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Thermodynamics of reactions catalyzed by PABA synthase

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Abstract

Microcalorimetry and high-performance liquid chromatography (HPLC) have been used to conduct a thermodynamic investigation of reactions catalyzed by PABA synthase, the enzyme located at the first step in the shikimic acid metabolic pathway leading from chorismate to 4-aminobenzoate (PABA). The overall biochemical reaction catalyzed by the PabB and PabC components of PABA synthase is: chorismate(aq) + ammonia(aq) = 4-aminobenzoate(aq) + pyruvate(aq) + H_2O(l). This reaction can be divided into two partial reactions involving the intermediate 4-amino-4-deoxychorismate (ADC): chorismate(aq) + ammonia(aq) = ADC(aq) + H_2O(l) and ADC(aq) = 4-aminobenzoate(aq) + pyruvate(aq). Microcalorimetric measurements were performed on all three of these reactions at a temperature of 298.15 K and pH values in the range 8.72–8.77. Equilibrium measurements were performed on the first partial (ADC synthase) reaction at $T = 298.15$ K and at pH = 8.78. The saturation molality of 4-aminobenzoate(cr) in water is (0.00382 ± 0.0004) mol kg^{-1} at $T = 298.15$ K. 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) = ADC^-(aq) + H_2O(l), $K = (10.8 \pm 4.2)$ and $\Delta_r H^o_m = -(35 \pm 15)$ kJ mol^{-1}. For the reaction: ADC^-(aq) = 4-aminobenzoate$^-$(aq) + pyruvate$^-$(aq) + H^+(aq), $\Delta_r H^o_m = -(139 \pm 23)$ kJ mol^{-1}. For the reaction: chorismate^{2-}(aq) + NH_4^+(aq) = 4-aminobenzoate$^-$(aq) + pyruvate$^-$(aq) + H_2O(l) + H^+(aq), $\Delta_r H^o_m = -(174 \pm 6)$ kJ mol^{-1}. Thermodynamic cycle calculations were used to calculate thermodynamic quantities for three

Abbreviations: ADC, 4-amino-4-deoxychorismate; ADIC, 2-amino-2-deoxyisochorismate; DEAE, diethylaminoethyl; DTT, DL-dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NADH, β-nicotinamide-adenine dinucleotide (reduced form); PABA, 4-aminobenzoic acid; PMSF, phenylmethylsulfonyl fluoride; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Tris, tris(hydroxymethyamino)methane.

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additional reactions that utilize L-glutamine rather than ammonia and that are pertinent to this branch point of the shikimic acid pathway. The quantities obtained in this 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: ADC; 4-aminobenzoic acid; 4-amino-4-deoxychorismate; Apparent equilibrium constant; Calorimetry; Chorismate; Enthalpy; PABA; Thermodynamics

1. Introduction

The first step in the branch of the shikimic acid metabolic pathway [1] that leads to the folate coenzymes is the reaction

chorismate(aq) + L-glutamine(aq)
= 4-aminobenzoate(aq)
+ L-glutamate(aq) + pyruvate(aq). (1)

This reaction is catalyzed in three steps in plants and microorganisms by an interacting set of enzymes, PabA, PabB and PabC, collectively referred to as *p*-aminobenzoate synthase, or PABA synthase. A transient complex between PabA and PabB catalyzes the first two steps of PABA synthesis by generating the intermediate substance 4-amino-4-deoxychorismate (ADC) according to the reactions

L-glutamine(aq) + H_2O(l)
= L-glutamate(aq) + ammonia(aq), (2)

chorismate(aq) + ammonia(aq)
= ADC(aq) + H_2O(l). (3)

The final step of PABA biosynthesis is catalyzed by the pyridoxal 5′-phosphate containing PabC according to the reaction

ADC(aq) = 4-aminobenzoate(aq)
+ pyruvate(aq). (4)

Anderson et al. [2] found that the proton NMR chemical shifts of the intermediate substance isolated from the above reactions were the same as chemically synthesized ADC thus confirming the nature of the intermediate. Recently, the X-ray crystal structures of the PabB aminodeoxychorismate synthase and the PabC aminodeoxychorismate lyase have been determined, respectively, by Parsons et al. [3] (PabB) and by Nakai et al. [4] and by Jensen et al. [5] (PabC). Magnesium ion is required for reactions (1) and (3) but is not required for the catalytic activity of PabC in reaction (4) [6]. The reactions catalyzed by the PabB and PabC subunits of PABA synthase are shown in Fig. 1 along with the other reactions that originate from chorismate, a substance that serves as the critical branch point in the shikimic acid metabolic pathway.

There is little thermodynamic information known about the above reactions other than a report of a value of the apparent equilibrium constant ($K' = 6.1$ at the temperature $T = 310.15$ K and pH = 8.6) for reaction (3) by Anderson et al. [2]. Earlier studies from this laboratory [7–10] have reported values of thermodynamic quantities for the other reactions shown in Fig. 1. Thus, obtaining a complete thermodynamic characterization of this final reaction at the branch point in the shikimic acid pathway is of obvious importance. However, such a study is complicated by the limited availability of the prerequisite enzymes and, perhaps more significantly, by the very labile nature of the ADC intermediate which undergoes a spontaneous transformation in the absence of an enzyme, to PABA and, possibly, a Claisen rearrangement analogous to that undergone by 2-amino-2-deoxyisochorismate (ADIC) [11]. Thus, working with ADC will clearly cause difficulties in the study of reactions (3) and (4). However, no such complications should arise if one can rapidly carry out the following reaction by using PabB and PabC

chorismate(aq) + ammonia(aq)
= 4-aminobenzoate(aq) + pyruvate(aq)
+ H_2O(l). (5)

