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Monofunctional Chorismate Mutase from *Bacillus subtilis*: Kinetic and ^{13}C NMR Studies on the Interactions of the Enzyme with Its Ligands†

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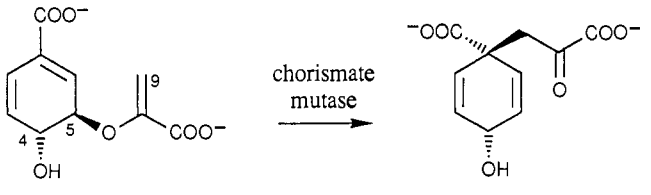
ABSTRACT: The interaction of the monofunctional chorismate mutase from *Bacillus subtilis* with chorismate and prephenate has been studied kinetically and by NMR spectroscopy with ^{13}C specifically labeled substrates. Prephenate dominates the population of enzyme-bound species, and the "off" rate constant ($\sim$60 s^{-1}) obtained from line-broadening experiments is close to the value of k_{cat} for chorismate (50 s^{-1}) determined kinetically. The calculated "on" rate constant for prephenate (8×10^5 M^{-1} s^{-1}) is similar to the value of k_{cat}/K_m for chorismate (5×10^5 M^{-1} s^{-1}). The kinetic parameters of the *Bacillus* mutase are remarkably insensitive to pH over a wide range and display no solvent isotope effect. These results suggest that the enzyme-catalyzed reaction may be encounter controlled (slowed from the diffusion limit by some feature of the enzyme's active site) and that k_{cat} for chorismate is determined by the product off rate. There is now no evidence to suggest that the skeletal rearrangement on the enzyme surface occurs by a pathway other than a pericyclic process.

The intramolecular rearrangement of chorismate (**1**) to prephenate (**2**) is catalyzed by chorismate mutase, the enzyme that lies at the branch point in the shikimate pathway that leads to the biosynthesis of the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan. The reaction is formally a Claisen rearrangement and constitutes the only example in primary metabolism of what appears to be a pericyclic process. The uncatalyzed rearrangement occurs readily in aqueous solution and seems to be a concerted, asynchronous reaction (Addadi et al., 1983). In contrast, little is known about the mechanism of the enzyme-catalyzed transformation, or about the origins of the 10^6-fold rate acceleration over the uncatalyzed process (Andrews et al., 1973; Görisch, 1978).



chorismate — prephenate

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Most mechanistic investigations of chorismate mutase have focused on the bifunctional "T" protein from *Escherichia coli*, in which the chorismate mutase activity is linked to that of prephenate dehydrogenase (Koch et al., 1971). This bifunctional enzyme is a homodimer of subunit M_r = 42 000, in which the mutase activity apparently derives from the N-terminal third of the protein (Hudson & Davidson, 1984; Maruya et al., 1987). The stereochemical course of the enzyme-catalyzed reaction has been determined and involves a transition state of chair-like geometry, as has also been found for the uncatalyzed transformation (Sogo et al., 1984; Copley et al., 1985). In contrast to the uncatalyzed reaction, the enzymic rearrangement is insensitive to tritium substitution either at C-5 or at C-9 of chorismate (**1**), suggesting that some transition state *before* that involving the chemical transformation limits the reaction rate when the substrate concentration is low (i.e., under V_{max}/K_m conditions; Addadi et al., 1983). These results do not illuminate the nature of the chemical rearrangement at the enzyme's active site, nor do they identify the rate-limiting transition state. The observation of a solvent deuterium kinetic isotope effect on V_{max} and of a small inverse secondary tritium isotope effect at C-4 of chorismate has been used to argue for a rate-limiting heterolysis on the enzyme surface (Guilford et al., 1987). This stepwise pathway was formulated as involving the attack of an enzymic nucleophile at C-5 of the substrate chorismate, thus generating a covalent enzyme-bound intermediate that subsequently collapses to prephenate in an S_N2'-like process. No observations bear directly on the nature of the pathway of the enzymic rearrangement, however, and this question must be regarded as unresolved. Much of the uncertainty derives from our ignorance both of the identity of the rate-limiting transition state(s) under saturating and subsaturating conditions and of the nature of the substrates when enzyme bound. The relatively large size of the bifunctional T protein, as well as the presence on it of multiple binding sites for substrates,

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products, and effectors, would compromise many direct approaches to the study of the interactions between chorismate mutase and its ligands. The complexity of this bifunctional mutase, both structural and kinetic, thus represents a barrier to a deeper understanding of the enzymic transformation.

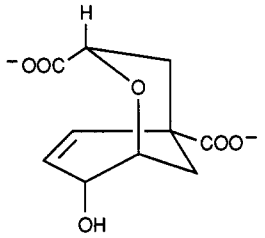
Resulting from our quest for a simpler system, we have recently reported the cloning of the *aroH* gene of *Bacillus subtilis* and the overexpression of the gene product in *E. coli* (Gray et al., 1990). The *aroH* gene encodes a *monofunctional* chorismate mutase that is a homodimer of subunit M_r = 14 500 and that possesses no other enzyme activities. This enzyme is not allosterically regulated by any of the end-product amino acids, and it follows classical Michaelis–Menten kinetics with parameters typical of the other bacterial mutases for which such data are available (Gray et al., 1990).

