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Avoiding the Road Less Traveled: How the Topology of Enzyme−Substrate Complexes Can Dictate Product Selection

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ABSTRACT

Enzymes are remarkable not only in their ability to enhance reaction rates, but also because they do so selectively, directing reactive intermediates toward only one of multiple potential products. 1-Aminocyclopropane-1-carboxylate (ACC) synthase and 7,8-diaminopelargonic acid synthase are pyridoxal 5′-phosphate-dependent enzymes that utilize *S*-adenosyl-L-methionine as a substrate but yield different products. The former produces ACC by α,γ-elimination, while the latter makes *S*-adenosyl-4-methylthio-2-oxobutanoate by transamination. The mechanisms of these two reactions are the same up to the formation of a quinonoid intermediate, from which they diverge. This Account explores how the active-site topology of the enzyme−intermediate complexes decides this pathway bifurcation.

Pyridoxal Phosphate Enzymes Provide Important Insight into Substrate and Reaction Specificities

Two of the central problems in mechanistic enzymology are substrate recognition and reaction specificity. The first is clearly that of optimal complementary juxtaposition of the recognition elements of the enzyme active site with the major specificity determinants of the substrate. This aspect has been studied extensively since the dawn of the discipline and will only be addressed selectively in this Account. The second issue is one of directing a reactant, potentially subject to a variety of metabolic fates, to the desired outcome in the face of alternate, more favorable thermodynamic and kinetic barriers. For example, *S*-adenosyl-L-methionine (SAM)[1] may donate a methyl group to a nucleophile or undergo decarboxylation, α,γ- or β,γ-elimination, or transamination. The last four reactions all depend on formation of a carbanion at the α position (Scheme 1), a situation that is most often facilitated in Nature by the cofactor pyridoxal 5′-phosphate (PLP). This role is assumed by a serine-derived pyruvoyl group in a small number of enzymes, including SAM decarboxylase. We will consider here only the PLP-dependent reactions. The formation of the normally thermodynamically inaccessible α-carbanion is promoted by condensation of the substrate amino group with the aldehyde moiety of PLP, which allows for resonance delocalization of the negative charge through the π orbitals of the cofactor (Scheme 2). This delocalized anion is referred to as the quinonoid intermediate.

As a result of this capacity to stabilize anions at positions adjacent to an amino group, PLP-dependent enzymes are able to catalyze a remarkably diverse set of reactions, a few of which have already been mentioned. These enzymes also utilize a wide variety of substrates, primarily amino acids, but also amino sugars[2] and other amine-containing metabolites. The ENZYME database (http://us.expasy.org/enzyme/) has more than 100 EC numbers assigned to PLP enzymes, making this group one of the largest classes of cofactor-dependent enzymes. Reflecting this diversity, PLP-dependent enzymes are found to participate in many essential cellular processes, particularly in the biosynthesis and transformation of amino acids and amino acid-derived metabolites. Because of their prominent role in metabolism, many of these enzymes have been identified as candidate drug targets. For example, inhibitors of γ-aminobutyrate aminotransferase are used to treat epilepsy and may also prove useful for treatment of other serious neurological disorders,[3] and serine hydroxymethyltransferase has been identified as a target for anti-cancer therapy.[4]

Sequence and structural analyses of PLP enzymes have revealed that they can all be classified in one of five families or fold types, at least two of which are evolutionarily related (for recent reviews, see refs 5−7). The fold type does not define the chemistry, as a number of reaction types are found in each. This combination of structural similarity and functional diversity within each fold type makes PLP enzymes ideal for the investigation of the sources of enzyme substrate and reaction specificity.

The first hypothesis concerning the control of reaction specificity by PLP enzymes was put forth by Dunathan in 1966.[8] He postulated that the enzymes bind their substrates such that the bond that is to be broken to form the carbanion is aligned in a plane that is perpendicular to the plane of the pyridine ring. This alignment allows the nascent π orbital to overlap with the π system of the cofactor and distinguishes, for instance, α-deprotonation (leading to a variety of products) from decarboxylation of an α-amino acid. All structures of PLP enzymes that have been determined support this hypothesis. A particularly interesting example is dialkylglycine decarboxylase, which catalyzes both a decarboxylation and a transamination

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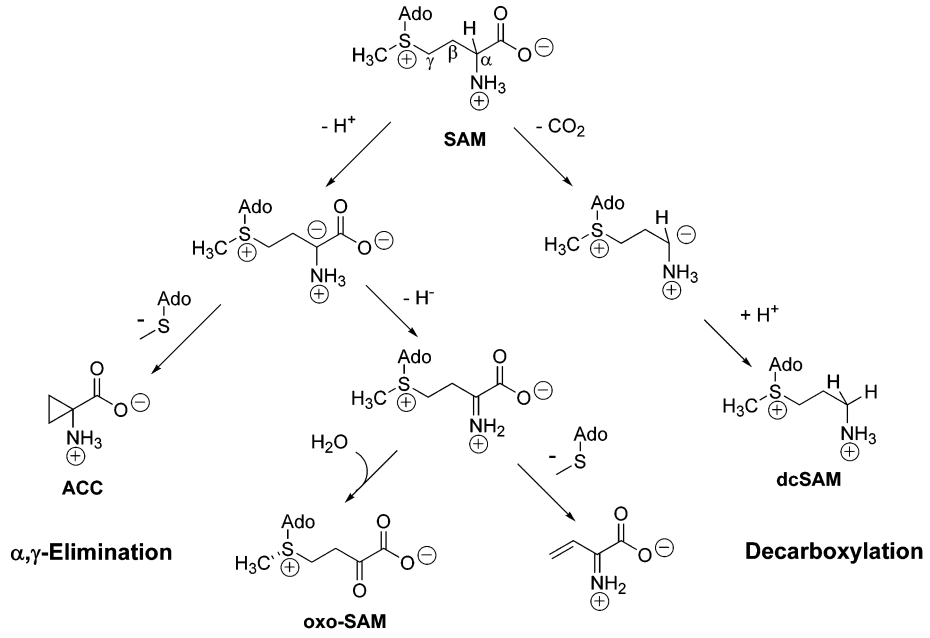
Scheme 1. The Metabolic Fates of SAM[a]



