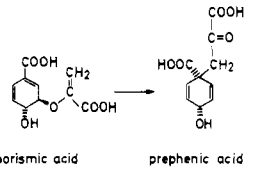
On the Mechanism of the Chorismate Mutase Reaction†

Helmut Gorisch

ABSTRACT: The conversion of chorismic acid to prephenic acid is formally analogous to a Claisen rearrangement. The activation parameters of this reaction are determined when catalyzed by the enzymes chorismate mutase (EC 5.4.99.5) from *Streptomyces aureofaciens* and chorismate mutase-prephenate dehydrogenase (EC 1.3.1.12) from *Aerobacter aerogenes*. The activation enthalpies are found to be 14 500 and 15 900 cal/mol, respectively. The rate constants, with which the enzyme-substrate complexes break down to product and free enzyme, permit calculation of the entropies of activation. For both enzymes, the entropy of activation is found to be about ±1 eu. Comparison of these data with those determined for the spontaneous thermal rearrangement of chorismate to prephenate (Andrews, P. R., Smith, G. D., & Young, J. G. (1973) *Biochemistry 12,* 3492) reveals that for the enzymatic reactions the activation enthalpy is decreased by 6200 and 4800 cal/mol, respectively, and the activation entropy is decreased by about 13 eu to a value of virtually zero. Chorismate mutase from *S. aureofaciens* is competitively inhibited by inorganic anions, suggesting that electrostatic forces contribute to the binding of the substrate. When arranged according to increasing inhibitory action, the anions form a Hofmeister series. Studies with substrate analogues further indicate that chorismate is bound by ionic interaction(s) with its carboxyl group(s) and possibly by the formation of a hydrogen bond in which the hydroxyl group of the ring participates. In contrast to aliphatic carboxylic acids, aromatic carboxylic acids are potent inhibitors which may indicate that hydrophobic forces or π-electron interactions contribute to the binding of the substrate.

The main branch point in the biosynthesis of the aromatic amino acids is chorismic acid. The intramolecular rearrangement of chorismic acid to prephenic acid (Scheme I),

SCHEME I



which is formally analogous to a Claisen rearrangement, is the first reaction of the terminal steps in the biosynthesis of phenylalanine and tyrosine. The spontaneous thermal rearrangement of chorismic acid proceeds at a measurable rate in the absence of enzyme. It appears that the one substrate-one product chorismate mutase reaction is one of the few examples where the enzymatic and the nonenzymatic reaction can be compared directly. Edwards & Jackman (1965) first put forward the idea that the enzyme chorismate mutase, which catalyzes the conversion of chorismic acid to prephenic acid, may invert the ring of the chorismate molecule to the energetically less favored axial conformation and orient the enolpyruvyl side chain correctly which facilitates formation of the transition state. The nonenzymatic rearrangement of chorismic acid was studied in detail by Andrews et al. (1973). In this communication the enthalpy and entropy of activation of the enzymatic conversion of chorismate to prephenate were studied.

Materials and Methods

Chemicals. The inorganic chemicals used were of analytical reagent grade. Acetic acid, succinic acid, adipic acid, phenol, anthranilic acid, 4-aminobenzoic acid, salicylic acid, phthalic acid, 2,4-dinitrophenylhydrazine, and dithiothreitol were obtained from Merck, Darmstadt, Germany. Shikimic acid was from Roth, Karlsruhe, Germany, and 3-hydroxybenzoic acid and terephthalic acid were from Fluka, Buchs, Switzerland. Sodium benzoate, 4-hydroxybenzoic acid, and the disodium salt of ethylenediaminetetraacetate (EDTA) were obtained from Riedel de Haen, Hannover, Germany, and 4-nitrobenzoic acid was from Schuchardt, München, Germany. Isophthalic acid, glutaric acid, and adamantane-1-carboxylic acid were from Ega-Chemie, Steinheim, Germany, and 4-hydroxyisophthalic acid and 5-hydroxyisophthalic acid from ICN Pharmaceuticals, Plainview, N.Y. Sephadex G-100 and G-200 were obtained from Pharmacia, Uppsala, Sweden. Chorismic acid was isolated according to Gibson (1970). Prephenate was prepared as described previously (Görisch, 1977).

Organisms and Growth Conditions. Streptomyces aureofaciens Tü 24 was grown as described previously (Görisch & Lingens, 1974). *Aerobacter aerogenes* poly 3 was a gift of Dr. Gibson, Canberra, Australia, and was grown as described by Koch et al. (1970a). The cells were harvested 12 h after inoculation. Buffers used were: (A) 0.2 M potassium phosphate, pH 7.5, 10^{-4} M EDTA, 10^{-4} M dithiothreitol; (B) 0.1 M Tris-HCl, 10^{-3} M EDTA, 10^{-3} M dithiothreitol, 10^{-3} M tyrosine, pH 8.1. The pH values of the Tris-HCl buffers were adjusted at room temperature, and the actual pH at any other temperature was calculated using a $pK_a/°C$ of −0.031 as given by Good et al. (1966).

