Inhibition of Glutamine Synthetase by Methionine Sulfoximine. Studies on Methionine Sulfoximine Phosphate

W. Bruce Rowe, Robert A. Ronzio,† and Alton Meister

ABSTRACT: The irreversible inhibition of glutamine synthetase by methionine sulfoximine is associated with the tight binding to the enzyme of adenosine diphosphate and methionine sulfoximine phosphate; the latter compound can be cleaved nonenzymatically and also by phosphatases to equimolar amounts of methionine sulfoximine and inorganic phosphate. Methionine sulfoximine phosphate is oxidized by L-amino acid oxidase to yield a phosphate-containing product. The chemical synthesis of methionine sulfoximine phosphate was achieved by treating methionine sulfoximine methyl ester with cyanoethyl phosphate and dicyclohexylcarbodimide; the product was isolated from the reaction mixture and crystallized. The enzyme- and chemically synthesized methionine sulfoximine phosphate products are identical as judged by paper chromatography in five solvent systems, paper electrophoresis at pH 2.8, 5.5, 7.5, and 9.0, relative stability at various values of pH, and the rates of cleavage in 1 N HCl at 100° and by phosphatases. Treatment of methionine sulfoximine phosphate with hydrosulfite gave a product exhibiting the properties of methionine sulfoximine phosphate, indicating that the phosphoryl moiety of methionine sulfoximine phosphate is attached to the sulfoximine nitrogen atom. L-Methionine sulfoximine phosphate inhibits glutamine synthetase in the absence of added nucleotide and metal ions; however, L-methionine sulfoximine phosphate inhibits the enzyme much more effectively in the presence of Mg²⁺ and adenosine diphosphate (and to a lesser extent adenosine triphosphate).

Previous studies in this laboratory (Ronzio et al., 1969a) have shown that the irreversible inhibition of glutamine synthetase from sheep brain by L-methionine sulfoximine in the presence of ATP and Mg²⁺ (or Mn²⁺) is associated with the tight binding to the enzyme of close to 8 moles each of methionine sulfoximine phosphate and ADP. It was established that the methionine sulfoximine phosphate derivative obtained from the enzyme (by brief heating at 100° or by treatment with perchloric acid) could be converted by treatment with acid or with several phosphatases into equimolar amounts of methionine sulfoximine and P. The present work, which was undertaken in order to further characterize the enzyme-bound methionine sulfoximine derivative, indicates that the phosphoryl moiety of this compound is attached to the sulfoximine nitrogen atom. The chemical synthesis of L-methionine sulfoximine phosphate has been achieved, and studies on the inhibition of glutamine synthetase by this compound have been carried out.

* From the Department of Biochemistry, Cornell University Medical College, New York, New York 10021. Received February 25, 1969. Supported in part by the National Science Foundation and the National Institute of Health, U. S. Public Health Service.
† Present address: Department of Biochemistry, University of Washington, Seattle, Wash.
Experimental Section

Materials. Glutamine synthetase was isolated from sheep brain as described by Ronzio et al. (1969b). L-Methionine sulfoximine, L-[\textsuperscript{14}C, \textsuperscript{32}P]methionine sulfoximine phosphate (enzyme synthesized), phosphatases, ATP, ADP, and other compounds were obtained and characterized as stated previously (Ronzio et al., 1969a). Methionine sulfoximine was prepared by treatment of methionine with hydrazoic acid (W. B. Rowe and A. Meister, 1969, in preparation). We are indebted to Dr. Daniel Wellner for the crystalline L-amino acid oxidase (Crotalus adamanteus), to Dr. Gerhard Schmidt for the yeast, prostate, and intestine phosphatases, and to Dr. R. T. Simpson and Dr. Bert L. Valle for the Escherichia coli phosphatase. Dicyclohexylcarbodiimide was obtained from Schwarz Bio-Research Inc. 3-Cyanoethyl phosphate was obtained as the barium salt from California Corp. for Biochemical Research. L-Ethionine sulfoximine was prepared as described by Bentley et al. (1951).

