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Thermodynamics of reactions catalyzed by anthranilate synthase

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Abstract

Microcalorimetry and high performance liquid chromatography have been used to conduct a thermodynamic investigation of reactions catalyzed by anthranilate synthase, the enzyme located at the first step in the biosynthetic pathway leading from chorismate to tryptophan. One of the overall biochemical reactions catalyzed by anthranilate synthase is: chorismate(aq) + ammonia(aq) = anthranilate(aq) + pyruvate(aq) + H_2O(l). This reaction can be divided into two partial reactions involving the intermediate 2-amino-4-deoxyisochorismate (ADIC): chorismate(aq) + ammonia(aq) = ADIC(aq) + H_2O(l) and ADIC(aq) = anthranilate(aq) + pyruvate(aq). The native anthranilate synthase and a mutant form of it that is deficient in ADIC lyase activity but has ADIC synthase activity were used to study the overall ammonia-dependent reaction and the first of the above two partial reactions, respectively. Microcalorimetric measurements were performed on the overall reaction at a temperature of 298.15 K and pH 7.79. Equilibrium measurements were performed on the first partial (ADIC synthase) reaction at temperatures ranging from 288.15 to 302.65 K, and at pH values from 7.76 to 8.08. The results of the equilibrium and calorimetric measurements were analyzed in terms of a chemical equilibrium model that accounts for the multiplicity of ionic states of the reactants and products. These calculations gave thermodynamic quantities at the temperature 298.15 K and an ionic strength of zero for chemical reference reactions involving specific ionic forms. For the reaction: chorismate^{2-}(aq) + NH_4^+(aq) = anthranilate$^-$(aq) + pyruvate$^-$(aq) + H^+(aq) + H_2O(l), $\Delta_r H_m^o = -(116.3 \pm 5.4)$ kJ mol^{-1}. For the reaction: chorismate^{2-}(aq) + NH_4^+(aq) = ADIC$^-$(aq) + H_2O(l), $K = (20.3 \pm 4.5)$ and $\Delta_r H_m^o = (7.5 \pm 0.6)$ kJ mol^{-1}. Thermodynamic cycle calculations were used to calculate thermodynamic quantities for three additional reactions that are pertinent to this branch point of the chorismate pathway. The quantities obtained in this

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study permit the calculation of the position of equilibrium of these reactions as a function of temperature, pH, and ionic strength. Values of the apparent equilibrium constants and the standard transformed Gibbs energy changes $\Delta_r G'^{o}_m$ under approximately physiological conditions are given.

Keywords: Anthranilate synthase; Apparent equilibrium constant; Chorismate; Enthalpy; Entropy; Gibbs free energy; Thermodynamics

1. Introduction

The chorismate metabolic pathway is of substantial interest because of its potential for the manufacture of aromatic amino acids and other bulk commodity chemicals [1,2]. This pathway is unique to plants and bacteria and therefore is an attractive target for potential herbicides and antibiotics. The first step in the branch of the chorismate pathway [3] that leads to the synthesis of tryptophan is

$$\text{chorismate(aq)} + \text{L-glutamine(aq)} = \text{anthranilate(aq)} + \text{L-glutamate(aq)} + \text{pyruvate(aq)} \quad (1)$$

This reaction is catalyzed by anthranilate synthase (EC 4.1.3.27). The anthranilate synthase from *Salmonella typhimurium* is a tetrameric complex of two subunits each of TrpE and TrpD, the products of the first two genes (*trpE* and *trpD*) of the *S. typhimurium trp* operon [4]. The magnesium ion is an essential cofactor of the enzyme. In *S. typhimurium* anthranilate synthase, the TrpD subunit is actually a bifunctional molecule containing two independent structural domains with separate activities. The amino-terminal domain is a glutamine amidotransferase that catalyzes the release of an amido group from glutamine. The amido group is then transferred to chorismate in the anthranilate synthase reaction catalyzed by TrpE. The carboxy-terminal domain of TrpD is a phosphoribosyl pyrophosphate transferase that catalyzes the second step of the tryptophan pathway; this step involves formation of *N*-(5′-phosphoribosyl)-anthranilate, a compound that is unstable in solution. TrpE by itself, or as part of the $(TrpE)_2(TrpD)_2$ complex, can catalyze an ammonia-dependent anthranilate synthase reaction, but the entire complex is required for the glutamine-dependent anthranilate synthase reaction (1) above. Thus, native anthranilate synthase from *S. typhimurium* is multifunctional and catalyzes the following reactions in addition to reaction (1):

$$\text{chorismate(aq)} + \text{ammonia(aq)} = \text{anthranilate(aq)} + \text{pyruvate(aq)} + \text{H}_2\text{O(l)} \quad (2)$$

$$\text{L-glutamine(aq)} + \text{H}_2\text{O(l)} = \text{L-glutamate(aq)} + \text{ammonia(aq)} \quad (3)$$

$$\text{5-phosphoribosyl-}\alpha\text{-pyrophosphate(aq)} + \text{anthranilate(aq)} = \textit{N}\text{-(5′-phosphoribosyl)-anthranilate(aq)} + \text{pyrophosphate(aq)} \quad (4)$$

Reactions (1) and (2) proceed via the intermediate compound *trans*-6-amino-5-[(1-carboxyethenyl)oxy]-1,3-cyclohexadiene-1-carboxylic acid, more commonly known as 2-amino-2-deoxyisochorismate or ADIC [5]. Reaction (2) can therefore be divided into two reactions involving the formation and breakdown of ADIC:

$$\text{chorismate(aq)} + \text{ammonia(aq)} = \text{ADIC(aq)} + \text{H}_2\text{O(1)} \quad (5)$$

$$\text{ADIC(aq)} = \text{anthranilate(aq)} + \text{pyruvate(aq)} \quad (6)$$

When native anthranilate synthase is used, ADIC does not accumulate in the reaction mixture because the native enzyme has both ADIC

synthase activity [reaction (5)] and ADIC lyase activity [reaction (6)]. However, Morollo and Bauerle [6] have constructed a mutant form of anthranilate synthase ($TrpE^{H398M}$) that allows accumulation of ADIC because its ADIC lyase activity is defective (less than 1% of the activity of the native enzyme). This mutant anthranilate synthase is essentially an ADIC synthase and thus can be used to prepare ADIC, a compound that is interesting and important in its own right as a potential precursor for substances with antibiotic activity [6].

Thermodynamic results for the reactions at this important branch point in the chorismate metabolic pathway are sparse. Yet, results of this type are needed to establish the energetics of both the individual reactions and of the chorismate pathway as a whole. 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 [7,8]. For the above reasons, this study was undertaken. The overall aim was to obtain a more thorough understanding of the thermodynamics of the anthranilate synthase catalyzed reactions [see Fig. 1 for the structures of the substances in reactions (1), (5), and (6)]. Earlier studies [9–13] from this laboratory have dealt with the thermodynamics of reactions in other branches of the chorismate pathway.

In this study, microcalorimetry was used to measure the enthalpy change for reaction (2) catalyzed by native anthranilate synthase. Several experiments were carried out with mutant ($TrpE^{H398M}$) anthranilate synthase to determine the apparent equilibrium constant [14] for reaction (5) as a function of temperature. The results of the calorimetric and equilibrium measure-

