Monofunctional Chorismate Mutase from *Bacillus subtilis*: FTIR Studies and the Mechanism of Action of the Enzyme†

Joseph V. Gray‡ and Jeremy R. Knowles*

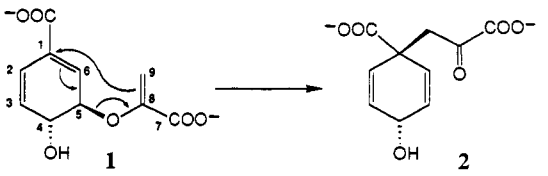
Department of Chemistry, Department of Biochemistry and Molecular Biology, and the Graduate Program in Biophysics, Harvard University, Cambridge, Massachusetts 02138

Received February 2, 1994; Revised Manuscript Received May 27, 1994®

ABSTRACT: The Fourier transform infrared (FTIR) spectrum of the complex between prephenate and the monofunctional chorismate mutase from *Bacillus subtilis* displays one prominent band at 1714 cm^{-1}. Using isotopically-labeled ligand, we have shown that this band corresponds to the ketonic carbonyl stretching vibration of enzyme-bound prephenate. The frequency of this carbonyl vibration of prephenate does not change significantly on binding to the protein. These data indicate that *chorismate mutase does not use electrophilic catalysis in the rearrangement of chorismate.* A comparison of the resolution-enhanced FTIR spectra of the unliganded mutase and of the protein complexed with its ligands reveals marked differences in the amide I′ vibration band. These changes suggest that structural alterations in the protein occur upon binding prephenate. When combined with information from the crystal structure of the enzyme and its complexes, it appears that significant ordering of the C-terminal region occurs upon ligand binding. These changes at the active site may be important for efficient catalysis and likely influence the association and dissociation rates of the enzyme and its ligands. The enzymic rearrangement of chorismate evidently proceeds via a pericyclic process, and much, if not all, of the rate acceleration derives from the selective binding of the appropriate conformer of the substrate, with some additional contribution possible from electrostatic stabilization of the transition state.

Chorismate mutase catalyzes the rearrangement of chorismate (**1**) to prephenate (**2**) (see Scheme 1), a transformation at the branch point in the shikimate pathway that leads to the biosynthesis of the aromatic amino acids in plants, fungi, and bacteria (Bentley, 1990; Poulsen & Verpoorte, 1991). The transformation of chorismate is formally a Claisen rearrangement (Ziegler, 1988) and is the only characterized example of a pericyclic process in primary metabolism. The uncatalyzed reaction proceeds rapidly in aqueous solution as a concerted asynchronous transformation in which bond breaking leads bond making at the transition state (Addadi et al., 1983). The enzyme catalyzes the rearrangement by a factor of more than 10^6 (Andrews et al., 1973; Görisch, 1978; Gray et al., 1990b). How chorismate mutase achieves this remarkable acceleration is not known. Most mechanistic studies on the enzymic transformation have used the bifunctional "T" protein from *Escherichia coli*, in which the activity of chorismate mutase and that of prephenate dehydrogenase lie in different active sites on the same polypeptide chain (Koch et al., 1971). The mutase activity is thought to derive from the N-terminal third of the protein (Hudson & Davidson, 1984; Maruya et al., 1987). Both the enzymic and the nonenzymic reactions proceed via transition states of chair-like geometry (Sogo et al., 1984; Copley & Knowles, 1985). In contrast to the nonenzymic reaction, the rate of the enzyme-catalyzed rearrangement is insensitive to tritium substitution either at C-5 or at C-9 of chorismate, suggesting that some transition state *before* that involving the chemical transformation limits the reaction rate at low substrate concentration (*i.e.*, under V_{max}/K_m conditions: Addadi et al., 1983). The observation of a small *inverse* secondary tritium kinetic isotope effect at C-4 of chorismate suggests that the substrate is in

Scheme 1



a pseudo-diaxial conformation at this rate-limiting transition state (Guilford et al., 1987). The large size of the *E. coli* enzyme and the complexity of its reaction kinetics have led to difficulties in the execution and interpretation of experiments aimed at illuminating the mutase reaction.

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., 1990b). The *aroH* gene encodes a monofunctional chorismate mutase of subunit M_r = 14 000. No cofactors are required for the reaction (Gray, 1991; Chook et al., 1993). This enzyme is not allosterically regulated and follows classical Michaelis–Menten kinetics with parameters typical of the other chorismate mutases that have been studied (Gray et al., 1990a,b; Gray, 1991). These kinetic parameters are insensitive to pH over a broad range and display no solvent kinetic isotope effect (Gray et al., 1990a). In NMR studies with specifically-labeled ^{13}C-substrates, we have shown that prephenate dominates the population of enzyme-bound species at equilibrium (Gray et al., 1990a). The "off" rate constant for prephenate dissociation from the active site (60 s^{-1}) is almost identical to the turnover number for chorismate (50 s^{-1}). These data suggest that the rate-limiting transition state at substrate saturation (*i.e.*, under V_{max} conditions) is that in which the reaction product dissociates from the active site. The chorismate mutase transformation appears, therefore, to be limited by the rate of formation of a productive enzyme–substrate complex (*i.e.*, a transition state *before* the chemical rearrangement) at low substrate concentration, and by

† This work was supported by the National Institutes of Health.

‡ Present address: Department of Biochemistry and Biophysics, School of Medicine, University of California, San Francisco, CA 94143-0448.

® Abstract published in *Advance ACS Abstracts,* July 15, 1994.

0006-2960/94/0433-9953$04.50/0

dissociation of the product (*i.e.*, a transition state *after* the chemical rearrangement) at substrate saturation.

