BIOORGANIC CHEMISTRY **25,** 43–61 (1997)
ARTICLE NO. BH961048

CONFERENCE PROCEEDINGS

Sixteenth Midwest Enzyme Chemistry Conference

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Received November 6, 1996

The 16th annual midwest conference on enzyme chemistry, organized by Ronald Viola (Program Chair) and David Lynn (Site Chair), was held on October 12, 1996, on the campus of the University of Chicago. The meeting was organized into three sessions on the general topics: (i) Enzyme Structure, (ii) Metals and Radicals, and (iii) Drugs and Diseases. An evening poster session was also held, with over 70 posters presented, covering a wide range of enzymology. A brief synopsis of each of the major talks is given, including some relevant references to provide an entry into the current work in these areas. A complete listing of the titles and authors of the poster presentations is also included.

ENZYME STRUCTURES

In the initial presentation of the meeting, Dr. Len Banasack from the University of Minnesota presented the work from his research group on the structural characterization of fumarase C from *Escherichia col*i. Fumarase C, which catalyzes the interconversion of L-malate and fumarate as part of the citric acid cycle, is homologous with the non-metal-containing fumarases in eukaryotic cells (*1*). There is also significant sequence homology with a larger group of proteins, including the enzymes L-aspartase (*2*), adenylosuccinate and argininosuccinate lyases (*3*), and the lens protein δ-crystallin (*4*).

The x-ray crystal structure of native fumarase C has been determined and refined to about 2 Å resolution (*5*). Their examination of this structure reveals that the native tetramer is formed by an unusual core of 20 α-helices, with 5 helices coming from the central domain of each subunit. In addition to the central five helical core, two other important structural domains have been identified. The active site, identified from the structure of enzyme–inhibitor complexes with both citrate and pyromellitate, occurs in a crevice formed at the interface of three of the four subunits. The active site has been analyzed in terms of potential catalytic side chains in the enzyme–inhibitor complexes, and the side chains of a histidine, glutamate, lysine, and asparagine along with a water molecule have been found to be in position to interact with the bound inhibitors (*5*). As seen in Fig. 1, the carboxylates (C1 and C4) of a citrate molecule bound at the active site are labeled along with the important amino acid sidechains. The black circle in the middle of the figure is a

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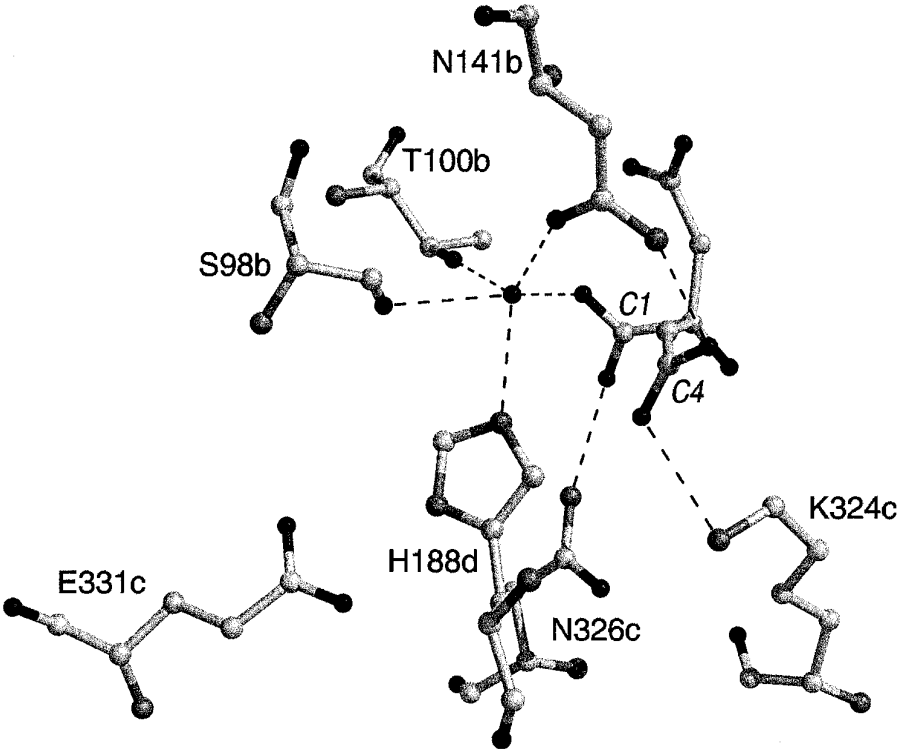


FIG. 1. Ball and stick representation of citrate bound at the active site of fumarase from *Escherichia coli.* The active site is formed by amino acids from three different subunits (labeled a–c) of the tetramer (figure produced by Todd Weaver).

tightly bound water molecule that has been proposed to play an important role in the catalytic process. A second site, located approximately 10–12 Å from the active site, has also been identified in the electron density maps. This site contains a bound malate interacting with an arginine, a histidine, and an unusual π-helix. Replacement of this histidine by site-directed mutagenesis results in the loss of malate binding, but has no effect on the catalytic activity of the enzyme. These results have lead this group to suggest that this site might be a previously unidentified regulatory site in fumarase C.