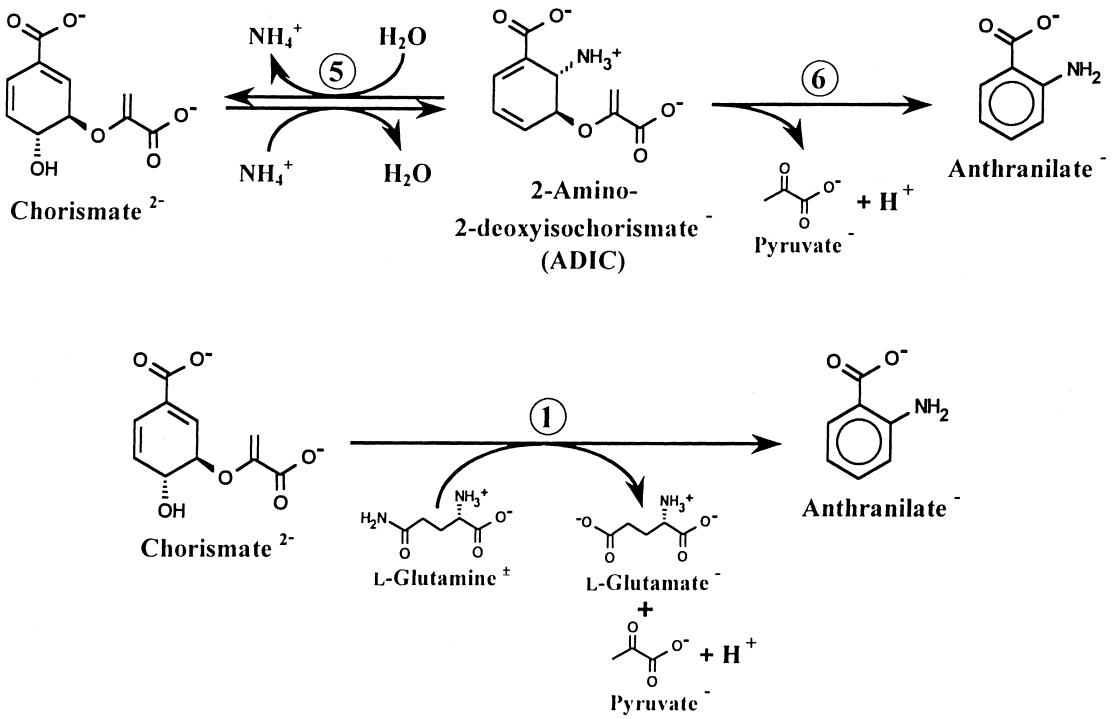

Fig. 1. Reactions catalyzed by anthranilate synthase from *Salmonella typhimurium*. The structures of all compounds are those of the predominant ionic forms (reference species) at the pH range of interest (7–8). ADIC synthase activity [reaction (5)] is present in both the native and mutant anthranilate synthase. ADIC lyase activity [reaction (6)] is present in native anthranilate synthase but is defective (less than 1% of the activity in the native form) in the mutant $TrpE^{H398M}$ anthranilate synthase. Reaction (1) is catalyzed by the native anthranilate synthase.

Table 1
Principal substances used in this study with their Chemical Abstracts Service (CAS) registry numbers, empirical formulae, relative molecular masses M_r, mass fraction moisture contents w determined by Karl Fischer analysis, mole fraction purity x as stated by supplier (Fi = Fisher, Fl = Fluka, M = Mallinckrodt, S = Sigma), and method used to determine x[a]

Substance	CAS registry number	Formula	M_r	w	Supplier	x	Method[b]
2-Amino-2-deoxyisochorismate	214403-80-2	$C_{10}H_{11}NO_5$	225.20		[c]		
Ammonium chloride	12125-02-9	NH_4Cl	53.49	0.0093	S	> 0.99	Titration with $AgNO_3$
Anthranilic acid	118-92-3	$C_7H_7NO_2$	137.14	0.0017	Fl	> 0.995	HPLC
Anthranilate synthase (native)			$227.6 \cdot 10^3$		[d]	≈ 0.90	1-D SDS-PAGE gel electrophoresis
Anthranilate synthase (mutant)			$227.6 \cdot 10^3$		[d]	≈ 0.99	1-D SDS-PAGE gel electrophoresis
Chorismate lyase			$18.8 \cdot 10^3$		[d]	> 0.95	1-D SDS-PAGE gel electrophoresis
Chorismic acid[e]	617-12-9	$C_{10}H_{10}O_6$	226.18		S	0.975	HPLC
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
Pyruvic acid, sodium salt	113-24-6	$C_3H_3O_3Na$	110.04	0.0073	S	0.994	$HClO_4$ titration
Sodium hydroxide	1310-73-2	NaOH	39.997		Fi	> 0.99	Acid titration
Tricine[f]	5704-04-1	$C_6H_{13}NO_5$	179.18		S	> 0.99	Acid titration

[a] The mole fraction purities are exclusive of the amount of water or, in the case of chorismate, diethyl ether.

[b] The HPLC methods described above (see Section 2) were used as checks on the purities of the anthranilic acid and chorismic acid. The other methods are those used by the vendor(s) to determine the purities of these substances.

[c] Prepared in situ.

[d] Prepared for this study. See Section 2.

[e] Two chorismate samples were used. The mass fraction of water in the first sample was 0.130; it also contained diethyl ether ($w = 0.022$) and other unidentified impurities ($x \approx 0.025$); the mass fraction of water in the second sample was 0.116; it also contained diethyl ether ($w = 0.041$) and other unidentified impurities ($x \approx 0.025$).

[f] Tricine is *N*-[tris(hydroxymethyl)methyl]glycine.

ments were analyzed in terms of an equilibrium model [15] that accounted for the multiplicity of ionic states of reactants and products. This analysis yielded values of the standard molar enthalpy changes for chemical reference reactions appropriate for reactions (2) and (5). A value of the equilibrium constant for the chemical reference reaction appropriate for reaction (5) was also obtained. The results of this study were combined with some results from the literature and some reasonable estimates of entropies in thermochemical cycle calculations to obtain a complete thermodynamic picture of reactions (1), (2), (5), and (6).

2. Materials and methods

2.1. Chemicals

Relevant information on the substances used in this study is given in Table 1.[1] The purities of anthranilic acid and chorismic acid were examined by using the chromatographic procedures described below and were found to be consistent with the purities stated by the vendor. Karl Fischer analysis was used for the determinations of the mass fractions of water in the various samples. The Karl Fischer procedure is similar to that previously described [16] except that the calibration now relies on the value of the solubility of water in 1-octanol reported by Margolis [17]. The mass fraction moisture contents w are judged to be reliable to within $\approx 0.03 \cdot w$ and were applied as corrections in all subsequent calculations.

2.2. Preparation of enzymes

Native anthranilate synthase was overexpressed in *E. coli* CB694 (tryptophan auxotroph) containing the phagemid pSTS23, which carries the *trpD* and *trpE* genes from native *S. typhimurium* [6]. The mutant anthranilate synthase (TrpD plus $TrpE^{H398M}$), which is deficient in ADIC lyase activity, was expressed from the phagemid pSTM25 in *E. coli* CB694 cells [6]. Both phagemids have a ColE1 origin of replication and the *S. typhimurium trpE* (or mutant *trpE*) and *trpD* genes under control of the *trp* promoter and terminator. Both pSTS23 and pSTM25 maintained in CB694 were gifts from Professor Ronald Bauerle at the University of Virginia, Charlottesville, USA. CB694 cells harboring pSTS23 or pSTM25 were grown in a minimal salts medium supplemented with glucose (2.5 g l^{-1}), casamino acids (2.0 g l^{-1}), ampicillin (0.10 g l^{-1}) and tryptophan (0.025 g l^{-1}). The mass concentration of tryptophan was high enough to allow protein synthesis, but not high enough to significantly inhibit expression of anthranilate synthase. Cell cultures were incubated with gentle shaking in baffled flasks at 37°C, then harvested when the absorbance at the wavelength $\lambda = 600$ nm reached approximately 1.8.

The procedure for purification of both the native and mutant anthranilate synthase was after Bauerle et al. [4] with some modifications, and is described here. First, after harvesting the cells by centrifugation at $5000 \times g_n$ ($g_n = 9.80665$ m s^{-2}), cell pellets were brought up in TE buffer [Tris (10 mM) + EDTA (1.0 mM) + DL-dithiothreitol (DTT) (1.0 mM) adjusted to pH 7.4 with HCl] and then centrifuged again. The pellets were then resuspended in a lysis buffer [MOPS (20 mM) at pH 7.4 + sodium acetate (8.3 mM) + EDTA (1.0 mM) + DTT (1.0 mM)]; the ratio of the volume of the buffer to the mass of the pellet was 4:1. The cellular suspension was frozen and stored at −20°C until needed for the next step. The frozen cell suspension was then thawed and the pH adjusted to 8.0. The cells were lysed using lysozyme (0.1 g l^{-1}) and their DNA degraded using DNAase I (1.0 mg l^{-1}) in the presence of magnesium sulfate (10 mM). Alternatively, the cells were lysed using lysozyme and agitated using a bead-beater (Biospec Products). Cellular debris was removed by centrifugation ($43\,000 \times g_n$ and at 4°C for 1 h). The salt $(NH_4)_2SO_4$ was then added

[1] Certain commercial equipment, instruments, or materials are identified in this paper to specify the experimental procedures adequately. Such identification is neither 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.

to the clear cell lysate so that the concentration of $(NH_4)_2SO_4$ was 38% of its solubility in water at 0°C. The protein precipitated by the $(NH_4)_2SO_4$ was collected by centrifugation and resuspended in a minimal volume of buffer A [potassium phosphate (0.1 M) + EDTA (0.1 mM) + DTT (1.0 mM), pH 7.0], dialyzed against several changes of buffer A to remove the salt, and concentrated via ultrafiltration at 4°C in an Amicon stirred cell. The mass concentration of the protein was adjusted to 5.0 mg ml^{-1} or less by dilution with buffer A. This solution was then loaded at a flow rate of 0.2 ml min^{-1} onto a DyeMatrex Orange-A (Amicon) affinity column that had been pre-equilibrated with buffer A. The column was washed (1 ml min^{-1}) with two bed volumes of buffer A, and anthranilate synthase was eluted at the same flow rate with buffer A containing tryptophan (50 μM). Both native and mutant *S. typhimurium* anthranilate synthase bind loosely but quite specifically to the Orange-A column. Thus, care must be taken to load the column slowly. Once the protein solution is loaded on the column, however, elution with tryptophan results in a peak of essentially pure anthranilate synthase. Fractions having the highest activity and purity (as established by SDS-PAGE analysis) were pooled. Tryptophan was removed from the pooled fractions through several cycles of dilution with buffer A and concentration with an Amicon-stirred cell. The mass concentrations of the purified proteins were 1.4 mg ml^{-1} for the native anthranilate synthase and 5.0 mg ml^{-1} for the mutant anthranilate synthase. The purities of the native and mutant anthranilate synthase were, respectively, ≈ 90 and ≈ 99%. These purities were determined by using densitometry of the anthranilate synthase bands on a Coomassie Blue-stained SDS-PAGE gel. The purified enzymes were stored at −80°C in buffer A. The preparation of the recombinant *E. coli* chorismate lyase used in the 'proof-of-reversibility' experiment (see Section 2.6) has been described previously [11].