Protein Determination. Protein was determined by the method of Groves et al. (1968). With extracts of *S. aureofaciens* the isoabsorbance wavelengths 224 and 235 nm were used (Görisch & Lingens, 1973).

Enzyme Preparations. Chorismate mutase from *S. aureofaciens* was purified 12-fold by $(NH_4)_2SO_4$ fractionation and chromatography on Sephadex G-200 to a specific activity of 0.66 unit/mg as described (Görisch & Lingens, 1974). The enzymatic activity was determined in buffer A. One unit of enzyme represents the formation of 1 μmol of prephenate per min at a temperature of 30 °C in an incubation mixture containing 0.35 μmol of chorismate in a total volume of 1 mL.

† From the Institut für Mikrobiologie der Universität Hohenheim, 7 Stuttgart 70, Germany. *Received February 22, 1978.* Part of these results has been reported at the Frühjahrstagung der Gesellschaft für Biologische Chemie, Regensburg, March 21-23, 1977 (Görisch, 1977a).

0006-2960/78/0417-3700$01.00/0

Chorismate mutase-prephenate dehydrogenase from *A. aerogenes* poly 3 was purified 17-fold by protamine sulfate precipitation, $(NH_4)_2SO_4$ fractionation, and Sephadex G-100 chromatography to a specific activity of 3.3 units/mg according to Koch et al. (1970a). The enzymatic activity was determined in buffer B. One unit of enzyme represents the formation of 1 μmol of prephenate per min at a temperature of 37 °C in an incubation mixture containing 2.5 μmol of chorismate in a total volume of 1 mL. This unit corresponds to 200 "units" as defined by Koch et al. (1970a).

Enzymatic Test. The activity of chorismate mutase was determined by estimating the prephenate formed from chorismate in a total volume of 1 mL. The temperature was held constant to ±0.05 °C. Correction for the nonenzymatic conversion of chorismate to prephenate during incubation was made by including an appropriate blank. After incubation 0.5 mL of 2 N HCl was added and incubation continued for a further 10 min at 30 °C to convert prephenic acid to phenylpyruvic acid. The formation of product was linear with time and proportional to the concentration of the enzymes at all conditions used.

Determination of Phenylpyruvic Acid. (A) The phenylpyruvic acid formed by the acid treatment of prephenate was measured at 320 nm after the addition of 1 mL of 3 N NaOH. A molar extinction coefficient of 17 000 M^{-1} cm^{-1} was used (Nishioka & Woodin, 1972). (B) In the presence of salicylic acid, 3-hydroxybenzoic acid, 4-hydroxyisophthalic acid, 5-hydroxyisophthalic acid, anthranilic acid, and 4-nitrobenzoic acid, the concentration of phenylpyruvic acid was estimated by the absorbance of its 2,4-dinitrophenylhydrazone as described elsewhere (Görisch, 1978). Samples containing a known amount of prephenate were used for standardization.

Results

Chorismate Mutase from S. aureofaciens: pH Optimum and Stability. The influence of hydrogen ion concentration upon the chorismate mutase reaction with the enzyme from *S. aureofaciens* was studied between pH 7 and 8. The maximal velocity V_{max} at each pH value was obtained by a least-squares analysis of substrate saturation curves according to eq 1 given by Hanes (1932)

$$\frac{S}{V} = \frac{1}{V_{max}} \times S + \frac{K_m}{V_{max}} \quad (1)$$

where v, S, and K_m are the initial velocity, the substrate concentration, and the apparent Michaelis constant, and V_{max} is the maximal velocity. The enzyme exhibits a broad pH optimum. No variation of the maximal velocity is observed in the pH range studied, neither at 30 °C nor at 10 °C. It was assured that within 1 h no inactivation of the enzyme occurs at all conditions used. No variation of the maximal velocity, V_{max}, was found for chorismate mutase from *S. aureofaciens* when the enzymatic activity was tested in phosphate buffers (pH 7.5), the concentrations of which vary from 0.05 to 0.3 M.

Determination of the Activation Parameters. The one-substrate-one-product reaction catalyzed by chorismate mutase will be analyzed by the simple model

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P$$

for which V_{max} is directly proportional to k_2. It is assumed that the breakdown of the enzyme-product complex to product and free enzyme is fast in comparison with the conversion of the enzyme-substrate to the enzyme-product complex.

