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GLUTAMATE SYNTHASE (NADH) FROM LUPIN NODULES

SPECIFICITY OF THE 2-OXOGLUTARATE SITE

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Summary

The binding of substrate analogues including potential alternative substrates, to glutamate synthase (NADH) (L-glutamate: NAD^+ oxidoreductase (transaminating) E.C. 1.4.1.14) has been investigated by studying competitive inhibition with respect to 2-oxoglutarate. Binding requires two terminal carboxyl groups on a C_5 straight chain molecule although some C_4 molecules bind weakly. Bulky substituents at C_2 decrease or prevent binding. Glutarate, the most potent inhibitor, binds much less tightly than the substrate. A 2-oxo group in a molecule other than the substrate does not appear to contribute significantly to binding. None of the analogues was able to act as an alternative substrate.

Assimilation of ammonia from fixed nitrogen in legume nodules has been shown to occur by means of reactions catalysed by the enzymes glutamine synthetase and NADH-dependent glutamate synthase [1—3]. NADH-dependent glutamate synthase catalyses the de novo synthesis of an amino acid, (glutamate), by the reductive amination of the corresponding keto acid. Unusual amino acids of unknown origin, have often been found in legume nodules [4,5], and the possibility was considered that these might arise from the reductive amination by glutamate synthase of keto acids excreted by the

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

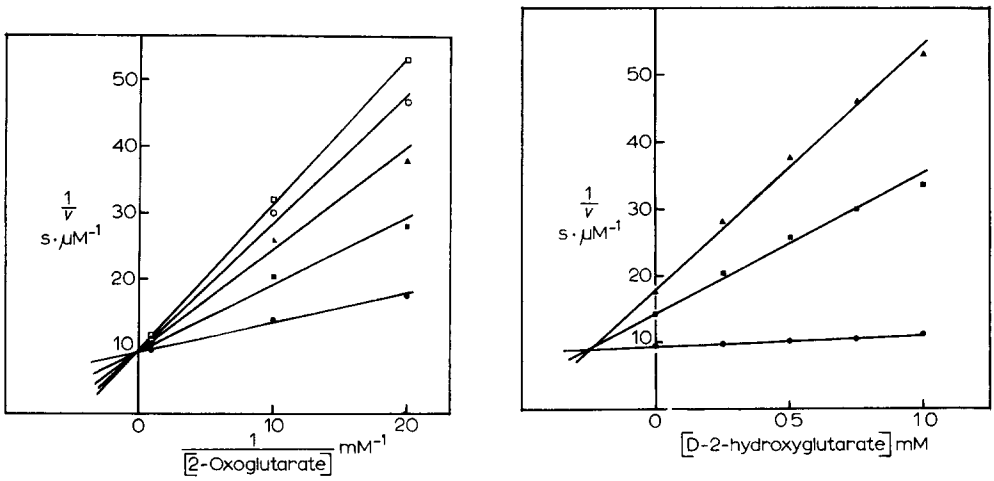

Fig. 1. Double-reciprocal plot showing inhibition by D-2-hydroxyglutarate of glutamate synthase, competitive with respect to 2-oxoglutarate. Reaction conditions are given in the text. Inhibitor concentrations were 0 (●——●), 0.25 mM (■——■), 0.5 mM (▲——▲), 0.75 mM (○——○) and 1 mM (□——□).

Fig. 2. Dixon plot for inhibition of glutamate synthase by D-2-hydroxyglutarate. Reaction conditions are given in the text. Concentrations of 2-oxoglutarate were 1 mM (●——●), 100 μM (■——■) and 50 μM (▲——▲).

bacteroids. In this work we have explored the requirements for binding at the 2-oxoglutarate site by investigating competitive inhibition by substrate analogues, and shown that glutmate synthase has a very high specificity for 2-oxoglutarate and the amination of other keto acids is thus unlikely.

Glutamate synthase was prepared as previously described [6,7] and substrates and substrate analogues were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Initial rate measurements were carried out by observing the rate of disappearance of NADH absorbance as previously described [7], in 50 mM Hepes-KOH buffer, pH 8.5/1% 2-mercaptoethanol.

Reaction mixtures comprised 3 nM enzyme/60 μM NADH/400 μM glutamine and contained varying concentrations of 2-oxoglutarate in the presence of a variety of concentrations of each substrate analogue. Glutamine was maintained at a concentration approximately equal to its K_m value [6,7], so that any inhibition competitive with that substrate would be seen as non-competitive inhibition with respect to 2-oxoglutarate.

Rates were checked for the nature of inhibition using double-reciprocal plots (Fig. 1), and K_i values were determined from plots of reciprocal velocity vs. inhibitor concentration (Fig. 2). Any substrate analogue that gave less than 10% inhibition at a concentration of 10 mM (or 1 mM in the case of 5-glutamyl glutamate) in the presence of 50 μM 2-oxoglutarate was assigned a K_i value of much greater than 10 mM (1 mM).

