Identification of L-Methionine s-Sulfoximine as the Diastereoisomer of L-Methionine SR-Sulfoximine That Inhibits Glutamine Synthetase*

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ABSTRACT: The diastereoisomers of L-methionine SR-sulfoximine phosphate have been separated as the corresponding α-*N*-L-leucyl derivatives by ion-exchange chromatography on an amino acid analyzer; the diastereoisomers of L-methionine SR-sulfoximine and L-methionine SR-sulfoxide can be separated without derivatization. Chromatographic study of the L-methionine sulfoximine phosphate obtained from sheep brain glutamine synthetase inhibited by treatment with L-methionine SR-sulfoximine, adenosine triphosphate, and Mg^{2+} showed the presence of only one diastereoisomer. Studies with preparations of L-methionine S-sulfoximine and L-methionine R-sulfoximine indicate that only the former diastereoisomer is phosphorylated and inhibits the enzyme.

Methionine sulfoximine has long been known as a convulsant agent (Bentley *et al.*, 1951) and as a potent inhibitor of brain glutamine synthetase (Pace and McDermott, 1952). In more recent studies it was established that L-methionine SR-sulfoximine inhibits ovine brain glutamine synthetase irreversibly and that such inhibition is associated with the tight binding to the enzyme of close to 8 moles each of methionine sulfoximine phosphate and ADP (Ronzio and Meister, 1967, 1968; Ronzio *et al.*, 1969a,b; Rowe *et al.*, 1969). After it was found that D-methionine SR-sulfoximine does not inhibit glutamine synthetase (Ronzio *et al.*, 1969a,b) it became of interest to learn whether a specific configuration about the asymmetric sulfur atom of L-methionine sulfoximine is required for inhibition. In an effort to answer this question, L-methionine sulfoximine phosphate (obtained from enzyme inhibited by incubation with L-methionine SR-sulfoximine, ATP, and Mg^{2+}) was converted into the α-*N*-L-leucyl derivative and examined chromatographically by the procedure of Manning and Moore (1968). Interpretation of the results is facilitated by the recent work of Christensen *et al.* (1969), who have separated the diastereoisomers of L-methionine SR-sulfoximine by separation of their salts with (+)-camphor-10-sulfonic acid and established their absolute configurations by X-ray crystallography.[1]

Experimental Section

Materials

Glutamine synthetase was isolated from sheep brain (Ronzio *et al.*, 1969a,b). L-Methionine SR-sulfoximine and L-methionine SR-sulfoximine phosphate were prepared as described by Rowe *et al.* (1969). L-Leucine-*N*-carboxyanhydride was obtained from Pilot Chemical Co., Watertown, Mass. (+)-Camphor-10-sulfonic acid was obtained from Eastman Kodak Co., and recrystallized from glacial acetic acid.

The authors wish to thank Dr. Anders Kjaer of the Technical University of Denmark, Lyngby, Denmark, for providing us with initial samples of L-methionine R-sulfoximine and L-methionine S-sulfoximine prepared from L-methionine SR-sulfoximine by the method of Christensen *et al.* (1969). The preparation of L-methionine R-sulfoximine used in the present studies was obtained in a similar manner as follows. The crystalline salt obtained from a mixture of (+)-camphor-10-sulfonic acid and L-methionine SR-sulfoximine in warm ethanol–ethyl acetate (1:3, v/v) (Christensen *et al.*, 1969) was recrystallized from warm ethanol–ethyl acetate (1:2) and then from warm ethanol–ethyl acetate (1:1). The crystalline material obtained from the latter solvent was dissolved in water and placed on a column of Dowex 2-acetate. The free methionine sulfoximine was eluted with two column volumes of water and the effluent was evaporated to dryness *in vacuo*. The residue obtained was crystallized from 75% ethanol and dried in a vacuum desiccator over NaOH pellets. This material was converted again into the (+)-camphor-10-sulfonic acid salt, and after three crystallizations of the salt carried out as described above, the free L-methionine R-sulfoximine was recovered by Dowex 2-acetate chromatography in about 10% yield.

L-Methionine S-sulfoxide and L-methionine R-sulfoxide were obtained by separation as the picrates as described by Lavine (1947).

The other compounds used in these studies were obtained as previously described (Rowe *et al.*, 1969).

Methods

Separation of the Diastereoisomers of L-*Methionine* SR-

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[1] We are greatly indebted to Dr. Anders Kjaer of the Technical University of Denmark for informing us of this research prior to publication and for providing us with generous samples of the two diastereoisomers.

