*PHOSPHORYLATION OF METHIONINE SULFOXIMINE BY GLUTAMINE SYNTHETASE**

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Studies in this laboratory on the activity of ovine brain glutamine synthetase toward a variety of methyl- and hydroxy-derivatives of glutamic acid, β-glutamic acid, and α-aminoadipic acid have led to the conclusion that the L-glutamate molecule is bound to the active site of glutamine synthetase in an extended conformation in which the α-hydrogen atom of the substrate is directed away from the enzyme.[1-7] The data also indicate that the binding site on the enzyme for ammonia is very close to the site of attachment of the γ-carboxyl carbon atom of L-glutamate. (Stereophotographs of models showing the proposed conformation have been published.[3-5, 7]) We have considered these findings in relation to the inhibitory effect of methionine sulfoximine on glutamine synthetase. Methionine sulfoximine can assume a conformation similar to that proposed for L-glutamate and may bind to the enzyme by attaching to both the glutamate and the ammonia binding sites—essentially as a bifunctional reagent. This is schematically indicated in Figure 1 in which possible sites of attachment to the enzyme of glutamate, ammonia, and methionine sulfoximine are indicated by arrows.

Methionine sulfoximine has long been known as a convulsion-producing agent,[8, 9] and it has been known for some time that methionine sulfoximine as well as methionine sulfone and methionine sulfoxide can act as glutamic acid antagonists and as relatively effective inhibitors of glutamine synthetase.[10-13] The possibility that there is a relationship between the striking inhibition of cerebral glutamine synthetase by methionine sulfoximine and the dramatic effect of this amino acid on the central nervous system has inspired a number of interesting speculations and experiments.[9, 14-22] Indeed, it seems highly probable that glutamine synthetase performs a significant function in the central nervous system and therefore that inhibition of this enzyme would be associated with substantial physiological effects.

The present communication is concerned with the mechanism of inhibition of glutamine synthetase by methionine sulfoximine. There appear to be at least two steps in the inhibition of glutamine synthetase by methionine sulfoximine; the first of these is affected by glutamate while the second is not.[23] Thus, incubation of the enzyme with methionine sulfoximine (in the presence of ATP and metal ion) leads to irreversible inhibition; the rate at which such inhibition occurs is decreased by glutamate, but once established, inhibition is not reversed by glutamate. We have previously reported that inhibition is associated with the binding of methionine sulfoximine to the enzyme.[23] We now report that a derivative of methionine sulfoximine is released when the inhibited enzyme is denatured by heating or by treatment with perchloric acid. The new compound

can be converted to methionine sulfoximine by treatment with strong mineral acid at 100° or by incubation with alkaline phosphatase. This paper presents data which show that methionine sulfoximine is phosphorylated in the presence of glutamine synthetase, magnesium (or manganous) ions, and adenosine 5′-triphosphate (ATP). The findings indicate that inhibition of glutamine synthetase by methionine sulfoximine is associated with tight binding of methionine sulfoximine phosphate and adenosine 5′-diphosphate (ADP) to the enzyme.

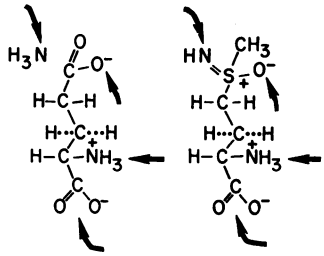

FIG. 1.—Schematic representation of the orientations of glutamate, ammonia, and methionine sulfoximine on the enzyme.

Materials and Methods.—Glutamine synthetase was isolated from sheep brain by a new method which will be described in a subsequent publication.[24] This procedure gives a preparation that is identical to that described earlier[25] with respect to specific activity, electrophoretic behavior, ultracentrifugal criteria,[26] electron microscopic[27] appearance, substrate specificity, and various kinetic parameters. Concentration of enzyme is expressed here in terms of molarity; the enzyme (mol wt, 525,000) exhibits a specific activity of 200 units per milligram of protein using the standard γ-glutamyl hydroxamate synthetase assay.[28]

The calf intestine phosphatase preparation used in these studies was the generous gift of Professor Gerhard Schmidt.[29]