2.3. Enzyme activity assays

The glutamine-dependent activity of the native anthranilate synthase was measured spectrophotometrically in an assay that couples reduction of the product pyruvate from reaction (1) with the oxidation of NADH catalyzed by lactate dehydrogenase. The decrease in absorbance of NADH at the wavelength λ = 340 nm was followed with a Beckman DU-650 UV/visible spectrophotometer at 22°C. A typical assay mixture consisted of [KH_2PO_4 (42 mM) + K_2HPO_4 (58 mM) at pH 7.0 + $MgCl_2$ (10 mM) + L-glutamine (20 mM) + chorismate (0.1 mM) + NADH (0.20 mM) + porcine lactate dehydrogenase (0.02 mg ml^{-1}) + anthranilate synthase (0.05 mg ml^{-1})]. The lactate dehydrogenase (Cal-Biochem, activity = 347 IU mg^{-1}00) was present in a substantial excess to ensure that it was not rate limiting. The amount of anthranilate synthase added to the assay mixture was adjusted to maintain the linearity of NADH consumption throughout the assay which lasted from 1 to 5 min. The ammonia-dependent anthranilate synthase activity was assayed as just described except that Tricine buffer (50 mM) at pH 8.0 was used instead of phosphate buffer; also $(NH_4)_2SO_4$ (50 mM) was used instead of L-glutamine.

Because pyruvate is not produced in the ADIC synthase reaction, the measurement of mutant ($TrpE^{H398M}$) anthranilate synthase activity required a different procedure. This procedure utilized the difference between the molar absorption coefficients of ADIC and chorismate at λ = 278 nm (8750 M^{-1} cm^{-1}). Thus, the increase in the absorbance with time at this wavelength was followed as ADIC was formed in the reaction mixture. A typical reaction mixture for the assay contained chorismate (0.50 mM), $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (50 mM), and mutant anthranilate synthase (0.05 mg ml^{-1}) in Tricine buffer (50 mM, adjusted to pH 8.0 with NaOH). The above assays used the barium salt of chorismate (Sigma Chemical Co.). The barium ion was removed from the solution by precipitation with excess sodium sulfate.

2.4. HPLC methods

The substances pertinent to the study of reaction (2), i.e. chorismate, anthranilate, and pyruvate, were separated by using a Hewlett-Packard

model 1100 HPLC equipped with a UV detector set at the wavelength $\lambda = 215$ nm and a Hewlett-Packard Hypersil C-18 column (4 mm i.d. $\times$ 250 mm long) thermostatted at 40°C. The mobile phase consisted of (I) [tetrabutyl ammonium hydroxide (0.01 mol l^{-1}) + ammonium phosphate (0.025 mol l^{-1}), adjusted to pH 7.0 with NaOH + acetonitrile (volume fraction $\phi = 0.01$)]; and (II) acetonitrile. The following gradient of these two mobile phases was formed: $\phi(I) = 0.90$ and $\phi(II) = 0.10$ at time $t = 0$; $\phi(I) = 0.85$ and $\phi(II) = 0.15$ at $t = 10$ min; $\phi(I) = 0.50$ and $\phi(II) = 0.50$ at $t = 20$ min. The flow rate was 0.8 ml min^{-1}. The approximate retention times were: pyruvate, 5.2 min; chorismate, 11.0 min; and anthranilate, 14.0 min.

The substances pertinent to reaction (5), i.e. chorismate, ADIC, 4-hydroxybenzoate, anthranilate, and pyruvate, were separated by using a HPLC method similar to that described above except that the wavelengh was set at $\lambda = 280$ nm and the mobile phase consisted of (I) [water + trifluoroacetic acid ($\phi = 0.001$)] and (II) [acetonitrile + trifluoroacetic acid ($\phi = 0.001$)]. The following linear gradient of these two mobile phases was formed: $\phi(I) = 1.00$ and $\phi(II) = 0$ from $t = 0$ to $t = 2$ min; $\phi(I) = 0.50$ and $\phi(II) = 0.50$ at $t = 17$ min. The approximate retention times were: pyruvate, 3.0 min; ADIC, 10.4 min; chorismate, 11.3 min; 4-hydroxybenzoate, 12.0 min; and anthranilate, 12.9 min.

2.5. Determination of chromatographic response factors

Chromatographic response factors for anthranilate, chorismate, and pyruvate were obtained by injecting standard solutions of these substances into the HPLC. The response factor for ADIC was determined by means of a material balance between a known amount of chorismate present in a reaction mixture of reaction (5) and the amount of ADIC formed in the same reaction. Chorismate and ADIC decompose over time in solution (see below); this had to be taken into account in determining their response factors. In all cases, buffers used to prepare solutions were either the same as or very similar to those used in either the calorimetric or equilibrium measurements. Gravimetric procedures were employed in preparing all solutions.

Chorismate and ADIC each decompose in aqueous solution at a certain rate to form various products over time. For example: prephenate, β-phenylpyruvate, and 4-hydroxybenzoate are spontaneous (non-enzymatic) decomposition products of chorismate [9]; anthranilate and pyruvate are formed in small amounts via the residual ADIC lyase activity of the mutant enzyme; and a substance (designated as compound A) is formed by the spontaneous Claisen rearrangement of ADIC [6].

The fractional rate of decomposition of chorismate [i.e. $(1/n)\cdot(dn/dt)$ where n is the amount of substance and t is time] was determined by dissolving a sample of chorismate (molality $m = 0.00051$ mol kg^{-1}) in buffer B [Tricine ($m = 0.0483$ mol kg^{-1}) adjusted to pH 7.8 with NaOH + NH_4Cl ($m = 0.0951$ mol kg^{-1}) + $MgCl_2$ ($m = 0.0102$ mol kg^{-1})]. This solution was immediately placed in a water bath thermostatted at $T = 298.15$ K. The loss of chorismate was then monitored for 3 h by periodically injecting aliquots of the solution into the HPLC. It was found that the fractional rate of decomposition of chorismate to a mixture of prephenate, β-phenylpyruvate, and 4-hydroxybenzoate was 0.027 h^{-1} under the specified conditions. This is comparable to the fractional rate of decomposition of 0.022 h^{-1} found previously [9] using different buffers and pH values but the same temperature.

Determination of the fractional rate of decomposition of ADIC required the in situ preparation of ADIC which is now described. First, chorismate was dissolved ($m = 0.00051$ mol kg^{-1}) in buffer B. Mutant anthranilate synthase was then added to this solution; the mass fraction w of the enzyme solution in the reaction mixture was 0.002. The reaction mixture was allowed to proceed for 3 h in the bath thermostatted at $T = 298.15$ K. The mutant anthranilate synthase protein was then removed from the reaction mixture by filtration (Millipore ultra free 4 centrifugal filter with a molecular weight cut-off of 5000, centrifugation at $5000 \times g_n$ for ≈ 30 min). This in situ prepared solution of ADIC was then returned to the bath

thermostatted at $T = 298.15$ K and, at the same time, a sample of the solution was injected into the HPLC and the area of the ADIC chromatographic peak measured. Aliquots were periodically taken out and injected into the HPLC over a span of 4.5 h, and the area of the ADIC chromatographic peak measured for each injection. From these results it was found that the fractional rate of decomposition of ADIC was 0.034 h^{-1} under the specified conditions. Plots of the chromatographic peak areas of chorismate and of ADIC as a function of time were both linear.