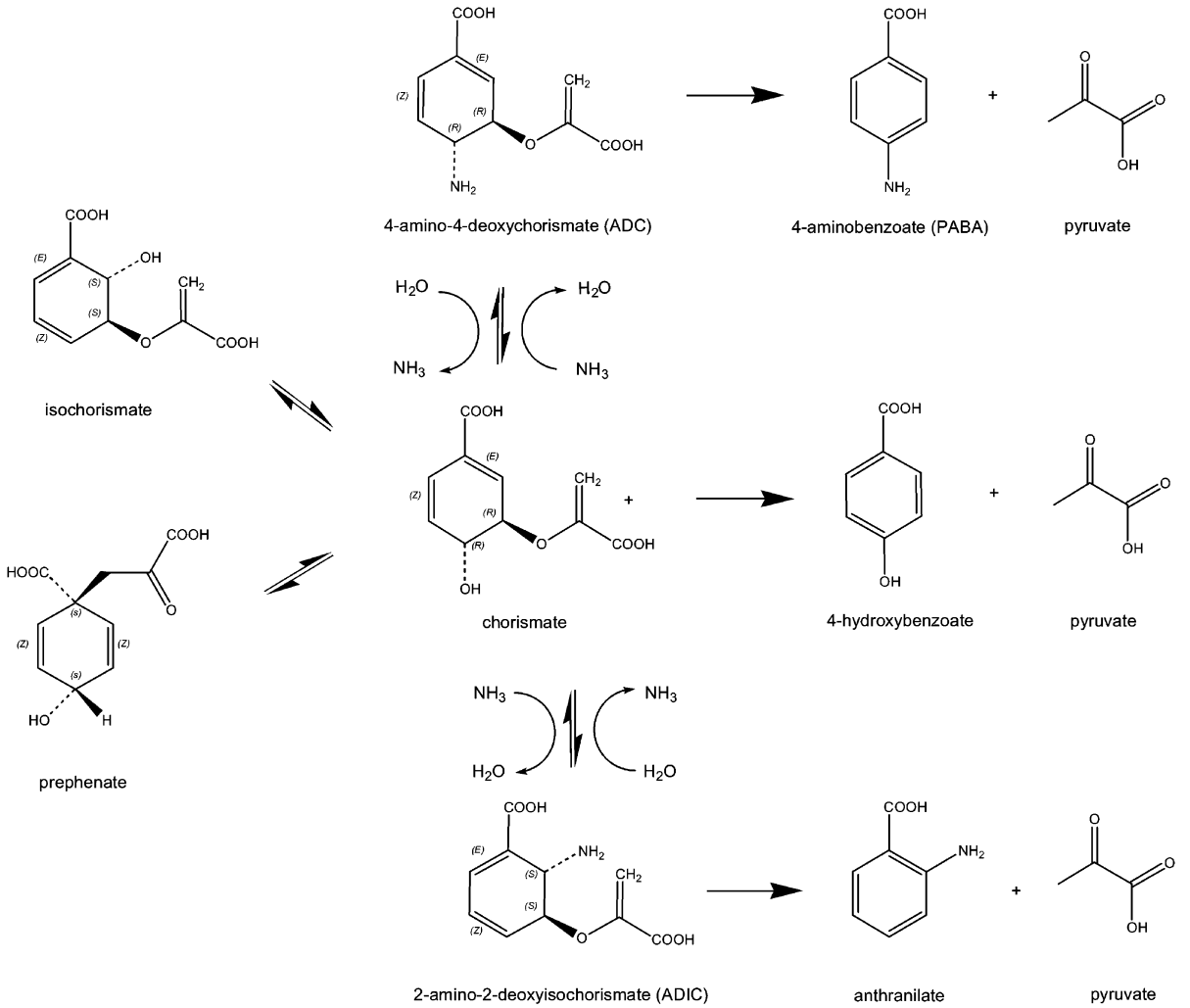

Fig. 1. The metabolic transformations that chorismate can undergo at the branch point in the chorismate metabolic pathway. The reactions that are of central interest to this study are in the top most portion of this figure. The neutral forms of the substances are shown.

This study reports an experimentally determined value of K' for reaction (3) as well as calorimetric enthalpies for reactions (3)–(5). These results are then used in an equilibrium model [12,13] together with pK values and enthalpies of ionization of the reactants and products to calculate standard thermodynamic quantities for chemical reference reactions [14] that correspond to the overall biochemical reactions (3)–(5). Use of additional thermodynamic results from the literature and an estimated entropy for chorismate^{2-}(aq) leads to an essentially complete thermodynamic description of all of the above reactions and the corresponding chemical reference reactions. This information serves to establish the thermodynamics of both the individual reactions and, in conjunction with earlier studies, this central branch point in the shikimic acid metabolic pathway.

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 and S, Sigma), and method used to determine x[a]

Substance	CAS No.	Formula	M_r	w	Supplier	x	Method[b]
4-aminobenzoic acid	150-13-0	$C_7H_7NO_2$	137.14	0.0009	S	>0.99	TLC
4-aminobenzoic acid, sodium salt	555-06-6	$C_7H_6NO_2Na$	159.12	0.029	S	>0.99	TLC and NaOH titration
4-amino-4-deoxychorismate (ADC)	97279-79-3	$C_{10}H_{11}NO_5$	225.20		[c]		
Ammonium chloride	12125-02-9	NH_4Cl	53.491		S	>0.99	Titration with $AgNO_3$
Chorismic acid	617-12-9	$C_{10}H_{10}O_6$	226.18	0.092[d]	[c]	>0.99	HPLC and enzymatic assay
Magnesium chloride	7786-30-3	$MgCl_2$	95.210		S	>0.98	EDTA and $AgNO_3$ titration
Sodium hydroxide	1310-73-2	NaOH	39.997		Fi	>0.99	Acid titration
PabB			5.10×10^4		[c]		
PabC			2.97×10^4		[c]		
Tricine	5704-04-1	$C_6H_{13}NO_5$	179.17		S	>0.99	Acid titration

[a] The mole fraction purities are exclusive of the amount of water or, in the case of chorismate, the organic solvents ether, methylene chloride, and hexane.
[b] The methods given here are those used by the vendor(s) to determine the purities of these substances. The samples of 4-aminobenzoic acid, chorismic acid, and ADC were also characterized as described in Section 2.
[c] Prepared for this study (see Section 2.1).
[d] The chorismic acid also contained ether ($w=0.004$), methylene chloride ($w=0.052$), and hexane ($w=0.029$) (see Section 2.1).

2. Materials and methods

2.1. Chemicals

Information on the substances used in this study is given in Table 1.[3] The purities of the 4-aminobenzoic acid, ADC, and chorismic acid were examined by using chromatographic procedures described below (see Section 2.3). Karl Fischer analysis (Metrohm Model 633 automatic titrator; calibration done by using a water-saturated octanol solution [15]) was used for the determination of the mass fractions of water in the various substances.

ADC was prepared from ammonia and chorismate by using the PabB component of ADC synthase (see Section 2.2). A typical synthesis was carried out with 2 cm^3 of a solution containing [chorismate (0.10 M) + $(NH_4)_2CO_3$ (0.80 M) + PabB (20 μM), pH = 8.7]. After equilibration (≈2 h at 37 °C), the reactants and products were separated by using the procedure described by Anderson et al. [2] except that the HPLC conditions used were those of Green and Nichols [16]. ADC was identified by its absorbance at the wavelength λ = 272 nm and its ability to produce 4-aminobenzoate after the addition of ADC lyase. The purified ADC was vacuum-dried, resuspended in water, and stored at −80 °C. The fractional rate of decomposition of ADC in Tricine buffer (0.19 M, pH = 8.70) at 25 °C was determined using the HPLC procedure described below (see Section 2.3) and found to be 0.065 h^{-1}.

Chorismic acid was prepared and purified from *E. coli* KA12, a chorismate mutase mutant, according to the protocol of Grisostomi et al. [17]. A modification to the protocol was the addition of the following trace mineral supplement to the growth media: [$FeCl_2\cdot4H_2O$ (10 mg dm^{-3}) + $ZnCl_2$ (2.0 mg dm^{-3}) + $NaMoO_4\cdot2H_2O$ (3.0 mg dm^{-3}) + $CaCl_2$ (2.0 mg dm^{-3}) + $MnCl_2\cdot4H_2O$ (1.0 mg dm^{-3}) + H_3BO_3 (0.10 mg dm^{-3}) + $CoCl_2\cdot6H_2O$ (0.10 mg dm^{-3}) + $CuSO_4\cdot5H_2O$ (0.10 mg dm^{-3})]. As a final purification step, chorismate was crystallized according to the protocol of Addadi et al. [18]; the solvent used was (methylene chloride + hexane + ether, volume ratio = 1:2:1). Crystals of chorismate were dis-

[3] 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.

solved in the minimum amount of ether, crystallized, filtered two more times, and then dried in a vacuum desiccator for 1.5 h. Analysis of the purified chorismic acid was accomplished by using gas chromatography (GC) for solvents, HPLC for biochemical impurities, and Karl Fischer titration for water content. GC analysis was performed by using a Hewlett-Packard 5890A GC with a HP5 column thermostatted at 35 °C; the head pressure of the helium carrier gas was 0.28 MPa. From this GC analysis the following mass fraction (w) impurities were found: ether, $w=0.004$; methylene chloride, $w=0.052$; and hexane, $w=0.029$. The HPLC analysis was performed with a Hewlett-Packard 1100 LC [Bio-Rad fermentation monitoring (a resin-based ion-exchange column in the hydrogen form) column (7.8 mm i.d.$\times$150 mm long) thermostatted at 40 °C; mobile phases: I = H_2SO_4 (0.005 M) and II = acetonitrile; gradient: volume fraction ϕ(II) = 0.04 at time $t=0$; ϕ(II) = 0.04 at $t=4$ min; and ϕ(II) = 0.30 at $t=30$ min; flow rate = 0.8 cm^3 min^{-1})]. Response factors were measured for chorismate, pyruvate, phenylpyruvate, and 4-hydroxybenzoate. It should be noted that any prephenate in the sample is spontaneously converted to phenylpyruvate due to the very acidic conditions used in the HPLC. Using this HPLC method, the mole fraction purity of the chorismate was >0.99 based on the quantitative determinations of the amounts of pyruvate, phenylpyruvate, and 4-hydroxybenzoate in the final sample. This purity excepts the amounts of water and organic solvents, the quantitation of which have been discussed above. This purity assessment was verified by using the measured absorbance of chorismate at $\lambda=275$ nm and its molar absorption coefficient $\varepsilon=2.63\times10^4$ dm^2 mol^{-1} [18]. An additional confirmation of the purity was obtained by performing an enzymatic conversion of chorismate to (4-hydroxybenzoate + pyruvate). Pyruvate concentration was measured by converting it to lactate with (lactate dehydrogenase + NADH). The change in the concentration of NADH was measured spectrophotometrically ($\lambda=340$ nm, $\varepsilon=6.292\times10^4$ dm^2 mol^{-1} [19]). The concentration of chorismate determined in this way was (0.04 ± 0.5) percent higher than its concentration based on the results of the Karl Fischer and GC analyses. The essential agreement obtained with these several methods for purity determination lends some confidence to the accuracy of the characterization of the chorismate sample. The fractional rate of decomposition of chorismate in Tricine buffer (0.19 M, pH = 8.70) at 25 °C was determined using the HPLC procedure described below (Section 2.3) and found to be 0.029 h^{-1}. This rate is close to the value 0.024 h^{-1} found with Tris buffer (0.063 M, pH = 7.70) and phosphate buffer (0.67 M, pH = 6.93) at 25 °C [7].

