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CONFERENCE PROCEEDINGS

Tenth Enzyme Mechanisms Conference

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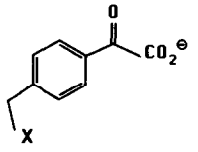
The tenth biennial conference on enzyme mechanisms was organized by T. Fink (chairman), R. Abeles, W. Cleland, B. Metcalf, and N. Oppenheimer. It was held at the Asilomar Conference center in Pacific Grove, California. Session topics included: 1, Bioorganic mechanisms; 2, structural mechanisms; 3, mechanisms through mutagenesis; 4, mechanism-based inhibition; 5, mechanisms—general; and 6, bioinorganic mechanisms. In addition to the lectures, two poster sessions were held which included topics from several areas of mechanistic enzymology. A brief summary of the lectures and a few references are presented below. As in the past, the authors and titles of the posters will also be listed.

BIOORGANIC MECHANISMS

In the first presentation, J. Kozarich, from the University of Maryland, discussed research efforts designed to identify enzyme-generated quininoid intermediates which could be chemically intercepted. To this end, the reactions of halogenated substrate analogs for three enzymes were described. Each of these enzymes was chosen for its ability to stabilize a carbanion intermediate during catalysis. Elimination of a halide from the resulting benzylic anion would result in a quinoidal intermediate, the existence of which could be supported through product analysis. Based on the leaving group potential of the halide, differential reactivity was observed in each case.

p-Bromomethylbenzoylformate (**I**) was found to be a potent inhibitor of benzo-



X= Br, Cl, F

I

ylformate decarboxylase from *Pseudomonas putida* (*1*). Deacylation, bromide elimination, tautomerization, and rearomatization of the thiamine pyrophosphate

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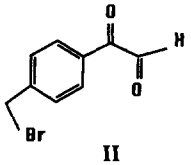
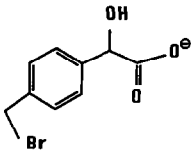
(TPP) adduct resulted in formation of *p*-methylbenzoyl-TPP. This species is converted to *p*-methylbenzoate by one of two mechanisms: either a slow, direct hydrolysis or a faster TPP-catalyzed process. In contrast, the fluorobenzoylformate is a substrate that is converted to the corresponding *p*-fluoromethyl aldehyde; the chloro analog partitions between both reaction pathways. Glyoxylase I, which is thought to be able to stabilize a carbanion intermediate in its catalytic mechanism (*2*), is similarly inactivated by *p*-bromomethylphenylglyoxal (**II**).



II

Here, enzyme-induced bromide elimination leads to a quininoid intermediate which can partition between product formation as *p*-methylbenzoylformate and enzyme inactivation.

Studies by G. Kenyon and co-workers (*3–5*) have suggested the involvement of a metal-stabilized carbanion intermediate in the reaction catalyzed by mandelate racemase. From this precedent Dr. Kozarich proposed that an enzyme-catalyzed elimination would be observed with bromomethylmandelate (**III**) as substrate.



III

This compound (λ_{max} = 234 nm), which is hydrolyzed nonenzymatically to *p*-hydroxymethylmandelate (λ_{max} = 222 nm) at pH 7.0, is enzymatically converted to a product identified to be methylbenzoylformate (λ_{max} = 264 nm). With fluoromethylmandelate, a good substrate for the enzyme, no elimination occurs. In the case of the bromide eliminations, electrophilic trapping of the resulting quininoid intermediate occurs by tautomerization and rearomatization of the activated complex.

These studies provide proof of enzymatic formation of quinoidodimethane intermediates, and establish minimal requirements of leaving group potential to generate such species. As emphasized by Dr. Kozarich, the similar behavior of the three enzymes, utilizing individual modes of intermediate stabilization, argues strongly for carbanionic intermediates of comparable reactivity.

Continuing her research efforts in the pathways of *de novo* purine biosynthesis, J. Stubbe, from the University of Wisconsin, presented results regarding the characterization and interactions of phosphoribosylamine (PRA) as a substrate for one of these activities, glycinamide ribonucleotide (GAR) synthetase, a protein

which recently was discovered to be able to catalyze three chemical transformations (*6–8*). PRA, the first intermediate in the purine biosynthetic pathway, has been reported to be of variable stability (*9, 10*). Professor Stubbe and her coworkers have characterized PRA for the first time using ^{13}C, ^{15}N, and two dimensional ^{13}C–^{1}H NMR spectroscopy and have studied its chemical properties. Incubating [1-^{13}C]ribose 5-phosphate (ribose 5-P) with $^{15}NH_3$ produced a 60:40 mixture of $\beta:\alpha$ PRA, α,β-ribose-5-P, and dimeric compounds. The K_{eq} for ribose 5-P and NH_3 with PRA was determined to be 2.5 M^{-1} by three independent methods. This K_{eq} was best calculated by separately measuring the rate of formation (k_f) of PRA from ribose 5-P and NH_3 via a coupled assay with cloned *Escherichia coli* GAR synthetase and its rate of decomposition (k_d). Both rates were measured as functions of pH and temperature. The half-life for decomposition of PRA at pH 7.5 and 37°C was determined to be 38 s, in agreement with the earlier study by Nierlich and Magasanik (*9*).

The rates of interconversion of the α and β anomers of PRA were also measured using saturation transfer and inversion transfer NMR spectroscopic techniques as a function of pH and temperature. At 37°C and pH 8.0, the rate constant for anomerization was found to be 27 s^{-1}, which can be compared to a turnover number of 14 s^{-1} for GAR synthetase determined under identical conditions.

Dr. Stubbe also discussed the purification to homogeneity of the *E. coli* GAR synthetase obtained from a cloned and overexpressed system. A kinetic analysis of the reaction catalyzed by this 110,000-Dal protein indicated that PRA adds before MgATP, which adds in rapid equilibrium prior to glycine. The K_m values for PRA, MgATP, and glycine are 75, 177, and 185 μM, respectively. From these kinetic constants, product distribution and similar data for PRPP amidotransferase, it was proposed that PRA does not exist free in solution, but is transferred or "channeled" directly between PRPP-amidotransferase and GAR synthetase.

One of the questions surrounding serine proteases is whether or not a hydrogen bond really exists between the serine and histidine of the active site. W. Bachovchin of Tufts University addressed this question using ^{15}N NMR spectroscopy with α-lytic protease. X-ray structures of α-lytic proteases suggest that His-57 and Ser-195 are too far apart to form a hydrogen bond. However, based on ^{15}N chemical shift data, it was concluded that a strong hydrogen bond exists between these two residues in the resting state of the enzyme (*11*). Furthermore, the hydrogen bond can be broken at low pH in complexes of the enzyme with diisopropylfluorophosphate or phenylmethylsulfonyl fluoride which function as tetrahedral intermediate analogs (*12*). The amino acids in the catalytic triad of Asp–His–Ser are strongly hydrogen-bonded. The Asp–His couple can activate the Ser-195 hydroxyl group for nucleophilic attack on substrate. The imidazole ring of His-57 may move from its position of serine activation to facilitate the formation of the transition state intermediate and subsequently to the acyl enzyme during the catalytic process. Thus the catalytic mechanism of this serine protease may involve direct movement of the imidazole ring of His-57 (*13*).