Methods. Glutamine synthetase was determined by the \gamma-glutamyl hydroxamate assay method (Wellner and Meister, 1966) using salt-free hydroxylamine. Paper electrophoresis was carried out as previously described (Ronzio et al., 1969a). In the experiments designed to determine ATP formation, 0.025 M sodium citrate buffer (pH 3.7) was used; at this value of pH AMP, ADP, ATP, and P, are separated. Paper chromatography (ascending) was carried out with the following solvents: solvent I, isobutyric acid-water-concentrated NH\textsubscript{4}OH (66:33:1, v/v/v); solvent II, concentrated formic acid-sec-butyl alcohol-water (70:15:15, v/v/v); solvent III, 95\% ethanol-1 N ammonium acetate (pH 5.5) (70:30, v/v); solvent IV, \(\alpha\)-butyl alcohol-acetic acid water (50:25:25, v/v/v); and solvent V, \(r\)-butyl alcohol-methyl ethyl ketone-formic acid-water (40:30:15:15, v/v/v). The RF values for methionine sulfoximine and methionine sulfoximine phosphate were, respectively, I, 0.64 and 0.26; II, 0.13 and 0.09; III, 0.37 and 0.14; IV 0.25 and 0.14; and V, 0.27 and 0.15. The position of P, and compounds containing phosphate was determined after chromatography by spraying the chromatograms with ammonium molybdate (Bandurski and Axelrod, 1952). Adenosine compounds were located by examining the chromatograms under ultraviolet light, and amino acids were located by spraying with ninhydrin.

\[^{14}\text{C}\] and \[^{32}\text{P}\] were determined with a Nuclear-Chicago liquid scintillation counter using double-isotope technique. Liquid samples were counted in methyl Cellosolve-toluene (1:2, v/v). \[^{14}\text{C}\] and \[^{32}\text{P}\] on dry Whatmann No. 3MM paper strips (0.5-1.0 cm wide \times 1.8 cm long) were measured in a toluene solution containing 0.05 g of p-bis[2-(5-phenyl oxazolyl)]benzene and 4 g of 2,5-diphenyloxazole per l. Determinations were made with a precision of \(\pm 5\%\).

P, was determined by the Leloir and Cardini (1957) modification of the method of Fiske and Subbarow (1925) except as noted.

Results

Oxidation of L-[\textsuperscript{14}C, \textsuperscript{32}P]Methionine Sulfoximine Phosphate by L-Amino Acid Oxidase. Previous studies indicated that the phosphoryl moiety of methionine sulfoximine phosphate is not attached to the \(\alpha\)-amino or \(\alpha\)-carboxyl groups of methionine sulfoximine (Ronzio et al., 1969a); thus, methionine sulfoximine phosphate was found to be relatively stable to acid. Additional evidence for this conclusion has come from studies in which it was found that enzymatically produced \[^{14}\text{C}, \[^{32}\text{P}\]methionine sulfoximine phosphate is oxidized by L-amino acid oxidase (without formation of \[^{32}\text{P}\]) to a product containing both \[^{14}\text{C}\] and \[^{32}\text{P}\]. In these studies, a reaction mixture (volume, 0.05 ml) containing crystalline Crotalus adamanteus venom L-amino acid oxidase (0.02 mg), Tris-HCl buffer (0.1 M; pH 7.75), and L-[\textsuperscript{14}C, \textsuperscript{32}P]methionine sulfoximine phosphate (0.2 mM \[^{32}\text{P}\], \(1.11 \times 10^6\) cpm/\(\mu\)mol; \[^{14}\text{C}, \(1.24 \times 10^6\) cpm/\(\mu\)mol) was incubated in air at 25\° for 24 hr. The entire reaction mixture was spotted on a strip of Whatman No. 3MM paper and subjected to paper electrophoresis (with carrier P,) at pH 2.56 at 55 V/cm and 20\° for 60 min. Under these conditions, methionine sulfoximine phosphate exhibited a mobility of \(-8.9\) cm and the only radioactive product of L-amino acid oxidase activity exhibited a mobility of \(-20.7\) cm. The product, which contained 24.6\% of the total radioactivity present, overlapped the area of the strip containing carrier P,. Therefore, the product was eluted from the strip with water and subjected to electrophoresis at pH 4.1 (0.05 M sodium acetate buffer) using the same conditions given above. The product moved 20.1 cm from the origin and was completely separated from the P, area (18 cm), which was devoid of radioactivity. The \[^{32}\text{P}/[^{14}\text{C}\] ratios of the product and the methionine sulfoximine phosphate were, within experimental error, the same. These findings show that, although L-methionine sulfoximine phosphate is a poor substrate for L-amino acid oxidase, about 25\% of the added L-methionine sulfoximine phosphate was oxidized under these conditions. As expected, L-methionine sulfoximine was oxidized more rapidly by the oxidase and was thus completely deaminated in 4 hr under the same conditions.

