ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF MOLECULAR BIOLOGY

F. F. Nord, Founding Editor

AMINO ACID METABOLISM, Part A

Volume 72

Edited by DANIEL L. PURICH
University of Florida College of Medicine
Gainesville, Florida

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PUBLISHER'S FOREWORD

Many of the most notable achievements in the molecular life sciences were initiated by enzymologists. The assertion of Hopkins that cell life is "an ordered sequence of events governed by specific catalysts" has become a truism in the analytical investigation of all life processes. The field of enzymology has grown by leaps and bounds over the past half-century, and with explosive growth of molecular and cell biology, new enzymes and enzymatic activities are still routinely found. Terms such as "ribozyme," "abzyme" (or catalytic antibodies), "molecular motors" (e.g., dynein and kinesin), and "polyprotein proteinases" have entered the biochemical literature in just the past decade or so.

Professor F. F. Nord began *Advances in Enzymology* in 1941 after he had been appointed Professor of Biochemistry at Fordham University. *Advances* is the successor to *Ergebnisse der Enzymforschung*, a periodic review series started in 1930, while he was still a chemistry professor in Berlin. His idea was to identify areas of enzymology that had undergone significant recent growth. He then sought out expert opinions of others to find an appropriate author to communicate the nature of those strides and to illustrate how these findings could be of broader interest.

This tradition of excellence was maintained by Professor Alton Meister who, with the pubication of Volume 35, succeeded Dr. Nord as the series editor. A man of enormous intellect and awareness of the discipline, Dr. Meister produced 37 volumes of *Advances in Enzymology*. He was proud of the chapters in this series, not so much as a testimony to his good judgment (though certainly his instincts and insights about enzymology were always on the mark), but as contributions of intrinsic worth to other practitioners of a field that has sprung up around enzyme catalysis and regulation. His last effort appeared as Volume 71 about a year after his untimely death in 1995.

The series will now be continued by Daniel L. Purich, Professor of Biochemistry and Molecular Biology at the University of Florida College of Medicine.

New York, New York
March 1998

John Wiley & Sons, Inc.

PREFACE

Advances in Enzymology is now nearing its seventh decade as the leading periodic and authoritative review of the latest scientific achievements in enzymology. This field concerns itself with the multifaceted nature of enzymes—their reaction properties; their kinetic behavior; their catalytic mechanisms; their regulatory interations; their expression from genes; their zymogen and other storage forms; their mutant forms (both naturally occurring and man-made); their associated pathophysiology; and their virtually limitless use in agriculture, nutrition, biomedicine, and biotechnology. As the new editor of this series, I shall endeavor to maintain the standard of excellence so masterfully established by Dr. Nord and so skillfully pursued by the late Alton Meister.

The value of *Advances in Enzymology* ultimately will only endure if practicing enzymologists judge its chapters to add value to their own pursuits. Accordingly, this volume marks the advent of several changes that should appeal to its readers. This and many subsequent volumes will be thematically organized so that readers of one chapter will be more likely to be interested in several or all of the other contributions. This monograph-within-a-monograph approach places added burdens on the contributors and editor for the timely appearance of each volume, but the final product should provide broader and more integrated perspective on any particular topic. Another new feature will be the inclusion of an abstract describing the scope and content of each chapter (the abstracts can be found in the front matter of the volume). While this was previously an unnecessary feature of earlier volumes in this series, abstracts allow users of information retrieval services to identify material of interest. In the future we may also be able to provide such information in advance of the actual publication date, thereby minimizing the lag time between a volume's first appearance and wider public awareness of its contents.

In seeking to present readers with the latest and most accurate information about enzyme action, I invite other enzymologists to contact me about their interests, their criticisms, and above all their ideas for improving the impact of this series.

Gainesville, Florida
March 1998

DANIEL L. PURICH

ABSTRACTS

Advances in the Enzymology of Glutamine Synthesis

DANIEL L. PURICH

Meister's proposal of a γ-glutamyl-P intermediate in the glutamine synthetase reaction set the scene for understanding how the stepwise activation of the carboxyl group greatly increased its susceptibility toward nucleophilic attack and amide bond synthesis. Topics covered in this review include: the discovery of the enzymatic synthesis of glutamine; the role of glutamine synthetase in defining the thermodynamics of ATPases; early isotopic tracer studies of the synthetase reaction; the proposed intermediacy of γ-glutamyl-phosphate; the mechanism of methionine sulfoximine inhibition; stereochemical mapping of the enzyme's active site; detection of enzyme reaction cycle intermediates; borohydride trapping of γ-glutamyl-P; positional isotope exchanges catalyzed by glutamine synthetase; regulation of bacterial enzyme; and a brief account of how knowledge of the atomic structure of bacterial glutamine synthetase has clarified ligand binding interactions. Concluding remarks also address how the so-called "Protein Ligase Problem" may be solved by extending the catalytic versatility of carboxyl-group activating enzymes.

Hepatic Glutamine Transport and Metabolism

DIETER HÄUSSINGER

Although the liver was long known to play a major role in the uptake, synthesis, and disposition of glutamine, metabolite balance studies across the whole liver yielded apparently contradictory findings suggesting that little or no net turnover of glutamine occurred in this organ. Efforts to understand the unique regulatory properties of hepatic glutaminase culminated in the conceptual reformulation of the pathway for glutamine synthesis and turnover, especially as regards the role of sub-acinar distribution of glutamine synthetase and glutaminase. This chapter describes these processes as well as

the role of glutamine in hepatocellular hydration, a process that is the consequence of cumulative, osmotically active uptake of glutamine into cells. This topic is also examined in terms of the effects of cell swelling on the selective stimulation or inhibition of other far-ranging cellular processes. The pathophysiology of the intercellular glutamine cycle in cirrhosis is also considered.

Enzymes Utilizing Glutamine as an Amide Donor

HOWARD ZALKIN AND JANET L. SMITH

Amide nitrogen from glutamine is a major source of nitrogen atoms incorporated biosynthetically into other amino acids, purine and pyrimidine bases, amino-sugars, and coenzymes. A family comprised of at least sixteen amidotransferases are known to catalyze amide nitrogen transfer from glutamine to their acceptor substrates. Recent fine structural advances, largely as a result of X-ray crystallography, now provide structure-based mechanisms that help to explain fundamental aspects of the catalytic and regulatory interactions of several of these aminotransferases. This chapter provides an overview of this recent progress made on the characterization of amidotransferase structure and mechanism.

