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Biochemistry

Characterization of composite aminodeoxyisochorismate synthase and aminodeoxyisochorismate lyase activities of anthranilate synthase

(chorismate/tryptophan biosynthesis/aminodeoxyisochorismate)

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ABSTRACT **Anthranilate synthase [chorismate pyruvate-lyase (amino-accepting), E.C. 4.1.3.27] catalyzes the formation of anthranilate (*o*-aminobenzoate) and pyruvic acid from chorismate and glutamine. A mutant form of the enzyme from *Salmonella typhimurium* accumulates a compound that we had isolated and identified as *trans*-6-amino-5-[(1-carboxyethenyl)-oxy]-1,3-cyclohexadiene-1-carboxylic acid, commonly called aminodeoxyisochorismate (ADIC). Here we report that ADIC is formed by a reversible, Mg^{2+}-dependent ADIC synthase activity of anthranilate synthase that can be functionally uncoupled from a Mg^{2+}-dependent ADIC lyase activity of the enzyme by single amino acid substitutions in the TrpE subunit of the anthranilate synthase complex of *S. typhimurium*. Both of the component activities of the enzyme are sensitive to feedback inhibition by L-tryptophan. Purified ADIC is quantitatively converted to anthranilate and pyruvic acid by the ADIC lyase activity of wild-type anthranilate synthase. ADIC also serves as a substrate for the formation of chorismate by the enzyme in the absence of glutamine and $(NH_4)_2SO_4$. The rate of ADIC formation by the mutant enzyme and the steady-state parameters for ADIC utilization by the wild-type enzyme are consistent with a role for ADIC as an enzyme-bound intermediate that does not accumulate during the course of the anthranilate synthase reaction. The altered catalytic specificity of mutant anthranilate synthase enzymes suggests a potential role for ADIC in secondary metabolism.**

The biosynthesis of most aromatic compounds in bacteria, fungi, and plants begins with the shikimate pathway branchpoint compound, chorismate. Five chorismate-utilizing enzymes catalyze the initial reactions of pathways leading to the formation of the major aromatic compounds of the cell (Fig. 1). Three of these enzymes, anthranilate (Anth) synthase, aminodeoxychorismate (ADC) synthase and isochorismate (IC) synthase, comprise a family of enzymes that have diverged from a common ancestor (1–3). Although a number of common mechanisms for catalysis by the enzymes have been proposed (4, 5), common intermediates have not yet been identified. Possible structural similarities of the active sites cannot yet be assessed because of the absence of comparative tertiary structural information.

There are substantial differences in catalytic function among the enzymes (Fig. 1). As the first step in L-tryptophan biosynthesis, Anth synthase (E.C. 4.1.3.27) catalyzes the formation of the aromatic product Anth in a reaction that involves elimination of pyruvic acid from chorismate. In contrast, ADC synthase and IC synthases form diene products that retain the *enol*pyruvyl group of chorismate. Current models for the Anth synthase catalytic mechanism propose the existence of an aminocyclohexadiene reaction intermediate, *trans*-6-amino-5-[(1-carboxyethenyl)oxy]-1,3-cyclohexadiene-1-carboxylic acid (6, 7), commonly known as aminodeoxyisochorismate (ADIC). ADIC is an isomer of ADC, the product of ADC synthase (8), and is the amino analogue of IC, the IC synthase product (9). Attempts to detect ADIC during enzymatic turnover of chorismate by Anth synthase have been unsuccessful (4); however, synthetic preparations of ADIC have been shown to be converted to Anth by Anth synthase at rates comparable to the rate of conversion of chorismate to Anth (10, 11). In addition, a lactylaminocyclohexadiene of undetermined stereochemistry was isolated from incubations of Anth synthase with a lactyl analogue of chorismate and was shown to be converted slowly by the enzyme to Anth (12).