The availability of purified monofunctional mutase has facilitated the crystallization and subsequent structure determination of the enzyme (Chook et al., 1993). In contrast to an earlier report (Gray et al., 1990b), the *Bacillus* enzyme is a symmetric trimer (Chook et al., 1993; Rajagopalan et al., 1993). The protein has a pseudo-$\alpha\beta$-barrel architecture, with a significant content of 3_{10}-helix. The C-terminal 12 residues are disordered in the crystals of the unliganded protein (Chook et al., 1994).

The binding of prephenate (Chook et al., 1994) or of Bartlett's transition-state analogue (Bartlett & Johnson, 1985; Chook et al., 1993) identifies three active sites, each at the interface of pairs of subunits in the trimer. Interestingly, the functional groups in the active site of the mutase appear only to be involved in ligand binding. There is therefore strong structural (Chook et al., 1993, 1994) as well as functional (Gray et al., 1990a; Gray, 1991) evidence against the covalent participation of the enzyme in the catalyzed reaction. Prephenate is bound in a conformation in which the pyruvoyl side chain lies over the ring, consistent with the known stereochemistry of the transformation (Sogo et al., 1984). Interestingly, there is an active site residue (arginine-90) that coordinates the side-chain ketonic carbonyl oxygen of prephenate or the ether oxygen of Bartlett's inhibitor (which is equivalent to the side-chain ether oxygen of chorismate) (Chook et al., 1993, 1994). It is possible that this residue could act as an electrophilic catalyst, perhaps facilitating a dissociative mechanism (via a tight ion pair) over the pericyclic process that operates for the uncatalyzed reaction. Alternatively, this residue may be involved in substrate binding (determining the substrate specificity, or the substrate orientation, or both). Our earlier NMR experiments (Gray et al., 1990a) did not indicate electrophilic participation by the enzyme, but the interpretation of chemical shift changes of ligand resonances upon binding to proteins is fraught with uncertainty (Rajagopalan et al., 1993). In this paper we investigate the chorismate mutase active site by FTIR spectroscopy of enzyme–ligand complexes.

From crystallographic studies it is evident that the gross structure of the enzyme is not significantly different from that of the unliganded protein when prephenate or a putative transition-state analogue is bound. There is, however, one notable change upon ligand binding. Much of the C-terminal region, which is disordered in crystals of the unliganded enzyme, becomes highly ordered upon complex formation (Chook et al., 1994). This C-terminal segment is immediately adjacent to the active site, with leucine-115 in van der Waals contact with the side chain of the bound ligand. Yet these structural changes in the protein upon complexation could be artifacts of the crystalline state. Indeed, only 8 of the 12 monomers in the asymmetric unit bind prephenate when it is diffused into crystals of the free enzyme (Chook et al., 1994). This finding may reflect restrictions imposed by the crystal lattice on the structure of some or all of the monomers. In this paper, we report resolution-enhanced FTIR spectroscopic studies, supported by ^{1}H NMR results, on the effects of ligand binding on the structure of chorismate mutase in solution.

EXPERIMENTAL PROCEDURES

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). The endo-oxabicyclic inhibitor (Bartlett & Johnson, 1985) was a generous gift of Professor Paul Bartlett. [U-^{13}C]Fructose 1,6-bisphosphate was kindly provided by Dr. Elizabeth Komives. Shikimate 3-phosphate was a generous gift of Dr. Jonathan Burbaum. [7,8,9-$^{13}C_3$]Chorismate was synthesized according to Gray et al. (1990a). Deuterium oxide (>99.9 atom %) and ^{18}O-enriched deuterium oxide (>97% ^{18}O, 86% ^{2}H) were purchased from MSD Isotopes (Montreal, Canada).

The monofunctional chorismate mutase from *B. subtilis* was purified from the overexpression strain *E. coli* XL1-Blue(pBSCM2) according to Gray et al. (1990a), except that the enzyme was passed twice over a column of QAE-Sephadex. The preparation was assayed for prephenate dehydrogenase and prephenate dehydratase, and only those fractions containing undetectable levels of these activities (<1 unit of contaminant per 10^7 units of chorismate mutase) were used. The purified bifunctional *E. coli* chorismate mutase–prephenate dehydrogenase was a generous gift of Dr. Shelley Copley.

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

Enzyme Assays. Chorismate mutase activity was assayed by monitoring the disappearance of chorismate spectrophotometrically at 275 nm in 50 mM potassium phosphate buffer, pH 7.5, at 30 °C, as described by Gray et al. (1990b). Contaminating prephenate dehydratase in chorismate mutase preparations was determined by quantifying, at 320 nm in NaOH (1.4 N), the amount of phenylpyruvate produced after incubation of the chorismate mutase fractions with chorismate (300 μM) at 30 °C for 30 min in *N*-ethylmorpholine–2-(*N*-morpholino)ethanesulfonic acid buffer (200 mM), pH 7.5. Contaminating prephenate dehydrogenase activity in the chorismate mutase fractions was assayed by monitoring the conversion of NAD^+ (2 mM) to NADH at 340 nm in 50 mM potassium phosphate buffer, pH 7.5, after the addition of chorismate (300 μM).

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

^{1}H NMR Spectroscopy of Chorismate Mutase. High-field Fourier transform NMR studies were performed on a Bruker AM-500 NMR spectrometer using a 5-mm probe (^{1}H, ^{13}C, ^{15}N, ^{32}P). Deuterium was used as the field lock. Chemical shifts were determined relative to the internal standard (trimethylsilyl)[2,2,3,3-$^2H_4$]propanoate in 50 mM potassium phosphate buffer, pD 8.2, in deuterium oxide. Solvent suppression was performed using a presaturation pulse. All spectra were obtained at ambient temperature under the following conditions: 01 = 9150 Hz, recycle time = 2.7 s, pulse width = 5 ms, and size = 32 000 data points.