Mechanistic Issues in Asparagine Synthetase Catalysis

NIGEL G. J. RICHARDS AND SHELDON M. SCHUSTER

The enzymatic synthesis of asparagine is an ATP-dependent process that utilizes the nitrogen atom derived from either glutamine or ammonia. Despite a long history of kinetic and mechanistic investigation, there is no universally accepted catalytic mechanism for this seemingly straightforward carboxyl group activating enzyme, especially as regards those steps immediately preceding amide bond formation. This chapter considers four issues dealing with the mechanism: (a) the structural organization of the active site(s) partaking in glutamine utilization and aspartate activation; (b) the relationship of asparagine synthetase to other amidotransferases; (c) the way in which ATP is used to activate the β-carboxyl group; and (d) the detailed mechanism by which nitrogen is transferred.

Mechanisms of Cysteine *S*-Conjugate β-Lyases

ARTHUR J. L. COOPER

Mercapturic acids are conjugates of *S*-(*N*-acetyl)-*L*-cysteine formed during the detoxification of xenobiotics and during the metabolism of such endogenous agents as estrogens and leukotrienes. Many mercaturates are formed from the corresponding glutathione *S*-conjugates. This chapter focuses on (a) the discovery of the cysteine *S*-conjugate β-lyases; (b) the involvement of pyridoxal-5-phosphate; (c) the influence of the electron-withdrawing properties of the group attached to the sulfur atom; and (d) the potential of cysteine *S*-conjugates as pro-drugs.

γ-Glutamyl Transpeptidase: Catalytic Mechanism and Gene Expression

NAOYUKI TANIGUCHI AND YOSHITAKA IKEDA

The γ-glutamyl transpeptidases are key enzymes in the so-called γ-glutamyl cycle involving glutathione synthesis, the recovery of its constituents, and in the transport of amino acids. This membrane-bound ectoenzyme thus serves to regulate glutathione synthesis. This chapter deals with the active site chemistry of γ-glutamyl transpeptidase, including the role of side-chain groups on the light subunit as well as several serine residues in the catalytic process. Also considered are genomic studies indicating (a) the presence of a single gene in mouse and rat; (b) the occurrence of multiple genes in humans; (c) the involvement of multiple promoters for gene expression; and (d) how these multiple promoters may play a role in the tissue-specific expression of γ-glutamyl transpeptidases.

Enzymology of Bacterial Lysine Biosynthesis

GIOVANA SCAPIN AND JOHN S. BLANCHARD

Bacteria have evolved three strategies for the synthesis of lysine from aspartate via formation of the intermediate diaminopimelate (DAP), a metabolite that is also involved in peptidoglycan formation. The objectives of this chapter are descriptions of mechanistic studies on the reactions catalyzed by dihydrodipicolinate synthase,

dihydrodopicolinate reductase, tetrahydrodipicolinate *N*-succinyltransferase, *N*-succinyl-*L,L*-DAP aminotransferase, *N*-succinyl-*L,L*-DAP desuccinylase, *L,L*-DAP epimerase, *L,L*-DAP decarboxylase, and DAP dehydrogenase. These enzymes are discussed in terms of kinetic, isotopic, and X-ray crystallographic data that allow one to infer the nature of interactions of each of these enzymes with its substrate(s), coenzymes, and inhibitors.

Collagen Hydroxylases and the Protein Disulfide Isomerase Subunit of Prolyl 4-Hydroxylases

KARI I. KIVIRIKKO AND TAINA PIHLAJANIEMI

Prolyl 4-hydroxylases catalyze the formation of 4-hydroxyproline in collagens and other proteins with an appropriate collagen-like stretch of amino acid residues. The enzyme requires Fe(II), 2-oxoglutarate, molecular oxygen, and ascorbate. This review concentrates on recent progress toward understanding the detailed mechanism of 4-hydroxylase action, including: (a) occurrence and function of the enzyme in animals; (b) general molecular properties; (c) intracellular sites of hydroxylation; (d) peptide substrates and mechanistic roles of the cosubstrates; (e) insights into the development of antifibrotic drugs; (f) studies of the enzyme's subunits and their catalytic function; and (g) mutations that lead to Ehlers-Danlos Syndrome. An account of the regulation of collagen hydroxylase activities is also provided.

ADVANCES IN ENZYMOLOGY

AND RELATED AREAS OF
MOLECULAR BIOLOGY

Amino Acid Metabolism, Part A

Volume 72

Edited by DANIEL L. PURICH

This volume is dedicated to the late Professor Alton Meister, the immediate past Editor of *Advances in Enzymology*, in recognition of his major contribuitons to amino acid metabolism. This is Part A in a subseries, entitled "Amino Acid Metabolism." Topics in Part A should be of immediate interest to those who are broadly concerned with amino acid assimilation and metabolism. Investigators interested in enzyme mechanism and regulation will also find this volume to be especially valuable.

Reprint of Meister's Classic Paper from Journal of Biological Chemistry, 1960, PC39 (by permission of American Society for Biochemistry and Molecular Biology):

ACTIVATED GLUTAMATE INTERMEDIATE IN THE ENZYMATIC SYNTHESIS OF GLUTAMINE

By P. R. KRISHNASWAMY, VAIRA PAMILJANS, and ALTON MEISTER, *Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts*

Advances in Enzymology and Related Areas of Molecular Biology, Volume 72: Amino Acid Metabolism, Part A, Edited by Daniel L. Purich
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Activated Glutamate Intermediate in the Enzymatic Synthesis of Glutamine*

P. R. KRISHNASWAMY, VAIRA PAMILJANS, AND ALTON MEISTER

From the Department of Biochemistry, Tufts University School of Medicine, Boston, Massachusetts

(Received for publication, May 31, 1960)

The enzyme that catalyzes glutamine synthesis (Reaction 1), also catalyzes a transfer reaction in which γ-glutamylhydroxamate is formed from glutamine and hydroxylamine in the presence of catalytic amounts of orthophosphate, or arsenate, and adenosine diphosphate; (see Meister (1) for review of literature).

$$\text{Glutamate} + NH_4^+ + \text{ATP} \xrightleftharpoons{Mg^{++}} \text{glutamine} + \text{ADP} + P_i \quad (1)$$

No intermediates have been found in these reactions and partial reactions have not been demonstrated. Glutamate is not an obligatory intermediate in the transfer reaction (2), and studies with synthetic γ-glutamylphosphate did not provide evidence for intermediate participation of this compound, although an enzyme-bound anhydride is not excluded (3). Studies on the synthesis of D- and L-glutamines and γ-glutamylhydroxamates suggested a mechanism involving an initial activation of glutamate of relatively low optical specificity, followed by a more specific reaction of activated glutamate with NH_4^+ (4). The observation that pyrrolidone carboxylate was formed, when a mixture of L-glutamate, ATP, Mg^{++}, and enzyme was heated (5), provided a significant clue to an activated glutamate intermediate.