In this report we describe (1) the accumulation of ADIC by a mutant form of Anth synthase from *Salmonella typhimurium*, (2) the detection of ADIC during catalytic turnover of chorismate by wild-type Anth synthase, and (3) characterization of the enzymatic conversion of purified ADIC to Anth. The results show that the Anth synthase reaction is a net activity of sequential ADIC synthase and ADIC lyase activities. All evidence indicates that the ADIC synthase activity is functionally analogous to the IC synthase and ADC synthase reactions, whereas the ADIC lyase activity is mechanistically distinct from the chorismate lyase (13, 14) and ADC lyase (15) reactions. We anticipate that the structural similarities shared by Anth synthase, IC synthase, and ADC synthase will be manifest in features of a common mechanism for the synthase reactions.

MATERIALS AND METHODS

Chemicals and Enzymes. Chorismic acid was prepared (16, 17) and assayed (18) as described. ADIC was prepared enzymatically from chorismic acid as described elsewhere (19). Anthranilic acid (Sigma) was recrystallized four times from H_2O. NADH, lactate dehydrogenase, Tris base, EDTA, L-tryptophan, and L-glutamine were from Sigma. Tricine was from Calbiochem. The mutagenic oligonucleotide (5′-CGCTATTCATACGTAATCNNG/CCTGGTTTC-CCGG-3′) was synthesized by Oligos Etc. (Guilford, CT). All other chemicals were analytical reagent grade.

Overproduction and Purification of Enzymes. Wild-type and mutant $TrpE^{H398M}$ Anth synthase complexes were overexpressed from phagemids pSTS23 and pSTM25, respectively, which were maintained in host strain *Escherichia coli* CB694 [W3110 Δ*trpE-A2 tna2 bglR*/F′ *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn*10*(Tet^r)]. pSTS23 is a 8.7-kb ColE1 replicon

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Abbreviations: Anth, anthranilate; ADIC, aminodeoxyisochorismate; ADC, aminodeoxychorismate; IC, isochorismate; $TrpE^{H398M}$ and $TrpE^{E358K}$, TrpE subunits with amino acid changes His-398 → Met and Glu-358 → Lys, respectively.
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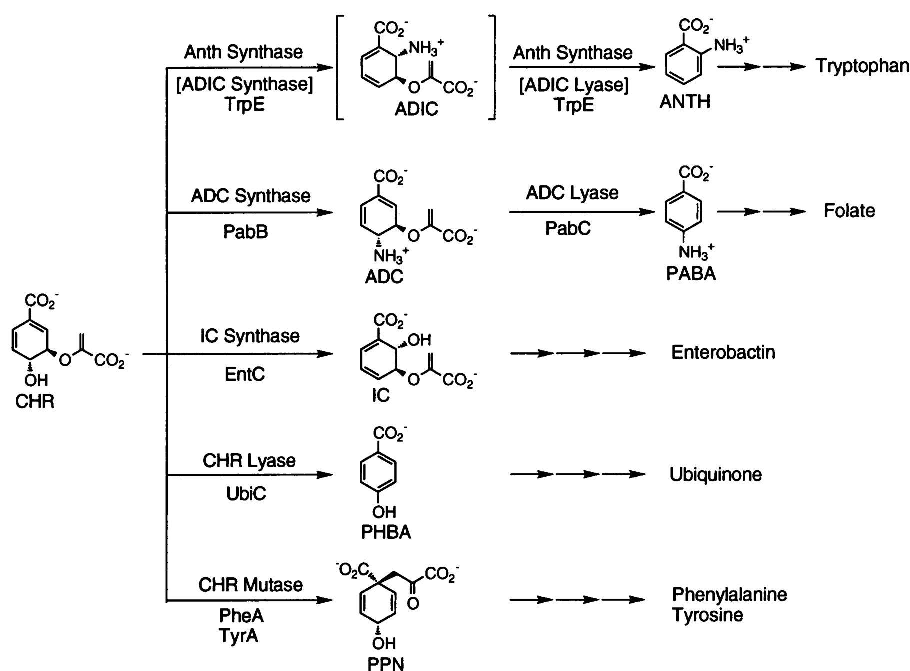