Chemical Synthesis of Methionine Sulfoximine Phosphate. The phosphorylation of methionine sulfoximine was attempted with phosphorus oxychloride as described by Neuhaus and Korkes (1958), who used this reagent for the synthesis of O-phosphoserine. No ninhydrin-positive compounds which migrated as anions on paper electrophoresis at pH 2.6 were formed in this reaction whether examined before or after treatment with 9 M ammonium hydroxide. Similarly negative results were obtained in attempts to phosphorylate methionine sulfoximine with a mixture of phosphoric acid and phosphorus pentoxide (Peterson et al., 1953). On the other hand, a successful synthesis was achieved by carrying out the phosphorylation with cyanomethyl phosphate in the presence of dicyclohexylcarbodiimide by the general procedure of Tener (1961).

L-Methionine sulfoximine methyl ester was prepared by passing hydrogen chloride into a suspension of the free L-amino acid (2 g, 11.2 mmoles) in 50 ml of methanol for 30 min. The mixture was refluxed for 2 hr and then concentrated in vacuo at 50\° to yield a syrup. This was dissolved in anhydrous methanol and the treatment with hydrogen chloride was repeated. After evaporation in vacuo with methanol, the ester was obtained as a syrup which was stored in a vacuum desiccator at 26\° over sodium hydroxide pellets for 18 hr.

Anhydrous pyridine (50 ml) was added to the ester and the suspension was warmed to 37\° with agitation until most of the ester dissolved. Pyridinium cyanomethyl phosphate (22.4 mmoles) dissolved in 22 ml of anhydrous pyridine was added to the ester solution and 50 ml of anhydrous pyridine con-
Acetic acid in ethanol. After electrophoresis, the radioactivity present in the Pi area of the dried paper strips was determined with a scintillation counter. At the point indicated by the arrow additional enzyme activity was washed with water (400 ml); the combined filtrate and washings were evaporated at 30° to about 20 ml. Examination of an aliquot of this solution by paper electrophoresis at pH 2.6 revealed four major ninhydrin-positive spots. One of these (30-40% of the ninhydrin-positive material present) was identified as methionine sulfoximine. Two anionic compounds were present; the major and less anionic of these exhibited a mobility consistent with methionine sulfoximine phosphate. The fourth ninhydrin-positive product did not migrate at pH 2.6 but after hydrolysis with 9 M ammonium hydroxide, disappeared with a concomitant increase in methionine sulfoximine phosphate. The amount of this compound decreased considerably during the chromatography described below; we believe that it is probably the cyanoethyl derivative of methionine sulfoximine phosphate.

The solution containing the products was repeatedly evaporated to dryness in the presence of ethanol. The residue thus obtained was washed with two 50-ml portions of warm (40°) ethanol and then dissolved in about 50 ml of water. The pH of this solution was adjusted to pH 1.5 by addition of HCl. The acidified aqueous solution was added to the top of a column of a Dowex 50 (H+, 4.5 X 15 cm) which had previously been equilibrated with 0.01 N HCl. The sample was washed into the column with 100 ml of 0.01 N HCl. Elution was carried out with 0.05 N acetic acid; fractions of 25 ml were collected. Methionine sulfoximine phosphate emerged from the column in a broad band (between 750 and 1750 ml of effluent). The more anionic ninhydrin-positive compound found on paper electrophoresis was eluted from the column between 650 and 750 ml of effluent. The third fraction, which contained mainly the presumed cyanoethyl derivative of methionine sulfoximine phosphate as well as some methionine sulfoximine phosphate, emerged between 2000 and 3000 ml of effluent. The fractions containing only methionine sulfoximine phosphate were combined and evaporated in vacuo to dryness at 30°. The residue was dissolved in the minimal volume of water and methionine sulfoximine phosphate was precipitated as a slightly yellow oil by addition of four volumes of acetone. The oil was dissolved in the minimal volume of warm 50% ethanol and allowed to crystallize at -20°. The crystals thus obtained were recrystallized from water to yield 210 mg of electrophoretically pure methionine sulfoximine phosphate. The white crystalline product (mp 149-152°) was stored in a vacuum desiccator at 26°.