[a] An α-carbanion can be formed either by decarboxylation or deprotonation. Reprotonation of the decarboxylated intermediate yields *S*-adenosyl-(5′)-3-methylthiopropylamine (dcSAM), an intermediate in polyamine biosynthesis.[35] Formation of the α-carbanion by deprotonation is a prelude to α,γ-elimination, β,γ-elimination, and transamination. The α-carbanions are stabilized by enzyme-bound PLP (not shown; see text and Scheme 2). The mechanisms of these three reactions are presented in more detail in Scheme 3.

reaction, depending on the orientation of the bound substrate.[9] Little work, however, has been directed to the understanding of the divergence to different products after the initial bond breakage, although it is known that minor changes in active sites of PLP enzymes often result in substantial increases in the rates of undesirable side-reactions (for example, see refs 10−13). Limitation of these side reactions, therefore, is often as important as enhancement of the desired reaction.[11]

In this Account, we show how two PLP-directed reactions of SAM, both of which depend on C_α proton labilization, yield different products. The enzymes catalyzing these two reactions are 1-aminocyclopropane-1-carboxylate (ACC) synthase, which preferentially catalyzes an α,γ-elimination, and 7,8-diaminopelargonic acid (DAPA) synthase, which effects a transamination reaction (Scheme 1).

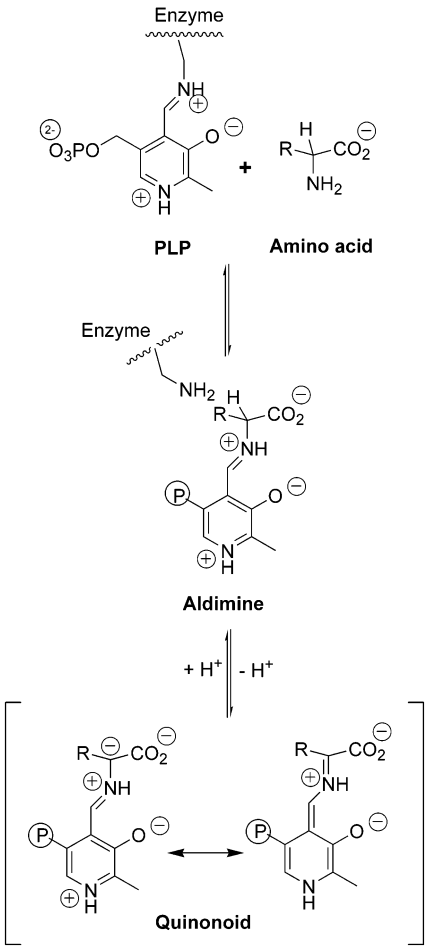
ACC Synthase: An Apple among Oranges

ACC is a cyclic amino acid produced by an α,γ-elimination of 5′-deoxy-5′-methylthioadenosine (MTA) from SAM (Scheme 1). This reaction, catalyzed by ACC synthase, represents the committed and rate-determining step in the biosynthesis of the gaseous hormone ethylene in flowering plants.[14] In addition to its agricultural importance, this enzyme attracted our attention because of its close relation to the well characterized PLP enzyme aspartate aminotransferase (AATase). Initial sequence analysis[15,16] indicated that ACC synthase is closely related to aminotransferaes. Subsequent structure determination[17] confirmed its predicted membership in Fold Type I of PLP enzymes, specifically in aminotransferase subclass I. This group includes AATase, tyrosine (aromatic) aminotransferase, and histidinol phosphate aminotransferase,[5,7,18] all of which catalyze transamination reactions. Although the sequence identity shared between ACC synthase and AATase is quite low (∼15% depending on species), the C_α atoms of apple ACC synthase and pig cytosolic AATase superimpose with an rmsd of 3.2 Å.[17] Considering the close structural similarity of ACC synthase to aminotransferases, we have attempted to determine what features of the enzyme allow it to discriminate between the desired elimination reaction and the potential aminotransferase reaction to generate *S*-adenosyl-4-methylthio-2-oxobutanoate (oxo-SAM, Scheme 1).

The mechanisms of both reactions have an important feature in common in that they share intermediates up to the quinonoid (Scheme 2). The steps after quinonoid formation are depicted in Scheme 3. In order for the enzyme to drive the α,γ-elimination reaction, it must increase the ratio k_{elim}/k_{trans}, where k_{elim} and k_{trans} are, respectively, the rate constant for α,γ-elimination of MTA and the net rate constant for transamination from the quinonoid. Much of the insight into this process has come from comparison of the reactions of ACC synthase with its natural substrate to those of the same enzyme with alternate substrates.

1. Reaction of ACC Synthase with SAM. The absorbance maxima of the different forms of the cofactor (indicated in Scheme 3) often allow intermediates in the reaction pathways of PLP enzymes to be distinguished spectrophotometrically and to be followed individually

Scheme 2. The Formation of the α-Carbanion Is Promoted by Condensation of a Substrate Amino Acid with Enzyme-Bound PLP to Form an Aldimine[a]



[a] The carbanion formed by subsequent deprotonation is resonance delocalized across the cofactor in the quinonoid intermediate.

during the course of a reaction monitored under pre-steady-state conditions. A strong absorbance at ~500 nm, indicative of the quinonoid intermediate, forms rapidly upon mixing SAM with ACC synthase (Figure 1).[19] This intermediate decays with a rate constant equal to the steady-state k_{cat} value for the reaction, indicating that it is one of the species involved in the equilibrium preceding the rate-determining step, which is presumed to be the irreversible α,γ-elimination of MTA. These data indicate that the quinonoid is a stable intermediate in the reaction, which is unusual. This intermediate does not accumulate in most transamination reactions, such as that of AATase with aspartate, although it is observed during the reaction of AATase with the alternative substrate cysteine sulfinic acid (CSA).[20]

2. Reaction of ACC Synthase with the Inhibitors Aminoethoxyvinylglycine and sincefungin. L-Aminoethoxyvinylglycine (AVG; Figure 2) is a potent inhibitor of ACC synthase, with a calculated K_d of 10−20 pM.[21] Spectrophotometric and crystallographic studies have shown that the inhibition is due to the formation of a stable ketimine, which is not hydrolyzed. The required quinonoid intermediate is not observed (Figure 3),[21] indicating that it does not accumulate during the reaction.

