J. Chem. Thermodynamics **2000**, 32, 1057–1070
doi:10.1006/jcht.2000.0677
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A thermodynamic study of the conversion of chorismate to isochorismate

Yadu B. Tewari, Andrew M. Davis, Prasad Reddy, and Robert N. Goldberg[a]

Biotechnology Division, National Institute of Standards and Technology, Gaithersburg, MD 20899, U.S.A.

Microcalorimetry and high performance liquid chromatography were used to conduct a thermodynamic investigation of the biochemical reaction chorismate(aq) = isochorismate(aq). This reaction occurs at the branch point of the chorismate metabolic pathway that leads to the synthesis of enterobactin. Isochorismate synthase, the enzyme that catalyzes this reaction, was prepared for this study by using molecular biology techniques. The equilibrium and calorimetric measurements were performed at $T = 298.15$ K and at the respective pHs 7.34 and 6.93. For the chemical reference reaction chorismate^{2-}(aq) = isochorismate^{2-}(aq), the equilibrium constant $K = (0.84 \pm 0.04)$ and the standard molar enthalpy of reaction $\Delta_r H^o_m = -(0.81 \pm 0.27)$ kJ · mol^{-1} at $T = 298.15$ K and ionic strength $I_m = 0$. Under approximately physiological conditions, the apparent equilibrium constant $K' = 0.83$ and the standard transformed Gibbs energy change $\Delta_r G'^o_m = 0.48$ kJ · mol^{-1} for the overall biochemical reaction.

KEYWORDS: apparent equilibrium constant; chorismate; enthalpy; entropy; Gibbs free energy; isochorismate synthase; transformed thermodynamic quantities

1. Introduction

The first step in the branch of the chorismate metabolic pathway[1] that leads to the synthesis of enterobactin is

$$\text{chorismate(aq)} = \text{isochorismate(aq)}. \quad (1)$$

The structural difference between chorismate and isochorismate involves only the location of the hydroxyl group and of the double bonds (see figure 1). Therefore, as a first approximation, one would guess the value of the equilibrium constant for this reaction to be near unity and the absolute value of the molar enthalpy change to be small.

[a]To whom correspondence should be addressed. Robert N. Goldberg, Biotechnology Division, Mail-stop 8312, National Institute of Standards and Technology, Gaithersburg, MD 20899, U.S.A. (E-mail: robert.goldberg@nist.gov).

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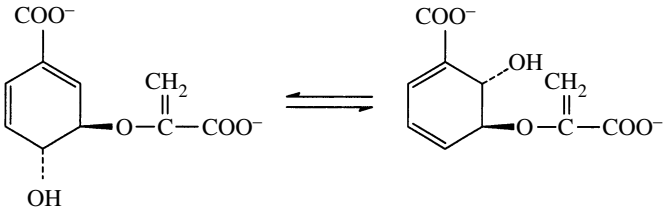

FIGURE 1. Structures of the substances in reaction (1). The predominant ionic forms at pH = 7.0 are shown.

The reversibility of this reaction, which is catalyzed by isochorismate synthase (EC 5.4.99.6), has been established and values of the apparent equilibrium constant K' as well as kinetic constants from both directions of reaction have been reported.[2, 3] However, the result of Kozlowski *et al.* for K' for reaction (1) was described by the authors[3] as being a "rough estimation". Also, there were relatively few details given by Liu *et al.*[2] in their study and no error estimate was attached to their result. The enthalpy change for reaction (1) has not been reported.

Accurate thermodynamic results are needed to establish the energetics of this important and interesting biochemical reaction which occurs at a branch point of the chorismate metabolic pathway. More specifically, metabolic control theory requires both thermodynamic and kinetic data for the reactions in a pathway as well as a knowledge of the metabolite concentrations *in vivo*.[4, 5] Also, there is significant interest in the chorismate metabolic pathway because of its potential for the manufacture of aromatic amino acids and other bulk commodity chemicals.[6, 7] Furthermore, since this pathway is unique to plants and bacteria, it is an attractive target for potential herbicides and antibiotics. Accordingly, this study was undertaken to obtain a more thorough understanding of the thermodynamics of this reaction. Earlier studies[8–13] from this laboratory have dealt with the thermodynamics of reactions in other branches of the chorismate pathway.

This study also required some care in regards to several matters pertinent to the chemistry of chorismate and isochorismate and the enzymology. First, since both isochorismate synthase and isochorismate were not available, both had to be prepared. Isochorismate synthase was prepared by using molecular biology methods; isochorismate was then prepared *in situ* by using the isochorismate synthase. Since both chorismate and isochorismate undergo spontaneous decompositions to prephenate and isoprephenate,[1] respectively, it was necessary to determine these rates of decomposition and to make appropriate corrections to the experimental results. This was particularly important in establishing the chromatographic response factor of isochorismate. Finally, the experiments used to obtain a value of the apparent equilibrium constant for reaction (1) were designed and executed to conclusively demonstrate that the reaction had in fact reached equilibrium.

2. Experimental

CHEMICALS

Relevant information on the substances used in this study is given in table 1.[b] The purity of the sample of chorismic acid was assessed as described previously.[8] The combined mass fraction w of all impurities (including water) in this sample was 0.182. Appropriate corrections for the presence of these impurities were applied in all subsequent calculations involving this sample.