As mentioned above, the response factor of ADIC was determined by using a procedure that relied upon a material balance between a known amount of chorismate initially present in a reaction mixture of reaction (5) and the principal product of the reaction, namely ADIC. The amount of chorismate in solution at a given time was determined by using its HPLC peak area and response factor. At the same time, the area of the peak corresponding to ADIC was measured. Then, by material balance, the amount of ADIC in the solution was determined and the response factor of ADIC calculated. At the outset, the reaction mixture used for the procedure contained chorismate ($m = 0.00051$ mol kg^{-1}) and mutant anthranilate synthase ($w = 0.002$) in buffer B incubated in the water bath at $T = 298.15$ K. As the reaction progressed, aliquots were taken out and injected into the HPLC. This was done at 30 min intervals for a period of 3 h. The chromatographic results obtained from these periodic injections were analyzed as follows. First, the molalities of chorismate and anthranilate were calculated by using the areas of their respective chromatographic peaks and their known response factors. The sum of the molalities of the decomposition products of chorismate (prephenate, β-phenylpyruvate, and 4-hydroxybenzoate) was calculated by using the known fractional rate of decomposition of chorismate. The sum of the molalities of ADIC and of compound A (the Claisen rearranged form of ADIC) were calculated by material balance. The known fractional rate of decomposition of ADIC to compound A was applied as a correction to the molality of ADIC present at a given time. The response factor for ADIC was then calculated by using the corrected molality of ADIC together with the measured area of the ADIC chromatographic peak. The average value of the response factor F obtained from these experiments had a statistical uncertainty (two estimated standard deviations of the mean) of $0.05 \cdot F$. Response factors obtained from each separate injection of the reaction mixture into the HPLC showed no systematic trend with time of injection.

The assignment of the retention time of ADIC as 10.4 min was based on the assumption that the newly-formed peak seen in the chromatogram corresponded to a product, namely ADIC, that was being produced in reaction (5) by the action of the mutant anthranilate synthase on the substrates chorismate and ammonia. That this newly-formed peak corresponds to ADIC is a reasonable assumption since the mutant enzyme used in the reaction is of high purity, and has been previously used to produce a compound that was definitively shown by NMR spectroscopy to be ADIC [5]. Additional evidence for the assignment of the peak at 10.4 min to ADIC was provided by the experiment described in the following section.

2.6. Proof-of-reversibility experiment

An initial experiment was performed to investigate whether or not reaction (5) could be approached from both reverse and forward directions, i.e. whether or not it was reversible. This experiment, termed the 'proof-of-reversibility' experiment, was important not only because it provided initial proof of the reversibility of reaction (5), but also because it further substantiated the assignment of the peak at 10.4 min to ADIC. The experiment is now described. First, mutant anthranilate synthase (mass fraction $w = 0.002$) was added to a mixture of the substrates (chorismate + ammonia) in buffer B (see Section 2.4). After 65 min of reaction, the mutant anthranilate synthase protein was removed by filtration through a Millipore ultra-free 4 centrifugal filter with a molecular weight cut-off of 5000 (centrifugation was at $5000 \times g_n$ for 30 min at 3°C). This left chorismate and ADIC, along with some of their decomposition products, in the filtrate. Choris-

mate lyase ($w = 3 \times 10^{-5}$), which removes chorismate by converting it to 4-hydroxybenzoate, was then added to the filtrate. HPLC confirmed that most (73%) of the chorismate was removed in this manner. The reaction mixture was then filtered through a centrifugal filter as described above to remove the chorismate lyase. The resulting mixture contained mostly ADIC, but also contained some chorismate as well as 4-hydroxybenzoate and decomposition products. By this procedure, ADIC was prepared enzymatically. Mutant anthranilate synthase ($w = 0.001$) was added back to this final ADIC solution. An increase in the chorismate peak and a simultaneous decrease in the assigned ADIC peak was observed, providing strong evidence that reaction (5) is indeed reversible and that the peak that formed in the forward reaction and diminished in the reverse reaction corresponded to ADIC. An approximate value of K' for reaction (5) was also calculated.

2.7. Determination of apparent equilibrium constants

The procedure used for determining the apparent equilibrium constant for reaction (5) is now described. The first step was the preparation of the reaction mixture (chorismate + ammonia) in the buffer (Tricine + NaOH) and the addition of mutant anthranilate synthase. Following a suitable time for chemical equilibrium (2.5 h at $T =$ 302.75 K and 4 h at $T = 288.15$ K), values of the reaction quotient Q' for reaction (5) were determined at several time points by using the HPLC. The reaction mixture was then divided into two very nearly equal portions. Some chorismate was then added to one portion and some mutant anthranilate synthase was added to the other portion. Following a suitable period of time for achievement of chemical equilibrium (0.5–2 h), values of Q' for reaction (5) were again measured by using the HPLC to analyze these two separate portions at several time points. The agreement of the values of Q' obtained from the two portions (i.e. for both addition experiments) with each other and with the value of Q' obtained from the original reaction mixture prior to any of the additions is good evidence that equilibrium had been reached and that Q' can be identified as K'. All equilibrations of solutions were performed by placing the bottles (glass with Teflon caps) containing the solutions in a thermostatted shaker bath (≈ 50 rev.min^{-1}).

An attempt was made to measure the apparent equilibrium constant K' for reaction (2) by measuring the extent of the reaction with equilibrium being approached from both directions. The solution used for the forward direction of reaction contained the mixture [chorismate ($m = 0.0018$ mol kg^{-1}) + NH_4Cl ($m = 0.0043$ mol kg^{-1})] in the buffer C: [K_2HPO_4 ($m = 0.099$ mol kg^{-1}) + H_3PO_4 ($m = 0.023$ mol kg^{-1}) + $MgCl_2$ ($m =$ 0.0010 mol kg^{-1}), pH 7.20]. The solution used for the reverse reaction contained [anthranilate ($m =$ 0.0025 mol kg^{-1}) + pyruvate ($m = 0.0035$ mol kg^{-1})] in buffer C. Native anthranilate synthase ($w = 0.0004$) was added to these two solutions. These two solutions were then placed in a bath thermostatted at $T = 298.15$ K. Following equilibration for 24 h, the concentrations of chorismate and anthranilate were measured by using the chromatographic procedure described above.

2.8. Microcalorimetry

Three heat-conduction microcalorimeters were used for the measurement of the enthalpy of reaction (2). 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 previously [18,19]. However, some significant changes have been made recently in the method of data collection. Specifically, the voltages of the thermopiles of the microcalorimeters are now measured with Hewlett-Packard model 34 420A 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

and ≈ 0.40 cm^3 of solution. The substrate solution was placed in the 0.55 cm^3 compartment and the enzyme solution was placed in the 0.40 cm^3 compartment. While a direct measurement of the enthalpy change for reaction (1) would have been desirable, a direct measurement of this quantity was seriously complicated by the inherent glutaminase activity of anthranilate synthase [4]. To circumvent this problem and to obtain an enthalpy change for reaction (1), reaction (2) was studied by microcalorimetry. Later (see Section 3), a thermochemical cycle that uses the calorimetric results obtained for reaction (2) together with calorimetric results for reaction (3) [16] will be used to calculate the enthalpy change for reaction (1). A phosphate buffer containing both $MgCl_2$ and NH_4Cl was used for the calorimetric experiments. The substrate solution was prepared by the addition of chorismate to a portion of this buffer. The molality of the NH_4Cl was ≈ 12 times greater than the molality of the chorismate. The enzyme solution was prepared by the addition of native anthranilate synthase to a portion of the same stock buffer solution 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. Reaction (2) was then allowed to proceed for 75–133 min after mixing, at which point the vessels were removed from the microcalorimeters. The HPLC was promptly used to determine the mole fraction of chorismate remaining in solution. The 'blank' enthalpy change for mixing of the substrate solution with the buffer was 0.40 mJ. For the mixing of the enzyme solution with the buffer, the 'blank' enthalpy was 0.33 mJ. These 'blank' enthalpies of mixing were applied as corrections to the measured calorimetric enthalpies which were ≈ -360 mJ.

It was judged that a reliable value of the calorimetric molar enthalpy of reaction $\Delta_r H_m$(cal) for reaction (5) could not be obtained using microcalorimetry. One reason is that the calorimetry would require the measurement of a small amount of heat produced over a relatively long period of time (≈ 3 h). This would make the heat measurement very uncertain. Additionally, there are several side reactions involving chorismate and ADIC (see above) that seriously complicate the analysis of calorimetric results for reaction (5). For these reasons it was decided that the best approach for obtaining the enthalpy change for reaction (5) was to measure the temperature dependency of the apparent equilibrium constant of this reaction. Temperatures of 288.15, 293.15, 298.15, and 302.75 K were chosen.

2.9. 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 pH values of the solutions used in this study. All pH measurements pertain to the temperature of interest.

3. Results and discussion

3.1. Thermodynamic formalism

The treatment of the results requires first, the introduction of a thermodynamic formalism that makes the important distinction between overall biochemical reactions that involve sums of species and reactions that involve specific species [14]. Having made this distinction, the results are then analyzed in terms of an equilibrium model [15] that is used to calculate the fractions of the various forms of the pertinent species. This model is also used to relate measured quantities [apparent equilibrium constants K' and calorimetrically determined molar enthalpies of reaction $\Delta_r H_m$(cal)] to standard thermodynamic quantities (equilibrium constants K and standard molar enthalpies of reaction $\Delta_r H_m^o$).

