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DANIEL L. PURICH

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AND RELATED AREAS OF MOLECULAR BIOLOGY

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PREFACE

Today's fast-paced growth of mechanistic information on enzyme reactions even astonishes those who have personally conducted decades of research on biological catalysis. The confluence of structural and dynamic information now provides a highly efficient means for discerning subtle features of enzyme catalysis, and the lessons learned from studies on a particular enzyme can no longer be viewed in isolation. Instead, these lessons serve as beacons that will illuminate and guide future explorations, often on entire classes of enzyme-catalyzed reactions. Part B in this Advances in Enzymology subseries entitled "Mechanism of Enzyme Action" continues the mission to provide new and valuable information about the nature of enzyme intermediates, the stepwise organochemical transformations of substrate to product, as well as the nature of barriers to interconversion of the various enzyme-bound species. Presented here are authoritative accounts by leading scientists who have taken this opportunity to share insights that are the product of highly imaginative and productive research. As has become the custom for Advances in Enzymology, these authors were encouraged to acknowledge the relevant findings from many research groups, while focusing greater emphasis on the scaffolding of logic that guided the research accomplished in their own laboratories. In this respect, the editor bears full responsibility for discouraging authors from providing exhaustive treatments that recapitulate findings already well described in earlier reviews on a particular topic.

Daniel L. Purich
Gainesville, Florida
February 2000

ABSTRACTS

Phosphotriesterase: An Enzyme in Search of Its Natural Substrate

The phosphotriesterase from *Pseudomonas diminuta* catalyzes the hydrolysis of a broad range of organophosphate nerve agents. The kinetic constants, k_{cat} and k_{cat}/K_m, for the hydrolysis of paraoxon, diethyl p-nitrophenyl phosphate, are 2200 s^{-1} and 4×10^7 $M^{-1}s^{-1}$, respectively. The utilization of substrates that are chiral at the phosphorus center has demonstrated that the overall hydrolytic reaction occurs with inversion of configuration. The native enzyme contains Zn^{2+} bound to the active site and catalytic activity is retained upon substitution with Mn^{2+}, Ni^{2+}, Cd^{2+}, or Co^{2+}. NMR and EPR spectroscopy has revealed the presence of an antiferromagnetically coupled binuclear metal center. X-ray crystallography shows the enzyme to exist as a homodimer and the protein adopts an $\alpha\beta_8$ folded structure. The binuclear metal center is ligated to the protein via four histidine residues, an aspartate, and a carbamoylated lysine residue that serves as a bridge between the two metal ions. The overall structure is very similar to the binuclear nickel center in urease. A naturally occurring substrate has not been identified for this enzyme but this protein has received considerable attention as a possible catalyst for the detoxification of organophosphate insecticides and military chemical weapons.

Phosphorivulokinase: Current Perspectives on the Structure/Function Basis for Regulation and Catalysis

Phosphoribulokinase (PRK), an enzyme unique to the reductive pentose phosphate pathway of CO_2 assimilation, exhibits distinctive contrasting properties when the proteins from eukaryotic and prokaryotic sources are compared. The eukaryotic PRKs are typically dimers of ~39 kDa subunits

while the prokaryotic PRKs are octamers of ~32 kDa subunits. The enzymes from these two classes are regulated by different mechanisms. Thioredoxin f mediated thiol-disulfide exchange interconverts eukaryotic PRKs between reduced (active) and oxidized (inactive) forms. Allosteric effectors, including activator NADH and inhibitors AMP and phosphoenolpyruvate, regulate activity of prokaryotic PRK. The effector binding site has been identified in the high resolution structure recently elucidated for prokaryotic PRK and the apparatus for transmission of the allosteric stimulus has been identified. Additional contrasts between PRKs include marked differences in primary structure between eukaryotic and prokaryotic PRKs. Alignment of all available deduced PRK sequences indicates that less that 10% of the amino acid residues are invariant. In contrast to these differences, the mechanism for ribulose 1,5-bisphosphate synthesis from ATP and ribulose 5-phosphate (Ru5P) appears to be the same for all PRKs. Consensus sequences associated with M^{++}-ATP binding, identified in all PRK proteins, are closely juxtaposed to the residue proposed to function as general base catalyst. Sequence homology and mutagenesis approaches have suggested several residues that may potentially function in Ru5P binding. Not all of these proposed Ru5P binding residues are closely juxtaposed in the structure of unliganded PRK. Mechanistic approaches have been employed to investigate the amino acids which influence $K_{m\ Ru5P}$ and identify those amino acids most directly involved in Ru5P binding. PRK is one member of a family of phospho or sulfo transferase proteins which exhibit a nucleotide monophosphate kinase fold. Structure/function correlations elucidated for PRK suggest analogous assignments for other members of this family of proteins.