Methyl-labeled C^{14}-L-methionine sulfoximine was prepared from C^{14}-L-methionine (Schwarz BioResearch, Inc.) as described by Bentley *et al.*[30] P^{32}-(β,γ)-ATP was obtained from Schwarz BioResearch, Inc.; this preparation contained more than 99.9 per cent of its radioactivity in the β- and γ-phosphoryl positions as determined by hydrolysis in 1 *N* HCl at 100° (7 min). The labeled ATP preparation contained less than 3 per cent ADP and inorganic phosphate. Unlabeled ATP, ADP, and adenosine 5′-phosphate (AMP) were obtained from Sigma Chemical Corporation. Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Inc.

Paper electrophoresis was carried out on strips of Whatman 3 MM paper (effective length, 42 cm) at 23° and 2400 v with the following buffer systems: pH 2.50: 0.5 *N* acetic acid, 0.4 *N* formic acid; at pH 2.80: 0.5 *N* acetic acid.

Paper chromatography was carried out on Whatman no. 1 paper with the following solvent systems. Solvent (1): 95 per cent ethanol–1 *N* ammonium acetate (pH 5.5), 70:30. The ammonium acetate solution was prepared by dissolving ammonium acetate (38.5 gm) in 400 ml of water and adjusting the pH to 5 by adding glacial acetic acid. After addition of 0.488 gm ethylenediaminetetraacetate (EDTA), the solution was made to 500 ml. (2) Isobutyric acid: water–conc. NH_4OH, 66:33:1. (3) Tert-butanol–concentrated formic acid–water, 70:15:15. The R_F values for methionine sulfoximine phosphate, methionine sulfoximine, and inorganic phosphate are, respectively: solvent (1),

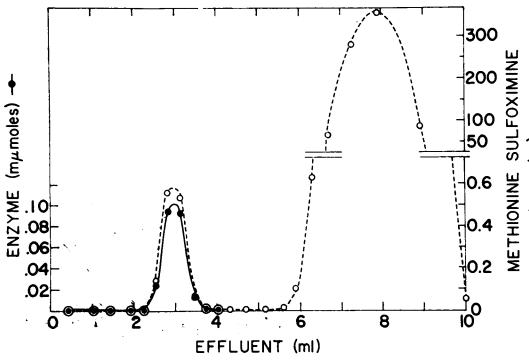

FIG. 2.—Binding of C^{14}-methionine sulfoximine to the enzyme. A mixture (vol, 0.2 ml) containing enzyme (1.24 μM), $MnCl_2$ (0.001 *M*), Na ATP (60 μM), KCl (0.15 *M*), EDTA (0.075 mM), C^{14}-L-methionine sulfoximine (5 mM), and Tris-HCl buffer, pH 7.2 (0.02 *M*) was incubated at 37° for 15 min. Under these conditions the enzyme was completely inhibited. The solution was then added to a column (0.8 × 18 cm) of Sephadex G-50. The column was eluted at 22–24° with a solution containing Tris-HCl buffer, pH 7.2 (0.005 *M*). Fractions of 0.3–0.4 ml were collected at 0° and aliquots were taken for determinations of protein and radioactivity.

0.14, 0.37, and 0.29; solvent (2), 0.26, 0.64, and 0.31; solvent (3), 0.19, 0.29, and 0.47.

Determinations of radioactivity were performed with a Nuclear-Chicago scintillation counter using double isotope technique.

Results.—Earlier studies showed that the irreversible inhibition of glutamine synthetase by methionine sulfoximine is associated with tight binding of this amino acid to the enzyme.[23] A representative experiment demonstrating binding of C^{14}-methyl-L-methionine sulfoximine to glutamine synthetase is described in Figure 2. In this study, the enzyme was incubated with C^{14}-methionine sulfoximine, ATP, and $MnCl_2$. After incubation for 15 minutes at 37°, no enzymatic activity could be detected, and the reaction mixture was placed on a column of Sephadex G-50. The protein-containing peak contained 0.22 mμmole of protein and 1.71 mμmole of C^{14}-methionine sulfoximine, indicating that about 8 moles of inhibitor were bound per mole of inactivated enzyme. A number of experiments of this type have been carried out with similar results; the range of values for the binding of methionine sulfoximine per mole of enzyme in five experiments was 7.4–8.9.