FTIR Spectroscopy. Spectra were obtained using an FTS-40 infrared spectrometer (Digilab, Cambridge, MA) equipped with an MCT detector (cooled with liquid N_2) and a temperature-controlled micro Circle cell (Spectra-Tech, Stamford, CT). The spectrometer was purged with a continuous flow of dry nitrogen gas (Middlesex Welding Co., Medford, MA) both before (for approximately 12 h) and during data collection. Spectra were calculated from a total of 1024 scans obtained at 2-cm^{-1} resolution over a 20-min acquisition period. Each FTIR spectrum is the result of ratioing to a background spectrum determined for the deuterated buffer on the same day. The spectrum of water vapor, collected on the same day, was subtracted from the

sample spectra when required. (Even under continuous purging of the spectrometer with dry N_2, the level of water vapor in the light path was never reduced to zero and varied slightly over the course of a day.) All samples in the Circle cell were maintained at 8 °C during data acquisition.

Protein samples were first concentrated to approximately 150–200 mg/mL, using Centriprep 10 concentrators (Amicon, Danvers, MA). The samples were then exchanged by centrifugation into deuterated buffer (containing 100% ^{16}O, or 80%/20% $^{18}O/^{16}O$, where appropriate) that had been prepared by lyophilizing 50 mM potassium phosphate buffer, pH 8.2 in H_2O, and dissolving the residue in either $D_2{}^{16}O$ or $D_2{}^{18}O$. Concentrated samples of chorismate and of the inhibitor (3) (100 and 300 mM, respectively) were prepared by dissolving the solids in the appropriate deuterated buffer. Prephenate solutions were prepared from chorismate solutions by the addition of catalytic amounts of the purified monofunctional chorismate mutase in the appropriate buffer, followed by centrifugation through 5-kDa-cutoff membranes (Millipore) to remove protein. The sample volume required to fill the Circle cell and its connecting tubing was approximately 70 μL.

The concentration of prephenate in the chorismate mutase samples was quantitated (both before and after data acquisition) by adding a portion to a solution containing the bifunctional *E. coli* chorismate mutase–prephenate dehydrogenase and excess NAD^+. The extent of conversion of NAD^+ to NADH at equilibrium, monitored spectrometrically at 340 nm, directly corresponds to the amount of prephenate in the sample. The concentration of phenypyruvate in the mutase FTIR samples was quantitated by monitoring A_{320nm} of a sample in NaOH (1.4 M).

Calculation of the Resolution-Enhanced FTIR Spectra. Fourier self-deconvolution was performed on the FTIR spectra of chorismate mutase using a bandwidth at half-height (σ) of 15 cm^{-1}, a resolution enhancement factor (K) of 2.0, and triangular apodization. Second derivative spectra were calculated from the parent FTIR spectra using a bandpass of 0.35. The above values for σ, K, and the bandpass were found empirically to be optimal for the *Bacillus* chorismate mutase. (Note: To facilitate the direct comparison between spectra, one set of parameters was used in the calculation of all the resolution-enhanced spectra presented herein.)

The resolution-enhanced spectra of yeast triosephosphate isomerase in the presence or absence of dihydroxyacetone phosphate were obtained as above, using the raw FTIR data of Komives et al. (1991). (These spectra had been acquired by Dr. E. Komives on the same machine as and under conditions similar to those described above.)

RESULTS

FTIR Studies on Enzyme-Bound Prephenate. The carbonyl region of the infrared spectra of chorismate, of prephenate, and of phenylpyruvate are presented in Figure 1. The ketonic carbonyl stretching vibration is evident at ~1711 cm^{-1} in the spectra of prephenate and of phenylpyruvate. The bands at 1614 cm^{-1} and at 1576 cm^{-1} can tentatively be assigned to vibrations of the side-chain and the exocyclic carboxylates of prephenate, respectively (Williams & Flemming, 1987). The FTIR spectrum of the unliganded *B. subtilis* chorismate mutase shows no vibration bands between 1700 and 1800 cm^{-1} (data not shown).

The FTIR spectrum of chorismate mutase in the presence of prephenate is shown in Figure 2B. The spectrum of the unliganded protein was subtracted to yield as flat a base line

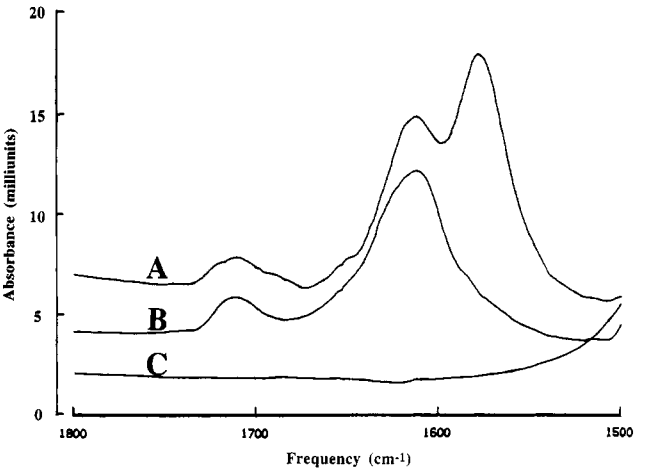


FIGURE 1: FTIR spectra of (A) prephenate, (B) phenylpyruvate, and (C) chorismate in D_2O buffer.