FIG. 1. Branch point reactions of chorismic acid in aromatic biosynthesis. Polypeptide designations are indicated beneath the names of the enzymes. Structures are shown for the products of the initial enzymatic transformations in each pathway. ADIC, the proposed Anth synthase reaction intermediate, is shown in parentheses. PABA (*p*-aminobenzoate), the product of the second enzyme in the folate biosynthetic pathway, is also shown. PHBA, *p*-hydroxybenzoate; PPN, prephenate; Chr, chorismate.

carrying the wild-type *S. typhimurium trpE* and *trpD* genes under the control of the *trp* promoter and terminator. pSTM25 is identical to pSTS23 with the exception of four site-directed nucleotide changes, constructed by standard techniques (20, 21), which result in a methionine substitution at His-398 (CAT → ATG) of the TrpE subunit of the Anth synthase complex of *S. typhimurium* as well as a silent codon change (GTG → GTA) at Val-396. All nucleotide regions of pSTM25 synthesized *in vitro* were sequenced (21) prior to use of the phagemid for enzyme expression. Overproducing strains were cultured essentially as described (18, 22) except that the tryptophan supplement of the minimal-salts medium was increased to 25 μg/ml and the cultures were harvested when the OD_{550} reached ≈2.0. Purification of the wild-type and mutant $TrpE^{H398M}$ Anth synthase complexes and determination of the absolute protein concentrations of the purified preparations was performed as described (18, 22).

Enzymatic Assays. The Anth synthase and ADIC lyase reaction mixtures contained 50 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 50 mM $(NH_4)_2SO_4$ (when included), wild-type or mutant Anth synthase complex, and variable amounts of chorismate or ADIC. ADIC concentration was determined spectrophotometrically by using an extinction coefficient of 11,500 $M^{-1}\cdot cm^{-1}$ at 280 nm in H_2O (see *Results*). Continuous spectrofluorometric assay of Anth synthase and ADIC lyase activities were performed as described (18). For kinetic experiments, wild-type Anth synthase complex was used at 0.8 μg/ml and substrates (chorismate and ADIC) were varied within the range of 1 μM and 100 μM. Apparent k_{cat} and K_m values were determined by using the Enzfitter nonlinear regression data analysis software (Elsevier, New York). Experiments were repeated at least once, and k_{cat} and K_m values did not vary from those reported by more than ±10%. Kinetic constants reported were derived from a single experiment with each data point representing an average of three determinations. The ADIC synthase-specific activity of the mutant $TrpE^{H398M}$ Anth synthase was determined by using the same reaction conditions as the Anth synthase assay with 250 μM chorismate as a substrate and 50 μg of enzyme per ml. The initial rate of ADIC formation was monitored by continuous spectrophotometric assay with a Hewlett-Packard 8452A diode array spectrophotometer. A difference coefficient of 8750 $M^{-1}\cdot cm^{-1}$ at 278 nm between ADIC and chorismate (5) was used. Pyruvate release from chorismate or ADIC was assayed by a lactate dehydrogenase-coupled assay in which the reaction mixture contained the same components as the fluorometric assay plus 0.2 mM NADH and 20 μg of lactate dehydrogenase per ml. The oxidation of NADH was followed at 22°C by the continuous monitoring of A_{340} with a Varian DMS200 dual beam spectrophotometer.

Analytical HPLC. Aliquots (200 μl) of the indicated reaction mixture were mixed with 4 vol of 0.1% CF_3COOH, and 0.5 ml of this mixture was separated on an analytical C_{18} column (Beckman ODS Ultrasphere 5 μm × 4.6 mm × 25 cm) by using a Beckman System Gold HPLC system. The following elution protocol was used: 0–2 min with 100% solvent A; 2–17 min with 0–50% solvent B; 17–19 min with 50% B; 19–21 min with 50–100% B; 21–23 min with 100% to 0% B; 23–31 min with 100% A (where solvent A is 0.1% CF_3COOH in distilled H_2O and B is 0.1% CF_3COOH in acetonitrile). Peaks were detected by monitoring the absorbance of the column effluent at 280 nm. Peak assignments were based on the coincidence of retention times and absorption spectra of unknowns with those of purified chorismate, Anth, ADIC, and *p*-hydroxybenzoate standards.