When solutions containing the inhibited enzyme, obtained as described in Figure 2, were treated with perchloric acid or were heated briefly at 100° followed by removal of the protein by centrifugation, virtually all of the radioactivity was found in the supernatant solution. Examination of such solutions by paper chromatography and paper electrophoresis showed that most or all of the radioactivity was associated with a new compound which was not identical with methionine sulfoximine, methionine sulfone, methionine sulfoxide, or methionine. However, after treatment of the new compound with 6 *N* HCl for two hours at 100°, all of the radioactivity could be recovered as methionine sulfoximine.

An experiment similar to that described in Figure 2 was carried out in which C^{14}-methionine sulfoximine and P^{32}-(β,γ)-ATP were employed. The inhibited enzyme was obtained by gel filtration and was found to contain both ^{14}C (equivalent to about 8 moles of C^{14} per mole of enzyme) and P^{32} (equivalent to about

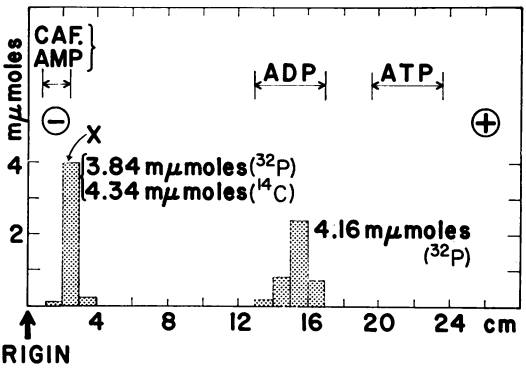

FIG. 3.—Separation of radioactive compounds obtained after denaturation of the inhibited enzyme. A mixture (vol, 0.3 ml) containing enzyme (8.5 μM), C^{14}-L-methionine sulfoximine (8.5 mM; 835 cpm/mμmole), P^{32}-(β,γ)-ATP (1.67 mM; 370 cpm/mμmole phosphate), KCl (0.1 *M*), imidazole–HCl buffer, pH 7.2 (0.01 *M*), EDTA (0.1 mM) was incubated for 30 min at 37°; enzymatic activity was completely inhibited. The solution was added to a column of Sephadex G-50 (0.8 × 18 cm) previously equilibrated with NH_4HCO_3 (pH 8.0; 0.05 *M*) and 0.1 mM EDTA. The column was eluted with the same buffer and the fractions containing the protein (between 2.0 and 4.0 ml) were combined and heated for 3 min at 100°. The denatured protein was removed by centrifugation and the supernatant solution was lyophilized. The residue was dissolved in 0.1 ml of water and aliquots of this solution were subjected to paper electrophoresis at pH 2.50 as described in the text. AMP, ADP, ATP, and caffeine (CAF), 0.25 μmole each, were added as markers to the sample. After electrophoresis, 1-cm sections of the paper strip were cut out for determinations of radioactivity. The only radioactive components found are those shown in the figure.

16 moles of P^{32} per mole of enzyme). After heat denaturation and removal of the protein, all of the radioactivity was found in solution. When this material was subjected to paper electrophoresis at pH 2.5, the results described in Figure 3 were obtained. The new compound (*x*), containing about equivalent amounts of P^{32} and C^{14}, moved slightly ahead of the uncharged marker (caffeine) and a standard of AMP. Under these conditions, methionine sulfoximine, methionine sulfone, and methionine sulfoxide move to the left of the origin (not shown in Fig. 3). In addition, an equimolar amount of P^{32} was found as ADP. No AMP, ATP, or Pi could be detected in this or similar experiments.

