Do enzymes obey the Baldwin rules? A mechanistic imperative in enzymatic cyclization reactions

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It is commonly assumed that enzymes have evolved to abide by the same energetic and stereoelectronic principles that govern reactions in solution. The principles formulated for organic ring-closure reactions can be used to develop a hypothesis for analysis of enzyme-catalyzed cyclization reactions.

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During the past several billion years, the forces of natural selection have refined and improved the properties of biological macromolecules so that they best contribute to the survival of their host organism [1,2]. These adaptive forces have engendered enzymes with the ability to catalyze reactions at rate accelerations of up to 10^{16}-fold. It is commonly assumed that natural selection favors enzymes that have evolved active-site arrangements that conform to underlying chemical principles (i.e. the energetic factors that govern structure and reactivity in solution also govern the basic features of enzyme-catalyzed reactions) [3]. Some features of contemporary enzymes might reflect the haphazard accidents of evolution, however, rather than adaptation for optimal catalytic power. Distinguishing between these possibilities is essential to our understanding of how enzymes achieve their enormous rate enhancements, and to our ability to design and manipulate the structure and catalytic activity of biological macromolecules [2].

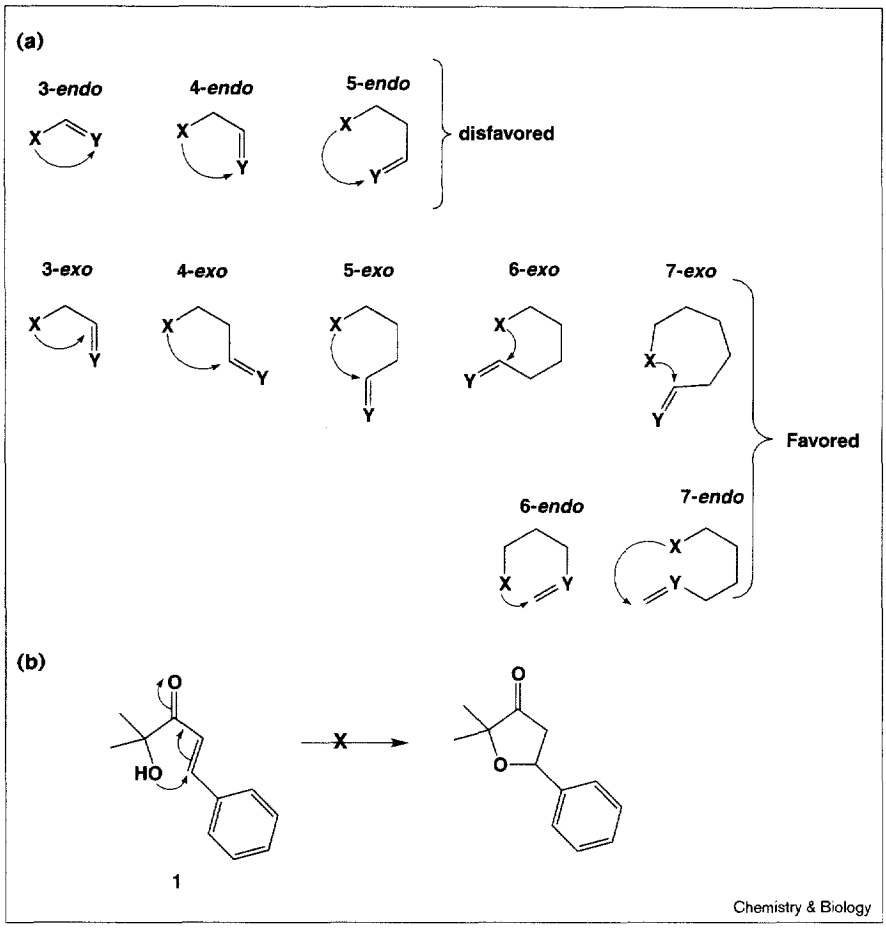
One type of enzymatic reaction that has not received much attention from mechanistic biochemists is cyclization. A series of rules formulated by Baldwin [4,5] makes explicit statements regarding modes of ring closure in organic systems. For those reactions involving nucleophilic attack at sp^2 (trigonal) centers, the rules state that 3-, 4-, 5-, 6- and 7-*exo*-trigonal attacks are favored ('allowed') processes, as well as 6- and 7-*endo*-trigonal attacks, whereas 3-, 4- and 5-*endo* attacks are disfavored ('violations'; *endo*, endocyclic; *exo*, exocyclic; Figure 1a) [4]. Although these rules are not absolute, there are many examples in the organic literature that substantiate them. One noteworthy example is the unsuccessful attempt to cyclize the hydroxy-enone system **1** (Figure 1b) to the furanone under a variety of basic conditions [5]. Yet, the enone was shown to be an intrinsically good Michael acceptor: it reacted readily with methoxide to yield the β-methoxy ketone [5]. These rules, especially the prohibition of 5-*endo*-trigonal processes, are therefore very reliable in the realm of organic reactivity. If a correlation between cyclization principles in organic systems and cyclization principles in enzymatic systems can be established, it could allow predictions regarding the catalytic strategies used by cyclization enzymes, stimulate new avenues of research, and guide biological chemists in elucidating biochemical pathways and intermediates.

