Thermodynamics of the Conversion of Chorismate to Prephenate: Experimental Results and Theoretical Predictions

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A thermodynamic investigation of the Claisen rearrangement of chorismate^{2-}(aq) to prephenate^{2-}(aq) has been performed using microcalorimetry and high-performance liquid chromatography. The study used a well-characterized monofunctional chorismate mutase from *Bacillus subtilis* that was devoid of prephenate dehydrogenase and prephenate dehydratase activities. The calorimetric measurements led to a standard molar enthalpy change $\Delta_r H^\circ_m = -(55.4 \pm 2.3)$ kJ mol^{-1} at 298.15 K for this reaction. An estimated value of the standard molar entropy change $\Delta_r S^\circ_m = 3$ J K^{-1} mol^{-1} for the above reaction was used together with the experimental value of $\Delta_r H^\circ_m$ to obtain a standard molar Gibbs free energy change $\Delta_r G^\circ_m \approx -56$ kJ mol^{-1} and an equilibrium constant $K \approx 7 \times 10^9$ for the conversion of chorismate^{2-}(aq) to prephenate^{2-}(aq) at 298.15 K. Thus, for all practical purposes, this reaction can be considered to be "irreversible". Quantum mechanics (Gaussian 94 with a B3LYP functional and a 6-31G* basis set) was used to calculate values of absolute and relative energies for the chorismic acid and prephenic acid species having charge numbers 0 and −2 both in the gas phase and in aqueous solution. The structures of prephenic acid and its dianion were also obtained along with values of thermodynamic reaction quantities. The effects of water solvation and solvent polarization were accounted for by using a self-consistent isodensity polarized continuum model (SCI-PCM). The calculated value of $\Delta_r H^\circ_m$ for the conversion of chorismate^{2-}(aq) to prephenate^{2-}(aq) at 298.l5 K was −46.4 kJ mol^{-1}. This very good agreement between theory and experiment suggests that the energetics of this Claisen rearrangement are well understood and that the SCI-PCM method is capable of adequately describing the increased solvent−solute interaction of prephenate^{2-} relative to chorismate.$^{2-}$

1. Introduction

The conversion of chorismate to prephenate (see Figure 1), catalyzed by chorismate mutase (EC 5.4.99.5), is a rare example of a biochemical Claisen rearrangement. This reaction also occupies a central position in the shikimic acid pathway leading to the biosynthesis of aromatic amino acids.[1,2] Consequently, the kinetics and mechanism of this reaction have received much attention.[3−11] Recent determinations of the crystal structures of three entirely different unrelated chorismate mutases (two natural enzymes[12−14] and a catalytic antibody[15]) complexed with a transition state analogue have greatly contributed to our understanding of the catalytic mechanism.[16] A variety of mutagenesis experiments[10,11,17−19] indicate the importance of electrostatic and hydrogen-bonding interactions in this reaction. The results of these studies are also supported by a number of quantum mechanical calculations on the reaction at the active site of the enzyme.[20−26]

However, thermodynamic information is also needed to complete our understanding of the energetics of this reaction. Since the only information available on the thermodynamics of the conversion of chorismate to prephenate is a report[3] that the apparent equilibrium constant (temperature $T = 298.15$ K, pH = 7.5) for the reaction is >600, it was decided to perform a thermodynamic investigation aimed primarily at the determination of the enthalpy change and the equilibrium constant for this reaction. This thermodynamic information provides the essential data needed for the calculation of the extent of reaction and how it can be affected by changes in the conditions of reaction. This study also provided an excellent opportunity to perform a comparison of the results of a quantum mechanical calculation with the experimental results on the thermodynamics of an enzyme-catalyzed reaction. This theoretical calculation provides molecular insight into the structure and energetics of the reaction that is not available from classical thermodynamics. It will be seen that the results obtained from the quantum mechanical calculations are validated by comparisons with the definitive experimental data. Thus, the thermodynamic and quantum mechanical studies complement each other so as to

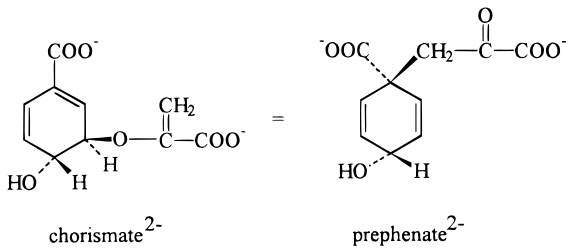

Figure 1. Claisen rearrangement of chorismate^{2-} to prephenate.$^{2-}$

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give a very detailed understanding of this biochemical Claisen rearrangement.