The structure of enzymes in nonaqueous solvents was the subject of the presentation by Dr. Gregory Farber from Penn State University. Enzymes often exhibit increased thermostability or altered substrate specificity in organic solvents (*6, 7*), and in these solvents enzymes are able to catalyze reactions that are either kinetically or thermodynamically impossible in water (*8*). An additional advantage of exploring nonaqueous enzymology is that it is possible to trap enzyme substrate complexes in organic solvents that have only a very short lifetime in aqueous solutions. Dr. Farber's group has solved a number of crystal structures in organic solvents in an

attempt to explain the altered properties which have been observed (*9*). Transfer of enzyme crystals grown in aqueous solution to nonpolar organic solvents, such as hexane, benzene, and toluene, can be accomplished with no apparent damage to the crystals. The structure of γ-chymotrypsin in a solution of hexane and isopropanol has suggested an explanation for the altered substrate specificity that has been observed (*10*). The inclusion of low levels of isopropanol results in an increase in the number of protein-associated water molecules, thus setting up conditions to allow the reaction to proceed in the hydrolytic direction. A series of structures of subtilisin in various concentrations of dimethylformamide has provided a rationale for the lower catalytic activity that is often observed as a consequence of moving into an enzyme into an organic solvent (*11*). While no gross structural changes in subtilisin have been observed in going from 0 to 50% DMF, the active site histidine does have a larger range of motion at the higher organic solvent levels. Structural evidence was presented that indicated the lengthening of a short hydrogen bond between this histidine and the active site aspartate at the higher DMF levels and a possible rotation in the histidine ring assisted by additional water molecules bound in the active site. The power of this experimental approach was further demonstrated by the use of several different organic solvents to trap and directly examine the structures of all of the important intermediates in the reaction catalyzed by chymotrypsin.

METALS AND RADICALS

Xuejun Zhong, in the laboratory of Dr. Ming-Daw Tsai at Ohio State University, has been studying how conformational changes can control the fidelity of DNA polymerase β. The fidelity of DNA polymerases is largely attributable to a two-step nucleotide binding mechanism (*12*). These researchers have identified two conformational changes in rat DNA polymerase β that are induced by the consecutive binding of nucleotide, followed by Mg^{2+} ion binding. Two phases of fluorescence changes were observed in the stopped-flow fluorescence assay for dTTP incorporation (Fig. 2) by using a synthetic DNA primer/template containing a fluorescent 2-aminopurine nucleotide analog at the template position opposite the incoming dTTP (*13*). The results from Mg^{2+} and dTTP concentration dependencies of the observed rate constant, and from experiments with a DNA substrate containing a dideoxynucleotide at the 3′-end of the primer, indicate that both phases result from conformational changes. Enzyme fidelity can be explained with this multiple conformational change mechanism. Binding of the correct nucleotide efficiently induces a rapid initial conformational change in the enzyme which then allows a catalytic Mg^{2+} ion to bind at the catalytic site in the enzyme–DNA–dNTP complex. Completion of this quarternary complex induces a second rate-limiting conformational change in the enzyme that is required before catalysis can occur. Similar rate-limiting conformational changes have been observed in T7 DNA polymerase (*14*), although some differences have been observed in the structures of various DNA polymerase-template complexes (*15*). Binding of an incorrect nucleotide can still induce the initial conformational change at a similar rate, but only when present

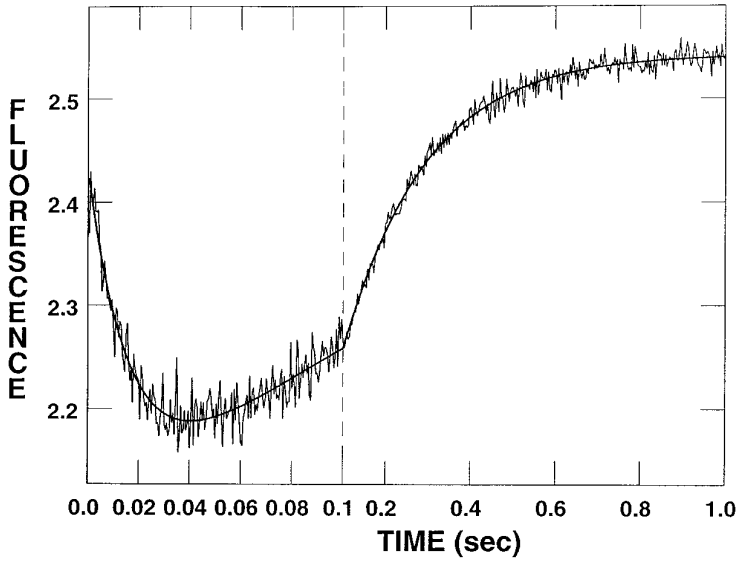


FIG. 2. Two phases of fluorescence changes observed in a stopped-flow fluorescence assay of dTTP incorporation by DNA polymerase β.