Although empirical analysis of organic reactions revealed these rules, their physical basis lies in stereoelectronic theory [6]. Stereoelectronic effects control the geometric path by which a nucleophile attacks an sp^2 center [7]. Presumably, the systems that are not favored for cyclization (i.e. 3-, 4- and 5-*endo*-trigonal processes) are unable to attain the proper geometry for nucleophilic attack.

Enzyme active sites appear to have evolved to conform to stereoelectronic principles, especially when the stereoelectronic preference contributes on the order of 10 kcal/mol to transition-state stability [3]. There is evidence, however, that enzymes might even reflect more subtle stereoelectronic effects on the order of 1–2 kcal/mol [3,8]. The barriers for the 'disfavored' ring-closure reactions are expected to decrease as the ring size increases; that is, $\Delta G^{\ddagger}$ (*endo*) 3>4>5>6 (allowed). The energy barriers are large enough, however, even in the 5-*endo*-trigonal process, to expect that enzymes would conform to these principles. Assuming that proteins have functionally adapted to reflect stereoelectronic principles, these rules governing solution reactivity might serve as useful guidelines for elucidating mechanisms of enzymatic ring formation. It would therefore be interesting to know if enzymatic cyclization systems, and perhaps even biochemical pathways, have evolved to reflect the Baldwin constraints.

The chemical structures of most cyclic metabolites contain five- and/or six-membered rings. In the case of six-membered-ring formation, the mechanistic rule is not so useful because both *endo* and *exo* ring-closure pathways are allowed by the Baldwin rules, although a given enzyme might favor one reaction course over another. In the case of three-, four-, and five-membered-ring formation, however, the hypothesis offered here can have predictive utility. Because 3, 4 and 5-*endo*-trigonal processes are not favored in organic chemistry, a functional model predicts that these ring-closure mechanisms are not operative in enzymatic systems, and hence these rings are formed either by *exo*

Figure 1



Baldwin's rules for for ring closure at sp^2-hybridized (trigonal) centers [4,5]. **(a)** Ring-closure reactions are classified by three terms: the size of the ring formed, whether the bond broken is exocyclic (*exo*) or endocyclic (*endo*) with respect to the newly formed ring, and the hybridization of the carbon atom undergoing attack in the cyclization reaction – tetrahedral (sp^3), trigonal (sp^2) or digonal (sp). The rules for ring closure reactions predict strong kinetic barriers for 3-, 4- and 5-*endo*-trigonal cyclizations. **(b)** Although the furanone system **1** is reactive as a Michael acceptor, it will not undergo intramolecular ring closure [5], presumably due to the large kinetic barrier for the 5-*endo*-trigonal process required to form the ring.