In this study we have used a monofunctional *Bacillus subtilis* chorismate mutase (a homotrimer of subunit molecular weight $M_r = 14\,500$) overproduced in an engineered *Escherichia coli* host strain that is devoid of prephenate dehydrogenase and prephenate dehydratase.[9,10] This eliminates the possibility of having any contamination of prephenate-degrading enzyme activities in the chorismate mutase preparation and thus is advantageous for the calorimetric measurements. In addition to its intrinsic chemical and biochemical importance, the conversion of chorismate to prephenate is also of relevance to biotechnology where there is significant interest in the use of the shikimic acid pathways for the manufacture of aromatic amino acids.[27,28]

2. Methods

2.1. Materials. Chorismic acid ($C_{10}H_{10}O_6$, $M_r = 226.19$, Chemical Abstracts Services (CAS) registry number 617-12-9) was obtained from Sigma.[29] Karl Fischer analysis {Brinkmann model 633 Karl Fischer Automat and model 665 Dosimat; Hydranal composite 2; methanol solvent; calibrated with (octanol + water)} of the chorismic acid showed that its mass fraction moisture content was (0.102 ± 0.006). All uncertainties in this paper, unless stated otherwise, are equal to two estimated standard deviations of the mean. The optical rotation α of the chorismate was stated by the vendor to be α(wavelength $\lambda =$ 589.3 nm, 20 °C, 0.2 g dm^{-3} in H_2O, path length = 10 cm) = −277°. The vendor also stated that the following impurities were present in the sample: diethyl ether (mass fraction $w =$ 0.0225 by GC), 4-hydroxybenzoate (mole fraction $x = 0.007$), prephenate ($x = 0.002$), and β-phenylpyruvate ($x = 0.006$). The amounts of these latter three impurities were determined by the vendor using HPLC with a Sperasorb C-18 column (25 cm × 4.6 mm) and a UV detector set at $\lambda = 215$ nm. The mobile phase was a gradient formed from {solution I (10 mM tributylammonium phosphate + 25 mM ammonium phosphate) at pH = 5.5 and solution II (acetonitrile)}: volume fraction ϕ(I) = 0.85 at time $t = 0$, ϕ(I) = 0.80 at $t = 10$ min, ϕ(I) = 0.70 at $t = 20$ min, and ϕ(I) = 0.50 at $t = 30$ min. The flow rate was 1.5 cm^3 min^{-1}. Several smaller unidentified peaks were also observed in the chromatogram that corresponded to a combined mole fraction impurity of ≈0.015. The mass fractions of the identified impurities are: 4-hydroxybenzoate, 0.0043; prephenate, 0.0024; β-phenylpyruvate, 0.0044. The unidentified impurities, besides having unknown molecular weights, also have unknown molar absorption coefficients, which makes their quantification uncertain. In any case, we assume that the molecular weights and molar absorption coefficients of the unidentified impurities are approximately equal to that of chorismate; on this basis, the mass fraction of these impurities in the chorismate sample is ≈0.015. The combined mass fraction of all impurities (including water) in the sample is therefore 0.151. Appropriate corrections for the presence of these impurities were applied in the calculation of the final calorimetric results. Upon receipt from the vendor, the chorismate was kept at −80 °C until ready for use; at which time it was transferred to another freezer at −20 °C. The chorismate was removed from the latter freezer for brief periods (1−2 min) to accomplish the necessary transfers of the sample during the course of the experiments.

The stability of chorismate in solution was examined using the chromatographic procedure described below. Accordingly, a sample of chorismate was dissolved in the two buffers (phosphate and Tris) used in this study, and the area of the chorismate chromatographic peak was measured as a function of time. Thus, it was found that 2.4% of the chorismate had spontaneously converted to a mixture of prephenate, β-phenylpyruvate, and 4-hydroxybenzoate after 65 min in both buffers at 25 °C. This is in good agreement with literature values on the decomposition of chorismate determined under similar conditions and extrapolated to 25 °C.[30] Since the calorimetric experiments required 15 min for loading of solutions and 50 min for equilibration of the vessels in the microcalorimeters, it was necessary to apply this 2.4% correction to the calorimetric results to account for this spontaneous conversion of chorismate to inert reactants (see below).

The sample of prephenate used in the equilibrium measurements was the barium salt ($C_{10}H_8O_6Ba$, $M_r = 361.50$, CAS no. 2931-08-0), which was obtained from Sigma. It had an estimated mole fraction purity of 0.78 based upon UV spectral analysis. For the potentiometric titrations (see below), a second prephenate sample with an estimated mole fraction purity of 0.48 based upon ^{1}H NMR analysis was used. This sample was the disodium salt of prephenic acid ($C_{10}H_8O_6Na_2$, $M_r = 270.15$, CAS no. 60311-19-5) which was directly isolated from an engineered *E. coli* strain deficient in prephenate dehydrogenase and prephenate dehydratase but proficient for chorismate mutase.[31] While the purity of both prephenate samples was rather low, they were satisfactory for the purposes of the specific experiments.

The sample of *B. subtilis* chorismate mutase used in this study was purified and assayed as previously described.[9] Based on scanning of Coomassie Blue-stained protein bands that were electrophoretically separated on a sodium dodecyl sulfate-containing polyacrylamide gel, the sample of chorismate mutase is estimated to have a mass fraction purity of ≈0.95. The value of the catalytic constant k_{cat} was ≈ 50 s^{-1} at 30 °C. The chorismate mutase was present at a mass fraction of 0.0014 in a solution containing {(50 mM glycine + NaOH, pH = 8.9), 1 mM DL-dithiothreitol, 2-propanol (volume fraction $\phi = 0.05$), glycerol ($\phi = 0.10$), 100 mM NaCl, and NaN_3 (mass fraction $w = 0.0002$)}. This solution was stored at ≈3 °C until just prior to use in the calorimetric experiments. At that time it was diluted (volumetric ratio of buffer to enzyme solution was ≈18:1) with either phosphate or Tris buffers to prepare the enzyme solution used in those experiments. The K_2HPO_4, H_3PO_4, Tris, and HCl used in the preparation of the buffers were reagent grade chemicals having mole fraction purities >0.995.