PREPARATION OF ISOCHORISMATE SYNTHASE

Escherichia coli BL21 (DE3) cells harboring the plasmid pJLT5053[2] (a gift from Professor Paul A. Bartlett), which encodes isochorismate synthase, were grown in a rotary shaker bath at the temperature $T = 310$ K in 2.5 dm^3 of Luria Bertani broth containing ampicillin (mass concentration $\gamma = 0.05\ g \cdot dm^3$). The incubation was continued until the absorbance of the culture was 0.4 at the wavelength $\lambda = 650$ nm. Isopropyl β-**D**-thiogalactopyranoside (IPTG) was added to this culture to a final concentration of $1 \cdot 10^{-4}\ mol \cdot dm^{-3}$ to induce the expression of isochorismate synthase. This culture was further incubated at $T = 310$ K for 3.5 h. Cells were pelleted by centrifugation at $1.13 \cdot 10^{-4} \cdot \boldsymbol{g}_n$ ($\boldsymbol{g}_n = 9.80665\ m \cdot s^{-2}$) for ≈15 min and then resuspended in 40 cm^3 of buffer A {Tris (concentration $c = 0.05\ mol \cdot dm^{-3}$) + **DL**-dithiothreitol (DTT) ($c = 1 \cdot 10^{-3}\ mol \cdot dm^{-3}$) + ethylenediaminetetraacetic acid (EDTA) ($c = 1 \cdot 10^{-3}\ mol \cdot dm^{-3}$) + phenylmethylsulfonyl fluoride (PMSF) ($c = 1 \cdot 10^{-4}\ mol \cdot dm^{-3}$) adjusted with HCl to pH = 7.5}. The resuspended cells were passed through a French Press twice at the pressure $p = 69$ MPa and then centrifuged at $10^5 \cdot \boldsymbol{g}_n$ for 1.5 h. The supernatant was then loaded onto a diethylaminoethyl (DEAE) anion-exchange column (volume $V = 80\ cm^3$) that had been pre-equilibrated with buffer A. The column was eluted with a linear gradient of NaCl that was formed by using (I) buffer A and (II) {buffer A containing NaCl ($c = 0.5\ mol \cdot dm^{-3}$)}. The gradient was: volume fraction $\phi(\mathrm{I}) = 1.00$ and $\phi(\mathrm{II}) = 0$ at time $t = 0$; $\phi(\mathrm{I}) = 0$ and $\phi(\mathrm{II}) = 1.00$ at $t = 14$ h. The protein fractions were run on a sodium dodecylsulfate polyacrylamide gel (SDS-PAGE, mass fraction $w = 0.12$) and visualized by staining with Coomassie brilliant blue. Those protein fractions corresponding to the molar mass of isochorismate synthase were pooled and loaded onto a G75 Sephadex column ($V = 500\ cm^3$) equilibrated with buffer B {KH_2PO_4 ($c = 0.025\ mol \cdot dm^{-3}$)+ K_2HPO_4 ($c = 0.025\ mol \cdot dm^{-3}$) + DTT ($c = 1 \cdot 10^{-3}\ mol \cdot dm^{-3}$) + EDTA ($c = 1 \cdot 10^{-3}\ mol \cdot dm^{-3}$) + PMSF ($c = 1 \cdot 10^{-4}\ mol \cdot dm^{-3}$) + NaCl ($c = 0.1\ mol \cdot dm^{-3}$), pH = 7.5}. The column was run at a flow rate of $8.3 \cdot 10^{-9}\ m^3 \cdot s^{-1}$. Fractions containing isochorismate synthase were again pooled and assayed for activity according to previously published protocols.[14] The activity (rate of conversion/mass of enzyme) was found to be $1.3 \cdot 10^{-4}\ mol \cdot s^{-1} \cdot g^{-1}$ by carrying out the assay[14] with $2 \cdot 10^{-7}$ mol of chorismate in the buffer {Tris ($c = 0.050\ mol \cdot dm^{-3}$) + $MgCl_2$ ($c = 2.5 \cdot 10^{-6}\ mol\ dm^{-3}$), pH = 8.0}

[b]Certain commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedures adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for the purpose.

TABLE 1. Principal substances used in this study with their Chemical Abstracts Service (CAS) registry numbers, empirical formulae, molar masses M, mass fraction moisture contents w determined by Karl–Fischer analysis, mole fraction purity x as stated by supplier (M = Mallinckrodt, S = Sigma), and method used to determine x

Substance	CAS registry number	Formula	M / g · mol^{-1}	w	Supplier	x	Method
Chorismic acid	617-12-9	$C_{10}H_{10}O_6$	226.18	0.116	S	0.975[a]	h.p.l.c.
Chorismate lyase			$18.8 \cdot 10^3$				
Isochorismic acid	22642-82-6	$C_{10}H_{10}O_6$	226.18		[b]		
Isochorismate synthase			$42.5 \cdot 10^3$		[c]		
Magnesium chloride	7786-30-3	$MgCl_2$	95.21		S	>0.98	EDTA and $AgNO_3$ titration
Phosphoric acid	7664-38-2	H_3PO_4	98.00		M	>0.995	(acid + base) titration
Potassium phosphate, dibasic	7758-11-4	K_2HPO_4	174.18		S	>0.995	(acid + base) titration

[a] Chorismate also contained diethyl ether ($w = 0.041$) and other unidentified impurities ($x \approx 0.025$). The mole fraction purity of the chorismate given above is exclusive of the amount of water and diethyl ether in the sample. [b] Prepared *in situ*. See Experimental. [c] Prepared for this study. See Experimental.

at $T = 310$ K. The mole fraction purity of the protein was found to be >0.95 by using SDS-PAGE. The mass fraction of the protein in buffer B was 0.014.