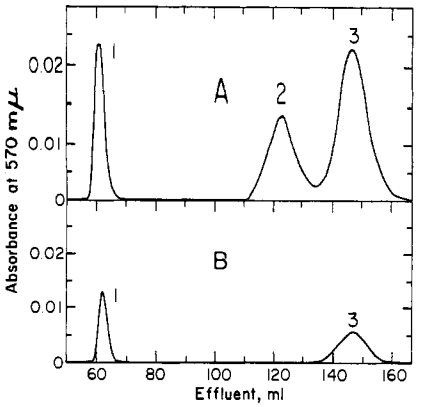

FIGURE 1: Chromatographic separation of the diastereoisomers of α-*N*-L-leucyl-L-methionine SR-sulfoximine phosphate (see the text). (A) α-*N*-L-Leucyl-L-methionine SR-sulfoximine phosphate (synthetic); (B) α-*N*-L-leucyl-L-methionine sulfoximine phosphate (from the inhibited enzyme). 1, Unreacted methionine sulfoximine phosphate; 2 and 3, diastereoisomers of α-*N*-L-leucyl-L-methionine sulfoximine phosphate.

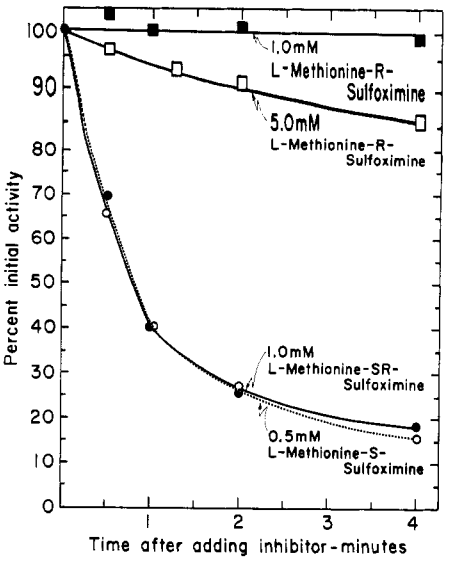

FIGURE 2: Inhibition of glutamine synthetase by the diastereoisomers of L-methionine sulfoximine. The reaction mixtures contained enzyme (8.6 μM), imidazole-HCl buffer (pH 7.2; 0.05 M), 2-mercaptoethanol (0.001 M), ATP (0.005 M), $MgCl_2$ (0.01 M), and L-methionine sulfoximine as indicated; final volume, 0.05 ml. After 0.5, 1, 2, and 4 min, aliquots (2.5 μl) were removed and enzyme activity was determined in the standard 1-ml γ-glutamyl hydroxamate assay system.

Sulfoximine Phosphate as the α-N-L-Leucyl Derivatives. L-Methionine SR-sulfoximine phosphate (0.5 μmole) was coupled with L-leucine-*N*-carboxyanhydride (Manning and Moore, 1968) and the dipeptide derivative was chromatographed on the 0.9 × 62 cm column of the automatic amino acid analyzer (Spackman *et al.*, 1958; Spackman, 1963) packed with Beckman-Spinco AA-15 resin. The eluent was 0.20 N sodium citrate (pH 1.99); the temperature of the column was maintained at 35°. The column was eluted for at least 1 hr before application of the sample to ensure equilibration. Analysis of the methionine sulfoximine phosphate obtained after treatment of the enzyme with L-methionine SR-sulfoximine, ATP, and $MgCl_2$ was carried out in the same manner.

Separation of the Diastereoisomers of L-Methionine SR-Sulfoximine. L-Methionine SR-sulfoximine and the separate L diastereoisomers of this compound were chromatographed directly on the 0.9 × 62 cm column of the automatic amino acid analyzer.[2] The eluent was 0.20 N sodium citrate (pH 3.15); the temperature was maintained at 40°. L-Methionine was included as a marker.

Separation of the Diastereoisomers of L-Methionine SR-Sulfoxide. These isomers have been separated previously on the 150-cm column of the amino acid analyzer (Spackman *et al.*, 1958). In the present study the separate diastereoisomers of established absolute configuration (Christensen and Kjaer, 1965), were chromatographed on the 0.9 × 62 cm column using 0.20 N sodium citrate (pH 2.80) as the eluent at 52°.