2.2. Preparation of enzymes

PabB and PabC were prepared from enzyme overproducing strains of *E. coli*. PabB was expressed from plasmid pNPB [6] and purified from *E. coli* BL21 cells as described by Parsons et al. [3]. The *pabC* gene encoding ADC lyase from *E. coli* was amplified from chromosomal DNA using primers designed according to the sequence published by Green et al. [20], cloned into a variant of pKK233-2 (Pharmacia) for expression [20,21], and purified from cultures of *E. coli* strain JV30 [ara, Δ(*lac-proAB*), thi, strA, (ø 80 *lacZ* ΔM15), Δ(srl-*recA*) 306::Tn*10* (tet^r) [*F'*: tra D36, *proAB*, $lacI^q$ Z ΔM15]] as described by Jensen et al. [5].

2.3. Chromatography and response factors

The substances pertinent to this study were separated by using a Hewlett–Packard model 1100 HPLC equipped with a UV detector set at $\lambda=280$ nm and a Hewlett–Packard Zorbax Extend-C18 (4.6 mm i.d.$\times$150 mm long) thermostatted at 35 °C. The isocratic mobile phase was acetic acid (volume fraction $\phi=0.05$) run at a flow rate of 0.8 cm^3 min^{-1}. The approximate retention times were: ADC, 5.7 min; 4-aminobenzoate, 10.7 min; and chorismate, 16.2 min.

The response factor of chorismate was determined by weighing a known mass of chorismic acid(cr) into a known mass of buffer [Tricine (0.18 M), pH = 8.71]. This solution was kept in an ice bath to minimize any degradation. Several injections of a portion of this solution were made into the HPLC. The response factor of 4-amino-

benzoate was also determined by using the same procedure. The concentration of the ADC solution was determined by converting a portion of it rapidly to 4-aminobenzoate by using PabC. The concentration of 4-aminobenzoate in this reacted solution was then determined with the HPLC and the known response factor of this substance. Since the conversion of ADC was rapid and quantitative, the concentration of ADC was readily determined from the measured concentration of 4-aminobenzoate. The response factor of ADC was then determined by performing several injections of the unreacted ADC stock solution, which had been kept in an ice bath to minimize any degradation, into the HPLC. The ratio of the response factor of chorismate to the response factor of ADC was 1.024.

2.4. Equilibrium measurements

The method used for equilibrium measurements on reaction (3) involved the preparation of a series of solutions that contained all three reactants (chorismate, ammonia, and ADC) at molalities that resulted in apparent reaction quotients Q' [$Q' = m(\mathrm{ADC})/\{m(\mathrm{chorismate})m(\mathrm{NH_4Cl})\}$, where m is the molality of the indicated substance] not too far from the estimated value of the apparent equilibrium constant K' under the conditions of measurement. A portion of each of these solutions was then injected into the HPLC and the areas corresponding to the chorismate and ADC were measured. PabB was then added to the remaining solution and the reaction mixture was promptly placed in a water bath thermostatted at $T = 298.15$ K. This reaction mixture was agitated by a gentle lateral shaking motion (≈ 1 shake s^{-1}). After 20 min, a portion of the reaction mixture was injected into the HPLC and the areas corresponding to the chorismate and ADC were measured. The already measured response factors of chorismate and of ADC permit the calculation of the molalities of these substances. The molality of ammonia in solution is known from the molality of the NH_4Cl used in the preparation of the initial solutions and from the subsequent weighings performed after the addition or removal of any solutions from the reaction mixture. Thus, one has sufficient information to calculate values of $Q'(\mathrm{initial})$, the initial reaction quotient prior to addition of PabB, and of $Q'(\mathrm{final})$, the final reaction quotient that resulted after the addition of PabB and 20 min of equilibration. Since the molality of ammonia was two orders of magnitude larger than the molalities of chorismate and of ADC and since the reaction mixtures were started not too far from equilibrium, the error made by neglecting any reaction of ammonia will have a negligible effect on the calculated value of the final apparent reaction quotient.

The apparent reaction quotients Q' were selected so that $Q'(\mathrm{min}) < K' < Q'(\mathrm{max})$, where K' is the presumed value of the apparent equilibrium constant and $Q'(\mathrm{min})$ and $Q'(\mathrm{max})$ are, respectively, the minimum and maximum values of Q' used in the experiments. The experimental results were then used to construct a plot of $Q'(\mathrm{initial})$ vs. the change in the reaction quotient $\Delta Q' = Q'(\mathrm{final}) - Q'(\mathrm{initial})$. In such a plot, or in a fit of $Q'(\mathrm{initial})$ against $\Delta Q'$, the value of $Q'(\mathrm{initial})$ corresponding to $\Delta Q' = 0$ is K'. This method presupposes a knowledge of an approximate value of K', and it will not work if the presumed value of K' is not in the aforementioned range. In this study, an initial estimate of K' was obtained from the previous value of K' in the literature [2] for reaction (3) as well as from preliminary experiments in which equilibrium was approached from the direction (chorismate + ammonia). However, even if the condition $Q'(\mathrm{min}) < K' < Q'(\mathrm{max})$ is not met, the results of an unsuccessful experiment will lead to a better estimate of K' and, very likely, to a successful next experiment. This approach was adopted because of the high rate of decomposition of ADC (Section 2.1) and also because of the very limited amounts of both this substance and of PabB. The severe limitations on the amounts of these substances necessitated working with small reaction volumes (≈ 0.20 cm^3).

An attempt was made to measure the apparent equilibrium constant K' for reaction (5) by measuring the extent of the reaction with equilibrium being approached from both directions. The solution used for the forward direction of reaction contained [chorismate (molality $m = 0.0017$ mol kg^{-1}) + NH_4Cl ($m = 0.0024$ mol kg^{-1}) + $MgCl_2$

($m = 0.0021$ mol kg^{-1})] in Tricine buffer ($m =$ 0.19 mol kg^{-1}, pH = 8.70). The solution used for the reverse reaction contained [4-aminobenzoate ($m = 0.0021$ mol kg^{-1}) + pyruvate ($m = 0.0024$ mol kg^{-1}) + $MgCl_2$ ($m = 0.0021$ mol kg^{-1})] also in the same buffer. PabB (mass fraction $w = 3 \times 10^{-5}$) and PabC ($w = 1.5 \times 10^{-4}$) were added to these two solutions which were placed in a bath thermostatted at $T = 298.15$ K. Following equilibration for 24 h, the concentrations of chorismate, 4-aminobenzoate, and pyruvate were measured by using the HPLC (see Section 2.3).

2.5. Calorimetry

The heat-conduction microcalorimeters used for the enthalpy of reaction measurements were calibrated electrically with a high stability d.c. power supply, calibrated digital voltmeter, standard resistor, and time-interval counter. Descriptions of the microcalorimeter(s) and its performance characteristics, the data-acquisition system, and the computer programs used to treat the results have been given by Steckler et al. [22,23]. The electric potential differences ΔV of the thermopiles in the microcalorimeters are measured with Hewlett–Packard model 34420A Nanovolt Meters. The values of ΔV are then recorded on a microcomputer and the areas of the thermograms are calculated by numerical integration. Currently, HP-VEE is being used for data acquisition and C++ for the calculations.