at about 50-fold higher concentration. The free energy differences of binding for correct vs incorrect base pairs accounts for the selection through productive binding in the base-pairing step (the fast conformational change). The second conformation with the addition of Mg^{2+} still takes place in the presence of the incorrect nucleotide, but at a rate which is about 500-fold slower. The initial conformational change induced by incorrect nucleotide binding results in a ''bad fit'' and greatly raises the kinetic energy barrier of the second conformational change step (the rate limiting step) (*16*). Discrimination between correct and incorrect nucleotides at the second conformational change step plays a major role in maintaining fidelity (*17*).

The next talk, by John Hlavaty at the University of Notre Dame, presented his work from the laboratory of Dr. Tom Nowak on the characterization of the metal ion sites in avian liver mitochondrial phosphoenolpyruvate carboxykinase (PEPCK). This enzyme is a 67-kDa monomeric gluconeogenic enzyme that catalyzes the reversible GTP-dependent conversion of OAA to PEP and CO_2 (*18*). PEPCK has an absolute requirement for divalent cations for activity and Mn^{2+} is the best activator (*19*). Mixed metal kinetic studies show a dual cation role for PEPCK, with one cation activating the enzyme through a direct interaction with the protein at site n_1, and the second cation acting in the cation–nucleotide complex that serves as a substrate at site n_2 (*20*). This talk reported the preparation of an active Co^{3+}–enzyme complex at site n_1 that has provided a stable complex for examining the kinetic, mechanistic, and binding properties at the n_2 metal site (*21*). EPR studies performed on the Co^{3+}–enzyme–GTP complex have determined a K_d of 5 μM for the binding of a single Mn^{2+}. Water proton relaxation rate (PRR) studies

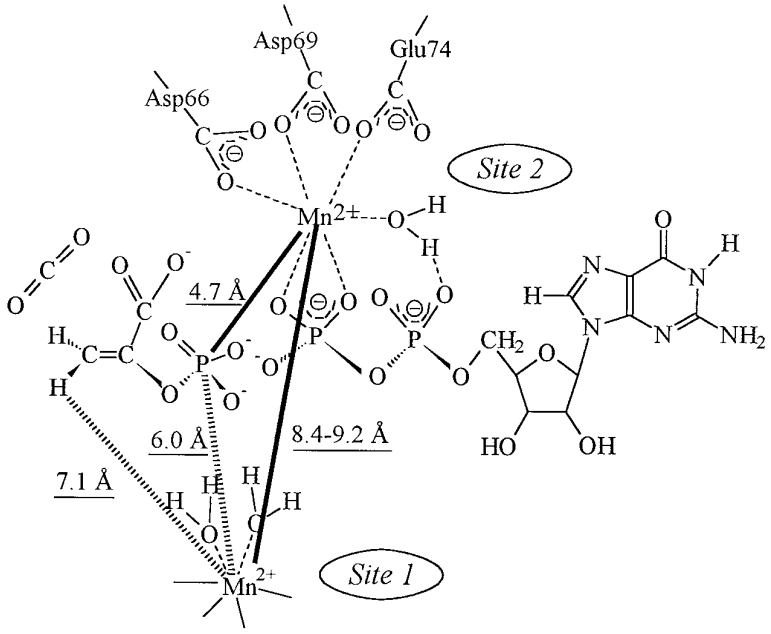


FIG. 3. Active site structure of the divalent metal ion binding sites in phosphoenol-pyruvate carboxykinase.

show a significant enhancement for the Co^{3+}–enzyme–MnGDP complex in the presence of PEP, but not with OAA or CO_2, suggesting that PEP interacts with the second metal ion. A proton relaxation rate (PRR) study for both the GTP and the GDP quarternary complexes as a function of frequency led to an estimated hydration number of about one for the n_2 metal site. A temperature-dependence study showed that the water protons at this site are in fast exchange with an activation energy of about 2 kcal/mol. The metal–metal distance between the enzyme-bound Mn^{2+} at site n_1 and Cr^{3+}–GTP at site n_2 was determined by PRR techniques (*22*) to be 8.4 Å. To determine the location of the n_2 cation site on PEPCK, the Co^{3+}–PEPCK complex was incubated with Co^{2+} and GTP, followed by oxidation with peroxide to create a doubly labeled and inactive complex. This complex was then digested by LysC and the two cobalt-containing peptides were purified by using reverse-phase HPLC. The first cobalt-containing peptide has previously been identified as the n_1 site (*21*). Amino acid sequencing on the second cobalt-containing peptide identified the region from tyrosine-57 to lysine-76 of PEPCK. This is a highly conserved region located in the N-terminal of PEPCK near the putative PEP binding site. Capillary electrophoresis analyses of tryptic and chymotryptic digests of the second cobalt-containing peptide suggest that aspartate-66, aspartate-69 and glutamate-74 may serve as ligands to the metal at the n_2 site. The identification of these ligands, along with the metal–metal and metal–substrate distances, have provided a detailed picture of the active site of PEPCK (Fig. 3).