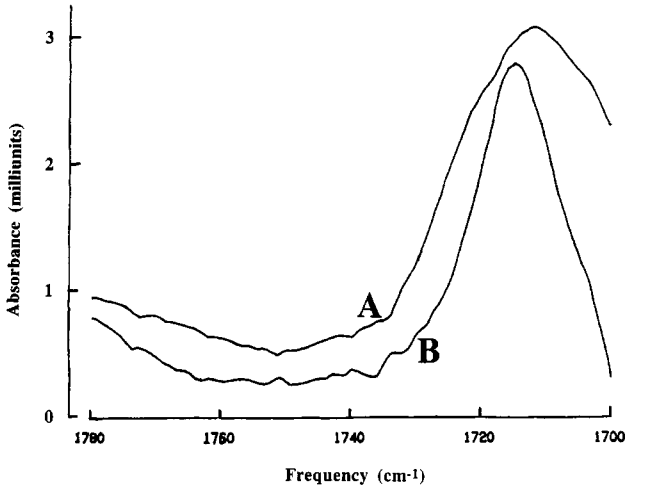


FIGURE 2: (A) FTIR spectrum of unbound prephenate (10 mM). (B) The corresponding region of the difference spectrum generated by subtraction of the FTIR spectrum of unliganded chorismate mutase from that of chorismate mutase (10 mN) in the presence of prephenate (10 mM). (The region below 1700 cm^{-1} is not presented due to the relatively large intensity of the amide I′ vibration band of the protein.)

as possible. A single band is evident at ~1714 cm^{-1}, which is close to the frequency of the carbonyl stretch vibration of unbound prephenate (Figure 2A), even though, under the conditions of the experiment, more than 95% of the prephenate is bound to the enzyme's active site (Gray et al., 1990a). The absorption band of the complex is half the width (~14 cm^{-1} at half-height) of the band for free prephenate (~30 cm^{-1} at half-height). The band does not derive from breakdown products of prephenate since less than 15% of the prephenate had decomposed during data acquisition (as determined by assaying both prephenate and phenylpyruvate before and after the spectral accumulation). Indeed, the spectrum of the enzyme in the presence of phenylpyruvate displays a broad band at ~1711 cm^{-1} that is cleanly eliminated by subtraction of the spectrum of free phenylpyruvate. The formation of an enzyme–prephenate complex under the experimental conditions is supported by analysis of the amide I′ band of the protein spectrum in the presence and in the absence of prephenate (see below). The sharp band at ~1714 cm^{-1} in the spectrum of prephenate–enzyme mixtures (Figure 2B) results, therefore, from the interaction of the ligand with the mutase. The 1714-cm^{-1} band could, however, be due to a new vibration of the protein in the complex rather than to liganded prephenate. To address this question, the effect of isotopic

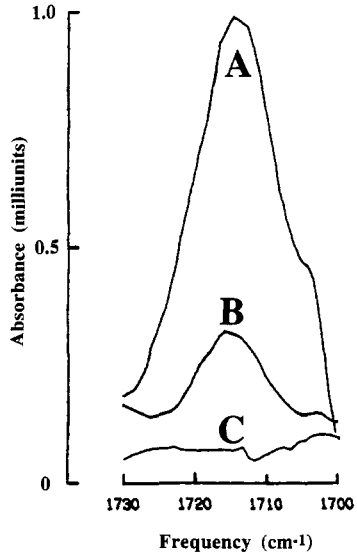


FIGURE 3: The difference spectrum generated by the subtraction of the FTIR spectrum of unliganded chorismate mutase from that of the mutase (10 mN) in the presence of prephenate (10 mM) (A) in $D_2{}^{16}O$ buffer and (B) in $D_2{}^{18}O$ buffer; or (C) in the presence of [7,8,9-$^{13}C_3$]prephenate (5 mM) in $D_2{}^{16}O$ buffer.

substitution at the carbonyl group of prephenate on the frequency of this 1714-cm^{-1} band was determined.

The carbonyl stretching frequency of free prephenate shifts from ~1711 cm^{-1} (in $D_2{}^{16}O$) to ~1679 cm^{-1} (in $D_2{}^{18}O$), consistent with the simple harmonic oscillator approximation that predicts a shift of 40 cm^{-1} to lower frequency on substitution of ^{18}O for ^{16}O in the carbonyl group (Atkins, 1983). Spectra of the unliganded protein, and of the protein in the presence of prephenate, were acquired in $D_2{}^{16}O$ and $D_2{}^{18}O$ (~80% ^{18}O atom % excess), under conditions where only the ketonic carbonyl oxygen of prephenate exchanges with solvent. These spectra are presented in Figure 3. Equivalent amounts of prephenate and of protein were present in these experiments, and the intensity of the 1714-cm^{-1} band of the mutase–prephenate complex in $D_2{}^{18}O$ is reduced to approximately 20% of the value in $D_2{}^{16}O$, consistent with the ~20% level of $D_2{}^{16}O$ in the $D_2{}^{18}O$ samples. The 1714-cm^{-1} band in the spectrum of the chorismate mutase–rephenate complex can, therefore, be assigned to the carbonyl stretch of the enzyme-bound ligand. Consistent with this assignment is the observation that the 1714-cm^{-1} band is absent in the corresponding spectrum of the complex between the mutase and [7,8,9-$^{13}C_3$]prephenate (Figure 3C).

Analysis of the Amide I′ Region of the FTIR Spectra of Chorismate Mutase and of Its Complexes with Ligands. The FTIR spectrum of unliganded chorismate mutase is shown in Figure 4A. The amide I′ band is broad with a maximum at 1637 cm^{-1}, with a shoulder at ~1650 cm^{-1}. The spectrum of the complex of chorismate mutase with prephenate is shown in Figure 5A, and while the amide I′ region of the spectrum of the complex differs somewhat from that of the unliganded protein, direct analysis is not possible. Resolution-enhancement procedures were applied to investigate these differences further.