Substrate saturation curves were measured at pH 7.5 between 10 and 30 °C. The maximal velocity V_{max} at each temperature was obtained by a least-squares analysis of the data according to the eq 1. The temperature dependence of the maximal velocity, V_{max}, which is proportional to the velocity constant k_2, i.e., the turnover number of the enzyme, is analyzed according to the Arrhenius equation (eq 2)

$$k_2 = Ae^{-E_a/RT} \quad (2)$$

where k_2 is the velocity constant, A is the frequency factor, E_a the activation energy, and R and T are the gas constant and the absolute temperature, respectively. From the slope of the Arrhenius diagram, the activation energy was calculated to be 15 100 ± 400 cal/mol. According to eq 3

$$\Delta H^{\ddagger} = E_a - RT \quad (3)$$

the enthalpy of activation $\Delta H^{\ddagger}$ was found to be 14 500 ± 400 cal/mol. The most highly purified chorismate mutase preparation from *S. aureofaciens* shows a specific activity of 120 μmol of prephenate per min per mg at a concentration of 0.35 mM chorismate[1] and a temperature of 30 °C. The apparent Michaelis constant was determined as 5.2×10^{-4} M (Görisch & Lingens, 1974). Using a molecular weight of 60 000 for chorismate mutase from *S. aureofaciens* and assuming a single catalytic site per enzyme molecule, the turnover number is calculated to be 300 s^{-1}. The enzyme is composed of three or four subunits (Görisch & Lingens, 1974). Assuming four subunits per enzyme molecule and one active site per subunit, the turnover number reduces to 75 s^{-1}.

According to transition state theory, the temperature dependence of the velocity constant k_2 is given by eq 4

$$k_2 = \frac{RT}{N\hbar} e^{-((\Delta H^{\ddagger}/RT)-(\Delta S^{\ddagger}/R))} \quad (4)$$

where $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, N is Avogadros number, $\hbar$ is Planck's constant, and R and T are the gas constant and the absolute temperature. Using the estimated rate constants with which the enzyme-substrate complex breaks down to free enzyme and product, and the known enthalpy of activation the entropy of activation can be calculated. Depending on the number of active sites assumed per enzyme molecule, its value is 0.6 to −2 eu (Table I).

Inhibition by Inorganic Anions and Cations. Monovalent anions in a concentration of 2×10^{-2} M inhibit the enzymatic activity of chorismate mutase from *S. aureofaciens* as is shown in Table II. The inhibition is effective immediately on mixing the salt solution with the enzyme and is readily reversed by dilution. When arranged in the order of increasing inhibitory action the anions form a Hofmeister series: Cl^-, $CN^- < Br^- < NO_3^- < I^- < ClO_4^-$, SCN^-. Inorganic cations like K^+, Na^+, and Li^+ in concentrations of 2×10^{-2} M do not inhibit the enzymatic activity of chorismate mutase from *S. aureofaciens* when tested in buffer B.

Figure 1A shows substrate saturation curves determined in the absence and presence of various inorganic anions and plotted according to eq 1. The apparent Michaelis constant is strongly influenced by the addition of inhibiting monovalent anions, while the slopes of the lines ($1/V_{max}$) are reduced insignificantly by 10% to 25%. The inhibition is therefore regarded as competitive.

To establish the number of anions bound per active site, inhibition data of several anions (Figure 2A) were analyzed according to eq 5

[1] In Görisch & Lingens (1974) the concentration of chorismate in the standard test was erroneously given as 2.5 mM. The concentration used was 0.35 mM as described earlier (Görisch & Lingens, 1973).

TABLE I: Activation Parameters of the Chorismate Mutase Reaction.

source of enzyme	no. of catalytic sites per molecule assumed	k_2 (s^{-1})	$\Delta H^{\ddagger}$ (cal/mol)	$\Delta S^{\ddagger}$ (eu)
spontaneous rearrangement[a]		1.24×10^{-5} (30 °C) 2.6×10^{-5} (37 °C)	20 700 ± 350	−12.9 ± 0.4
S. aureofaciens	1	300[b]		0.6
	2	150	14 500 ± 350	−0.8 ± 1.1
	4	75		−2.2
Aerobacter aerogenes	1	50[c]		0.3 ± 1.2
	2	25	15 900 ± 400	−1.1

[a] Determined by Andrews et al. (1973). [b] Determined at 30 °C; Görisch & Lingens (1974). [c] Determined at 37 °C; Koch et al. (1970a).

