

ENZYME CATALYSIS: LESSONS FROM STEREOCHEMISTRY

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Abstract - Stereoselectively labeled samples of *E*- and *Z*-[9-²H, ³H]-chorismate have been synthesized from *E*- and *Z*-[3-²H, ³H]phosphoenolpyruvate by the enzymes of the shikimate pathway. The position of the tritium label at the 9-position of chorismate has been determined, from which it is evident that in the reaction catalyzed by 3-phosphoshikimate 1-carboxyvinyltransferase, the addition step has the opposite stereochemical course from the elimination step. These samples of [9-²H, ³H]chorismate have been used to determine the stereochemical course of the reaction catalyzed by chorismate mutase. It is evident that the mutase proceeds via a transition state of chair-like geometry.

The stereochemical course of enzyme-catalyzed reactions has been a fruitful source of information about the mechanisms of enzymic catalysis, and has both provided important generalizations and posed challenging questions on the nature of these processes. For example, the use of the chiral [¹H, ²H, ³H]methyl group and the chiral [¹⁶O, ¹⁷O, ¹⁸O]-phospho group has demonstrated that whenever methyl or phospho transfers are mediated by enzymes, the reactions proceed via in-line associative paths that invert the configuration at carbon (or phosphorus) at each transfer. Such findings allow us more tightly to define the nature of the transition state for the catalyzed process, and usefully constrain our mechanistic postulates. In other areas, stereochemical investigations have generated interesting puzzles. For instance, why should enzymic aldol condensations seemingly always proceed with retention of configuration, yet the closely related Claisen condensations apparently always go with inversion? The reasons behind such evident stereochemical imperatives will surely, when we understand them, illuminate the larger question of why enzymes are such dramatically good catalysts.

In this paper, we focus on one stereochemical problem, that relates to the mechanism of chorismate mutase. This enzyme catalyzes the reaction shown in Fig. 1, which is

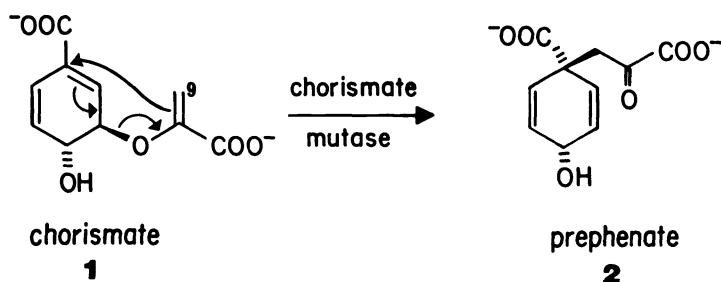


Fig. 1. The 3,3-sigmatropic rearrangement of chorismate (1) to prephenate (2), catalyzed by the enzyme chorismate mutase.

formally a Claisen rearrangement. Indeed, the mutase is the only characterized example of an enzyme that catalyzes what is -- superficially at least -- a pericyclic reaction. While there are a number of transformations in alkaloid biosynthesis that involve the Claisen-like rearrangement of dimethylallyl ethers, the enzymes that mediate these reactions have never been isolated. The stereochemical problem posed by chorismate mutase concerns the geometry of the transition state, and there are two possible formulations: 'chair-like' and 'boat-like' (Fig. 2). While unbiased non-enzymic systems generally prefer a chair geometry, this preference is rather marginal in energetic terms, and the energy difference is certainly small (2-4 kcal/mol) when compared with the large

difference (of nearly 10 kcal/mol) in the activation free energies of the enzymic and the non-enzymic processes (Ref. 1).