The Fourier self-deconvoluted spectrum of unliganded chorismate mutase and the second derivative spectrum are presented as spectra B and C, respectively, of Figure 4. Both methods of spectral analysis yield bands at similar wavenumbers in the amide I′ region. These bands occur with excellent reproducibility in analyses of spectra acquired on different days and using batches of protein that had been

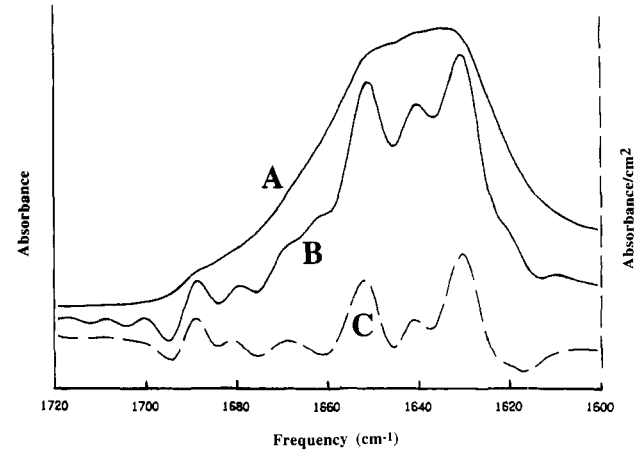


FIGURE 4: The amide I′ region of the vibration spectrum of unliganded chorismate mutase (10 mN): (A) the raw FTIR spectrum, (B) the deconvoluted spectrum, and (C) the second derivative spectrum.

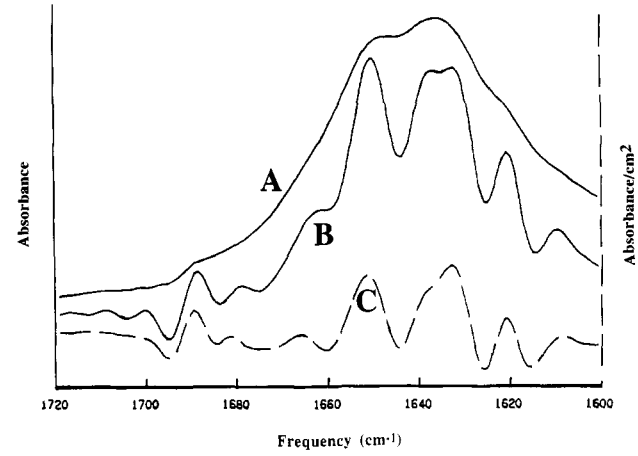


FIGURE 5: The amide I′ region of the vibration spectrum of chorismate mutase (10 mN) in the presence of prephenate (10 mN): (A) the raw spectrum, (B) the deconvoluted spectrum, and (C) the second derivative spectrum.

independently isolated and purified (data not shown). Moreover, the number and positions of the bands in the resolution-enhanced spectra are relatively insensitive to the values of the parameters used in the data manipulations. The major bands evident in the resolution-enhanced spectra of the unliganded enzyme (Figure 4B) are (tentative secondary structure assignments are included in parentheses: Byer & Sushi, 1986; Wantyghem et al., 1990; Surewicz et al., 1993): 1631 cm^{-1} (β-sheet), 1641 cm^{-1} (random coil, and perhaps 3_{10}-helix), 1651 cm^{-1} (α-helix), and 1689 cm^{-1}, with very minor bands at 1662 cm^{-1}, at 1668 cm^{-1}, and at 1679 cm^{-1}. These band assignments give a qualitative picture of the secondary structure of the protein that is in good agreement with that derived from the crystal structure of the enzyme (Chook et al., 1993) in spite of the uncertainty in assigning bands for 3_{10}-helices (Holloway & Mantsch, 1989).

The amide I′ regions of the Fourier self-deconvoluted and the second derivative spectra of the complex of chorismate mutase with prephenate are shown in spectra B and C, respectively, of Figure 5. Resolution enhancement clearly defines the differences between the spectra of the liganded and of the unliganded protein. The major bands at 1631 cm^{-1} and at 1641 cm^{-1} are absent and have been replaced by two new major bands at 1633 cm^{-1} (β-sheet) and at 1638 cm^{-1} (coil, or 3_{10}-helix, or both). New bands appear at 1621 cm^{-1} (β-sheet, or the prephenate carboxylate) and at 1610 cm^{-1} (aromatic side chains) in the spectrum of the complex. These

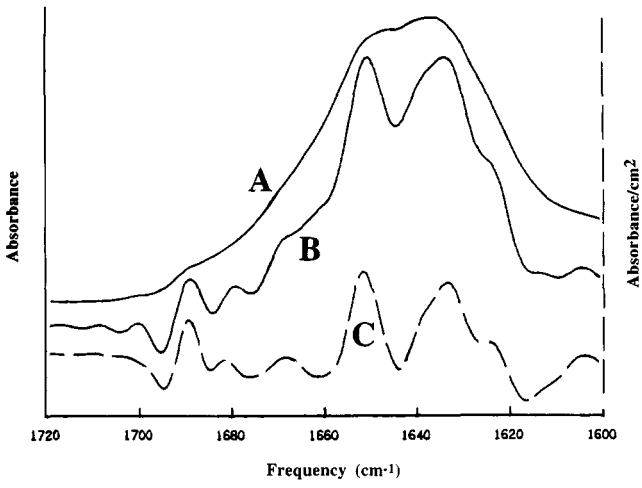


FIGURE 6: The amide I′ region of the vibration spectrum of chorismate mutase (5 mN) in the presence of Bartlett's inhibitor (3) (7.5 mM): (A) the raw spectrum, (B) the deconvoluted spectrum, and (C) the second derivative spectrum.

changes cannot be simulated by the addition of the spectra of unbound prephenate and of unbound enzyme (data not shown).

The Fourier self-deconvoluted and second derivative spectra of the complex between chorismate mutase and Bartlett's oxabicyclic transition-state analogue are shown in Figure 6. The amide I′ regions of these spectra differ significantly both from the unliganded mutase and from the enzyme–prephenate complex.