The second half of the lecture addressed the nature of boronic acid and peptide–boronic acid-inhibitor complexes of α-lytic protease. Boronic acid inhibitors can be divided into two categories. Type I inhibitors satisfy the primary specificity

requirements for the enzyme; in the complex both protons of the histidine-57 are hydrogen-bonded to the aspartate and serine of the catalytic triad. In the second case, Type II boronic acid inhibitors do not meet the specificity requirements of the enzyme and a covalent bond is formed between N-1 of His-57 and the boron atom of the inhibitor. Type I inhibitors are also "slow binding" but the nature of the histidine involvement in this process is not understood.

N. Pace from the University of Indiana spoke about RNase P, an enzyme made of nucleotides instead of amino acids which functions to cleave 5'-precursor segments during tRNA maturation. *In vivo,* this enzyme consists of two subunits; one RNA and the other protein (P). The two subunits can be dissociated by high concentrations of salt. The RNA portion alone can achieve catalysis, but high concentrations of cations (NH_4^+, Mg^{2+}) need to be present for catalysis *in vitro,* possibly to prevent anionic repulsion between the substrate and enzyme RNA. However, shielding anionic repulsion cannot be the only function since small cations (Na^+, Li^+) which bind nucleic acid more tightly than larger ions (K^+, NH_4^+) do not fully restore catalysis at any concentration (*14*). Small quantities of ethanol enhance the reaction at suboptimal ionic strength, consistent with simple electrostatic interactions promoting catalysis since solvents affect the dielectric constants.

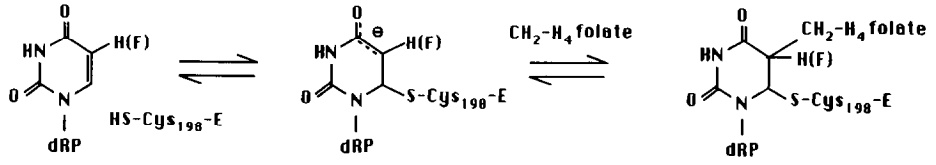
The sequences for RNase from several different sources were compared and the secondary structure is currently being determined by using a phylogenetic comparison of base-pairing possibilities. A comparison of secondary structures appears to be more relevant than the widely divergent primary sequences. The secondary structures for *Bacillus subtilis* and *E. coli* are very different, such that the "stems" are of different lengths and the sizes of the loops vary. Thus one must wonder if the RNA may have additional functions besides RNase P activity.

It appears that the tertiary structure may be very important in determining the mechanism of recognition. All the tRNAs in a cell have the general L-shaped tertiary structure. There are minor changes in structure, such as in the variable loops but RNase P must handle all 50 or so of these tRNAs. The *B. subtilis* RNase P RNA has been cloned and sequenced (*15*). Reconstruction of fragments of the RNA suggested that full tertiary structure and not a simple nucleotide sequence is required for full catalytic activity. Exactly why an enzyme is made of nucleotides instead of amino acids is still unknown but the RNA structure is possibly more fluid and more mobile than a protein would be at adjusting to meet the needs of such a variety of substrates.

STRUCTURAL MECHANISMS

In the opening lecture of this session, D. Santi and R. Stroud of the University of California, San Francisco presented the results of mechanistic and structural studies of the thymidylate synthase of *Lactobacillus casei.* Professor Santi summarized his group's efforts of nearly two decades to elucidate the chemical mechanism of the enzymatic reaction (*16–19*). From structural and mechanistic studies using the mechanism-based inhibitor 5-fluoro-2'-deoxyuridine 5'-monophosphate

(FdUMP; (*20–22*)) the conjugate addition of an enzymatic nucleophile (Cys-198) (*23, 24*) to the C-6 carbon elicits a carbanion at C-5 (**IV**) which attacks the methylene group of the cofactor, $N^{5,10}$-methylenetetrahydrofolate (CH_2–H_4folate) to form a covalent ternary enzyme–(F)dUMP–CH_2–H_4 folate complex (**V**).



IV V

Covalent modification studies have also provided insight into the primary structure of the enzyme's active site. The binding of dUMP to thymidylate synthase affords protection against both thiol- and arginine-modifying reagents. The former result is expected since it has been shown that Cys-198 attacks the C-6 carbon of dUMP, and a charged arginine residue(s) most likely engages the dianionic 5′-phosphate group of the substrate.

Thymidylate synthase is found as a dimer of identical 35-kDa subunits in species ranging from bacteria and bacteriophages through the vertebrates. The primary structure of the enzyme from eight different sources reveals that thymidylate synthase is even more conserved than the cytochromes, demonstrating one residue change for every 23 million years (*25*).

Professor Stroud described efforts to elucidate the atomic structure of *L. casei* thymidylate synthase (*25*). X-ray crystallography of hexagonal crystals of the protein produced a structural solution at 3 A resolution. The interface between the subunits of the dimer results from associations of five β-sheets found in each monomer. Residues from both subunits contribute to each active site, explaining the inactivity of monomers and suggesting a model for the well-characterized negative cooperativity of substrate binding (*26*). A very hydrophobic and highly conserved helical core element exists in the center of the protein, and may contribute to binding of the folate cofactor. Insertions or deletions of residues relative to a consensus conserved sequence are found on the outside surface of the enzyme, and may not appreciably affect its catalytic action.

The active site, containing Cys-198, was shown to be a cleft between the monomers. The proximal and invariant Arg-218 may form a hydrogen bond or ion pair with this sulfhydryl to render it more nucleophilic. This arginine is within 5.5 A of the Arg-179 of the other monomer; the two guanidinium groups are probably those which engage the 5′-phosphate group of dUMP as revealed by chemical modification studies.

R. Almassy presented the results of ongoing efforts in D. Eisenberg's laboratory at University of California, Los Angeles to solve the structure of glutamine synthetase (GS) from *Salmonella typhimurium*. This complex bacterial protein is a dodecamer consisting of identical 52-kDa subunits arranged in two stacked rings of six monomers (*27*). *S. typhimurium* glutamine synthetase, in a completely

unadenylylated form, was crystallized in the presence of $MnCl_2$, and its structure was solved to 3.5 A resolution (*28*). Among the interesting structural features of the protein are: (a) the two stacked hexameric rings (outer diameter of one hexamer = 143 A) are nearly eclipsed; (b) the polypeptide chains of each subunit are composed of α-helices mainly in the N-terminal domains and the centers, which run perpendicular to antiparallel β-sheets found in the C-terminal domains; (c), a polypeptide "loop" (amino acids 156–173) within each monomer extends into and partially blocks the central channel (or "hub") of the dodecamer with a separation of 24 A; and (d) the active sites, clearly distinguishable by the presence of two Mn ions, occur at the interface of two coplanar subunits and are formed by two polypeptide chains. The polypeptide loops, exposed to medium within the central cavity, are extremely sensitive to proteolysis; a molecule of trypsin actually fits into the 40-A-diameter central cavity of the protein.

The enzyme's active sites are formed mainly by six β-strands of the C-terminal domain of one subunit and two β-strands of the N-terminal domain of another. The two manganese ions are separated by 5.8 A (confirming the value previously reported (*29*)). That an N-terminal region is integrated into the active site is substantiated by chemical labeling studies using 5′-*p*-fluorosulfonylbenzoyladenosine (FSBA), which modifies Lys-47 (*30*). A difference map of FSBA-treated GS reveals this linkage to Lys-47, and also shows that the central polypeptide loops move upon binding of the nucleotide analog.

The crystal structure of GS that had been pretreated with ATP and the irreversible inhibitor, methionine sulfoximine (MetSox; (*31*), revealed significant differences from that of the free enzyme. Density changes observed in the region of the active site(s) indicate that the binding site of MetSox is just above the metal ions and in the interface between the subunits of each hexameric ring. Moreover, the binding of MetSox pulls regions of density of the N-terminal domain of one subunit on top of the C-terminal domain of its neighbor, thereby corroborating the notion that the active site exists at the subunit–subunit interface. In a sense, the formation of an enzyme–ATP–MetSox complex "glues" the subunits together, perhaps in order to exclude solvent from the active site during catalysis.