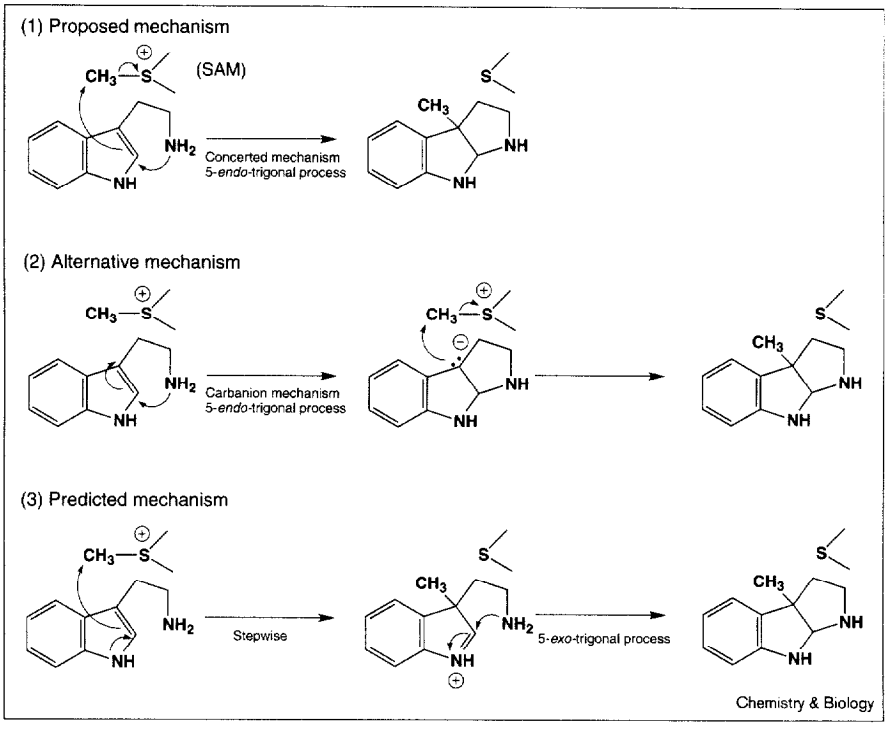
processes or by some other mechanism such as an S_N2 process (see below). Some examples follow that illustrate how this functional model can be useful as an intellectual tool for distinguishing mechanistic details.

A survey of biological ring-closure reactions reveals no well-documented cases of *endo* attack in enzymatic systems, but there are some cases where *exo* attack is operative. Proposals in which *endo* attack is invoked to explain product formation can be found in the literature, however. Such proposals are particularly relevant in the biosynthesis of secondary metabolites. In the biosynthesis of the indole alkaloid physostigmine it was suggested that concerted ring closure followed by nucleophilic attack on *S*-adenosyl-L-methionine (SAM) appears reasonable (Figure 2, mechanism 1) [9]. There are two other possible mechanisms, however: a stepwise process in which the nucleophilic amino group attacks at C-2 resulting in the formation of a carbanion that would be stabilized by the aromatic system, followed by nucleophilic attack on SAM (Figure 2, mechanism 2); or a stepwise process involving first methylation of the double bond by SAM to form the iminium type intermediate, followed by nucleophilic *exo* attack (Figure 2, mechanism 3). Both the concerted and the carbanionic mechanisms violate the Baldwin rule for 5-*endo* attack [10], but the stepwise mechanism (mechanism 3) conforms to the rule. The mechanistic imperative proposed here therefore predicts mechanism 3 to be the operative one.

Analogous arguments can be applied to analyze proposals for the formation of the cyclohexadienol ring of gliotoxin (Figure 3), which is thought to involve, at some undetermined point in the biosynthetic pathway, an arene oxide intermediate such as **2** [11,12]. A proposed mechanism for the formation of the cyclohexadieneol ring system of **3** invokes a disfavored 5-*endo*-trigonal process involving internal nucleophilic addition at C-3 of the arene oxide ring with concomitant opening of the epoxide ring. An enzymatic system that is functionally adapted to conform to stereoelectronic principles as a means of achieving catalytic optimality would probably have a different catalytic strategy than that proposed. For example, an enzyme–substrate covalent intermediate shown in Figure 3 is a means of avoiding a Baldwin 'violation'. An active-site sulfhydryl

Figure 2

Possible mechanisms for one of the ring-closure reactions in the biosynthesis of physostigmine.



group adds in nucleophilic fashion to C-3, opening the epoxide ring. Subsequent S_N2 attack by the amino group would displace the enzymatic nucleophile to give intermediate **5**. There is precedence for an enzyme-catalyzed sulfhydryl addition/displacement mechanism in the conversion of 2′-deoxyuridine-5′-phosphate to thymidine-5′-phosphate catalyzed by thymidylate synthase [13]. Alternatively, natural selection could, in principle, operate at the level of the pathway to produce an isomeric arene oxide **4** that could react in an intramolecular fashion to produce **3** directly, without a violation of Baldwin's rules. To the best of my knowledge, it is not known which of the two isomeric oxides is actually involved in the formation of the cyclohexadienol ring system of gliotoxin, nor whether the enzyme responsible for the conversion contains an active-site cysteine. Additional mechanistic and biochemical data regarding this system are required before the discussion can be carried further.