To underscore the degree of functional similarity among the various chorismate mutases, we have determined the K_i values for competitive inhibitors of the *Bacillus* mutase and compared these data with those determined for other mutases. Further, to gain insight into the nature of the rate-limiting transition state(s) of the enzymic process and the characteristics of the bound substrates, we have investigated the variation of the Michaelis–Menten parameters of the *Bacillus* mutase with pH and with deuteration of the solvent, and have performed ^{13}C NMR studies on the interactions between the enzyme and several of its ligands.

Experimental Procedures

Materials

Chemicals and Enzymes. All chemicals were purchased from Fischer Scientific (Fair Lawn, NJ), Sigma Chemical Co. (St. Louis, MO), or Mallinckrodt Chemical Co. (Paris, KY), unless otherwise stated. Chorismic acid was purified from *Klebsiella pneumoniae* 62-1 (American Type Culture Collection, Rockville, MD) according to the method of Addadi et al. (1983). Shikimate 3-phosphate and 5-enolpyruvoylshikimate 3-phosphate (EPSP) were generous gifts from Dr. Jonathan Burbaum. The *endo*-oxabicyclic inhibitor (**3**)



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(Bartlett & Johnson, 1985) was a generous gift from Professor Paul Bartlett. [U-^{13}C]Fructose 1,6-diphosphate was prepared from [U-^{13}C]glucose by Dr. Elizabeth Komives according to Belasco and Knowles (1980). [^{13}C]Formaldehyde was purchased from Cambridge Isotopes (Cambridge, MA). Deuterium oxide (>99.9 atom % ^2H) was purchased from MSD Isotopes (Montreal, Canada). Most enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Chorismate synthase from *Neurospora crassa* was a generous gift from Professor N. Amrhein, and EPSP synthase was a generous gift from Dr. Karen Anderson. The monofunctional chorismate mutase from *Bacillus subtilis* was purified from the overexpression strain *E. coli* XL1-Blue.pBSCM2 as described by Gray et al. (1990) and was stored in 50 mM potassium phosphate buffer, pH 7.5, at 4 °C.

[7,8,9-$^{13}C_3$]Chorismate. [U-^{13}C]Fructose 1,6-diphosphate was initially converted enzymatically to 3-phospho[U-^{13}C]-glycerate. This reaction was performed in 50 mM triethylammonium hydrochloride buffer, pH 7.7, containing EDTA (5 mM), Na_3AsO_4 (5 mM), NAD^+ (3 mM), dithiothreitol (2 mM), bovine serum albumin (0.1 mg mL^{-1}), sodium pyruvate (30 mM), [U-^{13}C]fructose 1,6-diphosphate (5 mM), aldolase (60 units), glyceraldehyde-3-phosphate dehydrogenase (300 units), yeast triosephosphate isomerase (12 000 units), and lactate dehydrogenase (600 units), in a total volume of 10 mL at 30 °C. The progress of the reaction was monitored by assaying portions of the reaction mixture for the loss of pyruvate (using lactate dehydrogenase/NADH). When the reaction was complete, the mixture was diluted with cold water (40 mL) and loaded onto a column (25 mL) of AG1X8 (Cl^- form) at 4 °C, which had been preequilibrated with 0.2 mM HCl. The column was washed with 0.2 mM HCl (500 mL) and eluted with a linear gradient (200 mL plus 200 mL) of HCl (0.2–200 mM). The fractions containing 3-phospho[U-^{13}C]glycerate were pooled and lyophilized. Cyclohexylamine (3 equiv) was added, and the sample was repeatedly lyophilized from added water. The yield of 3-phospho[U-^{13}C]glycerate was quantitative. The product was characterized by ^{13}C NMR spectroscopy and by coupled enzyme assay.

3-Phospho[U-^{13}C]glycerate was converted to [7,8,9-$^{13}C_3$]-EPSP in a single reaction of 20 mM triethanolamine hydrochloride buffer, pH 7.7, containing $MgCl_2$ (6 mM), EDTA (2 mM), dithiothreitol (1 mM), shikimate 3-phosphate (6 mM), 2,3-diphosphoglycerate (0.01 mM), 3-phospho[U-^{13}C]glycerate (2.5 mM), phosphoglycerate mutase (100 units), enolase (2.5 units), and EPSP synthase (10 units), in a total volume of 20 mL at 30 °C. The progress of the reaction was followed by monitoring A_{240nm} ($\Delta\epsilon$ = 2.5 mM^{-1} cm^{-1}). When the reaction was complete, the solution was boiled for 4 min and then centrifuged at 3000*g* for 10 min. The supernatant was diluted to 200 mL with water and loaded onto a column (80 mL) of DEAE-cellulose (DE52) at 4 °C, which had been preequilibrated in 20 mM triethylammonium bicarbonate buffer, pH 8.0. The column was washed initially with 20 mM triethylammonium bicarbonate buffer, pH 8.0 (200 mL), followed by 200 mM buffer (200 mL), and developed with a linear gradient (400 mL + 400 mL) of triethylammonium bicarbonate buffer, pH 8.0 (200–400 mM). The fractions were assayed for the presence of EPSP with a coupled assay of EPSP synthase and lactate dehydrogenase/NADH. Fractions containing EPSP were pooled and concentrated to a small volume. The mixture was neutralized with NaOH and repeatedly evaporated from 2-propanol/water (50:50 v/v). The tetrasodium salt of [7,8,9-$^{13}C_3$]EPSP was isolated in 72% yield and was found to be homogeneous by both ^{13}C NMR spectroscopy and by coupled enzyme assay.