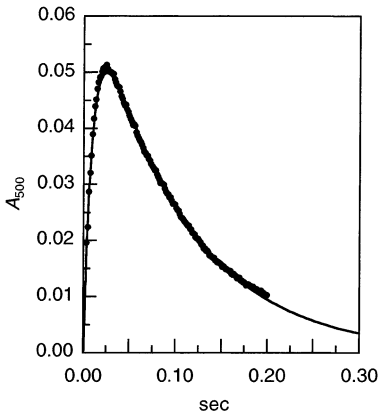


FIGURE 1. Formation and decay of the quinonoid intermediate during the reaction of ACC synthase with (*S*,*S*)-SAM. Excess ACC synthase (25 μM) was reacted with 5 μM (*S*,*S*)-SAM in reaction buffer (25 mM potassium phosphate, 2.5 μM PLP, 0.5 mM EDTA, 0.1 mM DTT, 7.5% glycerol, pH 8.3), and the absorbance at 500 nm was followed for 200 ms. [Reprinted from ref 19 with permission. Copyright, American Chemical Society, 2000].

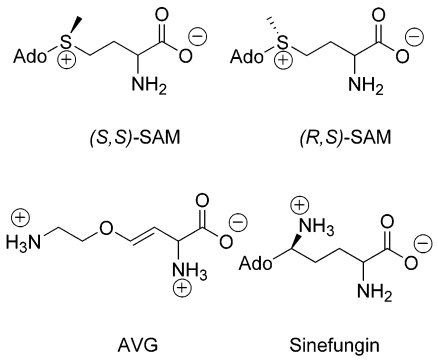


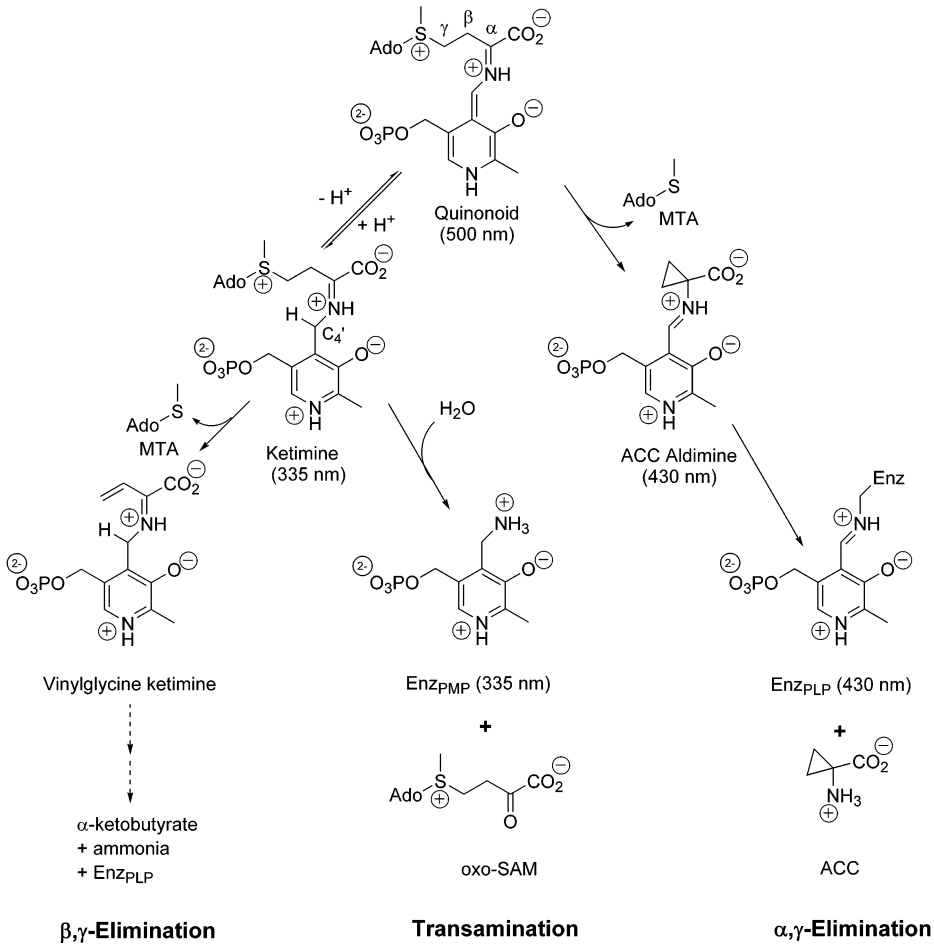
FIGURE 2. The structures of (*S*,*S*)-SAM, (*R*,*S*)-SAM, AVG, and sinefungin.

A similar result is observed in the reaction of ACC synthase with the close substrate analogue sinefungin (Figure 2). This compound also forms a ketimine that is not hydrolyzed (M. White and A. Eliot, unpublished results).

Although the results of the investigations employing these inhibitors do not necessarily extend to SAM, the close structural similarity of sinefungin to the natural substrate suggests that a SAM ketimine, if formed, would also be recalcitrant to hydrolysis. Further, the fact that different intermediates accumulate depending on which inhibitor or substrate is involved indicates that substrate structure sharply influences the thermodynamic and/or kinetic accessibility.

3. Reaction of ACC Synthase with (*R*,*S*)-SAM. The physiological diastereomer of SAM has the (*S*,*S*) configuration, but the (*R*,*S*) diastereomer (Figure 2) is both a substrate and a mechanism based inhibitor of ACC synthase.[22,23] Although the latter compound can suffer the same α,γ-elimination as the natural (*S*,*S*) diastereomer, it more often eliminates the same leaving group in a β,γ-process to produce a vinylglycine-related intermediate (Scheme 3). The ratio of the elimination rate constants $k_{\alpha,\gamma}/k_{\beta,\gamma}$ is 0.4,[24] as compared to ~60 for the (*S*,*S*) diastereomer.[25] This result proves that the topology of the (*S*,*S*)-

Scheme 3. Possible Fates of the Quinonoid Intermediate[a]