Comparison of Enzyme- and Chemically Synthesized Meth-
TABLE I: Inhibition of Glutamine Synthetase by Methionine Sulfoximine Phosphate.

<table>
<thead>
<tr>
<th>Concentration of Methionine Sulfoximine Phosphate (mM)</th>
<th>Methionine Sulfoximine Phosphate (+ ATP + Mg2+) Inhibition (%)</th>
<th>Methionine Sulfoximine Phosphate (+ ATP + Mg2+) Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>0.50</td>
<td>6</td>
<td>95</td>
</tr>
<tr>
<td>0.10</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td>5.0</td>
<td>58</td>
<td>92</td>
</tr>
</tbody>
</table>

* Mixtures (volume, 0.2 ml) containing imidazole-HCl buffer (0.05 M; pH 7.2), enzyme (0.143 μM), and either L-methionine sulfoximine phosphate or L-methionine sulfoximine and, as indicated, ATP (10 mM), and MgCl2 (20 mM) were incubated for 15 min at 37°C; aliquots (10 μl) were then removed and added to the standard γ-glutamyl hydroxamate assay solution (final volume, 1 ml) and glutamine synthetase activity was determined. The same results were obtained (in separate experiments) in the presence and absence of one of the following additions: MgCl2 (20 mM), ATP (10 mM), and ADP (10 mM). L-Threonine sulfoximine.

ionine Sulfoximine Phosphate. Paper chromatography of L-[14C, 32P]methionine sulfoximine phosphate obtained from the enzyme and of the chemically synthesized product was carried out in the five solvent systems given above; the products gave identical $R_F$ values in each of the several solvents. The mobilities of the two products on paper electrophoresis at pH values of 2.8, 5.5, 7.5, and 9.0 were also the same. The formation of inorganic phosphate in a mixture of L-[14C]methionine sulfoximine phosphate (from the enzyme) and chemically synthesized L-methionine sulfoximine phosphate was studied in 1 N HCl at 100°C (Figure 1). The rates of P1 liberation from both products were the same within experimental error.

Both products were completely hydrolyzed to yield equimolar amounts of P1 and methionine sulfoximine by yeast acid phosphatase, prostate acid phosphatase, E. coli alkaline phosphatase, and calf intestine alkaline phosphatase under the conditions previously described (Ronizio et al., 1969a; Table IV). The rates of hydrolysis were the same for the product obtained from the enzyme and for the chemically synthesized compound. Thus, as indicated in Figure 2, the formation of unlabeled P1, and of 32P1, was parallel with yeast acid phosphatase. In similar studies with prostate phosphatase, the values for per cent 32P1 and 14P1, liberated by 0.019 unit of enzyme in 10, 20, and 30 min were, respectively, 14.0 and 16.5, 21.0 and 25.5, and 35.0 and 29.0.

The stability at 60°C of the enzyme- and chemically synthesized methionine sulfoximine phosphate products were, within experimental error, the same over a broad range of pH (Figure 3).