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 solutions consisted either of chorismic acid(cr) dissolved in Tricine buffer that contained ($NH_4Cl + MgCl_2$) and was used for the study of reactions (3) and (5) or of [ADC(aq) + Tricine buffer] which was used for the study of reaction (4). An exchange (Amicon nitrogen gas pressure exchange cell with an Amicon YM10 membrane) with the same Tricine buffer used to prepare the respective substrate solution(s) was done for the PabB and PabC enzymes used in the calorimetric measurements.

In performing the measurements of the enthalpy changes for reactions (3)–(5), the times that the vessels and their contents were allowed to thermally equilibrate in the microcalorimeters prior to the mixing of the enzyme and substrate solutions were ≈ 65 min for reactions (3) and (5), and 60–90 min for reaction (4). Appropriate corrections were made for the pre-reaction of chorismate [reactions (3) and (5)] and of ADC [reaction (4)] by using the measured rates of decomposition of these substances (see Section 2.1). After mixing in the microcalorimeters, ≈ 60 min was allowed for reaction (3), ≈ 35 min for reaction (4), and ≈ 95 min for reaction (5). Following each reaction, the vessels were removed from the microcalorimeters and the HPLC (see Section 2.3) was promptly used to determine the extent(s) of reaction. In this way, it was found that the mole fractions of unreacted chorismate were 0.0022 for reaction (5) and 0.418–0.441 for reaction (3); the mole fraction of unreacted ADC was < 0.0001 in reaction (4). Appropriate corrections were made for unreacted substrates. In the case of reaction (3), the HPLC was also used to measure the amounts of ADC and 4-aminobenzoate formed. The 'recovery', defined as the sum of the final molalities of chorismate, ADC, and 4-aminobenzoate divided by the known initial molality of chorismate in a reaction vessel, was 0.995 ± 0.022. This value of the recovery is very close to unity and demonstrates that the products of the reaction are well accounted for. Additionally, it shows that the fraction of ADC that undergoes a Claisen rearrangement [11] during the time allowed and for the reaction conditions used is very small. However, the fraction of chorismate converted to 4-aminobenzoate was 0.038–0.055 and it was necessary to make a correction for the enthalpy change attributable to reaction (4). Since the molar enthalpy change for reaction (4) is very large, this was a substantial correction [(0.265–0.349)ΔH(measured)]. In calculating this correction, the enthalpy change for reaction (4) was first estimated by using the calorimetrically determined molar enthalpy change $\Delta_r H_m$(cal) for reaction (3) at pH = 8.77. This correction value was later

improved by using our equilibrium model and the calculated value of the standard molar enthalpy change for the reference reaction corresponding to reaction (4). The consequence of this change was only 0.2 kJ mol^{-1} in the final measured value of $\Delta_r H_m$(cal). Thus, no additional iterations were needed.

'Blank' enthalpy changes $\Delta_{mix}H$ were determined in control experiments. Thus, for mixing of the chorismate solution with buffer $\Delta_{mix}H = -(0.1 \pm 1.4)$ mJ; for mixing of the ADC solution with buffer $\Delta_{mix}H = (0.2 \pm 0.1)$ mJ; and for mixing of the enzyme solutions with buffer $\Delta_{mix}H = (2.0 \pm 1.7)$ mJ. The uncertainties given here are equal to two estimated standard deviations of the mean. These values of $\Delta_{mix}H$ were applied as corrections to the measured enthalpies $\Delta_r H$(cal) which were ≈ -45 mJ for reaction (3), ≈ -40 mJ for reaction (4), and ≈ -300 mJ for reaction (5). We judge that the total correction applied for the blank enthalpy changes leads to uncertainties of $\pm 0.044\Delta_r H_m$(cal) for reaction (3), $\pm 0.05\Delta_r H_m$(cal) for reaction (4), and $\pm 0.007\Delta_r H_m$(cal) for reaction (5).

2.6. Saturation molality measurements

The saturation molality of 4-aminobenzoic acid(cr) was determined by preparing two separate solutions of this substance in water. In both cases, sufficient 4-aminobenzoic acid(cr) was present to insure that a solid phase was also present. These two sample were contained in 20 cm^3 bottles sealed with Teflon-lined caps and were allowed to equilibrate with a gentle lateral shaking motion (≈ 1 shake s^{-1}) in baths thermostatted at the respective temperatures $T = 288.15$ K and $T = 310.15$ K. After 3 days, the molality of 4-aminobenzoic acid in each solution was determined by HPLC (see Section 2.3). The results were $m \approx 0.026$ mol kg^{-1} at $T = 288.15$ K and $m \approx 0.061$ mol kg^{-1} at $T = 310.15$ K. These two bottles were then placed in a thermostat at $T = 298.15$ K and allowed to equilibrate for an additional 2 days. A portion of the clear solution above the solid phase was removed from each of the two bottles. Each portion was then diluted with water by a factor of ≈ 20. This dilution was done gravimetrically. These two diluted solutions were then quantitatively analyzed for 4-aminobenzoic acid by HPLC. The response factor of 4-aminobenzoic acid was determined by using an aqueous solution containing a known molality of this substance that had been prepared gravimetrically and also set to be close to the molalities of the solutions being injected into the HPLC. The state of hydration of the 4-aminobenzoic acid(cr) was investigated by gently passing air for a period of 20 h over a sample of the solid phase of this substance that had been removed by filtration from the solution used in the saturation molality measurements. The mass fraction of water in this sample was then determined by using Karl Fischer titration and found to be 0.0010.

2.7. pH measurements

The 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 of pH = (4.005, 7.00 and 9.18). All pH measurements pertain to the temperature of interest.

3. Results and discussion

3.1. Thermodynamic formalism

The thermodynamic formalism and treatment of results is similar to that used in previous studies from this laboratory [7–10]. For overall biochemical reactions, the measured quantities are apparent equilibrium constants K' and calorimetrically determined molar enthalpies of reaction $\Delta_r H_m$(cal). From these quantities, one can use an equilibrium model that considers the contributions from the various species and reactions in solution to calculate values of the equilibrium constant K and the standard molar enthalpy of reaction $\Delta_r H^o_m$ for a selected reference reaction. Since water is a reactant in reaction (3) and also since some of the reactions are not symmetrical in regards to the charges on the reactants and products, it is particularly important to define the equilibrium constants and standard states to avoid any ambiguities in the definitions of these quantities. In this regard, the

apparent equilibrium constants for reactions (3), (4) and (5), respectively, are

$$K' = m(\text{ADC})/[m(\text{chorismate})m(\text{ammonia})] \quad (6)$$

$$K' = m(\text{4-aminobenzoate})m(\text{pyruvate})/m(\text{ADC}), \quad (7)$$

$$K' = m(\text{4-aminobenzoate})m(\text{pyruvate}) / [m(\text{chorismate})m(\text{ammonia})]. \quad (8)$$

The molalities m in the above equations are the total molalities of the various ionic forms of the respective species. The respective reference reactions corresponding to the overall biochemical reactions (3), (4) and (5) are

$$\text{chorismate}^{2-}(\text{aq}) + \text{NH}_4^+(\text{aq}) = \text{ADC}^-(\text{aq}) + \text{H}_2\text{O}(\text{l}), \quad (9)$$

$$\text{ADC}^-(\text{aq}) = \text{4-aminobenzoate}^-(\text{aq}) + \text{pyruvate}^-(\text{aq}) + \text{H}^+(\text{aq}), \quad (10)$$

$$\text{chorismate}^{2-}(\text{aq}) + \text{NH}_4^+(\text{aq}) = \text{4-aminobenzoate}^-(\text{aq}) + \text{pyruvate}^-(\text{aq}) + \text{H}_2\text{O}(\text{l}) + \text{H}^+(\text{aq}). \quad (11)$$

The choice of reference reactions is arbitrary and other reactions could have been selected. The equilibrium constants for the respective reference reactions are

$$K = m(\text{ADC}^-)/[m(\text{chorismate}^{2-})m(\text{NH}_4^+)], \quad (12)$$

$$K = m(\text{4-aminobenzoate}^-)m(\text{pyruvate}^-) m(\text{H}^+)/[m(\text{ADC}^-)], \quad (13)$$

$$K = m(\text{4-aminobenzoate}^-)m(\text{pyruvate}^-) m(\text{H}^+)/[m(\text{chorismate}^{2-})m(\text{NH}_4^+)]. \quad (14)$$

In this study, the standard state for the solute is the hypothetical ideal solution of unit molality ($m^\circ = 1$ mol kg^{-1}) and the standard state for the solvent is the pure solvent; the standard pressure $p^\circ = 0.1$ MPa.

