Computer Analysis of the Active Site of Glutamine Synthetase*

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ABSTRACT: A mathematical method has been designed for study of the active site of brain glutamine synthetase. Earlier work, which showed that this enzyme interacts with L- and D-glutamate, α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, *threo*-γ-methyl-L-glutamate, β-glutamate, *cis*-1-amino-1,3-dicarboxycyclohexane, L-methionine-(*S*)-sulfoximine, and L- and D-methionine sulfone, but not with a number of closely related compounds, led to the hypothesis that L-glutamate attaches to the enzyme in an extended conformation in which the α-hydrogen atom is directed away from the active site. In the present approach the three-dimensional coordinates of the substrates and inhibitors were calculated with a computer and, using certain assumptions, an "active site" was mathematically designed. The results support the earlier hypothesis as well as several additional new conclusions. Thus, it appears that the enzyme has a specific binding site for uncharged NH_3, and that the *S*-methyl groups of methionine sulfone and methionine sulfoximine bind to this site; the findings further indicate that these inhibitors act as analogs of the tetrahedral intermediate formed in the reaction rather than of glutamate itself.

The present work also seems to offer insight into the relationships between the rates of amide synthesis and the structures of the several amino acid substrates, the specificity of the reverse and γ-glutamyl transfer reactions, the strict stereospecificity of D-β-glutamine synthesis, and the specificity of the inhibition by the methionine derivatives. This work provides an explanation in steric terms for the phosphorylation of a specific isomer of methionine sulfoximine. Finally, the findings offer a more detailed approach to an understanding of the mechanism of the reaction in terms of the formation and reaction of γ-glutamyl phosphate on the enzyme.

Recent studies in this laboratory on brain glutamine synthetase have led to the hypothesis that L-glutamate attaches to the active site of this enzyme in an extended conformation in which the α-hydrogen atom of the substrate is directed away from the enzyme (Kagan *et al.*, 1965; Kagan and Meister, 1966a,b; Meister, 1968; Gass and Meister, 1968, 1970). This hypothesis has developed from and has been supported by experiments in which the ability of various glutamate analogs to interact with the enzyme was studied. Thus, α-methyl-L-glutamate, *threo*-β-methyl-D-glutamate, and *threo*-γ-methyl-L-glutamate are substrates, whereas other monomethyl glutamate derivatives are not. Both the L and D isomers of glutamate are substrates (Levintow and Meister, 1953, 1954; Wellner and Meister, 1966); β-glutamate is also a substrate and is converted only into D-β-glutamine (Khedouri *et al.*, 1964; Khedouri and Meister, 1965). The finding that the cyclic analog of glutamate, 1-amino-1,3-dicarboxycyclohexane (cycloglutamate), is an excellent substrate affords strong support for the hypothesis expressed above (Gass and Meister, 1968, 1970). Glutamine synthetase is inhibited irreversibly by the convulsant methionine sulfoximine. Only one of the four stereoisomers of this compound (the 2(*S*),*S*(*S*) isomer) inhibits the enzyme and is phosphorylated on the enzyme to give L-methionine-(*S*)-sulfoximine phosphate (Ronzio and Meister, 1968; Ronzio *et al.*, 1969; Rowe *et al.*, 1969; Manning *et al.*, 1969). On the other hand, both optical isomers of methionine sulfone inhibit glutamine synthetase; in this reaction, methionine sulfone is converted into a new compound which does not contain phosphate (Rowe and Meister, 1969, unpublished data).

In earlier communications from this laboratory explanations were offered for a number of the experimental observations. Thus, it was postulated that the amino acid substrate is bound to the enzyme in an extended conformation by at least three groups (α-carboxyl, γ-carboxyl, α-amino). It was shown that these three groups of D-glutamate can be made coincident with the respective groups of L-glutamate by rotating the D isomer through an angle of 69° about a line passing through carbon atoms 1, 3, and 5 (Kagan and Meister, 1966a). In the proposed active site conformation of L- and D-glutamate, the methyl groups of the enzymatically susceptible analogs lie on the same side of the molecule and are therefore presumably directed away from the enzyme. The studies on inhibition of the enzyme by methionine sulfoximine have led to the conclusion that this molecule attaches to both the enzyme sites for glutamate and ammonia, thus serving as a bifunctional reagent. Other explanations for the observed phenomena (*e.g.*, the relative rates of amide synthesis with various glutamate analogs) have also been put forth.

These conclusions and considerations have been derived largely from study of molecular (space filling and skeletal) models of the various substrates, nonsubstrates, and inhibitors. The present work represents an extension of these developments in which an attempt has been made to define by means of a computer individual points of attachment between the natural amino acid substrate (as well as analogs and inhibitors) and the active site of the enzyme. Thus, the three-dimensional coordinates of the amino acid substrates and inhibitors were calculated and an "active site" was mathematically designed. In this way an attempt has been made to achieve more quantitative explanations of the experimental observations. The results of this effort, given

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here, also support several new conclusions about the mechanism of action of glutamine synthetase.

Experimental Section

General Method. The three-dimensional coordinates of the amino acid substrates and inhibitors were calculated with an IBM 360/40 computer and plotted as stereographs on a Calcomp 1627 on-line plotter using programs written in Fortran IV (Gass, 1969).[1] Unless otherwise noted, standard tetrahedral angles (109.5°) and standard bond lengths were used (carbon–carbon, 1.54 Å; carbon–hydrogen, 1.1 Å; carbon–nitrogen, 1.47 Å; carboxyl carbon–oxygen single bond, 1.43 Å; double bond, 1.21 Å; O–C–O angle, 120°).

The basic initial assumption in these calculations is that the active site of the enzyme is constructed to interact optimally with the natural substrate, L-glutamic acid. Thus, the mathematical design of the active site is based on the assumed binding positions of specific atoms of L-glutamate. It is not assumed that the enzyme is inflexible, but the assumption is made that at some point during the course of the enzyme-catalyzed reaction, the spatial relationships between the requisite groups on the enzyme vary only within very narrow limits. Therefore the assumptions and conclusions have no necessary relevance to the state of the enzyme in the absence of substrates. It is assumed that L-glutamate is bound to the enzyme in the fully extended conformation and that the other substrate and inhibitors attach to the active site by noncovalent linkages involving the α-amino and α-carboxyl group as well as by the atoms attached to the C-5 carbon atom, or to the sulfur atom.

Summary of Calculations. The computer program was designed to calculate the coordinates of any molecule in any conformation if sufficient data are supplied to establish a unique conformation. Much of the program was designed to manipulate the coordinates of molecules; for example, rotation, translation, rotation of part of a molecule around a given bond, and finding the position of an atom such that the distance from this atom to a given point is at a minimum. The complete program listing and a discussion of the program are given elsewhere (Gass, 1969); a summary of the major calculations performed is given below.

In the calculations of the coordinates of the atoms of a molecule, each molecule is treated as a basic "carbon skeleton" with attached substituents. The first carbon atom of each molecule (C_1) is placed on the origin of the axes (0,0,0), C_3 is placed on the positive y axis, and C_2 is placed on one of the orthogonal planes. Figure 1 represents the carbon skeleton of glutamate in which C_2 has been placed on the yz plane. The triangle C_1–C_2–C_3 can be readily solved since the lengths of the two sides, A and B, and the included angle, β, are known. The y coordinate of C_3 (y_3) is equal to the length of the third side, $y_2 = A \cos \alpha$, and $z_2 = A \sin \alpha$.