Analysis of the Amide I′ Region of the FTIR Spectra of Yeast Triosephosphate Isomerase (TIM) and of Its Complex with Dihydroxyacetone Phosphate (DHAP). The Fourier self-deconvoluted spectrum and the second derivative spectrum of unliganded TIM from yeast yield bands at similar frequencies in the amide I′ region of the spectrum. The resolution-enhanced spectrum of TIM in the presence of DHAP is identical to that of the unliganded enzyme, both before and after resolution enhancement (data not shown), even though >60% of the protein should be liganded under the experimental conditions used (Komives et al., 1991). Subtraction of the spectrum of the unliganded protein prior to resolution enhancement, so as to remove any contribution of the unbound protein (~40%) in the sample, does not alter the appearance of the spectrum. When the raw spectrum of unliganded TIM is subtracted from that of the complex, the weak ligand carbonyl group vibration bands of the bound DHAP are evident between 1700 and 1780 cm^{-1} [as reported by Komives et al. (1991)]. This observation confirms that dihydroxyacetone phosphate is indeed bound to the enzyme under the conditions of the experiment.

^{1}H NMR Spectra of Chorismate Mutase and of Its Complex with Prephenate. The ^{1}H NMR spectrum of unliganded chorismate mutase and the spectrum of the enzyme in the presence of excess prephenate show relatively narrow linewidths, reflecting the lack of aggregation of the protein under the experimental conditions. There are clear differences between the spectrum of the enzyme and that of the enzyme–prephenate complex, and these are especially evident in the aromatic and in the upfield-shifted resonances (Figure 7).

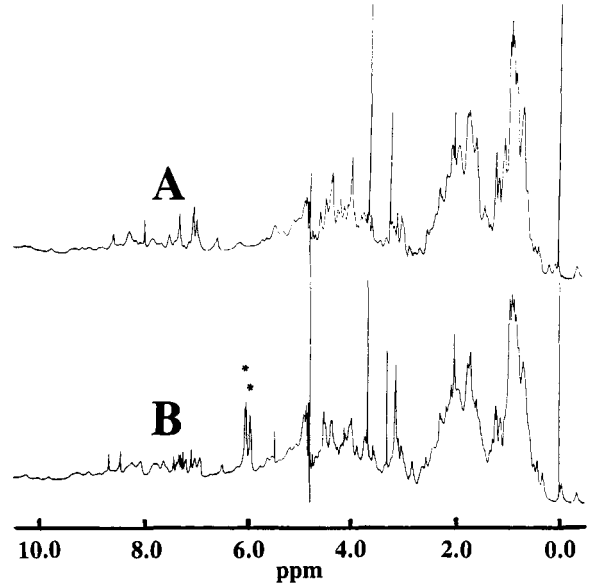


FIGURE 7: The ^{1}H NMR spectrum of chorismate mutase (2 mN) in the absence (A) and in the presence (B) of excess prephenate (≥10 mN). Asterisks indicate resonances from prephenate itself.

DISCUSSION

FTIR Studies on Enzyme-Bound Prephenate. In the difference spectrum of chorismate mutase in the presence and absence of prephenate, the only band above 1700 cm^{-1} is centered at 1714 cm^{-1} (see Figure 2). This frequency is close to, but not identical with, that of the carbonyl stretching vibration of unbound prephenate in aqueous solution. The 1714-cm^{-1} band can be assigned to the stretching vibration of the ketonic carbonyl group of enzyme-bound prephenate on the following basis. First, little decomposition of the prephenate occurs during the time of spectral acquisition, and on the basis of the known K_i, more than 95% of the prephenate is bound to the enzyme under the conditions used (Gray et al., 1990a). Second, it is clear from ^{13}C NMR experiments (Gray et al., 1990a), and from equilibrium-quenching experiments (J. V. Gray, unpublished work), that prephenate dominates the population of enzyme-bound species. Third, the linewidth of the infrared band in the spectrum of this complex is much narrower than for uncomplexed prephenate. Such a decrease in linewidth of a ligand carbonyl band upon binding to a protein has been noted earlier (Fisher et al., 1980; Belasco & Knowles, 1980; Kurz & Drysdale, 1987). Finally, the band at 1714 cm^{-1} shifts to lower frequency (where it is obscured by the amide I′ band of the protein) upon labeling of the prephenate carbonyl group with either ^{18}O or ^{13}C (Figure 3). The band at 1714 cm^{-1} clearly derives from the carbonyl stretch vibration of enzyme-bound prephenate.

The 1714-cm^{-1} band is the *only* substrate ketonic carbonyl band evident in the spectrum of the mutase–prephenate complex, and its integrated band intensity corresponds well with that for an equivalent amount of prephenate free in aqueous solution. This band therefore derives from the predominant form of enzyme-bound prephenate, which is structurally competent (Chook et al., 1994) as well as kinetically competent (Gray et al., 1990a) to lie directly on the reaction coordinate for the enzymic transformation of chorismate.

From the small shift in the carbonyl stretching frequency of prephenate when bound to the active site of the mutase, we conclude that the carbonyl group is slightly less polarized on the enzyme than in water. The small upfield change in chemical shift of the ^{13}C NMR resonance of the carbonyl carbon upon binding to the protein (Gray et al., 1990b) is consistent with this conclusion. [In the case of citrate synthase, where both ^{13}C NMR and FTIR data are also available for the ketonic carbonyl group of bound and free substrate, the carbonyl stretch of oxaloacetate shifts to lower frequency by

21 cm^{-1}, and there is a large concomitant ^{13}C chemical shift change (+6.8 ppm, downfield) for the carbonyl carbon (Kurz & Drysdale, 1987).] The combined FTIR and NMR data on the enzyme–product complex suggest that the monofunctional *chorismate mutase does not use electrophilic catalysis* to accelerate the skeletal rearrangement of chorismate. This finding does not imply that there is no hydrogen-bonding to the carbonyl oxygen by groups in the active site of the enzyme, but simply that the interactions on the enzyme are roughly equivalent to those in water. From crystallographic analysis of the monofunctional mutase and the two ligand complexes, the guanidino group of arginine-90 is within hydrogen-bonding distance of the ether oxygen of the bound inhibitor (Chook et al., 1993) and of the carbonyl oxygen of bound prephenate (Chook et al., 1994). This hydrogen-bonding interaction evidently aids in substrate specificity and orientation. Whether the enzyme effects some rate acceleration by electrostatic stabilization of the dipolar transition state (for example, by the charged guanidino group of Arg-90 being close to the migrating enolpyruvate moiety, and by the carboxylate group of Glu-78 being close the cyclohexadienyl skeleton) will have to await studies on mutant enzymes in which these interactions are modified.