R. Huber from the Max Planck Institute described the apparatus and principles for the conversion of light energy to chemical energy in photosynthetic bacteria. The crystal structures of the reaction center of *Rhodopseudomonas viridis* (*32, 33*) and the light-harvesting complexes (*34*) of cyanobacteria provide the basis for understanding the chemical principles of this conversion. The structures of the various components of the photosynthetic apparatus were described in some detail (*35*) along with a general description of overall structure based on previous work by electron microscopy (*36*).

The reaction center is composed of four subunits, L, M, H, and *c*-type cytochrome, which have all been crystallized. The L and M subunits each form five membrane spanning helices, and are associated in pseudo-twofold symmetry. Together, they contain four molecules of BChl-b and two of BPh-b, which constitute the pigments required for electron transfer. These act as an electron donor pathway for the associated quinone acceptor (Q_A), which reduces a second quinone (Q_B) with mediation by a single non-heme iron. Two of the BCh-b mole-

cules are closely associated, forming the "special pair," which is reduced by the cytochromes contained in the cytochrome *c* subunit. This subunit is associated with one side of the L–M pair, so that electron transfer is directional through the cytochrome subunit and the L–M pair. The H subunit is associated with the other side of the L–M pair, but its function is unclear. The rate-limiting step in the ultimate reduction of Q_B is the iron-mediated electron transfer from Q_A to Q_B; all other steps in the electron transfer sequence are extremely rapid (on the order of picoseconds).

The reaction center is the central portion of the photosynthetic apparatus, which also includes the light-harvesting and peripheral light-harvesting complexes (LHC and PLHC, respectively). The building block components of the PLHC also have been crystallized. These consist of homologous α and β chains assembled as a trimer of dimers, which are then stacked in various arrangements to give the PLHC. The overall structure of a dimer resembles that of myoglobin, with similar attachment of the chromophores, which are extended linear tetrapyrrole structures. Each chromophore in the trimer has a different λ_{max} due to its microenvironment and thus there is a directionality for electron transfer in these components as well as in the reaction centers. The basic theme of the light-harvesting process in bacteria is directional energy transfer through rigidly arranged protein components containing chromophores with modulated redox potentials. The proteins provide the spatial arrangement, alter redox potential, and assist in electron transfer. Thus, a surprisingly complete picture of this complex process is rapidly emerging from the combination of crystallography, chemistry, and electron microscopy.

MECHANISMS THROUGH MUTAGENESIS

The session on site-directed mutagenesis included discussions on both techniques and applications. The technology of specific amino acid replacement is being used to study very detailed questions of enzyme mechanism and structure–function relationships, as well as to engineer proteins with new activities and different stability or specificity properties.

M. Smith from the University of British Columbia opened the session by introducing the techniques of site-directed mutagenesis. He outlined the most commonly used methods for *in vitro* mutagenesis, including random mutagenesis, oligonucleotide-directed mutagenesis, and partial or total gene synthesis. These techniques have been extensively reviewed by Smith and others (*37–39*).

He then presented work in which oligonucleotide-directed mutagenesis was used to make a number of mutants of cytochrome *c* peroxidase (CCP) and cytochrome *c* in order to study specific properties of the mutant enzymes. In CCP, mutants at Trp-51 and Met-174 (*40*) were made to try to determine the origin of the $g = 2$ EPR signal arising when CCP reacts with hydrogen peroxide to form compound ES, an oxidized Fe(IV) form of the enzyme. A signal of this type is not observed in the oxidized state of horseradish peroxidase, and the origin and importance of the signal is unclear. Ser-172 and Cys-172 mutant enzymes were

both fully active, and both exhibited EPR signals, although the signals appeared sharper than that in the wild-type enzyme, apparently because a broad component of the signal was missing. A Phe-51 mutant enzyme exhibited increased activity and a shifted activity optimum with respect to pH. This enzyme also retained the EPR signal observed in the native protein, although the temperature dependence of the signal was different. Finally, it was shown that the sharp component of the signal represents only 10% of the iron spin density, and it was suggested that the signal is heme-centered and is modulated by the adjacent amino acids Trp-51 and Met-172.

Phe-87 is strictly conserved in all cytochrome *c* sequences known, and has been suggested to mediate electron transfer as it forms part of a hydrophobic shell on one heme edge. The redox potentials and electron-transfer rates to and from the heme were measured on Gly- Ser-, and Tyr-87 mutants of yeast cytochrome *c* (*41*). All of the mutants were functionally active with respect to CCP, with rates of oxidation from 20 to 70% that of wild-type enzyme. The results were interpreted to suggest that an aromatic, or possibly lipophilic, residue is required at this position to correctly modulate the redox potential, but Phe-87 is not required for electron transfer.

The substrate specificity of an enzyme is determined by a complex amalgam of steric, hydrophobic, hydrogen-bonding, and electrostatic interactions between enzyme and substrate. J. Wells of Genentech described the use of site-directed mutagenesis to probe these interactions in the serine protease, subtilisin (*42*). For subtilisin, the P1 site of a given peptide (H_2N–P_n . . . P2–P1–C(O)NH–P1′–P2′ . . . P_n'–COOH) is the dominant determinant for substrate specificity. The P1 binding cleft in the enzyme contains Gly-166, so that the wild-type enzyme can accommodate bulky residues such as tyrosine at P1. To probe the steric and hydrophobic interactions between the P1 residue of the substrate and this P1 binding cleft in the active site, two residues within the cleft of *B. amyloliquefactiens* subtilisin, Gly-166 and Glu-156, were mutagenized separately and in combination (*42*) by making use of cassette mutagenesis (*43*). The P1-variable subtilisin substrate employed was succinyl-Ala–Ala–Pro–P1-*p*-nitroanilide (sucAAPXNA), in which P1 was the amino acid Tyr.

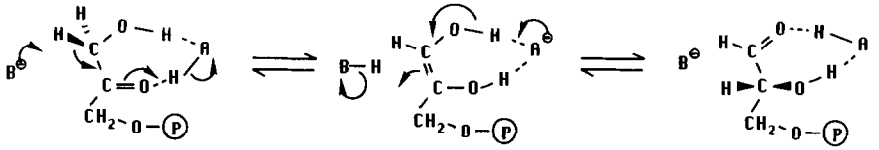
The mutagenesis of Gly-166 to other residues resulted in the expected alterations in substrate specificity at P1. As the residue substitution at position 166 increased in steric bulk (Gly → Thr → Leu → Trp), the k_{cat}/K_m value for sucAAPYNA (P1 = Tyr) decreased linearly. Coordinately, subtilisin mutants containing bulkier groups at position 166 preferred smaller residues at P1 (e.g., P1 = Ala was a much better substrate than P1 = Tyr for Trp-166). As the hydrophobicity of the mutant residue at position 166 increased, so did the preference for a hydrophobic residue at P1. When position 166 was varied with isosteric side chains of increasing hydrophobicity (Asp → Asn → Leu), there was a commensurate preference for a substrate with a hydrophobic residue at P1. The selection of these isosteric side chains eliminated steric effects.