We now report evidence for formation, in the absence of NH_4^+, of enzyme-bound γ-carboxyl-activated L- and D-glutamates associated with cleavage of ATP. It is well known that γ-glutamyl derivatives cyclize more readily than glutamate when heated. We reasoned that if an enzyme-bound γ-activated glutamate were formed, it might cyclize *very* rapidly on heating. Therefore, we incubated large amounts of highly purified sheep brain enzyme with ATP, C^{14}-L-glutamate and Mg^{++}; when this mixture was heated at 100° for 5 minutes, pyrrolidone carboxylate was formed (Table I). Much less pyrrolidone carboxylate was

* The authors acknowledge the generous support of the National Heart Institute, National Institutes of Health, and the National Science Foundation. We thank Mr. Daniel Wellner for assistance in the ultracentrifugation studies.

TABLE I *Formation of pyrrolidone carboxylate*

Experiment No.	Reaction mixtures*	Pyrrolidone carboxylate formed	
		With L-glutamate†	With D-glutamate†
		mμmoles	*mμmoles*
1	Glutamate + ATP + Mg^{++} + enzyme	16.8	17.2
2	Glutamate + ATP + Mg^{++} + enzyme‡	0.7	0.5
3	Glutamate + Mg^{++} + enzyme	0.5	0.2
4	Glutamate + ATP + enzyme	0.7	0.5
5	Glutamate + ATP + Mg^{++}	0.7	0.5
6	Glutamate + ATP + Mg^{++} + enzyme + NH_4^+	0.6	4.3

* The reaction mixtures (0.5 ml) contained enzyme, 5 mg (see footnote†); glutamic acid-1-C^{14} (L: 0.17 μmole, 910,000 c.p.m.; D: 0.19 μmole, 92,000 c.p.m.); ATP, 5 μmoles; $MgCl_2$, 25 μmoles; NH_4Cl, 0.05 μmole; and 2-amino-2-(hydroxymethyl)-1,3-propanediol-HCl buffer, pH 7.2, 25 μmoles. After 10 minutes (37°), they were heated at 100° for 5 minutes and then cooled; 0.5 ml of ethanol was added, and the precipitated protein was removed by centrifugation. The supernatant solution was analyzed for pyrrolidone carboxylate and glutamine by paper strip chromatography (*n*-butanol-acetic acid-H_2O; 4:1:1) and electrophoresis (pH 5.0), respectively. Sections of the strips were counted in a gas flow counter.

† The enzyme was about 70% pure as judged by ultracentrifugation studies. It was purified about 2000-fold from sheep brain by isoelectric precipitation, adsorption on $Ca_3(PO_4)_2$ gel, and differential heat inactivation. The latter step makes use of the interesting fact that ATP (+Mg^{++}) protects the enzyme against denaturation at 55°. Preparations of greater purity were obtained by $(NH_4)_2SO_4$ fractionation; however, this step was not employed for enzyme used in these studies, and precautions were taken to eliminate NH_4^+ from all solutions and reagents employed. The purified enzyme contained small amounts of material that absorbed at 260 mμ, the nature of which is being studied. Added ribonuclease did not inhibit the enzyme.

‡ The enzyme was inactivated before the experiment by heating at 100° for 5 minutes.

formed with heat-denatured enzyme, when enzyme, ATP, or Mg^{++} was separately omitted, when ATP was replaced by AMP, ADP, CTP, ITP, UTP, or GTP, or when enzyme was replaced by other proteins. When preincubation was reduced to 10 seconds, there was a 30% decrease in the pyrrolidone carboxylate formed. Heating at 100° for 1 to 20 minutes gave the same

results, and about 70% as much pyrrolidone carboxylate was formed when the mixture was heated at 55° for five minutes. This suggests a highly reactive intermediate that undergoes cyclization about 600 times more rapidly than glutamine which does not cyclize appreciably in 5 minutes at 100° at pH 7.2. The amounts of pyrrolidone carboxylate formed were of the same order as that of the enzyme; however, exact stoichiometry has not yet been attempted. Only traces of, or no, glutamine were formed. When NH_4^+ was added to the system, glutamine was formed and pyrrolidone carboxylate formation was reduced to the blank level. Studies with D-glutamate gave similar results; however, pyrrolidone carboxylate formation in the presence of NH_4^+ was not reduced to blank values. This suggests that under these conditions orientation of the D-glutamate intermediate on the enzyme may be less favorable with respect to reaction with NH_4^+ than that of L-glutamate.[1] Although the α-amino group of glutamate may be considered to replace NH_4^+ in the cyclization reactions, the rates of cyclization (at 55°) do not differ markedly for the two isomers of glutamate. It seems probable that the cyclization reactions, like the reactions with hydroxylamine (*cf.* Levintow and Meister (4)), reflect presence of an activated carboxyl group. When a mixture equivalent to that of 1 in Table I was centrifuged in a separation cell (7), there was a loss of C^{14} corresponding to 17.1 mμmoles of L-glutamate from the upper portion of the cell, when the enzyme passed below the separation plate; there was no sedimentation of glutamate in the controls. In ultrafiltration studies of these reaction mixtures, with Schleicher and Schuell membranes, C^{14}-glutamate remained associated with the protein which did not pass the membrane, whereas this result was not observed in the controls. The amount of glutamate bound to protein in these studies was equal to the pyrrolidone carboxylate formed in the heating experiments. Experiments with ATP^{32} similar to those reported in Table I, except that less ATP was used, are described in Table II. The mixtures were treated with ethanol and centrifuged to remove protein. Only traces of phosphate were found in the absence of glutamate, whereas significant amounts of phosphate were formed with L- or D-glutamates. No glutamine was formed in controls with C^{14}-glutamates. Pyrrolidone carboxylate in amounts equivalent to phosphate was found in parallel heating experiments. We have found that the purified brain

[1] It is possible that activation of D-glutamate occurs in the intact animal; conversion to D-pyrrolidone carboxylate might occur in the presence of relatively low physiological concentrations of ammonia (*cf.* Ratner (6)).