The above discussion suggests that chorismate mutase does not catalyze a dissociative reaction. The results favor a simple asynchronous pericyclic process of the kind that is followed by the uncatalyzed reaction. Indeed, the possibility exists that the enzyme accelerates the Claisen rearrangement of chorismate merely by selectively binding the reactive conformer of the substrate, the subsequent chemistry (perhaps aided by some electrostatic stabilization of the dipolar transition state) following from the nature of chorismate itself. Such a proposal is consistent with the structural and kinetic simplicity of the mutase (Gray et al., 1990a,b; Gray, 1991; Chook et al., 1993, 1994) and with the fact that the rate-limiting kinetic barriers are physical (as distinct from chemical) under both saturating and subsaturating conditions (Addadi et al., 1983; Gray et al., 1990a).

FTIR Studies on the Conformation States of Chorismate Mutase. The binding of prephenate to the enzyme induces significant changes in the amide I′ region of resolution-enhanced FTIR spectrum (Figures 4 and 5). Qualitatively, these changes indicate little alteration in α-helical structure of the enzyme upon binding prephenate, but there are significant shifts in the bands corresponding to random coil/3_{10}-helix and to β-sheet structures. The vibration band attributed to aromatic amino acid side chains is also intensified. Although these changes cannot be interpreted in detail from vibrational spectroscopy alone, they do not occur when the enzyme is incubated with phenylpyruvate, nor can they be simulated by superposition of the spectra of unliganded enzyme and of unliganded prephenate.

The following observations suggest that the enzyme undergoes a significant structural change upon ligand binding in solution. First, many protein residues contribute to the main random coil/3_{10}-helix band (at 1641 cm^{-1} in the unliganded enzyme). These residues must, on average, suffer considerable environmental changes in order to shift the mean frequency of the band by 3 cm^{-1}. Second, the frequency of the β-sheet vibration band at 1631 cm^{-1} shifts 2 cm^{-1} on complexation, again indicating some alteration in these structural elements. (The band at 1621 cm^{-1} in the complex probably derives from enzyme-bound prephenate.) [In comparison, the amide I′ regions of the resolution-enhanced FTIR spectra of triosephosphate isomerase and of its complex with dihydroxyacetone phosphate are indistinguishable (data not shown), *even though* a surface loop of 9 residues near the active site is known to move by 10 Å on complex formation (Banner et al., 1976; Alber et al., 1981; Pompliano et al., 1990).] Third, extensive changes upon binding prephenate are seen in the mutase throughout the ^1H NMR spectrum. These alterations are especially striking in the aromatic region and in the upfield resonances (see Figure 7).

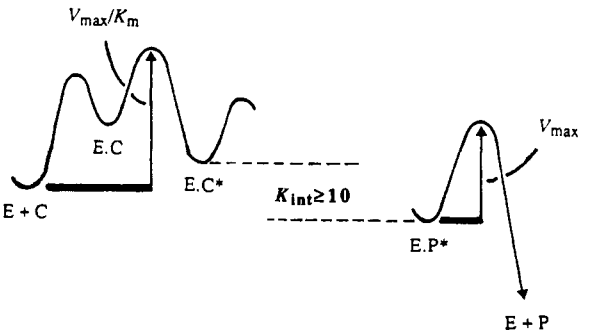


FIGURE 8: Putative kinetic barrier diagram (Burbaum et al., 1989) for the chorismate mutase reaction, where the rate-limiting transition state under V_{max}/K_m conditions involves the binding of chorismate, from the initial enzyme–chorismate encounter complex (E·C), to the active site. In the productive complex (E·C*), chorismate (C) is partially enveloped by the active site and is bound in a pseudo-diaxial conformation. The rearrangement then occurs (of E·C* to E·P*, $K_{int} \geq 10$) in an asynchronous pericyclic process. The departure of prephenate from the complex (E·P*) is rate-limiting under V_{max} conditions.

From the crystallographic analyses of the enzyme and its complexes (Chook et al., 1994) much of the C-terminus of the protein (which is disordered in the unliganded state) becomes ordered upon ligand binding. (Interestingly, this change involves alterations in a 3_{10}-helix, which may account directly for most of the changes seen in the FTIR spectrum.) These results clearly support the possibility that chorismate mutase is not a rigid template for ligand binding in solution. The binding of prephenate evidentally causes a significant alteration in the structure of the mutase, largely because of changes at the C-terminus, with more subtle changes (seen in the ^1H NMR spectra) possibly extending throughout the active site region.

The ligand-induced changes may play a critical role in the enzyme-catalyzed reaction. Part of the C-terminal region is in direct contact with the side chain of bound ligand. Ordering the C-terminal appears partially to sequester the bound ligand from bulk solvent (Chook et al., 1994). The slow binding of ligands to the mutase (Gray et al., 1990a), the rate-limiting steps of the catalytic cycle, and the unusually high specificity of the active site (Gray, 1991) probably derive from this sequestration. Finally, a naturally-occuring mutant allele of the *aroH* gene has been characterized (Gray, 1991) in which alanine-112 is replaced by valine. The mutant protein is stably folded but displays little or no enzymatic activity *in vivo* or *in vitro*. In the crystal structure of the wild-type enzyme (Chook et al., 1993, 1994) alanine-112, although not part of the active site, is at a contact point between the ordered C-terminus and the body of the protein. Substitution of the bulkier valine for alanine-112 would likely interfere with the positioning of the C-terminus.