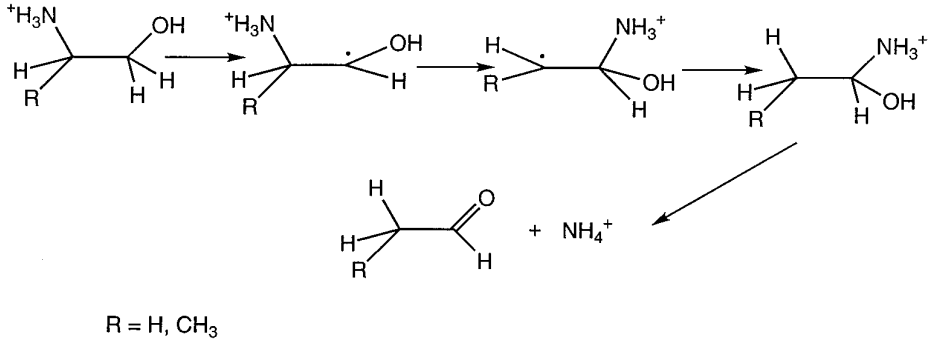
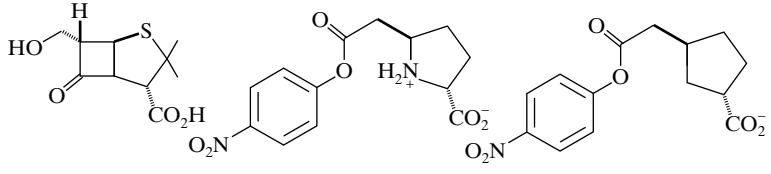


FIG. 4. Proposed radical mechanism for the deamination of ethanolamine catalyzed by ethanolamine ammonia-lyase.

The last talk in the morning session was given by Vahe Bandarian, reporting his work on the radical enzymology of ethanolamine ammonia-lyase (EAL) in the laboratory of Dr. George Reed at the University of Wisconsin. EAL catalyzes the deamination of ethanolamine to form acetaldehyde and ammonia—a transformation that requires coenzyme B_{12} (*23–25*). The function of coenzyme B_{12} is to abstract a hydrogen atom from carbon-1 of the substrate ethanolamine to generate a free radical intermediate. This intermediate then rearranges to form a product radical which then reacquires the hydrogen that had been abstracted from carbon-1 (Fig. 4). Deamination of the carbinolamine affords acetaldehyde and ammonia. The presence of free radicals during catalysis by EAL was shown in a seminal paper by Babior and collaborators (*26*) in which the authors demonstrated that upon incubation of the enzyme-coenzyme B_{12} complex with the slow substrate propanolamine an EPR signal was observed which is consistent with formation of a substrate-based free radical. This signal was called the ''radical doublet'' signal (*27*), and similar signals have been observed in a number of other coenzyme B_{12} enzyme systems. While these EPR signals were observed over 20 years ago, the exact identity of the species giving rise to the EPR signals have remained unsolved mysteries. The identity of the free radical giving the ''radical doublet'' EPR signal observed with EAL was the focus of this presentation. The kinetic competence of the signal has been established by rapid freeze–quench EPR in which the rate of production of the radical was shown to proceed at least as fast as catalytic turnover. A large number of isotopically labeled substrate analogs have been synthesized and incubated with EAL and with coenzyme B_{12} and studied by EPR (*28*). The spectra have been extensively analyzed and the simulation parameters have been used to identify the intermediate. The structure of the intermediate shows considerable unpaired spin density on carbon-1, allowing its identification as a substrate-like radical species. Furthermore, the structure of the radical emerging from the EPR studies provides some insight into how an enzyme deals with unstable species in its active site. This presentation showed the progress that has been made in this laboratory toward assignment of the ''radical doublet'' signal observed with EAL.