TABLE II *Formation of orthophosphate*

Reaction mixtures*	Orthophosphate formed	
	L-glutamate	D-glutamate
	mμmoles	*mμmoles*
Glutamate + ATP^{32} + Mg^{++} + enzyme	6.6	5.6
ATP^{32} + Mg^{++} + enzyme	0.2	0.1

* The reaction mixtures (0.2 ml) contained ATP, β, γ-P^{32}, 60,000 c.p.m., 0.1 μmole; enzyme, 3.8 mg.; $MgCl_2$, 0.5 μmole; buffer, 10 μmoles; and glutamate, 0.068 μmole; incubated at 37° for 1 minute and then treated with 0.2 ml of ethanol. After centrifugation, the supernatant solution was chromatographed on paper (isopropyl ether-formic acid, 3:2) and the orthophosphate was determined.

enzyme does not catalyze exchange between orthophosphate and ATP in the presence of glutamate and Mg^{++} (NH_4^+ absent); the mild ethanol treatment may release phosphate from a labile bound form.

The data indicate that binding of glutamate to the enzyme requires ATP and Mg^{++}. Binding is associated with cleavage of ATP and the formation of γ-carboxyl-activated glutamate; these events take place in the absence of NH_4^+ and without formation of glutamine. These findings should be considered in relation to the suggestions that (*a*) glutamate and NH_4^+ react before reaction with ATP (8), (*b*) that ATP cleavage and reaction with NH_4^+ occur essentially simultaneously (9, 10), and (*c*) that ATP is cleaved to orthophosphate before reaction with glutamate (11). The present studies suggest binding of ATP and Mg^{++} before binding of glutamate and favor stepwise arrangement of reactants on the enzyme with NH_4^+ reacting last. When considered in the light of O^{18} data (12, 13), our results are consistent with but do not prove formation of enzyme-bound γ-glutamyl-phosphate. It seems probable that the same intermediate is formed in the synthesis, reversal of synthesis (14), and transfer reactions. The requirement for ADP in the transfer reaction suggests that ADP is also bound to the enzyme, and that ADP may be required for binding of glutamine when the reaction proceeds in the reverse direction as well as for the integrity of the activated glutamate intermediate. Further studies on the intermediate and on the role of ADP are being carried out.

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ADVANCES IN THE ENZYMOLOGY OF GLUTAMINE SYNTHESIS

By DANIEL L. PURICH, *Department of Biochemistry and Molecular Biology, University of Florida College of Medicine, Gainesville, Florida 32610*

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I. Introduction

Few would quarrel with the assertion that glutamine is a phenomenally versatile metabolite—a building block for proteins, an amide nitrogen donor in numerous biosynthetic reactions, and interorgan carrier of latent ammonia, a wound healing adjuvant, an effector in cellular hydration, and a glial cell metabolite with roles in neuronal nutrition and signal transmission. What may be less well recognized today is the extent to which glutamine synthetase itself has factored so centrally in the development of modern biochemistry, particularly mechanistic enzymology. While enzymologists had for years recognized that amide bond formation requires activation of glutamine's γ-carboxylate to generate a good leaving group at physiologic pH, notions of enzymic catalysis had for a long time centered on fully concerted mechanisms in which the bond-making and bond-breaking steps occurs virtually simultaneously. Indeed, even with Racker's earlier demonstration of a thiolester intermediate in the glyceraldehyde-3-P dehydrogenase reaction, there was lingering resistance to the general concept that bond breaking and bond making are among the most facile steps of enzymic catalysis. Meister's proposal of a γ-glutamyl-phosphate intermediate set the scene for understanding the stepwise activation of carboxyl groups, thereby increasing their susceptibility toward nucleophilic attack and amide bond synthesis. The goal of this chapter is to consider many of the milestones in characterizing the intermediates formed in the glutamine synthetase reaction, a field largely pioneered by the late Alton Meister. Those efforts helped establish fundamental organizing principles of contemporary enzymology, and they set the scene for the systematic investigation of other carboxyl group activating reactions.

II. Meister's Legacy to the Enzymology of Glutamine Synthesis

Meister authored upward of 500 scientific papers, and his enormous and sustained productivity, as well as the fundamental nature of the work itself, entreats one to consider the characteristics of his investigative style. As one familiar with his research career, I am reminded of the writings of the biophysicist Platt, who in the 1964 published an essay entitled "Strong Inference." Platt's goal was to consider the tenets of scientific investigation that had brought such

great strides in such seemingly disparate fields as modern physics and molecular biology. He attributed this singular success to the ability to frame and solve problems concisely and decisively, and he asserted that these rapidly burgeoning fields shared a particularly effective method of doing scientific research, progressing systematically through a cumulative approach based on inductive inference, a method for which he coined the term strong inference (Platt, 1964). Tracing the elements of strong inference to the inductive approaches of Roger Bacon, Platt argued that the strategy involves the application of the following steps "to every problem in science, formally and explicitly and regularly: (a) devising alternative hypotheses; (b) devising a crucial experiment (or several of them), with alternative possible outcomes, each of which will, as nearly as possible, exclude one or more of the hypotheses; (c) carrying out the experiment so as to get a clean result; and (d) recycling the procedure, making subhypotheses or sequential hypotheses to refine the possibilities that remain; and so on." Platt suggested that many scientists get too wrapped up in the doing of experiments, rather than carefully considering the logic that maps the most effective path for solving problems, most often through recursive rounds of inference and critical experimentation. Enzymologists were among the first biochemists to recognize that systematic investigations of catalysis and regulation are also often greatly facilitated though the use of models. In principle, model building offers at least three advantages: first, because models are pictographic representations of a chemical process, such depictions often reveal gaps in one's understanding; second, the best models are minimalistic, quickly identifying essential mechanistic features; and third, models are generative, facilitating formulation of hypotheses about the nature of rival mechanistic schemes.

Anyone familiar with Meister's systematic attack on the enzymology of carboxyl group-activating enzymes, especially those of us who have sought to make our own contributions to this same endeavor, is well aware that he applied his own special brand of strong inference throughout his illustrious research career. Starting with his early work on transamination and extending throughout his exhaustive inquiry into the enzymology of glutamine and glutathione, he routinely created and tested models for enzyme mechanisms as well as developed entirely new metabolic pathways. Never one to adopt a single line of inquiry or a single test of a hypothesis, Meister's

systematic and multiple attack on a problem, as well as his masterful talent of keeping a line of inquiry on course, allowed him to fulfill far more than the aspiration of making scientific discoveries of lasting value. The approach allowed him to pursue a life-long love affair with the biochemistry of amino acids.