3.2. Results of experiments

The inability to detect any chorismate in an analysis of the products in either the forward or reverse direction of reaction (5) prevented the determination of a value of K' for reaction 1. However, by using the approximate detection limit of chorismate ($m \approx 3\times10^{-7}$ mol kg^{-1}) by the HPLC, we obtain the result $K' > 4000$ at $T = 298.15$ K and pH = 8.70. Later it will be seen that the value of K' for reaction (5) is $\approx 10^{32}$.

The results of the reaction quotient measurements for reaction (3) and the calorimetric measurements for reactions (3), (4) and (5) are given in Tables 2–5, respectively. A plot of the apparent reaction quotient Q'(initial) at the start of an experiment vs. the change in the apparent reaction quotient, $\Delta Q' = Q'(\text{final}) - Q'(\text{initial})$, is shown in Fig. 2. A fit of the equation $Q'(\text{initial}) = a + b\Delta Q'$ to the data leads to the values $a = (6.3 \pm 1.3)$ and $b = -(24.5 \pm 7.7)$. Since K' is the value of Q'(initial) corresponding to $\Delta Q' = 0$, we have the result $K' = (6.3 \pm 1.3)$ for reaction (3) at $T = 298.15$ K and pH = 8.78. The uncertainties in this value of K' and in the values of $\Delta_r H_m$(cal) given in Tables 2–5 are equal to two estimated standard deviations of the mean and do not reflect possible systematic errors which are now considered. We estimate that the values of Q' are reliable to $\approx \pm 0.05Q'$ with most of the uncertainty being attributable to the value of the response factor of ADC which, in turn, is based on the analysis of the concentration of this substance in its stock solution. This uncertainty in Q' translates directly into an uncertainty of $\pm 0.05K'$.

We judge that reasonable estimates of possible systematic error in the values of $\Delta_r H_m$(cal) for reaction (3) are: $\pm 0.01\Delta_r H_m$(cal) due to possible errors in the analysis of the chorismic acid(cr); $\pm 0.006\Delta_r H_m$(cal) due to uncertainties in the corrections made for the decompositions of the chorismate prior to reaction; $\pm 0.03\Delta_r H_m$(cal) due to the uncertainty in the correction made for the formation of 4-aminobenzoate; $\pm 0.02\Delta_r H_m$(cal) due to possible errors in the extent of reaction; and $\pm 0.023\Delta_r H_m$(cal) due to possible errors in the calorimetric measurements including the 'blank' enthalpies. For reaction (4) the estimates of possible systematic error are: $\pm 0.03\Delta_r H_m$(cal) due to errors in the determination of the molality of the ADC in solution; $\pm 0.013\Delta_r H_m$(cal) due to uncertainty in the correction made for decomposition of the ADC in solution prior to reaction; $\pm 0.0001\Delta_r H_m$(cal) due to possible errors in the

Table 2
Results of apparent reaction quotient Q' measurements at $T = 298.15$ K and pH = 8.78 for reaction (3): chorismate(aq) + ammonia(aq) = ADC(aq) + H_2O(l)[a]

m(Tricine) (mol kg^{-1})	m(NaOH) (mol kg^{-1})	$10^3 m$($MgCl_2$) (mol kg^{-1})	m(NH_4Cl) (mol kg^{-1})	$10^3 m$ (chorismate) (mol kg^{-1})	$10^3 m$ (ADC) (mol kg^{-1})	I_m (mol kg^{-1})	Q' (initial)	Q' (final)	$\Delta Q'$
0.129	0.134	1.94	0.131	1.639	0.586	0.25	2.65	2.72	0.075
0.123	0.128	1.85	0.125	1.800	0.759	0.24	3.34	3.37	0.027
0.0759	0.0792	1.76	0.0773	0.973	1.055	0.20	14.29	14.02	−0.27
0.133	0.139	1.98	0.136	0.880	0.484	0.26	3.97	4.04	0.077
0.108	0.113	2.11	0.110	0.706	0.579	0.23	7.43	7.43	0.00
0.112	0.117	2.28	0.114	0.770	0.525	0.23	5.87	5.96	0.093
0.106	0.110	2.08	0.108	0.668	0.607	0.23	8.46	8.43	−0.031
0.0913	0.0953	1.97	0.0931	0.637	0.816	0.22	14.15	13.77	−0.37

[a] The molalities m are those of the substances in solution at the end of an experiment and which are equal to the sums of the indicated substances in their various ionic forms. The following substances were also present in the reaction mixtures at the approximate molalities: MOPS [3-(*N*-morpholino)propanesulfonic acid], 0.0081 mol kg^{-1}; KCl, 0.0081 mol kg^{-1}; and DTT (DL-dithiothreitol), 3.2×10^{-4} mol kg^{-1}. The mass fraction of the enzyme PabB in solution was ≈ 0.002. The ionic strength I_m is calculated. The values of the apparent reaction quotient Q' at the initiation and at the end of each experiment (see Section 2.4) are given in columns 8 and 9, respectively. The quantity $\Delta Q' = Q'(\text{final}) - Q'(\text{initial})$, where $Q' = m(\text{ADC})/\{m(\text{chorismate})m(NH_4Cl)\}$.

Table 3
Results of calorimetric measurements at the temperature $T=298.15$ K, pH = 8.72, and ionic strength $I_m=0.29$ mol kg^{-1} on reaction (3): chorismate(aq) + ammonia(aq) = ADC(aq) + H_2O(l)[a]

m(Tricine) (mol kg^{-1})	m(NaOH) (mol kg^{-1})	$10^3 m(MgCl_2)$ (mol kg^{-1})	$m(NH_4Cl)$ (mol kg^{-1})	$10^3 m$(chorismate) (mol kg^{-1})	$\Delta_r H_m$ (cal) (kJ mol^{-1})
0.0999	0.120	1.73	0.200	1.805	−32.9
0.0999	0.120	1.76	0.200	1.881	−34.9
0.0999	0.120	1.72	0.200	1.774	−32.1
0.0999	0.120	1.71	0.200	1.734	−31.8
$\langle\Delta_r H_m(cal)\rangle = -(32.9\pm1.4)$ kJ mol^{-1}					

[a] All molalities m 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. Appropriate corrections were made both for the pre-reaction of chorismate (using its measured rates of decomposition) and for the amounts of 4-aminobenzoate formed. The values of the ionic strength (molality basis) I_m of the final reaction mixtures have been calculated. The mass fraction of the PabB in the final reaction mixtures was ≈0.0010. The uncertainty is equal to two estimated standard deviations of the mean. An estimate of total error for $\Delta_r H_m$(cal) of ±3.2 kJ mol^{-1} is assigned in Section 3.2

extent of reaction; and $\pm0.025\Delta_r H_m$(cal) due to possible errors in the calorimetric measurements including the 'blank' enthalpies.

For reaction (5) the estimates of possible systematic error are: $\pm0.01\Delta_r H_m$(cal) due to possible errors in the analysis of the impurities (including water and organic solvents) in the sample of chorismic acid(cr); $\pm0.006\Delta_r H_m$(cal) due to uncertainty in the correction made for decomposition of the chorismate in solution prior to reaction; $\pm0.001\Delta_r H_m$(cal) due to possible errors in the extent of reaction; and $\pm0.009\Delta_r H_m$(cal) due to possible errors in the calorimetric measurements including the 'blank' enthalpies. These estimates of possible systematic error are combined in quadrature together with the statistical uncertainty in the measured values of K' and of $\Delta_r H_m$(cal) expressed as one estimated standard deviation of the mean, to obtain combined standard uncertainties [24] for each result. These combined standard uncertainties are then multiplied by two to arrive at the final results: $K'=(6.3\pm1.5)$ for reaction (3) in Tricine buffer ($T=298.15$ K, pH = 8.78, $\langle I_m\rangle$ = 0.23 mol kg^{-1}), where $\langle\rangle$ denotes an average value; $\Delta_r H_m(cal)=-(32.9\pm3.2)$ kJ mol^{-1} for reaction (3) in Tricine buffer ($T=298.15$ K, pH = 8.72, $I_m=0.29$ mol kg^{-1}); $\Delta_r H_m(cal)=-(171\pm14)$ kJ mol^{-1} for reaction (4) in Tricine buffer ($T=298.15$ K, pH = 8.77, $\langle I_m\rangle=0.070$ mol kg^{-1}); and $\Delta_r H_m(cal)=-(178.9\pm5.4)$ kJ mol^{-1} for reaction (5) in Tricine buffer ($T=298.15$ K, pH = 8.74, $I_m=0.29$ mol kg^{-1}).