The locus of the atom C_4 is a circle with center P lying in a plane perpendicular to the C_3–C_2 bond. This plane shall be referred to as the locus plane. Equation 1 is the general equation of a plane and eq 2 is the normal form of a plane where g is the perpendicular distance from the origin to the

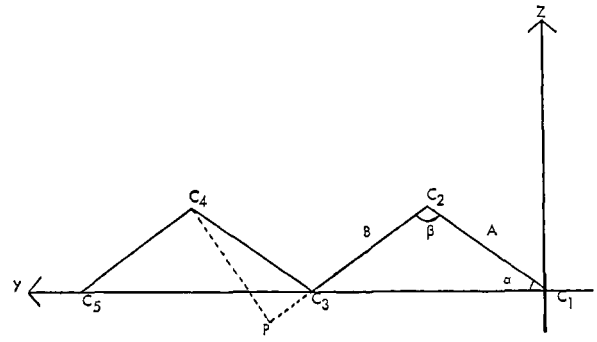

FIGURE 1: Representation of glutamate in the extended conformation (see the text).

$$Ax + By + Cz + D = 0 \quad (1)$$

$$x \cos \alpha + y \cos \beta + z \cos \gamma - g = 0 \quad (2)$$

plane, and α, β, γ are the direction angles of that perpendicular. The perpendicular distance from a point C_3 (x_3,y_3,z_3) to a plane $Ax + By + Cz + D = 0$ is given by eq 3.

$$d = |(Ax_3 + By_3 + Cz_3 + D)/(A^2 + B^2 + C^2)^{1/2}| \quad (3)$$

Since the coordinates of C_2 and C_3 are known, the direction angles can be calculated and the distance d which is the line C_3–P in Figure 1 can be found by the solution of the right triangle C_3–C_4–P. Thus, the equation of the locus plane of C_4 is

$$x \cos \alpha + y \cos \beta + z \cos \gamma + D = 0$$

where

$$D = -(x_3 \cos \alpha + y_3 \cos \beta + z_3 \cos \gamma) + d$$

To establish the position of C_4 one of its coordinates must be supplied. For example, in the extended conformation of glutamate, all of the carbon atoms are coplanar. Thus the x coordinate of each atom is zero. The plane $x = 0$ intersects the circular locus of C_4 at two points both of which are valid positions for C_4. The point having the greater z coordinate will give the extended conformation of glutamate. The succeeding carbon atoms are calculated in an identical manner.

After the coordinates of the carbon skeleton of a molecule have been calculated, the coordinates of the substituents on each carbon atom can be calculated quite simply. A substituent on C_2 in Figure 1 lies on a circle whose plane is perpendicular to the C_1–C_2 bond and on a circle whose plane is perpendicular to the C_3–C_2 bond. These two circles intersect at two points. If the bond lengths of the two substituents are equal, these two points are the coordinates of the two substituents. For glutamic acid a nitrogen atom and a hydrogen atom are attached to C_2 at different distances. In this case the calculations must be done for each substituent. If the point having the greater x coordinate is assigned to the nitrogen atom, L-glutamate will result, while the point having the smaller x coordinate will give D-glutamate.

[1] A program similar to the ORTEP Program (Johnson, 1965) was written for drawing the stereographs.

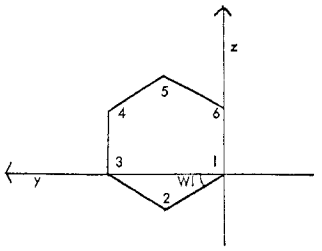

FIGURE 2: Representation of cyclohexane (see the text).

In the calculations of the coordinates of the atoms of cyclohexane (numbered as in Figure 2), the first three atoms are calculated as described above, and C_6 is then calculated by choosing a value of its x coordinate between the limits ± 1.2576. Since the positions of C_4 and C_5 of cyclohexane are dependent upon the position of the other four atoms, each x coordinate chosen for C_6 results in a unique set of conformations of the molecule.

In the calculation of the coordinates of C_4, it can be seen that the distance between C_4 and C_6 is a constant. This distance (d) can be calculated from the triangle C_4–C_5–C_6. The distance between these two atoms is given by eq 4. By conversion into polar coordinates

$$d^2 = (x_6 - x_4)^2 + (y_6 - y_4)^2 + (z_6 - z_4)^2 \qquad (4)$$

this equation can be simplified to eq 5 which is

$$x_6x_4 + y_6y_4 + z_6z_4 + (d^2 - \rho_4^2 - \rho_6^2)/2\rho_4\rho_6 = 0 \qquad (5)$$

the equation of a plane (referred to as the distance plane). In eq 5 ρ_4 is the distance from C_4 to the origin and ρ_6 is the distance from C_6 to the origin. If the axes are translated to place C_3 at the origin, ρ_4 is the C_3–C_4 bond length and ρ_6 can be calculated since the position of C_6 is known. The distance plane intersects the circular locus of C_4 at two points both of which are valid positions of C_4. These two positions of C_4 lead to two different conformations of cyclohexane resulting from the chosen position of C_6.

The coordinates of C_5 are calculated in a manner identical with that used for the calculation of C_4 since the distance from C_5 to C_6 is known. Thus, for each position of C_4 there should result two positions of C_5 for a total of four valid conformations.

Rotation of the axes is accomplished by successive planar rotations about two given axes. The coordinates of a point (x,y,z) become (x',y',z') according to the eq 6–8 by a rotation about the x axis by an angle α.

$$x' = x \qquad (6)$$

$$y' = y \cos \alpha - z \sin \alpha \qquad (7)$$

$$z' = z \cos \alpha + y \sin \alpha \qquad (8)$$

In placing glutamate, methionine sulfoximine, and the other substrates and inhibitors on the mathematically defined "active site" of the enzyme, the molecule is manipulated such that the distances between designated atoms and "enzyme sites" are

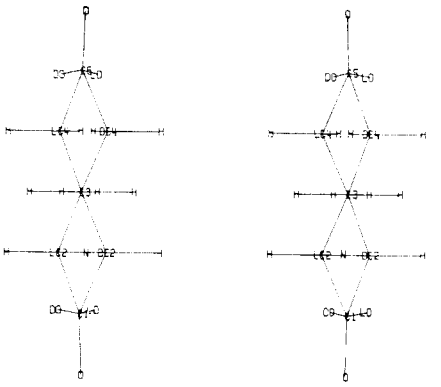

FIGURE 3: Stereograph of D- and L-glutamic acid in an extended conformation. The C-1 and C-5 carbon atoms and the C-2 nitrogen atom of D- and L- glutamate have been made coincident.

a minimum. One important restriction of such manipulations is that the molecules must remain in an extended conformation, *i.e.*, rotation about carbon–carbon bonds is not allowed. An example of this kind of manipulation is the rotation of the C_5 carboxyl group of glutamate around the C_4–C_5 bond such that the distance between an oxygen atom and a point P is a minimum. The locus of this oxygen atom is a circle around the C_5 carbon atom lying in the locus plane. It can be shown that the minimum distance between a circle with center P_1 from a point P not lying on the circle is the point of intersection of the circle and a plane passing through P_1 and P perpendicular to the plane of the circle. This plane passes through C_4 and C_5 as well as P since the locus plane is perpendicular to the C_4–C_5 bond and the center of the circle lies on the extension of this bond. The coefficients of this plane through these three points can be determined by the simultaneous solution of three equations of a plane (eq 1).

In placing the molecules into the "active site" the entire molecule is allowed to rotate about a line from the C_1 carboxyl carbon atom to the C_2 nitrogen atom.[2] Minimization of the distance from a C_5 oxygen atom to an "enzyme site" is accomplished by the simultaneous rotation of the entire molecule about this line and the rotation of the oxygen about the C_4–C_5 bond; such minimization is accomplished by successive approximation. Thus, the axes are translated to place the C_1 carboxyl carbon atom at the origin and rotated to place the C_2 nitrogen atom on the x axis. The entire molecule is then rotated to place the C_5 carbon atom well above the point P (the "enzyme site"). The oxygen atom is then placed at a minimum distance to point P by rotation about the C_4–C_5 bond and this distance is calculated. The entire molecule is then rotated downward by an increment of 1° and the oxygen atom is again placed at a minimum distance to point P. If this distance is less than the previous distance, the molecule is rotated again by 1°. This is repeated until the distance is greater than the previous distance, indicating that the point of minimum distance has been passed. The molecule is then rotated upward by two increments and the increment

[2] This line was chosen rather than one from the C-2 nitrogen atom to an oxygen atom because there is no reasonable basis at this time for making an assumption as to the positions of the C-1 carboxyl oxygen atoms.