Determination of Enzyme Activity. The 1-ml standard γ-glutamyl hydroxamate assay system was employed for the determination of enzyme activity (Ronzio *et al.*, 1969a,b).

Results

Isolation and Characterization of L-Methionine Sulfoximine Phosphate from the Inhibited Enzyme. Attempts to obtain separation of the diastereoisomers of chemically synthesized L-methionine SR-sulfoximine phosphate on the amino acid analyzer were unsuccessful because this compound was only slightly retarded by the resin. The α-*N*-L-leucyl dipeptides were prepared in order to obtain derivatives which would be significantly retarded on the column and which therefore might be separable. Separation of the α-*N*-L-leucyl-L-methionine SR-sulfoximine phosphate isomers was achieved in this manner as shown in Figure 1A. It is important to note that the preparation of dipeptides by the *N*-carboxyanhydride method proceeds in about 90% yield without measurable racemization or stereochemical selectivity (Manning and Moore, 1968; Denkewalter *et al.*, 1966). The difference in the size of peaks 2 and 3 may reflect a slight difference between the relative amounts of the two diastereoisomers present in the preparation of L-methionine SR-sulfoximine phosphate, or in the ninhydrin color yields of the respective α-*N*-L-leucyl derivatives.

The following experiment was then carried out in order to determine whether one or both diastereoisomers of L-methionine sulfoximine phosphate was formed on the enzyme. A reaction mixture (final volume, 2 ml) containing glutamine synthetase (5 μM), sodium ATP (0.01 M), magnesium chloride (0.02 M), 2-mercaptoethanol (0.05 M), imidazole-HCl buffer (pH 7.2, 0.05 M), and L-[^{14}C]methionine SR-sulfoximine (0.01 M) was incubated at 37° for 60 min. The enzyme, which was 97% inhibited, was applied to a column of Sephadex G-50; elution was carried out with 0.05 M potassium phosphate buffer (pH 7.2). The fractions containing the protein were combined and analyzed for protein, enzymatic activity, and radioactivity as previously described (Ronzio and Meister, 1968). A total of 10 mμmoles of inactivated enzyme was collected containing 79.6 mμmoles of [^{14}C]methionine sulfoximine phosphate. This solution was placed at 100° for 2 min and the supernatant solution obtained after centrifugation

[2] The ninhydrin color yield of each diastereoisomer is 1.73 times that of leucine, when determined with the manual ninhydrin reagent and a heating time of 9 min.

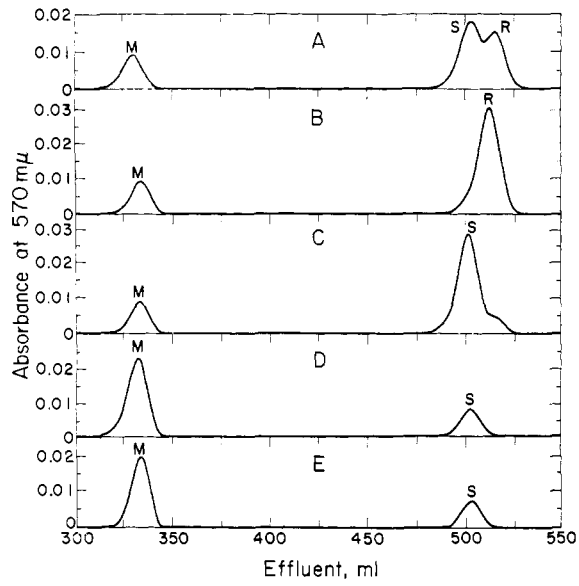

FIGURE 3: Chromatographic separation of the diastereoisomers of L-methionine SR-sulfoximine. Methionine (M) was added as a marker. In expt D and E reaction mixtures (final volume, 1.5 ml) containing glutamine synthetase (4.3 μM), NaATP (0.005 M), $MgCl_2$ (0.01 M), 2-mercaptoethanol (0.005 M), Tris-HCl buffer (pH 7.2; 0.01 M), KCl (0.04 M), and methionine sulfoximine (20 mM L-methionine R-sulfoximine and 1 mM L-methionine S-sulfoximine) were incubated at 37° for 90 min. The enzyme mixture (92% inhibited with L-methionine S-sulfoximine; 88% inhibited with L-methionine R-sulfoximine) was applied to a column of Sephadex G-50 and elution was carried out with 0.01 M potassium phosphate buffer (pH 7.2). The fractions containing the protein were combined and placed at 100° for 2 min. After centrifugation, the supernatant solutions were lyophilized. The residues were dissolved in 0.5 ml of 0.1 M sodium acetate buffer (pH 4.0) and yeast acid phosphatase (1.6×10^{-4} unit) was added. The phosphatase digest was placed at 25° for 16 hr, and then lyophilized. The dried residue was dissolved in 0.2 ml of water and chromatographed as described under Methods. (A) L-Methionine SR-sulfoximine; (B) L-methionine-R-sulfoximine; (C) L-methionine S-sulfoximine; (D) L-methionine sulfoximine from enzyme incubated with L-methionine S-sulfoximine; (E) L-methionine sulfoximine from enzyme incubated with L-methionine R-sulfoximine.