The conversion of [7,8,9-$^{13}C_3$]EPSP to [7,8,9-$^{13}C_3$]chorismate was carried out at 35 °C in a reaction mixture of 50 mM triethanolamine hydrochloride buffer, pH 7.0, containing dithiothreitol (1 mM), FMN (10 μM), NADPH (20 μM), KCl (50 mM), [7,8,9-$^{13}C_3$]EPSP (18 μmol), and chorismate synthase (0.35 unit), in a total volume of 10 mL. Prior to the addition of the enzyme, the solution had been purged with argon, and the reaction was conducted under a positive pressure of argon. The progress of the reaction was monitored by assaying portions (25 μL) for the presence of chorismate, with chorismate mutase. When the amount of chorismate had reached a steady value, the reaction was quenched by the addition of 1 N HCl (4 mL). The solution was chilled, and denatured protein was removed by centrifugation. The supernatant was repeatedly extracted with water-saturated diethyl ether (7 × 7.5 mL). The pooled ether extracts were

dried over magnesium sulfate, and the filtrate was evaporated to dryness and stored at −70 °C. The isolated yield of [7,8,9-$^{13}C_3$]chorismate was 21% on the basis of an assay using chorismate mutase. The product was characterized by ^{13}C NMR and by enzymatic assay, and was found to be approximately 95% pure. The major contaminant was [7,8,9-$^{13}C_3$]phenylpyruvate.

[9-^{13}C]Chorismate and [9-^{13}C]Prephenate. Racemic [9-^{13}C]chorismate was synthesized by the method of Pawlak and Berchtold (1987), with [^{13}C]paraformaldehyde. The labeled, racemic chorismate was then converted into a mixture of prephenate and the unnatural enantiomer of chorismate by incubation with purified *Bacillus* chorismate mutase. The prephenate was then purified on a Cyclobond 1 (β) HPLC column (Advanced Separation Technologies Inc., Whippany, NJ) according to Connelly and Siehl (1987) and stored as a lyophilized salt at −70 °C.

Methods

Protein Determination. Protein concentrations were determined by the method of Bradford (1976), with bovine serum albumin as the calibration standard.

Enzyme Assays. The chorismate mutase was assayed spectrophotometrically in 50 mM potassium phosphate buffer, pH 7.5, at 30 °C, as described by Gray et al. (1990). 3-Phosphoglycerate was assayed by coupling to lactate dehydrogenase. The assay was run in 100 mM Tris-HCl buffer, pH 8.0, containing $MgCl_2$ (3 mM), NADH (0.2 mM), ADP (1 mM), 2,3-diphosphoglycerate (10 μM), 3-phosphoglycerate ($\leq$0.2 mM), lactate dehydrogenase (10 units), pyruvate kinase (10 units), and enolase (2 units). EPSP was assayed either by coupling EPSP synthase to pyruvate kinase and lactate dehydrogenase as described by Boocock and Coggins (1983) or by conversion to chorismate with chorismate synthase as described by Lewendon and Coggins (1987).

Steady-State Kinetics of Chorismate Mutase. Immediately prior to the kinetic experiments, chorismic acid was recrystallized as described by Guilford et al. (1987). For pH-variation studies, concentrated buffers (either 0.5 or 1.0 M) were prepared and adjusted to the approximate pH values required. All kinetic measurements were made at buffer concentrations of 50 mM, with the reported pH values being measured for the reaction mixtures themselves. The reactions were followed spectrophotometrically as described above. The pH range was covered by use of a series of buffers chosen to maintain good buffering capacity over the entire range. Interfering buffer effects (inhibition, ionic strength variations, etc.) were screened by overlapping the pH ranges over which the buffers were used. Only buffers that gave no interfering effects were used in these studies.

The solvent deuterium kinetic isotope effect on the enzymic rearrangement was determined in 50 mM potassium phosphate buffer at 30 °C by the standard assay procedure described above. For reactions in H_2O, the pH was 7.5. The reactions in D_2O were run at pD = 7.5, the relation pD = meter reading + 0.4 (Glasoe & Long, 1960) being used.

All spectrophotometric measurements were made on a Hewlett-Packard 4582A diode array spectrophotometer.

^{13}C NMR Spectroscopy. High-field Fourier transform NMR studies were performed on a Bruker AM-500 NMR spectrometer (11.75 T, 500 MHz ^1H, 125 MHz ^{13}C), with a 5-mm probe (^1H, ^{13}C, ^{15}N, ^{32}P). The field was locked with deuterium as the lock signal. Chemical shifts were determined relative to the internal reference sodium 3-(trimethylsilyl)-[2,2,3,3-$^2H_4$]propanoate in 15% D_2O buffer. All spectra were proton decoupled with composite pulse decoupling so as to minimize sample heating. Spectra of the trilabeled substrates were obtained under the following conditions: 01 = 11 300 Hz, 02 = 9150 Hz, recycle time = 1.11 s, pulse width = 6 μs, and size = 32 000 data points. Spectra of enzyme-bound species were calculated from the free induction decay with a line broadening of 20 Hz. Spectra of the monolabeled prephenate used in line-broadening analysis were collected with 64 000 data points. Spectra of unbound species and for the line-broadening analysis were calculated with no applied line broadening.