DRUGS AND DISEASES

The last session of this meeting began with a presentation by Shahriar Mobashery from Wayne State University on the evolution of β-lactam hydrolase activity as a mechanism for drug resistance. The β-lactamase activity is the primary means for bacterial resistance to β-lactam antibiotics. These enzymes are presumed to have evolved from the primordial cell wall biosynthetic enzymes, the modern forms of which are referred to as penicillin-binding proteins (PBPs). Class A β-lactamases are most common among pathogens, with class C enzymes the next most common (*29*). Both class A and class C β-lactamases, as well as PBPs, undergo acylation at an active site serine residue by β-lactam antibiotics. The rate of deacylation of this acyl-enzyme intermediate from the active site of penicillin-binding proteins is slow; thereby the bacterium is deprived of the biosynthetic function of these enzymes, an event that results in bacterial death. However, β-lactamases are capable of undergoing deacylation in a facile manner, completing the turnover necessary for hydrolysis of the β-lactam antibiotic. From the four different classes of β-lactamases these researchers have investigated the mechanistic details of the deacylation step in both class A and class C enzymes. A molecular probe, 6α-hydroxymethylpenicillanate **(I),** was designed in a computer-aided process with the help of the crystal structure for the *Escherichia coli* TEM-1 β-lactamase, a prototypical class A enzyme. This molecule was designed to prevent the approach of the presumed hydrolytic water from the α-face of the acyl-enzyme intermediate. The compound acylated the purified enzyme readily, but resists deacylation, as expected (*30*). The crystal structure for the acyl-enzyme intermediate, the first for any acyl-enzyme intermediate for turnover of a substrate by a native class A β-lactamase, supported the design paradigms, indicating that the approach of the hydrolytic water is from the α-face, and is promoted by glutamate-166 as a general base (*31*). Interestingly, there is no counterpart to glutamate-166 in class C β-lactamases. Earlier work from this laboratory with the *Enterobacter cloacae* P99 β-lactamase, a prototypic class C β-lactamase had suggested that the approach of the hydrolytic water may be from the β-face of the acyl-enzyme intermediate in this case (*32*). If this were the case then the formerly β-lactam nitrogen, now a secondary amine at the acyl-enzyme intermediate stage, would be ideally positioned to serve as the general base in promoting a water molecule for approach to the acyl carbonyl from the β-face. To test this possibility two molecules were synthesized, *p*-nitrophenol (2*R*,5*R*)-5-prolylacetate **(II)** and *p*-nitrophenol (1*S*,3*S*)-3-carboxy-cyclopentylacetate **(III).**



I **II** **III**

Compound **II** acylates the active site serine of the P99 enzyme, and the intermediate then undergoes deacylation to compound **V** (Fig. 5). On the other hand, compound

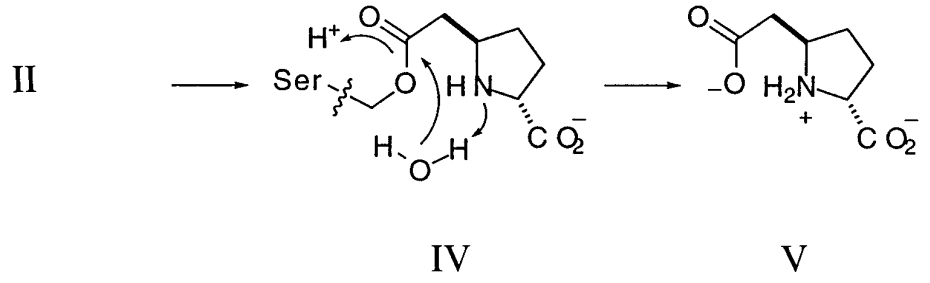


FIG. 5. Proposed mechanism for the acylation and subsequent deacylation of the active site serine of P99 β-lactamase by *p*-nitrophenyl (2*R*,5*R*)-5-propylacetate **(II).**

III only acylates the active site and, not having the requisite amine in its structure, the intermediate resists deacylation. Both compounds serve as substrates for the class A TEM-I β-lactamase, as was expected. These researchers concluded that substrate-assisted catalysis, provided from the penicillin base in the acyl-enzyme intermediate, is the mechanism for the class C β-lactamases. Based on these results they further propose that the evolution of classes A and C β-lactamases proceeded independently from the primordial penicillin binding proteins.