[a] The irreversible α,γ-elimination of MTA (right) proceeds directly from the quinonoid, followed by release of the product ACC to regenerate the PLP form of the enzyme. The transamination (middle) and β,γ-elimination (left) reactions require initial protonation of the quinonoid at C_4' on the cofactor to generate the ketimine. This step is likely reversible (see text). The transamination reaction is completed by hydrolysis of the ketimine to release the oxo-acid product, leaving the enzyme in the PMP form. The irreversible β,γ-elimination of MTA occurs from the ketimine because the β-proton acidity is increased by the presence of the adjacent imine. The resulting vinylglycine ketimine is converted to a vinylglycine aldimine by proton transfer from C_4' to C_α. The resulting *ene*amine is discharged from the enzyme and decomposes spontaneously to α-ketobutyrate and ammonia.[22]

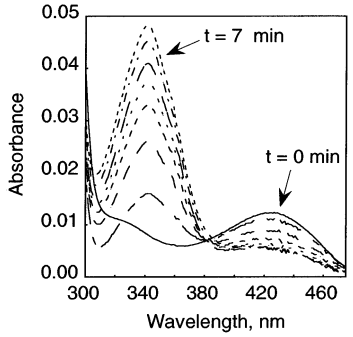


FIGURE 3. Formation of a ketimine following addition of AVG to WT ACC synthase. ACC synthase (3 μM) was added to 3.9 μM AVG in pH 6.3 buffer containing 62.5 mM MES, 62.5 mM MOPS, 125 mM 4-hydroxy-*N*-methylpiperidine, and 10% glycerol at 25 °C. Spectra were recorded every 60 s. The experiment was carried out at low pH to slow the rate of conversion of the PLP cofactor from its aldimine to ketimine form. The quinonoid intermediate is not observed during the reaction. [Reprinted from ref 21 with permission. Copyright, American Society of Biochemistry and Molecular Biology, 2002].

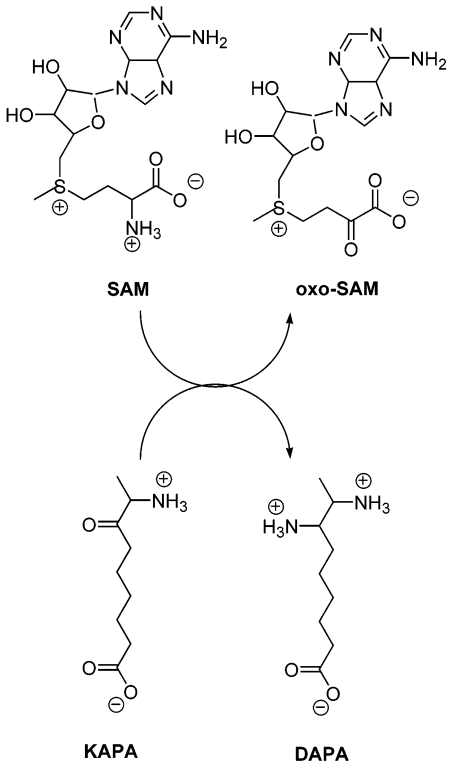
SAM complex is crucial for directing the nearly exclusive α,γ-elimination.

β,γ-Elimination requires a reasonably acidic C−H bond at the β position (Scheme 3). This increased acidity is expected to occur in the ketimine intermediate because the β proton is adjacent to an electron-withdrawing imine moiety; therefore, the β,γ-elimination likely proceeds from this intermediate as shown in Scheme 3. Spectrophotometric monitoring of the reaction with the (*R*,*S*) diastereomer reveals that, as in the reaction with the (*S*,*S*) compound, the most populated steady-state species is the quinonoid,[22] indicating that ketimine formation is either rate-determining or that the latter species is in equilibrium with the more populated quinonoid.

DAPA Synthase: A Physiological SAM Aminotransferase

7,8-Diaminopelargonic acid (DAPA) synthase is a PLP-dependent aminotransferase that transfers the amino group from SAM to 7-amino-8-ketopelargonic acid (KAPA) to produce DAPA and the oxo-acid of SAM (Scheme 4).[26] This enzyme catalyzes the second of four steps in the biosynthesis of biotin and is the only enzyme known to utilize SAM as a substrate in an aminotransferase reaction.

Scheme 4. The Reaction Catalyzed by DAPA Synthase[a]



[a] The amino group is transferred from SAM to KAPA to generate DAPA.

This enzyme, like ACC synthase and AATase, is a member of fold type I of PLP enzymes, but it is classified by its structure in aminotransferase subclass II.[5,7] The subclasses differ primarily in the conformation of the N-terminal portion of the protein,[27] but the region around the cofactor is similar in both.

Since the transamination reaction catalyzed by DAPA synthase proceeds from the same SAM quinonoid as the ACC synthase-catalyzed elimination, this enzyme must interact with the substrate in such a way that the partitioning of the intermediate down the possible reaction pathways instead favors the transamination. As in the case of ACC synthase, analysis of the reaction of the enzyme with different substrates has provided insight into the control of this partitioning.

1. Reaction of DAPA Synthase with SAM. In contrast to ACC synthase, DAPA synthase does not react with the (*R*,*S*) diastereomer of SAM, nor is that compound an inhibitor of the enzyme;[28] therefore, we will only consider the half-reaction with the (*S*,*S*) diastereomer. Spectrophotometric observation of this reaction (Figure 4)[28] reveals that the quinonoid intermediate forms rapidly and decays with a rate constant equal to k_{max} (the first-order rate constant for the half-reaction at saturation), just as in the reaction of ACC synthase with SAM. However, the products in this case are the 335 nm-absorbing pyridoxamine (PMP) form of the enzyme and oxo-SAM. No ACC is formed (A. Eliot, unpublished results, 2002), indicating that the barrier to α,γ-elimination is substantially higher than it is in the reaction of SAM with ACC synthase. The transamination reaction catalyzed by DAPA synthase, although favored, proceeds slowly, with a k_{max} of 0.02 s^{-1},

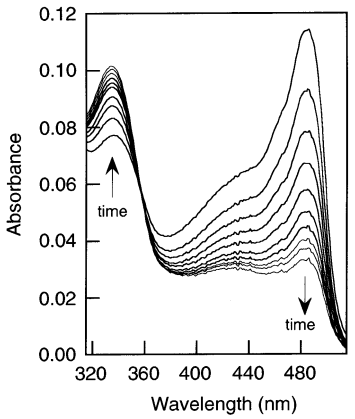


FIGURE 4. The quinonoid intermediate accumulates during the reaction of DAPA synthase with SAM. DAPA synthase (4.5 μM) was incubated with 1 mM SAM in reaction buffer (50 mM AMPSO, 20% glycerol, pH 9.0). Spectra were recorded every 30 s. The strong absorbance at 485 nm is characteristic of a quinonoid intermediate. [Reprinted from ref 28 with permission. Copyright, American Chemical Society, 2002].