In Fig. 1, the inhibition shown is clearly competitive. There is no evidence for competition with glutamine, which would give the inhibition a mixed character. The Dixon plot, (Fig. 2) has straight lines, indicating that only one inhibitor molecule is binding.

TABLE I

INHIBITION OF GLUTAMATE SYNTHASE BY C_5 ANALOGUES OF 2-OXOGLUTARATE

$R_1\text{-}\overset{\overset{R_3}{\|}}{C}\text{-}(CH_2)_2\text{-}R_2$

	R_1	R_2	R_3	K_i (mM)
2-Oxoglutarate	COOH	COOH	=O	0.024 *
Glutarate	COOH	COOH	H_2	0.35
D-2-Hydroxyglutarate	COOH	COOH	H, OH	0.28
L-2-Hydroxyglutarate	COOH	COOH	OH, H	0.25
D-Glutamate	COOH	COOH	H, NH_2	0.6
L-Glutamate	COOH	COOH	NH_2, H	0.6 **
2-Methyl-D-L-glutamate	COOH	COOH	NH_2, CH_3	>>10
5-Glutamyl-glutamate	COOH	COOH	H, glutamate	>> 1
Levulinate	CH_3	COOH	=O	>>10
2-Oxovalerate	COOH	CH_3	=O	>>10
D-Norvaline	COOH	CH_3	H, NH_2	>>10
L-Norvaline	COOH	CH_3	NH_2, H	>>10

* K_m for substrate [5].
** cf. 0.7 mM [4].

Structures and K_i values of all substrate analogues tested are given in Tables I and II.

Some clear patterns can be seen. The most potent competitive inhibitors are glutarate and D- and L-2-hydroxyglutarate, with K_i values of 0.25—0.35 mM, while substrates lacking either of the carboxyl groups showed no appreciable inhibition at concentrations of 10 mM. The effect of substituents on C_2 can be gauged by comparing K_i values for glutarate, D- and L-2-hydroxyglutarate, D- and L-glutamate and D,L-2-methyl glutamate. Although a hydroxyl group has no effect on binding, a 2-amino group increases the K_i value about 2-fold, and an additional 2-methyl group increases the K_i value by at least two orders of magnitude. Both D- and L-isomers appear to bind equally well.

5-Glutamyl glutamate was tested as a potential inhibitor in case it might resemble an intermediate in the reaction, but no inhibition was observed at levels up to 1 mM. The C_4 substrate analogues inhibit less strongly: the K_i

TABLE II

INHIBITION OF GLUTAMATE SYNTHASE BY C_4 ANALOGUES OF 2-OXOGLUTARATE

$R_1\text{-}\overset{\overset{R_3}{\|}}{C}\text{-}CH_2\text{-}R_2$

	R_1	R_2	R_3	K_i (mM)
Oxaloacetate	COOH	COOH	=O	5 *
L-Aspartate	COOH	COOH	NH_2, H	2.7 *
L-Asparagine	COOH	$CONH_2$	NH_2, H	14 *
Succinate	COOH	COOH	H_2	>>10
2-Oxobutyrate	COOH	CH_3	=O	>>10 *

* Data from Ref. 4.

value for aspartate is 4-times that for glutamate, and succinate does not appear to inhibit at all. The inhibition by oxaloacetate is of particular interest since it contains the same functional groups as the substrate; however, the K_i value is more than two orders of magnitude greater than the K_m value for 2-oxoglutarate.

The main requirement for binding at the 2-oxoglutarate site is for two carboxyl groups. This is reasonable, since two carboxyl groups are the only functional groups of 2-oxoglutarate not necessarily involved in the reaction. Since the K_m value for 2-oxoglutarate is an order of magnitude smaller than the K_i value of the tightest binding inhibitor, and there is evidence that the dissociation constant is an order of magnitude smaller than the K_m value [7], this 2-oxo group must play an important part in binding. The C_4 analogue, oxaloacetate, and C_5 analogues containing a 2-oxo group but lacking one of the carboxyl groups, show very poor or undetectable binding. It therefore seems likely that the tight binding of 2-oxoglutarate occurs not as a function of binding of the 2-oxo group itself, but rather as a result of some change triggered by that group in the presence of the two carboxyl groups, either a coupled partial reaction or a conformational change in the enzyme.

So far, no inhibitor competitive with glutamine has been found. Since the K_m value for glutamine is at least an order of magnitude higher than that for 2-oxoglutarate, it seems likely that K_i values for inhibitors would be similarly higher and thus, inhibition would not be seen unless concentrations were considerably higher than those used in this work.

None of the 2-oxo substrate analogues showed appreciable reaction as a substitute for 2-oxoglutarate; the specificity for this substrate appears to be absolute. In view of this specificity it seems unlikely that any amino acid other than glutamate is synthesized in a reaction catalysed by NADH-dependent glutamate synthase.

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