The Molecular Evolution of Pyridoxal-5'-Phosphate-Dependent Enzymes

The pyridoxal-5′-phosphate-dependent enzymes (B_6 enzymes) that act on amino acid substrates are of multiple evolutionary origin. The numerous common mechanistic features of B_6 enzymes thus are not historical traits passed on from a common ancestor enzyme but rather reflect evolutionary or chemical necessities. Family profile analysis of amino acid sequences supported by comparison of the available three-dimensional (3-D) crystal structures indicates that the B_6 enzymes known to date belong to four independent evolutionary lineages of homologous (or more precisely paralogous) proteins, of which the α family is by far the largest. The α family (with aspartate aminotransferase as the prototype enzyme) includes en-

zymes that catalyze, with several exceptions, transformations of amino acids in which the covalency changes are limited to the same carbon atom that carries the amino group forming the imine linkage with the coenzyme (i.e., Cα in most cases). Enzymes of the β family (tryptophan synthase β as the prototype enzyme) mainly catalyze replacement and elimination reactions at Cβ. The D-alanine aminotransferase family and the alanine racemase family are the two other independent lineages, both with relatively few member enzymes. The primordial pyridoxal-5′-phosphate-dependent enzymes apparently were regio-specific catalysts that first diverged into reaction-specific enzymes and then specialized for substrate specificity. Aminotransferases as well as amino acid decarboxylases are found in two different evolutionary lineages. Comparison of sequences from eukaryotic, archebacterial, and eubacterial species indicates that the functional specialization of most B_6 enzymes has occurred already in the universal ancestor cell. The cofactor pyridoxal-5′-phosphate must have emerged very early in biological evolution; conceivably, organic cofactors and metal ions were the first biological catalysts. In attempts to simulate particular steps of molecular evolution, oligonucleotide-directed mutagenesis of active-site residues and directed molecular evolution have been applied to change both the substrate and reaction specificity of existent B_6 enzymes. Pyridoxal-5′-phosphate-dependent catalytic antibodies were elicited with a screening protocol that applied functional selection criteria as they might have been operative in the evolution of protein-assisted pyridoxal catalysis.

O-Acetylserine Sulfhydrylase

O-Acetylserine sulfhydrylase (OASS) is a pyridoxal 5'-dependent enzyme that synthesizes L-cysteine in enteric bacteria, such as *Salmonella typhimurium* and *Escherichia coli,* and plants. OASS is a member of the β-family of PLP-dependent enzymes that specifically catalyze β-replacement reactions. Enzymes in this class include the β-subunit of tryptophan synthase (β-TRPS), cystathionine β-synthase, β-cyanoalanine synthase, and cysteine lyase. Other than OASS, only β-TRPS has been extensively studied, and thus mechanistic comparisons will be limited to it. This review focuses on the structure that has been solved recently, kinetic and acid-base chemical mechanisms, and spectroscopic studies using ^{31}P NMR, UV-visible, rapid-scanning stopped-flow phosphorescence, static and time-resolved fluorescence techniques. In addition, kinetic isotope effects and stereochemistry of the OASS reaction are discussed.