indicating that either ketimine formation or its hydrolysis is hindered, similar to the ACC synthase-catalyzed reaction.[28]

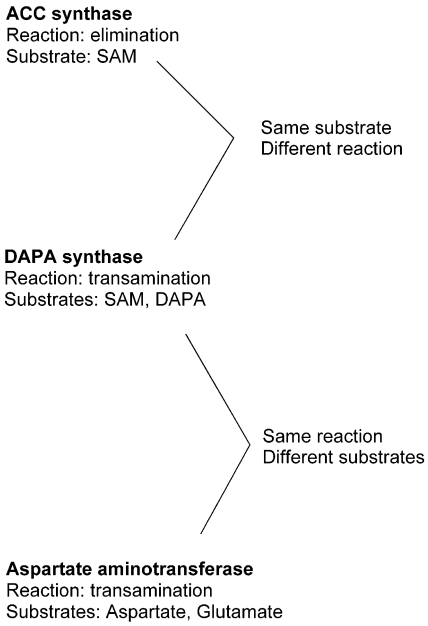
2. Reaction of DAPA Synthase with DAPA. The PMP form of DAPA synthase reacts with KAPA to generate DAPA and the PLP form of the enzyme in the physiological aminotransferase reaction (Scheme 4). The kinetics of the PLP form of DAPA synthase can be investigated with both SAM and DAPA. The value of k_{max} for DAPA is 0.8 s^{-1}, 40-fold greater than that for SAM. Moreover, the quinonoid intermediate does not accumulate during the reaction with DAPA, as opposed to that with SAM.[28] Instead, only the aldimine and ketimine are observed. Thus, substrate structure is a major determinant of the thermodynamic and/or kinetic accessibility of the quinonoid and ketimine intermediates, as was observed for ACC synthase.

Major Differences between the Reactions Catalyzed by the Three Enzymes

This comparison of the reactions catalyzed by three enzymes—ACC synthase, AATase, and DAPA synthase—which differ in either substrate utilized, reaction catalyzed, or both (Scheme 5), provides insight into the sources of the reaction specificity of these enzymes. Their reactions with alternative substrates and substrate analogues have been particularly revealing. Specifically, the obtained results identify three important factors that contribute to the different reaction specificities of ACC synthase and DAPA synthase.

1. Determinants of the Regiospecificity of Elimination. The fact that the differing stereochemistry of the sulfonium pole of the (*R*,*S*) diastereomer of SAM is sufficient to favor β,γ- over α,γ-elimination on an otherwise identical substrate shows that the topological relation of the groups attached to the stereocenter to the active site residues is sufficient to decide the regiochemistry of the elimination reaction. This result suggests that simple binding geometry interactions prevent α,γ-elimination

Scheme 5[a]



[a] ACC synthase, AATase, and DAPA synthase allow a comparison of three closely related, PLP-dependent enzymes that utilize the same substrate in different reactions (i.e., ACC synthase and DAPA synthase) or catalyze the same reaction with different substrates (i.e., DAPA synthase and AATase). Although the primary focus is on the different fates of SAM in the ACC synthase- and DAPA synthase-catalyzed reactions, AATase provides an important third point of comparison because of its close structural similarity to ACC synthase and the extensive kinetic and mechanistic studies that have been performed with it.

from the quinonoid formed from SAM in the reaction with DAPA synthase.

2. Control of Ketimine Hydrolysis. Although ketimine hydrolysis is partially rate-determining in the AATase-catalyzed transamination reaction,[29] this reaction still proceeds quickly (k_{cat} = 170 s^{-1}).[30] The DAPA synthase-catalyzed transamination reaction rate constants are substantially less, but ketimine hydrolysis must occur with a rate constant that is at least as great as k_{cat} (0.013 s^{-1}).[28] In contrast, the ketimines formed in the active site of ACC synthase are not hydrolyzed.

3. Quinonoid Stabilization. The quinonoid intermediate is calculated to be 1.7 kcal/mol less stable than the aldimine intermediate in the reaction of AATase with aspartate,[30] and it is not observed during the reaction in either steady-state or single-turnover kinetics. The ketimine is the most stable intermediate among the aldimine, quinonoid, ketimine trinity, and it accumulates during the reaction. The reaction of DAPA synthase with DAPA superficially appears similar, but it is the quinonoid intermediate that accumulates in the reaction of SAM with either ACC synthase or DAPA synthase. This stabilization of the quinonoid intermediate during the reactions with SAM may contribute to enhancing the rate of the α,γ-elimination reaction (see below).

How Do These Differences Relate to the Reaction Specificity of Each Enzyme?

It is reasonable that substrate binding geometry might be important for the α,γ-reaction, because the S_N2 closure of the cyclopropane ring requires alignment of the nucleophilic α-carbon atom of the quinonoid, the electrophilic γ-carbon atom, and the sulfur atom of the leaving group. It is also clear that prevention of hydrolysis of a ketimine formed on ACC synthase blocks a suicidal transamination reaction and thereby allows the substrate to be directed down the desired α,γ-elimination pathway. The hydrolysis reaction may be limited by exclusion of solvent as a result of closure of the enzyme around the bound substrate. A precedent can be found in AATase, which is known to exist in a closed form when substrate is bound.[31] The recently solved crystal structure of ACC synthase with AVG bound shows evidence of a similar, but less dramatic, closure.[21] In AATase, the closure is not sufficient to exclude solvent completely, but it has been suggested that it acts to prevent unwanted side reactions such as racemization resulting from protonation of the quinonoid by solvent.[32]