Conversion of Methionine Sulfoximine and Methionine Sulfoximine Phosphate into the Corresponding Sulfimines. Methionine sulfoxide can be readily reduced to methionine by treatment with sulfite at 38°C (Michael and Schmitz, 1939). We have carried out an analogous reaction with methionine sulfoximine; when methionine sulfoximine is treated with sulfite a new product is formed, which is identical on paper chromatography with methionine sulfoximine (CH3SH(C═NH)CH2CHNNH2COOH). Methionine sulfoximine gives a blue color on paper chromatograms that have been treated with ninhydrin. A diagrammatic representation of the results obtained with methionine sulfoximine and methionine sulfoximine phosphate is given in Figure 4. After methionine sulfoximine phosphate was treated with Na2S2O4 and then hydrolyzed for 2 hr in 6 N HCl at 100°C, the product obtained exhibited both the $R_F$ value and the blue color characteristic of methionine sulfoximine; the P1 formed was identified by a yellow spot after spraying an identical chromatogram with molybdate reagent. These observations, which indicate that methionine sulfoximine phosphate can be converted on mild reduction into methionine sulfoximine phosphate, provide evidence that the phosphoryl moiety of methionine sulfoximine phosphate is attached to the sulfoximine nitrogen atom of methionine sulfoximine rather than to its oxygen atom.

Inhibition of Glutamine Synthetase by Methionine Sulfoximine Phosphate.
In earlier studies, several attempts were made to reverse the inhibition produced by incubating the enzyme with L-methionine sulfoximine, ATP, and Mn²⁺; gel filtration, dialysis, and other procedures failed to restore activity (Ronzio et al., 1969a). However, the inhibition produced by incubating the enzyme with L-methionine sulfoximine phosphate is reversible. Thus, as indicated in Table III, when the inhibited enzyme is dialyzed or subjected to gel filtration, significant activity returns (expt 1, 2, and 5); under the conditions employed, gel filtration was more effective than dialysis. In contrast, when the enzyme was inhibited by incubation with L-methionine sulfoximine phosphate, ATP, and Mg²⁺ (expt 3, 4, and 6), neither gel filtration nor dialysis restored activity. The findings, which indicate that the presence of nucleotide and metal ion promotes the binding of L-methionine sulfoximine phosphate to the enzyme, are in accord with the studies described in Figure 5 which show that the presence of ADP (or ATP) plus Mg²⁺ enhances the inhibition produced by L-methionine sulfoximine phosphate.

**Attempt to Reverse the Phosphorylation Reaction.** An attempt was made to determine whether the enzyme could catalyze the synthesis of ATP from L-methionine sulfoximine phosphate and ADP. A reaction mixture (final volume, 0.16
Table III: Reversal of Inhibition of Glutamine Synthetase (GS) by Dialysis and Gel Filtration.

<table>
<thead>
<tr>
<th>Expt</th>
<th>Conditions of Inhibition</th>
<th>Inhib %</th>
<th>Treatment</th>
<th>Inhib %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GS + MSOP</td>
<td>73</td>
<td>Dialysis vs. imidazole</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>GS + MSOP</td>
<td>70</td>
<td>Dialysis vs. phosphate buffer</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>GS + MSOP + ATP + Mg^{2+}</td>
<td>100</td>
<td>Dialysis vs. phosphate buffer</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>GS + MSOP + ATP + Mg^{2+}</td>
<td>97</td>
<td>Gel filtration; phosphate buffer</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>GS + MSOP</td>
<td>82</td>
<td>Gel filtration; phosphate buffer</td>
<td>9</td>
</tr>
<tr>
<td>6</td>
<td>GS + MSOP + ATP + Mg^{2+}</td>
<td>95</td>
<td>Gel filtration; imidazole buffer</td>
<td>95</td>
</tr>
</tbody>
</table>

* In expt 1–3, glutamine synthetase (GS) (5.8 μM), L-methionine sulfoximine phosphate (MSOP) (0.5 mM), imidazole-HCl buffer (pH 7.2, 0.05 M), 2-mercaptoethanol (0.05 M), ATP (0.01 M), and MgCl₂ (0.02 M) were incubated as indicated in the table for 60 min at 37°C; final volume, 1 ml. Dialysis was carried out for 18 hr with stirring at 4°C against 1 M buffer (pH 7.2). In expt 4 and 5, glutamine synthetase (7.5 μM), L-methionine sulfoximine phosphate (2 mM), imidazole-HCl buffer (pH 7.2, 0.01 M), 2-mercaptoethanol (0.05 M), and as indicated, ATP (0.01 M), and MgCl₂ (0.02 M) were incubated for 30 min at 37°C. Experiment 6 was carried out with ATP (0.001 M) and MgCl₂ (0.002 M); otherwise the conditions were as given for expt 4 and 5. Gel filtration was carried out at 4°C with Sephadex G-50 and 0.05 M buffer (pH 7.2).