When the new compound (*x*) was treated with 6 *N* HCl at 100° for two hours,

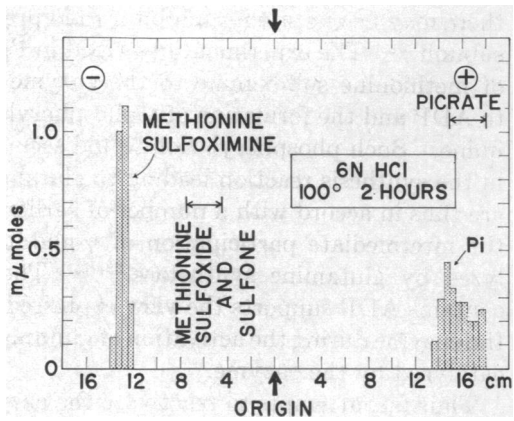

FIG. 4.—Cleavage of the C^{14}-P^{32}-methionine sulfoximine derivative to methionine sulfoximine and inorganic phosphate by 6 *N* HCl. A sample of the compound (*x*) obtained in the experiment described in Fig. 3 was mixed with an equal volume of 12 *N* HCl and placed at 100° for 2 hr. After cooling, the solution was evaporated to dryness and transferred with approximately 0.1 ml of water to a paper electrophoresis strip. Electrophoresis was carried out at pH 2.8 as described in the text. (Carrier methionine sulfoximine and Pi were added.) Within experimental error, all of the radioactivity applied to the paper was recovered as methionine sulfoximine (C^{14}, 2.56 mμmoles) and inorganic phosphate (P^{32}, 2.41 mμmoles).

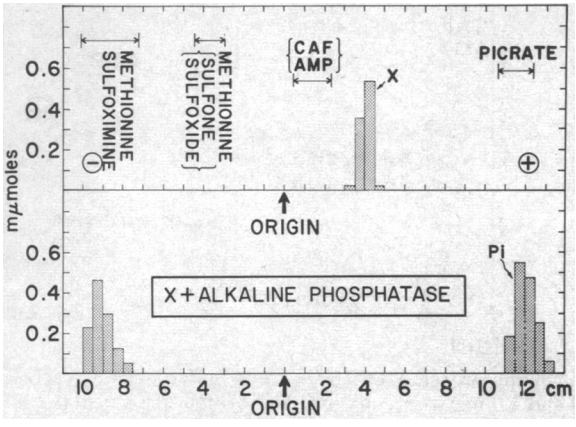

FIG. 5.—Hydrolysis of the C-^{14}P^{32}-methionine sulfoximine derivative (x) by calf intestine alkaline phosphatase. A sample of the compound (x) obtained as described in Fig. 3 was incubated with ethanolamine HCl buffer (pH 9.0; 0.05 M), $MgCl_2$ (0.001 M), and 0.033 mg of calf intestine alkaline phosphatase in a final volume of 0.05 ml for 90 min at 37°. The reaction mixture was then applied to a paper strip and subjected to electrophoersis at pH 2.8 as described in the text. A control experiment was carried out in which alkaline phosphatase was omitted (upper portion of the chart); markers were added as indicated. In the control, equivalent amounts of P^{32} (0.85 mμmole) and C^{14} (0.83 mμmole) were found in the peak labeled x; this represents all of the radioactivity added. After treatment with alkaline phosphatase (lower portion of the chart), only two radioactive compounds were found: C^{14}-methionine sulfoximine (1.14 mμmoles) and P^{32}-inorganic phosphate (1.57 mμmoles).

close to equivalent quantities of methionine sulfoximine (2.56 mμmoles) and inorganic phosphate (2.41 mμmoles) were obtained (Fig. 4).

Figure 5 describes the effect of alkaline phosphatase from calf intestine on the new compound. The upper portion of the chart describes the paper electrophoretic separation of the new compound in a control experiment in which phosphatase was omitted; markers of methionine sulfoximine, methionine sulfone, methionine sulfoxide, caffeine (CAF), picrate, and AMP were added before electrophoresis. After treatment with alkaline phosphatase, the new compound was found to have disappeared completely and equivalent amounts of methionine sulfoximine (C^{14}) and inorganic phosphate (P^{32}) were formed.

