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FOLATES AS INHIBITORS OF GLUTAMATE DEHYDROGENASE

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Summary

Folates and tetrahydrofolates inhibit beef liver glutamate dehydrogenase (EC 1.4.1.2). Double reciprocal plots indicate a competitive inhibition for α-ketoglutarate-glutamate by folic acid and methotrexate and a complex or mixed type for NAD-NADH site. Pteroic acid is not inhibitory at the concentrations studied. The addition of up to four γ-linked glutamyl residues to folic and tetrahydrofolic acids increases the inhibition. Further chain elongation of the γ-peptide had no effect on the inhibitory activity. The *p*-aminobenzoate poly-γ-glutamates were less inhibitory than the corresponding folyl polyglutamates.

Introduction

Glutamate dehydrogenase (EC 1.4.1.2) is known for its susceptibility to a wide range of effectors which makes it a useful model for studying enzyme regulation. Some years ago Vogel et al. [1,2] reported that folate as well as the folate analogs aminopterin and amethopterin (methotrexate) were inhibitors of glutamate dehydrogenase and yeast alcohol dehydrogenase. They did not report whether the inhibition results from binding at the active site or an allosteric site; nor did they determine the part of the folate molecule (pteridine ring, *p*-aminobenzoyl moiety or terminal glutamate) responsible for the inhibition. Recently the primary sequence of dihydrofolate reductase has been determined and several homologies between it and glutamate dehydrogenase observed [3].

We have used isophthalic azides as photoaffinity labels for the active site of glutamate dehydrogenase, but these substrate analogs bind too weakly to provide extensive labeling of the enzyme (K_i values approx. 10^{-2}) [4]. If the fo-

lates bind more tightly to the enzyme then some carbene or nitrene precursor patterned after these folates could be useful in elucidating the structure and mechanism of this enzyme and provide a means of studying its homology with dihydrofolate reductase as well. With this goal in mind, we have studied the folate structural specificity for inhibition of glutamate dehydrogenase.

Materials and Methods

A glycerol solution of glutamate dehydrogenase Sigma lot No. 83c-1630, NADH 98% Sigma lot No. 34c-6210 and α-ketoglutarate Sigma lot No. 51c-5250 were used without further purification. Folic acid was also purchased from Sigma Chemical Co. and purified by butanol extraction as described by Blakley [5]. Methotrexate was obtained from Dr. Robert Piper of Southern Research Institute. It moved as a single spot on DEAE-cellulose thin-layer chromatography, and its infrared, ultraviolet and NMR were satisfactory. Carbon, hydrogen and nitrogen elemental analysis was consistent with the trihydrate.

Polyglutamates of folic acid and *p*-aminobenzoate were synthesized by methods reported previously [6]. The tetrahydro derivatives were obtained by catalytic hydrogenation [7]. All compounds tested, except methotrexate and folic acid in the K_i determinations, were dissolved initially in approx. 5 M 2-mercaptoethanol in 0.1 M phosphate, pH 7.3, and diluted subsequently to the proper concentrations which were determined spectroscopically using the following molar extinctions. The folates and tetrahydrofolates were stable in these solutions for the duration of the experiment.

	λ_{max} (nm)	$\epsilon_m \times 10^{-3}$	pH
Folic acid and polyglutamates *	341	7.2	7
Tetrahydrofolic acid and polyglutamates	295	29.5	7
p-Aminobenzoyl polyglutamates	275	17.6	11
Methotrexate	370	7.1	13
Pteroic acid	365	8.9	13

* Lengthening the polyglutamyl chain does not alter de ϵ_m at the wavelengths used.

For K_i determinations of folic acid and methotrexate a stock solution of 0.1 M phosphate, pH 7.3, 0.05 M NH_4Cl and 1.2 μg/ml glutamate dehydrogenase was prepared. The inhibitors were dissolved in this solution and concentrations determined spectroscopically. 1 ml of solution was pipetted into a 0.5 cm path length cuvette. For the α-ketoglutarate, plot aliquots of α-ketoglutarate were pipetted from a 0.1 M stock solution (phosphate buffer, pH 7.3). 30 s after α-ketoglutarate was added, the reaction was initiated by addition of 10 μl of 0.011 M NADH. A similar procedure was followed for the NADH plot except that α-ketoglutarate was added to the stock to a final concentration of $5 \cdot 10^{-5}$ M. Reaction was initiated by addition of an aliquot of NADH from the 0.011 M stock.