ml) containing glutamine synthetase (3.7 μM), magnesium chloride (1.8 × 10⁻³ M), 2-mercaptoethanol (0.05 M), [8-¹⁴C]-ADP (9.2 × 10⁻⁵ M; 30 mCi/mmol), and L-methionine sulfoximine phosphate (0.015 M) was incubated at 37°C for 60 min. A control in which methionine sulfoximine phosphate was omitted was also carried out. After incubation, carrier AMP, ADP, and ATP were added and the solution was placed at 100°C for 2 min. The precipitated protein was removed by centrifugation and the supernatant solution was lyophilized and dissolved in 0.05 ml of water. Aliquots of this solution were subjected to paper electrophoresis at pH 3.6 and the radioactivity present in the areas corresponding to the nucleotides was determined. About 2% of the total radioactivity added initially was found as [¹⁴C]ATP in both the experiment and the control. These studies do not show ATP synthesis from ADP and L-methionine sulfoximine phosphate; however, it is possible that this reaction might be demonstrated under other experimental conditions. Under the conditions used here, more than 95% of the initial glutamine synthetase activity was inhibited.

Discussion

The present studies provide strong evidence that the phosphoryl moiety of methionine sulfoximine phosphate is attached to the sulfoximine nitrogen atom of this molecule; the following structures may therefore be considered:

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_3 \\
O=\text{S}=\text{NP}_2\text{O}_3\text{H}^+ & \quad O=\text{S}=\text{NHPO}_2\text{H}^+ \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CH}_2 & \quad \text{CH}_2 \\
\text{CHNH}_2\text{H}^+ & \quad \text{CHNH}_2\text{H}^+ \\
\text{COO}^- & \quad \text{COO}^-
\end{align*}
\]

Compounds possessing sulfur–nitrogen–phosphorus linkages of this type do not appear to have been reported previously. Methionine sulfoximine phosphate exhibits considerable stability to cleavage by acid and thus differs substantially in this respect from such compounds as phosphocreatine, phosphoarginine, and α-N-phosphorylarnino acids. It will be of interest to examine the properties of other compounds possessing phosphorylated sulfoximine moieties. It is possible that substrate analogs bearing sulfoximine or similar groups can undergo analogous phosphorylation reactions with other enzymes, e.g., γ-glutamylcysteine synthetase and succinyl thio kinase. For example, the product obtained in the oxidation of L-methionine sulfoximine by L-amino acid oxidase in the absence of catalase (CH₃S(=NH)(=O)CH₂CH₂COOH) might react in an analogous manner with succinyl thio kinase; this enzyme catalyzes a reaction that seems to involve enzyme-bound succinyl phosphate (Nishimura and Meister, 1965; Nishimura, 1967; Grinnell and Nishimura, 1969).

It is significant that methionine sulfoximine phosphate binds to the enzyme and produces inhibition in the absence of added nucleotide or metal ions. Since previous studies (Krishnaswamy et al., 1962) indicate that the binding of both glutamate and glutamine require nucleotide and metal ions, it would seem that the phosphoryl moiety of methionine sulfoximine phosphate promotes attachment of this compound to the enzyme. It is notable, however, that the addition of ADP and metal ions increases the inhibition and the tightness of binding of methionine sulfoximine phosphate. The finding that ATP is less effective than ADP in facilitating the binding of methionine sulfoximine phosphate suggests that the phosphoryl moiety of methionine sulfoximine phosphate may occupy the same enzyme site that normally attaches to the terminal phosphoryl moiety of ATP. In this connection it is interesting to note that P₃, protects the enzyme against inhibition by methionine sulfoximine phosphate in the presence of ADP, but not in the presence of ATP. It may also be noted that dialysis against phosphate buffer was apparently more effective than dialysis against imidazole buffer in removing methionine sulfoximine phosphate from the enzyme. In earlier studies it was found that glutamate plus ammonia protected the enzyme from inhibition by methionine sulfoximine (Ronzi et al., 1969a). In the present work neither glutamate nor ammonia (or a combination of these) protected very greatly...
against inhibition by methionine sulfoximine phosphate. This result would seem to be in accord with the postulate that the phosphoryl moiety of methionine sulfoximine phosphate binds strongly to a separate enzyme site. The data also indicate that the binding of methionine sulfoximine phosphate is influenced by the presence of various substrates separately and in combinations. Although more detailed studies of these phenomena are needed before definite conclusions can be drawn, it seems probable that the results thus far obtained reflect the ability of methionine sulfoximine phosphate to interact with the sites on the enzyme that normally combine with glutamate, ammonia, and the terminal phosphoryl moiety of ATP.