TABLE II: Inhibition of Chorismate Mutase from *S. aureofaciens* by Inorganic and Organic Anions.

compound added (2×10^{-2} M)	act.[a] (%)	r[b]	K_i[c] (M)
none	100		
KNO_3	67	1.13 ± 0.05	20×10^{-3}
KI	55	1.21 ± 0.1	10×10^{-3}
$NaClO_4$	48	1.06 ± 0.1	8×10^{-3}
KSCN	44	1.22 ± 0.1	6×10^{-3}
adamantane-1-carboxylic acid	79	1.93 ± 0.15	
phenol	77	2.5 ± 0.3	
benzoic acid	77	1.34 ± 0.1	28×10^{-3}
4-nitrobenzoic acid	64	1.37 ± 0.15	15×10^{-3}
salicylic acid	47	1.23 ± 0.11	6×10^{-3}
3-hydroxybenzoic acid	72	1.26 ± 0.04	19×10^{-3}
4-hydroxybenzoic acid	68	1.36 ± 0.1	18×10^{-3}
phthalic acid	72	1.1 ± 0.11	19×10^{-3}
isophthalic acid	64	1.09 ± 0.05	14×10^{-3}
4-hydroxyisophthalic acid	45	0.95 ± 0.1	6×10^{-3}

[a] Determined in buffer A. KCl, acetic acid, succinic acid, glutaric acid, adipic acid, and shikimic acid inhibit less than 4%, KCN, KBr, anthranilic acid, 4-aminobenzoic acid, terephthalic acid, and 5-hydroxyisophthalic acid inhibit less than 20% and therefore are omitted from the table. [b] r is the number of inhibitor molecules bound at the active site as determined by eq 5. [c] K_i is the inhibitor constant determined by eq 6. The K_i values have to be regarded as rough estimates since in evaluating the data it was neglected that V_{max} is 10–20% higher in the presence of inhibitors. In addition in some cases the number of inhibitor molecules bound at the active site apparently is larger than one.

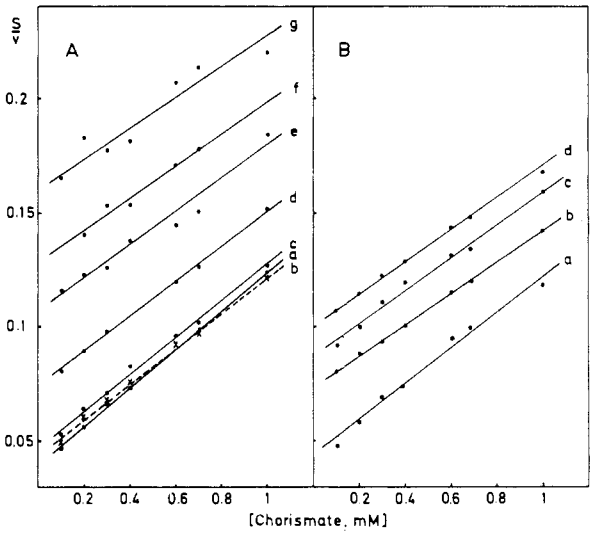


FIGURE 1: (A) Competitive inhibition of chorismate mutase from *S. aureofaciens* by inorganic anions. The reaction mixtures contained 3.8 mU of enzyme, inhibitor, and chorismate in 1 mL of buffer A. (a) Without inhibitor; (b) KCl, 2×10^{-2} M; (c) KBr, 2×10^{-2} M; (d) KNO_3, 2×10^{-2} M; (e) KI, 2×10^{-2} M; (f) $NaClO_4$, 2×10^{-2} M; (g) KSCN, 2×10^{-2} M. The data are plotted according to eq 1. (B) Competitive inhibition of chorismate mutase from *S. aureofaciens* by organic anions. The reaction mixtures contained 4 mU of enzyme, inhibitor, and chorismate in 1 mL of buffer A. (a) Without inhibitor; (b) benzoic acid, 2×10^{-2} M; (c) 4-hydroxybenzoic acid, 2×10^{-2} M; (d) isophthalic acid, 2×10^{-2} M. The data are plotted according to eq. 1.

$$\log (v_i/(v_0 - v_i)) = -r \log I + C \quad (5)$$

where v_i and v_0 are the initial velocity in the presence and absence of inhibitor, I is the inhibitor concentration, and C is a constant. The slopes r of the lines, which give the number of moles of inhibitor bound per mole of sensitive site (Nygard, 1961), are given in Table II.

For the determination of the inhibition constants, K_i, the substrate saturation curves in the presence and absence of inhibitors were replotted according to eq 6 given by Hunter & Downs (1945) for competitive inhibitors.

$$Iv_i/(v_0 - v_i) = K_i + SK_i/K_m \quad (6)$$

v_0 and v_i are the initial velocities in the absence and presence of inhibitors, I and S are the inhibitor and substrate concentrations, respectively, and K_i and K_m are the inhibitor constant and the apparent Michaelis constant. The K_i values shown in Table II were determined by a least-squares analysis of the data.