CHROMATOGRAPHY

The substances pertinent to the study of reaction (1), *i.e.* chorismate and isochorismate, were separated by using a Hewlett-Packard 1100 by high performance liquid chromatogram (h.p.l.c.) equipped with a u.v. detector set at the wavelength $\lambda = 215$ nm and a HP Hypersil C-18 column (4 mm i.d., 250 mm long) thermostatted at $T = 313$ K. The mobile phase consisted of (III) {tetrabutylammonium hydroxide ($c = 0.01$ mol $\cdot$ dm^{-3})+ ammonium phosphate ($c = 0.025$ mol $\cdot$ dm^{-3}) + acetonitrile ($\phi = 0.01$)} adjusted to pH = 7.0 with NaOH and (IV) acetonitrile. The following gradient of these two mobile phases was formed: ϕ(III) = 0.95 and ϕ(IV) = 0.05 at time $t = 0$; ϕ(III) = 0.90 and ϕ(IV) = 0.10 at $t = 10$ min; ϕ(III) = 0.80 and ϕ(IV) = 0.20 at $t = 20$ min; and ϕ(III) = 0.50 and ϕ(IV) = 0.50 at $t = 30$ min. The flow rate was $1.33 \cdot 10^{-8}$ m$^3 \cdot$ s^{-1}. The approximate retention times were 16.1 min for chorismate and 18.3 min for isochorismate. While not participating in reaction (1), the retention time of 4-hydroxybenzoate (10.0 min) will also be seen to be pertinent to this study. Solutions having known molalities of chorismate were used to determine the chromatographic response factor of this substance.

DETERMINATION OF CHROMATOGRAPHIC RESPONSE FACTORS

It should be noted that a sample of isochorismate was not available. Thus, the retention time of this substance was based initially on the supposition that, following addition of isochorismate synthase to chorismate and a suitable equilibration time, the newly formed peak on the chromatogram corresponded to isochorismate. This is a reasonable assumption in that the isochorismate synthase was prepared from the plasmid containing the cloned gene for this enzyme (see above) and was also in a highly purified form. Additional confirmation of the correctness of this assumption was obtained by the demonstration of the reversibility of this reaction as was seen from the equilibrium and control experiments that are described below.

The absence of a sample of isochorismate made it necessary to use a procedure for the determination of its response factor which relied on a material balance between the initially known amount of chorismate in the reaction mixture and the product of reaction (1), namely isochorismate. Clearly, the amount of chorismate in the reaction mixture (chorismate + isochorismate) at a given time can be measured by using h.p.l.c. and the known response factor of chorismate. The area of the peak corresponding to isochorismate is also measured in the same experiment . Then, by material balance, the amount of isochorismate in the solution is known and the response factor of isochorismate can be calculated. However, this procedure is complicated by the fact that additional products are also formed. Firstly, prephenate is formed by the spontaneous (non-enzymatic) Claisen rearrangement of chorismate.[8] Prephenate can further spontaneously decompose to (β-phenylpyruvate + carbon dioxide). This decomposition, however, has a half life $t_{1/2} = 130$ h at $T = 298.15$ K and pH = 7.0[15, 16] and therefore is not a concern for short-term experiments. Also possible is the spontaneous decomposition of chorismate

to (4-hydroxybenzoate + pyruvate). Finally, isoprephenate is formed by the spontaneous Claisen rearrangement of isochorismate.[1] Thus, accounting for the loss of chorismate and of isochorismate via these spontaneous reactions had to be made a part of the material balance calculation used to determine the molality of isochorismate in solution.

The fractional rate of decomposition of chorismate to prephenate had already been measured under very similar conditions and found to be $6.11 \cdot 10^{-6}$ s^{-1}.[8] The determination of the fractional rate of decomposition of isochorismate required its *in situ* preparation which is now described. First, a solution of chorismate (molality $m = 7.1 \cdot 10^{-4}$ mol $\cdot$ kg^{-1}) in buffer C {K_2HPO_4($m = 0.103$ mol $\cdot$ kg^{-1}) + H_3PO_4 ($m = 0.0169$ mol $\cdot$ kg^{-1}) + $MgCl_2$ ($m = 1.88 \cdot 10^{-3}$ mol $\cdot$ kg^{-1}), pH = 7.24} was prepared. Isochorismate synthase (mass fraction $w \approx 2 \cdot 10^{-3}$) was added to this solution which was then allowed to equilibrate at $T = 298.15$ K for ≈2 h. This reaction mixture was filtered (centrifugation at an acceleration of $4350 \cdot \boldsymbol{g}_n$ for 50 min at $T = 276$ K in a Centricon-10 tube having a relative molecular mass cut-off of $1 \cdot 10^4$) to remove the isochorismate synthase. Chorismate lyase ($w \approx 0.0004$) was then added to bring about the conversion of the chorismate to (4-hydroxybenzoate + pyruvate). After a suitable equilibration time (≈2 h), h.p.l.c. was used to confirm that the removal of chorismate was complete {m(chorismate) $< 3 \cdot 10^{-7}$ mol $\cdot$ kg^{-1}}. The chorismate lyase was then removed by using the same filtration procedure as described above. The final solution thus contains isochorismate, 4-hydroxybenzoate, and pyruvate but is free of chorismate and of any enzymes. Thus, one has accomplished the *in situ* preparation of isochorismate. This solution was then allowed to equilibrate at $T = 298.15$ K. A portion of this mixture was injected into the h.p.l.c. at 30 min intervals (a total of six such measurements were made) and the area corresponding to isochorismate was determined. In this way, the fractional rate of decomposition of isochorismate was found to be $8.89 \cdot 10^{-6}$ s^{-1}.