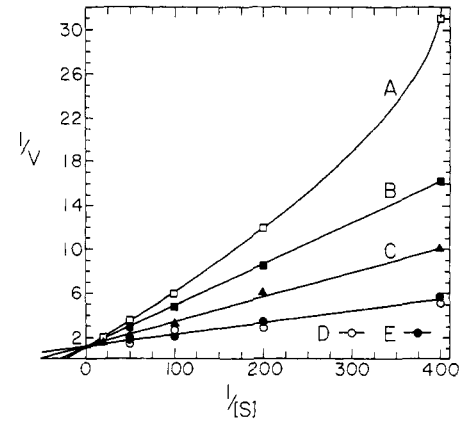

FIGURE 4: Inhibition of glutamine synthetase by L-methionine S-sulfoximine and L-methionine R-sulfoximine. The reaction mixtures contained enzyme (11.5 mμM); sodium L-glutamate (0.0025–0.1 M, as indicated), NH_2OH (0.10 M), NaATP (0.01 M), $MgCl_2$ (0.02 M), imidazole-HCl buffer (pH 7.2; 0.05 M), 2-mercaptoethanol (0.025 M), L-methionine sulfoximine; final volume, 1.0 ml. Curve A: 2 mM L-methionine S-sulfoximine; curve B: 0.5 mM L-methionine S-sulfoximine; curve C: 10 mM L-methionine R-sulfoximine; curve D: 2 mM L-methionine R-sulfoximine; curve E: no methionine sulfoximine; incubated at 37° for 15 min. Ordinate: (μmoles of γ-glutamyl hydroxamate formed)$^{-1}$; abscissa: (L-glutamate concentration (M))$^{-1}$.

was lyophilized. The residue was dissolved in 0.5 ml of water and converted into the α-*N*-L-leucyl derivative and chromatographed as described under Methods. As indicated in Figure 1B, only one diastereoisomer was found.

Identification of the Diastereoisomer of L-*Methionine* SR-*sulfoximine that Inhibits Glutamine Synthetase.* Glutamine synthetase was incubated in separate experiments with L-methionine S-sulfoximine and L-methionine R-sulfoximine in the presence of ATP and magnesium ions; after incubation, the reaction mixtures were diluted 400-fold and assayed for enzymatic activity. As indicated in Figure 2, 0.5 mM L-methionine S-sulfoximine produced within experimental error as much irreversible inhibition as observed with 1 mM L-methionine SR-sulfoximine. On the other hand, little if any inhibition was observed with 1 mM L-methionine R-sulfoximine. However, when the concentration of L-methionine R-sulfoximine was increased to 5 mM, a small but definite inhibition of the enzyme was observed. These observations clearly indicate that L-methionine S-sulfoximine is much more inhibitory than L-methionine R-sulfoximine. The studies described below show that the relatively small extent of inhibition by L-methionine R-sulfoximine may be ascribed to the presence in this preparation of a small amount of L-methionine S-sulfoximine.