RESULTS

Detection of an Anth Synthase Reaction Intermediate. Native Anth synthase complex from *S. typhimurium* is composed of two dissimilar subunits, TrpE and TrpD, assembled as an $\alpha_2\beta_2$ heterotetramer. TrpE subunit alone is able to catalyze the NH_3-dependent Anth synthase reaction, but the participation of the glutamine amidotransferase domain of TrpD is required to utilize glutamine as the amide donor (22). Chemical modification studies of the TrpE subunit of Anth synthase have been consistent with the existence of a single

active site histidine, and possible acid and base functions for this residue in the Anth synthase reaction have been suggested (23). His-398, one of the highly conserved histidine residues in the polypeptide, is a likely candidate for this role (4).

To test this idea further, we engineered a collection of mutant Anth synthase enzymes by site-directed mutagenesis of the cloned *trpE* gene with all possible amino acid substitutions for His-398. Mutant enzymes were overexpressed, and the Anth synthase activity of each mutant enzyme was determined. While many of the enzymes were inactive, some exhibited a low level of Anth synthase activity (between 0.1% and 10% of wild type), whether assayed in the standard NH_3-dependent or glutamine-dependent reactions (22). The Anth synthase activity of all partially active mutant enzymes was nonlinear, increasing with time and never reaching a steady-state level, in marked contrast to the linear activity of the wild-type enzyme under the same conditions. In addition, dynamic changes in the UV absorption spectra of the reaction mixtures of mutant enzymes were noted (Fig. 2), suggesting the accumulation of a new molecular species with an extinction coefficient greater than that of the substrate, chorismate. Of the mutant enzymes that have been analyzed, these features were most dramatic in reactions catalyzed by the $TrpE^{H398M}$ enzyme, which has a residual Anth synthase activity that is $\leq 1\%$ of the wild-type level.

Fractionation of reactions catalyzed by the mutant $TrpE^{H398M}$ Anth synthase by HPLC revealed the presence of a new molecular species that was eluted earlier than either chorismate or Anth (Fig. 3 *A* and *B*). Control experiments established that chorismate, amino donor, Mg^{2+}, and enzyme were absolute requirements for the appearance of the new compound. In the early stages of the reaction, accumulation paralleled the consumption of chorismate and eventually reached a level as high as 15 mol % of the initial chorismate concentration. The initial rate at which the new compound was formed (0.4 nmol/sec per nmol of enzyme) was almost 100 times greater than the initial rate of Anth formation. After prolonged incubation ($\approx$8 h), none of the new species remained and all of the substrate chorismate was accountable as Anth. Essentially identical results were obtained with either glutamine-dependent or NH_3-dependent assay conditions. Unless indicated otherwise, all subsequent experiments were performed with NH_3 as a substrate.

Formation of the new species was also detected in reaction mixtures of the wild-type enzyme, although at greatly reduced levels. The amount present in reactions incubated at 22°C (Fig. 3*C*, solid line), although too small for precise measurement by HPLC assay, was substoichiometric to the

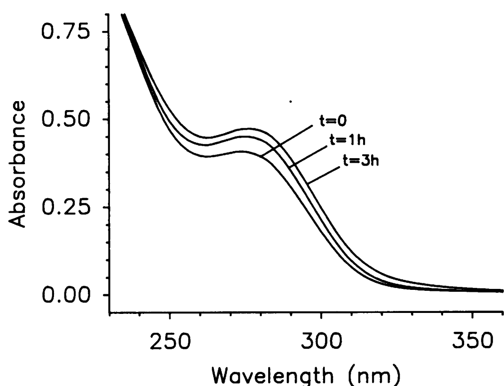

FIG. 2. Spectrophotometric monitoring of Anth synthase reaction mixture with mutant $TrpE^{H398M}$ complex. Standard NH_3-dependent conditions were used with chorismate at 150 μM and enzyme at 5 μg/ml. Spectra were recorded at room temperature with a Hewlett–Packard diode array spectrophotometer.