2.2. Chromatography. Analyses for amounts of chorismate and prephenate in solution were done with a Hewlett-Packard 1100 HPLC, a Bio-Rad Aminex Fermentation Monitoring column (150 mm × 7.8 mm) thermostated at 40 °C and a UV−visible detector set at $\lambda = 225$ nm. Solutions I (5 mM H_2SO_4) and II (methanol) were used for the mobile phase. The following linear gradient was used: solution I (volume fraction $\phi = 1.00$) at $t = 0$; at $t = 20$ min the mobile phase consisted of solution I ($\phi = 0.85$) and solution II ($\phi = 0.15$). The flow rate was 0.8 cm^3 min^{-1}. Under acidic conditions, prephenate is transformed rapidly (half-life $t_{1/2} = 1.0$ min in 1 mol dm^{-3} HCl at 25 °C)[32,33] to (β-phenylpyruvate + carbon dioxide + H_2O). Thus, the prephenate is quantitatively converted to β-phenylpyruvate, which has a retention time of 7.5 min. With results from Gibson[34] and from Andrews et al.,[30] we estimate that chorismate has a $t_{1/2}$ of ≈ 4 h under the conditions used for the HPLC analysis. Since the retention time of the chorismate is 11.2 min, its instability is insignificant. Most importantly, the essential feature of the chromatographic analyses performed for the equilibrium and calorimetric measurements was the detection of chorismate. Therefore, the

results are not affected by the aforementioned chemical transformations. Using chorismate solutions having known concentrations, we found that the lower limit of detectability with this chromatographic procedure was $\approx 3 \times 10^{-7}$ mol dm^{-3}.

2.3. Equilibrium Measurements. The aim in the equilibrium measurements was to measure the extent of the reaction with equilibrium being approached from both directions of reaction. The solution used for the forward direction of reaction contained chorismate at a molality $m = 0.0029$ mol kg^{-1} in phosphate buffer {K_2HPO_4 ($m = 0.050$ mol kg^{-1}) adjusted to pH = 7.2 with H_3PO_4}. The solution used for the reverse direction of reaction contained prephenate ($m = 0.0018$ mol kg^{-1}) in the same phosphate buffer. Chorismate mutase solution (see above) was added to these two solutions so that the mass fraction of the enzyme in these solutions was 1.7×10^{-5}. These solutions were placed in 20 cm^3 Teflon capped glass bottles and gently shaken at ≈50 rpm in a water bath thermostated at 25 °C. Following equilibration for 7 h, the concentrations of chorismate and prephenate in these solutions were measured using the chromatographic procedure described above.

2.4. Calorimetry. Three heat-conduction microcalorimeters were used for the enthalpy of reaction measurements. They were calibrated electrically with a high stability dc power supply, calibrated digital voltmeter, standard resistor, and time-interval counter. Descriptions of the microcalorimeters and their performance characteristics, the data-acquisition system, and the computer programs used to treat the results are given in refs 35 and 36. The sample vessels, fabricated from high-density polyethylene, contained two compartments that held ≈0.55 and ≈0.40 cm^3 of solution. Two different buffers were used in these measurements: {Tris ($m = 0.0991$ mol kg^{-1}) adjusted to pH = 8.1 with HCl} and {K_2HPO_4 ($m = 0.0996$ mol kg^{-1}) adjusted to pH = 7.5 with H_3PO_4}. The substrate solution (placed in the 0.55 cm^3 compartment) contained chorismate dissolved in one of these buffers. The enzyme solution, which consisted of the chorismate mutase solution (see above) dissolved in the same buffer used for the substrate solution was placed in the 0.40 cm^3 compartment.

The vessels and their contents were allowed to equilibrate in the microcalorimeters for ≈50 min before the enzyme and substrate solutions were mixed. Following reaction in the microcalorimeter for ≈60 min, the reaction vessels were removed from the microcalorimeters and their contents were promptly analyzed with the HPLC. Thus, it was found that the mole fraction of unreacted chorismate was <0.0008 for the reactions carried out in phosphate buffer and ≈0.0015 for the reactions carried out in Tris buffer. Small corrections were applied for incomplete reaction. The "blank" enthalpy changes for the mixing of the substrate solutions with the buffer ranged from 0.11 to 1.31 mJ; for the mixing of the enzyme solutions with the buffer these enthalpies ranged from −0.65 to −1.13 mJ. These "blank" enthalpies of mixing were also applied as corrections to the measured calorimetric enthalpies ΔH(cal), which were in the range −596 to −683 mJ for the conversion of chorismate to prephenate. Corrections were also applied for the impurities (moisture and other chemicals) in the chorismate and for the aforementioned spontaneous conversion of chorismate to other substances in solution prior to the actual calorimetric measurements. In applying these latter corrections, it was assumed that these impurities were "inert" reactants and that they made no contribution to the measured enthalpy changes (see below).