A partial separation of L-methionine SR-sulfoximine into its diastereoisomers was achieved by chromatography on the amino acid analyzer (Figure 3A). Since ammonia is eluted from this column just after methionine sulfoximine it was not possible to use an eluent of lower pH. However, the separation achieved was sufficient to make possible studies with the separate diastereoisomers. Chromatography of the L-methionine S-sulfoximine preparation indicated the presence of a small amount of L-methionine R-sulfoximine (Figure 3C). Similar examination of the preparation of L-methionine R-sulfoximine gave a single component possessing a slight but definite asymmetry suggesting the presence of a small amount of L-methionine S-sulfoximine (Figure 3B). These chromatographic findings therefore indicate that the preparations of L-methionine S-sulfoximine and L-methionine R-sulfoximine contain small amounts of the corresponding diastereoisomers. In order to establish whether the inhibition observed with high concentrations of L-methionine R-sulfoximine is produced by this diastereoisomer or by the small amount of contaminating L-methionine S-sulfoximine, a chromatographic study of the enzyme-bound methionine sulfoximine was carried out. Relatively large amounts of the enzyme were incubated (a) with 1 mM L-methionine S-sulfoximine and (b) with 20 mM L-methionine R-sulfoximine in the presence of ATP and magnesium ions; the inactivated enzyme preparations were isolated by gel filtration (see legend, Figure 3) and the methionine sulfoximine phosphate was liberated from the enzyme by brief heating at 100° (Ronzio and Meister, 1968). The protein-free solutions were then treated with yeast acid phosphatase (Ronzio *et al.*, 1969a,b) and chromatographed. As indicated in Figure 3D, the diastereoisomer which was obtained from

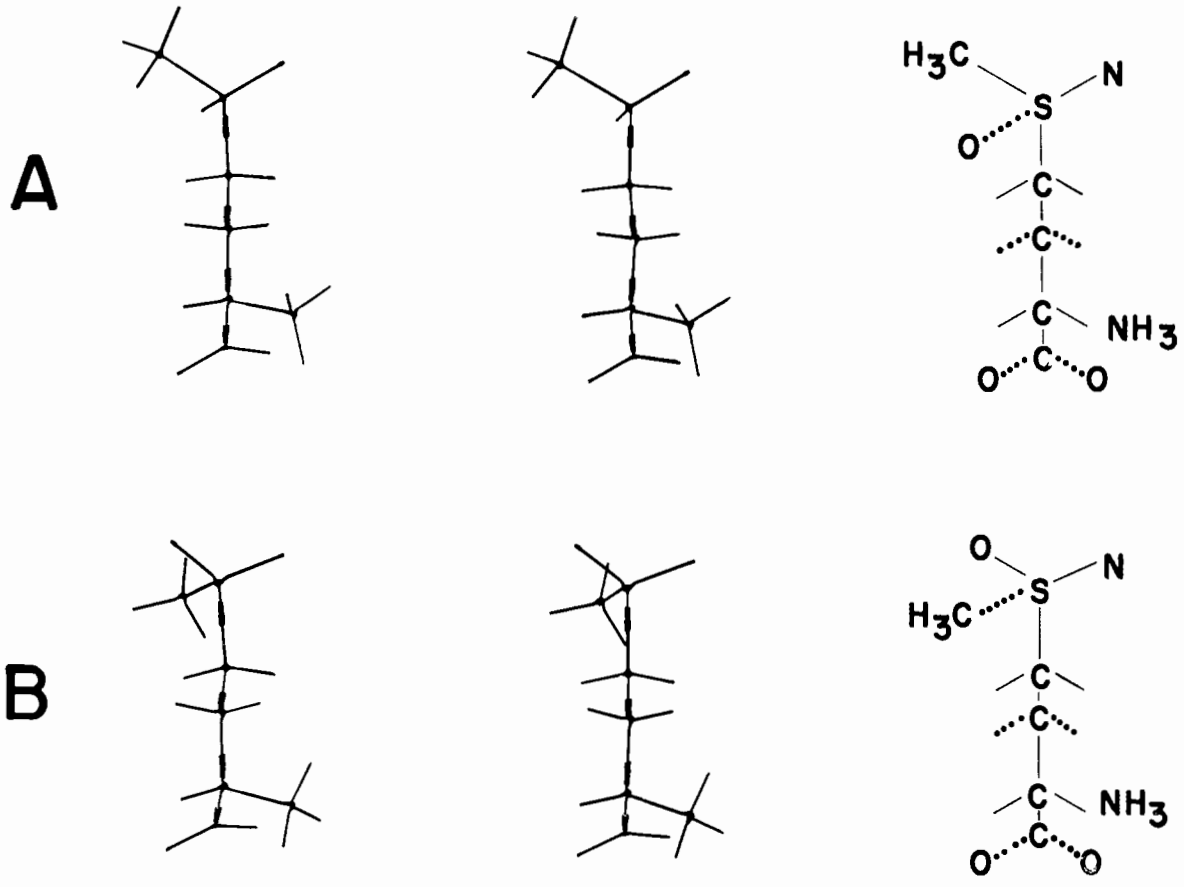

FIGURE 5: Stereophotographs of Dreiding models of L-methionine S-sulfoximine (A) and L-methionine R-sulfoximine (B) (see the text).

the enzyme inhibited by incubation with L-methionine S-sulfoximine corresponded to L-methionine S-sulfoximine. Similar chromatography of the isomer obtained from the enzyme inhibited with L-methionine R-sulfoximine revealed that this also was L-methionine S-sulfoximine. The findings therefore indicate that only one isomer, L-methionine S-sulfoximine, inhibits glutamine synthetase irreversibly and that the inhibition observed with the preparation of L-methionine R-sulfoximine is explained by the presence in this preparation of a small amount of L-methionine S-sulfoximine.

