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MECHANISM OF THE ANTHRANILATE SYNTHETASE REACTION

EVIDENCE AGAINST AN INTRAMOLECULAR HYDROGEN TRANSFER

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SUMMARY

The mechanism of the anthranilate synthetase reaction was studied using chorismic acid specifically labeled with ^{14}C and ^{3}H and the partially purified enzyme from *Salmonella typhimurium*. The third hydrogen atom of the methyl group of pyruvate formed in the reaction does not originate from C-2 nor from any other ring position of chorismate. ^{3}H at C-2 of chorismate is not exchanged by the enzyme in the absence of glutamine. The data rule out an intramolecular hydrogen transfer from C-2 of chorismate to C-3′ of the side-chain, which had been proposed earlier, and strongly suggest that water is the ultimate source of the third hydrogen of the pyruvate methyl group. The observed low incorporation of ^{3}H from ^{3}HOH into the methyl group of pyruvate is explained by an isotope effect.

INTRODUCTION

Anthranilate synthetase catalyzes the first specific reaction in tryptophan biosynthesis, the glutamine[1]- or NH_3-dependent[2] conversion of chorismate into anthranilate (Diagram 1). In this complex reaction, the enolpyruvyl group at C-3 of chorismate and the hydroxyl group at C-4 are eliminated and an amino group is introduced at C-2. In its native state, in *Salmonella typhimurium*, anthranilate synthetase exists as an aggregate with the next enzyme of the tryptophan biosynthetic pathway, anthranilate–5-phosphoribosyl-1-pyrophosphate phosphoribosyltransferase[3]. The enzyme aggregate can utilize either glutamine or NH_3 for anthranilate synthesis. The anthranilate synthetase protein can be obtained free from anthranilate–5-phosphoribosyl-1-pyrophosphate phosphoribosyltransferase. This protein has been termed anthranilate synthetase Component I and will only use NH_3 as amino donor for anthranilate synthesis[3,4].

In a previous study of the reaction, catalyzed by anthranilate synthetase Compo-

nent I, it was observed that very little tritium was incorporated into the pyruvate when the reaction was carried out in tritiated water[5]. On the basis of this result, it was concluded that the third hydrogen atom of the methyl group of pyruvate originated from chorismate, and as a possible mechanism it was suggested that the proton from C-2 of the ring of chorismate underwent an intramolecular shift to the methylene group of the leaving enolpyruvate during the terminal elimination step. It was suggested that this intramolecular shift could be mediated by a basic group on the enzyme that is susceptible to alkylation by bromopyruvate, which accepts the proton and, without exchange with the protons of the medium, transfers it to the methylene group.

In the present paper we report results showing that the hydrogen atom from C-2 of the ring of chorismate is not incorporated into the pyruvate formed. The data also indicate that the third hydrogen of the pyruvate methyl group does not originate from any of the other ring positions of chorismate. A preliminary account of this work has been presented[6].

MATERIALS AND METHODS

Chemicals and enzymes

Reagent grade chemicals were used whenever possible. Chorismic acid was prepared according to the method of GIBSON[7]. Anthranilate synthetase was partially purified from *S. typhimurium*. Most experiments were carried out with a preparation of the native enzyme aggregate purified through the DEAE-cellulose column step[16], which had a specific activity of 678 units/mg protein. Glutamine was always used as the amino donor with this enzyme. A number of experiments were duplicated using the purified NH_3-dependent anthranilate synthetase Component I (380 units/mg. protein)[5]. Lactate dehydrogenase from rabbit muscle (400 units/mg) was obtained from Calbiochem.

Labeled compounds

Tritiated water (1 C/ml) was purchased from New England Nuclear Corp., deuterated water (99.7% ^2H) from Columbia Organic Chemical, Co., and DL-[1,6-^{14}C]-shikimic acid (8.7 μC/μmole) from Schwarz Bio Research. [7-^{14}C]Anthranilic acid (1.3 μC/μmole) was a gift from Prof. I. A. Spenser, McMaster University, Hamilton. [1,2-^{14}C]-, [7-^{14}C]- and [2-^3H]chorismic acids were prepared from [1,6-^{14}C]-, [7-^{14}C]- and [6-S, 6-^3H]shikimic acids, respectively, using a cell-free extract of *Aerobacter aerogenes* mutant 62-1 (ref. 8). The same procedure was also followed in the preparation of [*side chain*-1-^{14}C]- and [3-^3H]chorismic acids from non-labeled shikimic acid and [1-^{14}C]- and [(Z)-3-^3H]phosphoenolpyruvate[8,9]. The double-labeled preparations of chorismic acid were obtained by mixing the appropriate singlely labeled species. The specific activities of these preparations were usually not exactly known, but were high enough to ensure that the amount of material introduced with the radioactivity was negligible compared with the amount of carrier substrate.