Figure 3

Possible pathways for formation of the cyclohexadieneol ring of gliotoxin **3**.

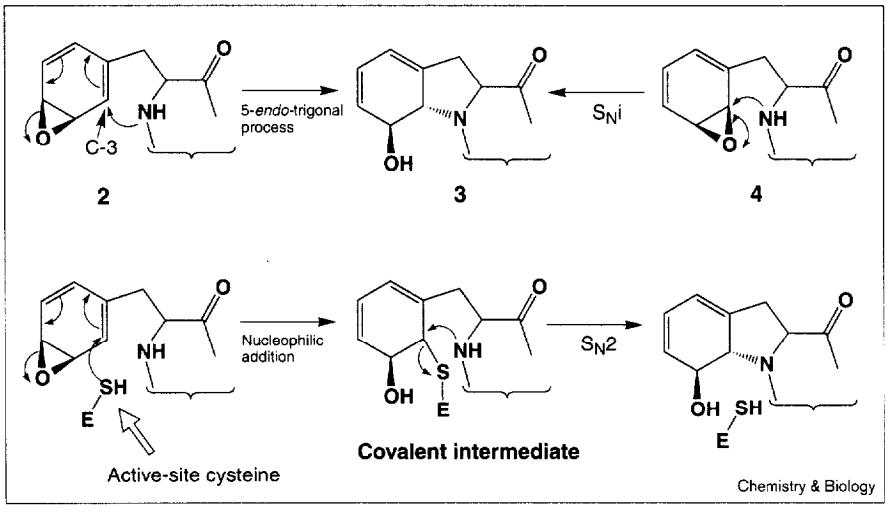
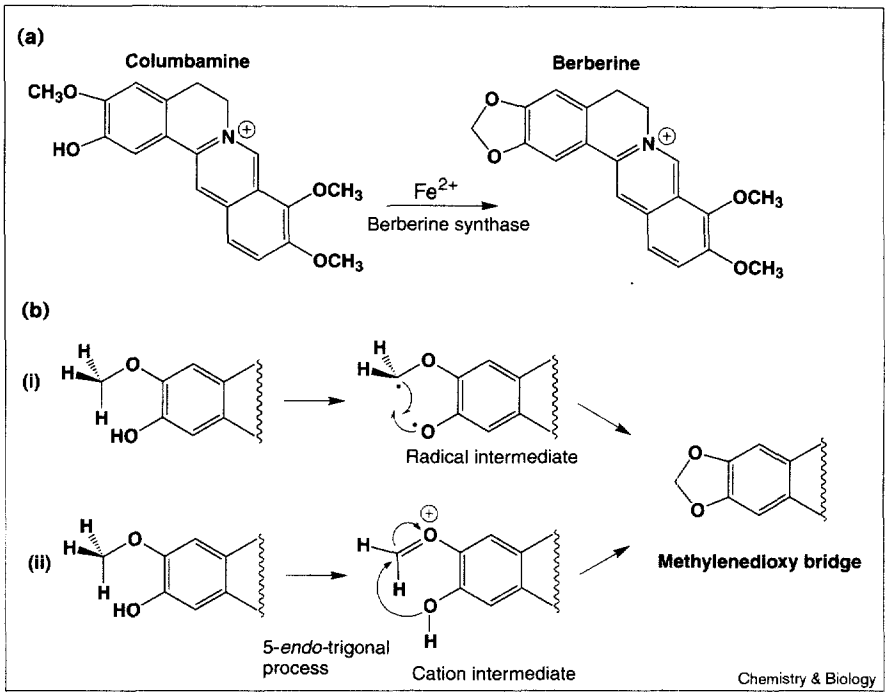