Charge substitutions at position 166 increased affinity for substrates containing complementary charges at P1 while residues bearing the same charge were rejected (*44*). The Asp-166 mutant effectively utilized a substrate in which P1 repre-

sents Lys. Using this approach, it was possible to nearly interchange the substrate specificity profiles of the subtilisin of *B. amyloliquefactiens* with that of *B. licheinformis* by exchange of residues in the P1 binding cleft.

Triosephosphate isomerase (TIM) catalyzes the isomerization of glyceraldehyde 3-phosphate (GAP, **VII**) to dihydroxyacetone phosphate (DHAP, **VI**) at the limit of diffusion control ($k = 4 \times 10^8$ M^{-1} s^{-1}), indicating that the binding of GAP to enzyme comprises the highest transition state of the reaction (*45–48*). *In vivo*, the lowest ground state in the free-energy profile of this reaction is free enzyme plus the more stable substrate, DHAP. The enzyme constitutes a perfect enzymatic catalyst in that it is incapable of reducing the greatest difference in free energy for the reaction, and has therefore reached an endpoint in its evolution. As such, a genetically engineered mutation of any of the enzyme's catalytic residues would result in silent or deleterious effects on the catalytic power of the enzyme. The research group of J. Knowles at Harvard University has destroyed the catalytic perfection of TIM via site-directed mutagenesis in order to study the evolutionary reversion of the resulting "imperfect" isomerases to perfect catalysts.

In the reaction catalyzed by TIM, a single enzymatic base (B), identified as Glu-165, abstracts a proton from both DHAP and GAP (*49–53*). Site-directed mutation of Glu-165 of the chicken liver isomerase to an aspartyl residue results in a mutant enzyme with a specific activity which is 0.1% of that of the wild-type TIM (*54*). Apparently, by removal of a methylene unit in the residue, the ability of this essential carboxylic group to abstract protons from either DHAP or GAP is greatly diminished in the mutant. While the respective free energies of the enzyme-bound reaction intermediates are unaltered, the transition state barriers separating them have been greatly increased. The rate-limiting step of this mutant isomerase is no longer diffusion of the substrate through solvent but the interconversion of DHAP and the *cis*-enediol (**VIII**) on the enzyme. Accordingly, the



VI VIII VII

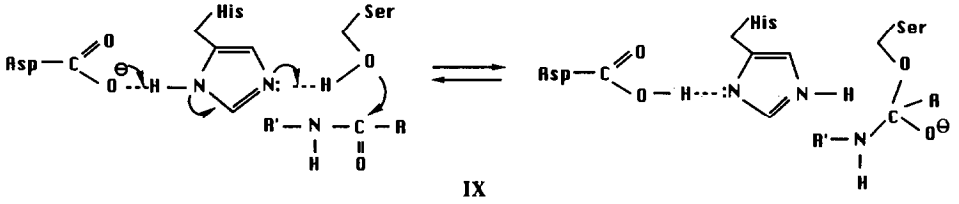
transition state barrier for this reaction is too high, and so the reversion of this mutant isomerase back to catalytic perfection requires a mutational event that restores the more uniform binding of ground states and transition states observed in the wild-type enzyme.

To accomplish this reversion, *E. coli* containing the gene for the Glu-165 → Asp mutant was subjected to random mutagenesis using nitrous acid, formic acid, and hydrazine in order to effect changes in all four bases. *E. coli* containing wild-type triosephosphate isomerase, but not those that are TIM-deficient can grow in media containing only lactate or glycerol. The mutant colonies bearing "sluggish" isomerases could be selected from TIM-deficient *E. coli* by their ability to grow on

media containing lactate alone, but not glycerol alone. Using this selection method, 10 revertant colonies were found out of nearly 13,000 colonies of mutagenized bacteria. Six of these were shown to be true revertants in that Asp-165 had reverted back to Glu-165. Characterization of the other revertants revealed an isomerase which is 25-fold more active than the Glu-165 → Asp mutant. This "pseudo" revertant still possesses an aspartyl residue at position 165, but the mutation of the serine at position 95 in the wild-type enzyme to proline had bestowed upon the "pseudo" revertant a 20-fold increase in activity over the Glu-165 → Asp mutant. Future kinetic and structural characterization of this Glu-165 → Asp/Ser-95 → Pro mutant should reveal those aspects of the reaction energetics that have been improved by this new mutation.

MECHANISM-BASED INHIBITION

The afternoon session on mechanism-based inhibition focused on the design and structural characteristics of inhibitors for serine and zinc proteases. Classically, serine proteases are thought to contain an active site triad consisting of Asp–His–Ser (**IX**), which also has been referred to as a charge relay system.



IX

Despite extensive work, the precise involvement of these three residues in the catalytic mechanism is not completely understood for any serine protease.
In addition to the metal ion, glutamyl and histidyl residues are thought to be involved in the catalytic machinery of zinc proteases.

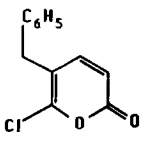
P. Bartlett from the University of California at Berkeley spoke about phosphorus-containing transition state inhibitors of zinc proteases. Several potent inhibitors of thermolysin have been made in which the carbon atom of the scissile peptide linkage has been replaced with phosphorus. The substitution of a tetrahedral phosphorus in several phosphonamidate or phosphonate ester peptide analogs has led to inhibitors which are transition state or intermediate state analogs (*55*). A series of carbobenzoxy-Gly–Leu–*X* inhibitors was prepared in which the Gly–Leu peptide linkage was replaced with either a phosphonate ester or a phosphonamidate. Each phosphonate ester binds about 840-fold weaker than the corresponding phosphonamidate, indicating a loss of 4 kcal/mol in binding energy (*56–57*). The X-ray structures are virtually identical for the Cbz–Gly^{P}-(O or NH)-Leu–Leu pair (*58*). Thus the enzyme–inhibitor complexes are essentially identical except for the presence or absence of a specific hydrogen bond.

Incorporation of an α-substituted phosphonic acid analog yielded inhibitors that exhibit slow-binding kinetics. In the case of Cbz–(L)–Phe^{P}-(O)-Leu–Ala binding to thermolysin, the inhibitor is bound so tightly that the $t_{1/2}$ for dissociation of the enzyme–inhibitor (EI) complex is 116 days. How can one account for slow-binding of an inhibitor? One possible mechanism for slow-binding of protease inhibitors may be their extrusion of a water molecule in the active site. When a normal fast-binding inhibitor binds, several water molecules are displaced. It is postulated that when a slow-binding inhibitor binds, an additional water molecule is ejected. Expulsion of this specific water molecule is a rare event but once the inhibitor binds in the absence of this water molecule, a very tight EI complex is formed.

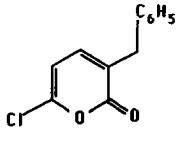
The basic inhibitor design strategy for thermolysin was to determine the best substrate for a particular enzyme and make the corresponding phosphorus-containing tetrahedral intermediate analogs. This design concept has been extended to other zinc proteases. While these phosphorus amino acid and peptide analogs are not very good inhibitors of leucine aminopeptidase, they are very potent inhibitors of carboxypeptidase A.

D. Ringe from the Massachusetts Institute of Technology spoke about the structures of the complexes of inhibitors bound to serine proteases. Elastase is an endopeptidase in animals which is specific for the structural protein elastin, the main component of tendons, ligaments, bronchi, and arterial walls. An inhibitor of this enzyme could have important pharmacological and therapeutic applications. In an effort to design a very specific inhibitor for elastase the following plan was adopted: (1) use chymotrypsin as a model to establish ground rules for designing inhibitors; and (2) attempt to apply the resulting empirical rules toward the inhibition of elastase.