Chromatography systems

All separations were carried out on Whatman 3MM paper, which had been washed with distilled water for 24 h and air-dried. The following solvent systems were used: (1) ethyl acetate–isopropanol–conc. ammonia (45:35:20, by vol.) (anthranilate

R_F 0.3; lactate R_F 0.1); (2) ethanol–conc. ammonia–water (80:4:17, by vol.) (lactate and anthranilate R_F 0.6, phosphoenolpyruvate, phosphoglycerate, pyruvate, shikimate all between R_F 0 and 0.1).

Radioactive bands were eluted with distilled water and the solutions were concentrated by lyophilization.

Assay procedures

The anthranilate synthetase reaction was assayed in either or both of two ways: (1) by measuring the increase in fluorescence at 400 nm ($\lambda_{exc.}$ 325 nm) due to the anthranilate formed[5]. These measurements were carried out in an Aminco–Bowman spectrofluorometer. (2) By recording the decrease in the absorbance at 340 nm due to consumption of reduced pyridine nucleotide, when the reaction was carried out in the presence of NADH and lactate dehydrogenase. A Zeiss PMQ II spectrophotometer combined with a Sargent SRG linear recorder was used for these determinations. Method 1 was also used to determine the concentration of solutions of chorismate. In some experiments when [*ring*-^{14}C]substrate was used, the minimum conversion of chorismate to anthranilate was determined from the amount of [^{14}C]-anthranilate recovered from the reaction mixture.

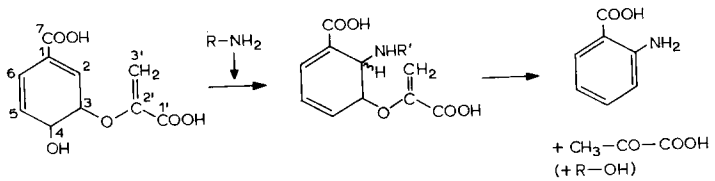

Diagram 1. Conversion of chorismate to anthranilate showing a hypothetical intermediate. R and R′ can be H or γ-glutamyl.

Radioactivity determinations

Radioactive substances on paper chromatograms were located by scanning the chromatogram in a Packard Model 7201 chromatogram scanner. The radioactivity of substances in solution was determined in a Beckman LS 100 liquid scintillation counter using 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazolyl-2)-benzene in toluene–ethanol as the scintillator solution. ^{3}H/^{14}C ratios were determined by counting the samples in two channels, one of which did only register ^{14}C but not ^{3}H. Counting efficiencies as well as the spillover of ^{14}C into the ^{3}H channel were determined by recounting each sample after the addition of internal standard.

Individual experiments

For reactions using the NH_3-dependent anthranilate synthetase activity of anthranilate synthetase Component I previously described conditions were used[5]. Reaction mixtures for the glutamine-dependent activity of the anthranilate synthetase enzyme aggregate are described in the tables. Unless stated otherwise, incubations were carried out at 25° until the fluorescence due to anthranilate no longer increased. In the experiments of Tables I and III, the reaction mixture was immediately lyophilized and the recovered water was analyzed for tritium. In the experiments of Table III, the residue of the lyophilization was dissolved in water and its ^{3}H/^{14}C ratio was meas-

TABLE I

ELIMINATION OF THE ^3H FROM C-2 OF CHORISMATE IN THE ANTHRANILATE SYNTHETASE REACTION

The reaction mixture contained in a volume of 1 ml: Expts. 1–3: 1 μmole chorismic acid (approx. 0.05–0.1 μC of ^{14}C), 5 μmoles glutamine, 6 μmoles $MgCl_2$, 50 μmoles potassium phosphate buffer (pH 7.4), and 46 μg protein. Expt. 4: 1 μmole chorismic acid (0.1 μC of ^{14}C), 50 μmoles $(NH_4)_2SO_4$, 10 μmoles $MgCl_2$, 50 μmoles triethanolamine–HCl buffer. (pH 8.9), 2 μmoles thioglycerol, 450 μg protein.