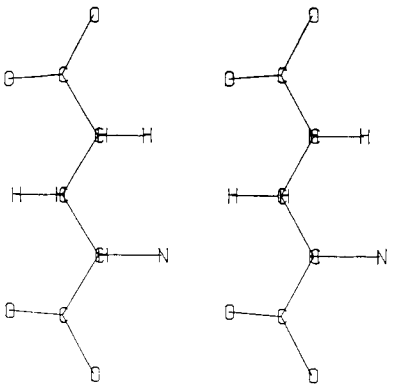

FIGURE 4: L-Glutamic acid rotated from the position shown in Figure 3 so that the plane of the paper is made the plane of symmetry between D- and L-glutamic acid.

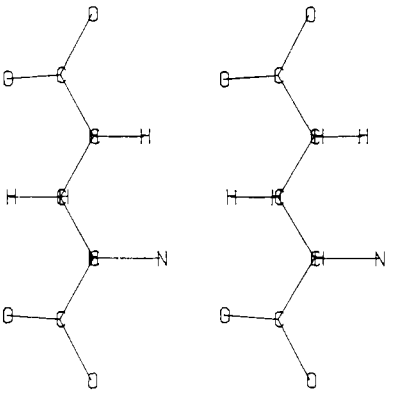

FIGURE 5: D-Glutamic acid (see Figure 4).

is divided by 10. This procedure is then repeated with 0.1° incremental rotations. These manipulations are performed repeatedly until the rotational increment is less than 10^{-8}°.

When the distances between two atoms attached to C_5 and two "enzyme sites" are minimized the procedure is the same except the distance considered is the sum of the distances between the two atoms and the two "enzyme sites." Thus, one achieves the best fit of both atoms to the respective enzyme sites.

Results

Binding of L-*Glutamate to the Active Site.* When L-glutamate is reflected across a plane containing the α- and γ-carboxyl carbon atoms and the α-amino nitrogen atom, D-glutamate is obtained (Figure 3). The three atoms that lie in the reflecting plane are common to both molecules and are coincident; thus they may bind to the same enzyme sites. As shown in Figure 3, three hydrogen atoms (α-L, *threo*-γ-L, *threo*-β-D) uniquely extend to the left, while the other hydrogen atoms extend to the right, top, or bottom. It is notable that only the three hydrogen atoms which extend to the left may be substituted by methyl groups with retention of enzymatic susceptibility. Furthermore, it is only in the extended conformation of glutamate that this difference among these hydrogen atoms is so pronounced. In the diequatorial conformation of cycloglutamate (which corresponds to the extended conformation of glutamate), the three methylene groups of the ring also extend to the left. If the reflecting plane is made the *xy* plane of a three-dimensional orthogonal coordinate system and placed in the plane of the paper these three hydrogen atoms (and the ring of *cis*-L-cycloglutamate) extend directly upward in the positive *Z* direction (Figures 4–6). It is as though the substrates bind in a cleft in the enzyme such that there is room for substitution by bulky groups only directly above, away from the enzyme.

Nucleophilic attack of a C-5 oxygen atom of glutamate upon the terminal phosphate group of ATP leads to formation of the intermediate enzyme-bound acyl phosphate. It is likely that the phosphate group of the acyl phosphate binds to a specific enzyme site and that this site binds the phosphate groups of both L- and D-γ-glutamyl phosphate. Thus, the oxygen atoms of L- and D-glutamate which are phosphorylated are, at some time during the course of the reaction, coincident in space. As indicated in Figure 3 any atom of L-glutamate coincident with an atom of D-glutamate must lie in the reflecting plane. Since the plane of the carboxyl group is different from the reflecting plane, it is possible to place one, but not both, of the oxygen atoms of each carboxyl group in the reflecting plane. The C-5 oxygen atom that extends upward in Figure 3 and to the right in Figures 4 and 5 was placed in the reflecting plane and designated as the oxygen to be phosphorylated. (As shown below, the choice of this oxygen atom is not arbitrary.) We now define the positions of the C-5 oxygen atoms of L-glutamate as enzyme binding sites; the oxygen atom lying in the reflecting plane is defined as the "phosphorylation site" (OP site), and the position of the remaining oxygen as the "oxygen binding site" (OB site). These sites are labeled "ENZ" in subsequent stereographs. There are at present no criteria useful for assignment of the positions of the C-1 carboxyl oxygen atoms, and therefore they have been arbitrarily placed.

The Tetrahedral Intermediate. We may now consider the steric requirements of the enzyme-bound tetrahedral addition compound formed in the reaction of ammonia with the acyl phosphate. It seems reasonable to assume that the oxygen atom and phosphate group of the tetrahedral intermediate will be very near their respective positions in the acyl phosphate. (If this were not so, these bonds would have to be broken as the reaction proceeded from the acyl phosphate

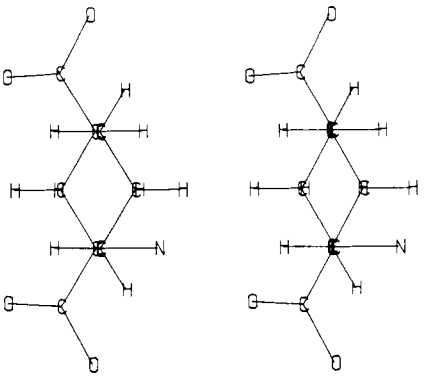

FIGURE 6: *cis*-L-Cycloglutamic acid (see Figure 4).

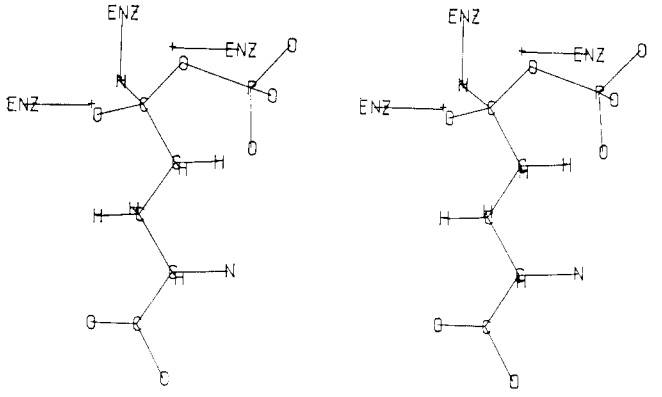

FIGURE 7: The tetrahedral addition compound formed in the reaction of L-γ-glutamyl phosphate with ammonia. Distances: O–C-5, 1.42 Å; O–P, 1.61 Å; P=O, 1.47 Å; P–OH, 1.55 Å. The angles around the C-5 carbon atom and the phosphorus atom are 109.5°. C_5–O–P angle, 115.9°.

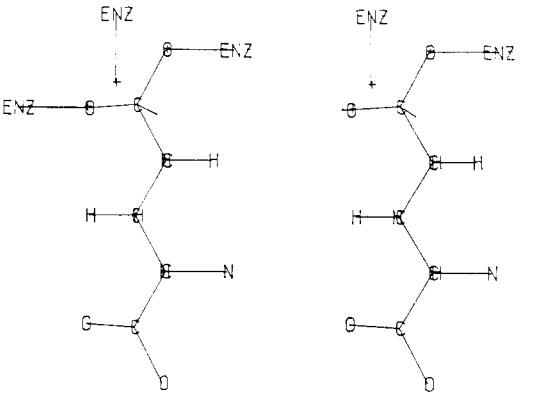

FIGURE 9: D-Glutamic acid showing the line perpendicular to the C-5 carboxyl group.

to tetrahedral intermediate.) It would thus appear that the conformation of the tetrahedral intermediate will be one in which the distances between its two oxygen atoms and the two oxygen reference points on the enzyme will be at a minimum (*i.e.*, as shown in Figure 7). The oxygen atoms of the tetrahedral intermediate are 0.42 Å and 0.25 Å from the OP site and OB site, respectively. A third reference point on the enzyme can also be defined (the central "ENZ" in Figure 7). It is likely that ammonia is bound to the enzyme at a position close to the nitrogen atom in the tetrahedral intermediate, and the nitrogen atom indicated in Figure 7 is designated as the ammonia binding site of the enzyme. The maximum rate of reaction of the carboxyl group with a nucleophile is achieved when the attack is from a position perpendicular to the plane containing the carbon atom and the two oxygen atoms of the carboxyl group (Bender, 1960). Such perpendiculars are shown in Figures 8 and 9 for L- and D-glutamate, respectively. It is notable that with L-glutamate the position of the nitrogen atom in the tetrahedral intermediate is only 4.3° from the perpendicular to the C-5 carbon atom.