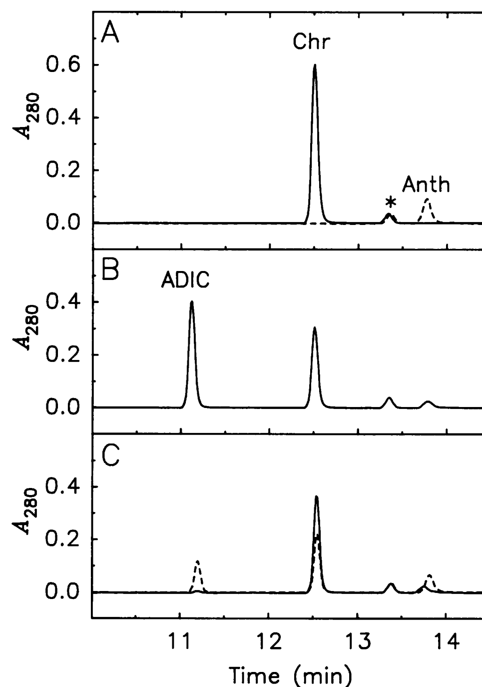

FIG. 3. HPLC fractionation of wild-type and mutant $TrpE^{H398M}$ Anth synthase reaction mixtures. Standard NH_3-dependent reaction conditions were used with chorismate (Chr) at 250 μM and enzymes at 100 μg/ml except where noted. (*A*) Chromatogram of reaction mixture prior to the addition of enzyme (——) and 3 min after the addition of wild-type Anth synthase (- - -). The peak indicated by the asterisk is *p*-hydroxybenzoate, an impurity present in some preparations of chorismate. (*B*) Chromatogram of a reaction mixture after 165 min incubation with mutant $TrpE^{H398M}$ Anth synthase. (*C*) Chromatogram of a reaction mixture after a 30-s incubation with wild-type Anth synthase (——) and after a 90-min incubation with wild-type Anth synthase at 0°C (- - -).

amount of enzyme present (400 nM). However, accumulation was markedly enhanced when the reaction temperature was lowered, increasing to $\approx$2 mol % of the input chorismate at 0°C (data not shown).

These observations are consistent with the conclusion that the wild-type and mutant $TrpE^{H398M}$ enzymes form an intermediate in the Anth synthase reaction and that the mutant enzyme accumulates this intermediate as a result of an impairment in its ability to convert this compound to Anth. We have isolated and purified this putative intermediate and identified it as aminodeoxyisochorismate (ADIC) by $^1H,^1H$ and $^1H,^{13}C$ COSY (correlated spectrography) two-dimensional NMR analysis (19).

Characterization of ADIC as a Substrate for Wild-Type Anth Synthase. Purified ADIC was found to serve as substrate for quantitative formation of Anth and pyruvate by wild-type Anth synthase in the presence of $(NH_4)_2SO_4$ or glutamine. This ADIC lyase activity was strictly Mg^{2+} dependent. No Anth was formed in the absence of Mg^{2+} or when 10 mM EDTA was included in standard reaction mixtures. Neither $(NH_4)_2SO_4$ or glutamine was required for the utilization of ADIC by Anth synthase; however yields of Anth and pyruvate were 25% higher when either was included in the reaction mixture. Analysis of ADIC lyase reaction mixtures by HPLC revealed that, in the absence of $(NH_4)_2SO_4$, 80% of the ADIC was converted to Anth and the remaining 20% to chorismate. When $(NH_4)_2SO_4$ was added to the completed reaction, the preformed chorismate was rapidly and quantitatively converted to Anth and pyruvate. Thus, the ADIC synthase activity of the wild-type enzyme is reversible, with the final product ratio between the forward lyase reaction and

the reverse synthase reactions being about 4:1 in the absence of $(NH_4)_2SO_4$ or glutamine.