The response factor of isochorismate was determined by using a reaction mixture of chorismate ($m = 7.1 \cdot 10^{-4}$ mol $\cdot$ kg^{-1}) in buffer C to which isochorismate synthase ($w \approx 0.002$) was added. This mixture was placed in a thermostatted water bath at $T = 298.15$ K. A sample of this reaction mixture was then injected into the h.p.l.c. at 30 min intervals for a period of 3 h. The molality of chorismate was calculated by using its known response factor and the area of the chorismate peak. The sum of the molalities of the decomposition products of chorismate was calculated by using the known fractional rate of decomposition of chorismate. The average molality of chorismate in solution during a specific time interval was used to perform this part of the calculation. Next, the sum of the molalities of isochorismate and of isoprephenate was calculated by using a material balance. The known fractional rate of decomposition of isochorismate to isoprephenate was then applied as a correction and the molality of isochorismate present at a given time was obtained. Finally, the response factor F for isochorismate was calculated by using the molality of isochorismate together with the measured area of the isochorismate chromatographic peak. The average value of F obtained from these experiments had a statistical uncertainty (two estimated standard deviations of the mean) of $0.012 \cdot F$. Also, the value of F obtained from each of the separate injections of the reaction mixture into the h.p.l.c. showed no systematic trend with the time of injection. This constancy lends some confidence to the value of F that was obtained.

DETERMINATION OF APPARENT EQUILIBRIUM CONSTANTS

The procedure used for the equilibrium measurements is now described. First, chorismate ($m = 8.4 \cdot 10^{-4}$ mol $\cdot$ kg^{-1}) was dissolved in buffer C. Isochorismate synthase was then added to this solution. This solution was allowed to equilibrate for ≈3 h in a bath thermostatted at $T = 298.15$ K. Injections into the h.p.l.c. of portions of this solution were then made at ≈30 min intervals and the areas of the respective peaks corresponding to chorismate and isochorismate were determined. The ratio R of the peak areas was found to be reasonably constant ($\pm 3 \cdot 10^{-3} \cdot$ R). Then, with the already determined response factors of chorismate and isochorismate, a value of the apparent reaction quotient Q' was calculated. All equilibrations of solutions were performed by placing the bottles (glass with Teflon caps) containing the solutions in a thermostatted water bath and shaken at ≈50 rpm. To minimize spontaneous decompositions, the stock solutions of chorismate and of isochorismate were kept in an ice bath until needed for use in the equilibrium or calorimetric experiments.

The procedure used for the determination of a value of Q' from the reverse direction of the reaction follows. First, isochorismate ($m = 4 \cdot 10^{-4}$ mol $\cdot$ kg^{-1}) was prepared *in situ* by using the procedure described earlier in which chorismate lyase was used to remove all of the chorismate from the solution. Next, isochorismate synthase was added to this solution which was then allowed to equilibrate in the water bath thermostatted at $T = 298.15$ K. As above, periodic injections (every 30 min for a total equilibration time of 3 h) of the reaction mixture into the h.p.l.c. were performed and a value of Q' was determined.

MICROCALORIMETRY

Three heat-conduction microcalorimeters were used for the enthalpy of reaction measurements. They were calibrated electrically with a high stability d.c. 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 have been given by Steckler *et al.*[17, 18] However, some significant changes have been made subsequently in the method of data collection. Specifically, the voltages of the thermopiles of the microcalorimeters are now measured with Hewlett-Packard model 34420A Nanovolt Meters. These voltages are then recorded on a microcomputer with a data acquisition program written in Hewlett-Packard HP-VEE. The integration of the areas of the thermograms is done by using a code written in C++.

The calorimetric sample vessels were fabricated from high-density polyethylene. Each vessel had two compartments that held, respectively, ≈0.55 cm^3 and ≈0.40 cm^3 of solution. The substrate solutions were placed in the 0.55 cm^3 compartment and the enzyme solutions were placed in the 0.40 cm^3 compartment. The substrate solution held chorismate dissolved in a phosphate buffer that contained $MgCl_2$. The enzyme solution was prepared by the addition of isochorismate synthase to the stock buffer solution that was used for the preparation of the substrate solution. The purpose of this procedure was to minimize the "blank" enthalpies (see below).

The vessels and their contents were allowed to thermally equilibrate in the microcalorimeters for 60 min before the enzyme and substrate solutions were mixed. After this mixing, approximately 35 min was allowed for reaction (1). Following reaction, the vessels were removed from the microcalorimeters and the h.p.l.c. was promptly used to determine the molalities of the chorismate and isochorismate in the reaction mixture. The h.p.l.c. was also used to check for the presence of side reactions. Here, the presence of 4-hydroxybenzoate was of particular interest because of the large molar enthalpy of reaction for the formation of it and pyruvate from chorismate.[10] However, since there was no evidence in the chromatograms for 4-hydroxybenzoate, its molality was judged to be $<3 \cdot 10^{-7}$ mol $\cdot$ kg^{-1}; the amount of this substance in solution is $<3 \cdot 10^{-10}$ mol. Since the enthalpy change for the formation of this amount of 4-hydroxybenzoate from chorismate is <0.04 mJ, no correction for its formation was made. "Blank" enthalpy changes were determined in appropriate control experiments. Thus, the "blank" enthalpy change for mixing of the chorismate solution (the substrate solution) with the buffer was -0.74 mJ. For the mixing of the isochorismate synthase (the enzyme solution) with the buffer, the "blank" enthalpy change was -0.1 mJ. These "blank" enthalpies of mixing were applied as corrections to the measured calorimetric enthalpies which were ≈ -4 mJ. Thus, reaction (1) is close to being thermoneutral.

pH MEASUREMENTS

Measurement of pH was done with an Orion Model 811 pH meter and a Radiometer combination glass micro-electrode. The pH meter was calibrated with Radiometer standard buffers that bracketed the pHs of the solutions used in this study.