2.5. Measurement of pH and Potentiometric Titrations. Measurement of pH was done with an Orion Model 811 pH meter and a Radiometer combination glass microelectrode. The pH meter was calibrated with Radiometer standard buffers and with a standard phosphate buffer (pH = 7.42 at 25 °C). This latter buffer was prepared from KH_2PO_4 and Na_2HPO_4, standard reference materials 186-Id and 186-IId, respectively, from the National Institute of Standards and Technology. Potentiometric titrations of chorismic and prephenic acids were performed with 5.71 mM HCl from pH ≈ 11.3 to pH = 2.8. The chorismic acid was also titrated with 5.58 mM NaOH from pH = 2.2 to pH = 10.8. Although the resultant titration curves were not precise enough for the determination of accurate pK's, the derivative plots had a broad minimum at pH ≈ 3.0 indicative of pK's at this approximate value.

2.6. Quantum Mechanical Calculations. All calculations were performed with the Gaussian 94 programs[37] running on IBM SP1/SP2 parallel computers at the Office of Information Technologies at the University of Notre Dame. All structures were fully optimized using the B3LYP functional[38,39] and a 6-31G* basis set. This hybrid density functional has been shown to yield excellent results for a number of pericyclic reactions, including the Claisen rearrangement.[40,41] The structures were confirmed as minima on the energy hypersurface by harmonic frequency analysis. The force constants thus obtained were used to calculate the zero-point energies and the vibrational frequencies. The entropies, enthalpies, and the Gibbs free energies of the various chorismic acid and prephenic acid species were then calculated using standard methods of statistical mechanics.

Solvation effects were calculated using single-point calculations on the gas-phase optimized geometries. For the accurate calculation of the solvation energies of charged species such as chorismate^{2-} and prephenate,$^{2-}$ it is important to perform a self-consistent determination of the solvent polarization as well as the size and shape of the solute cavity.[42] We therefore used the self-consistent isodensity polarized continuum model (SCI-PCM)[43] and a continuum with a relative permittivity $\epsilon_r = 81.0$ and an isodensity surface of 0.0004 electrons Å^{-3}. Although this approach does not consider specific solute−solvent interactions, it has been shown[44] that this type of cavity method describes the electrostatic effects well and gives reasonable results for the solvation effects in the Claisen rearrangement. The solvation energies obtained with the SCI-PCM calculations include a correction for the zero-point energies and the thermal and entropic contributions. These calculations provide an estimate of the thermodynamic properties in solution.

3. Results and Discussion

3.1. Thermodynamic Formalism. In the discussion of the thermodynamics of biochemical reactions, it is important to distinguish between the overall biochemical reaction, which pertains to sums of pseudoisomer species,[45,46] and a chemical reference reaction, which pertains to specific species. The chemical reference reaction balances both the atoms and charges in the reaction while the overall biochemical reaction does not balance charge or, when pX (e.g. pH or pMg) is specified, the element X. Thus, for the conversion of chorismate to prephenate the overall biochemical reaction is

$$\text{chorismate(aq)} = \text{prephenate(aq)} \qquad (1)$$

The apparent equilibrium constant K' for this reaction is

$$K' = m(\text{prephenate})/m(\text{chorismate}) \qquad (2)$$

where the m's are the respective total molalities of all chorismate

TABLE 1: Results of Calorimetric Measurements at $T = 298.15$ K

Phosphate Buffer

expt	$m(K_2HPO_4)$[a]/(mol kg^{-1})	$m(H_3PO_4)$/(mol kg^{-1})	m(chorismate)/(mol kg^{-1})	pH	I[b]/(mol kg^{-1})	Δ_rH_m(cal)[c]/(kJ mol^{-1})
1	0.0553	0.005 71	0.0114	6.93	0.16	−56.1
2	0.0631	0.006 51	0.0132	6.93	0.19	−55.0
3	0.0612	0.006 32	0.0127	6.93	0.18	−55.7
4	0.0608	0.006 28	0.0126	6.93	0.18	−55.5
5	0.0641	0.006 61	0.0133	6.93	0.19	−55.1
					$\langle\Delta_rH_m(\text{cal})\rangle = -(55.5 \pm 0.4)$[d]	

Tris Buffer

expt	m(Tris)/(mol kg^{-1})	m(HCl)/(mol kg^{-1})	m(chorismate)/(mol kg^{-1})	pH	I/(mol kg^{-1})	Δ_rH_m(cal)/(kJ mol^{-1})
1	0.0623	0.0285	0.0137	7.70	0.071	−55.0
2	0.0624	0.0286	0.0137	7.70	0.071	−55.0
3	0.0649	0.0297	0.0144	7.70	0.074	−54.5
4	0.0622	0.0285	0.0129	7.70	0.069	−56.5
5	0.0631	0.0289	0.0131	7.70	0.070	−55.5
6	0.0615	0.0282	0.0127	7.70	0.068	−55.9
					$\langle\Delta_rH_m(\text{cal})\rangle = -(55.4 \pm 0.6)$	