For determinations of inhibition by the polyglutamyl folates and tetrahydrofolates a stock solution was prepared containing 0.1 M phosphate, pH 7.3, 0.1 M mercaptoethanol, 0.05 M NH_4Cl, $1.0 \cdot 10^{-4}$ M α-ketoglutarate and 0.6

μg/ml glutamate dehydrogenase. 0.7 ml of this solution was added to a semi-micro cuvette (1.0 cm path length), inhibitor added, and the reaction initiated by addition of 10 μl of NADH giving a final concentration of $1.3 \cdot 10^{-4}$ M.

All reactions were followed by monitoring change in absorbance at 340 nm.

Results

Lineweaver-Burk plots (Fig. 1) indicate that folic acid and methotrexate are competitive inhibitors of the α-ketoglutarate site with K_i values of $1 \cdot 10^{-4}$ and $3 \cdot 10^{-4}$, respectively. The inhibition is more complicated for the NADH site and essentially we present the data without comment (Fig. 2).

No inhibition was observed with pteroic acid up to $3 \cdot 10^{-4}$ M. Fig. 3 indicates the increase in inhibition observed with chain length for folate and tetrahydrofolate poly-γ-glutamates. Fig. 4 indicates the inhibition produced by *p*-aminobenzoyl polyglutamates.

The lack of inhibition of glutamate dehydrogenase by pteroic acid suggests that folate binding results principally from the glutamate attraction for the glutamate site. The inhibition by the *p*-aminobenzoyl polyglutamates, which lack the pteridine ring, supports this idea. The pteridine ring contributes to secondary binding effects, however, as indicated by the fact that in its presence the inhibitory activity increased (compare Figs. 3 and 4). Furthermore, reduction of the pteridine moiety to the tetrahydro form decreased the inhibition. Since the reduced folates were tested as a racemic mixture of the (+)-L and (—)-L dia-

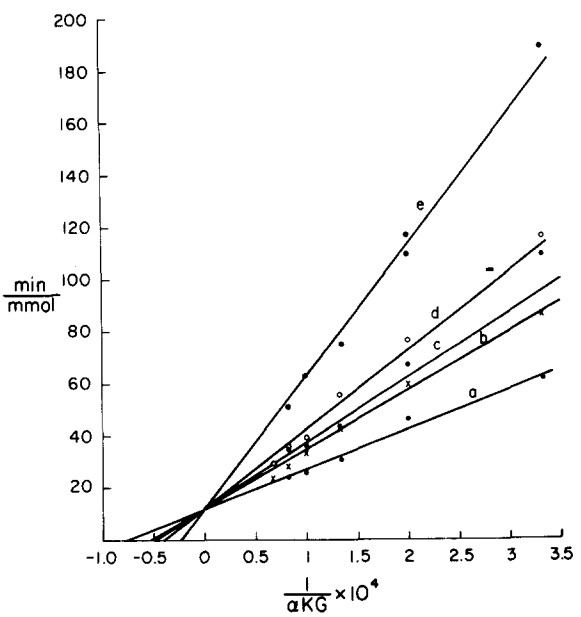

Fig. 1. Double reciprocal plots of initial activity of glutamate dehydrogenase vs. α-ketoglutarate concentrations at different folic acid and methotrexate concentrations. Initial conditions: (a) 0.1 M phosphate, pH 7.3, 0.05 M NH_4Cl, 1.2 μg/ml glutamate dehydrogenase, $1.1 \cdot 10^{-4}$ M NADH; plus: (b) $1.1 \cdot 10^{-4}$ M methotrexate, (c) $2.2 \cdot 10^{-4}$ M methotrexate, (d) $1.0 \cdot 10^{-4}$ M folic acid and (e) $2.01 \cdot 10^{-4}$ M folic acid.

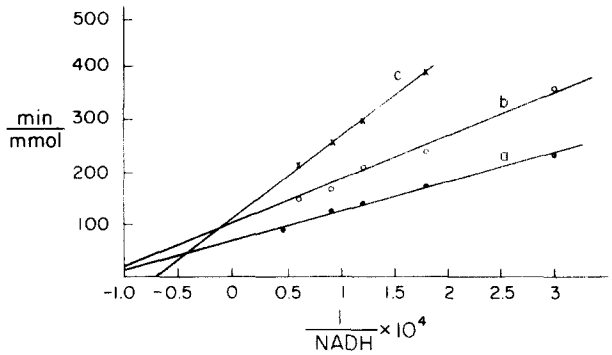

Fig. 2. Double reciprocal plots of initial activity of glutamate dehydrogenase vs. NADH concentration with folic acid and methotrexate inhibitors. Initial conditions: (a) 0.1 M phosphate, pH 7.3, 0.05 M NH_4Cl, 1.2 μg/ml glutamate dehydrogenase, $5.0 \cdot 10^{-5}$ M α-ketogluterate, plus (b) $2.2 \cdot 10^{-4}$ M methotrexate and (c) $2.0 \cdot 10^{-4}$ M folic acid.

steroisomers, the effect of the natural (—)-L form still needs to be investigated. Secondary binding effects attributable to the pteridine ring are also supported by the observation that methotrexate was slightly less inhibitory than folic acid.