Inhibition by Organic Anions. Carboxylic acids were also tested as potential inhibitors of chorismate mutase from *S. aureofaciens*. Aliphatic acids at concentrations of 2×10^{-2} M do not inhibit the enzyme, with the exception of adamantane-1-carboxylic acid. Aromatic carboxylic acids show appreciable inhibition (Table II). Also phenol, which is not ionized at the pH used, inhibits the activity of chorismate mutase from *S. aureofaciens*. The inhibition is effective immediately on mixing the inhibitor solutions with the enzyme and is readily reversed by dilution.

Substrate saturation curves were determined in the absence and presence of inhibitory anions. As is seen in Figure 1B, K_m is strongly influenced, while the slope of the lines ($1/V_{max}$) is reduced insignificantly by 10% to 20%. In the presence of 10^{-2} M adamantane-1-carboxylic acid and phenol, however, V_{max} increases by 40% and 50%, respectively, of its value as determined in their absence, and therefore these compounds cannot be regarded as competitive inhibitors. The number mf anions bound per active site are given in Table II. The dicarboxylic acids show a value of r close to 1 and the monocarboxylic acids a slightly higher value of about 1.3 (Figure 2B). Adamantane-1-carboxylic acid and phenol again behave different than the rest of the inhibitory compounds showing values of r of 1.9

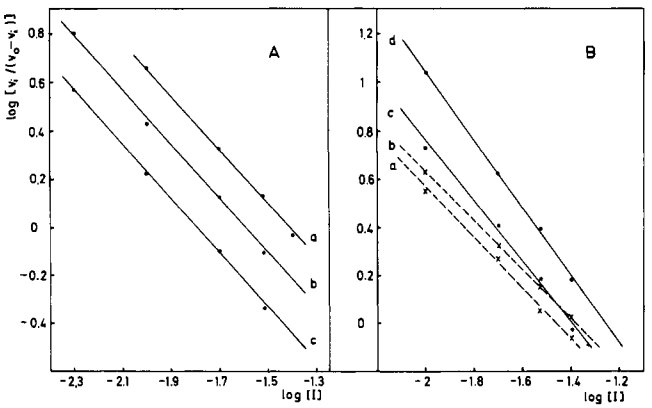


FIGURE 2: (A) Inhibition of chorismate mutase from *S. aureofaciens* by increasing concentrations of inorganic anions. The reaction mixtures contained 4.4 mU of enzyme, 0.5 μmol of chorismate, and inhibitor in 1 mL of buffer A. (a) KNO_3; (b) KI; (c) KSCN. The data are plotted according to eq 5. (B) Inhibition of chorismate mutase from *S. aureofaciens* by increasing concentrations of organic anions. The reaction mixtures contained 4.8 mU of enzyme, 0.5 μmol of chorismate, and inhibitor in 1 mL of buffer A. (a) Isophthalic acid; (b) phthalic acid; (c) 3-hydroxybenzoic acid; (d) benzoic acid. The data are plotted according to eq 5.

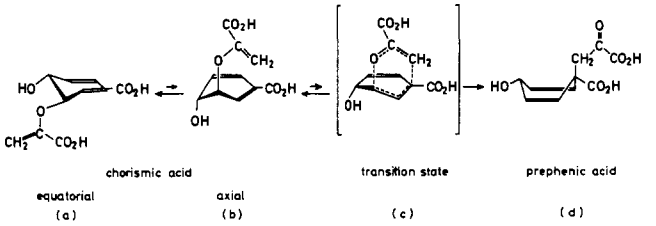


FIGURE 3: Sequence of steps involved in the rearrangement of chorismate to prephenate.

and 2.5, respectively. The inhibition constants K_i were determined according to eq 6 and are shown in Table II.

Chorismate Mutase–Prephenate Dehydrogenase from Aerobacter aerogenes: pH Optimum. The pH optimum of the conversion of chorismate to prephenate catalyzed by chorismate mutase–prephenate dehydrogenase (EC 1.3.1.12) from *A. aerogenes* was studied between pH 7.5 and 8.8. The maximal velocity, V_{max}, at each pH was obtained by a least-squares analysis of substrate saturation curves according to eq 1. At a temperature of 20 °C and a temperature of 37 °C, the enzyme exhibits a pH optimum between pH 8 and pH 8.2.

Determination of the Activation Parameters. Substrate saturation curves at chorismate concentrations from 0.1 to 1 mM were measured at pH 8.1 between 20 and 37 °C. The temperature dependence of the maximal velocity, V_{max}, is analyzed according to the Arrhenius equation (eq. 2). The enthalpy of activation was calculated to be 15 900 cal/mol. Using the specific activity for the most highly purified enzyme obtained by Koch et al. (1970a) and assuming a single catalytic site per molecule, Andrews et al. (1973) calculated a turnover number of 50.4 s^{-1}. According to Koch et al. (1970b), chorismate mutase–prephenate dehydrogenase from *A. aerogenes* consists of two apparently identical subunits. Assuming a catalytic site per subunit, the turnover number is reduced to 25 s^{-1}. The entropy of activation is thus found to be 0.3 to −1.1 eu (Table I).