Expt. No.	*Enzyme preparation*	*Substrate*	*^{14}C recovered as anthranilate (%)*	*^3H recovered as water (%)*	*$^3H/^{14}C$ ratio of*	
					Chorismate	*Anthranilate*
1	Native	[7-^{14}C,2-^3H]-Chorismate		82	5.2	0.02
2	Native	[7-^{14}C,2-^3H]-Chorismate		79	11.7	0.06
3	Native	[1,2-^{14}C,2-^3H]-Chorismate	59*	82	13.7	0
4	Component I	[1,2-^{14}C,2-^3H]-Chorismate	18	16	13.7	1.37**

* There was some loss of [^{14}C] anthranilate during the isolation.
** Maximum value. There was not enough material to purify to constant $^3H/^{14}C$ ratio.

ured. From the residues of the experiments in Table I, anthranilate was isolated by chromatography in System 1, followed by cocrystallization with carrier anthranilate from ethanol. In the experiments of Table II, the reaction mixture was chromatographed in System 2 and the lactate was rechromatographed in System 1. In the experiments in Table IV, the components of the reaction except chorismate were mixed in normal water at 0°, the solution was lyophilized, the residue dissolved in 0.95 ml tritium water and the chorismate was added as a solution in 0.05 ml normal water. In the experiments of Table V chorismate, glutamine, $MgCl_2$ and buffer were mixed, lyophilized, and dissolved in 0.97 ml of a mixture of triated water and normal water or deuterium water (99.7% ^2H). The remaining components of the reaction mixture were added as a solution in 0.03 ml of normal water. In both sets of experiments (Tables IV and V) the reaction was terminated by freezing and lyophilizing the mixture. The residues were repeatedly (4 times) dissolved in water at 0° and lyophilized. To

TABLE II

NON-INCORPORATION OF THE ^3H FROM C-2 OF CHORISMATE INTO THE PYRUVATE FORMED IN THE ANTHRANILATE SYNTHETASE REACTION

The reaction mixture contained in a volume of 1 ml: 0.1 μmole chorismic acid, 5 μmoles glutamine, 5 μmoles $MgCl_2$, 50 μmoles potassium phosphate buffer (pH 7.4), 0.5 μmole NADH, 50 μg lactate dehydrogenase, 34 μg native anthranilate synthetase.

Substrate	*Amount of ^{14}C used*	*$^3H/^{14}C$ ratio of*		*Retention of ^3H in pyruvate (%)*
		Chorismate	*Lactate*	
[1′-^{14}C,2-^3H]-Chorismic acid	0.075 μC	10.4	0.5	5
[1′-^{14}C,3′-^3H]-Chorismic acid	0.029 μC	1.27	1.16	92

TABLE III

NON-EXCHANGE OF THE HYDROGEN AT C-2 OF CHORISMATE BY ANTHRANILATE SYNTHETASE IN THE ABSENCE OF GLUTAMINE

The reaction mixture contained in a volume of 1 ml: 0.5 μmole [1,2-^{14}C,2-^{3}H]chorismic acid (0.03 μC of ^{14}C,^{3}H/^{14}C = 10.5), 5 μmoles glutamine, 6 μmoles $MgCl_2$, 50 μmoles potassium phosphate buffer (pH 7.4) and 80 μg native anthranilate synthetase. Incubation was for 40 min at 25°.

Reaction mixture	*Conversion into anthranilate (%)*	*^{3}H found in water (%)*	*^{3}H/^{14}C ratio of residue containing unreacted chorismate*
Complete	60	—**	—
— glutamine	—*	1.7	10.5
Heat-denatured enzyme			
— glutamine	—*	1.2	10.8

* Not determined, expected to be zero.
** Not determined, expected to be 60% (*cf.* data in Table I)

TABLE IV

NON-INCORPORATION OF ^{3}H FROM TRITIATED WATER INTO ANTHRANILATE IN THE ANTHRANILATE SYNTHETASE REACTION

The reaction mixtures were the same as in Table I, except that the chorismic acid was unlabeled, the solution contained tritiated water (about 20 mC), and a known amount of [^{14}C]anthranilate was added after termination of the reaction.

Enzyme	*Anthranilate formed (μmoles)*	*Anthranilate [^{14}C] added (disint./min)*	*^{3}H/^{14}C ratio*		*μatoms of H incorporated per μmole anthranilate*
			*Calculated**	*Found*	
Native	0.967	$1.67 \cdot 10^5$	2.23	0.01	0.004
Native	0.822	$1.45 \cdot 10^5$	2.60	0.04	0.02
Component I	0.24	$4.18 \cdot 10^4$	2.20	0.05	0.02
Component I	0.292	$4.15 \cdot 10^4$	2.70	0.22	0.08

* Calculated for incorporation of 1 μatom tritium per μmole anthranilate.