The next talk, by Crystal Sheppard working in the laboratory of Dr. Rowena Matthews at the University of Michigan, presented their work on an analysis of a mutation in methylenetetrahydrofolate reductase (MTHFR). Deficiencies in MTHFR have been correlated with increased risk of heart disease (*33*) and neural tube defects (*34*). Human MTHFR has been cloned and mutations have been identified in human patients with altered MTHFR activity (*35, 36*). A human MTHFR alanine to valine mutation was identified as a genetic risk factor for cardiovascular disease and neural tube defects (*37*). Noting that the *E. coli* MTHFR (ecMTHFR) has significant sequence homology to human MTHFR, and that mutations causing altered function in humans occur in regions of high identity, this research group constructed the corresponding changes in ecMTHFR. Mutations associated with loss of enzymatic activity in humans caused loss of activity in *E. coli*, thus confirming ecMTHFR as a useful model for studying the MTHFR family. Purified A177V enzyme has catalytic properties that are similar to wild-type ecMTHFR, but has impaired flavin binding. It appears that upon dilution the ecMTHFR tetramer dissociates and loses flavin (FAD) (Fig. 6). Although the wild-type and the A177V mutant ecMTHFR have similar activities, the mutant loses both flavin and activity at a faster rate than the wild-type ecMTHFR. These results suggest that in humans with this mutation, impaired flavin binding results in decreased amounts of active MTHFR enzyme. The rate of flavin and activity loss is reduced by the addition of folate, suggesting a possible therapy to overcome the impaired binding affinity (*38*). Further studies are in progress in this laboratory using both histidine-tagged wild-type and A177V enzymes. In addition, collaborative work with Dr. Brian Gunther and Dr. Martha Ludwig has led to reproducibly generated crystals of ecMTHFR that diffract to high resolution. Determination of

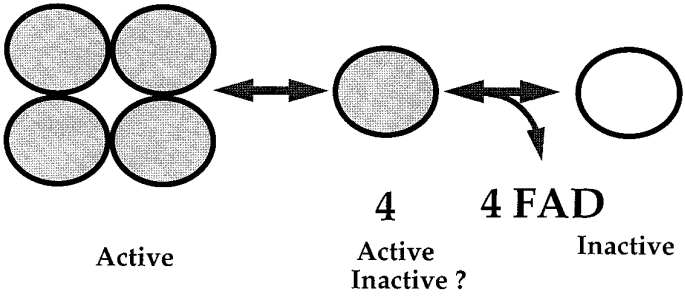


FIG. 6. Dissociation of the subunits of *Escherichia coli* methylenetetrahydrofolate reductase on dilution, with the subsequent loss of FAD.

the structure of this altered enzyme will provide new insights into the binding of flavin by MTHFR and into the relationship with cardiovascular disease.

Jim Peliska from the University of Michigan spoke on a collaborative project with Parke–Davis Pharmaceuticals on the characterization of new HIV-1 reverse transcriptase inhibitors. Over the last several decades, extensive research on the infection cycle of the retrovirus has revealed important details concerning its structure, molecular biology, enzymology and pathogenicity. An important milestone in the study of retroviruses came in 1970 with the discovery and isolation of viral RNA-dependent DNA polymerase (reverse transcriptase) by Temin and Baltimore (*39, 40*). Interest in retrovirology intensified in the mid 1980s with the discovery of the virus HIV as the required agent for acquired immunodeficiency syndrome (AIDS) (*41, 42*). A major emphasis of this research has been focused on developing anti-retroviral drugs that would affect particular stages of retroviral replication, with reverse transcriptase as a principle target. A significant complication in the development of a therapy for HIV is the high degree of genetic variation associated with its RNA genome. These variations result primarily from the low fidelity associated with proviral DNA synthesis by reverse transcriptase and the high frequency of genetic recombination occurring during reverse transcription (*43, 44*). This results in the spread of a broad population of genetically distinct HIV virion and the rapid selection of drug resistant viral strains. This collaborative project is developing strategies to elucidate the mechanistic details of one class of recombination events—termed forced copy-choice—that occurs during reverse transcription. Since these DNA strand transfer events occur frequently during reverse transcription (*45*), they make attractive new targets for therapeutic intervention. Such inhibitors could both inhibit viral replication directly, and also serve to curtail the level of genetic recombination that occurs during reverse transcription, thereby helping to stabilize the viral genome. Using a DNA strand transfer model system and a technique called scintillation proximity (*46, 47*), these researchers have applied drug screening technology developed in their laboratory to the identification of new inhibitors of HIV-1 reverse transcriptase (*48*). The screen was specifically designed to select inhibitors that target DNA strand transfer events catalyzed by the target enzyme

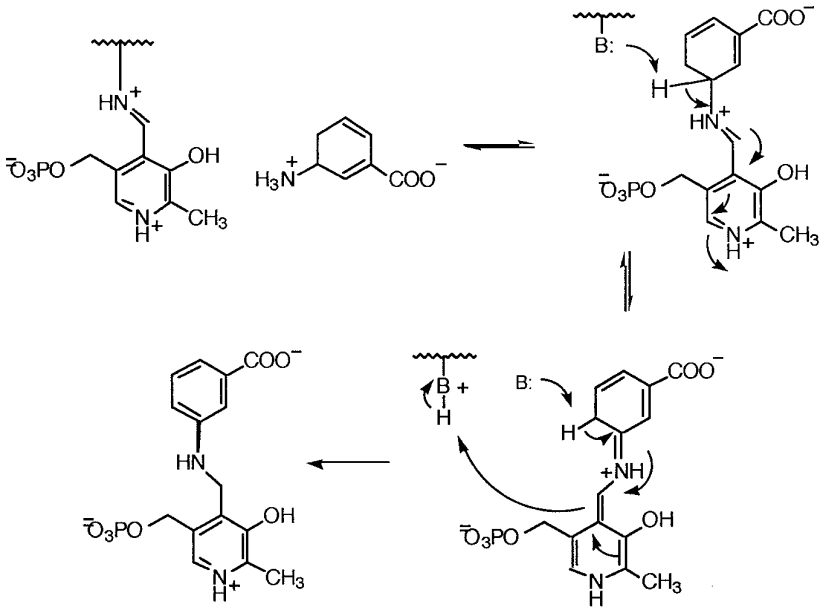
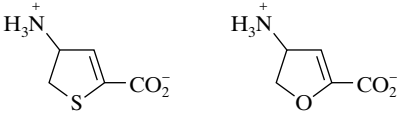