[a] The molalities m are those following mixing of the enzyme and substrate solutions and prior to any reaction. The following substances (originally in the chorismate mutase solution) were also present in the reaction mixtures at the following approximate molalities: glycerol, 0.051 mol kg^{-1}; glycine, 0.0018 mol kg^{-1}; NaCl, 0.0037 mol kg^{-1}; 2-propanol, 0.024 mol kg^{-1}. Trace amounts of other substances are also present (see experimental section). The mass fraction of the chorismate mutase in the reaction mixtures was $\approx 5 \times 10^{-5}$. [b] The ionic strength is a calculated quantity. [c] Δ_rH_m(cal) is the calorimetrically determined molar enthalpy of reaction. Appropriate corrections have been applied for moisture and impurities in the chorismate, for "blank" enthalpies of mixing, and for the spontaneous conversion of chorismate to other substances in solution prior to the start of the actual calorimetric measurements. [d] This statistical uncertainty in the average value of Δ_rH_m(cal) is equal to two estimated standard deviations of the mean. See Results and Discussion section for final estimate of uncertainty.

and prephenate species in solution. We now choose the following chemical reference reaction

$$\text{chorismate}^{2-}(\text{aq}) = \text{prephenate}^{2-}(\text{aq}) \quad (3)$$

for which the equilibrium constant is

$$K = m(\text{prephenate}^{2-})/m(\text{chorismate}^{2-}) \quad (4)$$

Here only the molalities of the species having a charge number of −2 are considered. However, at the pHs at which experiments were performed in this study (see below), the predominant (mole fraction $x > 0.996$) species of chorismic and prephenic acids are, respectively, chorismate^{2-} and prephenate.$^{2-}$ Since these species are predominant and since no protons are produced or absorbed in reaction 3, the change in binding of H^+ is zero and there is no need to apply a buffer protonation or any ionization corrections to the calorimetrically determined molar enthalpy of reaction Δ_rH_m(cal) in the calculation of the standard molar enthalpy $\Delta_rH^\circ_m$ for the chemical reference reaction 3. Since reaction 3 is charge symmetric, $\Delta_rH^\circ_m$ should be essentially constant at low ionic strengths ($I < 0.25$ mol kg^{-1}).

The basis for the assignment of the chorismate and prephenate species is now discussed. In the absence of values of the pK's of chorismic and prephenic acids, we use the pK's of substances that have the essential structural features of these two acids to make an approximate estimate of their pK's. Specifically, we have the following pK's ($T = 298.15$ K and $I = 0$):[47] propanoic acid, pK = 4.87; cyclohexane carboxylic acid, pK = 4.90; propenoic acid, pK = 4.26; 2-oxopropanoic acid, pK = 2.49; 2-oxopentanedioic acid, pK_1 = 1.9 and pK_2 = 4.44. Thus, it is seen that introduction of a double bond, a carbonyl, or a carboxyl group into an aliphatic acid correlates with a lower value of the pK. On this basis, we estimate the pK's of chorismic and prephenic acids to be <4.5. This estimate is consistent with the approximate results obtained from potentiometric titrations of these substances (see experimental section). Since the calorimetric experiments were done at pH = 6.93 and pH = 7.70, the difference |pH − pK| > 2.4 and the predominant species (mole fraction $x > 0.996$) in the calorimetric experiments are chorismate^{2-} and prephenate^{2-}.

3.2. Results of Calorimetric Experiments. The results of the calorimetric measurements are given in Table 1. It is seen that the values of the calorimetrically determined molar enthalpy of reaction Δ_rH_m(cal) are essentially the same in both the phosphate and Tris buffers. These two buffers have significantly different standard molar enthalpies of ionization: $\Delta_{ion}H^\circ_m = 47.48$ kJ mol^{-1} for Tris·H$^+$ [48] and $\Delta_{ion}H^\circ_m = 4.2$ kJ mol^{-1} for $H_2PO_4^-$.[49] Thus, the calorimetric results are consistent with no protons being produced or absorbed in the overall biochemical reaction. On this basis we combine the two sets of results and calculate the average $\langle\Delta_rH_m(\text{cal})\rangle = -55.4$ kJ mol^{-1} (estimated standard deviation of the mean $s = 0.18$ kJ mol^{-1}).

A 2.4% correction was applied to the calorimetric results to correct for the spontaneous conversion of chorismate to inert reactants. We now discuss why we can consider the products as being inert. First, since the enzyme was purified from an *E. coli* strain that was devoid of prephenate dehydratase and prephenate dehydrogenase, we can rule out the possibility that our chorismate mutase preparation was contaminated with any product-degrading activities. Second, we assessed the extent to which the following spontaneous decomposition of the product prephenate can occur

$$\text{prephenate(aq)} = \beta\text{-phenylpyruvate(aq)} + \text{carbon dioxide(aq)} + H_2O(l) \quad (5)$$

Previous kinetic results[32,33] show that $t_{1/2}$ for the decomposition of prephenate via this reaction is 130 h at $T = 298.15$ K and pH = 7.0. Thus, for the calorimetric experiments which lasted 1 h, the mole fraction of prephenate converted to (β-phenylpyruvate + carbon dioxide + H_2O) will be <0.0053. However, since the standard transformed molar enthalpy change $\Delta_rH'^\circ_m$ [45,46] for this biochemical reaction is not known, the proper correction to be applied is uncertain and none has been applied. We estimate that the systematic error in Δ_rH_m(cal) due to neglect of this correction will be no larger than $0.005\Delta_rH_m$(cal).