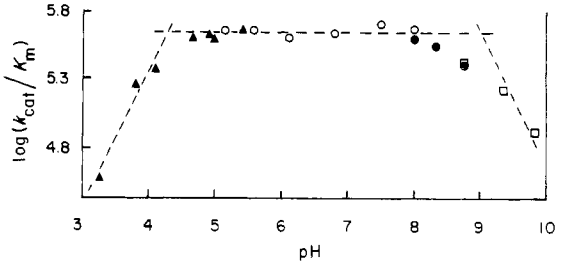

FIGURE 1: Plot of log (k_{cat}/K_m) versus pH for the monofunctional chorismate mutase from *B. subtilis*. The buffers used were as follows: sodium acetate (50 mM), pH 3.3–5.4; potassium phosphate (50 mM), pH 5.2–8.0; Tris-HCl (50 mM), pH 8.0–9.0; glycine–NaOH (50 mM), pH 9.0–9.9. The dashed lines have gradients of +1, 0, and −1.

Sample Preparation. NMR samples were prepared to final concentrations of 50 mM potassium phosphate buffer, pH 8.0, containing D_2O (15% v/v). Sample concentrations are detailed in the legends of the appropriate figures.

RESULTS

Inhibition. The sensitivity of the mutase to its product prephenate (**2**) and to the putative transition-state analogue (**3**) (Bartlett & Johnson, 1985) was investigated. Both compounds were found to be competitive inhibitors of the enzyme, with K_i values of 70 μM and 3 μM, respectively.

pH Variation. The steady-state reaction catalyzed by chorismate mutase was followed in a variety of buffers, and these data are shown in Figure 1. It is evident that k_{cat}/K_m is essentially invariant with pH between pH 5 and pH 9.

Solvent Deuterium Isotope Effect. The D_2O solvent isotope effect for the enzymic reaction catalyzed by the *Bacillus* mutase gives, at pH 7.5, the following: $^{D_2O}(V_{max})$, 1.01; $^{D_2O}(K_m)$, 1.03; and $^{D_2O}(V_{max}/K_m)$, 0.98.

NMR Studies with [7,8,9-$^{13}C_3$]Chorismate. The proton-decoupled ^{13}C NMR spectra of unbound [7,8,9-$^{13}C_3$]chorismate and [7,8,9-$^{13}C_3$]prephenate (generated by preincubation of labeled chorismate with catalytic amounts of the *Bacillus* chorismate mutase) are presented in Figure 2. For the trilabeled chorismate, three resonances are seen, and these have been assigned on the basis of chemical shift values and ^{13}C–^{13}C coupling constants: a doublet of doublets at 173.56 ppm (C-7), a doublet of doublets at 155.76 ppm (C-8), and a doublet of doublets at 95.68 ppm (C-9), with ^{13}C–^{13}C coupling constants of 75 Hz (C-7:C-8), 82 Hz (C-8:C-9), and 6 Hz (C-7:C-9). For the trilabeled prephenate three resonances are seen: a doublet of doublets at 172.43 ppm (C-7), a doublet of doublets at 206.13 ppm (C-8), and a doublet of doublets at 50.90 ppm (C-9), with ^{13}C–^{13}C coupling constants of 61 Hz (C-7:C-8), 39 Hz (C-8:C-9), and 12.5 Hz (C-7:C-9).

When the ^{13}C NMR spectrum of the trilabeled substrate is run in the presence of a large excess of monofunctional chorismate mutase and the resonances for the enzyme liganded with isotopically unlabeled substrate are subtracted, the data shown in Figure 3 are obtained. In this experiment, the enzyme concentration (3 mN) was sufficiently high to ensure

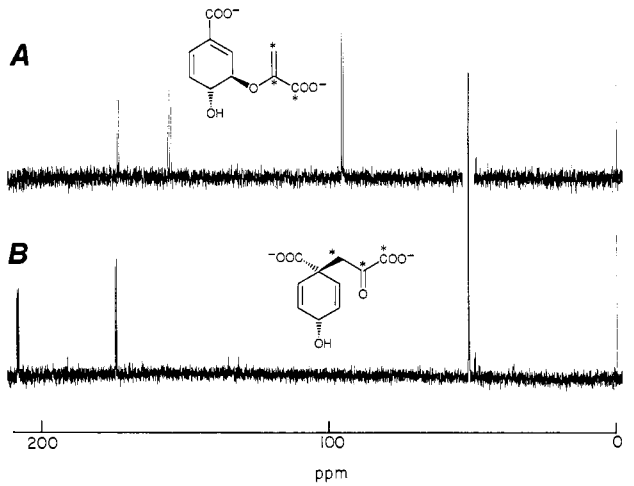

FIGURE 2: ^{13}C NMR spectra of (A) [7,8,9-$^{13}C_3$]chorismate (1 mM) and (B) [7,8,9-$^{13}C_3$]prephenate (1 mM) generated by addition of a catalytic amount of purified monofunctional chorismate mutase to sample A.