III. Milestones in Glutamine Biochemistry

In seeking to develop better tools for discriminating the detailed catalytic and regulatory mechanisms for this enzyme, Meister and other notable enzymologists (among them Krebs, Koshland, Boyer, Stadtman, and Rose) substantially advanced our understanding of fundamental concepts of enzyme catalysis and metabolic control. This task was all the more challenging because there is no chromophoric cofactor that allows one to examine directly the formation and turnover of catalytic intermediates. While space does not permit a full consideration of Meister's achievements, much less the work of those mentioned above, a brief retrospective of several milestones in the biochemistry of glutamine synthetase appears to be well warranted.

A. DISCOVERY OF GLUTAMINE SYNTHETASE ACTIVITY

Krebs (1935) first reported that the amino acid glutamine could be synthesized from ammonia and glutamate in tissue slices of pig and rabbit kidney. He had conducted a series of experiments to show that glutamate, more than any other α-amino acid, increases oxygen uptake and that addition of glutamate also results in a reduction of ammonium ion. The compound formed from ammonia and glutamate was acid labile, and Krebs carried out additional studies with brain cortex slices and retina to demonstrate the synthesis of what he concluded to be L-glutamine. In fact, Krebs also remarked that the synthesis of glutamine depended on tissue respiration, an observation that presaged the involvement of adenosine triphosphate (ATP). Later work by Speck (1949) demonstrated that pigeon liver extracts form glutamine from glutamate and ammonia in the presence of phosphate, magnesium ion, and molecular oxygen, and he postulated the involvement of ATP formed during respiration. The stoi-

chiometric reaction of ATP, glutamate, and ammonia was later directly demonstrated in aqueous extracts of pigeon liver and sheep brain acetone powders with the products identified as glutamine, adenosine diphosphate (ADP), and orthophosphate (P_i) (Elliott, 1951). Hydroxylamine can substitute for ammonia to form γ-glutamylhydroxamate, and glutamine synthetase also catalyzes the so-called γ-glutamyl transferase reaction even in the absence of added ATP:

$$\text{Glutamine} + NH_2OH \xrightleftharpoons{ADP,\ Me^{2+},\ As_i} \gamma\text{-Glutamylhydroxamate} + NH_3$$

where the divalent metal ion (Me^{2+}) can be magnesium or manganese. It is noteworthy that arsenate (As_i) can substitute for orthophosphate in this reaction, and the γ-glutamyl transferase reaction probably offered the first hint that the glutamine synthetase might involve transient activation of the γ-carboxylate.

B. ATPASE THERMODYNAMICS

Because ATP hydrolysis is the universal driving force in metabolism, membrane transport, motility, and memory processes, the thermodynamics of the ATPase reaction is of enduring fundamental importance. The Gibbs free energy of ATP hydrolysis (and likewise the corresponding values for the hydrolysis of the other nucleoside-5′-triphosphates) depends on several variables. These variables consist mainly of the hydrogen ion concentration, absolute temperature, the concentration of uncomplexed magnesium, the presence of other monovalent (viz., sodium and potassium ions), and divalent cations (particularly calcium ion), as well as the solution ionic strength. Nonetheless, the ATPase reaction favors product formation to such a great extent that one cannot directly obtain sufficiently accurate experimental determinations for ATPase mass action ratio (i.e., $[ADP][P_i]/[ATP]$) by starting from ATP in the presence of an ATPase. In this regard, early investigators recognized that the glutamine synthetase and glutaminase reactions offered special advantages for those seeking accuracy and precision in determinations of the equilibrium constant for ATP hydrolysis. In this case, summation of these reactions yields the ATPase mass action ratio:

$$\begin{aligned} \text{Glutamate} + NH_3 + \text{ATP} &= \text{Glutamine} + \text{ADP} + P_i \\ \text{Glutamine} + H_2O &= NH_3 + \text{Glutamate} \\ \hline \text{ATP} + H_2O &= \text{ADP} + P_i \end{aligned}$$

Alberty (1968; 1969) and Phillips (1969) were among the first to recognize that the ability of magnesium ion to form a higher affinity complex with the ATP^{4-} form than with $HATP^{3-}$ explains why the ATPase equilibrium is so strongly influenced (a) by the concentration of "free" or uncomplexed magnesium ion, (b) by the concentration of protons that alter the protonation state of the nucleotide, (c) by the ionic strength of the supporting electrolyte and buffer, and (d) by the presence of other monovalent and divalent cations. More recently, Alberty and Goldberg (1992) presented a detailed consideration of the *transformed* Gibbs energy as the chief criterion for chemical equilibrium at specified temperature, pressure, pH, concentration of free magnesium ion, and ionic strength. One begins by writing the apparent equilibrium constant in terms of the total concentrations of reactants, like ATP_{total}, rather than in terms of the concentrations of individual molecular species, such as $MgATP^{2-}$, ATP^{4-}, or $HATP^{4-}$. The standard transformed Gibbs energy can then be calculated from the Gibbs energy by using the apparent equilibrium constant as well as the temperature dependence of the apparent equilibrium constant at specified pressure, pH, concentration of free magnesium ion, and ionic strength. From the apparent equilibrium constants and standard transformed enthalpies of reaction that have been measured in the ATP series and the dissociation constants of the weak acids and magnesium complexes involved, Alberty and Goldberg (1992) showed that one can calculate standard Gibbs energies of formation and standard enthalpies of formation of the species involved at zero ionic strength. They followed the convention that the standard Gibbs energy of formation as well as the standard enthalpy of formation for adenosine in dilute aqueous solutions be set equal to zero. On the basis of this convention, standard transformed Gibbs energies of formation and standard transformed enthalpies of formation of ATP, ADP, AMP, and adenosine at 298.15 K, 1 bar, pH 7, a concentration of free magnesium ions of 10^{-3} *M*, and an ionic strength of 0.25 *M* have been calculated. It is also worth noting that Alberty and Cornish-Bowden (1993) recently presented a cogent

analysis of how this formalism allows one to understand the relationship between the apparent equilibrium constant "K'" for a biochemical reaction and the equilibrium constant, K, for a reference reaction written as a chemical equation.