Possible sources of systematic errors in the measurements and estimates of their effects on Δ_rH_m(cal) are heat measurements, $< 0.003\Delta_rH_m$(cal); incomplete reaction and possible side reactions, $<0.010\Delta_rH_m$(cal); moisture content in the chorismate,

TABLE 2: Absolute Energies (hartrees molecule^{-1}) and Entropies (J K^{-1} mol^{-1}) for the Chorismic Acid and Prephenic Acid Species Calculated at the B3LYP/6-31G* Level[a]

species	E(SCF)	E(solv)	E(ZP)	E(SCF) + E(ZP)	H	G	S
chorismic acid, pseudo diequatorial (g)	−838.370 52	−838.388 00	0.194 45	−838.176 07	−838.160 22	−838.219 64	523.1
chorismic acid, pseudo diaxial (g)	−838.366 66	−838.385 44	0.194 63	−838.172 03	−838.155 93	−838.217 33	540.6
prephenic acid (g)	−838.374 67	−838.397 09	0.194 32	−838.180 35	−838.164 0	−838.225 66	542.7
chorismate,$^{2-}$ pseudo diequatorial (g)	−837.185 76	−837.422 00	0.168 05	−837.017 71	−837.002 64	−837.060 41	508.8
chorismate,$^{2-}$ pseudo diaxial (g)	−837.166 13	−837.407 59	0.168 00	−836.998 13	−836.982 56	−837.041 66	520.5
prephenate^{2-} (g)	−837.194 98	−837.440 29	0.168 45	−837.026 53	−837.011 26	−837.0693 9	512.1

[a] E(SCF) is the self-consistent field energy; E(solv) is equal to E(SCF) plus the cavity contribution; E(ZP) is the zero-point energy; H, G, and S are, respectively, the enthalpy, Gibbs free energy, and entropy; 1 hartree = $4.359\,748\,2 \times 10^{-18}$ J. [b] The quantities E(SCF) and E(solv) refer to $T = 0$; E(ZP) and {E(SCF) + E(ZP)} refer to $T = 0$ and $p = 101.325$ kPa; H, G, and S refer to $T = 298.15$ K and $p = 101.325$ kPa.

$<0.005\Delta_r H_m$(cal); and impurities in the chorismate, $<0.017\Delta_r H_m$(cal). These estimates of possible systematic error are combined in quadrature together with the statistical uncertainties in the measured values of $\Delta_r H_m$(cal), expressed as one estimated standard deviation of the mean, to obtain a combined standard uncertainty of 1.1_4 kJ mol^{-1}. This combined standard uncertainty is then multiplied by 2 to arrive at a final estimate of uncertainty of ± 2.3 kJ mol^{-1}. Clearly, the largest possible source of systematic errors in these measurements can be attributed to possible impurities in the chorismate, particularly those that have not been identified. In summary, $\Delta_r H_m$(cal) = −(55.4 ± 2.3) kJ mol^{-1}. On the basis of the points made above, $\Delta_r H^\circ_m$ for the chemical reference reaction 3 also has this same value.

3.3. Results of Equilibrium Experiments. There was no measurable amount of chorismate in either the forward or reverse reaction mixtures. Thus, it was not possible to measure an equilibrium constant. Based upon the lower limit of the amount of chorismate detectable with our chromatographic procedure, we can state that $K'(T = 298.15$ K, pH = 7.2) > 9700 for the biochemical reaction 1. The decomposition of prephenate to (β-phenylpyruvate + carbon dioxide + H_2O) at pH = 7.2 and $T = 298.15$ K is too slow (see above) to invalidate this result. The aforementioned inequality also holds for the equilibrium constant for the chemical reference reaction 3 and is consistent with the earlier report by Addadi et al.[3] that the apparent equilibrium constant ($T = 303.15$ K, pH = 7.5) for this reaction is >600.

3.4. Results of Quantum Mechanical Calculations. The structures of prephenic acid and its dianion obtained from the quantum mechanical calculations are shown in Figure 2. Prephenate^{2-} in the gas phase is, like the diequatorial conformation of chorismate,$^{2-}$ stabilized by a strong intramolecular hydrogen bond.[25] This hydrogen bond is strong enough to distort the 1,4-cyclohexadienyl moiety into a boatlike conformation. It can also be expected that, in polar solvents, the hydrogen bond will be bridged by solvent molecules. The BLYP and Hartree−Fock structures of the pseudo diequatorial and the pseudo diaxial conformations of chorismic acid and chorismate have been described previously[22] and are not significantly different from the structures obtained with the B3LYP/6-31G* level that was used in this study.