Structure–Rate Relationships. We have thus constructed an "active site" based on the positions of five atoms of fully extended L-glutamate: (1) the C-1 carboxyl carbon atom, (2) the C-2 nitrogen atom, (3) the C-5 oxygen atom (OP site), (4) the other C-5 oxygen atom of L-glutamate (OB site), and (5) the ammonia binding site derived from the position of the nitrogen atom in the tetrahedral intermediate. We shall now place other substrates and inhibitors into this active site in an effort to explain experimentally observed phenomena.

Examination of Figures 8 and 9 illustrates the markedly different orientations of the carboxyl groups of L- and D-glutamate relative to the ammonia binding site of the enzyme. Thus, the line perpendicular to the C-5 carboxyl group of D-glutamate makes an angle of 39° with the perpendicular to the C-5 carboxyl group of L-glutamate. Since it would be expected that the rate of reaction would be influenced by this angle, it would be expected that D-glutamate would react with ammonia more slowly than L-glutamate; indeed, this is in general agreement with the experimental observations[3] (relative rates: L, 100; D, 27).

The nitrogen atom of the tetrahedral intermediate derived from L-glutamate is only 2.2 Å from the methyl carbon atom of *threo*-γ-methyl-L-glutamate and from the C-4 methylene carbon atom of *cis*-L-cycloglutamate. The sum of the van der Waals radii of the nitrogen atom (1.5 Å) and a methyl or methylene group (2.0 Å) is greater than the distance between the nitrogen atom of the tetrahedral intermediate and the γ-methyl and C-4 methylene groups. This would result in crowding in the tetrahedral intermediate and steric hindrance to the approach of the ammonia nitrogen atom. A slower rate of reaction would therefore be expected and has been observed (relative rates: L-glutamate, 100; *threo*-γ-methyl-L-glutamate, 27; *cis*-L-cycloglutamate, 29).

The tetrahedral intermediate derived from D-γ-glutamyl phosphate fits very well into the active site (Figure 10); the oxygen atoms are 0.14 Å and 0.25 Å from the OP site and OB site, respectively. However, the nitrogen atom is in a markedly different position from that of the L-tetrahedral intermediate (Figure 7). The distance between this nitrogen atom and the methyl carbon atom of *threo*-β-methyl-D-glutamate was calculated to be 2.4 Å, which is less than the

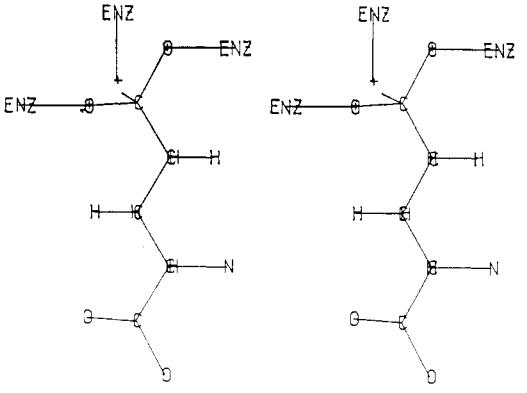

FIGURE 8: L-Glutamic acid showing the line perpendicular to the C-5 carboxyl group. The oxygen at the right is the OP site and the oxygen atom at the left is the OB site.

[3] The observed rate of D-glutamine synthesis is, based on these considerations, perhaps greater than expected, and it cannot be definitely concluded that other factors are not involved.

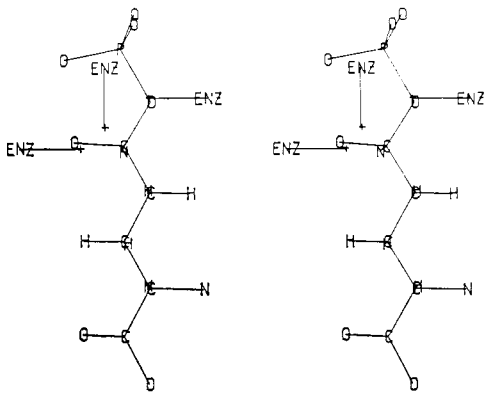

FIGURE 10: The tetrahedral addition compound formed by reaction of D-γ-glutamyl phosphate with ammonia (see Figure 7). (The position of the phosphate group is arbitrary.)

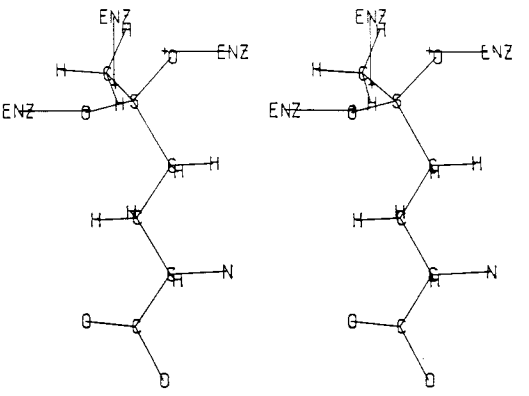

FIGURE 11: L-Methionine sulfone: distances: S–O, 1.48 Å; S–C, 1.75 Å. The angles around the sulfur atom are 190.5° (see Figure 13).

sum of the van der Waals radii (3.5 Å). These relationships were not apparent on examination of the space-filling models, but become evident from the calculations. It is clear that one would expect *threo*-β-methyl-D-glutamate to react with ammonia more slowly than does D-glutamate because of this steric hindrance; again, these considerations are in accord with the data (relative rates: D-glutamate, 27; *threo*-β-methyl-D-glutamate, 2.2).

Structure–Inhibition Relationships. The substituents of the sulfur atoms of methionine sulfone and methionine sulfoximine are arranged in an essentially tetrahedral manner, and therefore these methionine derivatives closely resemble the tetrahedral intermediate. The sulfone oxygen atoms can be placed at minimum distances from the OP and OB sites in a manner similar to that carried out with the tetrahedral intermediate. Figure 11 shows L-methionine sulfone in this relationship; the distances between the sulfone oxygen atoms and the OP site and OB site are, respectively, 0.22 and 0.06 Å. Study of an analogous presentation of D-methionine sulfone indicated that the corresponding respective distances are 0.12 and 0.48 Å. The calculations indicate that D-methionine sulfone does not fit as well as L-methionine sulfone, and this is in accord with the observation that the D isomer is not as good an inhibitor as the L isomer.

As shown in Figure 11, the methyl group of L-methionine sulfone is very close to the position of the nitrogen atom of the L-tetrahedral intermediate; it would appear that the methyl group of L-methionine sulfone must lie very close to the ammonia binding site. These considerations suggest that the site which binds the ammonia is a hydrophobic site and accordingly support the view that ammonia (rather than ammonium ion) is bound to the enzyme.