Discussion.—The irreversible inhibition of glutamine synthetase by methionine sulfoximine requires ATP and metal ions. Complete inhibition is associated with binding of 8 moles of methionine sulfoximine to the enzyme, suggesting that there may be one site for inhibitor (and probably for glutamate) on each enzyme subunit.[31] The experiment described in Figure 3 demonstrates that the binding of methionine sulfoximine to the enzyme is associated with cleavage of ATP to ADP and the formation of a phosphorylated derivative of methionine sulfoximine. Such phosphorylation would seem to be analogous to that which occurs in the synthesis reaction leading to glutamine formation. The present findings are thus in accord with a number of earlier observations which strongly indicate the intermediate participation of γ-glutamyl phosphate in the reactions catalyzed by glutamine synthetase.[32–35] The finding that the inhibited enzyme contains ADP supports the view expressed earlier that nucleotide is attached to the enzyme during the activation of glutamate and in the course of other reactions catalyzed by the enzyme.

Thus far, attempts to reactivate the enzyme have not succeeded and it would appear, at least under the conditions employed, that the tight binding of phos-

phorylated methionine sulfoximine and nucleotide to the enzyme effectively inhibit catalytic activity. It has been possible to dissociate the inhibitor from the enzyme by procedures that denature the enzyme, suggesting that the linkage between phosphorylated methionine sulfoximine and enzyme is not covalent.

Although the structure of methionine sulfoximine phosphate remains to be established, presently available data render unlikely attachment of phosphate to the α-amino and α-carboxyl groups. Thus, the stability of methionine sulfoximine phosphate is notable; it is not split by prolonged incubation at 37° or by brief heating at 100° at pH 7.5. It is relatively stable at 26° in strong acid solution. Studies on methionine sulfoximine phosphate obtained from the inactivated enzyme and also on a preparation of an apparently identical compound obtained by a chemical synthesis[36] indicate that methionine sulfoximine phosphate is about 50 per cent hydrolyzed when heated at 100° in 1 *N* HCl for 85 minutes. Such stability seems inconsistent with α-N-phosphoryl or carboxyl phosphate derivatives, since such compounds are very susceptible to hydrolysis. If, as discussed above, the imino nitrogen atom of methionine sulfoximine attaches to the ammonia binding site of the enzyme, it seems reasonable to tentatively propose that the phosphate group of methionine sulfoximine phosphate is linked to the sulfoximine oxygen atom, i.e.,

$$[HOOC—CH(NH_2)—CH_2CH_2]\ [HN=]\ [CH_3]\ \overset{+}{S}—OPO_3H^-.$$

The susceptibility of the new compound to the action of alkaline phosphatase would seem to be consistent with this type of linkage. Additional work on the characterization of methionine sulfoximine phosphate is in progress.

The present studies provide a more detailed explanation than has been available previously for the irreversible inhibition of glutamine synthetase by methionine sulfoximine (and possibly also for the physiological action of a convulsion-producing agent). The findings are in accord with the "acyl-phosphate" mechanism of action of glutamine synthetase. The tightness of the binding of methionine sulfoximine phosphate and nucleotide to glutamine synthetase suggests practical approaches to the study of the chemical nature of the active site of this enzyme. The possibility that substrate analogues possessing sulfoximine groups can be of general value in the study of enzymes that catalyze phosphorylation reactions seems worthy of exploration.

Summary.—Ovine brain glutamine synthetase interacts with methionine sulfoximine, ATP, and Mn^{++} (or Mg^{++}) to yield an inhibited enzyme which contains close to 8 moles each of methionine sulfoximine phosphate and ADP. The occurrence of this phosphorylation reaction is in accord with previous findings that indicate formation of enzyme-bound γ-glutamyl phosphate as an intermediate in the reactions catalyzed by glutamine synthetase. The tight binding of methionine sulfoximine phosphate and ADP to the enzyme appears to explain the irreversible inhibition produced by methionine sulfoximine *in vitro* and *in vivo*. Methionine sulfoximine phosphate is released by heat denaturation of the enzyme or by precipitation of the enzyme with perchloric acid. Methionine sulfoximine phosphate is split to methionine sulfoximine and inorganic

phosphate when heated in 6 *N* HCl at 100° for two hours, or when treated at 37° with alkaline phosphatase.

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