The arguments outlined above indicate that chorismate mutase suffers ligand-induced transitions. Chorismate mutase appears, indeed, to be a classic example of induced fit (Koshland, 1958). The dynamics of the C-terminal tail motion may influence the structure (and reactivity) of bound ligand, as well as limiting the diffusion of ligands into and out of the active site (see Figure 8).

Chorismate mutases are unlike all other chorismate-utilizing enzymes, both in terms of primary sequence and in terms of the degree of phylogenetic conservation (Gray, 1991). This singularity may reflect the unique nature of bound chorismate in its complexes with the mutase and supports the possibility that the correct binding of chorismate is effectively synonymous with catalysis of the Claisen rearrangement (Haynes et al., 1994). Whether the enzyme also exploits electrostatic stabilization of the transition state remains to be determined. Ironically, out of the large literature on this enzyme and from all the scrutiny that its workings have enjoyed, chorismate mutase may well be one of the simplest of enzymes in mechanistic terms.

ACKNOWLEDGMENT

We gratefully acknowledge Dr. Elizabeth Komives, Dr. Shelley Copley, Dr. Jonathan Burbaum, and Professor Paul Bartlett for their generous gifts of materials. We thank Dr. Elizabeth Komives for her advice and help throughout much of this work. Finally, we thank Yuh Min Chook and Professor William Lipscomb for sharing their structural data with us, and for many helpful discussions.

REFERENCES

Addadi, L., Jaffe, E. K., & Knowles, J. R. (1983) *Biochemistry 22*, 4494–4501.

Alber, T., Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Rivers, P. S., & Wilson, I. A. (1981) *Philos. Trans. R. Soc. London, B 293*, 159–171.

Andrews, P. R., Smith, G. D., & Young, I. G. (1973) *Biochemistry 12*, 3492–3498.

Atkins, P. W. (1983) *Physical Chemistry*, 2nd ed., Oxford University Press, Oxford.

Banner, D. W., Bloomer, A. C., Petsko, G. A., Phillips, D. C., Pogson, C. I., & Wilson, I. A. (1976) *Nature 255*, 609–614.

Bartlett, P. A., & Johnson, C. R. (1985) *J. Am. Chem. Soc. 107*, 7792–7793.

Belasco, J. G., & Knowles, J. R. (1980) *Biochemistry 19*, 472–477.

Bentley, R. (1990) *CRC Crit. Rev. Biochem. Mol. Biol. 25*, 307–384.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Burbaum, J. J., Raines, R. T., Albery, W. J., & Knowles, J. R. (1989) *Biochemistry 28*, 9293–9305.

Byer, D. M., & Sushi, H. (1986) *Biopolymers 25*, 469–487.

Chook, Y.-M., Ke, H.-M., & Lipscomb, W. N. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 8600–8603.

Chook, Y.-M., Gray, J. V., Ke, H.-M., & Lipscomb, W. N. (1994) *J. Mol. Biol. 240*, 476–500.

Copley, S. D., & Knowles, J. R. (1985) *J. Am. Chem. Soc. 107*, 5306–5308.

Fisher, J., Belasco, J. G., Khosla, S., & Knowles, J. R. (1980) *Biochemistry 19*, 2895–2901.

Görisch, H. (1978) *Biochemistry 17*, 3700–3705.

Gray, J. V. (1991) Ph.D. Thesis, Harvard University, Cambridge, MA.

Gray, J. V., Eren, D., & Knowles, J. R. (1990a) *Biochemistry 29*, 8872–8878.

Gray, J. V., Golinelli-Pimpaneau, B., & Knowles, J. R. (1990b) *Biochemistry 29*, 376–383.

Guilford, W. J., Copley, S. D., & Knowles, J. R. (1987) *J. Am. Chem. Soc. 109*, 5013–5019.

Haynes, M. R., Stura, E. A., Hilvert, D., & Wilson, I. A. (1994) *Science 263*, 646–652.

Holloway, P. W., & Mantsch, H. H. (1989) *Biochemistry 28*, 931–935.

Hudson, G. S., & Davidson, B. E. (1984) *J. Mol. Biol. 180*, 1023–1051.

Koch, G. L. E., Shaw, D. C., & Gibson, F. (1971) *Biochim. Biophys. Acta 229*, 795–804.

Komives, E. A., Chang, L. C., Lolis, E., Tilton, R. F., Petsko, G. A., & Knowles, J. R. (1991) *Biochemistry 30*, 3011–3019.

Koshland, D. E., Jr. (1958) *Proc. Natl. Acad. Sci. U.S.A. 44*, 98–104.

Kurz, L. C., & Drysdale, G. R. (1987) *Biochemistry 26*, 2623–2627.

Maruya, A., O'Connor, M. J., & Backman, K. (1987) *J. Bacteriol. 169*, 4852–4853.

Pompliano, D. L., Peyman, A., & Knowles, J. R. (1990) *Biochemistry 29*, 3186–3194.

Poulsen, C., & Verpoorte, R. (1991) *Phytochemistry 30*, 377–386.

Rajagopalan, J. S., Taylor, K. M., & Jaffe, E. K. (1993) *Biochemistry 32*, 3965–3972.

Sogo, S. G., Widlanski, T. S., Hoare, J. H., Grimshaw, C. E., Berchtold, G. A., & Knowles, J. R. (1984) *J. Am. Chem. Soc. 106*, 2701–2703.

Surewicz, W. K., Mantsch, H. H., & Chapman, D. (1993) *Biochemistry 32*, 389–394.

Wantyghem, J., Baron, M.-H., Picquart, M., & Lavialle, F. (1990) *Biochemistry 29*, 6600–6609.

Williams, D. H., & Fleming, I. (1987) *Spectroscopic Methods in Organic Chemistry*, 4th ed., McGraw-Hill, London, U.K.

Ziegler, F. E. (1988) *Chem. Rev. 88*, 1423–1452.