Inactivation of chymotrypsin by 5-benzyl-6-chloro-2-pyrone (**X**) and 3-benzyl-6-chloro-2-pyrone (**XI**) has been shown to occur in a mechanism-based manner (*59*).



X



XI

The unsubstituted chloropyrones are not inactivators of chymotrypsin and hence, the position of the benzyl group affects its specificity. During inactivation of chymotrypsin by **X**, the chlorine is lost. Dr. Ringe described crystallographic studies indicating that the pyrone ring remains intact in the enzyme–inhibitor complex. A covalent bond is formed between C-6 of **X** with the β-oxygen of serine while the 5-benzyl group of **X** residues in the hydrophobic specificity pocket of the enzyme (*60*). The rings of **X** in the unbound state are perpendicular to each other. Upon binding, the rings become coplanar. The active site serine moves downward, and a tryptophan also moves to close in the specificity pocket. Thus (1) the inhibitor accommodates the enzyme and (2) the enzyme accommodates the inhibitor.

During inactivation by **XI**, the pyrone ring is opened and a covalent adduct is formed that will slowly deacylate. Crystallographic analysis shows that the benzyl group is again held in the hydrophobic pocket of the enzyme but the free carboxylate forms a salt bridge with the active site histidine which prevents water from attacking the acyl linkage (*61*). The reactivation process can be explained by reorientation of the terminal carboxylate group so that the salt bridge to histidine-57 is broken followed by rapid deacylation of the complex to regenerate free enzyme and 4-benzyl-2-pentanoic acid.

As pointed out by Dr. Ringe, crystallographic results can be both misleading and enlightening. α-Trifluoroketones, another class of serine protease inhibitors, are proposed to interact with serine to form a tetrahedral, intermediate-state analog. When such an inhibitor was crystallized with chymotrypsin, however, the structural analysis was ambiguous since a second enzyme molecule altered the anticipated positioning of the bound inhibitor. In addition, tri-L-Ala, a known elastase inhibitor, was shown to coordinate to an active site tyrosine, not to any of the amino acids of the catalytic triad. Ac-L-Ala-L-Pro-L-Ala-*p*-nitroanilide is a substrate of elastase resulting in acylation of the active-site serine by the C-terminal alanyl residue. Two molecules of the substrate are bound in the crystals of the complex, one in each direction of the two inhibitor grooves. Ac-L-Ala-L-Ala-L-Pro-L-Ala-CH_2Cl was proposed as an elastase inhibitor in which the α-chloroketone might alkylate an active-site histidine. Initially, upon soaking crystals of elastase in solutions containing the inhibitor, the crystals fractured. Therefore, the crystals were stabilized and then soaked in the presence of the inhibitor. Subsequent structural studies indicated that the inhibitor does react with histidine; however, preferential binding occurs in the specificity pocket, not in the catalytic site. This "crystallographic artifact" is due to a second molecule of elastase partially blocking the region where inhibitor binding is anticipated, explaining why the crystals fractured upon binding of the inhibitor in the presumably preferred catalytic site.

The presentation by Dr. Ringe emphasized that (1) protein and inhibitor interactions can result from mutual structural accommodations and (2) in view of the unexpected positioning of the inhibitors, results obtained from crystallographic studies must be coupled with an understanding of enzyme–ligand interactions to identify potential crystallographic artifacts.

D. Hangauer from Merck, Sharp, and Dohme Research Labs continued on the theme of the afternoon session by describing design strategies toward the inhibition of the zinc proteases thermolysin and angiotensin-converting enzyme (ACE). The goals of these efforts were to design inhibitors of thermolysin based on enzyme–ligand interactions predicted by molecular modeling, and to use these results to aid in the design of other zinc-dependent proteases, such as ACE. Using interactive computer graphics and the crystal structure of thermolysin (*62*), the cleavage mechanism of a model substrate, Z-Phe–Phe–Leu–Trp (Z = benzyloxycarbonyl), was studied. Several binding modes for the Michaelis complex and the tetrahedral intermediate in the thermolysin active site were discussed by Dr. Hangauer. The proposed trajectory for proceeding from the Michaelis complex to the tetrahedral intermediate was described to involve the simultaneous

activation of a zinc-bound water molecule with attack on the scissile peptide.

Using crystallographic data of thermolysin with phosphonamidate (*63*) and peptide (*64*) inhibitors, two potential sites of binding of a tetrahedral intermediate were defined. Dr. Hangauer compared the binding of these analogs with the zinc endopeptidase inhibitor design developed at Merck, represented by *N*(1-carboxy-3-phenylpropyl)-Leu–Trp. The design of several compounds as potential inhibitors based on predictive interactions for thermolysin was presented. In contrast to a parallel ordering of the inhibitor potency to substrate efficiency for structurally analogous compounds as previously described by P. Bartlett for the phosphonate inhibitors, the inhibitors described by Dr. Hangauer did not follow the same structural trends. Several additional energetically favorable interactions were discussed, including the displacement of an active-site water molecule, coordination of the inhibitor to the zinc as a sixth ligand, and coordination of the inhibitor to an active site histidine. Subsequent crystallographic analysis (*65*) indicated that a Michaelis complex, which does not include coordination of the scissile peptide to the zinc atom, was consistent with a tetrahedral intermediate complex wherein the two oxygen atoms of the hydrated scissile peptide straddle the zinc in a bidentate fashion. From these observations, Dr. Hangauer suggested that the Merck inhibitor design is an analog of the transition state for breakdown of the tetrahedral intermediate.

Several newly designed thermolysin inhibitors also were tested as inhibitors of ACE. The results of these experiments indicated that thermolysin is good as a qualitative model for ACE, but the inhibition potencies for the two enzymes were not parallel. Dr. Hangauer concluded that this differential response to alterations in some of the compounds is indicative that ACE might have a Glu or Asp near the S_1 subsite, whereas thermolysin does not. Through the combination of crystallographic information and computer graphic modeling, Dr. Hangauer and his colleagues are now able to better understand potential interactions with the binding sites of these two zinc proteases.

MECHANISMS—GENERAL

The research of Professor S. A. Bernhard's group at the University of Oregon is aimed at understanding the mechanisms of transfer of substrates and products by contiguous enzymes in a metabolic pathway. In yeast, the concentrations of glycolytic enzymes (32–1400 μM) are comparable to those of their corresponding substrates (20–26,000 μM). With suitably low binding constants the concentrations of unbound glycolytic metabolites *in vivo* may be negligible, such that transfer of a glycolytic intermediate from one enzyme to the next may be direct, that is, does not involve dissociation and random diffusion through the solvent. The rapid combined turnover number of the coupled phosphoglycerate kinase–glyceraldehyde-3-phosphate dehydrogenase (PGK-G3PDH) system significantly exceeds the maximum rate of dissociation of 1,3-diphosphoglycerate (DPG) from PGK ($K_d <$ 10 n*M*), suggesting direct transfer of DPG from PGK to G3PDH (*66*).

Likewise, the transfer of NADH from G3DPH to liver alcohol dehydrogenase (LADH) is proposed to proceed by a direct transfer mechanism. Using an excess of G3PDH (E_1) over NADH, the concentration of free NADH was rendered negligibly low, such that the observed rapid rate of LADH-catalyzed oxidation of NADH was proposed to occur by a direct enzyme–metabolite (E_1–M) to enzyme (E_2) transfer, not by dissociation/diffusion of NADH (*67*). The kinetics of these and other E_1–M–E_2 coupled enzyme systems also suggest direct transfer and conform to the Michaelis–Menten equation. An excess of E_1 over E_1–M constitutes competitive inhibition. From these studies it is clear that some structural features of the E_1–M complex contain the correct determinants for recognition by E_2.