In previous studies evidence was obtained that under conditions of equilibrium gel filtration, nucleotide was bound to the enzyme only in the presence of methionine sulfoximine phosphate (Ronzio et al., 1969a). It thus appears that methionine sulfoximine phosphate, nucleotide, and the enzyme interact to form a tight complex. The lack of reversibility observed in the present studies (Table III) attests to the stability of this complex. However, there is evidence that the enzyme undergoes additional change when it is incubated with methionine sulfoximine, ATP, and Mg++. Thus, it has been observed by ultracentrifugal procedures that the native octameric form of the enzyme aggregates in the presence of methionine sulfoximine, ATP, and Mg++ to yield forms of the enzyme possessing higher molecular weights; furthermore, electron microscope studies indicate that there is a linear aggregate of the octameric units under these conditions (Wilk et al., 1968).

The present studies on the inhibition of glutamine synthetase by methionine sulfoximine and methionine sulfoximine phosphate have developed from earlier work in this laboratory on the unusual specificity of this enzyme; however, additional work is needed in order to fully integrate the present studies with the earlier ones. Previous studies have shown that both L- and D-glutamate are good substrates for the synthesis of the corresponding isomers of glutamine and y-glutamyl hydroxamate (Levintow and Meister, 1953; Meister, 1968) and of pyrrolidonecarboxylate (Krishnaswamy et al., 1960, 1962). On the other hand, only the L isomer of glutamine is significantly active in the y-glutamyl transfer and arsenolysis reactions catalyzed by sheep brain glutamine synthetase (Meister, 1962; Wellner, 1963); in addition, there is evidence that the enzyme binds D-glutamine very much less effectively than L-glutamine (Krishnaswamy et al., 1962). This marked difference between the optical specificity exhibited by the enzyme toward glutamine and glutamate seems analogous to the finding that L-methionine sulfoximine is an effective inhibitor while D-methionine sulfoximine is not (Ronzio et al., 1969a), and to the observation that both the L and D isomers of methionine sulfone are effective inhibitors (W. B. Rowe and A. Meister, 1969, in preparation). It would appear that the binding sites on the enzyme for the y-carboxyl oxygen atoms of glutamate and for the sulfone oxygen atoms of methionine sulfone are so situated as to be able to interact with the respective L and D isomers. On the other hand, the binding of the amide nitrogen atom of L-glutamine and of the sulfoximine nitrogen atom of L-methionine sulfoximine seem to involve an enzyme site (perhaps the same site that normally binds ammonia), which is unfavorably located for attachment to the corresponding nitrogen atoms of the respective D isomers.

The observation that only one stereoisomer of L-methionine sulfoximine (L-methionine s-sulfoximine) is phosphorylated (Manning et al., 1969) indicates clearly that the sulfoximine moiety of this molecule is oriented in a specific manner on the enzyme. This result suggests that interaction of the sulfoximine nitrogen atom with a specific site on the enzyme is required for binding and phosphorylation.

References
Manning, J. M., Moore, S., Rowe, W. B., and Meister, A. (1969), Biochemistry 8, 2861 (this issue; paper III).
Meister, A. (1962), Enzymes 6, 443.
Micheel, F., and Schmitz, H. (1939), Ber. 72, 992.
Nishimura, J. S. (1967), Biochemistry 6, 1094.
Wellner, V. P. (1963), Ph.D. Dissertation, Tufts University, Boston, Mass.
Wellner, V. P., and Meister, A. (1966), Biochemistry 5, 872.