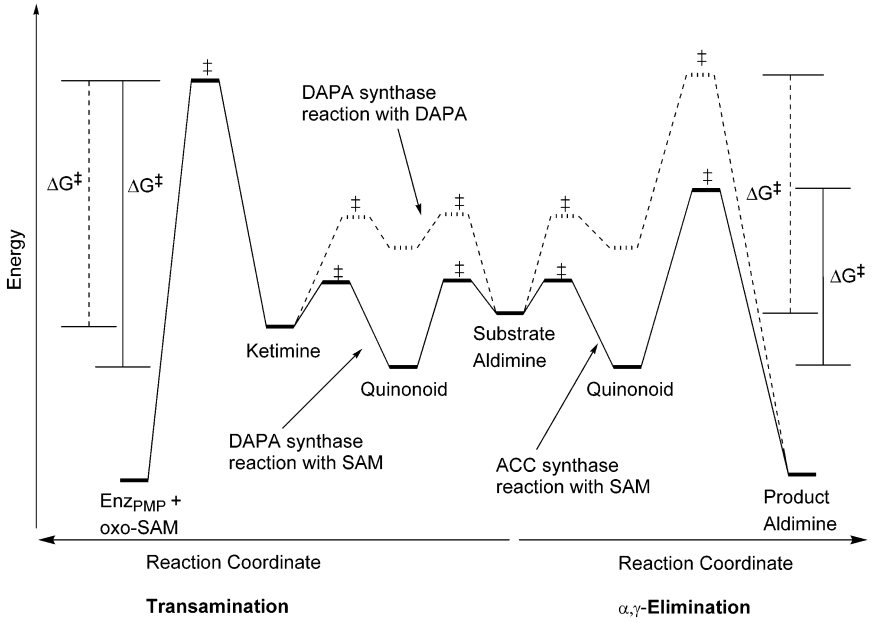
Accumulation of the quinonoid intermediate can also contribute to reaction specificity. Both transamination and elimination reactions must proceed via the quinonoid intermediate. The product distribution is decided by the relative barrier height leading from the quinonoid to the transamination products via ketimine versus that leading from the quinonoid to the α,γ-elimination products. ACC synthase selectively favors the latter reaction with SAM by preferential stabilization of the quinonoid and of the transition state leading to the ACC aldimine (Scheme 6), while the barrier to transamination is less affected. Quinonoid stabilization may even increase the overall barrier to transamination, because the quinonoid can act as a kinetic trap (Scheme 6).

It is intriguing to note that the stabilized quinonoid is a property of the substrate and not specific to the enzyme involved, since the quinonoid accumulates in the reaction of at least one substrate with each enzyme.

Why Is the Quinonoid Intermediate Stabilized by Certain Substrate Structures?

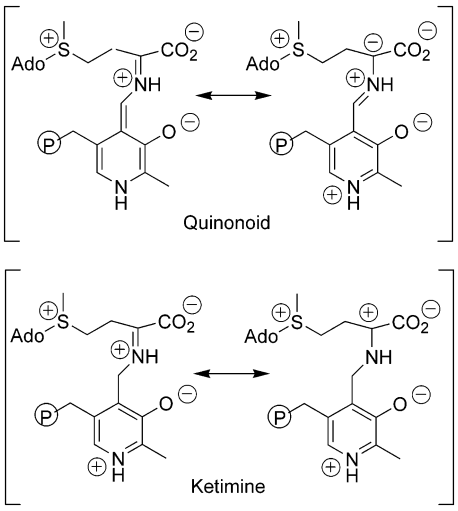
It is interesting to speculate why the structures of certain substrates help to stabilize the quinonoid intermediate. An electron-withdrawing side chain is the common attribute shared by the two substrates whose structures result in quinonoid stabilization. These are a sulfinic acid in the case of CSA and a sulfonium ion for SAM. An explanation for quinonoid stabilization is provided by the structures of the quinonoid and ketimine intermediates. The C_α position in the quinonoid is nucleophilic and partially anionic, while the C_α position of the ketimine is electrophilic and partially cationic (Scheme 7). An electron-withdrawing moiety in the side chain is thus expected to stabilize the electron-rich quinonoid, while destabilizing the electron-deficient ketimine. Ketimines are observed in the ACC synthase AVG and sinefungin complexes because the side chain of AVG is much less electron-withdrawing than that of SAM, and the formal positive charge of sinefungin is one atom more remote from the C_α carbon atom than is that of SAM.

Scheme 6. The Effect of Quinonoid Stabilization on the α,γ-Elimination (Right) and Transamination (Left) Reactions[a]



[a] Both are depicted as proceeding from the substrate aldimine (center). The solid lines represent the free-energy profile for the reactions where the quinonoid is the most stable intermediate, as is observed for the reactions of SAM with either ACC synthase or DAPA synthase. The dashed lines represent the cases where the quinonoid is an unstable intermediate relative to the ketimine (transamination only) and aldimine (both), as in the reaction of DAPA synthase with DAPA and other aminotransferases with their physiological substrates. The stabilization of the quinonoid and the rate-determining transition state between it and the product aldimine in ACC synthase results in a decrease in the overall barrier to the α,γ-elimination reaction. The reaction of SAM with DAPA synthase also proceeds via the stabilized quinonoid, resulting in an increased barrier for transamination, which accounts at least in part for the low rate constant ($k_{max} = 0.02\ s^{-1}$). In contrast, the quinonoid is not stabilized in the reaction of DAPA with the same enzyme, and the value of k_{max} (0.8 s^{-1}) is correspondingly higher. The reactions of well characterized aminotransferases follow the same dashed reaction coordinate pathway.

Scheme 7. Resonance Forms of the Quinonoid (Top) and Ketimine (Bottom) Intermediates[a]



[a] The quinonoid is partially anionic at $C_α$, while the ketimine has a partial positive charge at the same position.

Conclusions

DAPA synthase and ACC synthase are PLP-dependent enzymes that utilize SAM as a substrate but produce different products. DAPA synthase transaminates SAM to produce oxo-SAM and the PMP form of the enzyme, while ACC synthase yields ACC and MTA by α,γ-elimination. A quinonoid intermediate is formed in both reactions by α-deprotonation of the substrate, but the reaction pathways diverge from this common intermediate. The ability of each enzyme to direct the intermediates down the desired reaction pathway is complete; neither enzyme catalyzes the other reaction to a measurable extent. Pre-steady-state and steady-state kinetic analyses of the reactions of these enzymes with a variety of substrates and inhibitors have been particularly useful in helping to understand how they achieve such specificity. Further comparisons with the structurally related and well-characterized enzyme AATase have added insight into this question.