Although the geometrical arrangement of atoms about the sulfur atom of methionine sulfoximine is essentially the same as that of methionine sulfone, only one of the four isomers of methionine sulfoximine, L-methionine-(*S*)-sulfoximine, is a strong inhibitor (Manning *et al.*, 1969). Since both D- and L-methionine sulfone are inhibitors and can presumably bind to the two oxygen binding sites, it is evident that considerations other than steric must explain the inhibition pattern observed with the isomers of methionine sulfoximine. If the imine nitrogen atom of methionine sulfoximine were assumed to bind to an oxygen binding site, then by analogy with the sulfones, it would be expected that both D- and L-methionine-(*S*)-sulfoximine would be phosphorylated. Since this does not occur, a different mode of binding of the sulfoximines must be sought. Two different approaches have been examined: (1) that the imine nitrogen atom of the sulfoximine binds to the ammonia binding site, and (2) that the methyl group binds to the ammonia binding site.

(1) If one assumes that the imine group is the only group attached to the sulfur atom of methionine-sulfoximine which binds to the enzyme, it becomes apparent that both the L and D isomers of methionine-sulfoximine can bind to the ammonia binding site of the enzyme. As shown in Figure 12, when D-methionine-sulfoximine is bound in this manner, the entire molecule lies above the binding site and the imine nitrogen atom extends down into the ammonia binding site. However, since both L and D isomers can bind, this approach does not seem to explain the observed specificity of inhibition. On the other hand, it may be assumed that in addition to the binding of the imine nitrogen atom to the ammonia binding site, an oxygen atom is also bound to an enzyme site. If the

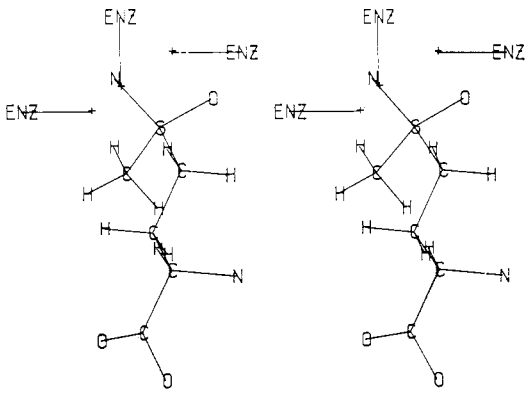

FIGURE 12: D-Methionine-(*R*)-sulfoximine. The distance between the imine nitrogen atom and the ammonia binding site has been minimized: distances: S–O, 1.48 Å; S–N, 1.56 Å; S–C, 1.75 Å. The angles about the sulfur atom are 109.5°. The O–S–N angle is reported to be 118° (Christensen *et al.*, 1969). This would increase the distance between the imine nitrogen atom and the oxygen atom by 0.12 Å.

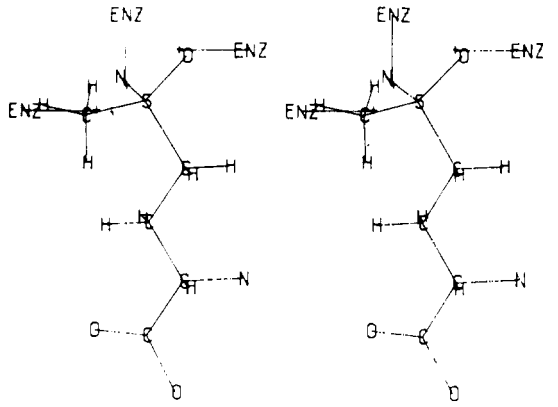

FIGURE 13: L-Methionine-(*S*)-sulfoximine. The distances between the imine nitrogen atom and the ammonia binding site and between the oxygen atom and the OP site have been minimized (see Figure 12).

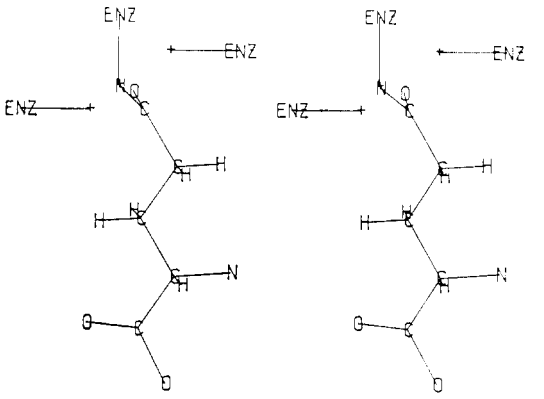

FIGURE 15: L-Glutamine. The distance between the amide nitrogen atom and the ammonia binding site has been minimized: distances: C–N, 1.28 Å, C=O, 1.27 Å. The angle N–C–O is 123°.

sum of the distances between the nitrogen and oxygen atoms and their respective enzyme sites is minimized, it may be calculated that the imine nitrogen atom of L-methionine-(*S*)-sulfoximine is 0.25 Å from the ammonia binding site and the oxygen atom is 0.22 Å from the oxygen binding site (Figure 13). The respective distances from the ammonia binding site and oxygen sites for D-methionine-sulfoximine are 0.55 Å and 0.13 Å. It appears unlikely, however, that L-methionine-(*R*)-sulfoximine could bind, since considerable steric interaction would be expected between the methyl group and the terminal phosphate moiety of ATP, which must be bound at a position close enough to react with the C-5 oxygen of L-glutamate. Furthermore, as indicated in Figure 13 it seems improbable that there would be sufficient room for the methyl group of L-methionine-(*S*)-sulfoximine in the vicinity of the OB site. In addition, the oxygen atom of L-methionine-(*S*)-sulfoximine would be very close to the OP site, and therefore this line of reasoning would suggest that the oxygen atom would be phosphorylated; it is, however, the nitrogen atom which is phosphorylated.

In summary, therefore, the postulate that the sulfoximine imine group binds to the ammonia binding site does not seem to offer a satisfactory explanation for the observed specificity of inhibition. If only the imine moiety binds, it would seem that both D and L isomers of methionine-sulfoximine could bind almost equally well. If in addition, an oxygen atom binds, the difference between the fit of the D and L isomers is very small and there would seem to be steric hindrance to the binding of the isomer known to inhibit. Furthermore, the observed phosphorylation of the imine nitrogen atom would not be explained.

(2) In an alternative approach, we may assume that the sulfoximine oxygen atom of methionine sulfoximine binds to an oxygen site on the enzyme and that the methyl group binds to the ammonia binding site as in the case of L-methionine sulfone. In this arrangement, L-methionine-(*S*)-sulfoximine is bound so that the sulfoximine nitrogen atom is in an excellent position to be phosphorylated (Figure 14). Indeed, L-methionine-(*S*)-sulfoximine fits well into these sites; the nitrogen atom of the imine group lies close to the OP site (0.24 Å), the oxygen atom is 0.06 Å from the OB site, and the methyl carbon atom is 0.02 Å from the methyl carbon atom of L-methionine sulfone. It can now be seen that the oxygen atom of L-glutamate which was chosen as the site of phosphorylation can no longer be considered an arbitrary choice; consideration of the steric arrangement of L-methionine-(*S*)-sulfoximine requires that this oxygen atom of L-glutamate be bound to the OP site. If the sulfoximine oxygen atoms of the D isomers of methionine-sulfoximine are placed at a minimum distance from the OB site, and the carbon atom of the methyl group is placed at a minimum distance from the ammonia binding site, the methyl carbon atom can be placed no closer than 0.9 Å from the position of the methyl carbon atom of L-methionine sulfone; these considerations are in accord with the observation that the D-methionine-sulfoximine isomers are not bound. It is evident that L-methionine-(*R*)-sulfoximine can bind in a manner analogous to that postulated for L-methionine-(*S*)-sulfoximine (Figure 14); in this case the positions of the sulfoximine nitrogen and oxygen atoms are reversed and the sulfoximine oxygen atom is close to the OP site. In this arrangement it is difficult to see how the nitrogen atom could be phosphorylated; on the other hand, one might expect phosphorylation of the oxygen atom. However, since this has not been observed, we must

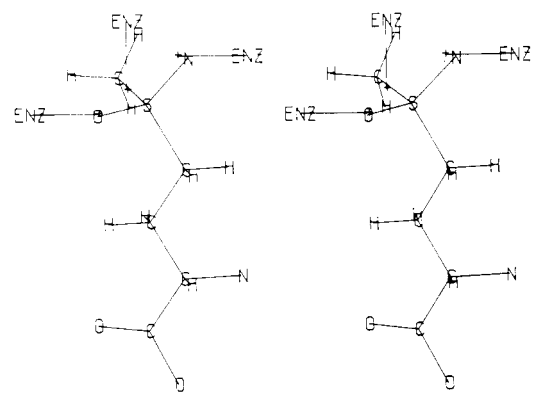

FIGURE 14: L-Methionine-(*S*)-sulfoximine. The distances have been minimized between the oxygen atom and the OB site and between the methyl carbon atom and the methyl carbon atom of L-methionine sulfone in the conformation shown in Figure 11 (see Figure 12).