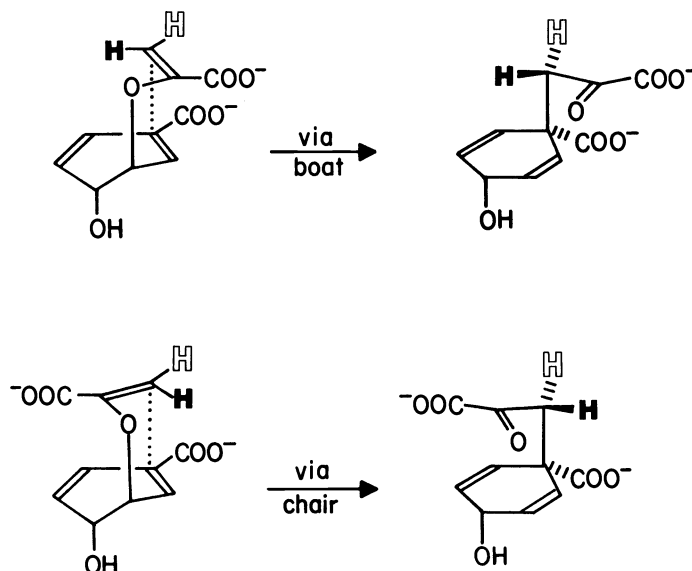
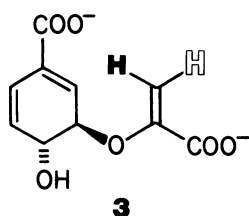


Fig. 2. The stereochemical consequences of the rearrangement of isotopically-labeled Z-chorismate, via a transition state of either boat or chair geometry.

As is illustrated in Fig. 2, rearrangement via a chair-like transition state has a different stereochemical outcome from rearrangement via a boat-like transition state, if the hydrogen atoms on carbon-9 in the starting chorismate are stereochemically distinct. If the black H represents a heavy isotope of hydrogen, the chorismate has the Z configuration. If rearrangement to prephenate then involves a transition state of boat-like geometry, the center bearing the isotopic label in prephenate has the R configuration. Yet if the rearrangement goes via a chair, the Z isomer of chorismate gives prephenate having the S-configuration at the isotopic carbon. To define the geometry of the transition state, therefore, we require the labeled species of chorismate (3). When we began this investigation, no chemical synthesis even of unlabeled chorismate had been reported, and we opted to generate 3 using the enzymes of the shikimate pathway



that are responsible for the biosynthesis of the aromatic amino acids, for which chorismate is an intermediate. [Late in 1982, two independent syntheses of unlabeled chorismate were published (Ref. 2 & 3), but neither of these was appropriate for the synthesis of stereospecifically labeled material. As discussed later, however, Berchtold's group has now achieved the synthesis of 3 by an ingenious modification (Ref. 4) of their earlier route.]

The biosynthetic step in which the carboxyvinyl group of chorismate is attached to the shikimate moiety is catalyzed by the enzyme 3-phosphoshikimate 1-carboxyvinyl-transferase [EC 2.5.1.19], and it had been suggested (Ref. 5 & 6) that this reaction involved the addition-elimination sequence illustrated in Fig. 3. At first sight, therefore, even if a stereospecifically-labeled sample of [3-³H]phosphoenolpyruvate were used in the transferase reaction (Fig. 3), the transient formation of a freely rotating monodeuteromethyl group would result in the formation of equal amounts of the Z and F isomers of the product 5-(1-carboxyvinyl)-3-phosphoshikimate. All stereochemical information in the labeled substrate would have been lost during the formation of the product. Yet we had earlier shown that in the transferase reaction, there is a primary

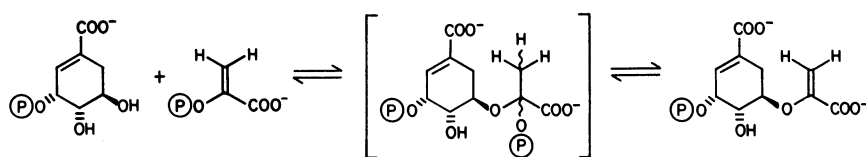


Fig. 3. The probable mechanism of the reaction catalyzed by 3-phosphoshikimate 1-carboxyvinyltransferase.

kinetic hydrogen isotope effect in the elimination step (Fig. 3), which has the result that a heavy hydrogen isotope is preferentially retained in the product (Ref. 5). If, therefore, doubly-labeled $[3-^2\text{H}, ^3\text{H}]$ phosphoenolpyruvate were used as substrate, the product should retain much of this stereochemical information. Because deuterium is used as a stoichiometric label whereas tritium is present only at tracer levels, the stereochemical consequences are as shown in Fig. 4. Deuterium will be located essentially equally at the