Kinetic Analysis of the ADIC Lyase Activity of Wild-Type Anth Synthase. Steady-state kinetic analysis of the ADIC lyase of the wild-type enzyme revealed an apparent k_{cat} of 15 sec^{-1} in the presence of $(NH_4)_2SO_4$ (Table 1). This is 1.6-fold higher than that for the NH_3-dependent Anth synthase activity of the enzyme assayed under identical conditions. In the absence of $(NH_4)_2SO_4$, the apparent k_{cat} for ADIC lyase was about 1.2-fold that of the NH_3-dependent Anth synthase. The K_m for ADIC in both the presence and absence of $(NH_4)_2SO_4$ was about 3 times the K_m for chorismate in the NH_3-dependent Anth synthase reaction. Thus, the catalytic efficiency (k_{cat}/K_m) of the enzyme for the Anth synthase reaction is about 2-fold higher than that for the ADIC lyase reaction.

The ADIC lyase activity in the absence of $(NH_4)_2SO_4$ remained linear for a much shorter period of time than either the ADIC lyase activity in the presence of $(NH_4)_2SO_4$ or the NH_3-dependent Anth synthase activity. This led us to test whether chorismate, which is formed in significant amounts from ADIC under NH_3-free conditions, might be acting as an inhibitor of the ADIC lyase activity. The results verified that chorismate is an effective inhibitor of ADIC lyase with a mixed pattern of inhibition. The concentration of chorismate that inhibits response by 50% was found to be ≈ 3 μM when 100 μM ADIC was used as substrate. The ADIC lyase was also found to be sensitive to feedback inhibition by tryptophan, with K_i^{Trp} about twice that of the NH_3-dependent Anth synthase reaction (Table 1). Kinetic plots of the data revealed cooperativity in tryptophan binding, as has been noted for the glutamine- and NH_3-dependent Anth synthase activities of the enzyme (18).

Properties of ADIC. The molar extinction coefficient for ADIC in H_2O at its absorption maximum of 280 nm (ε_{280}) was determined to be 11,500 $M^{-1} \cdot cm^{-1}$, based on the quantitative enzymatic conversion of ADIC to Anth by wild-type Anth synthase in the presence of $(NH_4)_2SO_4$. This value is higher than the ε_{280} of 5900 $M^{-1} \cdot cm^{-1}$ reported for synthetic ADIC (10) but is very similar to the ε_{278} of 12,800 $M^{-1} \cdot cm^{-1}$ determined for IC, the hydroxy analogue of ADIC (5), prepared by enzymatic synthesis.

ADIC is moderately unstable in solution; its half-life in Tris buffer at pH 8.0 and 22°C was found to be ≈ 34 hr. HPLC and fluorescence analysis revealed nonenzymatic conversion of ADIC to a variety of compounds, including Anth, which accounted for about 7% of the total products. We have commented elsewhere on the superior stability of ADIC prepared as a free acid relative to the CF_3COOH salt (19). ^1H NMR spectra showed that the major breakdown product of free acid and CF_3COOH salt preparations of ADIC is an adduct formed by Claisen rearrangement that has been shown to be an inhibitor of Anth synthase (11). We found that the maximal velocity of the ADIC lyase activity of Anth synthase was reduced by >40% when preparations of ADIC were used that had accumulated breakdown products accounting for no more than 10% of the starting ADIC.