TABLE V

INCORPORATION OF TRITIUM FROM ^{3}HOH AND ^{3}HO^{2}H INTO THE PYRUVATE FORMED IN THE ANTHRANILATE SYNTHETASE REACTION

The reaction mixture contained in a volume of 1 ml: 0.126 μmole [1′-^{14}C]chorismic acid (0.0021 μC), ^{3}HOH (about 20 mC) in normal water or $^{2}H_2O$ (final enrichment about 94% ^{2}H), 5 μmoles glutamine, 5 μmoles $MgCl_2$, 50 μmoles potassium phosphate buffer (pH 7.4), 0.5 μmole NADH, 50 μg lactic dehydrogenase, 34 μg native anthranilate synthetase. The conversion of chorismate was quantitative after 5 min as shown by spectrofluorimetry.

	^{3}H/^{14}C ratio of lactate calculated for incorporation of 1 μatom of H per μmole	*^{3}H/^{14}C ratio found*	*Atoms of H incorporated per μmole*
^{3}HOH in H_2O	11.1	1.65	0.149
^{3}HOH in $^{2}H_2O$	10.75	3.16	0.294

the experiments of Table IV, [7-^{14}C]anthranilate was then added and the anthranilate was isolated by chromatography in System 1 followed by crystallization with carrier. From the experiments of Table V, lactate was obtained by chromatography in Systems 2 and 1.

RESULTS

In a number of preliminary experiments (Table I) we used chorismic acid tritiated in the 2-position of the ring which also carried a ^{14}C label in this part of the molecule and which we had obtained in previous work[8,9]. As shown in Table I, the ^{3}H was largely or completely found in the water that was recovered from the reaction mixture. In agreement with this, the anthranilate formed was devoid of tritium as shown by the decrease in the ^{3}H/^{14}C ratio in the conversion of the chorismate into anthranilate. Complete loss of the hydrogen at C-2 of chorismate has to be expected, since SRINIVASAN[10] has shown that it is this carbon to which the amino group becomes attached. Recovering the ^{3}H from C-2 of chorismate in the water does, of course, imply that the pyruvate formed is not tritiated. Initial attempts to demonstrate this directly by adding a known amount of ^{14}C as pyruvate after the incubation and measuring the ^{3}H/^{14}C ratio of the isolated pyruvate, however, gave variable results. The difficulty lies in the chemical instability of pyruvate which made it impossible to determine accurately the amount of [^{14}C]pyruvate added. To overcome this problem, we prepared chorismate labeled with ^{14}C in the side chain, which was mixed with the 2-tritiated material and then subjected to the anthranilate synthetase reaction. In this way, the [^{14}C]pyruvate which served as the reference, was generated during the reaction itself. Table II shows that the pyruvate, which was enzymatically reduced and isolated as lactate, had a ^{3}H/^{14}C ratio corresponding to incorporation of not more than 5% of the ^{3}H. The control experiment with both ^{3}H and ^{14}C in the side chain showed that the ^{3}H from the 3′-position is largely recovered in the lactate, indicating that the non-incorporation of the ^{3}H from the 2-position of chorismate into pyruvate is not due to inadequate workup procedures.

In trying to reconcile the present finding that the ^{3}H from C-2 of chorismate is not incorporated into the pyruvate with the earlier observation of a low ^{3}H incorporation from ^{3}HOH into the pyruvate, we considered the possibility that the tritium at C-2 might be replaced during the binding of chorismate to the enzyme*. In this case, the enzyme could be expected to catalyze the exchange of the ^{3}H from C-2 of chorismate in the absence of the second substrate, glutamine. Using [1,2-^{14}C,2-^{3}H]-chorismate, it was, however, found that such an exchange does not take place (Table III). Under conditions which in the presence of glutamine gave a 60% conversion of chorismate into anthranilate, only 1.7% of the tritium was released into water compared to 1.2% in the control reaction with denatured enzyme, and the ^{3}H/^{14}C ratio of the chorismate did not change.

If, as the experimental results indicate, the third hydrogen atom of the pyruvate methyl group does not originate from the hydrogen at C-2 of chorismate, one has to consider the possibility that it comes from another position of the chorismate ring. Since none of the carbon-bound hydrogens of chorismate except the one at C-2 is

* This possibility has been pointed out by Dr. R. Somerville, Purdue University.

replaced by a different group in the conversion to anthranilate, a hydrogen transferred from a nuclear position to C-3' would have to be replaced by another hydrogen. This hydrogen would have to come from water, since the second substrate of the reaction, glutamine, is not an obligatory one but can be replaced by NH_3. Therefore, if this possibility were to hold true, tritium should be incorporated into the product anthranilate when the reaction is carried out in tritiated water. Several experiments using both native anthranilate synthetase and anthranilate synthetase Component I were carried out to examine this point. The reactions were conducted with non-labeled chorismate in tritiated water and the yield of anthranilate was determined fluorometrically. From it and the specific radioactivity of the water, the amount of ^3H corresponding to incorporation of one hydrogen atom into anthranilate was calculated. Known amounts of [^{14}C]anthranilate were added after the reaction had been terminated and the calculated $^3H/^{14}C$ ratios were compared to those found for the purified samples of anthranilate isolated from the incubations. As seen in Table IV, in no case was there any significant incorporation of tritium into the anthranilate and at least in one experiment the $^3H/^{14}C$ ratio as was low as 0.4% of the one calculated for the incorporation of one proton. We can therefore rule out the possibility that the third hydrogen atom of the methyl group of pyruvate originates from any of the ring positions of chorismate.