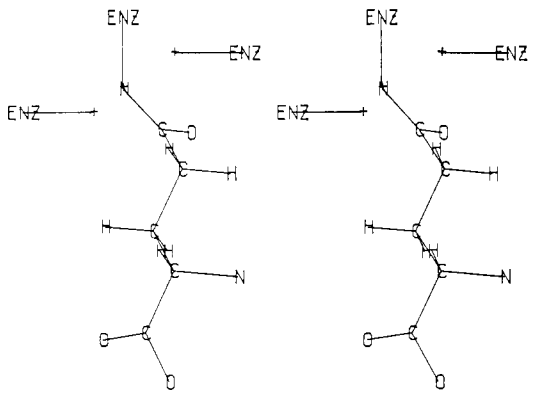

FIGURE 16: D-Glutamine. The distance between the amide nitrogen atom and the ammonia binding site has been minimized. For distances and angles see Figure 15.

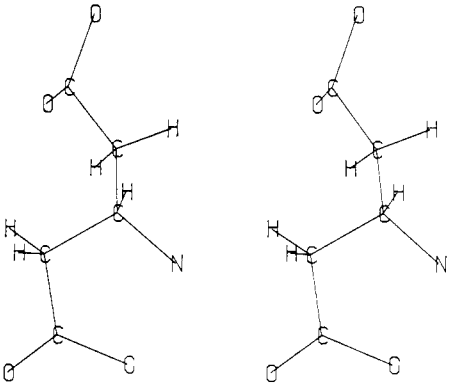

FIGURE 17: β-Glutamic acid in a conformation which would lead to the formation of D-β-glutamine.

consider the possibilities that the oxygen atom of methionine sulfoximine is too acidic to react with the terminal phosphate of ATP, that the two oxygen binding sites are sufficiently different so that the oxygen atom of the (*R*) isomer cannot bind to the OP site (although that of the (*S*) isomer can bind to the OB site), or that the imine group of the (*R*) isomer is repelled by the OB site.

Binding of Glutamine. In considering the reversal of synthesis and the transferase reaction, one would expect that glutamine might fit into the active site with its amide nitrogen atom in the ammonia binding site and its oxygen atom in the OB site. In this position, formation of the tetrahedral intermediate from glutamine and phosphate would be analogous to its formation from γ-glutamyl phosphate and ammonia. However, the restriction that the α-nitrogen atom and the C-1 carboxyl carbon atom must remain fixed prevents such a fit. The amide nitrogen atom can be placed into the ammonia binding site as shown in Figure 15, but in this conformation the oxygen atom of the amide group is 1.08 Å from the OB site and 1.30 Å from the OP site. The distance between the C-5 carbon atom of L-glutamine and the oxygen atom of the phosphate group is only 1.25 Å. Binding of glutamine in this conformation in the presence of phosphate would therefore lead to severe van der Waals interactions, which may well account for the very high K_m value of L-glutamine in the transferase reaction (Wellner, 1963), and for the greatly decreased binding of L-glutamine in the presence of phosphate (Krishnaswamy *et al.*, 1962).

D-Glutamine can also bind with its amide nitrogen atom in the ammonia site (Figure 16), but in this conformation the entire molecule lies above the binding sites with the amide nitrogen atom extending into the ammonia site. These considerations suggest that reaction with phosphate would be improbable, and this is consistent with the observation that D-glutamine is an extremely poor substrate in reversal of synthesis in the transferase reaction (Levintow and Meister, 1953, 1954).

If *threo*-γ-methyl-L-glutamine and *cis*-L-cycloglutamine were bound to the enzyme in a manner analogous to that of L-glutamine shown in Figure 19 below, the distance between the amide nitrogen atom and the methyl group of γ-methylglutamine or the C-4 methylene group of cycloglutamine would be 2.7 Å. This is 0.8 Å less than the sum of the van der Waals radii of the nitrogen atom (1.5 Å) and the methyl or methylene group (2.0 Å). This is in accord with the findings that neither of these analogs is a substrate in the transferase reaction.

β-Glutamate. Optically inactive β-glutamate forms only D-β-glutamine in the enzyme-catalyzed reaction. It is possible for this compound to attain a conformation in which the distance between the C-1 carboxyl carbon atom and the C-3 amino nitrogen atom is only 0.09 Å greater than the distance between the C-1 carboxyl carbon atom and the 2-amino nitrogen atom of L-glutamate. Thus, it is possible for β-glutamate to bind to the enzyme with its C-1 carboxyl carbon atom coincident with that of L-glutamate and its 3-amino nitrogen atom only 0.09 Å from the 2-amino binding site. In this conformation the distance between the C-5 oxygen atom of β-glutamate and the OP site is 0.43 Å.

Figure 17 shows the conformation of β-glutamate which would lead to the formation of D-β-glutamine. It may be seen that the C-4 carbon atom extends upward in the positive *z* direction. Figure 18 shows the conformation of β-glutamate which would result in the formation of L-β-glutamine; here the C-4 carbon atom extends in the negative *z* direction. We have shown above that there is steric hindrance by the enzyme in this direction; therefore β-glutamate would not be expected to bind in this conformation. Thus, only the conformation shown in Figure 17 can bind and yield D-β-glutamine.

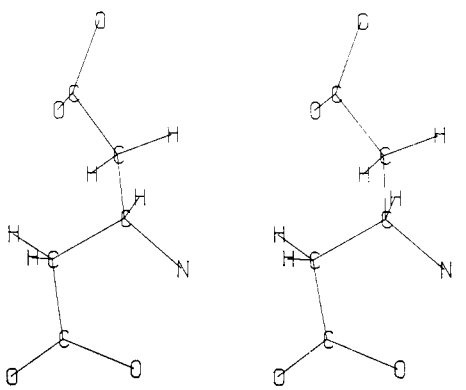

FIGURE 18: β-Glutamic acid in a conformation which would lead to the formation of L-β-glutamine.

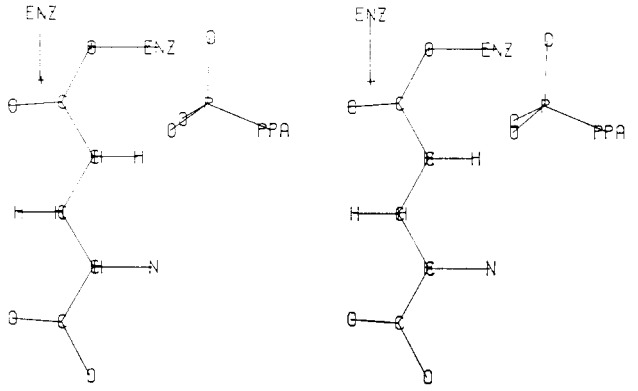

FIGURE 19: L-Glutamic acid showing the possible position of ATP. "PPA" represents the ADP portion of the ATP molecule. For the dimensions of the phosphate group see Figure 7.

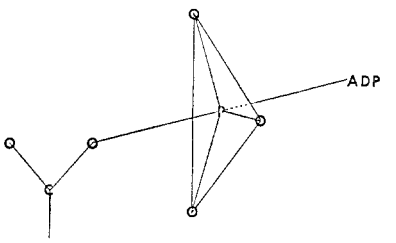

FIGURE 20: The trigonal-bipyramidal intermediate in the phosphorylation reaction (see the text).