Finally, lest one consider the usage of Gibbs free energy (G) in biochemistry to be a cut and dried issue, Welch (1985) suggested that the symbol 'ΔG', as discussed in most biochemical textbook calculations of free energy change (e.g., freeze-clamp studies of steady-state levels of metabolites in a particular tissue), can lead to erroneous conclusions about biological processes. To describe the thermodynamic behavior of metabolic reactions within cells, he favors the use of the instantaneous change, symbolized by the expression ($\Delta G/\Delta\xi$), where ξ is the degree of advancement of the reaction. His article offers mathematical and graphical analyses of a sample reaction to demonstrate the fundamental difference between ΔG and $\Delta G/\Delta\xi$.

C. EARLY ISOTOPIC TRACER STUDIES OF THE GLUTAMINE SYNTHETASE REACTION

There was general recognition early on that the synthesis of glutamine might conceivably involve the transient formation of a covalent intermediate. Two rival hypotheses emerged: (a) that an amidophosphate intermediate could first form from ATP and ammonia, followed by attack of the γ-carboxylate on phosphorus with the expulsion of ammonia; and (b) that a γ-glutamyl-phosphate compound formed by attack of the γ-carboxylate on a MgATP complex, thereby expelling P_i and allowing NH_3 attack on the acyl-P to form glutamine. While a number of investigators sought to demonstrate the formation of a covalent compound directly, this was confounded by the reactivity of an unstable intermediate as well as the difficulty in preventing the presence of trace amounts of ammonia.

A major lead was provided by Boyer et al. (1956) who used [γ-^{18}O]-glutamate to trace the entry of oxygen atoms during the catalysis of the overall reaction from glutamate, ATP, and ammonia. These investigators discovered that ^{18}O-orthophosphate was synthesized, and Kowalsky et al. (1956) likewise showed the stoichiometric conversion of ^{18}O-orthophosphate from [γ-^{18}O]glutamate, with little or no incorporation of labeled oxygen into ADP. This finding was

consistent with two possibilities: (1) a fully concerted mechanism whereby ammonia attack at the γ-carboxylate led to the direct transfer of one of the two carboxylate oxygen atoms to ATP to produce ADP and P_i; and (2) the stepwise synthesis of the γ-carboxyl-P from ADP and glutamate, followed by nucleophilic attack of NH_3 attended by release of P_i bearing an oxygen atom once held by the γ-carboxylate. These ideas also fit with the findings of Levintow and Meister (1954) who had examined the γ-glutamyl transferase reaction in the presence of radiocarbon-labeled glutamate. They concluded that the fact that no label appeared in either glutamine or the γ-glutamylhydroxamate argued that the amide and hydroxamate were sufficiently activated to form an unstable covalent intermediate, whereas glutamate was not.

While isotope exchange, especially that of Boyer et al. (1959) and Wedler and Boyer (1973), demonstrated that some of the steps proceeded at rates faster than the rate-determining steps, for some time equilibrium isotope exchange played no further role in determining the chemical processes involved in the catalytic mechanism of glutamine synthetase. On the other hand, equilibrium isotope exchange measurements did demonstrate promise for investigating how regulatory effectors alter the catalysis of enzymes (Wedler and Boyer, 1973; Purich and Allison, 1980). It remained for Rose to develop the positional isotope exchange protocol in the mid-1970s (see Section III.H).

D. INTERMEDIACY OF γ-GLUTAMYL-PHOSPHATE IN GLUTAMINE SYNTHETASE REACTION

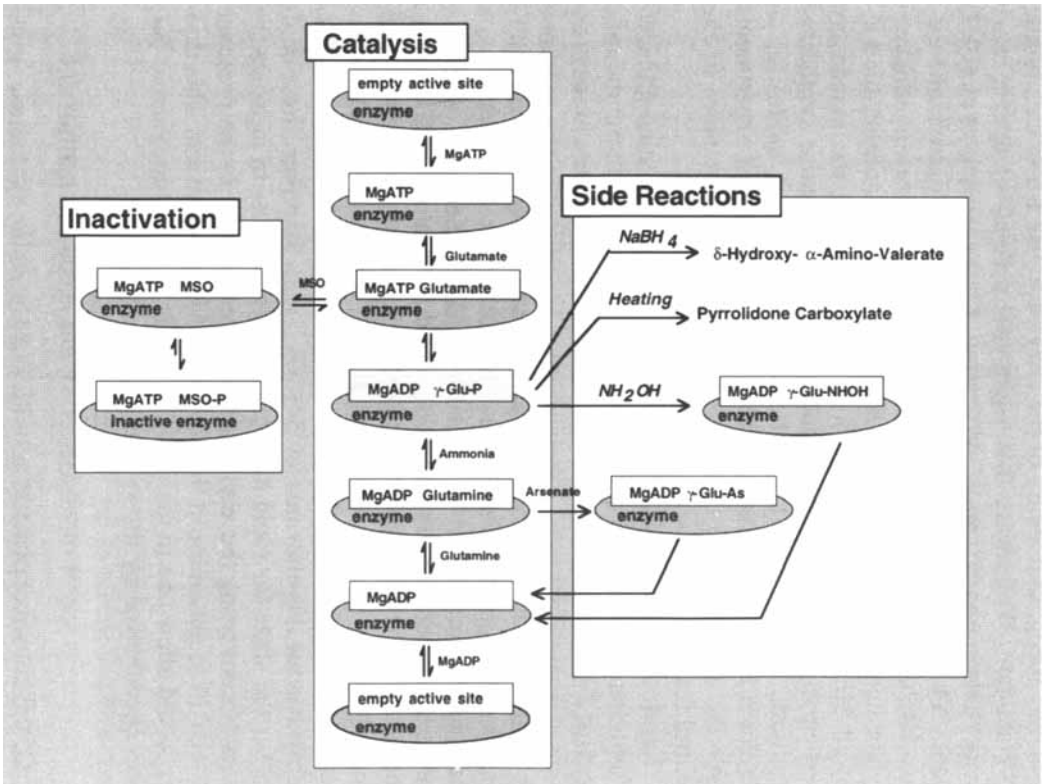
The next crucial step in elucidating the reaction mechanism was taken by Krishnaswamy et al. (1962) who reacted the enzyme with ATP and [^{14}C] glutamic acid in NH_3 depleted solutions. This procedure resulted in the synthesis of pyrrolidone carboxylate (also known as pyroglutamate and 5-oxoproline), a cyclic compound that can also be inefficiently formed in the nonenzymatic dehydration of glutamate or in the nonenzymatic displacement of the amide nitrogen of glutamine by nucleophilic attack of the α-amino group on the γ-amide. This suggested that exposure to the enzyme facilitated formation of a suitably activated covalent compound corresponding to the γ-acyl-P, which upon brief heating to 100°C would undergo intramolecular

attack to form the cyclic amino acid. Both D- and L-glutamate were effective in the enzyme-catalyzed synthesis of the D and L forms of pyrrolidone carboxylate, a finding that was consistent with the generally recognized ability of the enzyme to use either enantiomer of glutamate. Well aware that γ-glutamyl derivatives are far more susceptible to nonenzymatic cyclization, Krishnaswamy et al. (1962) went on to demonstrate that glutamine formed the pyrrolidone-carboxylate some 600 times more slowly than in the corresponding enzymic step observed in the above experiment. This observation allowed Meister (1962) to offer a scheme involving the mandatory participation of an acyl-P intermediate in the glutamine synthetase reaction. Remarkably, that scheme (now modified modestly, as shown in Fig. 1) has withstood the test of time, and all experiments over the ensuing three decades have verified one or more of the central features of Meister's γ-glutamyl-P model.