Tables 2 and 3 contain, respectively, calculated values of absolute energies and relative energies for the chorismic acid and prephenic acid species. The calculated thermodynamic reaction quantities are given in Table 4. The difference in the values of {E(SCF) + E(ZP)} between the chorismate^{2-} pseudo diequatorial and pseudo diaxial species in the gas phase is 51.4 kJ mol^{-1}; it is reduced to 35.5 kJ mol^{-1} by solvation. Although this result is in qualitative agreement with experiment,[5] a more accurate result would require either the reoptimization of the structure in the solvent cavity or free energy perturbation calculations.[26] Since the number of intramolecular hydrogen bonds before and after the Claisen rearrangement is unchanged, the aforementioned procedures are not needed. Thus, it can be expected that the simplified calculations used herein should give a reasonable estimate of the reaction energetics.

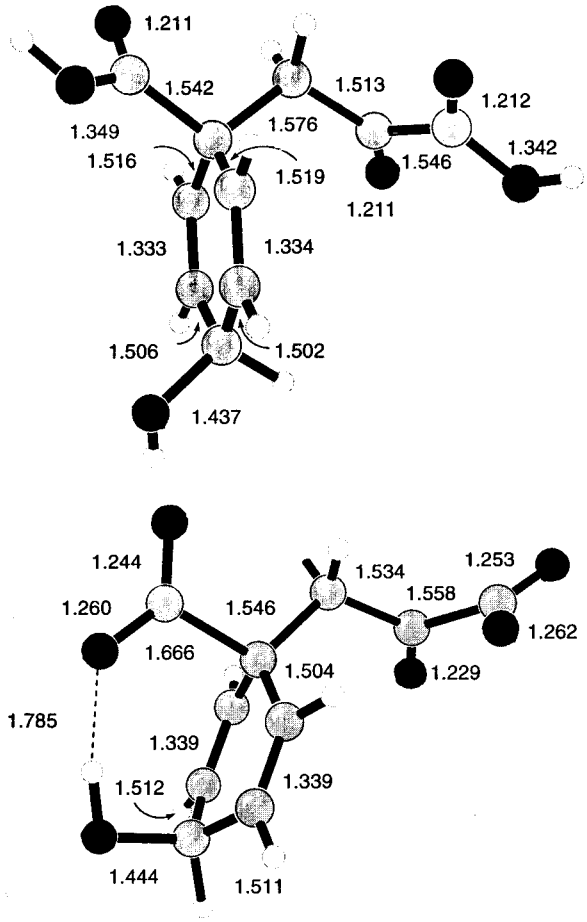

Figure 2. Structures of prephenic acid and prephenate^{2-} as obtained from quantum mechanical calculations. The distances are in angstroms. The Cartesian coordinates are given in Table S1 contained in the Supporting Information for this paper.

In the gas phase at $T = 0$ and $p = 101.325$ kPa, the conversion of chorismic acid to prephenic acid is exothermic by −11.3 kJ mol^{-1} and the corresponding reaction of the dianion is exothermic by −23.2 kJ mol^{-1}. This can be compared to the exothermicity of the parent Claisen rearrangement of allyl vinyl ether, which has been calculated[40] to be −72.4 kJ mol^{-1} at the BLYP/6-31G* level. The reduced exothermicity of 49.2 kJ mol^{-1} of the chorismate^{2-} reaction relative to the rearrangement of allyl vinyl ether can be attributed to reactant stabilization by the conjugated diene and by the two α,β-unsaturated carboxylate groups. In the product prephenate^{2-} all three of these interactions are eliminated.

Inclusion of solvation effects increases the calculated exothermicity of the reaction by 12.9 kJ mol^{-1} for chorismic acid

TABLE 3: Calculated Relative Energies (kJ mol^{-1}) for the Chorismic Acid and Prephenic Acid Species at $T = 298.15$ K and $p = 101.325$ kPa[a]

species	E(SCF) + E(ZP)	H	G	E(solv) + E(ZP)	E(solv) + E(corr)
chorismic acid, pseudo diequatorial (g)	0	0	0	0	0
chorismic acid, pseudo diaxial (g)	10.6	11.3	6.1	7.3	2.7
prephenic acid (g)	−11.3	−9.9	−15.8	−24.2	−28.7
chorismate,$^{2-}$ pseudo diequatorial (g)	0	0	0	0	0
chorismate,$^{2-}$ pseudo diaxial (g)	51.4	52.7	49.2	37.7	35.5
prephenate^{2-} (g)	−23.2	−22.6	−23.6	−47.0	−47.4

[a] E(SCF) is the self-consistent field energy; E(ZP) is the zero-point energy; H and G are, respectively, the enthalpy and Gibbs free energy; E(solv) is equal to E(SCF) plus a cavity contribution; E(corr) is the sum of E(ZP), thermal, and entropic contributions.