Thus, the apparent equilibrium constants for the two reactions [(2) and (5)] that were studied experimentally are

$$K' = m(\text{anthranilate}) \cdot m(\text{pyruvate}) / [m\ (\text{chorismate}) \cdot m(\text{ammonia})] \quad (7)$$

$$K' = m(\mathrm{ADIC}) \cdot m^{\circ} / [m(\mathrm{chorismate}) \cdot m(\mathrm{ammonia})] \quad (8)$$

The molalities m in the above equations are the total molalities of the various charged and uncharged species that are formed from the dissociation of the various substances in solution. The quantity m° has been used to keep the appropriate equilibrium constants dimensionless in the above equations. In discussing the thermodynamics of these reactions, it will prove useful to introduce the respective reference reactions that pertain to specific ionic forms:

$$\mathrm{chorismate}^{2-}(\mathrm{aq}) + \mathrm{NH}_4^+(\mathrm{aq}) = \mathrm{anthranilate}^-(\mathrm{aq}) + \mathrm{pyruvate}^-(\mathrm{aq}) + \mathrm{H_2O(l)} + \mathrm{H}^+(\mathrm{aq}) \quad (9)$$

$$\mathrm{chorismate}^{2-}(\mathrm{aq}) + \mathrm{NH}_4^+(\mathrm{aq}) = \mathrm{ADIC}^-(\mathrm{aq}) + \mathrm{H_2O(l)} \quad (10)$$

The equilibrium constants for reactions (9) and (10), respectively, are

$$K = m(\mathrm{anthranilate}^-) \cdot m(\mathrm{pyruvate}^-) \cdot m(\mathrm{H}^+) / \left[m(\mathrm{chorismate}^{2-}) \cdot m(\mathrm{NH}_4^+) \cdot m^{\circ} \right] \quad (11)$$

$$K = m(\mathrm{ADIC}^-) \cdot m^{\circ} / \left[m(\mathrm{chorismate}^{2-}) \cdot m(\mathrm{NH}_4^+) \right] \quad (12)$$

The standard state used in this study for the solutes is the hypothetical ideal solution of unit molality ($m^{\circ} = 1$ mol kg^{-1}); as a consequence of the Gibbs–Duhem equation, the limit of the activity of water a_w is 1 as the sum of the molalities of the solutes goes to 0.

3.2. Results of experiments

The result of the attempted measurement of K' for reaction (2) was that there was no chorismate detectable in either of the two solutions (forward and reverse directions of reaction) used in the experiments. Using the approximate limit of detection of chorismate ($m \approx 3 \cdot 10^{-7}$ mol kg^{-1}) by the HPLC and allowing for decomposition of chorismate, we obtain $K' > 3000$ from the experiments involving the forward and reverse directions of reaction. These results pertain to $T =$ 298.15 K and pH = 7.2. Later it will be seen that the value of K' for reaction (2) is approximately 10^{28}.

The results of the calorimetric measurements for reaction (2) and the equilibrium measurements for reaction (5) are given in Tables 2 and 3, respectively. It should be noted that there were three different types of experiments from which values of Q for reaction (5) were determined. These were a type 'P' experiment starting with (chorismate + NH_4Cl) and for which Q' has reached an essentially constant value (a plateau value); a type 'E' experiment in which additional enzyme (mutant anthranilate synthase) was added to the reaction mixture; and a type 'S' experiment in which additional substrate (chorismate) was added to the reaction mixture. The 'proof-of-reversibility' experiment (see Section 2.6) led to the result $K' \approx 8.65$ at $T = 298.15$ K, pH 7.9, and $I_m \approx 0.15$ mol kg^{-1}. This result is considered approximate, primarily because no attempt was made to determine the molality of NH_4Cl which may have changed somewhat due to the two filtrations that the solution underwent. Nevertheless, its agreement with the value $K' = 8.44$, obtained under very similar conditions for the 'P', 'S', and 'E' experiments at $T = 298.15$ K (see Table 3 and above), helps to confirm that chemical equilibrium had indeed been achieved.

3.3. Uncertainties in results

The uncertainties in the measured value of $\Delta_r H_m$(cal) and of K' represent only the random errors inherent in the measurements and do not reflect possible systematic errors which are now considered. We judge that reasonable estimates of the standard uncertainties [20] due to possible systematic errors in the measured value of $\Delta_r H_m$(cal) are: $0.02 \cdot \Delta_r H_m$(cal) due to impurities in the chorismate sample (this includes the uncertainty in the moisture content); $0.006 \cdot \Delta_r H_m$(cal)

Table 2
Results of the calorimetric measurements at $T = 298.15$ K for reaction (2): chorismate(aq) + ammonia(aq) = anthranilate(aq) + pyruvate(aq) + H_2O(l)[a]

Experiment	pH	m (K_2HPO_4) (mol kg^{-1})	$10^3 \cdot m$ (H_3PO_4) (mol kg^{-1})	$10^3 \cdot m$ ($MgCl_2$) (mol kg^{-1})	m (NH_4Cl) (mol kg^{-1})	$10^3 \cdot m$ (chorismate) (mol kg^{-1})	I_m (mol kg^{-1})	$\Delta_r H_m$(cal) (kJ mol^{-1})
1	7.81	0.1809	7.92	1.68	0.0438	3.31	0.54	−122.2
2	7.81	0.1806	7.91	1.68	0.0437	3.28	0.54	−123.4
3	7.81	0.1840	8.06	1.71	0.0445	3.66	0.55	−124.7
4	7.81	0.1859	8.14	1.73	0.0450	3.28	0.56	−123.3
5	7.81	0.1848	8.10	1.72	0.0447	3.13	0.55	−123.7
6	7.77	0.1928	8.44	1.79	0.0466	3.51	0.58	−123.1
7	7.77	0.1924	8.43	1.79	0.0465	3.44	0.58	−124.6
8	7.77	0.1903	8.34	1.77	0.0460	3.06	0.57	−124.9
9	7.77	0.1929	8.45	1.79	0.0466	3.52	0.58	−122.5
10	7.77	0.1903	8.34	1.77	0.0460	3.07	0.57	−124.2

[a]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. The values of the ionic strength I_m are calculated. The mass fraction w of the native anthranilate synthase in solution was ≈ 0.0001. The mole fraction of unreacted chorismate as determined by HPLC was < 0.0002. The results for two series of measurements done at slightly different pH values and ionic strengths have been pooled and used to calculate $\langle \Delta_r H_m(\text{cal}) \rangle = -(123.7 \pm 0.6)$ kJ mol^{-1} at $\langle \text{pH} \rangle = 7.79$ and $\langle I_m \rangle = 0.56$ mol kg^{-1}. The uncertainty given here is equal to two estimated standard deviations of the mean. An estimate of total error for $\Delta_r H_m$(cal) of ± 5.4 kJ mol^{-1} is assigned in the text (see Section 3.3).

Table 3
Results of equilibrium measurements for reaction (5): chorismate(aq) + ammonia(aq) = ADIC(aq) + H_2O(l)[a]

T (K)	Type of experiment	pH	m (Tricine) (mol kg^{-1})	m (NaOH) (mol kg^{-1})	m ($MgCl_2$) (mol kg^{-1})	$10^3 \cdot m$ (chorismate) (mol kg^{-1})	m (NH_4Cl) (mol kg^{-1})	$10^3 \cdot m$ (ADIC) (mol kg^{-1})	I_m (mol kg^{-1})	Q'	K'
288.15	P	8.08	0.0465	0.0310	0.0112	0.0708	0.09177	0.0617	0.15	9.50 ± 0.09	
288.15	E	8.08	0.0463	0.0308	0.0112	0.0519	0.09138	0.0446	0.15	9.41 ± 0.18	9.50 ± 0.08
288.15	S	8.08	0.0468	0.0311	0.0113	0.0473	0.09219	0.0418	0.15	9.58 ± 0.09	(± 2.2)
293.15	P	8.00	0.0467	0.0310	0.0112	0.0290	0.09206	0.0240	0.15	8.99 ± 0.12	
293.15	E	8.00	0.0465	0.0308	0.0112	0.0225	0.09167	0.0186	0.15	9.02	9.02 ± 0.08
293.15	S	8.00	0.0469	0.0312	0.0113	0.0209	0.09246	0.0176	0.15	9.10	(± 2.0)
298.15	P	7.86	0.0470	0.0295	0.00995	0.0458	0.09255	0.0342	0.14	8.07 ± 0.18	
298.15	E	7.86	0.0468	0.0294	0.00991	0.0152	0.09216	0.0119	0.14	8.47 ± 0.18	8.44 ± 0.26
298.15	S	7.86	0.0472	0.0296	0.00999	0.0365	0.09294	0.0298	0.14	8.79 ± 0.54	(± 1.9)
302.75	P	7.76	0.0471	0.0295	0.00997	0.0349	0.09274	0.0237	0.14	7.31 ± 0.07	
302.75	E	7.76	0.0469	0.0294	0.00992	0.0070	0.09226	0.0050	0.14	7.67	7.51 ± 0.22
302.75	S	7.76	0.0473	0.0297	0.01000	0.0182	0.09308	0.0131	0.14	7.72 ± 0.54	(± 1.7)