Discussion

The main function of an enzyme is to enhance the rate at which biological substrates are transformed into their products. In the absence of the enzyme chorismate mutase, chorismate spontaneously rearranges to prephenate at pH 7.5. The reaction follows first-order kinetics with a rate constant of 1.24×10^{-5} s^{-1} at 30 °C and 2.6×10^{-5} s^{-1} at 37 °C (Andrews et al., 1973). From the specific activity of the most highly purified preparation of chorismate mutase (EC 5.4.99.5) from *Streptomyces aureofaciens* (Görisch & Lingens, 1974), a turnover number of 300 s^{-1} at 30 °C can be calculated. The enzyme consists of three to four similar subunits so that two or even four catalytic sites per enzyme molecule are possible. Thus the turnover number reduces to 150 or 75 s^{-1}, respectively. A similar calculation was done by Andrews et al. (1973) with the most highly purified preparation of chorismate mutase–prephenate dehydrogenase (EC 1.3.1.12) from *Aerobacter aerogenes* (Koch et al., 1970a). The turnover number was calculated to be 50 s^{-1} at 37 °C. Since chorismate mutase–prephenate dehydrogenase from *A. aerogenes* is composed of two identical or almost identical subunits (Koch et al., 1970b), we may assume there are two catalytic sites per enzyme molecule. The turnover number is then reduced to 25 s^{-1}. The enzymes chorismate mutase from *S. aureofaciens* and chorismate mutase–prephenate dehydrogenase from *A. aerogenes* thus enhance the rate at which chorismate is converted to prephenate by roughly a factor of 10^7 and 10^6, respectively (Table I).

Andrews et al. (1973) studied the temperature dependence of the rate of the nonenzymatic conversion of chorismate to prephenate. They determined an enthalpy of activation of 20.7 kcal/mol and an entropy of activation of −12.9 eu. The conversion of chorismate to prephenate is formally analogous to a Claisen rearrangement. Similar reactions are the rearrangement of 1-cyclohexenylallylmalonitrile (Foster et al., 1947), vinyl allyl ether (Schuler & Murphy, 1950) and isopropenyl allyl ether (Stein & Murphy, 1952) with enthalpies of activation of roughly 25.2, 29.6, and 28.3 kcal/mol and entropies of activation of −11.7, −7.7, and −7.7 eu, respectively. While the enthalpies of activation are slightly higher, the entropies of activation are slightly smaller compared with the conversion of chorismate to prephenate. The sequence of steps probably involved in the rearrangement of chorismate depicted in Figure 3 is based on the results of Andrews et al. (1973).

It is reasonable to assume that the enzymatic conversion of chorismate involves the same transition state as the nonenzymatic reaction. Chorismate mutase from *S. aureofaciens* as well as chorismate mutase–prephenate dehydrogenase from *A. aerogenes* enhance the rate of the rearrangement of chorismate by reducing the entropy of activation to virtually zero. This means that in the enzyme–substrate complex the free rotation of the enolpyruvyl side chain is abolished and the side chain is oriented correctly to allow the transition state to form. In addition, the enzymes enhance the rate of reaction by reducing the enthalpy of activation by 5 to 6 kcal/mol. This value is close to the energy difference of 7 kcal calculated for the equatorial and axial conformation of chorismate by Andrews et al. (1973). Thus by comparing the enthalpy and entropy of activation of the chemical and enzymatic rearrangement of chorismate (Table I), one may conclude that the enzymes stabilize chorismate in its axial conformation and freeze the free rotation of the enolpyruvyl side chain by orienting it correctly. Of course the enzymes can achieve the reduction of the enthalpy of activation also by stabilizing the transition state by 5–6 kcal/mol. As can be seen in Figure 3 the transition state closely resembles the axial form of chorismate.

The most likely groups involved in bond formation between the substrate and the active site of the enzyme are the ring

TABLE III: Angles[a] of Chorismate Bound to the Proposed Active Site of Chorismate Mutase from *Streptomyces aureofaciens* Compared with Conformation of Free Chorismate.

	bound to chorismate mutase					angles calcd by extended Hückel theory from Andrews et al. (1973)				
	α	β	γ^b	μ^b	ν	α	β	γ^b	μ^b	ν
equatorial	120	270	170	0	40	120	90 270	0	0	300
axial	280	300	140	90	130	120	90 270	0	0	60

[a] The angles are labeled and defined according to Andrews et al. (1973), Figure 4. [b] $\gamma = 0 (= 180°)$; $\mu = 0 (= 180°)$.