TABLE 4: Thermodynamic Reaction Quantities Calculated Using Quantum Mechanics[a]

reaction	$\Delta_r H^\circ_m$/ (kJ mol^{-1})	$\Delta_r S^\circ_m$ (J K^{-1} mol^{-1})
chorismic acid (g) = prephenic acid (g)	−10.1	19.7
chorismic acid (aq) = prephenic acid (aq)	−23.0	
chorismate^{2-} (g) = prephenate^{2-} (g)	−22.6	3.3
chorismate^{2-} (aq) = prephenate^{2-} (aq)	−46.4	

[a] $\Delta_r H^\circ_m$ and $\Delta_r S^\circ_m$ are, respectively, the standard molar enthalpy and entropy of reaction at $T = 298.15$ K and $p = 101.325$ kPa.

and by 23.8 kJ mol^{-1} for chorismate^{2-} (see Table 4). The calculated value of $\Delta_r H^\circ_m = -46.4$ kJ mol^{-1} for reaction 3 differs by only 9 kJ mol^{-1} from the measured value of −(55.4 ± 2.3) kJ mol^{-1}. We consider this to be a remarkably good agreement which, however, may be due to a fortuitous cancellation of the solvation effects in the calculations. In any case, the important result is that the SCI−PCM method has now been demonstrated as being capable of adequately describing the increased solvent−solute interaction of prephenate^{2-} relative to chorismate^{2-}. It also helps to establish a basis for the possible success of quantum mechanical calculations on other biochemical reactions.

3.5. Estimated Thermodynamic Quantities. The Benson group additivity approach[50,51] can be used to estimate values of the standard molar enthalpies of formation $\Delta_f H^\circ_m$ and standard molar entropies S°_m of chorismic and prephenic acids in the gas and liquid phases. These enthalpies of formation and entropies can then be combined to obtain estimates of $\Delta_r H^\circ_m$ and $\Delta_r S^\circ_m$ for the reactions

$$\text{chorismic acid(g)} = \text{prephenic acid(g)} \qquad (6)$$

$$\text{chorismic acid(l)} = \text{prephenic acid(l)} \qquad (7)$$

Thus, using the Benson group values tabulated by Domalski and Hearing,[52] we obtain $\Delta_r H^\circ_m = -48$ kJ mol^{-1} and $\Delta_r S^\circ_m = 9$ J mol^{-1} K^{-1} for reaction 6 and $\Delta_r H^\circ_m = -26$ kJ mol^{-1} and $\Delta_r S^\circ_m = -9$ J mol^{-1} K^{-1} for reaction 7 at $T = 298.15$ K. In the Benson calculation, the major contribution to $\Delta_r H^\circ_m$ for reactions 6 and 7 comes from the rearrangement of the 2-alkylpropenoic acid in chorismic acid to the 2-oxopropanoic acid in prephenic acid. The difference in the enthalpies of formation of the cyclohexadiene rings is only one-third of the value of the contribution to $\Delta_r H^\circ_m$ attributable to the aforementioned rearrangement.

The quantum mechanical calculation gave $\Delta_r H^\circ_m = -10.1$ kJ mol^{-1} for the gas-phase reaction 6 and $\Delta_r H^\circ_m = -46.4$ kJ mol^{-1} for the reaction 3 involving the aqueous dianions. Thus, it is seen that, in this case, the result obtained from the quantum mechanical calculation is superior to the Benson estimate, which does not include any contributions from hydration or ionization. The Benson method gave an estimated $\Delta_r S^\circ_m = 9$ J K^{-1} mol^{-1} for reaction 6 while from the quantum mechanical calculations, a value of 19.6 J K^{-1} mol^{-1} was obtained. However, the quantum mechanical calculation also yielded a value of $\Delta_r S^\circ_m = 3.3$ J K^{-1} mol^{-1} for the reaction

$$\text{chorismate}^{2-}\text{(g)} = \text{prephenate}^{2-}\text{(g)} \qquad (8)$$

On the *assumption* that hydration effects cancel, we obtain an estimated value of $\Delta_r S^\circ_m = 3$ J K^{-1} mol^{-1} for reaction 3. This value is used together with the measured value of $\Delta_r H^\circ_m = -55.4$ kJ mol^{-1} to obtain an estimate of the standard molar Gibbs free energy change $\Delta_r G^\circ_m \approx -56$ kJ mol^{-1} and from which we obtain $K \approx 7 \times 10^9$ for reaction 3. If the estimated value of $\Delta_r S^\circ_m$ were in error by 30 J K^{-1} mol^{-1}, the value of K could be as large as 3×10^{11} and as small as 2×10^8. In any case, these estimates are consistent with our experimental result $K > 9700$. Thus, for all practical purposes, reaction 3 can be considered to be "irreversible". It would be useful to have both equilibrium and kinetic data for metabolically related reactions such as the conversion of chorismate to anthranilate. This information could provide a detailed explanation as to why one branch in the pathway leading to the synthesis of aromatic amino acids is favored over an alternative one and to what extent rigorous kinetic control at the chorismate branch point is required to direct metabolic flow.

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Supporting Information Available: One table containing the Cartesian coordinates of chorismic and prephenic acids, chorismate^{2-} and prephenate^{2-} as obtained from the quantum mechanical calculations (4 pages). Ordering information is given on any current masthead page.

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