Because of the close sequence and structural similarities of ACC synthase to AATase and the other subclass I aminotransferases, it is instructive to think of the question of reaction specificity from an evolutionary perspective; that is, how has ACC synthase evolved to prevent the aminotransferase reaction and to promote elimination? It is likely that this enzyme, which participates only in the biosynthesis of the regulator molecule ethylene in higher plants, evolved relatively late from broad specificity aminotransferases. Once affinity for SAM was acquired, the stability of the quinonoid intermediate formed in the reaction of that substrate contributed to the development of the enzyme's ability to catalyze the α,γ-elimination. Optimization of the geometry of the bound substrate for cyclopropane ring formation further increased specificity for this reaction over others, as did increased exclusion of water from the active site to prevent transamination. The latter process was also influenced by the choice of substrate, as the achievement of the solvent-excluded conformation is contingent on specific interactions with the substrate. The evolutionary heritage of ACC synthase

is manifested in the retained ability of the enzyme to catalyze slow transamination of substrates such as alanine.[19]

DAPA synthase, on the other hand, has evolved to bind SAM but has retained its function as an aminotransferase. The stable quinonoid formed from this substrate also increases the potential for catalysis of α,γ-elimination, but that reaction is presumably blocked by simply binding the substrate in a conformation that does not allow for closure of the cyclopropane ring. SAM remains a suboptimal substrate for transamination, as evidenced in the lower rate constant for turnover relative to DAPA, and this low rate constant is likely the result of the quinonoid stabilization, which creates a kinetic trap.

Although the conclusions presented derive from analyses of these two enzymes and their close relatives, it is likely that other enzymes utilize similar mechanisms to direct intermediates to the intended product and to limit possible side-reactions. An understanding of these mechanisms is critical to the success of protein engineering efforts to alter the specificities of enzymes and to engineer new catalytic activities.

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References

(1) Abbreviations: AATase, aspartate aminotransferase; ACC, 1-aminocyclopropane-1-carboxylate; AVG, L-aminoethoxyvinylglycine; AMPSO, *N*-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; CSA, cysteine sulfinic acid; DAPA, 7,8-diaminopelargonic acid (7,8-diaminononanoic acid); dcSAM, decarboxylated SAM (*S*-adenosyl-(5′)-3-methylthiopropylamine); DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; KAPA, 7-keto-8-aminopelargonic acid (8-amino-7-oxononanoic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MTA, 5′-deoxy-5′-methylthioadenosine; oxo-SAM, *S*-adenosyl-4-methylthio-2-oxobutanoate; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; SAM, *S*-adenosyl-L-methionine; WT, wild-type.

(2) He, X. M.; Liu, H. W. Formation of Unusual Sugars: Mechanistic Studies and Biosynthetic Applications. *Annu. Rev. Biochem.* **2002**, *71*, 701−754.

(3) Kleppner, S. R.; Tobin, A. J. GABA Signalling: Therapeutic Targets for Epilepsy, Parkinson's Disease and Huntington's Disease. *Emerging Ther. Targets* **2001**, *5*, 219−239.

(4) Snell, K.; Riches, D. Effects of a Triazine Antifolate (NSC 127755) on Serine Hydroxymethyltransferase in Myeloma Cells in Culture. *Cancer Lett.* **1989**, *44*, 217−220.

(5) Jansonius, J. N. Structure, Evolution and Action of Vitamin B_6-dependent Enzymes. *Curr. Opin. Struct. Biol.* **1998**, *8*, 759−769.

(6) Mehta, P. K.; Christen, P. The Molecular Evolution of Pyridoxal-5′-Phosphate-Dependent Enzymes. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1998**, *74*, 129−183.

(7) Schneider, G.; Käck, H.; Lindqvist, Y. The Manifold of Vitamin B_6 Dependent Enzymes. *Struct. Fold. Des.* **2000**, *8*, R1−R6.

(8) Dunathan, H. C. Conformation and Reaction Specificity in Pyridoxal Phosphate Enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **1966**, *55*, 712−716.

(9) Sun, S.; Zabinski, R. F.; Toney, M. D. Reactions of Alternate Substrates Demonstrate Stereoelectronic Control of Reactivity in Dialkylglycine Decarboxylase. *Biochemistry* **1998**, *37*, 3865−3875.

(10) Stover, P.; Zamorga, M.; Shostak, K.; Gautam-Basak, M.; Schirch, V. Escherichia coli Serine Hydroxymethyltransferase: the role of histidine 228 in determining reaction specificity. *J. Biol. Chem.* **1992**, *267*, 17679−17687.

(11) Vacca, R. A.; Giannattasio, S.; Graber, R.; Sandmeier, E.; Marra, E.; Christen, P. Active-site Arg → Lys Substitutions Alter Reaction and Substrate Specificity of Aspartate Aminotransferase. *J. Biol. Chem.* **1997**, *272*, 21932−21937.

(12) Graber, R.; Kasper, P.; Malashkevich, V.; Strop, P.; Gehring, H.; Jansonius, J. N.; Christen, P. Conversion of Aspartate Aminotransferase into an L-Aspartate β-Decarboxylase by a Triple Active-site Mutation. *J. Biol. Chem.* **1999**, *274*, 31203−31208.

(13) Jackson, L. K.; Brooks, H. B.; Osterman, A. L.; Goldsmith, E. J.; Phillips, M. A. Altering the Reaction Specificity of Eukaryotic Ornithine Decarboxylase. *Biochemistry* **2000**, *39*, 11247−11257.

(14) Yang, S. F.; Hoffman, N. E. Ethylene Biosynthesis and its Regulation in Higher Plants. *Annu. Rev. Plant Physiol.* **1984**, *35*, 155−189.

(15) Huang, P. L.; Parks, J. E.; Rottmann, W. H.; Theologis, A. Two Genes Encoding 1-Aminocyclopropane-1-Carboxylate Synthase in Zucchini (*Cucurbita pepo*) are Clustered and Similar but Differentially Regulated. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7021−7025.

(16) Mehta, P. K.; Christen, P. Homology of 1-Aminocyclopropane-1-carboxylate Synthase, 8-Amino-7-oxononanoate Synthase, 2-Amino-6-caprolactam Racemase, 2,2-Dialkylglycine Decarboxylase, Glutamate-1-semialdehyde 2,1-Aminomutase and Isopenicillin-N-epimerase with Aminotransferases. *Biochem. Biophys. Res. Commun.* **1994**, *198*, 138−143.

