyme con-

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Table 1. K_m and V_{max} of bovine liver glutamate dehydrogenase with formiminoglutamate and glutamate at pH 7.4 and 30°C
The test conditions are described in the text.

Substrate	With NAD ⁺		With NADP ⁺	
	K_m (mM)	V_{max} (μmol of NADH formed/ min per mg of protein)	K_m (mM)	V_{max} (μmol of NADPH formed/ min per mg of protein)
Glutamate	2.2	17.8	2.2	5.4
Formimino- glutamate	7.8	0.4	7.2	1.9

Table 2. Rate of reduction of NAD⁺ in the presence of mixtures of glutamate and formiminoglutamate

The conditions of incubations were as described for the measurements of K_m and V_{max} (phosphate buffer, pH 7.4, 30°C). NAD⁺ was used as the coenzyme. v is the initial linear rate; v_1/v is the ratio of the velocity in the presence of a mixture of glutamate and formiminoglutamate to the rate in the presence of glutamate alone (for the calculation see the text). Each cuvette contained 0.9 ml of 0.3 M-sodium phosphate buffer, pH 7.4, 0.2 ml of 75.5 mM-NAD⁺, 0.02 ml of 0.1 M-ADP, and the substrate concentrations shown in the Table, with water to give a final volume of 2 ml.

Substrates added (mM)		v (μmol/min per mg of protein)	$\frac{v_1}{v}$	
Formimino- glutamate	Glutamate		Measured	Calculated
8	2	6.3	0.66	0.67
8	4	9.1	0.83	0.73
16	4	6.6	0.61	0.64
0	2	9.5	—	—
0	4	10.9	—	—

centrations, K_m rising with falling coenzyme concentrations (Engel & Dalziel, 1970). In view of the high K_m and the relatively low V_{max} value, formiminoglutamate is not likely to react very much *in vivo*, although in the perfused liver the concentration may rise to over 20 mM (Krebs *et al.*, 1976).

Inhibition of glutamate dehydrogenase by formiminoglutamate

When both glutamate and formiminoglutamate were present, the rate of reduction of NAD⁺ or NADP⁺ was lower than with glutamate alone (Table 2). This inhibition of glutamate dehydrogenase by formiminoglutamate is expected to be competitive, and, on the simplifying assumptions that formiminoglutamate is merely an inhibitor that combines with the enzyme without reacting, and therefore treating K_m (formiminoglutamate) as K_i , the degree of inhibition can be calculated on the basis of Michaelis-Menten kinetics by the formula (see Dixon, 1953):

$$\frac{v_1}{v} = \frac{K_m + [S]}{K_m \left(1 + \frac{[I]}{K_i} \right) + [S]}$$

The measured and predicted inhibitions agreed within 10%. The simplifying assumption is a close

approximation with NAD⁺ as cofactor, because V_{max} for formiminoglutamate is only 2% of that for glutamate at pH 7.4.

The reaction between glutamate dehydrogenase and the two substrates was also studied under the conditions of the glutamate determination, i.e. at pH 9.3 with Tris/hydrazine buffer, but much lower enzyme concentrations (5 μg/ml) were used. The concentrations of both substrates were 0.20 mM and the concentration of NAD⁺ was 1.5 mM. Under these conditions the initial rate with glutamate was 0.64 μmol of NADH formed/min per mg of protein. The rate with formiminoglutamate was 0.34 μmol/min per mg. Thus at pH 9.3 the differences in the rate of reaction between the two substrates are much smaller than at pH 7.4.