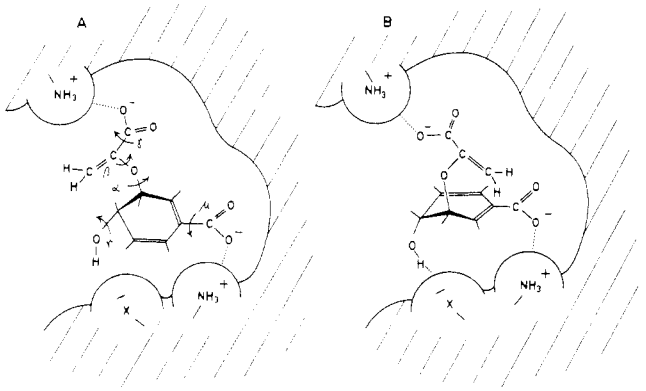


FIGURE 4: Proposed active site of chorismate mutase. The binding of the substrate chorismate is shown: (A) in its most stable equatorial conformation; (B) in its axial conformation (which closely resembles the assumed transition state) with the enolpyruvyl side chain oriented correctly to allow the transition state to form. This axial conformation is reached from A by inversion of the conformation of the ring and rotations around the bonds α, β, γ, μ, and ν, without breaking one of the ionic bonds in which the carboxyl groups are assumed to participate.

carboxyl, the hydroxyl, and the side chain carboxyl group of chorismate. These groups are all capable of forming strong ionic and hydrogen bonds with suitable partners. Inhibition studies with anions support this idea. Chorismate mutase from *S. aureofaciens* is inhibited by monovalent inorganic anions at concentrations of 2×10^{-2} M at which they do not destroy secondary and tertiary structure of enzymes (Von Hippel & Schleich, 1969). When the anions are arranged according to their inhibitory power, they form a Hofmeister series. A similar situation is described by Fridovich (1963) for the inhibition of acetoacetic acid decarboxylase by inorganic anions.

Aromatic carboxylic acids are also inhibitors of chorismate mutase from *S. aureofaciens*. Aromatic dicarboxylic acids like phthalic acid and isophthalic acid are stronger inhibitors than benzoic acid. The introduction of a hydroxyl group also results in a stronger inhibitory action as is demonstrated with salicylic acid, 3-hydroxy- and 4-hydroxybenzoic acid. The strongest inhibitor is 4-hydroxyisophthalic acid. The inhibition of chorismate mutase by inorganic and organic anions shows competitive behaviour with respect to the substrate, which may indicate that cationic groups at the active site of the enzyme are involved in the binding of chorismate. The strong inhibitory action of salicylic acid and 4-hydroxyisophthalic acid suggests that a hydrogen bond participates also.

That, in addition to ionic attraction and hydrogen bonding, hydrophobic forces or π-electron interactions may contribute to the binding of the substrate is suggested by the finding that aliphatic mono- and dicarboxylic acids like acetic acid, shikimic acid, succinic acid, glutaric acid, and adipic acid are only very weak inhibitors.

If the inhibition is studied with increasing concentrations of the inhibitors, the number of inhibitor molecules binding to the active site shows a tendency to be larger than one in the case of the monovalent inorganic anions and the monocarboxylic acids. This suggests that two inhibitor molecules can be bound. In case of the dicarboxylic aromatic acids, it was found within experimental error that only one molecule of inhibitor binds to the active site. These findings support the idea that there may be two cationic groups at the active site. Independent evidence for ionic bond formation stems from studies of Gething & Davidson (1977) with chorismate mutase-prephenate dehydratase (EC 4.2.1.51) from *Escherichia coli*. The chorismate mutase activity of this enzyme is completely lost by modification of a single lysine residue per subunit.

Evidence that the binding of the enolpyruvyl side chain at the active site is important in forming the enzyme-substrate complex stems from inhibitor studies by Ife et al. (1976) with chorismate mutase-prephenate dehydrogenase (EC 1.3.1.12) from *A. aerogenes*. They found that the methyl ester of the side chain carboxyl group of chorismic acid does not inhibit the activity of the enzyme. The 2,3-epoxide of chorismic acid and the 2,3-dihydrochorismic acid with unmodified carboxylic acid groups, however, show competitive inhibition with respect to the substrate. Recently Andrews et al. (1977) reported inhibitor studies with chorismate mutase-prephenate dehydrogenase (EC 1.3.1.12) from *E. coli*. Their results with hydroxybicyclononanedicarboxylic acids suggest that the enzymically catalyzed conversion of chorismate to prephenate proceeds through a chair-like transition state. Inhibitor studies with derivatives of adamantane reveal, in agreement with the results reported in this paper, that, in addition to the ionic attraction of carboxylic acid groups and the hydrogen bonding of a hydroxyl group, hydrophobic forces contribute to the binding of the inhibitors at the active site of the enzyme.