3. Results and discussion

THERMODYNAMIC FORMALISM

The apparent equilibrium constant[19] for reaction (1) is

$$K' = m(\text{isochorismate})/m(\text{chorismate}). \tag{2}$$

The molalities m in the above equation are the total molalities of the various charged and uncharged species that are formed from the dissociation of the various substances in solution. In discussing the thermodynamics of overall biochemical reactions, it is useful to introduce a reference reaction that pertains to specific ionic forms. Here we shall use

$$\text{chorismate}^{2-}(\text{aq}) = \text{isochorismate}^{2-}(\text{aq}). \tag{3}$$

The species selected are the predominant ionic forms at pH $= 7.0$. Since the choice of the reference reaction is arbitrary, other ionic forms could also have been chosen. The equilibrium constant for reaction (3) is

$$K = m(\text{isochorismate}^{2-})/m(\text{chorismate}^{2-}). \tag{4}$$

The standard state used in this study is the hypothetical ideal solution of unit molality ($m^{\circ} = 1$ mol $\cdot$ kg^{-1}). Since reactions (1) and (3) are symmetrical, the values of K' and K are independent of the choice of the scale (*i.e.* molality, concentration, and mole fraction) used to express the composition of the solution.

RESULTS OF EXPERIMENTS

The equilibrium measurements performed on reaction (1) yielded mean values of the apparent reaction quotient $\langle Q' \rangle = (0.846 \pm 0.003)$ from the forward direction of reaction and $\langle Q' \rangle = (0.834 \pm 0.005)$ from the reverse direction of reaction (see table 2). We judge the closeness of these results to be good evidence that, for practical purposes, equilibrium has been reached and that the Q's can be identified as K's. The combined result is $K' = (0.840 \pm 0.006)$ at $T = 298.15$ K, pH = 7.34, and ionic strength $I_m = 0.31 \text{ mol} \cdot \text{kg}^{-1}$. The above uncertainties and all subsequently stated uncertainties, unless indicated otherwise, are equal to two estimated standard deviations of the mean.

The results of the calorimetric measurements are given in table 3. The chromatographic analyses of the reaction mixtures that were done immediately following their removal from the calorimeter gave values of $Q' = 0.848$ and $Q' = 0.861$ for reaction (1). Each of these values is based upon a single injection of the sample into the h.p.l.c. The mean value $\langle Q' \rangle = (0.855 \pm 0.013)$ is in agreement with the already determined value $K' = (0.840 \pm 0.006)$. Since this latter result is based on 10 injections of reaction mixture into the h.p.l.c., it is considered to be the more reliable value for K'. Thus, the value $K' = 0.84$ was used to calculate the extent of reaction ($\xi = 0.4565$) for the calorimetric experiments. The result of the calorimetric measurements is $\langle \Delta_r H_m(\text{cal}) \rangle = -(0.81 \pm 0.12) \text{ kJ} \cdot \text{mol}^{-1}$ at $T = 298.15$ K, pH = 6.93, and $I_m = 0.23 \text{ mol} \cdot \text{kg}^{-1}$.

The uncertainties in the measured values of K' and $\langle \Delta_r H_m(\text{cal}) \rangle$ represent only the random errors inherent in the measurements and do not reflect possible systematic errors which are now considered. We judge that a reasonable estimate of the standard uncertainty[20] due to possible systematic errors in the values of K' is $0.03 \cdot K'$. This estimate is attributed to possible error in the determination of the response factors and in the chromatography and is inclusive of possible errors in the amounts of the impurities in the chorismate sample. Because of the closeness of the values of Q' determined from both directions of reaction, no error estimate has been made for a possible failure of reaction (1) to reach equilibrium. The estimate of possible systematic error $(0.03 \cdot K')$ is combined in quadrature together with the statistical uncertainty in the measured value of K', expressed as one estimated standard deviation of the mean, to obtain a combined standard uncertainty.[20] This combined standard uncertainty is then multiplied by two to arrive at the final value $K' = (0.84 \pm 0.05)$ for reaction (1) at $T = 298.15$ K, pH = 7.34, and $I_m = 0.31 \text{ mol} \cdot \text{kg}^{-1}$.

Similarly, we judge that reasonable estimates of error in the values of $\Delta_r H_m(\text{cal})$ for reaction (1) are: $0.02 \cdot \Delta_r H_m(\text{cal})$ due to impurities in the chorismate sample; $0.04 \cdot \Delta_r H_m(\text{cal})$ due to a possible error in the extent of reaction; $0.05 \cdot \Delta_r H_m(\text{cal})$ due to possible side reactions; $0.003 \cdot \Delta_r H_m(\text{cal})$ due to possible errors in the calorimetric calibrations; and $0.15 \cdot \Delta_r H_m(\text{cal})$ due to uncertainties in the "blank" enthalpies. Combining these standard uncertainties as described above, the final result is $\Delta_r H_m(\text{cal}) = -(0.81 \pm 0.27) \text{ kJ} \cdot \text{mol}^{-1}$ for reaction (1) at $T = 298.15$ K, pH = 6.93, and $I_m = 0.23 \text{ mol} \cdot \text{kg}^{-1}$.