[a] The molalities m are those of the substances in solution at equilibrium and which are each equal to the sums of the molalities of the indicated substances in their various ionic forms. The following substances were also present in the reaction mixtures at the specified approximate molalities: K_2HPO_4, $m \approx 0.003$ mol kg^{-1}; KH_2PO_4, $m \approx 0.002$ mol kg^{-1}; EDTA, $m \approx 5 \times 10^{-6}$ mol kg^{-1}; and DTT, $m \approx 0.00001$ mol kg^{-1}. The mass fraction w of the mutant anthranilate synthase in solution was ≈ 0.0003. The ionic strength I_m is calculated. The three different types of equilibrium experiments are described in the text (see Section 2) and are: P, an experiment starting with (chorismate + NH_4Cl) and for which the reaction quotient Q' has reached an essentially constant value; E, an experiment involving addition of further enzyme (mutant anthranilate synthase) to the reaction mixture; and S, an experiment involving addition of further substrate (chorismate) to the reaction mixture. The values of K' are the averages of all of the individual results for Q' at that temperature. The uncertainties in the values of Q' and of K' are equal to two estimated standard deviations of the mean. Where no uncertainty is given for a value of Q', the result is based on a single measurement. Estimates of total error for K' are given in parentheses (see Section 3.3) below the value of K'.

due to uncertainties in the calorimetric measurements (this includes uncertainties in the 'blank' enthalpies); and $0.005 \cdot \Delta_r H_m(\text{cal})$ due to possible uncertainties in the determination of the amount of unreacted chorismate and for possible side reactions. These estimates of possible systematic error are combined in quadrature together with the statistical uncertainty in the measured value of $\Delta_r H_m(\text{cal})$, 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 value: $\Delta_r H_m(\text{cal}) = -(123.7 \pm 5.4)$ kJ mol^{-1} for reaction (2) at $T = 298.15$ K, pH 7.79, and $I_m = 0.56$ mol kg^{-1}.

Similarly, we judge that reasonable estimates of error in the values of K' for reaction (5) are: $0.05 \cdot K'$ due to possible error in the determination of the response factors, particularly that of ADIC; and $0.10 \cdot K'$ due to possible chromatographic error in the determinations of the very small amounts of ADIC and chorismate. Because of the agreement of the values of Q' determined from the three different types of equilibrium measurements (see Table 3), the error due to a possible failure of the reactions to reach equilibrium is negligible. The final results for the values of K' for reaction (5) that include the above standard uncertainties are given in Table 3.

3.4. Ionization constants

The pK values and standard molar enthalpies for the H^+ and Mg^{2+} dissociation reactions of the reactants and of the buffers are needed to relate the experimental results for reactions (2) and (5) to thermodynamic quantities for the respective reference reactions (9) and (10). These pK values and standard molar enthalpies $\Delta_r H_m^o$ are given in Table 4. The pK values for L-glutamine and for L-glutamic acid will be used in subsequent calculations and are also included in Table 4. The pK and $\Delta_r H_m^o$ values for the ionization of anthranilate, L-glutamine, L-glutamic acid, pyruvic acid, and Tricine are from Martell and Smith [21]. Where necessary, the pK and $\Delta_r H_m^o$ values were adjusted to $I_m = 0$ by using an 'ion-size' parameter of 1.6 kg$^{1/2}$ mol$^{-1/2}$ in the extended Debye–Hückel equation used to estimate the activity coefficients of the aqueous species in solution. The thermodynamic quantities for the ionizations of NH_4^+ and $H_2PO_4^-$ are calculated, respectively, from Wagman et al. [22] and from Cox et al. [23]. The thermodynamic quantities for the dissociation of Mg^{2+} from $MgHPO_4$ are from Clarke et al. [24]. The pK of ADIC is estimated from the pK of L-*trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHAA) which was measured by Hefford et al. [25]. DHAA should be a suitable analog for ADIC since the only difference between DHAA and ADIC is that DHAA has a hydroxyl group in place of the pyruvate group present on ADIC. Hefford et al. [25] used a potentiometric titration to obtain the value pK = 8.56 for DHAA at $T = 298.15$ K and $I_c = 0.10$ M. Adjustment of this value for the effect of ionic strength leads to p$K = 8.78$ at $I = 0$. An additional check on the validity of DHAA as an analog for ADIC comes from use of the estimation method of Perrin et al. [26]. Specifically, use of their [26] Table 4.2 leads to the value p$K = 8.8$ for ADIC. Accordingly, for purposes of the calculations to be described below, we adopt p$K = 8.8$ for ADIC at $T = 298.15$ K and $I = 0$. By using known values [21] of the standard molar entropy changes $\Delta_r S_m^o$ for the ionizations of 3-aminopropanoic and 3-aminobutanoic acids, we estimate $\Delta_r S_m^o \approx -38$ J K^{-1} mol^{-1} for the ionization of ADIC. This estimated value of $\Delta_r S_m^o$ is combined with the estimated value of the pK of ADIC to obtain $\Delta_r H_m^o \approx 39$ kJ mol^{-1} for the ionization of ADIC. This value of $\Delta_r H_m^o$ must be considered as very approximate. On the basis of the known pK values of other substances that are structurally similar to chorismic acid, its two lowest pK values were both previously estimated [9] to be ≤ 4.5. This estimate was also found to be consistent with the approximate results obtained from a potentiometric titration that was performed [9] on chorismic acid. The values of the thermodynamic quantities given in Table 4 will be used in subsequent calculations.

3.5. Equilibrium model

The equilibrium model used for the calculation

Table 4
The pK values and standard molar enthalpy changes $\Delta_r H_m^o$ for the aqueous H^+ and Mg^{2+} dissociation reactions of substances pertinent to this study at $T = 298.15$ K and $I = 0$[a]

Reaction	pK	$\Delta_r H_m^o$ / kJ mol^{-1}
$ADIC^- = ADIC^{2-} + H^+$	8.8[b]	39[b]
Anthranilic acid$^+$ = anthranilic acid^0 + H^+	2.08	15
Anthranilic acid^0 = anthranilate$^-$ + H^+	4.96	11
Chorismate$^-$ = chorismate^{2-} + H^+	4.5[b]	
L-Glutamine$^+$ = L-glutamine$^\pm$ + H^+	2.19	3.0
L-Glutamine$^\pm$ = L-glutamine$^-$ + H^+	9.21	40
L-Glutamic acid^0 = L-glutamate$^-$ + H^+	4.30	3.0
L-Glutamate$^-$ = L-glutamate^{2-} + H^+	9.96	40
$NH_4^+ = NH_3 + H^+$	9.25	52.2
Pyruvic acid^0 = pyruvate$^-$ + H^+	2.48	12.1
$H_2PO_4^- = HPO_4^{2-} + H^+$	7.212	3.6
$MgHPO_4 = HPO_4^{2-} + Mg^{2+}$	2.71	−12.2
Tricine = tricine$^-$ + H^+	8.135	31

[a]The standard state for the solutes is the hypothetical ideal solution of unit molality. See Section 3.4 for the basis of these values.
[b]Estimated (see Section 3).

of the equilibrium constants K and standard molar enthalpies $\Delta_r H_m^o$ for the reference reactions from the measured values of K' and $\Delta_r H_m$(cal) has been described previously [15]. This model has been modified [27] so that it now uses Mathematica [28] to solve the chemical equilibrium equations and calculate the desired thermodynamic quantities. The Gibbs energy minimization uses an algorithm obtained from Alberty and Krambeck (personal communication; in review). The calculations also include corrections for non-ideality and are made self-consistent [15] with regard to ionic strength. The non-ideality corrections are based on the extended Debye–Hückel equation [15] in which the 'ion-size' parameter has been set at 1.6 kg$^{1/2}$ mol$^{-1/2}$.

By applying this model and by using the experimental result for $\Delta_r H_m$(cal) and the thermodynamic quantities given in Table 4, we calculate $\Delta_r H_m^o = -(116.3 \pm 5.4)$ kJ mol^{-1} at $T = 298.15$ K and $I = 0$ for the reference reaction (9). The equilibrium model was also used to calculate the change in binding of the hydrogen ion $\Delta_r N$ (H^+) to reactants and products. Thus, $\Delta_r N$ (H^+) = −0.976 for reaction (2) at $T = 298.15$ K, pH 7.79, and $I_m = 0.56$ mol kg^{-1}. This value of $\Delta_r N$ (H^+) was used in the buffer protonation correction [29] to calculate the value of $\Delta_r H_m^o$ for the reference reaction (9) from the experimentally determined value of $\Delta_r H_m$(cal) for the overall biochemical reaction (2).