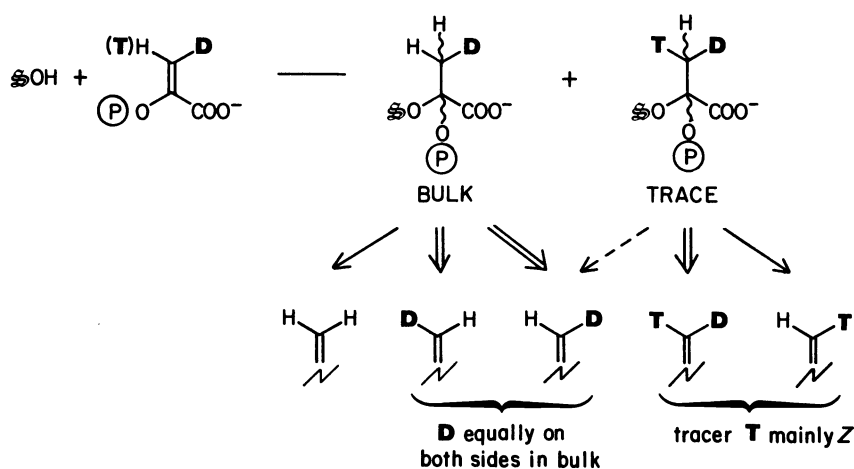


Fig. 4. The consequence of using doubly-labeled Z - $[3-^2\text{H}, ^3\text{H}]$ phosphoenolpyruvate as a substrate in the reaction catalyzed by 3-phosphoshikimate 1-carboxyvinyltransferase. 5OH represents the 5-hydroxyl group of 3-phosphoshikimate. The center hydrogen of the methyl group is removed for illustration only.

E and Z positions, whereas the tritium tracer will reside preferentially E or Z . Now, we do not know the stereochemical course of the addition and elimination steps of Fig. 3: if both these steps proceed with *anti* geometry (or if both are *syn*) then an E labeled phosphoenolpyruvate substrate will become a Z labeled product, whereas if the addition and elimination steps have different geometries (one *anti*, one *syn*) an E substrate will become an E product. It is necessary, therefore, first to determine the stereochemical course of the addition-elimination sequence shown in Fig. 3, in order to define the location of tritium in the 5-(1-carboxyvinyl)-3-phosphoshikimate product from which, by the 1,4-elimination of inorganic phosphate catalyzed by chorismate synthase, the stereo-selectively-tritiated samples of chorismate are made. This analysis was performed as shown in Fig. 5, which involves *i* aromatization of the carbocyclic ring, *ii* the *cis* reduction of the carboxyvinyl group, *iii* isolation of the R_S -lactic acid fragments by Birch reduction, *iv* enzymic removal of the S -lactate, and *v* determination of the configuration of the chiral methyl group (Ref. 6) in the remaining R -lactate. Since the configuration of the chiral methyl group is related to the configuration of the lactate at carbon-2 (because the reduction in *ii*, above, is *cis*), the finding of an S -methyl group requires that the original chorismate sample was Z .

Both Z and E isomers of $[3-^2\text{H}, ^3\text{H}]$ phosphoenolpyruvate were made from $[1-^3\text{H}]$ glucose and $[1-^3\text{H}]$ mannose, respectively, according to the method of Cohn et al. (7). Each of these samples was used as substrate in the enzymic condensation with 3-phosphoshikimate (Fig. 3), to produce what were found to be predominantly Z - $[9-^3\text{H}]$ and predominantly E - $[9-^3\text{H}]$ chorismates, respectively. That is, the carboxyvinyltransferase catalyzes the formation