Discussion

The present approach supports previously published considerations relating to the mapping of the active site of glutamine synthetase, and also leads to additional new conclusions. Thus, the mathematical treatment presented here, which rests on substantially the same postulates as originally stated (Kagan *et al.*, 1965), defines an active site equipped with two carboxyl and one amino group binding sites that can interact with L- and D-glutamate, *threo*-β-methyl-D-glutamate, α-methyl-L-glutamate, *threo*-γ-methyl-L-glutamate, β-glutamate, *cis*-L-cycloglutamate, L-methionine-(*S*)-sulfoximine, and L- and D-methionine sulfone, but not with a number of other closely related molecules. The collected evidence is in accord with an active site to which one portion of the substrate or analog can attach; the remainder of the molecule, which may have relatively bulky substituents, extends away from the enzyme. The calculations permit the selection of a phosphorylation site (by virtue of the coincidence in the positions of one of the γ-carboxyl oxygen atoms of L- and D-glutamate), and of an ammonia-binding site on the enzyme (derived from the calculated position of the nitrogen atom of the tetrahedral intermediate). The calculations indicate that the methyl groups of methionine sulfone and methionine sulfoximine attach to the ammonia-binding site of the enzyme, suggesting that this site, probably hydrophobic, is designed for un-ionized ammonia rather than the ammonium ion. The earlier suggestion that the sulfoximine nitrogen atom of methionine sulfoximine binds to this site (Ronzio and Meister, 1968) now seems much less likely. The present work also provides detailed steric explanations for the more rapid synthesis of L-glutamine as compared with D-glutamine, and for the relatively low rates of amide synthesis from *threo*-β-methyl-D-glutamate and *threo*-γ-methyl-L-glutamate, and *cis*-L-cycloglutamate; explanations for the specificity of inhibition by the methionine derivatives are also given.

The calculations are in accord with the conclusion that methionine sulfone and methionine sulfoximine are analogs of the tetrahedral intermediate rather than of glutamate. The arrangement of the atoms about the sulfur atoms of the methionine derivatives is essentially tetrahedral and closely resembles the geometry of the tetrahedral intermediate. Indeed it is unlikely that the active site of an enzyme can provide optimal binding for both the reactants and the products of a reaction; however, the site may be arranged as to provide optimal affinity for the transition state of an enzyme-catalyzed reaction. Thus, resistance to change in the structure of the active site would produce strain in the reactants upon binding (Jencks, 1966, 1969). Such strain would bring the substrate part of the way along the reaction coordinate toward the transition state. As shown here, it is impossible for the tetrahedral intermediate to fit exactly into the binding site for glutamate because the bond lengths and angles of the two molecules are different (Figure 7). If the active site were arranged to provide optimum binding for the oxygen atoms of the tetrahedral intermediate, L-glutamate would be strained on binding and this strain would tend toward the direction of the tetrahedral intermediate, thus accelerating its formation and facilitating the overall reaction.

The findings concerning the relative positions of phosphate and glutamate lead to considerations about the reaction of glutamate with the terminal phosphate group of ATP to form γ-glutamyl phosphate (Figure 19). It would seem that if two atoms are bound to the enzyme at a distance equal to or greater than the sum of their van der Waals' radii, then either the enzyme must move or the bonds to the enzyme must be broken when these atoms react and move to within their covalent bond distance. If on the other hand the active site binds the two atoms at a distance equal to their covalent bond length, then strain would be induced due to van der Waals' forces. The sum of the van der Waals' radii of the oxygen atom (1.4 Å) and the phosphorus atom (1.9 Å) is 3.3 Å. The resultant bond in the acyl phosphate and in the tetrahedral intermediate is about 1.6 Å (as reported for the O–P bond of phosphoserine (McCallum *et al.*, 1959)). If these atoms bind at their van der Waals' distance, they must move 1.7 Å toward each other. Since the synthesis of glutamine is reversible, we must account not only for the formation of the acyl phosphate from glutamate and ATP but also for the reaction of the acyl phosphate with ADP to form ATP in the reverse reaction. The 1.7 Å movement of the phosphate group would be toward glutamate in the forward reaction and toward ADP in the reverse direction. The reaction of the terminal phosphate group of ATP with a nucleophile such as the carboxyl oxygen atom of glutamate probably results in an inversion of the phosphate group by analogy with similar nonenzymatic reactions (Green and Hudson, 1963; see also Westheimer, 1968). In such an inversion the phosphate group passes through a trigonal-bipyramidal intermediate with the entering and leaving oxygen atoms at the apical positions and the phosphorus atom and remaining three oxygen atoms in the basal plane (Figure 20). To postulate

that the active site is designed to accelerate this reaction by straining the phosphate group toward this intermediate requires that the oxygen-binding sites favor the oxygen positions in the basal plan where the O–P–O angles are about 120°. It is difficult to design such an active site which will accelerate the reaction in both directions if the phosphate group must move 1.7 Å during the course of the reaction. However, if the oxygen atoms of the phosphate group remain fixed to sites on the enzyme, the inversion of the phosphate group causes the phosphorus atom to move approximately 1 Å toward the attacking oxygen atom. Moreover, the oxygen atom of L-glutamate moves 0.4 Å toward the phosphate group of ATP in going from glutamate to the tetrahedral intermediate.

If the ATP were bound as shown in Figure 19 such that the distance between the oxygen atom of L-glutamate and the phosphorus atom of ATP were equal to the sum of their van der Waals' radii (3.3 Å), and if the phosphate of the resulting tetrahedral intermediate were in the position shown in Figure 7, the inversion of the phosphate group combined with the movement of the oxygen atom of L-glutamate toward the position of the tetrahedral intermediate would bring the two atoms to within 0.3 Å of the required covalent bond distance (1.6 Å) without additional movement of the reactants or the enzyme. The final distance between the phosphorus atom of the tetrahedral intermediate and the oxygen of ADP is 3.0 Å assuming a shortening of the P–O bond from 1.6 Å to 1.5 Å in the P=O bond (Gurskaya, 1968). This distance is 0.3 Å less than the van der Waals' distance. The van der Waals' radius of an atom in a direction which makes a small angle with the direction of a covalent bond to that atom is smaller than in other directions. If the angle is less than 35°, the reduction in effective radius is about 0.5 Å (Pauling, 1948). The line from the oxygen atom of the tetrahedral intermediate to the phosphorus atom of ATP forms an angle of 54.7° with the bond between this phosphorus atom and the three oxygen atoms bound to it. While this angle is greater than 35°, the combined smaller effects of such an angle with the three bonds to the phosphorus atom may significantly reduce the effective van der Waals' radius of the phosphorus atom such that there would be little resistance to the binding of an oxygen atom at 3.0 Å or less. Thus, the possibility exists that glutamate and ATP may bind without van der Waals' interactions, and that by a "proximity by inversion," the phosphorus atom may move to the covalent bond distance without translation of the oxygen atoms attached to it. It is possible then to picture an active site designed so as to spread the oxygen atoms of the phosphate group toward the 120° angle of the intermediate; this would facilitate the reaction.

It is tempting to offer additional speculation concerning the mechanism of glutamine synthesis and the other reactions catalyzed by the enzyme, *e.g.*, formation of pyrrolidonecarboxylate. However, further information is clearly required; for example, the very low rate of pyrrolidonecarboxylate formation in the absence of ammonia (Krishnaswamy *et al.*, 1962) may become more explicable after the rate of release of the acyl phosphate from the enzyme and the rate of its cyclization are experimentally determined.

The mathematical approach used here seems to offer new insight into both the steric relationships between the reactants at the active site of glutamine synthetase and certain aspects of the reaction mechanism. It should be emphasized that the conclusions rest on the assumptions made and must therefore remain as tentative. However, it seems possible to put at least some of the findings to experimental test. It will be of special importance to integrate them with data about the chemical structure of the active site of the enzyme when such information becomes available. In this respect an approach of the type developed here would be expected to be particularly useful in the study of enzymes whose detailed structures are known.