Considering the sequence of steps which occur during the rearrangement of chorismate to prephenate one is tempted to construct a model of the active site of chorismate mutase using the axial conformation of the substrate, because it closely resembles the transition state (Figure 4). It is assumed that both carboxyl groups are bound by ionic interactions so that the oxygen atoms can move on a sphere with a radius of 2.8 Å around the assumed protonated amino groups. The length of the proposed hydrogen bond formed by the ring hydroxyl group with a suitable partner also is assumed to be 2.8 Å. This crude speculative model shows an interesting property insofar as chorismate may change from the axial conformation to an equatorial conformation by rotations of the angles α, β, γ, μ, and ν (Figure 4) without breaking one of the ionic bonds in which the carboxyl groups are assumed to participate. Moreover the equatorial conformation finally reached closely resembles the most stable conformation of free chorismate in

solution as predicted from molecular orbital calculations by Andrews et al. (1973). In Table III the angles of the most stable conformations of free chorismate are compared with the angles derived from the model study. One may speculate, therefore, that chorismate mutase binds its substrate in the equatorial form via ionic interactions with both carboxyl groups, thus abolishing the rotational freedom of the enolpyruvyl side chain (Figure 4A). The substrate can be inverted to the axial conformation, without breaking one of the ionic bonds, and the axial conformation then may be stabilized by the formation of a hydrogen bond (Figure 4B). This sequence of steps can explain how the entropy of activation is reduced by about 13 eu and the enthalpy of activation is decreased by 5 to 6 kcal/mol with respect to the nonenzymatic conversion of chorismate to prephenate.

Acknowledgments

The author wishes to thank Professor Dr. F. Lingens for his continuous interest in this work, financial support, and careful reading of the manuscript. He is grateful to Mrs. I. Beck for excellent technical assistance.

References

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

Andrews, P. R., Cain, E. N., Rizzardo, E., & Smith, G. D. (1977) *Biochemistry 16,* 4848.

Edwards, J. M., & Jackman, L. M. (1965) *Aust. J. Chem. 18,* 1227.

Foster, E. G., Cope, A. C., & Daniels, F. (1947) *J. Am. Chem. Soc. 69,* 1893.

Fridovich, I. (1963) *J. Biol. Chem. 238,* 592.

Gething, M.-J. H., & Davidson, B. E. (1977) *Eur. J. Biochem. 78,* 111.

Gibson, F. (1970) *Methods Enzymol. 17A,* 362.

Good, N. E., Winget, G. D., Winter, W., Conolly, T. N., Izawa, S., & Singh, R. M. M. (1966) *Biochemistry 5,* 467.

Görisch, H. (1977a) *Hoppe-Seyler's Z. Physiol. Chem. 358,* 237.

Görisch, H. (1977b) *Anal. Biochem. 82,* 177.

Görisch, H. (1978) *Anal. Biochem. 86,* 764.

Görisch, H., & Lingens, F. (1973) *J. Bacteriol. 114,* 645.

Görisch, H., & Lingens, F. (1974) *Biochemistry 13,* 3790.

Groves, W. E., Davis, Jr., F. C., & Sells, B. (1968) *Anal. Biochem. 22,* 195.

Hanes, C. S. (1932) *Biochem. J. 26,* 1406.

Hunter, A., & Downs, C. E. (1945) *J. Biol. Chem. 157,* 427.

Ife, R. J., Ball, L. F., Lowe, P., & Haslam, E. (1976) *J. Chem. Soc., Perkin Trans. 1,* 1776.

Koch, G. L. E., Shaw, D. C., & Gibson, F. (1970a) *Biochim. Biophys. Acta 212,* 375.

Koch, G. L. E., Shaw, D. C., & Gibson, F. (1970b) *Biochim. Biophys. Acta 212,* 387.

Nishioka, L., & Woodin, T. (1972) *Anal. Biochem. 45,* 617.

Nygard, A. P. (1961) *J. Biol. Chem. 236,* 2779.

Schuler, F. W., & Murphy, G. W. (1950) *J. Am. Chem. Soc. 72,* 3155.

Stein, L., & Murphy, G. W. (1952) *J. Am. Chem. Soc. 74,* 1041.

Von Hippel, P. H., & Schleich, T. (1969) in *Structure and Stability of Biological Macromolecules* (Timasheff, S. N., & Fasmann, G. D., Eds.) p 417, Marcel Dekker, New York, N.Y.