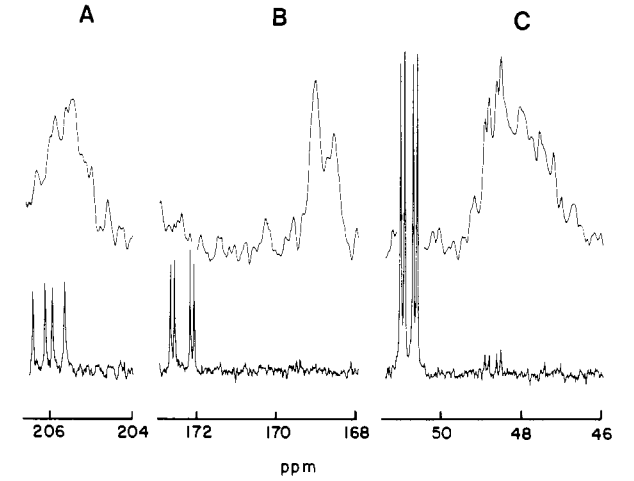

FIGURE 3: ^{13}C NMR resonances of (A) C-8, (B) C-7, and (C) C-9 carbons of [7,8,9-$^{13}C_3$]prephenate free in solution (lower traces) and bound to the monofunctional chorismate mutase (upper traces). The resonances of enzyme-bound species are from a spectrum of trilabeled substrate (1 mM) in the presence of excess enzyme (3 mN) (23 000 transients), from which the spectrum of a similar sample containing isotopically *un*labeled substrate bound to the enzyme has been subtracted. The sharp multiplet at 48.8 ppm arises from [7,8,9-$^{13}C_3$]-phenylpyruvate, which was a minor contaminant in the preparation of trilabeled chorismate.

that the resulting spectrum represents that of enzyme-bound species only (the K_m for chorismate is 100 μM, and the K_i for prephenate is 70 μM). Three new broad resonances centered at 48.1, 168.8, and 205.5 ppm are evident in this difference spectrum. (A small sharp doublet of doublets centered at 48.8 ppm is also seen, which corresponds to unbound ^{13}C-labeled phenylpyruvate, a minor contaminant in the substrate preparation used.) The three resonances in Figure 3 correspond quite closely to those of free prephenate, each having suffered a slight upfield shift (Table I) that is typical for ^{13}C NMR spectra of enzyme-bound species (Malthouse, 1986). To confirm that these resonances derive from prephenate bound to the active site of the enzyme rather than to some other site, the "transition-state" analogue (**3**), which is a competitive inhibitor of the enzyme with a K_i of 3 μM, was added in 1.5-fold molar excess over the enzyme, and the ^{13}C NMR spectrum was obtained. In the resulting spectrum, the three broad resonances from the enzyme-bound prephenate were replaced by three sharp multiplets corresponding precisely to

Table I: ^{13}C Chemical Shift Values for C-7, C-8, and C-9 of Chorismate and Prephenate

	carbon	δ(unbound) (ppm)	δ(enzyme bound) (ppm)	Δδ(upfield shift) (ppm)
chorismate	C-7	173.56		
	C-8	155.76		
	C-9	95.68		
prephenate	C-7	172.43	168.84	3.59
	C-8	206.13	205.51	0.62
	C-9	50.90	48.10	2.8

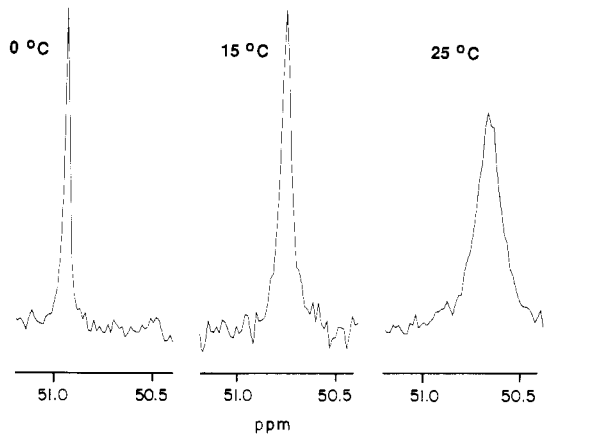

FIGURE 4: Temperature dependence of the spectrum of unbound [9-^{13}C]prephenate (0.4 mM total) in the presence of chorismate mutase (0.27 mN). The concentrations are such that the ratio of free to enzyme-bound prephenate is 1:1. The chemical shift values (ppm) are as follows: at 0 °C, 50.95; at 15 °C, 50.77; at 25 °C, 50.68.

the resonances of unbound prephenate. No resonances corresponding to free or to enzyme-bound chorismate were detected in any of these difference spectra.