The Aromatic Amino Acid Hydroxylases

The enzymes phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase constitute the family of pterin-dependent aromatic amino acid hydroxylases. Each enzyme catalyzes the hydroxylation of the aromatic side chain of its respective amino acid substrate using molecular oxygen and a tetrahydropterin as substrates. Recent advances have provided insights into the structures, mechanisms, and regulation of these enzymes. The eukaryotic enzymes are homotetramers comprised of homologous catalytic domains and discrete regulatory domains. The ligands to the active site iron atom as well as residues involved in substrate binding have been identified from a combination of structural studies and site-directed mutagenesis. Mechanistic studies with nonphysiological and isotopically substituted substrates have provided details of the mechanism of hydroxylation. While the complex regulatory properties of phenylalanine and tyrosine hydroxylase are still not fully understood, effects of regulation on key kinetic parameters have been identified. Phenylalanine hydroxylase is regulated by an interaction between phosphorylation and allosteric regulation by substrates. Tyrosine hydroxylase is regulated by phosphorylation and feedback inhibition by catecholamines.

L-Aspartase: New Tricks From an Old Enzyme

The enzyme L-aspartate ammonia-lyase (aspartase) catalyzes the reversible deamination of the amino acid L-aspartic acid, using a carbanion mechanism to produce fumaric acid and ammonium ion. Aspartase is among the most specific enzymes known with extensive studies failing, until recently, to identify any alternative amino acid substrates that can replace L-aspartic acid. Aspartases from different organisms show high sequence homology, and this homology extends to functionally related enzymes such as the class II fumarases, the argininosuccinate and adenylosuccinate lyases. The high-resolution structure of aspartase reveals a monomer that is composed of three domains oriented in an elongated S-shape. The central domain, comprised of five ¯-helices, provides the subunit contacts in the functionally active tetramer. The active sites are located in clefts between the subunits, and structural and mutagenic studies have identified several of the active site functional groups. While the catalytic activity of this enzyme has been known for nearly 100 years, a number of recent studies have revealed some interesting and unexpected new properties of this reasonably well-charac-

terized enzyme. The non-linear kinetics that are seen under certain conditions have been shown to be caused by the presence of a separate regulatory site. The substrate, aspartic acid, can also play the role of an activator, binding at this site along with a required divalent metal ion. Truncation of the carboxyl terminus of aspartase at specific positions leads to an enhancement of the catalytic activity of the enzyme. Truncations in this region also have been found to introduce a new, non-enzymatic biological activity into aspartase, the ability to specifically enhance the activation of plasminogen to plasmin by tissue plasminogen activator. Even after a century of investigation there are clearly a number of aspects of this multifaceted enzyme that remain to be explored.

CONTENTS

CLASSICS IN ENZYMOLOGY

Reprint of Chapter 7 from Advances in Enzymology and Related Subjects, Volume 2, Edited by F.F. Nord and C.H. Werkman. Published by Interscience Publishers, Inc., New York, 1942.

HETEROTROPHIC ASSIMILATION OF CARBON DIOXIDE

By C.H. Werkman and H.G. Wood, Department of Bacteriology, State University of Agriculture, Ames, Iowa

HETEROTROPHIC ASSIMILATION OF CARBON DIOXIDE*

By

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CONTENTS

I. Introduction

In 1935 heterotrophic assimilation of carbon dioxide was advanced by Wood and Werkman (1) as a definite and experimentally supported concept. They stated, "It has been established with several species of *Propionibacterium* that the total carbon dioxide liberated during fermentation of glycerol plus that remaining in the form of carbonate is less than the original carbon dioxide added as carbonate. This decrease is believed to result from utilization of carbon dioxide by the bacteria during their dissimilation of glycerol. Carbon and oxidation-reduction balances support this view." The unexpected finding of carbon dioxide utilization by such typically heterotrophic organisms as the propionic acid bacteria had been

* Presented in part at the Seminar of Organic Chemistry, Fordham University, New York, on February 4, 1942.

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first obtained some two years previous, but the unexpected nature of the results led to additional experiments in order to obtain convincing and, if possible, conclusive proof. It was for this reason that the authors in their initial proposal of heterotrophic utilization of carbon dioxide took a definite stand and have remained firm in their pronouncement notwithstanding considerable doubt and criticism expressed in private communications and in print.