References

Bender, M. L. (1960), *Chem. Rev. 60*, 53.

Christensen, B. W., Kjaer, A., Neidle, S., and Rogers, D. (1969), *Chem. Commun.*, 169.

Gass, J. D. (1969), Doctoral Dissertation, Department of Biochemistry, Cornell University Medical School, New York, N. Y.

Gass, J. D., and Meister, A. (1968), paper presented at the 155th National Meeting of the American Chemical Society, San Francisco, Calif., April 1, 1968, Abstract No. 5.

Gass, J. D., and Meister, A. (1970), *Biochemistry*, submitted for publication.

Green, M., and Hudson, R. F. (1963), *J. Chem. Soc.*, 540.

Gurskaya, G. V. (1968), The Molecular Structure of Amino Acids, Consultants Bureau, New York, N. Y., p 45.

Jencks, W. P. (1966), *in* Current Aspects of Biochemical Energetics, Kaplan, N. O., and Kennedy, E. P., Ed., New York, N. Y., Academic, p 290.

Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N. Y., McGraw-Hill, p 282.

Johnson, C. K. (1965), ORTEP: A Fortran Thermal-Ellipsoid Plot Program for Crystal Structure Illustrations, Oak Ridge National Laboratory.

Kagan, H. M., Manning, L. R., and Meister, A. (1965), *Biochemistry 4*, 1063.

Kagan, H. M., and Meister, A. (1966a), *Biochemistry 5*, 725.

Kagan, H. M., and Meister, A. (1966b), *Biochemistry 5*, 2423.

Khedouri, E., and Meister, A. (1965), *J. Biol. Chem. 240*, 3357.

Khedouri, E., Wellner, V. P., and Meister, A. (1964), *Biochemistry 3*, 824.

Krishnaswamy, P. R., Pamiljans, V., and Meister, A. (1962), *J. Biol. Chem. 237*, 2932.

Levintow, L., and Meister, A. (1953), *J. Amer. Chem. Soc. 75*, 3039.

Levintow, L., and Meister, A. (1954), *J. Biol. Chem. 209*, 265.

Manning, J. M., Moore, S., Rowe, W. B., and Meister, A. (1969), *Biochemistry 8*, 268.

McCallum, G. H., Robertson, J. M., and Sim, G. A. (1959), *Nature 184*, 1863.

Meister, A. (1968), *Advan. Enzymol. Rel. Areas Mol. Biol. 31*, 183.

Pauling, L. (1948), The Nature of the Chemical Bond, Cornell University Press, Ithaca, New York, p 192.

Ronzio, R. A., and Meister, A. (1968), *Proc. Nat. Acad. Sci. U. S. 59*, 164.

Ronzio, R. A., Rowe, W. B., and Meister, A. (1969), *Biochemistry 8*, 1066.

Rowe, W. B., Ronzio, R. A., and Meister, A. (1969), *Biochemistry 8*, 2674.

Wellner, V. P. (1963), Doctoral Dissertation, Department

of Biochemistry, Tufts University School of Medicine, Boston, Mass., p 48.

Wellner, V. P., and Meister, A. (1966), *Biochemistry 5*, 872.
Westheimer, F. H. (1968), *Accounts Chem. Res. 1*, 70.

Intramolecular Catalysis of Sulfate Ester Hydrolysis. A Model for Aryl Sulfate Sulfohydrolase*

Stephen J. Benkovic† and Leonard K. Dunikoski, Jr.

ABSTRACT: The hydrolysis of 2-(4(5)-imidazolyl)phenyl sulfate (I) was studied as a possible model for aryl sulfate sulfohydrolase. The pH–rate profile may be divided into two regions: (1) at pH 1–3 the compound undergoes conventional A-1 acid-catalyzed hydrolysis, (2) at pH 4–7 the neighboring imidazole moiety catalyzes the reaction. The compound which would result from intramolecular nucleophilic attack by imidazole, 4(5)-(2′-hydroxyphenyl)imidazole *N*-sulfate, was synthesized and its solvolytic behavior compared with the initial substrate. Although trapping experiments designed to detect the intermediacy of the *N*-sulfate do not entirely exclude that mode of catalysis, the hydrolysis of I at pH 4–7 appears to involve mainly intramolecular general acid catalysis. Comparison of I with salicyl sulfate and other compounds capable of hydrogen bonding indicates rate accelerations can be rationalized in terms of transition state stability as manifested by ΔpK_a for the corresponding phenolic products.

The arylsulfatase enzymes which catalyze the hydrolysis of aryl sulfates have been found in several microorganisms and in higher plants and mammals (Roy, 1960, and references therein). Although the arylsulfatases A and B of mammalian liver have been extensively studied (Roy, 1960, and references therein), the physiological functions of these aryl sulfatases remain unknown. Recent *in vitro* searches for natural substrates have led to the postulation that cerebroside 3-sulfate may be degraded *in vivo* by lysosomal arylsulfatases (Mehl and Jatzkewitz, 1968), but as yet there is only indirect evidence (Choy and Cravioto, 1968). The function of a related but distinct class of enzymes, the steroidal sulfatases, is more visible since their importance in the *in vivo* synthesis of estrogens *via* the simple hydrolysis of estrogen sulfates during pregnancy (Benagiano *et al.*, 1967) and in the normal state (Sandberg and Jenkins, 1966) has been demonstrated.

The nature of the catalytic groups at the active site of these enzymes is unknown. Detailed studies with the sulfatase of *A. metalcaligenes* (Dodgson, *et al.*, 1956) have revealed that both the rate of hydrolysis and binding of substrate to the enzyme are increased by the introduction of electron withdrawing substituents in the aryl sulfates, and that the O–S bond of the aryl sulfate is cleaved (Spencer, 1958). A recent study of the action of group-specific protein reagents on sulfatase A of ox liver (Jerfy and Roy, 1969) has shown that neither SH groups nor amino groups are involved in the hydrolysis reaction but that tyrosyl residues are essential for activity of the enzyme, which is inactivated by treatment with *N*-acetylimidazole or with tetranitromethane. Histidyl residues are implicated in the mechanism of action by spontaneously reversible inactivation of the enzyme with acetic anhydride and inactivation by photooxidation in the presence of Rose Bengal.

Some insight into the reaction catalyzed by sulfatase A might be gained by studying the hydrolysis of a molecular model which incorporates the essential features of tyrosyl and histidyl residues. For this reason, we have studied the hydrolysis of 2-(4(5)imidazolyl)phenyl sulfate.

Experimental Section

4-(2′-Hydroxyphenyl)imidazole was prepared by the method of Pandit and Bruice (1960), mp 179–180° uncorrected. *Anal.* Calcd for $C_9H_8N_2O$: C, 67.55; H, 5.04; N, 17.51. Found: C, 67.30; H, 5.07; N, 17.44. Selective sulfation of the phenolic oxygen or the imidazole nitrogen depends upon the conditions employed.

2-(4(5)-Imidazolyl)phenyl Sulfate. 4-(2′-Hydroxyphenyl)-imidazole (1.0 g, 6.2 mmoles) and fresh pyridine–sulfur trioxide complex (1.0 g, 6.2 mmoles) were dissolved in 20 ml of anhydrous dimethylformamide in a 35-ml round-bottom flask fitted with a drying tube, and warmed at 80°. After 3.5 hr, the solution was cooled in an ice bath and poured into 250 ml of ice water, and 5% KOH added dropwise to bring the solution to pH 8. Saturated barium hydroxide solution was added dropwise until all inorganic sulfate precipitated, which was filtered off and the filtrate washed with two 200-ml portions of ether. The aqueous phase was concentrated to 50 ml under vacuum, passed through an NH_4^+ form of a Rexyn-101 (H^+) column, and concentrated under vacuum to yield an orange liquid.

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