To determine if the free and enzyme-bound forms of prephenate are in fast or slow exchange, ^{13}C NMR spectra were obtained under conditions in which the ratio of enzyme-bound prephenate to free prephenate was approximately unity. At 4 °C, resonances corresponding to all three labeled carbons of *both* the bound and the free forms of prephenate are evident in the spectra (data not shown). The free and bound forms of prephenate are thus in slow exchange on the NMR time scale. In spectra of the same samples at 25 °C, there is, however, some line broadening of the resonances for unbound prephenate with very little concomitant chemical shift, and the exchange rate between bound and unbound prephenate is thus measurable by line-broadening analysis. Since the coupling between the labeled carbons of [7,8,9-$^{13}C_3$]prephenate compromises any quantitative analysis using this molecule (Nageswara Rao, 1989), the exchange rate was determined by analysis of spectra of a sample of [^{13}C]prephenate labeled only at C-9.

NMR Studies with 9-^{13}C Singly Labeled Substrate. The proton-decoupled ^{13}C NMR spectrum of unbound racemic [9-^{13}C]chorismate and of both bound and unbound [9-^{13}C]-prephenate yielded single resonances at the chemical shift values assigned to C-9 of the 7,8,9-$^{13}C_3$-trilabeled materials, as expected. In addition, under conditions in which approximately equal proportions of free and enzyme-bound prephenate were present, the line width of the resonance for the unbound form increased with increasing temperature with almost no concomitant chemical shift: see Figure 4.

To determine the "off" rate (k_{off}) of a ligand exchanging between two states, one bound to the protein (B) and the other unbound (UB), in the "slow-exchange" limit by line-broadening analysis, the following equation applies (Bovey, 1988):

$$k_{off} = \pi(F_{UB}/F_B)(\delta\delta\nu_{UB})$$

where k_{off} is the first-order rate constant for enzyme-bound prephenate leaving the active site, F_{UB} is the fraction of ligand unbound, F_B is the fraction of ligand bound to the protein, and $\delta\delta\nu_{UB}$ is the excess line width due to exchange (that is, the difference in line width of the unbound species in the presence and absence of exchange with the bound form). [It should be noted that in the present case the line width of the resonance corresponding to C-9 of *enzyme-bound* prephenate is too large to be accurately measured, and the line-broadening analysis necessarily relies on measurements made on the unbound species only.]

To obtain an accurate value for k_{off} for prephenate, two parameters must be determined. First, the line widths must be measured with precision. To achieve an accurate line shape for the C-9 resonance of unbound prephenate, the spectra were collected with 64 000 data points per spectrum. Second, to determine the excess line width due to exchange ($\delta\delta\nu_{UB}$), the resonance line widths of unbound prephenate in the presence and absence of exchange are required. Accordingly, the line width of the unbound species was first determined under conditions of exchange between approximately *equal* populations of free and enzyme-bound forms (these proportions being calculated from the measured K_i for prephenate of 70 μM). An excess of the competitive inhibitor **3** (K_i = 3 μM) was then added to the sample, and the ^{13}C resonance line width was redetermined. Under these conditions, more than 99% of the enzyme active sites are blocked by the inhibitor, and exchange of free and bound prephenate is eliminated. This spectrum yields the line width in the absence of exchange under precisely the same conditions of temperature, protein concentration, viscosity, etc., as the earlier measurement. The line-width analysis was performed at 20 °C. At this temperature, the excess line broadening is substantial and can be measured with some precision. The decomposition of prephenate (due in part to small amounts of prephenate dehydratase activity in the chorismate mutase preparation) is minimal under these conditions.

The data from one such experiment are shown in Figure 5. The value of k_{off} for prephenate at 20 °C was determined from three separate experiments to be 32 ± 13 s^{-1}.

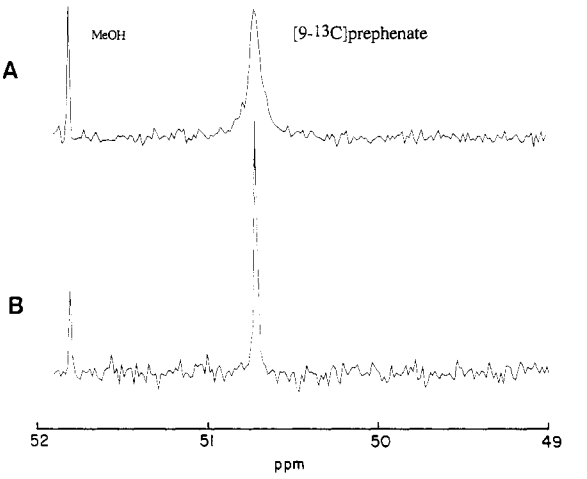

FIGURE 5: Partial spectrum of [9-^{13}C]prephenate (0.32 mM total) in the presence of chorismate mutase (0.23 mN). The concentrations are such that the ratio of free to enzyme-bound prephenate is 1:1. (A) Before and (B) after the addition of excess competitive inhibitor (**3**) (0.75 mM). A small amount of methanol is also present as an unbound standard.