An important question arises from these findings: do all dehydrogenases transfer their nicotinamide coenzymes via an E_1–M–E_2 complex? From studies with a variety of coupled dehydrogenases, the pattern that emerges is that direct transfer of NADH occurs whenever the two successive dehydrogenases possess opposite stereospecificity of hydride transfer (*67–68*). An A-dehydrogenase such as LADH transfers NADH directly to the B-specific glutathione reductase, but NADH transfer to other A-dehydrogenases such as malate dehydrogenase and alanine dehydrogenase requires dissociation and random diffusion of the coenzyme.

Molecular graphics analysis have shown that a rotation of 180° about the glycosidic bond of the coenzyme attends its transfer from an A-dehydrogenase to a B-dehydrogenase, permitting its proper conformation on the recipient enzyme (*69*). In this fashion, a B-dehydrogenase is structurally complementary to an A-dehydrogenase–NADH. This complementarity is further emphasized by the finding that A-dehydrogenases are predominantly negatively charged in the coenzyme binding site while the B-dehydrogenases are positively charged. As such the direct transfer mechanism for NADH may be driven by electrostatics.

In the second presentation of the session, A. Cerami from the Rockefeller University summarized work on cachectin, a macrophage protein that induces catabolic states in animals. Parasitic, bacterial, and viral infections in addition to malignant tumors in mammals often induce a condition known as cachexia (or wasting). This disease state is characterized by decreased food consumption, weight loss, degeneration of muscle and adipose tissues, anemia, and induction of a catabolic state. Although common in many parasitic diseases, the mechanism of this disorder has only recently begun to be unraveled. To this end, Dr. Cerami and his co-workers have identified and purified a 17.5-kD protein from endotoxin-stimulated RAW 264.7 macrophages; it is these cells (*70*) that may be responsible for cachexia and shock. This protein, cachectin, is produced by macrophages in response to endotoxin or several other bacterial or protozoal products. Upon release, cachectin acts as a hormone, binding to specific high affinity receptors to elicit a number of biological responses.

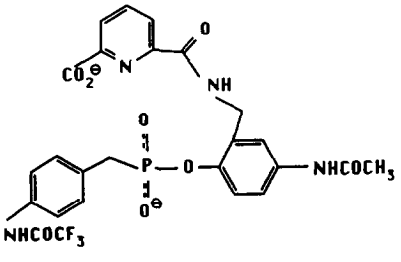
Dr. Cerami has shown, for example, that in the adipocyte, selective inhibition of mRNA production can selectively suppress several anabolic enzymes such as lipoprotein lipase (*71*). Glucose is no longer converted to fats, causing cell metabolism to switch to the breakdown of fatty acids. Cachectin-induced changes in muscle physiology include increases in muscle consumption and lactose produc-

tion with decreased resting membrane potential, glycogen storage, and protein mass (*72*). During the course of the characterization of cachectin, Dr. Cerami and his co-workers discovered that purified mouse cachectin resembled human tumor necrosis factor in amino acid composition, N-terminal amino acid sequence (14 of the first 19 amino acids were conserved), and biological activity toward actinomycin D-treated L-929 cells (*73*). This observation served to emphasize the extensive range of effects associated with this protein.

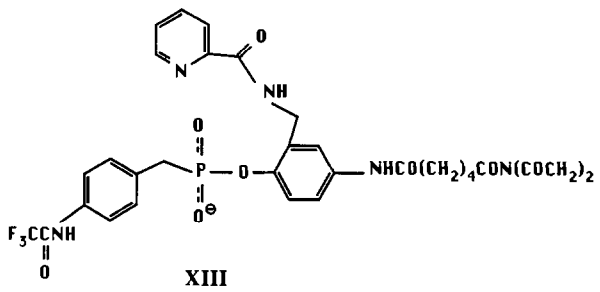
Cachectin also has many properties similar to those of interleukin-1 (IL-1), yet they bind to different receptors and the proteins lack structural homology. It was proposed that cachectin itself initiates pharmacological effects following which, IL-1 is released, inducing additional biological responses (*74*). It was proposed that low levels of cachectin are helpful to the host in removing invasive pathogens—with severe wasting and shock resulting from a normal condition of fighting infection that has become uncontrollable. This supposition was supported by decreased mortality *in vivo* upon treatment with antibodies raised against cachetin prior to cachectin challenge. These results have added to the understanding of the biological properties of cachectin, its production, and its role in cachexia and shock.

In bridging the disciplines of immunology and enzymology, R. Lerner of the Scripps Institute described the development of antibodies which can perform a specific catalytic chemical reaction. Traditionally, an antibody is considered to be a protein which specifically binds other molecules, but does not break any bonds or perform catalysis; thus, the immune system acts as a binding or flagging process. Previous attempts to use antibodies as templates for simulating an enzyme active site were met with limited success (*75–76*). Dr. Lerner and his co-workers, however, have extended this concept through implementation of the Pauling transition state theory of binding energy (*77*). It was thought that an antibody elicited to a hapten representing the presumed transition state in a catalytic reaction should lower the free energy of activation by stabilizing the transition state relative to reactants and products.

Dr. Lerner discussed using this concept in the development of a catalytic-antibody, termed an "abzyme," which could perform the hydrolysis of carboxylic esters. Peptides containing a tetrahedral phosphorus, as previously described by Bartlett (*55*), are known to be potent inhibitors of metalloproteases, presumably by acting as a transition state analog. From these results, Dr. Lerner and his colleagues employed the phosphonate esters (**XII** and **XIII**) as haptens for the

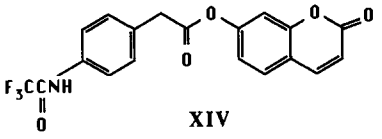


XII

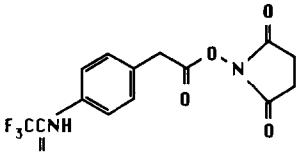


XIII

catalytic site. Two antibodies elicited to **XIII** demonstrated specific ester hydrolysis activity upon **XIV** and **XV** with concurrent acyl transfer to an essential residue

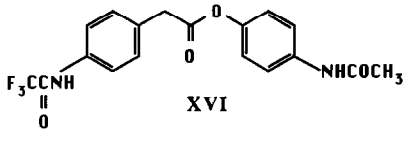


XIV

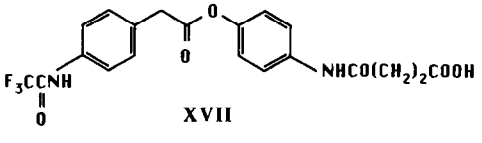


XV

of the antibody catalytic site (*78*). Dr. Lerner proposed this functionality to be a histidine, and that through a judicious choice of substrates, this process was demonstrated to be catalytic, not just stoichiometric. To test this hypothesis, the substrates were modified such that the expelled phenolate would be a better leaving group, as in **XVI** and **XVII**. Several of these compounds were substrates for the catalytic antibody, demonstrating enzyme-like saturation kinetics. In addition, the phosphonate-ligands were competitive inhibitors in Lineweaver–Burk analysis against substrate **XVI**.