Table 4
Results of calorimetric measurements at the temperature $T=298.15$ K and pH = 8.77 on reaction (4): ADC(aq) = 4-aminobenzoate(aq) + pyruvate(aq)[a]

m(Tricine) (mol kg^{-1})	m(NaOH) (mol kg^{-1})	$10^4 m$(ADC) (mol kg^{-1})	I_m (mol kg^{-1})	$\Delta_r H_m$(cal) (kJ mol^{-1})
0.0811	0.0637	3.17	0.069	−169.7
0.0812	0.0638	3.14	0.069	−170.6
0.0829	0.0651	2.59	0.070	−171.2
0.0832	0.0654	2.52	0.071	−172.5
$\langle\Delta_r H_m(cal)\rangle = -(171.0\pm1.2)$ kJ mol^{-1}				

[a] All molalities m 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. Appropriate corrections were made for the pre-reaction of ADC by using its measured rates of decomposition. The values of the ionic strength (molality basis) I_m of the final reaction mixtures have been calculated. The mass fractions of the PabC in the final reaction mixtures was ≈0.00035. The uncertainty is equal to two estimated standard deviations of the mean. An estimate of total error for $\Delta_r H_m$(cal) of ±14 kJ mol^{-1} is assigned in Section 3.2.

Table 5
Results of calorimetric measurements at the temperature $T=298.15$ K, pH = 8.74, and ionic strength $I_m=0.29$ mol kg^{-1} on reaction (5): chorismate(aq) + ammonia(aq) = 4-aminobenzoate(aq) + pyruvate(aq) + H_2O(l)[a]

m(Tricine) (mol kg^{-1})	m(NaOH) (mol kg^{-1})	$10^3 m(MgCl_2)$ (mol kg^{-1})	$m(NH_4Cl)$ (mol kg^{-1})	$10^3 m$(chorismate) (mol kg^{-1})	$\Delta_r H_m$(cal) (kJ mol^{-1})
0.0997	0.119	2.02	0.195	1.960	−180.9
0.0997	0.119	2.02	0.195	1.970	−177.2
0.0997	0.119	2.06	0.197	2.355	−179.9
0.0997	0.119	2.00	0.195	1.826	−177.5
0.0997	0.119	2.01	0.195	1.863	−178.1
0.0996	0.118	1.99	0.194	1.688	−179.8
$\langle\Delta_r H_m(\text{cal})\rangle = -(178.9 \pm 1.3)$ kJ mol^{-1}					

[a] All molalities m 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. Appropriate corrections were made for the pre-reaction of chorismate by using its measured rate of decomposition. The values of the ionic strength (molality basis) I_m of the final reaction mixtures have been calculated. The mass fractions of the PabB and PabC in the final reaction mixtures were ≈0.00024 and ≈0.00014, respectively. The uncertainty 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 Section 3.2.

The saturation molality m(sat) of 4-aminobenzoic acid(cr) was determined after it was found that the existing literature [25,26] values for this quantity differed substantially. Our results for m(sat) are given in Table 6. Since the results obtained from two experiments, which were started at temperatures 25 K apart, are close to each other, one has excellent evidence that equilibrium has been achieved. The results obtained with these two solutions have been combined to obtain the final value $m(\text{sat}) = (0.0382 \pm 0.0004)$ mol kg^{-1} at $T=298.15$ K. Since the mass fraction of water in the 4-aminobenzoic acid solid phase in equilibrium with the saturated aqueous solution was found to be 0.0010 (see Section 2.6), the stable phase of this substance at $T=298.15$ K is judged to be the anhydrous form.

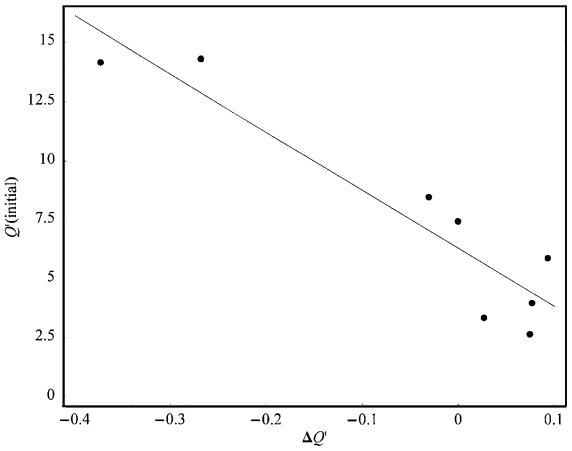

Fig. 2. The apparent reaction quotient Q'(initial) at the start of an experiment vs. the change in the apparent reaction quotient $\Delta Q' = Q'(\text{final}) - Q'(\text{initial})$.

3.3. Ionization constants

The pK values and standard molar enthalpies for the H^+(aq) dissociation reactions of the reactants and of the buffer are needed to relate the experimental results for reactions (3), (4) and (5) to thermodynamic quantities for their respective

Table 6
The saturation molality m(sat) of 4-aminobenzoic acid(cr) in water at $T=298.15$ K[a]

	m(sat) mol kg^{-1}
Solution A[b]	0.0382 ± 0.0004
Solution B[c]	0.0381 ± 0.0005
$\langle m(\text{sat})\rangle = (0.0382 \pm 0.0004)$ mol kg^{-1}[d]	

[a] The pH of the solution was 3.35. The first two uncertainties are equal to two estimated standard deviations of the mean and include a component of uncertainty for the determination of the chromatographic response factor. The final uncertainty has been expanded to include both sets of results.
[b] Solution initially thermostatted at $T=288.15$ K.
[c] Solution initially thermostatted at $T=310.15$ K.
[d] Mean value.

Table 7
The pKs and standard molar enthalpy changes $\Delta_r H^o_m$ at $T=298.15$ K and $I=0$ for the aqueous H^+ dissociation reactions of substances pertinent to this study and to the analysis of results from the literature[a]

Reaction		pK	$\Delta_r H^o_m$ (kJ mol^{-1})
Chorismate^{2-} = chorismate^{2-} + H^+	(15)	<4.5	
ADC^- = ADC^{2-} + H^+	(16)	9.0[b]	≈ 40[b]
NH_4^+ = NH_3 + H^+	(17)	9.245	51.95
4-aminobenzoic acid^0 = 4-aminobenzoate$^-$ + H^+	(18)	4.87	2.0
Pyruvic acid^0 = pyruvate$^-$ + H^+	(19)	2.48	12.1
Tricine$^\pm$ = Tricine$^-$ + H^+	(20)	8.135	31.37

[a] See Section 3.3 for the basis of these values. The standard state for the solutes is the hypothetical idealsolution of unit molality.
[b] Estimated (see Section 3.3).

reference reactions (9), (10) and (11). These pK values and standard molar enthalpies $\Delta_r H^o_m$ are given in Table 7. The basis of the selected values is now discussed. The pK and $\Delta_r H^o_m$ values for aqueous NH_4^+ and Tricine$^\pm$ are from a recent review [27]. The values for 4-aminobenzoic and pyruvic acids are from Martell et al. [28]. On the basis of the known pK values of other substances that are structurally similar to chorismate, its two lowest pK values have been estimated [7] to be ≤ 4.5. This estimate was also found to be consistent with approximate results obtained from a potentiometric titration that had been performed [7] on chorismic acid. The pK of ADC was obtained by using the estimation method of Perrin et al. [29]. This method had been found [9] to give a predicted value for the pK of 2-amino-4-deoxyisochorismate (ADIC) that was in excellent agreement with the pK value obtained from the substance L-*trans*-2,3-dihydro-3-hydroxyanthranilic acid (DHAA) that has a structure very similar to ADIC. We use the known [28] values of the standard molar entropy changes $\Delta_r S^o_m$ for the ionizations of 3-aminopropanoic and 3-aminobutanoic acids to estimate $\Delta_r S^o_m \approx -38$ J K^{-1} mol^{-1} for the ionization of ADC. Combination of this value with the estimated pK of ADC leads to the very approximate value $\Delta_r H^o_m \approx 40$ kJ mol^{-1} for the ionization of ADC. The values of the thermodynamic quantities given in Table 7 will be used in the calculations that follow.