Khedouri et al. (1964) chemically synthesized β-amino-glutaryl-P and demonstrated the enzymatic synthesis of β-amino-glutaramic acid (or β-glutamine) by ovine brain glutamine synthetase in the presence of ammonium ions. Likewise, they found that in the presence of hydroxylamine, β-amino-glutaryl-phosphate was enzymatically transformed into β-amino-glutaryl-hydroxamate. Moreover, when the synthetic acyl-P was incubated with ADP and enzyme, ATP was formed. None of these reactions occurred in the absence of enzyme, and β-aspartyl-P was completely ineffective in the above cited reactions. Later, studies by Allison et al. (1977) addressed the ter-reactant initial rate kinetics of β-glutamate, ATP, and hydroxylamine as substrates for this enzyme (see Section III.G). Together, these studies set the stage for rapid mix–quench experiments to measure the rates of converting the acyl-P intermediate to ATP in the presence of ADP or to glutamine in the presence of ammonium ion. This finding would allow one to identify the kinetic and thermodynamic barriers encountered by the synthetase.

E. MECHANISM OF METHIONINE SULFOXIMINE INHIBITION

Of the known enzymatic activities of glutamine synthetase, none is more revealing of the acyl-P intermediate than the inhibitory properties of methionine sulfoximine, or MSO (Meister, 1974). Of the four MSO stereomers, only L-methionine-*S*-sulfoximine irreversibly


Catalysis
empty active site
enzyme
MgATP
MgATP
enzyme
Glutamate
MgATP Glutamate
enzyme
MgADP γ-Glu-P
enzyme
Ammonia
MgADP Glutamine
enzyme
Glutamine
MgADP
enzyme
MgADP
empty active site
enzyme
Inactivation
MgATP MSO
enzyme
MSO
MgATP MSO-P
inactive enzyme
Side Reactions
NaBH 4
δ-Hydroxy- α-Amino-Valerate
Heating
Pyrrolidone Carboxylate
NH 2 OH
MgADP γ-Glu-NHOH
enzyme
Arsenate
MgADP γ-Glu-As
enzyme

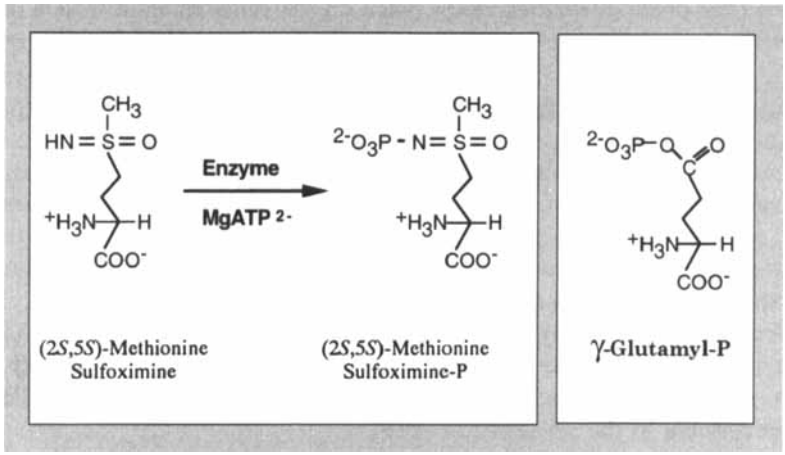


Figure 2. Irreversible inhibition of glutamine synthetase by sulfoximine methionine. Only the (*2S, 5S*)-diasteriomer of MSO acts as a suicide inhibitor; the other three diasteriomers bind reversibly, yet fail to undergo phosphorylation. (*Note*: The phosphorylated inhibitor mimics the geometrical configuration of the proposed covalent intermediate formed in the synthetase reaction.)

inhibits glutamine synthetase, and the phosphorylated inhibitor appears to mimic the transition state of the synthetase reaction (Fig. 2). Methionine sulfoximine competes with both ammonia and glutamate for the ovine enzyme, suggesting that it occupies both pockets within the enzyme's active site. Computer-assisted mapping of ligand interactions indicates that the sulfoximine oxygen atom of L-methionine-(*S*)-sulfoximine binds in place of the acyl group's oxygen and that the methyl group occupies the ammonia binding site. In fact, such geometric considerations allow one to rationalize why

Figure 1. Integrated mechanism for glutamine synthetase. The diagram shows the main route of catalysis as well as enzyme inactivation by methionine sulfoximine, and other side reactions that are now recognized as hallmarks of the formation of an acyl-P intermediate. (*Note*: Although the catalytic path implies an ordered binding mechanism with ATP as the leading substrate, later kinetic experiments have clearly established a random pathway for substrate addition.)

covalent phosphorylation takes place on the sulfoximine nitrogen of MSO. A similar steric orientation cannot be achieved with either of the D-isomers of MSO, and while L-methionine-(*R*)-sulfoximine does bind to the enzyme, the reversed positions of the sulfoximine nitrogen and oxygen atoms do not permit efficient phosphorylation. Meister (1974) also suggested (a) that the oxygen atom might be too acidic to attack ATP, and (b) that the observed binding of the methyl group in place of ammonia indicates that a hydrophobic interaction with un-ionized ammonia occurs, as opposed to ammonium ion. The latter would certainly favor nucleophilic attack on the acyl-P intermediary. Notably, inhibition by D- and L-methionine sulfone is reversible and can be easily reversed at higher glutamate concentrations; moreover, L- or D-methionine sulfone inhibition becomes more potent, but not irreversible in the presence of ATP.