XVI

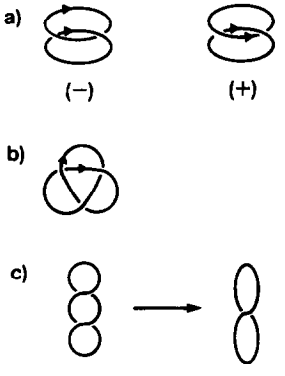


XVII

As typified with **XVI**, the K_m, K_i, V_m, and k_{cat} of the catalyzed reaction are 1.9 ± 0.2 μM, 0.16 ± 0.4 μM, 2.2 ± 0.2 × 10^{-9} M/s, and 0.027 s^{-1}, respectively, with a ratio of the catalyzed to the uncatalyzed hydrolysis rate of 960 (*79*). Interestingly, substrate specificity for the abzyme is greater than with typical metalloesterases.

The use of antigenic recognition coupled with other activities or properties could have widespread application in chemical synthesis, biology, and medicine. Dr. Lerner and his co-workers have begun to expand these horizons, focusing on the development of a new class of proteins with both antibody and enzyme characteristics. Further extrapolation of these techniques could result in the design and preparation of new antibody–antigen complexes capable of performing unique kinetic functions.

N. Cozzarelli from the University of California at Berkeley described the use of topological isomers of DNA in deducing enzymatic mechanisms for enzymes such as recombinases and topoisomerases (*80*). Topoisomers, which can only be interchanged by bond-breaking reactions, include knots, catenanes, and linking number isomers, illustrated below (**XVIII**). These isomers can be distinguished by gel electrophoresis or electron microscopy.



SCHEME **XVIII**. Types of topoisomers: (a) left- and right-handed singly linked catenanes; (b) left-handed knot; (c) −1 change in linking number (loss of one plectonemic supercoil). *Note:* arrows indicate the direction of the DNA primary sequence.

The type of information gained by studying topoisomers can include the way in which two recombination sites are brought together, the direction of strand rotation in the bond-breaking reaction, and the required structure of the DNA substrate. The use of catenane structures was illustrated by considering the products of the action of a resolvase on a circular DNA containing a duplicated transposon, with the two sites oriented in a head-to-tail fashion. The observed major product of the reaction is a singly linked catenane, with two left-handed crossovers, with each half of the catenane containing one copy of the transposon. The observation of this specific topoisomer, as opposed to a number of other theoretical possibilities, demonstrates that in the enzyme–DNA intermediate, the two recombination sites are brought together in a parallel rather than anti-parallel fashion, and that the DNA standards are rotated or crossed-over once in a left-handed sense.

The implications of linking number changes during enzyme reactions were illustrated by the reaction of the bacteriophage λ integrase (int). The products of recombination events with the int enzyme reflect the linking number due to plectonemic supercoils (the type illustrated above) of the substrate DNA. Experiments with int in whole cells suggest that only 40% of the DNA in *E. coli* is

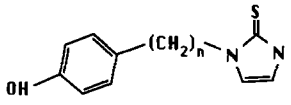
plectonemically supercoiled, unlike DNA in solution, which is completely plectonemic. The remainder of the DNA in cells must be supercoiled in a different, perhaps more closely packed structure, as yet to be determined. These examples illustrate the power of the study of topoisomers in understanding the structure and dynamics of DNA.

BIOINORGANIC MECHANISMS

H. Gray, from the California Institute of Technology, opened the session on bioinorganic mechanisms by describing one approach toward investigating long-range electron transfer in proteins. The mechanism(s) for electron transfer is not well-understood and data to test existing theory are slowly being gathered by a number of methods (*81–83*). Professor Gray has begun to address the problem of the distance dependence of electron transfer rates by measuring the rates of electron transfer in heme proteins in which the surface histidines are substituted with ruthenium complexes (*84*). The individual proteins possessing a single and unique ruthenium substitution were isolated resulting in the isolation of a series of ruthenium-substituted proteins. The rate of electron transfer from zinc-substituted heme to the ruthenium complex was then measured by energy transfer methods as a function of distance in a constant medium and between an identical donor–acceptor pair. This experiment is analogous to the energy transfer experiments developed by Hoffman and McLendon (*85–86*) to measure the rate of electron transfer in cytochrome complexes where one of the cytochromes was zinc-substituted. The electron transfer rates obtained from the zinc–ruthenium system were found to be linear over distance, for the small number of rates determined, unlike the rates obtained in zinc–iron cytochrome complexes. The one exception to the linear correlation was a faster than expected rate for a long-range distance (22 A). In this case, a Trp side chain is found in the electron transfer pathway, and might account for the observed rate. Also, a proportionality factor obtained from the linear correlation was more in agreement with predicted values than that obtained in the cytochrome complex experiments. Finally, the rates obtained for transfer over a known distance in the zinc–ruthenium system did not agree at all with the rates for a comparable predicted distance, based on modeling, in the cytochrome complexes. Gray thus proposed that either the predicted distance is incorrect, or the mechanism of transfer is different in protein–protein complexes. There may also be a change in mechanism at longer distances, explaining the deviation from linearity observed at 22 A. Gray is now collecting more data to establish the validity of this approach, and to clarify the apparent contradictions among the various approaches.

L. Kruse of Smith Kline & French Laboratories continued the session with a description of inhibitor and extended X-ray absorbance fine structure (EXAFS) studies on dopamine β-hydroxylase (DBH). The mechanism and properties of this enzyme have been extensively studied (*87*). Dr. Kruse described a potent bisubstrate inhibitor incorporating *p*-cresol and imidazole-2-thione as dopamine and oxygen (or metal-binding) analogs. Characterization of the bisubstrate inhibi-

tor (**XIX**) and its components (*p*-cresol and 1-methyl-imidazole-2-thione) by ki-



XIX

netic methods (*88*) showed (a) that *p*-cresol is both a substrate and an irreversible inhibitor, with a partition ratio of 1300:1; (b) that *p*-cresol and 1-methyl-imidazole-2-thione bind in a mutually exclusive manner; and (c) that the imidazole-2-thione is a competitive inhibitor with respect to oxygen, in the pH range where the binding of substrates is random (*89*). *p*-Cresol is unique as an irreversible inhibitor of DBH because it lacks the reactive group at the hydroxylation site common to most of the mechanism-based inhibitors of this enzyme. An active-site peptide containing [^{3}H]-*p*-cresol was then isolated and sequenced.

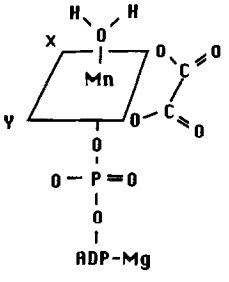
EXAFS studies suggested a coordination sphere for the active site copper(s) of approximately four nitrogen or oxygen ligands for the Cu(II) form of the enzyme. At least some of these ligands are histidine, based on the observed third-shell scattering, characteristic of histidine coordination. The Cu(I) form of the enzyme was suggested to have a coordination sphere of two or three nitrogen or oxygen ligands plus one sulfur ligand. In neither case was there any evidence for a Cu–Cu scattering shell, up to a distance of 4 A. It was therefore proposed that there are two types of coppers, with one supplying electrons to the second, dioxygen-binding copper at the site of dopamine hydroxylation.

George Reed from the University of Wisconsin spoke about the dual cation requirement of pyruvate kinase, describing function and relative proximity of the inorganic cofactors. Pyruvate kinase requires two divalent cations. These cations reside in separate sites with different affinities for different species of cation (*90*). Mn(II) residues in a protein-based site and Mg(II) resides in a nucleotide-based site.

In addition to the divalent cations, a monovalent cation is also required by pyruvate kinase. NMR studies with CrATP complexes at the nucleotide site have determined that the three cations and two substrates are all bound in close proximity (*91*).