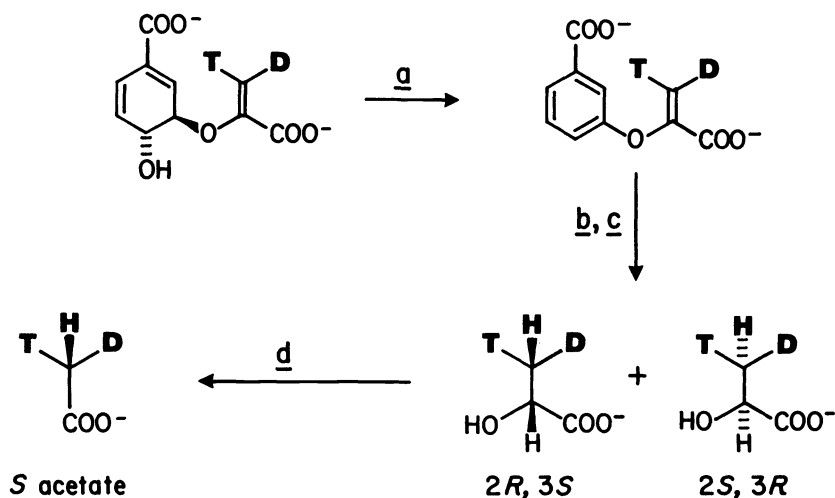


Fig. 5. Scheme for the analysis of the location of the tritium label at carbon-9 of chorismate. a free acid, dimethylsulfoxide, room temperature, overnight; b reduction with Wilkinson's catalyst, H_2 , 4 atm., in methanol-benzene, room temperature; c Birch reduction with Li/NH_3 , followed by mild acid; d removal of S-lactate using lactate dehydrogenase with 3-acetylpyridine adenine dinucleotide, pH 9.5. The R-lactate was purified by ion-exchange chromatography and then subjected to Kuhn-Roth oxidation.

of E product from E substrate, and Z product from Z substrate. This demonstrates that the addition and elimination steps shown in Fig. 3 have opposite geometries; one is syn and the other is anti (see Fig. 6). Further, the values found for the enantiomeric excess of

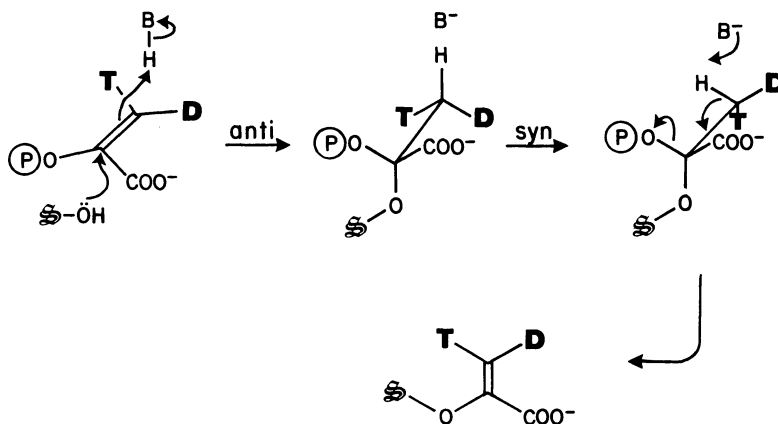


Fig. 6. One of the two possible stereochemical courses for the reaction catalyzed by 3-phosphoshikimate 1-carboxyvinyltransferase. Illustrated above is the 'anti-syn' sequence for the addition and elimination steps, but the results are equally compatible with a 'syn-anti' sequence. S-OH represents the 5-hydroxyl group of 3-phosphoshikimate.

the chiral methyl group of the lactate samples (Fig. 5) were close to those expected on the basis that some configurational integrity is lost in the transferase reaction [which loss can be estimated from the known isotope effect in this transformation (Ref. 5)]. We now have samples of chorismate that contain a tritium label at carbon-9 which is predominantly either E or Z. Deuterium and hydrogen are, for the reasons discussed earlier and illustrated in Fig. 4, randomly distributed between the E and Z positions, and

the location of these isotopes contains no stereochemical information. [A preliminary communication concerning the preparation of these samples has been published (Ref. 8).]

Concurrently with these experiments, Berchtold and his group (Ref. 4) devised a chemical approach to stereoselectively-labeled [9-²H]chorismate, the configuration of which could be assigned by NMR. Using this synthetic route, we have collaborated in the production of samples of both F and Z [9-²H]chorismate in which the F (or Z) deuterium has been doped with tritium. We have, therefore, two pairs of stereoselectively labeled chorismates. The enzymically prepared samples have a tritium label predominantly F or Z at carbon-9 (while deuterium and hydrogen are randomly distributed between these two positions), and the chemically synthesized samples have a deuterium label doped with tritium, either F or Z. The stereochemical analysis of the chorismate mutase reaction involves the use of these materials as substrates, and the determination of the location of the tritium tracer (R or S) at the isotopically-labeled carbon atom in the prephenate product (Fig. 2). To locate the tritium label in prephenate, we exploit the fact that prephenate is relatively unstable at low pH, and suffers spontaneous decarboxylative dehydration at pH values below 6. This produces phenylpyruvate (Fig. 7). Now, Retey and his collaborators have