Similarly, we used the equilibrium model together with the experimentally determined values of K' for reaction (5) and the values of the thermodynamic quantities given in Table 4 to calculate the following values of K for reference reaction (10) at $I = 0$: $K = 23.3 \pm 0.2$ at $T = 288.15$ K; $K = 22.0 \pm 0.2$ at $T = 293.15$ K; $K = 20.8 \pm 0.7$ at $T = 298.15$ K; and $K = 18.7 \pm 0.6$ at $T = 302.75$ K. The uncertainties given here are

based on the respective uncertainties in the experimentally determined values of K' for reaction (5). Application of the model of Clarke and Glew [30] to the temperature dependence of these equilibrium constants leads to $K = (20.3 \pm 0.6)$, $\Delta_r G^o_m = -(7.46 \pm 0.07)$ kJ mol^{-1} and $\Delta_r H^o_m = -(10.6 \pm 3.2)$ kJ mol^{-1} for reaction (10) at $T = 298.15$ K and $I = 0$. The uncertainties given here are equal to two estimated standard deviations of the mean and are based solely on the fit of the model to the data. An alternative calculation used a least-squares approach in which the quantity $\Sigma[K'\ (\text{measured}) - K'\ (\text{calculated})]^2$ was minimized by varying the values of K and $\Delta_r H^o_m$ for reaction (10). The results of this calculation were $K = 20.3$ and $\Delta_r H^o_m = -10.4$ kJ mol^{-1} for reaction (10) at $T = 298.15$ K and $I = 0$. The values of the deviations [K' (measured) $- K'$ (calculated)] from the fit are: -0.08 at $T = 288.15$ K; 0.07 at $T = 293.15$ K; 0.21 at $T = 298.15$ K; and -0.15 at $T = 302.75$ K. Thus, the results are well represented by the model. In the absence of the variance–covariance matrix for the least-squares minimization calculation, standard deviations for K and $\Delta_r H^o_m$ were not calculated. The difference of 0.2 kJ mol^{-1} between the values of $\Delta_r H^o_m$ calculated by these two methods is not considered significant; an average of the two values for $\Delta_r H^o_m$ will be used subsequently.

The uncertainties in the calculated values of $\Delta_r H^o_m$ and K for the respective reference reactions (9) and (10) have two components: the experimental uncertainties in the measured quantities [$\Delta_r H_m$(cal) and K'] and possible errors in the quantities used in the equilibrium model. This latter component of uncertainty was examined by perturbing each of the pertinent quantities in the model with an assumed possible error. Specifically, the appropriate pK values (see Table 4) were perturbed as follows: $ADIC^-$, ± 0.3; NH_4^+, ± 0.01; $H_2PO_4^-$, ± 0.01; $MgHPO_4$, ± 0.02; and Tricine, ± 0.02. The values of $\Delta_r H^o_m$ for the dissociation reactions were also perturbed: $ADIC^-$, ± 20 kJ mol^{-1}; NH_4^+, ± 0.5 kJ mol^{-1}; $H_2PO_4^-$, ± 0.2 kJ mol^{-1}; $MgHPO_4$, ± 0.5 kJ mol^{-1}; and Tricine, ± 1.0 kJ mol^{-1}. The 'ion-size' parameter used in the activity coefficient model was also perturbed by ± 0.3 kg$^{1/2}$ mol$^{-1/2}$. Since the pK values for the ionizations of anthranilic, chorismic, and pyruvic acids are far removed from the pH values at which experiments were performed, possible errors in the thermodynamic quantities for their ionizations have a negligible effect on the values of the calculated quantities.

The combined effect of these perturbations in the calculated value of $\Delta_r H^o_m$ for reaction (9) was relatively small (0.4 kJ mol^{-1}). As a consequence, the final uncertainty in the calculated value of $\Delta_r H^o_m$ for reaction (9) is the same as the uncertainty given above and is attributable to possible experimental errors. Thus, the final result is $\Delta_r H^o_m = -(116.3 \pm 5.4)$ kJ mol^{-1} for reaction (9) at $T = 298.15$ K and $I = 0$.

Similarly, the combined effects of the perturbations in the calculated value of K for reaction (10) was found to be ± 4.1. Here the principal source of possible error in the perturbation calculation is the uncertainty in the pK of $ADIC^-$. The second most significant possible error is the uncertainty in the value of the 'ion-size' parameter used to calculate the activity coefficients of the species in solution. The effect on the value of $\Delta_r H^o_m$ for reaction (10) due to the combined perturbations listed above was only 0.14 kJ mol^{-1}. This is because the value of $\Delta_r H^o_m$ is based on $(\partial \ln K/\partial T)_P$ which is changed only slightly by the perturbations. Combination of the uncertainties attributable to the model calculations with the experimental uncertainties leads to the final results for reaction (10) at $T = 298.15$ K and $I = 0$: $K = (20.3 \pm 4.5)$, $\Delta_r G^o_m = -(7.5 \pm 0.6)$ kJ mol^{-1}, $\Delta_r H^o_m = -(10.5 \pm 3.2)$ kJ mol^{-1}, and $\Delta_r S^o_m = -(10 \pm 11)$ J K^{-1} mol^{-1}.

3.6. Comparisons with values in the literature

The only result from the literature for any of the reactions studied herein is that of Kozlowski et al. [31] who reported the value $K' = 2.67$ at pH 8.0 for reaction (5). The buffer used in this study contained $(NH_4)_2SO_4$ ($c = 0.050$ M). The temperature was not reported but was most likely ≈ 298 K. We use their result with the equilibrium model to calculate the value $K = 6.3$ for reaction (10) at

$T = 298.15$ K and $I = 0$. This value does not appear to be in agreement with the result $K = (20.3 \pm 4.3)$ obtained in this study.

Ganem [32] estimated the value $\Delta_r H_m^o \approx -4.6$ kcal mol^{-1} $= -19$ kJ mol^{-1} for reaction (5). This very approximate value was based on a molecular mechanics calculation. While the state of ionization of the chorismate and of the ADIC were not specified in his [32] calculation, the result is proximate to the result obtained in this study, namely $\Delta_r H_m^o = -(10.5 \pm 3.2)$ kJ mol^{-1} for reaction (10) at $T = 298.15$ K and $I = 0$. As pointed out earlier, thermodynamic information on the reactions of interest is sparse and there are no other results from the literature with which additional comparisons can be made.

3.7. Thermochemical cycles

A surprising amount of useful information can be obtained by using thermodynamic cycle calculations coupled with a few judicious estimates. Kishore et al. [16] give values of K, $\Delta_r G_m^o$, and $\Delta_r S_m^o$ for the reaction

$$\text{L-glutamine}^{\pm}(\text{aq}) + \text{H}_2\text{O(l)} = \text{L-glutamine}^{-}(\text{aq}) + \text{NH}_4^+(\text{aq}) \quad (13)$$

The sum of reactions (10) and (13) is the reaction

$$\text{chorismate}^{2-}(\text{aq}) + \text{L-glutamine}^{\pm}(\text{aq}) = \text{ADIC}^{-}(\text{aq}) + \text{L-glutamate}^{-}(\text{aq}) \quad (14)$$

Thus, since the values of $\Delta_r G_m^o$, $\Delta_r H_m^o$, and $\Delta_r S_m^o$ for reactions (10) and (13) are known, these quantities can be also calculated for reaction (14). The results of this calculation are given in Table 5.

It is also possible to calculate the standard molar enthalpies of formation $\Delta_f H_m^o$ of anthranilate$^-$(aq), chorismate^{2-}(aq), and ADIC$^-$(aq). First, we have $\Delta_f H_m^o = -401.2$ kJ mol^{-1} for anthranilic acid(s) from Pedley et al. [33]. Also, Larsen and Magid [34] measured the standard molar enthalpy of solution $\Delta_{sol} H_m^o$ of anthranilic acid(s); their result was $\Delta_{sol} H_m^o = 26.7$ kJ mol^{-1} at $T = 298.15$ K. Although Larsen and Magid [34] did not report the pH of the final solution, we shall assume that it is sufficiently basic that the predominant species is anthranilate$^-$(aq). Thus, $\Delta_f H_m^o = -374.5$ kJ mol^{-1} for anthranilate$^-$(aq). This value with the result $\Delta_r H_m^o = -116.3$ kJ mol^{-1} for reaction (9) and with values of $\Delta_f H_m^o$ for H_2O(l) [23], for NH_4^+(aq) [23], and for pyruvate$^-$(aq) [35] leads to $\Delta_f H_m^o = -1007.0$ kJ mol^{-1}

Table 5
Equilibrium constants K, standard molar Gibbs energy changes $\Delta_r G_m^o$, standard molar enthalpy changes $\Delta_r H_m^o$, and standard molar entropy changes $\Delta_r S_m^o$ at $T = 298.15$ K and $I = 0$ for several reference reactions in aqueous solution that are pertinent to this study[a]