Figure 4

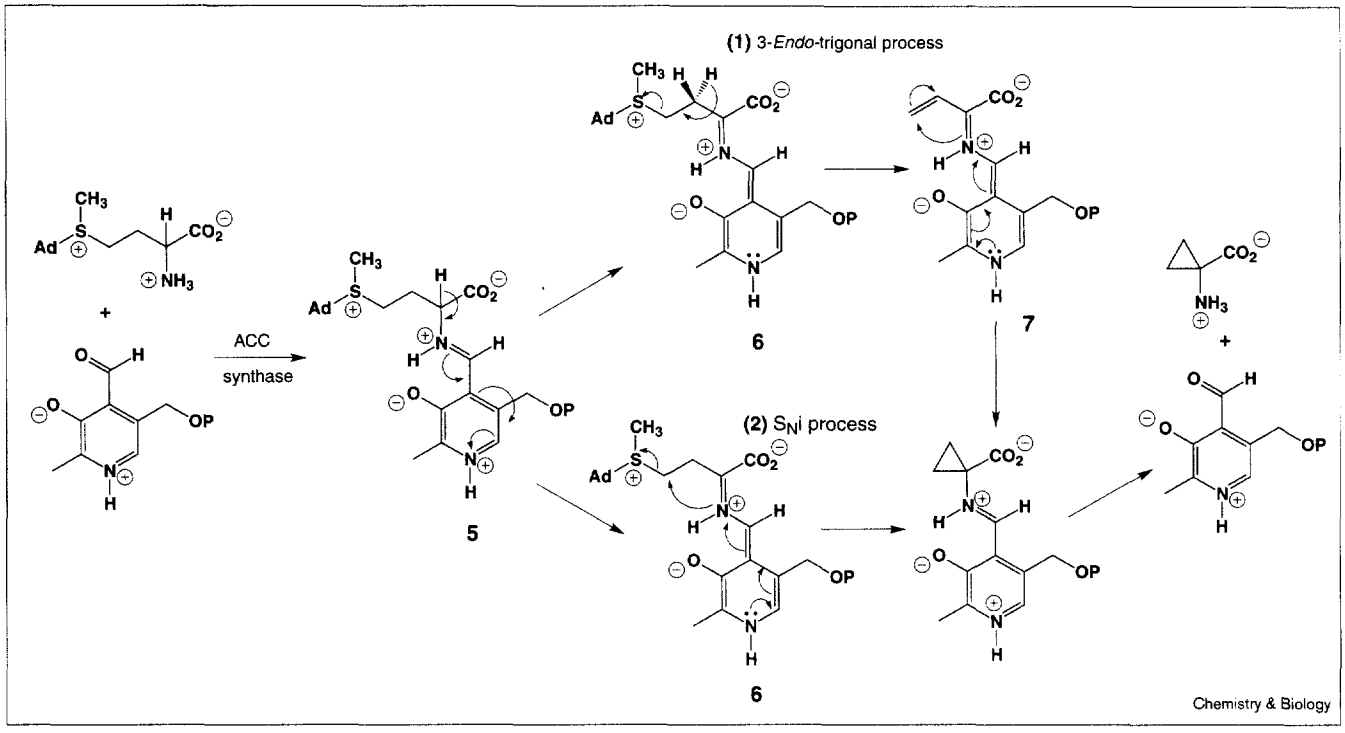


The methylenedioxy-bridge-forming reaction catalyzed by berberine synthase. **(a)** In *Berberis* cell cultures the last step in the biosynthesis of the benzylisoquinoline alkaloid berberine is catalyzed by an Fe^{2+}-dependent enzyme. **(b)** The mechanism is thought to involve either a (i) radical or (ii) cation intermediate [15]. The cationic mechanism is a violation of Baldwin's rules.

A system that has been studied more closely is the formation of the benzylisoquinoline alkaloid berberine. The terminal step in its biosynthetic sequence in *Berberis* cell cultures is the formation of berberine from columbamine, shown in Figure 4a, in a reaction catalyzed by the Fe^{2+}-dependent enzyme berberine synthase [14]. The reaction involves the formation of a methylenedioxy bridge by oxidative cyclization of an O-methoxyphenyl precursor [14]. Kobayashi *et al.* [15] obtained stereochemical results that were interpreted as consistent with two 'equally plausible' mechanisms (Figure 4b). In one mechanism, abstraction of a hydrogen atom from both the methyl group and the adjacent hydroxyl group gives a diradical, which collapses via C–O bond formation [15]. An alternative mechanism (Figure 4b) proceeds via a cationic intermediate [15]. Analysis of these mechanisms in the context of the proposed ring-closure hypothesis favors the radical mechanism for the following reasons. In the ionic mechanism, the methylene–oxygen bond has appreciable double bond character, as does the oxygen–phenyl bond. This ring closure is again a 5-*endo*-trigonal process and is expected to have a high energetic barrier. Baldwin's rules are also reliable for analysis of radical processes [16,17]. The same bonds postulated to have double-bond character in the ionic intermediate also have double-bond character in the radical intermediate, but the barriers to rotation for radical systems are considerably smaller (often as much as 15 kcal/mol), indicating that the radical has considerably less double-bond character than the cation. For example, the rotational barrier for the C1–C2 bond of an α-oxo radical is similar to that for a single bond (~9 kcal/mol), whereas the barrier for the corresponding enolate is greater than 27 kcal/mol [17]. The radical intermediate should therefore have a lower barrier for ring closure than the cationic intermediate. The presence of an active-site Fe^{2+} also supports a radical mechanism in berberine synthase.