Discussion

From the similarity in the values of k_{cat} and K_m for the monofunctional *B. subtilis* chorismate mutase [k_{cat}, 50 s^{-1}; K_m, 100 μM (Gray et al., 1990)] and the bifunctional *E. coli* (T) protein [k_{cat}, 72 s^{-1}; K_m, 42 μM (Davidson & Hudson, 1987)], it appears that these enzymes are functionally similar. This view gains further support from the inhibition studies reported here. The product prephenate (**2**) and the transition-state analogue (**3**) (Bartlett & Johnson, 1985) are competitive inhibitors of the *Bacillus* mutase, with K_i values (of 70 and 3 μM) comparable to those seen for the *E. coli* bifunctional enzyme (46 and 0.3 μM; Davidson & Hudson, 1987; Andrews & Heyde, 1979; Bartlett & Johnson, 1985). The close similarity of these steady-state kinetic parameters for the two enzymes is striking, given that the quantities being measured include dissociation constants for product (**2**) and for a transition-state analogue (**3**), apparent K_m values for substrate turnover (which reflect some combination of chorismate binding and partitioning of the enzyme–substrate complexes), and values of k_{cat}. There is, moreover, some similarity in the amino acid sequence of the N-terminal portions of the *E. coli* T protein and of the *Bacillus* mutase studied here (Gray et al., 1990). The monofunctional chorismate mutase from *B. subtilis* thus seems to be an ideal system for further probing the enzyme-catalyzed reaction, due to its small size, the lack of associated enzyme activities, and the absence of complicating effector binding sites.

A deeper understanding of the chorismate mutase system requires knowledge of the identities of the rate-limiting transition states for the enzymic process and of the nature of the enzyme-bound substrate and product. From the work of Addadi et al. (1983), it appears that at low substrate concentrations (i.e., under V_{max}/K_m conditions) the rate-determining transition state occurs *before* the chemical transformation. No evidence bears directly on the identity of this transition state, however, and we have no knowledge of the nature of the transition state that limits the rate at high substrate concentrations (i.e., under V_{max} conditions). To address these questions, we undertook steady-state kinetic investigations and ^{13}C NMR experiments on the interactions between the *Bacillus* mutase and its ^{13}C-labeled substrate and product.

The value of k_{cat}/K_m was found to be essentially invariant over a broad pH range, between pH 4 and pH 9 (see Figure 1). Although the interpretation of the pH dependence of the kinetic parameters of enzymes is an uncertain process (Knowles, 1976), the unusual pH *in*sensitivity of the *Bacillus* mutase is a provocative result. The simplicity of the pH profile between pH 4 and pH 9 suggests a functional simplicity for the step(s) of the catalytic cycle that involve the actual rearrangement and argues against the involvement of any catalytic group that ionizes in this pH range. Indeed, since the dependence of k_{cat}/K_m on pH is only affected by ionizations in free enzyme and in free substrate, it may be that the low apparent pK_a (below 5) derives from the substrate carboxyl groups and that the high apparent pK_a (above 9) arises from the deprotonation of cationic loci (possibly lysine ammonium ions) at the enzyme's active site.

The observation of a solvent isotope effect on k_{cat} for the *E. coli* bifunctional enzyme was part of the argument that the rearrangement mediated by this mutase is not a simple pericyclic process. Thus, while recognizing the dangers of making mechanistic deductions based upon solvent isotope effects, we proposed that the rate-limiting heterolysis of chorismate requires a proton transfer from a general acid at the active site (Guilford et al., 1987). However, no solvent isotope effect is

observed for the reaction catalyzed by the *Bacillus* mutase. The simplest interpretation of this observation is that the rate-limiting transition state (or transition states) of the reaction catalyzed by the monofunctional mutase do *not* involve a concomitant proton transfer. On this basis, the solvent isotope effect seen with the *E. coli* enzyme would be a consequence of subtle changes in protein structure, rather than of any characteristic of catalyzing functionality at the active site. The interpretation of solvent isotope effects on enzymic transformations is known to be problematic (Jencks, 1986), and the lack of a solvent isotope effect for the *Bacillus* enzyme undermines the interpretation of the effect shown by the bifunctional *E. coli* enzyme.

The NMR studies of the ^{13}C-labeled substrates and of their interactions with the *Bacillus* mutase first establish that prephenate dominates the population of enzyme-bound substrate forms. This material is completely liberated from the enzyme by the tight-binding transition-state analogue (**3**) and is therefore bound at the active site. Because of the limited sensitivity of the NMR method, a lower limit of 10 in favor of prephenate can be placed on K_{int}, the equilibrium constant of enzyme-bound chorismate and prephenate. The small upfield chemical shifts seen for the resonances of the three side-chain carbons of the trilabeled substrate, C-7, C-8, and C-9, upon binding to the active site, are typical for ^{13}C resonances of ligands bound to proteins (Malthouse, 1986). These shifts are consistent with the absence of any requirement for metals seen with this enzyme (J. Gray, unpublished results). The smaller upfield shift of the prephenate carbonyl carbon, C-8, relative to the shifts of C-7 and C-9, is consistent with (although it certainly does not prove) the existence of a hydrogen-bonding interaction between the enzyme and the carbonyl oxygen of prephenate in the active site. The existence of such an interaction has been implied by the relative dissociation constants of a pair of enzyme inhibitors that differ only in having a methylene group in place of the ether oxygen (Clarke et al., 1987; Bartlett et al., 1988).