Oxalate, a structural analog of the enolate form of pyruvate, has been used to mimic the reactive form of pyruvate in complexes with the enzyme, Mn(II), Mg(II), and ATP. Based on the superhyperfine coupling between the unpaired electron spin of Mn(II) and the nuclear spin of ^{17}O, EPR studies show oxalate is bound at the active site as a bidentate ligand with Mn(II). The Mn(II) is also coordinated to the γ-phosphate of ATP. Labeling the α- and β-phosphate groups of ATP with ^{17}O has no effect on the EPR spectrum. Further studies in ^{17}O water showed a single water ligand bound to the Mn(II). These data were interpreted to indicate that ATP bridges Mn(II) and Mg(II) at the active site. Further studies with two equivalents of Mn(II) ions at the active site also suggest close proximity of the divalent cations. Thus the active site contains ATP as a bridging ligand for the two divalent cations, and both oxalate and ATP (γ-phosphate) bind directly to

the Mn(II) at the protein-based site. An active-site structure consistent with these observations might be **XX**.



XX

Based on the enzyme–Mn(II)–oxalate–Mg(II)–ATP structure, one could envision the normal reverse reaction of pyruvate kinase in which the divalent cation at the protein-based site activates the keto-acid substrate through chelation and promotes phosphoryl-transfer by simultaneous coordination to the enolate oxygen and to an oxygen of the γ-phosphate of ATP.

ACKNOWLEDGMENTS

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POSTER PRESENTATIONS

I. *A Kinetic Study of a Compartmentalized Enzyme: Dopamine β-Hydroxylase in Chromaffin Granule Ghosts,* N. G. AHN AND J. P. KLINMAN, University of California, Berkeley, California.

II. *The Mechanism of Substrate Inactivation of E. Coli S-Adenosylmethionine Decarboxylase,* D. L. ANTON AND R. KUTNY, E. I. DuPont de Nemours Co., Wilmington, Delaware.

III. *Biosynthesis of 6,7-Dimethyl-8-ribityllumazine,* A. BACHER,* R. VOLK,* G. NEUBERGER,* K. SCHOTT,* R. LADENSTEIN,† R. HUBER,† H. G. FLOSS,‡ AND P. J. KELLER,‡ *Technische Universitat, Munich, West Germany, †Max-Planck Institute, Martinsreid, West Germany, and ‡Ohio State University, Columbus, Ohio.

IV. *Divalent Metal Ion: Phosphoryl Ligand Interaction in Yeast Inorganic Pyrophosphatase,* A. BANERJEE,* J. L. MCCRACKEN,† J. PEISACH,† AND B. S. COOPERMAN,* *University of Pennsylvania, Philadelphia, Pennsylvania, †Albert Einstein College of Medicine, Bronx, New York.

V. *Dihydrofolate Synthetase/Folypolyglutamate Synthetase: Simultaneous Demonstration of Acyl Phosphate Intermediates in Two Reactions Catalyzed by a Multifunctional Protein,* R. BANERJEE, B. SHANE, AND J. K. COWARD; Rensselaer Polytechnic Institute, Troy, New York.

VI. *An Artificial Acyl-Enzyme and an Artificial Enzyme,* M. L. BENDER, Northwestern University, Evanston, Illinois.

VII. *Inhibitor Studies with Mung Bean Glutamine Synthetase,* B. BOETTCHER AND E. ASHIZAWA, Chevron Chemical Co., Richmond, California.

VIII. *Structural Comparison of Rapid and Slow Forming Complexes of α-Lytic Protease with Peptide Boronic Acid Inhibitors,* R. BONE,* D. AGARD,* C. KETTNER,† AND A. SHENVI,† *University of California, San Francisco, California, and †E. I. DuPont de Nemours Co., Wilmington, Delaware.

IX. *Correlation of Copper Valency with Tyramine Hydroxylation in the Dopamine β-Monooxygenase Reaction,* M. BRENNER AND J. P. KLINMAN, University of California, Berkeley, California.

X. *Preliminary Studies on the Chemical Mechanism of E. Coli Pyruvate–Formate Lyase,* E. J. BRUSH, K. A. LIPSETT, AND J. W. KOZARICH, University of Maryland, College Park, Maryland.

XI. *Isotopically Sensitive Branching in the Enzymatic Cyclization of Geranyl Pyrophosphate to (−)-α- and (−)-β-Pinene,* D. CANE, Brown University, Providence, Rhode Island.

XII. *Reversal of Substrate Charge Specificity by Site-Directed Mutagenesis of Asparate Aminotransferase,* C. N. CRONIN, B. A. MALCOLM, AND J. F. KIRSCH, University of California, Berkeley, California.

XIII. *HASL: The Hypothetical Active-Site Lattice. I. Glyoxalase-I Studies,* A. M. DOWEYKO, Uniroyal Chemical Co., Naugatuck, Connecticut.

XIV. *Reversible, Slow, Tight-binding Inhibitors of Human Leukocyte Elastase,* R. P. DUNLAP,* AND R. H. ABELES,† *Eastman Kodak Research Lab., Rochester, New York, and †Brandeis University, Waltham, Massachusetts.

XV. *Evidence for Substrate Channeling in the E. coli Tryptophan Synthase Bi-enzyme Complex,* M. F. DUNN, B. ROBUSTELL, AND M. ROY, University of California, Riverside, California.

XVI. *Structural Characterization of β-Lactamase Inactivated by β-Lactam Sulfones,* L. M. ELLERBY, M. HSIEH, AND A. L. FINK, University of California, Santa Cruz, California.

XVII. *Resonance-Raman Spectroscopic Identification of the "Transient" Intermediate formed in the Native Ternary Complex with Thymidylate Synthase,* A. L. FITZHUGH,* S. FODOR,† AND T. G. SPIRO,† *National Cancer Institute, Frederick, Maryland, and †Princeton University, Princeton, New Jersey.

XVIII. *Dihydroxyacid Dehydratase Purified from Spinach is an Fe–S Protein,* D. H. FLINT AND M. H. EMPTAGE, E. I. DuPont de Nemours Co., Wilmington, Delaware.

XIX. *Degradation and Detoxification of Organophosphonates: Cleavage of the Carbon to Phosphorus Bond by Escherichia coli,* J. W. FROST, S. LOO, AND L. AVILA, Stanford University, Stanford, California.

XX. *Benzoylformate Analogues Exhibit Differential Rate-Determining Steps in the Benzoylformate Decarboxylase Reaction,* G. A. GARCIA,* P. M. WEISS,† P. F. COOK,‡ G. L. KENYON,* AND W. W. CLELAND,† *University of California, San Francisco, California, †University of Wisconsin, Madison, Wisconsin, and ‡North Texas State University, Denton, Texas.

XXI. *Observation of 5-Membered Cyclic Phosphates in Tissue,* R. A. GRAHAM, B. S. SZWERGOLD, W. J. THOMA, AND T. R. BROWN, Fox Chase Cancer Center, Philadelphia, Pennsylvania.

XXII. *NMR and Kinetic Studies of the Mechanism of Staphylococcal Nuclease: pH-Dependence of Active Site Amino Acids,* C. B. GRISSOM, E. L. ULRICH, W. B. HORTON, D. A. BENWAY, D. A. MILLS, AND J. L. MARKLEY, University of Wisconsin, Madison, Wisconsin.

XXIII. *Atomic Structure of Thymidylate Synthase: Target for Rational Drug Design,* L. W. HARDY, J. S. FINER-MOORE, W. R. MONTFORT, M. O. JONES, D. V. SANTI, AND R. M. STOUD, University of California, San Francisco, California.

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