The experiments described in Figure 4 were carried out in order to determine whether L-methionine R-sulfoximine inhibits the enzyme reversibly. As indicated in Figure 4 (curves D and E), no inhibition was observed with 2 mM L-methionine R-sulfoximine. In an experiment with 10 mM L-methionine R-sulfoximine, about 25% inhibition was observed (curve C); however, this extent of inhibition is about half of that observed with 0.5 mM L-methionine S-sulfoximine (curve B). Thus, the inhibition observed with 10 mM L-methionine R-sulfoximine could be explained by the presence of 2–3% L-methionine S-sulfoximine in the preparation of L-methionine R-sulfoximine. Such a degree of contamination is in accord with the data given in Figure 2 and the chromatographic findings (Figure 3B). The shape of the double-reciprocal plot obtained with 2 mM L-methionine S-sulfoximine (Figure 4A) is similar to that observed previously with L-methionine SR-sulfoximine (Ronzio *et al.*, 1969a,b) and reflects a mixed type of inhibition.

Separation and Identification of the Diastereoisomers of L-*Methionine Sulfoxide.* Previous studies have demonstrated that the diastereoisomers of L-methionine sulfoxide can be separated by ion-exchange chromatography (Spackman *et al.*, 1958). Recently, Christensen and Kjaer (1965) established the absolute configurations of these diastereoisomers; thus, the dextrorotatory isomer of L-methionine sulfoxide has been assigned the 2-S,*S*-S configuration. In the present study, the diastereoisomers of L-methionine sulfoxide were chromatographed as described under Methods; under these conditions, L-methionine S-sulfoxide is eluted just before L-methionine R-sulfoxide. The two optically pure sulfoxides were converted into methionine sulfoximine by treatment with hydrazoic acid (Bentley *et al.*, 1951);[3] subsequent chromatography (as described under Methods) revealed that in each case the product was a mixture of the diastereoisomers of L-methionine sulfoximine. It may therefore be concluded that epimerization occurs under the conditions of this reaction.

Discussion

The chromatographic studies described above demonstrate that only one diastereoisomer, L-methionine S-sulfoximine, is phosphorylated by glutamine synthetase and produces irreversible inhibition. The finding that L-methionine R-sulf-

[3] Carried out by Dr. Robert A. Ronzio.

oximine does not inhibit the enzyme (reversibly or irreversibly) indicates that this diastereoisomer does not bind effectively to the active site of the enzyme. Study of models of L-methionine S-sulfoximine and L-methionine R-sulfoximine in the light of the information available about the activity of the enzyme toward other substrates (Meister, 1968) suggests that the *S*-methyl group of L-methionine S-sulfoximine is directed away from the enzyme (analogous to the postulated position of the methyl group of *threo*-γ-methyl-L-glutamate (Kagan and Meister, 1966)). If this is the case, the sulfoximine nitrogen atom of L-methionine S-sulfoximine must be oriented toward the same side of the molecule as the α-amino nitrogen atom when the inhibitor is attached to the enzyme (Figure 5A). It is evident from inspection of the model of L-methionine R-sulfoximine (Figure 5B) that the sulfoximine nitrogen atom of this diastereoisomer can be brought to this position only if the methyl group is rotated to a position close to the postulated position of the methyl group of *threo*-γ-methyl D-glutamate, which is not a substrate (Kagan and Meister, 1966). These considerations suggest that the sulfoximine nitrogen atom of L-methionine R-sulfoximine cannot attach to the enzyme site which interacts with the corresponding nitrogen atom of L-methionine S-sulfoximine and that such interaction is required for phosphorylation. It seems possible that L-methionine S-sulfoximine can assume a conformation on the enzyme in which the essentially tetrahedral sulfoximine moiety is closely analogous to the tetrahedral intermediate formed normally in the course of glutamine synthesis.

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