TABLE 2. Results of equilibrium measurements at $T = 298.15$ K and pH = 7.34 for reaction (1): chorismate(aq) = isochorismate(aq). The molalities m are those of the substances in solution at equilibrium (see Results and discussion) and are equal to the sums of the molalities of the indicated substances in their various ionic forms. The measured reaction quotients Q' are given in the last column. The ionic strength I_m is calculated. The following substances were also present in the reaction mixtures at the specified approximate molalities: KH_2PO_4, $m \approx 0.003$ mol · kg^{-1}; EDTA, $m \approx 1.1 \cdot 10^{-4}$ mol · kg^{-1}; **DL**-dithiothreitol(DTT) $m \approx 1.1 \cdot 10^{-4}$ mol · kg^{-1}; NaCl, $m \approx 0.012$ mol · kg^{-1}; and phenylmethylsulfonyl fluoride (PMSF), $m \approx 1.1 \cdot 10^{-5}$ mol · kg^{-1}. The solution used for the reverse direction of reaction also contained 4-hydroxybenzoate ($m \approx 4 \cdot 10^{-4}$ mol · kg^{-1}) and pyruvate ($m \approx 4 \cdot 10^{-4}$ mol · kg^{-1}). The mass fraction w of the isochorismate synthase in solution was ≈0.0015. The uncertainties given below are equal to two estimated standard deviations of the mean. An estimate of total error for K' of ±0.05 is assigned in the text (see Results and discussion)

Direction of reaction	$\frac{m(K_2HPO_4)}{mol \cdot kg^{-1}}$	$\frac{m(H_3PO_4)}{mol \cdot kg^{-1}}$	$\frac{10^3 \cdot m(MgCl_2)}{mol \cdot kg^{-1}}$	$\frac{10^4 \cdot m(chorismate)}{mol \cdot kg^{-1}}$	$\frac{10^4 \cdot m(isochorismate)}{mol \cdot kg^{-1}}$	$\frac{I_m}{mol \cdot kg^{-1}}$	Q'
Forward	0.0980	0.0157	1.75	3.75	3.16	0.31	0.842
Forward	0.0980	0.0157	1.75	3.47	2.92	0.31	0.842
Forward	0.0980	0.0157	1.75	3.42	2.89	0.31	0.846
Forward	0.0980	0.0157	1.75	3.34	2.83	0.31	0.848
Forward	0.0980	0.0157	1.75	3.26	2.77	0.31	0.850
Reverse	0.0939	0.0149	1.65	2.02	1.67	0.30	0.828
Reverse	0.0939	0.0149	1.65	2.01	1.67	0.30	0.830
Reverse	0.0939	0.0149	1.65	1.95	1.63	0.30	0.836
Reverse	0.0939	0.0149	1.65	1.88	1.56	0.30	0.830
Reverse	0.0939	0.0149	1.65	1.68	1.42	0.30	0.847
				Forward: $\langle Q' \rangle = 0.846 \pm 0.006$			
				Reverse: $\langle Q' \rangle = 0.834 \pm 0.005$			
				$K' = \langle Q' \rangle = 0.840 \pm 0.006$			

TABLE 3. Results of the calorimetric measurements at $T = 298.15$ K, pH = 6.93, and ionic strength $I_m = 0.23$ mol · kg^{-1} for reaction (1): chorismate(aq) = isochorismate(aq). The molalities m are those obtained after mixing of the enzyme and substrate solutions and prior to any reaction. All molalities are equal to the sums of the molalities of the indicated substances in their various ionic forms. $\Delta_r H_m$(cal) is the calorimetrically determined molar enthalpy of reaction (1). The value of the ionic strength was calculated. The mass fraction w of isochorismate synthase in solution was ≈0.003. The uncertainty of ±0.12 kJ · mol^{-1} given below for $\Delta_r H_m$(cal) is equal to two estimated standard deviations of the mean. An estimate of total error for $\Delta_r H_m$(cal) of ± 0.27 kJ · mol^{-1} is assigned in the text (see Results and discussion)

Experiment	$\frac{m(K_2HPO_4)}{mol \cdot kg^{-1}}$	$\frac{m(H_3PO_4)}{mol \cdot kg^{-1}}$	$\frac{10^3 \cdot m(MgCl_2)}{mol \cdot kg^{-1}}$	$\frac{10^3 \cdot m(chorismate)}{mol \cdot kg^{-1}}$	$\frac{\Delta_r H_m(cal)}{kJ \cdot mol^{-1}}$
1	0.0838	0.0141	1.34	8.56	−0.95
2	0.0841	0.0141	1.34	8.67	−0.93
3	0.0837	0.0141	1.34	8.51	−0.65
4	0.0865	0.0145	1.38	8.61	−0.75
5	0.0861	0.0145	1.37	8.40	−0.64
6	0.0864	0.0146	1.38	8.54	−0.91
$\langle \Delta_r H_m(cal) \rangle = -(0.81 \pm 0.12)$ kJ · mol^{-1}					

EQUILIBRIUM MODEL

The pKs and standard molar enthalpies for the H^+(aq) and Mg^{2+}(aq) dissociation reactions of the reactants and of the buffers are needed to relate the experimental results for reaction (1) to thermodynamic quantities for the reference reaction (3). In a previous study,[8] the pKs of chorismic acid were judged to be <4.5. This was based upon the pKs of structurally related substances and was consistent with approximate results obtained from a potentiometric titration done on chorismic acid. Because isochorismic acid is similar in structure to chorismic acid (see figure 1), we use this same value of the pKs for isochorismic acid. Since the calorimetric and equilibrium experiments were done at pH = 6.93 and pH = 7.34, respectively, the difference $|pH - pK| > 2.7$ and the predominant species (mole fraction $x > 0.998$) in these experiments are chorismate^{2-}(aq) and isochorismate^{2-}(aq). Also needed are thermodynamic quantities for binding of H^+(aq) and Mg^{2+}(aq) to HPO_4^{2-}(aq). The thermodynamic quantities for the ionization of $H_2PO_4^-$(aq) were calculated from Cox *et al.*[21] The thermodynamic quantities for the dissociation of Mg^{2+}(aq) from $MgHPO_4$(aq) are from Clarke *et al.*[22] These pKs and related quantities are summarized in table 4.