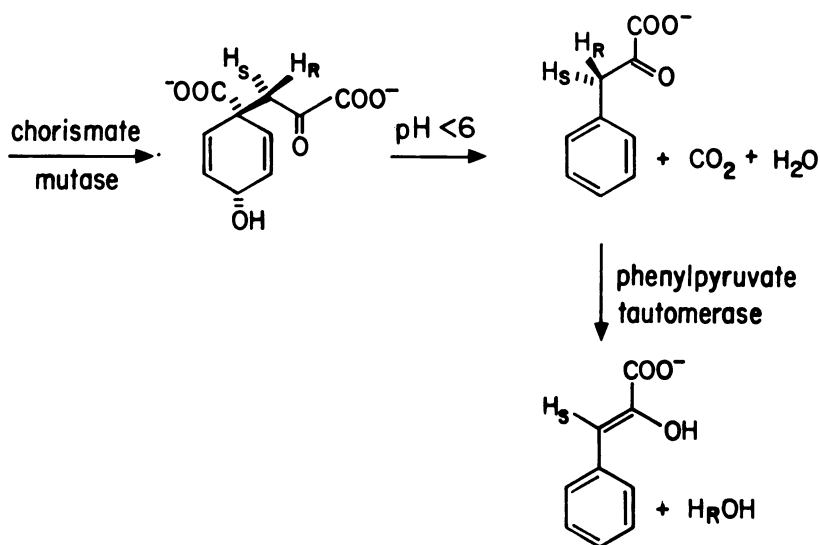


Fig. 7. Analysis of the location of tritium in prephenate. At pH values below 6, prephenate suffers decarboxylative dehydration to phenylpyruvate, and phenylpyruvate tautomerase catalyzes the more rapid loss of the pro-R hydrogen to the solvent (Ref. 9).

demonstrated (Ref. 9) that the enzyme phenylpyruvate tautomerase catalyzes the loss of the pro-R hydrogen at carbon-3 of phenylpyruvate, and this reaction can be used to determine the configuration at this center. In practice, since both chorismate mutase and phenylpyruvate tautomerase are catalytically active at pH values down to 5, the mutase reaction and the stereochemical analysis of the product prephenate can be run simultaneously. Stereoselectively-labeled chorismate is incubated with both mutase and tautomerase at pH 5.4 or below, and the proportion of volatile tritium to involatile tritium is determined in samples withdrawn over a period of several hours. [In some experiments, some [¹⁴C]chorismate was added, and the ³H:¹⁴C ratio was monitored. This avoids experimental scatter due to any variation in sample volume.] While the observed rate of loss of the pro-R hydrogen was only about ten times faster than the observed rate of loss of the pro-Shydrogen (enzyme-catalyzed loss of tritium is superposed upon the non-enzymic reaction), this difference is quite enough for a clean 'burst' of volatile tritium to be followed. When the slow reaction is extrapolated to time zero, the 'burst' sizes for enzymically-synthesized [Z-³H]chorismate, for randomly-labeled [FZ-³H]chorismate, and for enzymically-synthesized [F-³H]chorismate, were 20%, 51%, and 67%, respectively. For the racemic, chemically-synthesized samples of [Z-³H]chorismate and [E-³H]chorismate, 'burst' sizes of 13% and 35% were observed, which correspond for the natural isomer of chorismate to 26% and 70%, respectively (see Ref. 10).

Since [Z-³H]chorismate produces prephenate with only 20% (or 26%) of the tritium label in the pro-R position, and [E-³H]chorismate produces prephenate with 67% (or 70%) of tritium in the pro-R position, we can see (Fig. 2) that the enzyme proceeds via a transition state of chair-like geometry. These results provide unambiguous proof for the suggestion made earlier on the basis of studies on the inhibition of chorismate mutase by a number of dicarboxylic acids (Ref. 11).

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