The number of γ-glutamates attached to the folyl moiety significantly affected the inhibition (for the tetrahydro series, mono < tri < penta ≈ hepta).

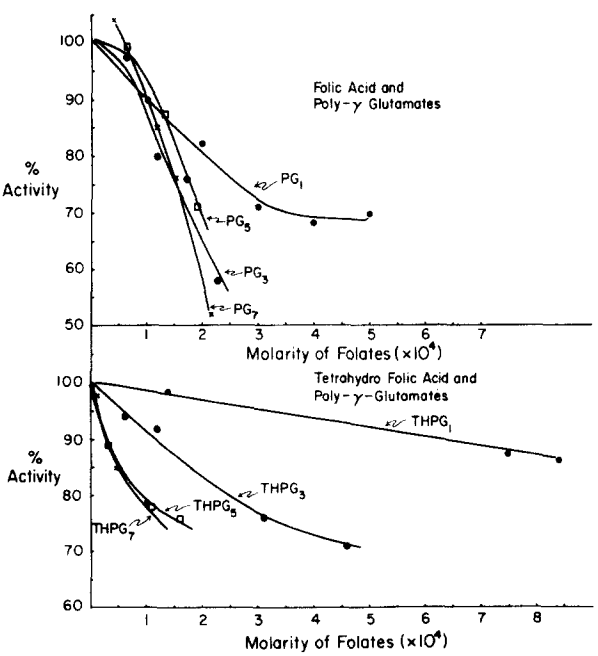

Fig. 3. Percent activity of glutamate dehydrogenase remaining measured as initial rate vs. concentration of selected folates. Initial conditions: 0.1 M phosphate, pH 7.3, 0.1 M mercaptoethanol, 0.6 μg/ml glutamate dehydrogenase, 0.05 M NH_4Cl, $1.0 \cdot 10^{-4}$ M α-ketoglutarate and $1.3 \cdot 10^{-4}$ M NADH. PG_1, pteroyl-γ-glutamate (folic); PG_3, pteroyl-tri-γ-glutamate; $THPG_5$, tetrahydropteroyl-penta-γ-glutamate, etc.

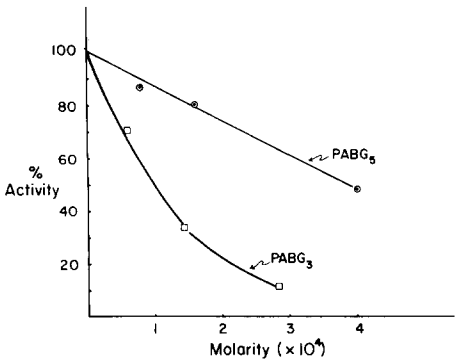

Fig. 4. Percent activity of glutamate dehydrogenase remaining as initial rate vs. concentration of selected *p*-aminobenzoates. Initial conditions: 0.1 M phosphate, pH 7.3, 0.6 μg/ml glutamate dehydrogenase, 0.05 M NH_4Cl, $1.0 \cdot 10^{-4}$ M α-ketoglutarate, and $1.3 \cdot 10^{-4}$ M NADH. $PABG_3$, *p*-amino-benzoyl-tri-γ-glutamate and $PABG_5$, *p*-amino-benzoyl-penta-γ-glutamate.

With the oxidized folyl polyglutamates maximum inhibition was reached with the triglutamate. The reason for the increase in inhibitory activity seen with elongation of the γ-glutamyl chain is not clear at present.

The biological significance, if any, of these findings is unknown. It is interesting that the activity of this important enzyme, which does not require a folate coenzyme, is affected in varying degrees by folyl polyglutamates of different chain lengths. To the best of our knowledge, the only other non-folate-requiring enzyme affected by the presence of folyl-polyglutamates is the cystathionine-γ-synthase of *Neurospora crassa* described by Selhub et al. [8]. This enzyme is activated by N^5-methyltetrahydrofolic acid, the activation increasing with the polyglutamates. The possibility of a regulatory role for the folyl-polyglutamates linking folate-dependent with folate-independent pathways is interesting, although the rather high ligand concentrations required detract from this possibility. Conversely, the interesting possibility must be considered that folate levels could be regulated by enzyme binding since tissue levels of glutamate dehydrogenase exceed those for the various folates. In rat liver mitochondria for example, glutamate dehydrogenase is about 5% of the total protein representing an enzyme site concentration greater than 10^{-4} M [9].

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