3.4. Equilibrium modeling calculations

The equilibrium model used has been previously described [12] and has also been implemented in terms of a Mathematica package [13] that allows for a relatively convenient calculation of the desired quantities. An important part of the Mathematica package is an efficient algorithm [30] that is used to solve the simultaneous non-linear equations that describe the chemical equilibria. We have used the extended Debye–Hückel equation with the 'ion-size' parameter set at 1.6 kg$^{1/2}$ mol$^{-1/2}$ to treat the non-ideality of the various species in solution. By applying this model to the experimental results obtained in this study we calculate $K=(10.8\pm0.6)$ and $\Delta_r H^o_m = -(33.4\pm3.2)$ kJ mol^{-1} for reaction (8), $\Delta_r H^o_m = -(134.7\pm14)$ kJ mol^{-1} for reaction (10), and $\Delta_r H^o_m = -(174.0\pm5.4)$ kJ mol^{-1} for reaction (11). The equilibrium model also yielded the following values for the change in binding of the hydrogen ion under the actual conditions of reaction: $\Delta_r N(H^+) = (-0.419, -0.468,$ and $0.182)$ for reactions (3), (4) and (5), respectively.

The uncertainties in the calculated values of $\Delta_r H^o_m$ are based on the respective uncertainties in the experimentally determined values of K' and $\Delta_r H_m$(cal). However, there is also a component of uncertainty due to uncertainties in the parameters 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. Thus, the pK values were perturbed as follows: ADC^-, ± 0.4; NH_4^+, ± 0.003; Tricine, ± 0.003. The values of $\Delta_r H^o_m$ for the dissociation reactions were also perturbed: ADC^-, ± 20 kJ mol^{-1}; NH_4^+, ± 0.2; Tricine, ± 0.2. 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 of chorismate and 4-aminobenzoic acid are far removed from the pH values at which experiments were performed, possible errors in the thermodynamic quantities for their ionization reactions have a negligible effect on the values of the calculated quantities. The final uncertainties in the values of the thermodynamic quantities for the reference reactions were obtained by combining, in quadrature, the experimental uncertainties with the estimated uncertainties attributable to the model. Thus, the results with expanded uncertainties are: $K=(10.8\pm 4.2)$ and $\Delta_r H^o_m = -(33\pm 13)$ kJ mol^{-1} for reaction (9), $\Delta_r H^o_m = -(135\pm 18)$ kJ mol^{-1} for reaction (10), and $\Delta_r H^o_m = -(174.0\pm 5.4)$ kJ mol^{-1} for reaction (11). It is seen that the final uncertainty in the value of $\Delta_r H^o_m$ for reaction (9) is substantially larger than the uncertainty attributable to possible experimental error. The reason for this is the large uncertainty in the values of pK and $\Delta_r H^o_m$ for the ionization of ADC. However, should reliable experimental values for these quantities become available, the results of our experiments have been reported in sufficient detail to permit a recalculation of thermodynamic quantities for the reference reactions.

It should also be noted that since reaction (11) is equal to [reaction (9) + reaction (10)], one has an additional way of checking the consistency of the measurements. Specifically, $[\Delta_r H^o_m(8) + \Delta_r H^o_m(10)] = -(168\pm 25)$ kJ mol^{-1} a value that is in agreement with the directly measured value $\Delta_r H^o_m(11) = -(174.0\pm 5.4)$ kJ mol^{-1}. Since the aforementioned relationship is exact, we now choose to make our results consistent with this relationship. Thus, by means of a weighted least-squares calculation we have the following final adjusted set of values: $\Delta_r H^o_m = -(34._9\pm 15)$ kJ mol^{-1}, $\Delta_r H^o_m = -(138._7\pm 23)$ kJ mol^{-1}, and $\Delta_r H^o_m = -(173._7\pm 5.6)$ kJ mol^{-1} for reactions (9), (10) and (11), respectively. The uncertainties have been increased due to the uncertainties associated with the adjustments in the values. These results will be rounded to the nearest 'kJ mol^{-1} in the remainder of this paper.

3.5. Results from the literature

The only result in the literature for any of the reactions studied herein is that of Anderson et al. [2] who reported $K'=6.1$ for reaction (3) at $T=310.15$ K and pH = 8.6. From the information given in this study [2], we calculate the ionic strength $I_m = 0.31$ mol kg^{-1}. Application of the equilibrium model to this result and with the value of $\Delta_r H^o_m$ for reaction (9) obtained in this study, we obtain the value $K=10.7$ for reaction (9) at $T=298.15$ K and $I=0$. This result is in excellent agreement with the result $K=(10.8\pm 4.2)$ of the present study.

The solubility of 4-aminobenzoic acid(cr) in H_2O(l) has been reported by Caronna [25] ($T=291.15$ K to $T=303.15$ K) and by Niazi and Mahmood [26] ($T=278.15$ K to $T=318.15$ K). We have calculated values of the saturation molality at $T=298.15$ K from the results of these studies. Thus, m(sat) = 0.435 mol kg^{-1} from the study of Caronna [25] and m(sat) = 0.00428 mol kg^{-1} from the study of Niazi and Mahmood [26]. In the calculation of m(sat) from the results of the latter workers [26] we have assumed that the densities of the saturated 4-amino benzoic acid solutions were equal to the density of H_2O(l) at the temperatures used by these investigators. Our result $m(\text{sat}) = (0.0382\pm 0.0004)$ mol kg^{-1} at $T=298.15$ K lies between these two results. Because of the methodology used, and in which the attainment of equilibrium was clearly demonstrated, we have some confidence in our result for m(sat). This value will be used in all subsequent calculations.

3.6. Thermochemical cycles

We now turn to some thermochemical cycle calculations that lead to values of the standard molar enthalpies of formation $\Delta_f H^o_m$ for 4-aminobenzoate$^-$(aq) and for chorismate^{2-}(aq) at $T=298.15$ K and $p=0.1$ MPa. First we have

$\Delta_f H^o_m = -413.2$ kJ mol^{-1} for 4-aminobenzoic acid(cr) from the combustion calorimetry measurements of Lebedeva et al. [31]. Also, Larsen and Magid [32] measured the standard molar enthalpy of solution $\Delta_{sol} H^o_m = 23.3$ kJ mol^{-1} for 4-aminobenzoic acid(cr) in H_2O(l). Assuming that the predominant species in their [32] solution calorimeter was 4-aminobenzoate^0(aq) and using the value of $\Delta_r H^o_m$ for the ionization of 4-aminobenzoate^0(aq) given in Table 7, we have $\Delta_f H^o_m = -387.9$ kJ mol^{-1} for 4-aminobenzoate$^-$(aq). This value together with the result $\Delta_r H^o_m = -(174 \pm 6)$ kJ mol^{-1} for reaction (11) and with the tabulated values of $\Delta_f H^o_m$ for H_2O(l) [33], for NH_4^+(aq) [33], and for pyruvate$^-$(aq) [69WIL] leads to $\Delta_f H^o_m = -962._7$ kJ mol^{-1} for chorismate^{2-}(aq). A value of $\Delta_f H^o_m = -1007._0$ kJ mol^{-1} for chorismate^{2-}(aq) was obtained by Byrnes et al. [9] using a thermochemical pathway involving anthranilate. The 45 kJ mol^{-1} difference in the $\Delta_f H^o_m$ values is larger than one would prefer and is indicative of some error(s) in the thermodynamic quantities in the pathways used to obtain these values.