The finding that the k_{off} for prephenate (32 s^{-1} at 20 °C; therefore, ~60 s^{-1} at 30 °C) bound to the active site of the *Bacillus* mutase is almost identical with the turnover number of the enzyme (k_{cat} = 50 s^{-1}, 30 °C) suggests that *the rate-limiting transition state of the enzymic process at substrate saturation* (i.e., under V_{max} conditions) *is that in which the product prephenate is lost from the active site.* This possibility is consistent with the lack of a solvent isotope effect and with the unusual pH *in*sensitivity of the catalyzed reaction. There is now no reason to suggest that anything other than a simple and rapid pericyclic process occurs at the active site of chorismate mutase.

Although the process that determines k_{cat} for the reaction catalyzed by chorismate mutase has now been indicated, the nature of the rate-determining transition state under k_{cat}/K_m conditions remains uncertain. As reported here, this transition state is insensitive to solvent deuteration and to large variations in the pH. These findings are consistent with the insensitivity of k_{cat}/K_m for the enzyme reaction to tritium substitution at C-5 or C-9 of chorismate (Addadi et al., 1983), and it seems possible that the overall catalyzed reaction is limited either by the rate of substrate encounter with the active site or by some conformation change that follows this association. Since the dissociation constant of prephenate from the active site is 70 μM and the rate constant k_{off} is ~60 s^{-1} at 30 °C, the k_{on} for prephenate binding to the enzyme is approximately 8 × 10^5 M^{-1} s^{-1}. This rate constant is significantly smaller than the estimated encounter rate of around 10^8–10^9 M^{-1} s^{-1} for

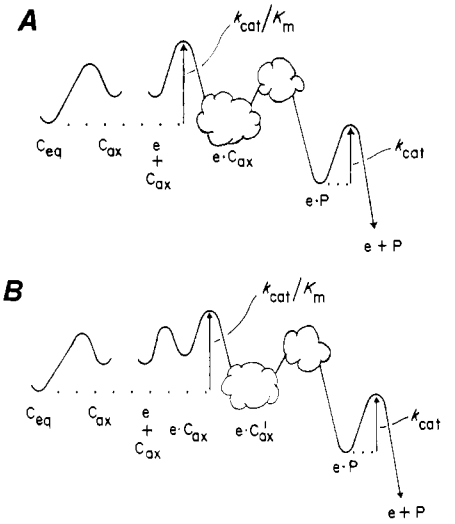

FIGURE 6: Possible kinetic barrier diagrams (Burbaum et al., 1989) for the chorismate mutase reaction, where the rate-limiting step under V_{max}/K_m conditions is (A) encounter between the free enzyme (e) and the pseudodiaxial conformer of free chorismate (C_{ax}) or (B) a conformation change of an initial, unproductive, enzyme–chorismate complex in which the enolpyruvoyl side chain is located for rearrangement. C_{eq} is the pseudodiequatorial conformer of chorismate, and P is prephenate.

many protein–ligand associations (Solc & Stockmayer, 1973; Schorr & Schmitz, 1976; Samson & Deutsch, 1978; Chou & Zhou, 1982). Yet this difference does not deny the possibility that the "on" rate for prephenate is encounter controlled, and it has been suggested that the necessary loss even of a single water molecule from an enzyme's active site can lower the rate of substrate binding very significantly (Bartlett & Marlowe, 1987). Interestingly, the value of k_{on} for prephenate is close to the value of k_{cat}/K_m for the substrate, chorismate, which is 5 × 10^5 M^{-1} s^{-1}. The simplest interpretation of these facts is that the value of k_{on} for *both* chorismate and prephenate is about 5 × 10^5 M^{-1} s^{-1} and that the mutase-catalyzed reaction of chorismate is encounter controlled. This attractively simple view does not, however, exclude the possibility that chorismate binds to the enzyme more rapidly than prephenate and that the enzyme–chorismate complex then undergoes a conformation change in which the chorismate is brought into a conformation appropriate for its rapid conversion into prephenate. Upon the slow release of prephenate, the unliganded enzyme would then revert to the predominant chorismate-binding form. In this scheme, the pseudodiaxial conformer of chorismate could be bound initially [as is consistent with the inverse secondary isotope effect seen upon tritium substitution at C-4 of chorismate (Guilford et al., 1987)] and be followed by the proper positioning of the enolpyruvyl side chain for the subsequent rearrangement. These relationships are illustrated in Figure 6, in which the kinetic barriers to reaction under k_{cat}/K_m and under k_{cat} conditions, are specified.

In summary, the chorismate mutases from *B. subtilis* and *E. coli* are functionally very close, while sharing only a slight similarity in primary sequence (Gray et al., 1990). The reaction catalyzed by chorismate mutase appears to be limited under subsaturating conditions by some step that occurs *before* the chemical transformation, and under saturating conditions by the diffusion of prephenate out of the active site. The rearrangement itself occurs relatively rapidly, and there is now no evidence to suggest that this occurs other than by a pericyclic process.

The monofunctional chorismate mutase from *B. subtilis* is proving to be an important system in the continuing search

for a deeper understanding of this fascinating enzymic transformation. We have crystallized the enzyme (J. Gray and H. Ke, unpublished work) and have found that these crystals diffract to 2.0-Å resolution. The results of these and other studies will be reported in due course.

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