The equilibrium model used for the calculation of the equilibrium constants K and standard molar enthalpies $\Delta_r H_m^o$ for the reference reaction (3) from the measured values of K' and $\Delta_r H_m$(cal) has been described previously.[23] This model has been modified[24] so that it now uses Mathematica[25] to solve the chemical equilibrium equations and calculate the desired thermodynamic quantities. The Gibbs free energy

TABLE 4. The pKs and standard molar enthalpy changes $\Delta_r H^o_m$ for the aqueous proton dissociation reactions of substances pertinent to this study at $T = 298.15$ K and $I_m = 0$. See Results and discussion for the basis of these values

Reaction	pK	$\frac{\Delta_r H^o_m}{kJ \cdot mol^{-1}}$
$chorismate^- = chorismate^{2-} + H^+$	<4.5	
$isochorismate^- = isochorismate^{2-} + H^+$	<4.5	
$H_2PO_4^- = HPO_4^{2-} + H^+$	7.212	3.6
$MgHPO_4 = HPO_4^{2-} + Mg^{2+}$	2.71	−12.2

minimization uses an algorithm obtained from Alberty and Krambeck.[26] The calculations also include corrections for non-ideality and are made self-consistent[23] with regard to the ionic strength. The non-ideality corrections are based on the extended Debye–Hückel equation[23] in which the "ion-size" parameter has been set at 1.6 $kg^{1/2} \cdot mol^{-1/2}$.

By applying the equilibrium model with the experimental results for K' and for $\Delta_r H_m$(cal) and the thermodynamic quantities given in table 4, we calculate $K = (0.84 \pm 0.04)$ and $\Delta_r H^o_m = -(0.81 \pm 0.27)$ kJ · mol^{-1} at $T = 298.15$ K and $I_m = 0$ for the reference reaction (3). Thus, the respective values of K and K' and of $\Delta_r H^o_m$ and $\Delta_r H_m$(cal) are the same. This could have been anticipated from both the large difference between the pKs and the pHs at which the experiments were performed and the charge symmetry of reaction (3). The equilibrium model also yielded the result that the change in binding of the hydrogen ion $\Delta_r N(H^+) = 0$ for reaction (1). Thus, the buffer protonation correction is zero. The standard molar Gibbs free energy and standard molar entropy changes for reaction (3) at $T = 298.15$ K and $I_m = 0$ are $\Delta_r G^o_m = (0.43 \pm 0.12)$ kJ · mol^{-1} and $\Delta_r S^o_m = -(4.2 \pm 1.0)$ J · K^{-1} · mol^{-1}, respectively.

COMPARISON WITH RESULTS IN THE LITERATURE

Values of K' for reaction (1) have been reported by Liu *et al.*[2] and by Kozlowski *et al.*[3] Liu *et al.*[2] used n.m.r. to measure the value $K' = 0.66$ for reaction (1). The buffer used was {potassium phosphate ($c = 0.050$ mol · dm^{-3}) + $MgCl_2$(c = $5 \cdot 10^{-3}$ mol · dm^{-3}), (pH = 7.5)}. The temperature, while not clearly stated, was either ambient (≈298.15 K) or 310.15 K. They also determined kinetic constants at $T = 310.15$ K for reaction (1) and then used a Haldane relationship to calculate the value $K' = 0.56$. Here the buffer was {Tris ($c = 0.10$ mol · dm^{-3}) + HCl + $MgCl_2$ ($c = 0.010$ mol · dm^{-3}), pH = 7.8}. The result obtained from the kinetic data is probably less certain than their[2] result obtained by n.m.r. Kozlowski *et al.*[3] used h.p.l.c. to obtain the value $K' = 0.55$ for reaction (1). The buffer they[3] used was {$(NH_4)_2SO_4$ ($c = 0.050$ mol · dm^{-3}), pH = 8.0}. The temperature was not stated but is assumed to be ambient (≈298 K). Kozlowski *et al.*[3] characterized their result for K' as a "rough estimation". Thus, the value of $K' = 0.66$ from the n.m.r. experiment of Liu *et al.*[2] is judged to be the most reliable of the earlier results. This result leads to $K = 0.66$ for the reference reaction (3) at $T = 298.15$ K and $I_m = 0$. If, however,

the n.m.r. experiments of Liu *et al.*[2] were carried out at $T = 310.15$ K, the adjustment of the value of K obtained to $T = 298.15$ K can be made by using the value of $\Delta_r H^o_m$ for reaction (3) determined in this study. In such a case, the result would be $K = 0.67$ for reaction (3) at $T = 298.15$ K. In the absence of assigned uncertainties, it is not possible to state whether the result of the present study ($K = 0.84 \pm 0.04$) is in agreement with the earlier results of Liu *et al.*[2] and of Kozlowski *et al.*[3]

APPARENT EQUILIBRIUM CONSTANTS UNDER APPROXIMATELY PHYSIOLOGICAL CONDITIONS

It is desirable to have a value of the apparent equilibrium constant K' for reaction (1) under approximately physiological conditions which are taken to be:[27] $T = 311.15$ K, pH = 7.0, pMg = 3.0, and $I_m = 0.25$ mol · kg^{-1}. By using the equilibrium model with the thermodynamic quantities obtained in this study for the reference reaction (3), we calculate $K' = 0.83$ and the standard molar transformed Gibbs free energy change $\Delta_r G'^o_m = 0.48$ kJ · mol^{-1} for reaction (1) under these conditions.