FIG. 7. Aromatization pathway for the mechanism-based inactivation of γ-aminobutyrate aminotransferase by isogabaculine.

during reverse transcription and recombination. Distinct classes of novel HIV-1 RT inhibitors have been identified by using these screening technologies on a very large compound database. Class I inhibitors act on both the strand transfer and the DNA polymerase activities, while class II inhibitors appear to affect only the strand transfer activity. The mechanism by which these new inhibitors function to inhibit recombination is under investigation using both biophysical and enzyme kinetic techniques.

In the last presentation of the meeting Mengmeng Fu from Northwestern University spoke about the synthesis and mechanistic studies of some potential anticonvulsant agents carried out in the laboratory of Dr. Richard Silverman. 4-Amino-4,5-dihydro-2-thiophene carboxylic acid **(VI)** and 4-amino-4,5-dihydro-2-furan carboxylic acid **(VII)** are rationally designed mechanism-based anticonvulsant agents (*49, 50*)



VI **VII**

based upon the known mechanism of the dihydroaromatic γ-aminobutyric acid aminotransferase (GABA-AT) inhibitors gabaculine **(VIII)** and isogabaculine **(IX)** (Fig. 7) (*51, 52*).

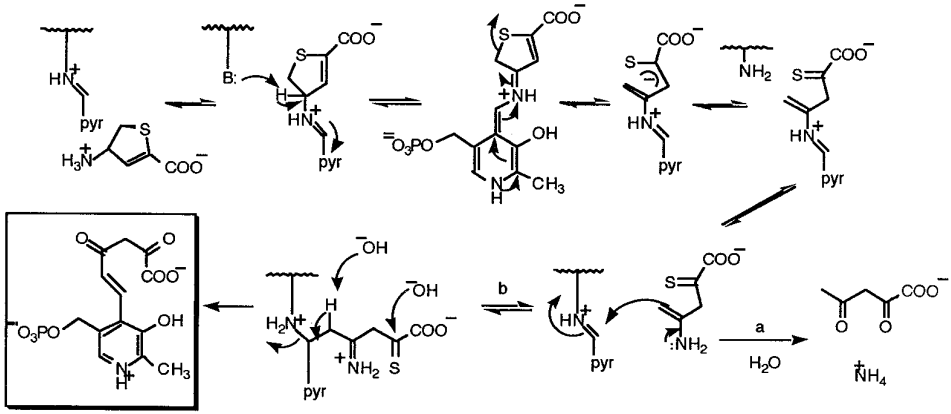
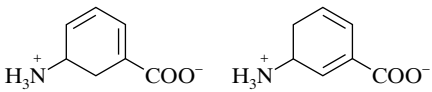
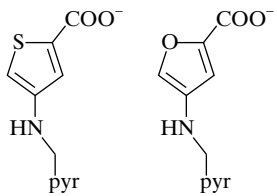


FIG. 8. Enamine pathway for the mechanism-based inactivation of γ-aminobutyrate aminotransferase by 4-amino-4,5-dihydro-2-thiophene carboxylic acid **(VI).**



VIII **IX**

It has been suggested that **VI** and **VII,** when incubated with GABA-AT, would be converted to aromatic pyridoxamine-5′-phosphate adducts **(X** and **XI).**



X **XI**

Studies in this laboratory of *R*-**VI,** *S*-**VI,** and *S*-**VII** have shown, however, that all of these compounds inactivate pig brain GABA-AT *via* mechanisms which are different from the proposed aromatization pathway. Despite the fact that GABA-AT has been shown to abstract only the 4-pro-S-hydrogen from the substrate GABA (*53, 54*), HPLC and mass spectrometric studies of metabolites formed have shown that both *R*-**VI** and *S*-**VII** inactivate GABA-AT by the same enamine mechanism (Fig. 8) out of several possibilities (*55*), with the inactivation by *R*-**VI** being significantly slower, as was predicted.