Shrake et al. (1982) investigated the interactions of *Escherichia coli* glutamine synthetase with the resolved L-(*S*)- and L-(*R*)-diastereoisomers of the substrate analogue L-methionine-(*SR*)-sulfoximine. Reversible binding of the (*S*) isomer to unadenylylated manganese enzyme showed a stoichiometry of 1 equiv per subunit and negative cooperativity with a Hill coefficient of 0.7. The affinity of this enzyme complex was (a) highest for the (*S*) isomer; (b) lowest with the (*R*) isomer; and (c) intermediate for an equimolar (*S*) and (*R*)-isomer mixture. The affinity for the (*S*)-isomer was enhanced greater than 35-fold by ADP and was decreased approximately threefold by adenylylation of the enzyme. Earlier work by these investigators showed that UV spectral perturbations were markedly different for the binding of commercial L-methionine-(*SR*)-sulfoximine to unadenylylated and adenylylated manganese enzymes (Shrake et al., 1980). Yet, essentially the same protein difference spectrum is obtained for binding the resolved (*S*) and (*R*) diastereoisomers, and equimolar mixture of (*S*) and (*R*) isomers, and the commercial (*S*)- and (*R*)-isomeric mixture to a particular enzyme complex.

F. STEREOCHEMICAL MAPPING OF LIGAND INTERACTIONS AT THE ACTIVE SITE OF GLUTAMINE SYNTHETASE

Although X-ray crystallography provides the most definitive information on the stereochemical considerations governing substrate

binding, atomic structures of enzyme complexes often fail to reveal the nature of ligand-induced conformational changes that strongly influence the kinetics and thermodynamics of enzyme–ligand interactions. Nonetheless, one can frequently infer stereochemical relationships by comparing observed binding constants, inhibition constants, and in some cases Michaelis constants, for interactions of inhibitors and substrates with enzymes (Gass and Meister, 1970; Purich et al., 1973). In the case of sheep brain glutamine synthetase, the goal was to discover why both D- and L-glutamate serve as substrates, and Gass and Meister (1970) studied these substrates along with 10 of their monomethyl derivatives. Of the latter, only three were active as substrates, and by making the assumption that amino acid substrates bind to glutamine synthetase in their most extended conformations, these investigators found that those hydrogens, whose methyl substituents yielded enzymatic activity, lie on one side of the molecule (i.e., the side that resides behind the plane for the structures of L-glutamate and D-glutamate as shown in Fig. 3). This finding led to the hypothesis that the enzyme approaches its amino acid substrate from above the plane of the paper, thereby permitting methyl groups of the three active methyl derivatives to project backward and away from the enzyme's surface. To test this proposal, these investigators prepared L-*cis*-1-amino-1,3-dicarboxycyclohexane as a conformationally restricted L-glutamate analogue wherein the two backward-extending hydrogens of D- and L-glutamate are connected by the three additional methylene groups within the cyclohexane ring (see Fig. 3). This analogue proved to be an effective substrate for the sheep brain enzyme, thereby fixing the stereochemistry of enzyme–substrate complexation without any atomic-level information on the enzyme's structure.

In retrospect, this conformationally restricted derivative would have also served as a valuable mechanistic probe of the mechanism of carboxyl group activation, because reaction of the analogue with ATP in an ammonia-free solution should form the corresponding acyl-P intermediate, and any intramolecular attack of the α-amino group to a pyroglutamate-like product (resembling a pyrrolidone carboxylate) should be far less likely. A similar argument applies to the use of β-glutamate, which should form an acyl-P compound that resists intramolecular attack by the amino group.

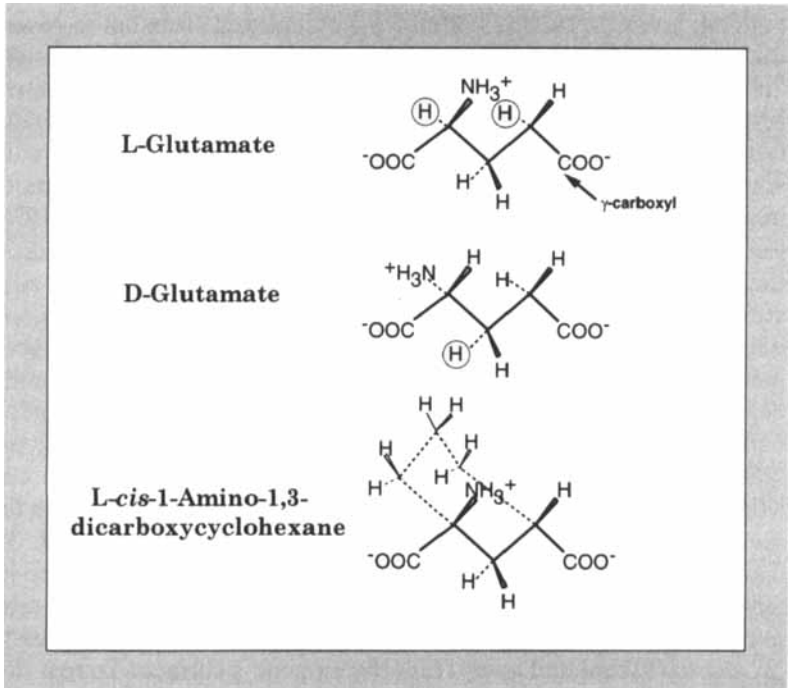


Figure 3. Stereochemical representation of L-glutamate, D-glutamate, and L-*cis*-1-amino-1,3-dicarboxycyclohexane. Both D- and L-glutamate as well as aminoadipic acid, are substrates for the enzyme, but only 3 of 10 monomethyl derivatives of D- and L-glutamate are substrates. Assuming that the substrates are completely extended when bound to the enzyme, active substrates were obtained when the circled hydrogens are substituted by a methyl group. These hydrogens lie behind the plane of the paper, and they most probably face outward from the enzyme-bound substrate toward the bulk solvent. L-*cis*- 1-Amino-1,3-dicarboxycyclohexane is an alternative substrate for glutamate in the glutamine synthetase reaction. Note that the cyclohexane ring is connected by three successive methylenes that bridge the two replaceable (circled) hydrogen atoms in L-glutamate.

G. THE KINETIC REACTION MECHANISM OF GLUTAMINE SYNTHETASE

The kinetic reaction mechanism of multisubstrate enzymes deals principally with the determination of the substrate binding order. Such information can often provide valuable evidence for the occurrence of ordered conformational changes that are evoked by sub-