(17) Capitani, G.; Hohenester, E.; Feng, L.; Storici, P.; Kirsch, J. F.; Jansonius, J. N. Structure of 1-Aminocyclopropane-1-carboxylate Synthase, a Key Enzyme in the Biosynthesis of the Plant Hormone Ethylene. *J. Mol. Biol.* **1999**, *294*, 745−756.

(18) Haruyama, K.; Nakai, T.; Miyahara, I.; Hirotsu, K.; Mizuguchi, H.; Hayashi, H.; Kagamiyama, H. Structures of *Escherichia coli* Histidinol-phosphate Aminotransferase and its Complexes with Histidinol-phosphate and *N*-(5′-Phosphopyridoxyl)-L-glutamate: Double Substrate Recognition of the Enzyme. *Biochemistry* **2001**, *40*, 4633−4644.

(19) Feng, L.; Geck, M. K.; Eliot, A. C.; Kirsch, J. F. Aminotransferase Activity and Bioinformatic Analysis of 1-Aminocyclopropane-1-carboxylate Synthase. *Biochemistry* **2000**, *39*, 15242−15249.

(20) Furumo, N. C.; Kirsch, J. F. Accumulation of the Quinonoid Intermediate in the Reaction Catalyzed by Aspartate Aminotransferase with Cysteine Sulfinic Acid. *Arch. Biochem. Biophys.* **1995**, *319*, 49−54.

(21) Capitani, G.; McCarthy, D. L.; Gut, H.; Grütter, M. G.; Kirsch, J. F. Apple 1-Aminocyclopropane-1-carboxylate Synthase in Complex with the Inhibitor L-Aminoethoxyvinylglycine. evidence for a ketimine intermediate. *J. Biol. Chem.* **2002**, *277*, 49735−49742.

(22) Feng, L.; Kirsch, J. F. L-Vinylglycine is an Alternative Substrate as Well as a Mechanism-based Inhibitor of 1-Aminocyclopropane-1-carboxylate Synthase. *Biochemistry* **2000**, *39*, 2436−2444.

(23) Satoh, S.; Yang, S. F. Inactivation of 1-Aminocyclopropane-1-carboxylate Synthase by L-Vinylglycine as Related to the Mechanism-based Inactivation of the Enzyme by *S*-Adenosyl-L-methionine. *Plant Physiol.* **1989**, *91*, 1036−1039.

(24) McCarthy, D. L.; Capitani, G.; Feng, L.; Gruetter, M. G.; Kirsch, J. F. Glutamate 47 in 1-Aminocyclopropane-1-carboxylate Synthase is a Major Specificity Determinant. *Biochemistry* **2001**, *40*, 12276−12284.

(25) This value is an extrapolation from the fact that ACC synthase is inactivated once per 30 000 turnovers of (*S*,*S*)-SAM[33,34] and is inactivated once every 500 turnovers of L-vinylglycine.[22] Assuming that the inactivation by SAM is entirely the result of β,γ-elimination to generate a vinylglycine-related intermediate and that this intermediate partitions just as in the reaction with free vinylglycine, then the ratio of α,γ- to β,γ-elimination in the reaction with SAM is approximately 30 000/500 = 60.

(26) Pai, C. H.; McLaughlin, G. E. Uptake of Pimelic Acid by *Escherichia coli* and *Pseudomonas denitrificans*. *Can. J. Microbiol.* **1969**, *15*, 809−810.

(27) Käck, H.; Sandmark, J.; Gibson, K.; Schneider, G.; Lindqvist, Y. Crystal structure of Diaminopelargonic Acid Synthase: Evolutionary Relationships Between Pyridoxal-5′-phosphate-dependent Enzymes. *J. Mol. Biol.* **1999**, *291*, 857−876.

(28) Eliot, A. C.; Sandmark, J.; Schneider, G.; Kirsch, J. F. The Dual-specific Active Site of 7,8-Diaminopelargonic Acid Synthase and the Effect of the R391A Mutation. *Biochemistry* **2002**, *41*, 12582−12589.

(29) Goldberg, J. M.; Kirsch, J. F. The Reaction Catalyzed by *Escherichia coli* Aspartate Aminotransferase Has Multiple Partially Rate-determining Steps, While that Catalyzed by the Y225F Mutant is Dominated by Ketimine Hydrolysis. *Biochemistry* **1996**, *35*, 5280−5291.

(30) Goldberg, J. M.; Swanson, R. V.; Goodman, H. S.; Kirsch, J. F. The Tyrosine-225 to Phenylalanine Mutation of *Escherichia coli* Aspartate Aminotransferase Results in an Alkaline Transition in the Spectrophotometric and Kinetic pK_a Values and Reduced Values of Both k_{cat} and K_m. *Biochemistry* **1991**, *30*, 305−312.

(31) Kirsch, J. F.; Eichele, G.; Ford, G. C.; Vincent, M. G.; Jansonius, J. N.; Gehring, H.; Christen, P. Mechanism of Action of Aspartate Aminotransferase Proposed on the Basis of its Spatial Structure. *J. Mol. Biol.* **1984**, *174*, 497−525.

(32) Kochhar, S.; Christen, P. Mechanism of Racemization of Amino Acids by Aspartate Aminotransferase. *Eur. J. Biochem.* **1992**, *203*, 563−569.
(33) White, M. F.; Vasquez, J.; Yang, S. F.; Kirsch, J. F. Expression of Apple 1-Aminocyclopropane-1-carboxylate synthase in *Escherichia coli*: Kinetic Characterization of Wild-type and Active-site Mutant forms. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 12428−12432.
(34) Satoh, S.; Yang, S. F. Specificity of *S*-Adenosyl-L-methionine in the Inactivation and the Labeling of 1-Aminocyclopropane-1-carboxylate Synthase Isolated from Tomato Fruits. *Arch. Biochem. Biophys.* **1989**, *271*, 107−112.
(35) Pegg, A. E. Recent Advances in the Biochemistry of Polyamines in Eukaryotes. *Biochem. J.* **1986**, *234*, 248−262.

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