Products of dehydrogenation of formiminoglutamate

The primary products of the interaction between glutamate dehydrogenase and formiminoglutamate are expected to be α-oxoglutarate and formamidine. The latter would arise as a singly charged cation. The formamidine free base is known to be unstable on account of decomposition to NH₄⁺ and formamide. No detailed information appears to be available on the stability of formamidine in neutral or

very weak alkaline solutions, and measurements of the stability were therefore carried out. These involve the determination of ammonia formed. Since the standard ammonia assay (Kun & Kearney, 1973) is carried out at pH 8, the stability of formamidine was determined at pH 7.4 and 8.0. At pH 7.4 (0.2M-sodium phosphate buffer) 4mM-formamidine formed less than 1% (mol/mol) ammonia in 20h at 37°C. At pH 8.0 in 0.5M-triethanolamine buffer the yield of ammonia was 12% after 20h at 37°C. Since the assay of ammonia is completed within 30min at room temperature (22°C), any breakdown of formamidine during the assay may be neglected. Under these conditions formamide (8mM) proved to be stable at pH 7.4, and to yield 6% ammonia at pH 8.0.

Addition of formamidine (4mM) or formamide (8mM) to rat liver homogenates or sonicated preparations in phosphate buffer did not increase ammonia formation measurably within 1h (pH 7.4; 37°C).

Equilibrium constant of the formiminoglutamate-glutamate dehydrogenase system

In view of the relative stability of formamidine at pH 7.4 it was possible to measure the equilibrium constant of the dehydrogenation of formiminoglutamate by NAD⁺. The formiminoglutamate and NAD⁺ preparations used in this work were of high purity, with no contamination by glutamate or ammonia. Thus, from the known initial concentrations of formiminoglutamate and NAD⁺ and the amount of NADH formed when equilibrium had been reached, the concentrations of all reactants could be calculated. Equal concentrations of formiminoglutamate and NAD⁺ were incubated with glutamate dehydrogenase in a solution containing the following: 1.0ml of 0.2M-potassium phosphate buffer, pH 7.4; 0.1ml of 0.1M-EDTA; 0.02ml of 0.1M-ADP; 0.2ml of 0.01M-NAD⁺; 0.2ml of 0.01M-glutamate or -formiminoglutamate. The final volume was 2.0ml, and the NADH formed was measured at 340nm in a Gilford spectrophotometer. The concentrations of oxoglutarate, formamidine and NADH were taken to be equal, and those of formiminoglutamate and NAD⁺, also equal, were calculated from the initial concentrations of NAD⁺ and the NADH formed.

The equilibrium constant at pH 7.0 as defined by the equation:

$$K_{eq} = \frac{[2\text{-oxoglutarate}][\text{NADH}][\text{formamidine}]}{[\text{formiminoglutamate}][\text{NAD}^+]}$$

was 0.017 μM at pH 7.0 (37°C). The value is about 200 times lower than reported for glutamate under the

same conditions (3.87 μM) (Williamson *et al.*, 1967). This implies that the reaction of glutamate dehydrogenase with formiminoglutamate stops when very little substrate has reacted. This, together with the larger K_m and lower V_{max} , accounts for the relative stability of formiminoglutamate within the cell.

Fate of formiminoglutamate in vivo

The kinetic data (the high K_m , the low reaction rate at pH 7.4 and an equilibrium constant unfavourable for the reaction) indicate that the reaction between glutamate dehydrogenase and formiminoglutamate is normally unimportant *in vivo* because the latter does not normally accumulate sufficiently.

Even in the exceptional situation of cobalamin and folate deficiency, where after histidine loading much formiminoglutamate can accumulate, this is shed by the liver and excreted in the urine (Chanarin, 1969). The reason for the non-decomposition of the formiminoglutamate can be derived from the equilibrium constant and the relative stability of formamidine at pH 7.4. In the mitochondria (the site of glutamate dehydrogenase) the ratio [free NAD⁺]/[free NADH] is about 10 (Williamson *et al.*, 1967) and the oxoglutarate concentration is about 0.12mM (Brosnan *et al.*, 1970). From these data and the equilibrium constant reported above it follows that at equilibrium the reaction between formiminoglutamate and glutamate dehydrogenase comes to a stop when the ratio [formiminoglutamate]/[formamidine] is about 700, in other words when a minute amount of formiminoglutamate has reacted.

This work was supported by grants from the Medical Research Council, U.K., the U.S. Public Health Service (grant no. 5 R01 AM 11748-07 MET). J. V. is Becario de la Institución 'Alfonso el Magnánimo' de la Diputación de Valencia.

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