Since we were unable to measure a value of K' for reaction (5) it was not possible to obtain a value of K for the corresponding reference reaction (11). However, an approximate value for this equilibrium constant is obtained by means of the following calculation. First, the standard molar entropy of 4-aminobenzoic acid(cr) is estimated to be 168 J K^{-1} mol^{-1} by using the Benson group-additivity parameters given by Domalski and Hearing [35]. This value is then used together with the entropies of C(s), H_2(g), N_2(g), and O_2(g) [33], the value of the saturation molality obtained in this study, the values of $\Delta_f H^o_m$ and of $\Delta_{sol} H^o_m$ for 4-aminobenzoic acid(cr), and the pK value for the ionization of PABA$^-$(aq) (see Table 7) to obtain the standard partial molar entropy $S^o_{2,m} \approx 133$ J K^{-1} mol^{-1} for 4-aminobenzoate$^-$(aq). We use values of $S^o_{2,m}$ for pyruvate$^-$(aq) [34], NH_4^+(aq) [33], and H_2O(l) [33] and our earlier [9] estimate of $S^o_{2,m}$ for chorismate^{2-}(aq) to calculate $\Delta_r S^o_m \approx -57$ J K^{-1} mol^{-1} for reaction (11). Combination of this value with the result $\Delta_r H^o_m = -174$ kJ mol^{-1} for this reaction leads to $\Delta_r G^o_m \approx -157$ kJ mol^{-1} and $K \approx 3 \times 10^{27}$ for reaction (11).

Thermodynamic quantities can now be calculated for the reactions that involve the utilization of L-glutamine in place of ammonia. Here the pertinent reference reactions are:

$$\text{chorismate}^{2-}(\text{aq}) + \text{L-glutamine}^{\pm}(\text{aq}) = \text{4-aminobenzoate}^-(\text{aq}) + \text{pyruvate}^-(\text{aq}) + \text{L-glutamate}^- + \text{H}^+(\text{aq}) \quad (20)$$

$$\text{chorismate}^{2-}(\text{aq}) + \text{L-glutamine}^{\pm}(\text{aq}) = \text{ADIC}^- + \text{L-glutamate}^-. \quad (21)$$

The calculation of the thermodynamic quantities for reactions (21) and (22) uses the results obtained in this study together with those of Kishore et al. [36] for the reaction

$$\text{L-glutamine}^{\pm}(\text{aq}) + \text{H}_2\text{O(l)} = \text{L-glutamate}^-(\text{aq}) + \text{NH}_4^+(\text{aq}). \quad (22)$$

Since reaction (21) is equal to [reaction (11) + reaction (23)] and since reaction (22) is equal to [reaction (9) + reaction (23)], one can calculate values of K, $\Delta_r G^o_m$, $\Delta_r H^o_m$, and $\Delta_r S^o_m$ for reactions (21) and (22). The results of these calculations are given in Table 8 which summarizes the thermodynamic quantities for the several reference reactions pertinent to this study.

3.7. Apparent equilibrium constants under approximately physiological conditions

The results obtained in this study allow for the calculation of apparent equilibrium constants K' and of standard molar transformed Gibbs energy changes $\Delta_r G'^o_m$ [14] for the various reactions considered herein under approximately physiological conditions. Here, physiological conditions are taken to be [37] $T = 311.15$ K, pH = 7.0, pMg = 3.0, and $I_m = 0.25$ mol kg^{-1}. The results of these calculations are given in Table 9.

4. Conclusion

This study, in conjunction with earlier ones [7–10] from this laboratory, provides the essential thermodynamic data for the reactions that occur at

Table 8
Equilibrium constants K, standard molar Gibbs energy changes $\Delta_r G^o_m$, standard molar enthalpy changes $\Delta_r H^o_m$, and standard molar entropy changes $\Delta_r S^o_m$ at $T=298.15$ K and $I=0$ for the several reference reactions in aqueous solution that are pertinent to this study[a]

Reaction	K	ΔrG^o_m (kJ mol^{-1})	ΔrH^o_m (kJ mol^{-1})	ΔrS^o_m (J K^{-1} mol^{-1})
(9) chorismate^{2-} + NH_4^+ = ADC$^-$ + H_2O	10.8 ± 4.2	$-(5.9 \pm 1.0)$	$-(35 \pm 15)$	$-(98 \pm 50)$
(10) ADC$^-$ = 4-aminobenzoate$^-$ + pyruvate$^-$ + H^+	$\approx 3\times10^{26}$	≈ -151	$-(139 \pm 23)$	≈ 41
(11) chorismate^{2-} + NH_4^+ = 4-aminobenzoate$^-$ + pyruvate$^-$ + H_2O + H^+	$\approx 3\times10^{27}$	≈ -157	$-(174 \pm 6)$	≈ -57
(21) chorismate^{2-} + L-glutamine$^{\pm}$ = 4-aminobenzoate$^-$ + pyruvate$^-$ + L-glutamate$^-$ + H^+	$\approx 7\times10^{29}$	≈ -170	$-(199 \pm 6)$	≈ -97
(22) chorismate^{2-} + L-glutamine$^{\pm}$ = ADIC$^-$ + L-glutamate$^-$	$\approx 2\times10^{3}$	≈ -19	$-(60 \pm 15)$	≈ -138
(23) L-glutamine$^{\pm}$ + H_2O = L-glutamate$^-$ + NH_4^+	≈ 200	≈ -13	$-(25.2 \pm 0.3)$	≈ -40

[a] The results for reaction (23) are from Kishore et al. [36]. The values for reactions (21) and (22) were calculated (see Section 3.6). In some cases the values are based on estimates; these values have been 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 uncertainties is given in Section 3.4.

Table 9
Apparent equilibrium constants K' and standard transformed molar Gibbs energy changes $\Delta_r G'^{o}_m$ under 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'	$\Delta rG'^{o}_m$ (kJ mol^{-1})
Chorismate + ammonia = ADC + H_2O	1.7	−1.4
L-glutamine + H_2O = L-glutamate + ammonia	250	−14.3
Chorismate + L-glutamine = ADC + L-glutamate	425	−15.7
ADC = 4-aminobenzoate + pyruvate	$\approx 4\times10^{32}$	≈ −194
Chorismate + ammonia = 4-aminobenzoate + pyruvate + H_2O	$\approx 7\times10^{32}$	≈ −196
Chorismate + L-glutamine = 4-aminobenzoate + pyruvate + L-glutamate	$\approx 2\times10^{35}$	≈ −210

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

the chorismate branch point in the shikimic acid metabolic pathway. Three of the five reactions that start with chorismate can be considered to be 'reversible' in the sense that the apparent equilibrium constants for these reactions are not large ($K' < 100$). However, two of these three reactions are immediately followed by reactions that have very large values of the apparent equilibrium constant ($K' > 10^{20}$) and which would be termed as 'irreversible' in the literature. The one remaining reaction, namely the conversion of chorismate to isochorismate, is followed by conversion first to 2,3-dihydro-2,3-dihydroxybenzoate(aq) and then to 2,3-dihydroxybenzoate(aq). While values of the apparent equilibrium constant are not known for these two reactions, it is expected that the conversion to 2,3-dihydroxybenzoate(aq) is 'irreversible' because this reaction results in the formation of an aromatic six-membered ring. In fact, it is the formation of aromatic six-membered rings that underlies the high exothermicity of this class of reactions and that are an important characteristic of the shikimic acid pathway. Also, it is clear that there must either be a thermodynamic driving force ($\Delta_r G'^{o}_m < 0$) if a pathway is to proceed in the direction that defines that pathway or that the product(s) of the pathway must be removed and thus pull the chain of reactions along according to Le Chatelier's principle. However, having such a driving force in the form of an 'irreversible' reaction at or near the beginning of a pathway serves the additional useful purpose of immediately committing the pertinent substrates to that pathway.

In addition to these qualitative observations, it is hoped that the results of this study will play an important role in a quantitative understanding of the shikimic acid pathway. A formalism for such an understanding is provided by metabolic control theory [38,39]. This theory requires thermodynamic and kinetic data for the reactions of interest and a knowledge of the metabolite concentrations in vivo. Now that an important portion of the thermodynamic data has been obtained, it is hoped that the remaining needed information will also become available. This would lead to a quantitative understanding of the flux of matter and energy for an important series of reactions as they occur in a living system.

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