Table 1. Steady-state kinetic constants for wild-type Anth synthase and ADIC lyase reactions

	Anth synthase	ADIC lyase	
	+ $(NH_4)_2SO_4$*	− $(NH_4)_2SO_4$	+ $(NH_4)_2SO_4$
k_{cat}, sec^{-1}	9.3	11	15
K_m, μM	4.0	11	14
k_{cat}/K_m, $\mu M^{-1} \cdot sec^{-1}$	2.3	1.0	1.1
K_i^{Trp}, μM	2.5	5.3	ND†

*No Anth synthase activity was detectable in the absence of $(NH_4)_2SO_4$.
†Not determined.

DISCUSSION

The results reported here and elsewhere (19) demonstrate the enzymatic synthesis of ADIC and establish its role as a reaction intermediate in the biosynthesis of Anth from chorismate by Anth synthase. ADIC is formed by a Mg^{2+}-dependent, reversible ADIC synthase activity that is functionally similar to the activities of the homologous IC synthase (24) and ADC synthase (8, 25). ADIC is then converted to Anth by a Mg^{2+}-dependent, ADIC lyase activity of Anth synthase. This activity appears to be mechanistically distinct from the activities of the recently characterized ADC lyase (15) and chorismate lyase (13, 14), since the former requires pyridoxal phosphate as cofactor and the latter is Mg^{2+}-independent.

Anth synthase converts ADIC to Anth at a rate that is about 60% greater than the rate for the overall conversion of chorismate to Anth under the same conditions, consistent with the observation that ADIC does not normally accumulate during Anth synthase catalysis (4). The quantities of ADIC detected during turnover of chorismate by the wild-type enzyme at 22°C are substoichiometric with respect to the amount of enzyme in the incubations, indicating that there is no release of ADIC to the bulk solution during the course of Anth synthase catalysis. Given the high effective concentrations of ADIC that would result if the intermediate remains enzyme-bound or otherwise sequestered by the enzyme, ADIC would not be expected to accumulate, even though the catalytic efficiency for the ADIC lyase reaction is approximately half that of the NH_3-dependent Anth synthase reaction. The observed chorismate inhibition of the ADIC lyase reaction also supports a catalytic model in which there is no release of ADIC to the bulk solution. Efficient carbon flow into the tryptophan biosynthetic pathway will only occur if the pool of chorismate in the cell does not inhibit the ADIC lyase activity of Anth synthase. ADIC lyase is also sensitive to feedback inhibition by tryptophan, with kinetics very similar to those of tryptophan inhibition of the overall NH_3-dependent Anth synthase reaction. The ADIC synthase activity of the mutant $TrpE^{H398M}$ Anth synthase, as well as the reverse reaction, the formation of chorismate from ADIC, are likewise sensitive to tryptophan inhibition, although inhibition constants have not yet been derived. The simplest model that accounts for these results holds that the active sites for both the ADIC synthase and ADIC lyase activities are overlapping or closely associated in a domain formed by the carboxyl-terminal segment of the TrpE polypeptide, where they are both subject to feedback control exerted by tryptophan binding to the amino-terminal regulatory domain (18).

Both of the constituent reactions catalyzed by Anth synthase occur at rates that appear to be greater than the analogous reactions in related enzymes. Even at a lower temperature of assay (22°C vs. 37°C), the velocity and catalytic efficiency of the ADIC lyase reaction are >10-fold higher than the k_{cat} (0.8 sec^{-1}) and k_{cat}/K_m (8.2×10^4 $M^{-1} \cdot sec^{-1}$) values reported for chorismate lyase (13). Kinetic constants for ADC lyase have not been reported. If we consider that the ADIC synthase reaction for the wild-type enzyme is effectively "coupled" to the ADIC lyase reaction, the rate of the NH_3-dependent Anth synthase reaction sets a lower limit for the velocity of the ADIC synthase reaction. Thus, the velocity and catalytic efficiency of ADIC synthase are greater than the k_{cat} (2.9 sec^{-1}) and k_{cat}/K_m (2.1×10^5 $M^{-1} \cdot sec^{-1}$) reported for IC synthase (24) as well as the k_{cat} (0.1 sec^{-1}) and k_{cat}/K_m (1.5×10^3 $M^{-1} \cdot sec^{-1}$) for ADC synthase (8). Although we have not yet characterized a mutant enzyme completely devoid of ADIC lyase activity,