The first experiment in Table V shows that the low incorporation of tritium from ^3HOH into pyruvate previously observed[5] is reproducible under the different experimental conditions of this study. In this experiment, chorismic acid labeled with ^{14}C in the enolpyruvyl side chain was subjected to the anthranilate synthetase reaction in tritiated water and the resulting pyruvate was enzymatically reduced to lactate, which was purified and analyzed for its $^3H/^{14}C$ ratio. The present experiment with native anthranilate synthetase resulted in incorporation of tritium corresponding to 15% of the theoretical amount equivalent to one hydrogen. Interestingly, this figure increased to 29% when the reaction was conducted with ^3HOH in 94% ^2H_2O (Table V, 2nd experiment).

DISCUSSION

The results of this study clearly show that the labeled hydrogen from C-2 of chorismate is not incorporated into the pyruvate formed in the anthranilate synthetase reaction. Likewise, none of the other carbon-bound hydrogens of chorismate can be the source of the third hydrogen atom of the pyruvate methyl group. ^3H at C-2 of chorismate is not labilized by anthranilate synthetase in the absence of the second substrate of the enzyme. Experiments with the glutamine analog 6-diazo-5-oxonorleucine indicate ordered binding of first chorismate and then glutamine (H. NAGANO AND H. ZALKIN, unpublished results). Therefore, chorismate binds to the enzyme under these conditions and consequently, exchange of the ^3H independent of the overall reaction taking place can not account for its failure to be incorporated into pyruvate. These findings do not substantiate the earlier suggestion[5] that the third hydrogen atom of the pyruvate methyl group comes from chorismate. They definitely exclude an intramolecular proton transfer from C-2 of chorismate to the side chain.

The low incorporation of tritium from ^3HOH into pyruvate in the anthranilate synthetase reaction needs to be explained. We suggest that this represents a "product

isotope effect * resulting from the discrimination against ^3H in the cleavage of ^3H–O–H. This kind of isotope effect is observed in reactions, chemical as well as enzymatic, involving incorporation of a proton from the medium, which are either themselves irreversible or are made irreversible by trapping the product. In the anthranilate synthetase reaction the observed product isotope effect (from data in Table V) is about 6.7, which lies in the general range found for other reactions. SIMON *et al.*[12], for example, determined the following values: phosphoglucose isomerase, 6.2; phosphomannose isomerase, 5.2; triosephosphate isomerase, 6.0; pyruvate kinase, 6.4. Aldolase and pyruvate decarboxylase had lower product isotope effects (both 2.5). A value of 7.1 has been observed in a purely chemical system[11], the hydration of isobutene. If a product isotope effect is indeed responsible for the low incorporation of ^3H into the pyruvate, more ^3H should be incorporated if the reaction is carried out with ^3HOH in ^2H_2O instead of H_2O. The experiment (Table V) shows that this is the case. The product isotope effect of ^3H in ^2H_2O is 3.2 (extrapolated for 100% ^2H_2O), a figure somewhat higher than that observed by GOLD AND KESSICK[11] in the isobutene hydration (1.9). In the experiments of ZALKIN AND KLING[5] the pyruvate kinase reaction was used as a positive control and incorporation of ^3H corresponding to 1.07 atoms of hydrogen per mole was found. However, since the pyruvate was not trapped as lactate and the enzyme is known to catalyze the enolization of pyruvate[13–15], it is to be expected that essentially any number of atoms of hydrogen incorporated per mole between 0.16 (the minimum dictated by the product isotope effect[12]) and 3 (for complete exchange) might be observed, depending on the conditions of the reaction.

Finally, the results of these experiments indicate a similar mechanism with respect to formation of pyruvate for the reactions catalyzed by the glutamine-dependent native enzyme aggregate and the NH_3-dependent anthranilate synthetase Component I.

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* A product isotope effect[11] is defined as the ratio of theoretical amount of hydrogen isotope incorporated in the absence of an isotope effect/observed hydrogen isotope incorporation.