CONCLUSION

The small absolute values of the thermodynamic quantities ($\Delta_r G^o_m$, $\Delta_r H^o_m$, and $\Delta_r S^o_m$) for reaction (3) reflect the subtle structural differences between chorismate and isochorismate (see figure 1). The fact that isochorismate has a cyclohexadiene moiety fully conjugated with the carboxylate, while chorismate is cross conjugated, might confer some additional stability on isochorismate.[1] However, steric effects and other interactions, particularly hydrogen bonding, would be more substantial for a hydroxy group located between a carboxyl and a pyruvyl group (*i.e.* isochorismate) than for a hydroxy group located adjacent to just a pyruvyl group (chorismate). Also, there is the additional complication that both chorismate and isochorismate exist as a mixture of the pseudo diaxial and pseudo diequatorial forms[1] which may not be the same for both substances. Therefore, a detailed molecular explanation of the measured thermodynamic quantities does not appear to be possible at this time.

We thank Dr Paul A. Bartlett (University of California at Berkeley) for providing the plasmid used to prepare the isochorismate synthase.

REFERENCES

1. Walsh, C. T.; Liu, J.; Rusnak, F.; Sakaitani, M. *Chem. Rev.* **1990**, 90, 1105–1129.
2. Liu, J.; Quinn, N.; Berchtold, G. A.; Walsh, C. T. *Biochemistry* **1990**, 29, 1417–1425.
3. Kozlowski, M. C.; Tom, N. J.; Seto, C. T.; Sefler, A. M.; Bartlett, P. A. *J. Am. Chem. Soc.* **1995**, 117, 2128–2140.
4. Fell, D. A. *Biochem. J.* **1992**, 286, 313–330.
5. Kashiwaya, Y.; Sato, K.; Tsuchiya, N.; Thomas, S.; Fell, D. A.; Veech, R. L.; Passonneau, J. V. *J. Biol. Chem.* **1994**, 269, 25502–25514.
6. Frost, J. W.; Draths, K. M. *Chem. Br.* **1995**, 31, 206–210.
7. Flores, N.; Xiao, J.; Berry, A.; Bolivar, F.; Valle, F. *Nature Biotechnol.* **1996**, 14, 620–623.
8. Kast, P.; Tewari, Y. B.; Wiest, O.; Hilvert, D.; Houk, K. N.; Goldberg, R. N. *J. Phys. Chem. B* **1997**, 101, 10976–10982.

9. Kishore, N.; Tewari, Y. B.; Akers, D. L.; Goldberg, R. N.; Miles, E. W. *Biophys. Chem.* **1998**, 73, 265–280.
10. Tewari, Y. B.; Chen, J.; Holden, M. J.; Houk, K. N.; Goldberg, R. N. *J. Phys. Chem. B* **1998**, 102, 8634–8639.
11. Tewari, Y. B.; Kishore, N.; Goldberg, R. N.; Luong, T. N. *J. Chem. Thermodynamics* **1998**, 30, 777–793.
12. Kishore, N.; Holden, M. J.; Tewari, Y. B.; Goldberg, R. N. *J. Chem. Thermodynamics* **1999**, 31, 211–227.
13. Byrnes, W. M.; Goldberg, R. N.; Holden, M. J.; Mayhew, M. P.; Tewari, Y. B. Thermodynamics of reactions catalyzed by anthranilate synthase. *Biophys. Chem* **2000**, 84, 45–64.
14. Tummuru, M. K. R.; Brickman, T. J.; McIntosh, M. A. *J. Biol. Chem.* **1989**, 264, 20547–20551.
15. Weiss, U.; Gilvarg, C.; Mingioli, E. S.; Davis, B. D. *Science* **1954**, 119, 774–775.
16. Zamir, L. O.; Tiberio, R.; Jensen, R. A. *Tetrahedron Lett.* **1983**, 24, 2815–2818.
17. Steckler, D. K.; Goldberg, R. N.; Tewari, Y. B.; Buckley, T. J. *J. Res. Natl. Bur. Stand (U.S.)* **1986**, 91, 113–121.
18. Steckler, D. K.; Goldberg, R. N.; Tewari, Y. B.; Buckley, T. J. *Natl. Bur. Stand. (U.S.) Tech. Note 1224*. Computer Software for the Acquisition and Treatment of Calorimetric Data. U.S. Government Printing Office: Washington, DC. **1986**.
19. Alberty, R. A.; Cornish-Bowden, A.; Gibson, Q. H.; Goldberg, R. N.; Hammes, G. G.; Jencks, W.; Tipton, K. F.; Veech, R.; Westerhoff, H. V.; Webb, E. C. *Pure Appl. Chem.* **1994**, 66, 1641–1666.
20. Taylor, B. N.; Kuyatt, C. E. *NIST Technical Note 1297*. Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results. U.S. Government Printing Office: Washington, DC. **1994**.
21. Cox, J. D.; Wagman, D. D.; Medvedev, V. A. *CODATA Key Values for Thermodynamics*. Hemisphere: New York. **1989**.
22. Clarke, H. B.; Cusworth, D. C.; Datta, S. P. *Biochem. J.* **1954**, 58, 146–154.
23. Goldberg, R. N.; Tewari, Y. B. *Biophys. Chem.* **1991**, 40, 241–261.
24. Akers, D. L.; Goldberg, R. N. Thermodynamics of biochemical reactions, *Mathematica J*., (in press).
25. Wolfram, S. *The Mathematica Book*. Cambridge University Press: Melbourne. **1996**.
26. Alberty, R. A.; Krambeck, F. J. Personal communication.
27. Veloso, D.; Guynn, R. W.; Oskarsson, M.; Veech, R. L. *J. Biol. Chem.* **1973**, 248, 4811–4819.

(Received 3 December 1999; in final form 17 February 2000)

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