the initial rate for the ADIC synthase activity of the mutant $TrpE^{H398M}$ Anth synthase, with an apparent rate of 0.4 sec^{-1} under standard assay conditions, is also comparable to the values reported for IC synthase and ADC synthase. The difference in the rates of the ADIC synthase activity of the wild-type and mutant $TrpE^{H398M}$ Anth synthase enzymes may reflect phenomena such as the leaving group ability, rather than a true difference in the k_{cat} for the elementary synthase step in the overall reaction.

Increases in both the yield and the rate of formation of Anth from ADIC in the presence of 50 mM $(NH_4)_2SO_4$ have also been reported in studies utilizing synthetic ADIC (10, 11). We do not yet know whether the presence of $(NH_4)_2SO_4$ acts to prevent the formation of chorismate altogether or whether chorismate is transiently formed and converted to Anth without accumulation. Attempts to trap chorismate during Anth synthase turnover of ADIC in the presence of $(NH_4)_2SO_4$ and further kinetic study of the forward and reverse synthase reactions should discriminate between the two possibilities.

ADIC is a compound of interest not only because of its role as an intermediate in the Anth synthase reaction as described here but also as a potential precursor for a number of other metabolites such as phenazine dyes (26), antibiotics (27), and *p*-aminophenylalanine (28). Our results suggest the possibility that enzymes may exist that synthesize ADIC as a precursor to metabolites other than tryptophan. In this regard, an Anth synthase homologue has been described in *Pseudomonas aeruginosa*, encoded by the *phnA* and *phnB* genes, which is involved in the synthesis of the phenazine pigment pyocyanin (29). Although a number of previous studies argued against Anth as a phenazine precursor (26), the authors concluded that the putative Anth synthase encoded by *phnA* and *phnB* catalyzed the formation of Anth as the initial step in phenazine biosynthesis. We suggest the alternative possibility that the enzyme encoded by *phnA* and *phnB* is an ADIC synthase rather than an Anth synthase, consistent with proposals that an aminated derivative of chorismate serves as a phenazine precursor (26).

We have shown here that a minor structural alteration in Anth synthase, the substitution of a single amino acid residue, is sufficient to uncouple its ADIC synthase activity from its ADIC lyase activity. Although further experiments will be required to define the precise role of $TrpE^{H398}$ in Anth synthase catalysis, our results are generally consistent with a potential role as an acid or base catalyst in the lyase reaction or possibly as a binding group necessary to stabilize ADIC or a transition state intermediate in the lyase reaction. Although the mutant $TrpE^{H398M}$ enzyme still retains some residual ADIC lyase activity, it is feasible that other mutations could render the enzyme a monofunctional ADIC synthase. Our observation that a single amino acid substitution at another position within the TrpE polypeptide ($TrpE^{E358K}$) also leads to the accumulation of ADIC by Anth synthase is consistent with this hypothesis (A.A.M. and R.B., unpublished data).

The results presented here demonstrate that Anth synthase is a bifunctional enzyme catalyzing sequential ADIC synthase and ADIC lyase reactions without release of ADIC to the bulk solution. All evidence to date suggests that both of these reactions occur at an active site(s) formed by the TrpE polypeptide alone. In this regard the structural basis for Anth synthase catalysis differs from tryptophan synthase, another bifunctional enzyme involved in tryptophan biosynthesis, which sequesters the intermediate indole by direct channeling between active sites that are located on two different subunits of the enzyme complex (30). The mutations that result in the observed alteration of the catalytic function of Anth synthase will provide insight into the structural basis for catalysis by Anth synthase and related enzymes and furnish an experimental path to direct tests of the proposed role of ADIC in secondary metabolism.

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