The mechanism of *S*-**VII** inactivation of GABA-AT is expected to be different from that of **VI,** because oxygen is not as good a leaving group as is sulfur. The metabolites formed from *S*-**VII** inactivation of [^{3}H]pyridoxal-5′-phosphate-GABA-AT coelute with cold pyridoxamine-5′-phosphate (PMP). The mass spectrum, how-

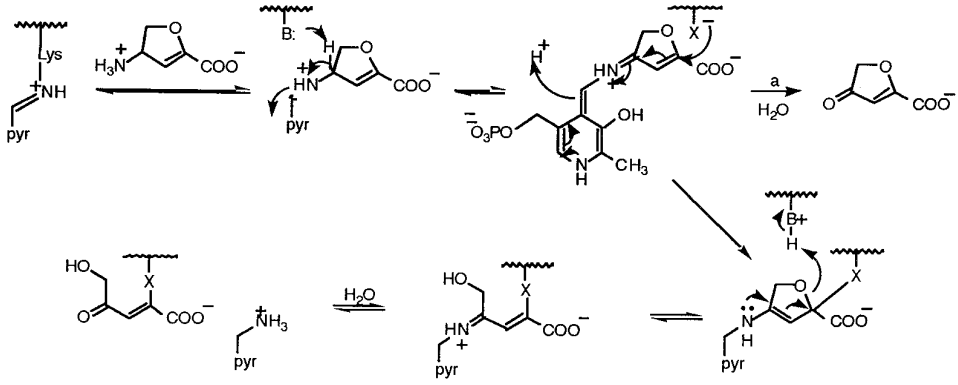


FIG. 9. Michael addition pathway for the mechanism-based inactivation of γ-amino-butyrate aminotransferase by 4-amino-4,5-dihydro-2-furan carboxylic acid **(VII).**

ever, showed the presence of PMP and also an unknown species with a mass 22 units greater than PMP. These results suggest the possible involvement of a Michael addition pathway (Fig. 9) for the inactivation of GABA-AT by *S*-**VII.**

APPENDIX: POSTER PRESENTATIONS

N^{ω}-Propargyl-L-arginine Is a Potent Inhibitor and Slow Substrate of Nitric Oxide Synthase, Walter Fast, Marc Levsky, Michael A. Marietta, and Richard B. Silverman, Department of Biochemistry, Molecular Biology, and Cell Biology and Department of Chemistry, Northwestern University, Evanston, IL 60208; and Interdepartmental Program in Medicinal Chemistry, College of Pharmacy and Department of Biological Chemistry, School of Medicine, University of Michigan, Ann Arbor, MI 48109.

T7 DNA Helicase Has Three Non-catalytic Nucleotide Binding Sites, A Possible General Feature for Hexameric Helicases, Kristen C. Moore, Manju M. Hingorani, M. Todd Washington, and Smita S. Patel, Department of Biochemistry, Ohio State University, Columbus, OH 43210.

T7 DNA Helicase: Mechanism of Nucleotide Triphosphate Hydrolysis and Similarities to the F_1-ATPase, M. Todd Washington, Manju M. Hingorani, Kristen C. Moore, and Smita S. Patel, Department of Biochemistry, Ohio State University, Columbus, OH 43210.

Towards the Discovery of Antiparasitic Drugs: Mechanism Based Design and Syntheses of Inhibitors for Glutathionylspermidine Synthetase, Shoujun Chen, David S. Kwon, Chun-Hung Lin, William P. Malachowski, Christopher T. Walsh, and James K. Coward, Interdepartmental Program in Medicinal Chemistry, College of Pharmacy and Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055; and Department of Biological Chemistry and Molecular Pharmacology, Harvard University Medical School, Boston, MA 02115.

Overexpression of S-Adenosyl-L-methionine Hydrolase, David L. Howe and Ronald W. Woodard, Interdepartmental Program in Medicinal Chemistry, University of Michigan, Ann Arbor, MI 48109-1065.

Further Studies of KDO 8-P Synthase: 3-Bromopyruvate Inactivation Studies and Further Site-Directed Mutagenesis, Mayur A. Patel, Christopher Adis, and Ronald W. Woodard, Interdepartmental Program in Medicinal Chemistry, University of Michigan, Ann Arbor, MI 48109-1065.

Probing the Active Site of Aspartate Aminotransferase Using NMR Methods, Emilia T. Mollova, David E. Metzler, and Agustin Kintanar, Department of Biochemistry and Biophysics, Iowa State University, Ames, IA 50011.

Preliminary Studies of the Cyclohydrolase of Histidine Biosynthesis, Robert L. D'Ordine, Thomas J. Klem, and V. Jo Davisson, Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN 47907-1333.

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ACKNOWLEDGMENTS

The author thanks each of the speakers for providing references and a summary of their presentation and appropriate illustrations. The help of Mrs. Rita Sabo in the preparation of this manuscript is acknowledged.

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