The concept of heterotrophic utilization of carbon dioxide was first proposed at the Spring (1935) Meeting of the North Central Branch of the Society of American Bacteriologists in connection with studies on the fermentation of glycerol by bacteria belonging to the genus *Propionibacterium*. These bacteria do not form sufficient carbon dioxide from the glycerol to mask the uptake of carbon dioxide. Therefore, in a medium containing carbonate to neutralize the acids formed from glycerol, *i. e.*, propionic and succinic with a trace of acetic, determination of the carbon balance indicated that the carbon dioxide at the end of the experiment was not equivalent to that of the original medium in the form of carbonate, and that the products of fermentation contained more carbon than was present in the glycerol fermented.

Table I taken from the original work of Wood and Werkman (2) clearly shows that carbon dioxide was utilized by four species of the heterotrophic propionic bacteria used in the experiment. It was pointed out at this time (1936) that:

> "The fact that chemical analysis shows a decrease of carbon dioxide (accountable as carbonate carbon dioxide) is, perhaps, proof enough of carbon dioxide utilization. However, the carbon and oxidation-reduction balances furnish additional evidence."

The authors then continued (1936), "This observation (carbon dioxide utilization) requires a reinterpretation of previous results. Investigators have not considered the possibility of carbon dioxide utilization in constructing schemes of dissimilation. If one considers the limited number of bacteria which have been shown to utilize carbon dioxide and also that such forms (autotrophic) differ markedly from the propionic acid bacteria, failure to consider the possibility of carbon dioxide utilization may be understood." The principle of heterotrophic carbon dioxide utilization was again presented before the Second International Congress of Microbiology, meeting in London during the Summer of 1936. It was not, however, readily accepted and opposing comments were made.

It is significant that the same authors (4) made the following comment in 1938 regarding the utilization of carbon dioxide by animal tissue.

"Krebs and Johnson (1937) have recently shown that citric acid is synthesized by avian tissue from oxalacetic acid and some unknown compound. It is possible that this synthesis involves utilization of carbon dioxide."

TABLE I

DISSIMILATION OF GLYCEROL BY PROPIONIC ACID BACTERIA

Culture	Glycerol fermented per liter, mM.	CO_2 utilized per 100 mM. of fermented glycerol, mM.	Products per 100 mM. of fermented glycerol			Carbon recovery		Oxidation-reduction index	
			Propionic acid, mM.	Acetic acid, mM.	Succinic acid,‡ mM.	Basis-glycerol *plus* CO_2, %	Basis-glycerol only, %	Basis-glycerol *plus* CO_2	Basis-glycerol only
49W	212.6	37.7	55.8	2.9	42.1	101.2	114.0	1.081	2.550
34W	209.0	43.2	59.3	2.0	34.5	93.1	106.6	0.925	2.270
52W*	112.0	20.0	78.4	5.9	8.7	94.6	101.0	0.918	1.386
11W†	218.4	1.1	89.3	2.6	3.9	96.5	96.8	1.135	1.162
15W	176.4	12.3	78.4	5.8	7.8	89.1	92.6	1.047	1.376

* 7.0 mM. of lactic acid produced per 100 mM. of fermented glycerol.

† 0.5 mM. of lactic acid produced per 100 mM. of fermented glycerol.

‡ Succinic acid identified by melting point and mixed melting point.

The experimental proof of carbon dioxide assimilation by animal tissue came in 1940 through the work of Evans and Slotin (3).

Since the isotopes of carbon have become available for use as tracers of fixed carbon dioxide, there has been a tendency to disregard the work done previously. It is true that with the advent of the tracer technique, detection of the fixation of carbon dioxide and its behavior in metabolism have been facilitated; nevertheless, fixation by heterotrophic forms already had been clearly demonstrated by quantitative data obtained with the propionic acid bacteria. Moreover, since all the products were aliphatic carbon compounds of two or more carbon atoms, fixation in a carbon to carbon linkage was shown to occur. Location of the fixed carbon among the products and its position within the molecule was a matter of speculation at that time. The isotopic investigations have been of particular service in clearing up these latter points.

Wood and Werkman (4) showed an equimolar relationship between the carbon dioxide fixed and the succinic acid formed, and found that inhibition of fixation by sodium fluoride (5) resulted in a corresponding reduction in succinic acid. As a result the proposal was made that the succinic acid was the result of a C_3 and C_1 synthesis. Pyruvic acid was suggested as the possible C_3 compound since it could be isolated from the fermentation (6).