In at least one instance, experimental data are available to suggest that enzymes have functionally adapted to avoid Baldwin violations. A pyridoxal phosphate-dependent enzyme (1-aminocylcopropane-1-carboxylic acid synthase) catalyzes the formation of 1-aminocyclopropane-1-carboxylic acid (ACC) from SAM [18]. Pyridoxal phosphate enables enzymes to catalyze reactions at the α, β or γ carbons of α-amino acids [19]; the pathway by which ACC synthase catalyzes the formation of ACC from SAM involves a reaction at the γ carbon of the methionine residue of SAM [18]. Most pyridoxal-phosphate-dependent enzymes that catalyze reactions at the γ carbon have a common mechanism: tautomerization of the aldimine **5** to the ketimine **6** facilitates elimination across the β, γ carbons to form the imine **7** [19]. Ramalingam *et al.* [20] noted that formation of ACC from concerted attack by C-α (which has carbanion character) at the β, γ double bond is a 3-*endo*-trigonal process that violates Baldwin's rule for three-membered-ring formation (Figure 5). In light of this consideration, the authors proposed an alternative 'Baldwin-allowed' process in which the α carbanion directly displaces the 5′-methylthio-5′-deoxyadenosine moiety at

Figure 5



Possible mechanisms for the pyridoxal phosphate dependent formation of ACC from SAM catalyzed by ACC synthase. Adapted from [20].

the γ carbon (Figure 5). Consistent with this S_Ni process, ACC-synthase-catalyzed ring closure occurs with inversion of stereochemistry at the γ carbon of methionine without exchange or isotopic discrimination of the hydrogens at the β carbon [20]. ACC synthase therefore appears to have adapted its active site so as to circumvent the β,γ-elimination pathway that commonly occurs for pyridoxal-phosphate-dependent reactions at the γ carbon of amino acids, thereby avoiding a violation of Baldwin's rules.

In summary, the intrinsic preferences for ring-closure reactions have been largely ignored in the analysis of enzymatic cyclization reactions. Although speculative, the hypothesis presented here appears reasonable *a priori*. If enzymes have evolved to be optimal catalysts, and if stereoelectronic factors facilitate one mode of reaction over another, it is likely that the enzyme will have evolved an active site that takes advantage of the stereoelectronic principle [3]. This is not to say that enzymes cannot catalyze disfavored reactions. Recently, Janda *et al.* [21] generated an antibody that promotes cyclization of a hydroxyalkyl epoxide via a disfavored 6-*endo*-tetrahedral pathway rather than the favored 5-*exo*-tetrahedral pathway. There may be instances, therefore, where a naturally occurring enzyme catalyzes a cyclization reaction by a 'disfavored' pathway, perhaps because of constraints imposed by the stereochemical properties of the reactants and products, or because the enzyme is constrained from adapting its active site to accommodate a Baldwin-allowed process. Alternatively, there might not be selective pressure to improve the catalytic rate such that an enzyme that catalyzes a reaction by a slower disfavored pathway could contribute as effectively to the survival of the host organism as an enzyme that catalyzes the reaction by a more expedient Baldwin-allowed process. To determine conclusively whether enzymes will obey the Baldwin rules in general, the behavior of individual enzymes must be examined. The examples outlined here are interesting systems with which to test functional hypotheses in enzymology, and illustrate how such hypotheses can provide a framework for evaluating the plausibility of proposed biosynthetic reaction pathways.

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