Reaction	K	$\Delta_r G_m^o$ / kJ mol^{-1}	$\Delta_r H_m^o$ / kJ mol^{-1}	$\Delta_r S_m^o$ / kJ mol^{-1}
(9) Chorismate^{2-} + NH_4^+ = anthranilate$^-$ + pyruvate$^-$ + H^+ + H_2O	$\approx 4 \times 10^{22}$	≈ -129	-116.3 ± 5.4	≈ 42
(10) Chorismate^{2-} + NH_4^+ = ADIC$^-$ + H_2O	20.3 ± 4.5	-7.5 ± 0.6	-10.5 ± 3.2	-10 ± 11
(13) L-Glutamine$^{\pm}$ + H_2O = L-glutamate$^-$ + NH_4^+	200	-13.1	-25.2 ± 0.3	-40
(14) Chorismate^{2-} + L-glutamine$^{\pm}$ = ADIC$^-$ + L-glutamate$^-$	4.1×10^3	-20.6	-35.7 ± 3.2	-52 ± 12
(15) Chorismate^{2-} + L-glutamine$^{\pm}$ = anthranilate$^-$ + Pyruvate$^-$ + L-glutamate$^-$ + H^+	$\approx 7 \times 10^{24}$	≈ -142	-141.5 ± 5.4	≈ 0
(16) ADIC$^-$ = anthranilate$^-$ + pyruvate$^-$ + H^+	$\approx 2 \times 10^{21}$	≈ -122	-105.8 ± 6.3	≈ 52

[a] The results for reaction (13) are from Kishore et al. [16]. The results for reactions (14), (15), and (16) were calculated (see Section 3). In some cases the values are based on estimates; these values are all designated as being approximate. The standard state for the solutes is the hypothetical ideal solution of unit molality; the activity of water $a_w \rightarrow 1$ as the sum of the molalities of the solutes $\rightarrow 0$. The basis of the uncertainities is given in the text (see Section 3).

for chorismate^{2-}(aq). Use of this value together with the result $\Delta_r H^o_m = -10.5$ kJ mol^{-1} for reaction (10) and the $\Delta_f H^o_m$ values of H_2O(l) and of NH_4^+(aq) [23], leads to $\Delta_f H^o_m = -864.9$ kJ mol^{-1} for ADIC$^-$(aq). All of these calculations pertain to $T = 298.15$ K and the pressure $P = 0.1$ MPa (1 bar = 0.986923 atm).

Ideally, one would like to calculate a value of K for reaction (9) from an experimentally measured value of K' for reaction (2). However, since the value of K' for reaction (2) was found to be greater than 3000, K' could not be measured and such a calculation of K was not possible. It was possible, however, to estimate a value of K for reaction (9) using a thermochemical cycle that relies on thermodynamic data from the literature and two reasonable estimates of entropies. In this calculation, which is now described, all quantities pertain to $T = 298.15$ K, $I = 0$, and $P = 0.1$ MPa. First, the Benson group-additivity method with parameters given by Domalski and Hearing [36] was used to estimate the standard molar entropy $S^o_m = 168$ J K^{-1} mol^{-1} for anthranilic acid(s). This value was then used together with the entropies of C(s), H_2(g), N_2(g), and O_2(g) [23], the saturation molality of anthranilic acid(s) [37], and values of $\Delta_f H^o_m$ [33] and of $\Delta_{sol} H^o_m$ [34] for anthranilic acid(s) to calculate the standard partial molar entropy $S^o_{2,m} \approx 231$ J K^{-1} mol^{-1} for anthranilate$^-$(aq). Wilhoit [35] gives $S^o_{2,m} = 171.5$ J K^{-1} mol^{-1} for pyruvate$^-$(aq) and accurate values of $S^o_{2,m}$ for NH_4^+(aq) and H_2O(l) are given in the CODATA Tables [23]. An estimate of $S^o_{2,m}$ for chorismate^{2-} is now needed to calculate $\Delta_r S^o_m$ for reaction (9). For this estimate, we rely upon the Benson method [36] and obtain $S^o_{2,m} \approx 320$ J K^{-1} mol^{-1} for chorismate^{2-}(aq). With the aforementioned entropies, we calculate $\Delta_r S^o_m = 42$ J K^{-1} mol^{-1} for reaction (9). Combination of this value with the result $\Delta_r H^o_m = -116.3$ kJ mol^{-1} for reaction (9) leads to $\Delta_r G^o_m - 129$ kJ mol^{-1} and $K \approx 4 \times 10^{22}$. Making a liberal assignment of possible error of ± 80 J K^{-1} mol^{-1} in the estimated value of $\Delta_r S^o_m$ for reaction (9), the value of K could be as low as 10^{18} or as high as 10^{26}. In any case, this result is consistent with the experimental finding that K' for reaction (2) is > 3000.

Thermodynamic quantities can now be calculated for the reactions

$$\text{chorismate}^{2-}(\text{aq}) + \text{L-glutamine}^{\pm}(\text{aq}) = \text{anthranilate}^{-}(\text{aq}) + \text{pyruvate}^{-}(\text{aq}) + \text{L-glutamate}^{-}(\text{aq}) + \text{H}^{+}(\text{aq}) \quad (15)$$

$$\text{ADIC}^{-}(\text{aq}) = \text{anthranilate}^{-}(\text{aq}) + \text{pyruvate}^{-}(\text{aq}) + \text{H}^{+}(\text{aq}) \quad (16)$$

Reaction (15) is equal to [reaction (9) + reaction (13)]. Also, reaction (16) is equal to [reaction (9) − reaction (10)]. The calculated thermodynamic quantities for these reactions are given in Table 5.

3.8. Apparent equilibrium constants under approximately physiological conditions

It is desirable to have values of apparent equilibrium constants K' for the several biochemical reactions under approximately physiological conditions. Here, physiological conditions are taken to be [38]: $T = 311.15$ K, pH = 7.0, pMg = 3.0, and $I_m = 0.25$ mol kg^{-1}. Thus, with the thermodynamic quantities obtained in this study for the reference reactions and with the equilibrium model, apparent equilibrium constants K' and standard molar transformed Gibbs energy changes $\Delta_r G'^o_m$ for the several reactions pertinent to this study have been calculated. The results of these calculations are given in Table 6. Reaction (1), which represents the glutamine-dependent activity of native anthranilate synthase, is of particular biochemical interest. For this reaction, $K \approx 5 \times 10^{30}$ and $\Delta_r G'^o_m \approx -183$ kJ mol^{-1}. This reaction can therefore, be classified as 'irreversible.' In contrast $K' = 4.8$ and $\Delta_r G'^o_m - 4.1$ kJ mol^{-1} for reaction (5) which is clearly reversible under approximately physiological conditions.

3.9. Conclusion

Chorismate occupies a key position in the metabolism of microorganisms. It can undergo five distinct biotransformations [31,39], each of which is the first step in a branch of the shiki-

Table 6
Apparent equilibrium constants K' and standard transformed molar Gibbs energy changes $\Delta_r G'^{o}_m$ at approximately physiological conditions ($T = 311.15$ K, pH 7.0, pMg = 3.0, and $I_m = 0.25$ mol kg^{-1}) for several biochemical reactions in aqueous solution that are pertinent to this study[a]

Reaction	K'	$\frac{\Delta_r G'^{o}_m}{\text{kJ mol}^{-1}}$
Chorismate + ammonia = ADIC + H_2O	4.8	−4.1
L-Glutamine + H_2O = L-glutamate + ammonia	255	−14.3
Chorismate + L-glutamine = ADIC + L-glutamate	1.2×10^3	−18.4
ADIC = anthranilate + pyruvate	$\approx 4 \times 10^{27}$	≈ -165
Chorismate + ammonia = anthranilate + pyruvate + H_2O	$\approx 2 \times 10^{28}$	≈ -169
Chorismate + L-glutamine = anthranilate + L-glutamate + pyruvate	$\approx 5 \times 10^{30}$	≈ -183

[a] The values of K' and of $\Delta_r G'^{o}_m$ for the last three reactions are very approximate.

mate–chorismate metabolic pathway. A potentially useful framework for understanding what happens at such a strategic point in a biochemical pathway is provided by metabolic control theory [7,8]. This theory, however, requires a knowledge of both the thermodynamics and the kinetics of the appropriate reactions. Earlier studies from this laboratory have provided values of the necessary thermodynamic quantities for two of the other branch points in this metabolic pathway, specifically the conversion of chorismate to prephenate [9] and of chorismate to (pyruvate + 4-hydroxybenzoate) [13]. The results obtained herein (see Tables 4–6) now provide the essential data needed for understanding the thermodynamics of the several biochemical reactions pertinent to one additional branch of this metabolic pathway. When complemented with the pertinent kinetic data and the results of in vivo substrate measurements, a quantitative understanding should emerge of just how this important metabolic branch point operates to control the flux of matter and energy between these different branches.

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